Published in final edited form as:

Alcohol. 2017 May ; 60: 95–101. doi:10.1016/j.alcohol.2017.01.016.

Mechanistic insights into epigenetic modulation of ethanol consumption

Igor Ponomarev^{a,b}, Claire E. Stelly^a, Hitoshi Morikawa^a, Yuri A. Blednov^a, R. Dayne Mayfield^a, and R. Adron Harris^{a,b}

^aWaggoner Center for Alcohol and Addiction Research

^bThe College of Pharmacy, The University of Texas at Austin, 2500 Speedway A4800, Austin, Texas 78712, USA

Abstract

There is growing evidence that small-molecule inhibitors of epigenetic modulators, such as histone deacetylases (HDAC) and DNA methyltransferases (DNMT), can reduce voluntary ethanol consumption in animal models, but molecular and cellular processes underlying this behavioral effect are poorly understood. We used C57BL/6J male mice to investigate the effects of two FDA-approved drugs, decitabine (a DNMT inhibitor) and SAHA (an HDAC inhibitor), on ethanol consumption using two tests: binge-like drinking in the dark (DID) and chronic intermittent every other day (EOD) drinking. Decitabine but not SAHA reduced ethanol consumption in both tests. We further investigated decitabine's effects on the brain's reward pathway by gene expression profiling in the ventral tegmental area (VTA), using RNA sequencing and electrophysiological recordings from VTA dopaminergic neurons. Decitabine-induced decreases in EOD drinking were associated with global changes in gene expression, implicating regulation of cerebral blood flow, extracellular matrix organization, and neuroimmune functions in decitabine actions. In addition, an *in vivo* administration of decitabine shortened ethanol-induced excitation of VTA dopaminergic neurons *in vitro*, suggesting that decitabine reduces ethanol drinking via changes in the reward pathway. Taken together, our data suggest a contribution of both neuronal and non-neuronal mechanisms in the VTA in the regulation of ethanol consumption. Decitabine and other epigenetic compounds have been approved for cancer treatment, and understanding their mechanisms of actions in the brain may assist in repurposing these drugs and developing novel therapies for central disorders, including drug addiction.

Keywords

alcohol; epigenetics; drug repurposing; behavior; transcriptome; neuronal activity

Address correspondence to: Igor Ponomarev, University of Texas at Austin, Waggoner Center for Alcohol and Addiction Research, 2500 Speedway A4800, Austin, TX 78712, USA, ponomarev@utexas.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors declare no conflict of interest.

Introduction

Mounting evidence points to a central role of chromatin (epigenetic) modifications in controlling gene expression and behavior in alcohol use disorders (AUD), but the exact mechanisms of this control are not well understood. Small-molecule inhibitors of enzymes that modify DNA and histones, such as DNA methyltransferase inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi), have the potential to change gene expression and downstream behaviors via regulation of chromatin structure (Abel & Zukin, 2008; Spanagel, 2009). These epigenetic drugs have been extensively studied in the treatment of somatic diseases, such as cancer, leading to their approval for use in patients (Copeland, Olhava, & Scott, 2010), and have recently emerged as potential therapeutics for neurodegenerative disorders and drug addiction (Coppedè, 2014; Ponomarev, 2013; Walker, Cates, Heller, & Nestler, 2015).

Several research groups demonstrated the effects of HDACis and DNMTis on different alcohol behaviors, including withdrawal-related anxiety (Pandey, Ugale, Zhang, Tang, & Prakash, 2008), locomotor sensitization (Sanchis-Segura, Lopez-Atalaya, & Barco, 2009), and consumption (Barbier et al., 2015; Tulisiak, Harris, & Ponomarev, 2016; Warnault, Darq, Levine, Barak, & Ron, 2013; Wolstenholme et al., 2011). High alcohol (ethanol) consumption is a prerequisite for developing AUD, and compounds that can reduce ethanol drinking are of special importance for drug development. For example, the FDA-approved drugs azacitidine (a DNMTi) and vorinostat (also known as SAHA, an HDACi) reduced chronic and limited-access binge ethanol consumption, respectively, in C57BL/6J male mice (Warnault et al., 2013). It has been hypothesized that these drugs act via changes in brain gene expression; however, specific molecular and cellular mechanisms underlying drug-induced changes in alcohol behaviors have not been determined. Understanding central mechanisms of actions of FDA-approved drugs that target somatic diseases may assist in repurposing these compounds for brain disorders, including drug addiction. In addition, mechanistic knowledge will help develop novel therapies targeting specific components of drug-induced changes. Some epigenetic drugs have toxic side effects, limiting their therapeutic window. Therefore, understanding their downstream effects on brain may point to novel less toxic compounds that may mimic these effects on brain and behavior.

The goal of the current study was two-fold: a) to replicate and extend previous findings on the effects of epigenetic drugs on ethanol intake, and b) to investigate the effects of selected drugs on gene expression and cellular physiology in the VTA, a critical region of the reward pathway. We used the alcohol-preferring C57BL/6J mice to test the HDACi, SAHA and the DNMTi, decitabine in two ethanol paradigms: a sub-chronic bingelike drinking and a chronic intermittent consumption. Central effects of decitabine, the only drug that reduced ethanol intake in both tests, were further investigated using gene expression profiling in VTA and recordings from VTA dopaminergic (DA) neurons. Mechanistic insights gained from this study may help develop new therapies for brain disorders, including AUD.

Materials and methods

Animals

C57BL/6J (B6) male mice were used in all experiments. Original breeders were purchased from Jackson Laboratories (Bar Harbor, ME) and a colony has been maintained at The University of Texas at Austin. Mice were initially group-housed 4–5 to a cage. Before ethanol-drinking experiments, animals were moved to the experimental room and remained there for at least 2 weeks to adapt to a reversed 12:12-hour light:dark cycle (lights off at 10:00 AM). When animals were approximately 60 days old, they were individually housed and ethanol-drinking procedures were begun. Food was available *ad libitum*. The temperature and humidity of the rooms were kept constant. Experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin.

Preparation of drug solutions

Suberanilohydroxamic acid (SAHA, vorinostat) and decitabine (5-aza-2'-deoxycytidine) were purchased from Selleckchem (Houston, TX). Both drugs were freshly prepared as suspensions in saline with 4–5 drops of Tween-80 (Sigma-Aldrich, St. Louis, MO) and injected intraperitoneally (i.p.) in a volume of 0.1 mL/10 g of body weight. Doses of 50 mg/kg for SAHA and 0.5 mg/kg for decitabine were used in all experiments. Selection of doses and time points for drug injections was based on previous studies reporting molecular and behavioral effects of the drugs (Liang et al., 2015; Warnault et al., 2013). Saline containing 4–5 drops of Tween-80 was used as a control.

Drinking In the Dark (DID) ethanol consumption

A modification of previously described one-bottle DID (Rhodes, Best, Belknap, Finn, & Crabbe, 2005) was used. Briefly, starting 3 h after lights-off, water bottles were replaced for 3 h with bottles containing a 20% (v/v) ethanol solution. Except for the 3-h period of ethanol exposure, mice had unlimited access to water. All mice were pre-trained to drink ethanol for 1 week to establish a stable level of consumption. Following pre-training, on days 0 and 1, all animals were injected with saline 2 h before the drinking session to habituate to the injection procedure. On days 2–4, the treatment-group animals were injected with one of the drugs (SAHA or decitabine) and the control-group animals were injected with vehicle ($n = 6–12$ per group) 2 h before the drinking session. Ethanol intake was calculated after each 3–h session.

Every Other Day (EOD) ethanol consumption

In a separate experiment, a modification of a two-bottle EOD procedure also known as chronic intermittent (CI) drinking (Osterndorff-Kahanek, Ponomarev, Blednov, & Harris, 2013) was used. Briefly, mice were given one bottle of 15% (v/v) ethanol and one bottle of water during a 24-h session every other day (test day), with continuous access to water only on non-ethanol days. On alternative ethanol days, bottle positions were alternated to control for position preference. All mice were pre-trained (EOD) to drink ethanol for 2 months to establish a stable level of consumption. Following pre-training, at the end of day 1, all animals were injected with saline 1 h before the start of the drinking session on test day 2 to

habituate to the injection procedure. Similarly, animals were injected with either SAHA, decitabine, or vehicle 1 h before drinking sessions on test days 4, 6, 10, and 12 ($n = 15$ – 16 per group). All mice were injected with vehicle before the drinking session on test day 8 to evaluate if potential effects of drugs on ethanol consumption during days 4 and 6 are long lasting. Ethanol intake, preference, and water intake were calculated after each 24-h session.

Gene expression profiling

Twenty-four hours after the end of the last EOD drinking session (test day 12), mice from the Decitabine and Vehicle groups ($n = 15$ per group) were killed by cervical dislocation. The brains were removed, flash-frozen in liquid nitrogen, and embedded in OCT compound for sectioning. Brain slices ($300\text{-}\mu\text{m}$ thickness) were prepared at $-13\text{ }^{\circ}\text{C}$ using a cryostat. The ventral tegmental area (VTA) was dissected using the brain-punch tissue set in accordance with coordinates from the mouse brain atlas (Franklin & Paxinos, 2008). Total RNA was isolated from tissue samples using MagMAXTM-96 Total RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA) and sent to the Genomic Sequencing and Analysis Facility (GSAF) at the University of Texas (<https://wikis.utexas.edu/display/GSAF/Home+Page>) for RNA-Seq library preparation. All libraries passed the quality control, and samples were sequenced on the Illumina HiSeq 2500 sequencer at ~ 30 million reads per sample (single-read, 1×50 bp). Quality assessment of data files was done using FastQC (v0.11.5). Reads were aligned to the mouse reference (GRCm38/mm10) using TopHat2 (v2.0.10) and mapped using Bowtie2 (v2.1.0.0). HTSeq (v0.6.1) was used to assemble transcripts and generate read counts per transcript using the output from TopHat2. Data were normalized, and genes that were differentially expressed between the Decitabine and Vehicle groups (DEGs) were determined using the DESeq2 package for R.

Bioinformatics analysis

Two DEG lists, one up- and one down-regulated by decitabine (nominal p value < 0.05), were subjected to an over-representation analysis for biological pathways using Enrichr (Chen et al., 2013) (<http://amp.pharm.mssm.edu/Enrichr>) based on two databases: Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Over-representation p value for each pathway was calculated based on the Fisher's exact test with the correction for false discovery rate. To test if DEGs are markers of specific cell types, we profiled the top 41 DEGs (p value < 0.0001) using a database for cell type-specific brain transcriptomes (http://web.stanford.edu/group/barres_lab/brain_rnaseq.html) (Zhang, Chen, et al., 2014). At least 3-fold enrichment, compared to a second highest expression, was used as the criterion for a specific cell marker. In addition, to identify specific markers of DA neurons affected by the decitabine treatment, we compared all DEGs to dopamine-enriched genes identified by our previous study investigating effects of ethanol binge drinking for 3 weeks on microdissected DA neurons (Marballi, Genabai, Blednov, Harris, & Ponomarev, 2016; Appendix S1). We also compared DEGs from the current study to all genes differentially expressed between ethanol and control groups from the 2016 study. Statistical significance for the overlap between gene lists was estimated using a hypergeometric probability test.

Electrophysiological experiment

In a separate experiment, mice were treated with either decitabine or vehicle (a single injection every other day for 8 days for a total of 4 injections). Twenty-four hours after the last injection, mice were decapitated under isoflurane anesthesia, and brains were rapidly dissected in ice-cold sucrose-substituted artificial cerebrospinal fluid (ACSF). Horizontal midbrain slices 200 μ m thick were cut on a vibrating microtome and recovered for 1 h at 34 °C in oxygenated ACSF (in 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 1.2 mM or 0.1 mM MgCl₂, 2.4 mM CaCl₂, 11 mM glucose, 21.4 mM NaHCO₃). During recording, slices were perfused with ACSF at 34 °C. Recording pipettes were filled with 150 mM NaCl and loose seals (10–20 M Ω) were formed. Putative DA neurons were identified by the presence of slow (1–5 Hz) spontaneous pacemaker-type firing of action potentials of >1.2-ms width in voltage-clamp mode. Firing rates were monitored in current-clamp $I = 0$ mode. Data were digitized at 10 KHz and filtered at 2 KHz. Spikes were detected and analyzed with the AxoGraphX event-detection utility. Basal firing rate was recorded from 59 neurons (28 saline, 31 decitabine; $n = 8$ mice per group). Firing rate response to 80-mM ethanol was recorded for 10 min from 13 neurons (seven after vehicle from four mice, six after decitabine from two mice) with basal firing rate in the 1.5–2.5 Hz physiological range. Ethanol-induced firing frequency was normalized as % baseline for data analysis.

Statistical Analysis

Behavioral data were analyzed using a two-way mixed analysis of variance (ANOVA) with Treatment as a between-subject factor and Time as a within-subject factor (GraphPad Prism, GraphPad Software, Inc., La Jolla, CA). For DID, ANOVAs were carried out for days 2–4 for each drug separately. For EOD, a single ANOVA was carried out for days 4–12. Electrophysiological data were analyzed using a t test for basal firing rate and I_h current and a two-way mixed ANOVA with Treatment and Time as factors. To compensate for the underpowering effect of multiple repeated measures, we analyzed three critical time points representing the peak of ethanol effect in the Decitabine group (~1 min), the peak of ethanol effect in the Vehicle group (~5 min), and returning to baseline for both groups (~9 min). Statistically significant interaction terms were followed by Bonferroni *post hoc* tests. All data are reported as the mean $\pm$ SEM.

Results

Behavior

We used a DID paradigm to determine the effects of two epigenetic drugs on binge drinking. SAHA did not affect ethanol intake in the DID procedure, as neither main effect of treatment nor the interaction term were statistically significant, while treatment with decitabine significantly reduced ethanol intake ($F[1,22] = 16.27$, $p < 0.001$) with no significant Treatment $\times$ Time interaction (Fig. 1). We then tested SAHA and decitabine in a longer EOD paradigm that represents a model of escalated ethanol consumption. Similar to the DID results, decitabine but not SAHA progressively reduced ethanol intake over the course of injections, showing a cumulative effect (Fig. 2A). Both main effect of Treatment ($F[2,43] = 9.18$, $p < 0.001$) and the interaction term ($F[8,172] = 7.79$, $p < 0.001$) were statistically significant, with the effect of decitabine on test days 10 and 12 reaching statistical

significance in the *post hoc* comparisons. In addition, decitabine reduced ethanol preference on the same days (Fig. 2B; main effect of Treatment ($F_{[2,43]} = 4.97$, $p < 0.05$); interaction [$F_{(8,172)} = 6.39$, $p < 0.001$]). Treatment with decitabine did not significantly affect water intake from the water bottle on test days (data not shown).

Gene expression

The main objective of this analysis was to identify molecular and cellular functions potentially involved in decitabine-induced reduction of ethanol intake and preference. Overall, 692 genes were differentially expressed (DEGs) between Decitabine and Vehicle groups at a nominal p value of <0.05 , and the top 41 DEGs ($p < 0.0001$) were all up-regulated (Supplemental Table 1). DEGs up-regulated in the Decitabine group (434 genes) were highly enriched in a variety of functional groups and molecular pathways (non-redundant functional groups with lowest p values are shown in Table 1). The majority of the over-represented biological groups were directly related to the functions of glial and endothelial cells and could be categorized into four biological domains: cerebral blood circulation, extracellular matrix, neuroimmune functions, and molecular pathways. DEGs down-regulated in the Decitabine group (258 genes) did not yield significantly over-represented functional groups. Consistent with the functional group analysis, 80% of top DEGs ($p < 0.0001$) were specific markers of either endothelial cells or glia, including astrocytes, microglia, and oligodendrocytes, while the other 20% were expressed in multiple cell types. Comparison of DEGs from the current study and dopamine-enriched genes from our previous work (Marballi et al., 2016) detected only 10 overlapping genes (out of 353), which were statistically under-represented ($p < 0.05$), and none of these genes were differentially expressed between ethanol and control groups in the 2016 study. Genes overlapping between current DEGs and all genes differentially expressed between ethanol and control groups from the 2016 study were also under-represented (14 out of 552; $p < 0.05$).

Electrophysiology

The main objective of this experiment was to investigate the effects of decitabine on cellular physiology without potentially confounding effects of ethanol consumption. Decitabine injections did not alter intrinsic pacemaking properties of DA neurons, as there was no difference in basal firing rate (Fig. 3A) or I_h current (data not shown). An *in vivo* administration of decitabine shortened ethanol-induced excitation (faster desensitization) of DA neurons *in vitro*, as indicated by main effect of Treatment ($F_{[1,11]} = 5.58$, $p < 0.05$), significant Treatment $\times$ Time interaction ($F_{[2,22]} = 3.67$, $p < 0.05$), and a significant group difference at a midpoint of ethanol treatment (Fig. 3C).

Discussion

Chemical compounds that alter gene expression via the regulation of chromatin structure are targets for drug development for brain disorders, including drug addiction. Here, we used a combination of molecular, cellular, and behavioral approaches to study central effects of the DNMT inhibitor, decitabine, which is FDA-approved for the treatment of myelodysplastic syndromes, a group of diverse bone marrow disorders that can progress to cancer

(Hollenbach et al., 2010). Decitabine (5-aza-2'-deoxycytidine) is a cytidine nucleoside analog that causes DNA hypomethylation *in vitro* and *in vivo* (Liang et al., 2015; Wilson, Jones, & Mompalmer, 1983) by direct incorporation into DNA during DNA replication, and subsequent inhibition of DNMTs via covalent inactivation (Ewald, Sampath, & Plunkett, 2008). DNA methylation in gene promoters is associated with transcriptional silencing, and decitabine's anticancer effects are attributed, in part, to its ability to inhibit DNA hypermethylation and to reactivate tumor suppressor genes (Jabbour, Issa, Garcia-Manero, & Kantarjian, 2008). Decitabine readily passes the blood-brain barrier (Karahoca & Mompalmer, 2013), which may result in changes in brain gene expression and downstream functions.

Our study is the first to show that decitabine reduces ethanol consumption in two different paradigms. This is consistent with two previous reports demonstrating similar effects of different DNMTis, azacitidine (5-azacytidine) in mice exposed to a chronic intermittent procedure (Warnault et al., 2013), and RG108 in a rat model of alcohol dependence (Barbier et al., 2015). In contrast to these findings, Qiang and colleagues reported an azacitidine-induced increase in ethanol intake and preference in mice exposed to ethanol vapor (Qiang et al., 2015). Also, in contrast to the Warnault et al. study, we did not find a statistically significant reduction of ethanol intake by SAHA administration, although a trend was observed (see Fig. 1A). These discrepancies may be explained, at least in part, by procedural differences between studies. For example, differences between our study and the 2015 report (Qiang et al., 2015) included drug (decitabine vs. azacitidine), ethanol exposure (EOD vs. vapor), and method of injection (i.p. vs. intracerebroventricular [i.c.v.]). Also, compared to the 2013 study, we used a different DID procedure and a different vehicle to prepare SAHA solutions. A detailed analysis of these conflicting results can be found in a review in this issue of *Alcohol* (Tulisiak et al., 2016). However, more studies are required to identify potential causes of this disparity. In our study, the effects of decitabine on blood ethanol concentration and sucrose consumption were not tested, although they are unlikely to occur, because a structurally similar molecule, azacitidine, did not affect either variable (Warnault et al., 2013). Based on our behavioral results, decitabine was selected for further investigation.

We tested the hypothesis that treatment with decitabine produces molecular and cellular changes in the VTA, which may underlie the drug-induced reduction in ethanol intake and preference. VTA DA neurons represent the first segment of the reward pathway, and manipulation of the VTA neurocircuits often results in changes in ethanol consumption (Gonzales, Job, & Doyon, 2004). Treatment with decitabine resulted in more up- than down-regulated genes in VTA, which is consistent with its mechanism of action as a DNA hypomethylating agent. Transcriptional activation of some of the up-regulated genes may be directly related to decitabine-induced decrease in promoter methylation, and this hypothesis will be tested in future experiments. One potential limitation of our study is the lower consumption of ethanol in the Decitabine group during the last two drinking sessions; therefore, it is possible that some DEGs were a result of differential ethanol consumption rather than treatment with decitabine. This possibility is rather unlikely, because mice were drinking ethanol for 2 months before the drug test and the overall consumption did not differ between the groups. In addition, gene expression was measured 24 h after the end of the last

drinking session; thus, there was no ethanol onboard. Because of the cumulative effect of decitabine on ethanol intake, we would expect that mice would consume different amounts of ethanol at that time point, and, therefore, differences in gene expression would represent molecular correlates of decitabine-induced behavioral changes. Finally, we did not find a significant overlap between DEGs in our study and ethanol-related genes from our previous work (Marballi et al., 2016), suggesting that changes in gene expression in the current study are more likely to be related to the treatment with decitabine rather than differential ethanol intake.

Functional group analysis of genes up-regulated by decitabine highlighted biological categories mainly associated with functions of glia and endothelial cells, a somewhat surprising result, given the focus of the majority of previous research on neuronal functions. Our results suggest that regulation of local blood circulation, organization of extracellular matrix (ECM), and neuroimmune pathways in the VTA are important mediators of decitabine-induced effects on ethanol consumption. To gain more insight into decitabine-induced changes, we tested whether DEGs are markers of specific cell types using databases for cell type-specific transcriptomes (Marballi et al., 2016; Zhang, Chen, et al., 2014). Consistent with the functional group analysis, the majority of top DEGs were specific markers of endothelial or glial cells, while neuron-specific genes were under-represented. Taken together, our data suggest that decitabine mainly affects glial and endothelial cells in the VTA, with little direct effects on gene expression in neurons. This is not particularly surprising, given that decitabine is mainly incorporated into newly synthesized DNA, implying that postmitotic neurons will be the least affected. There is still evidence that nucleoside analogs can be incorporated into DNA of non-dividing cells via a DNA repair mechanism (Cooper-Kuhn & Kuhn, 2002), which may lead to changes in DNA methylation and gene expression.

Despite the apparent shortage of neuron-specific transcriptional changes, an *in vivo* pretreatment with decitabine modulated the firing properties of VTA DA neurons, as they became less sensitive to the local application of ethanol *in vitro*. Ethanol potentiates firing of VTA DA neurons both *in vivo* (Gessa, Muntoni, Collu, Vargiu, & Mereu, 1985) and *in vitro* (Brodie & Appel, 1998), which is consistent with ethanol-induced increases in extracellular DA levels in the nucleus accumbens (Yim & Gonzales, 2000). The decitabine-induced desensitization of ethanol-induced potentiation may indicate a decreased salience of ethanol as a positive reinforcer, which may mediate the reductions in ethanol intake and preference (Berridge & Robinson, 1998). We hypothesize that these changes in DA neuron firing are mediated, at least in part, by decitabine-induced regulation of endothelial and glial functions, especially re-organization of ECM. ECM of the brain, a meshwork of secreted proteoglycans and glycoproteins of neuronal, glial, and endothelial origin, occupies intercellular space and surrounds and integrates neurons into the tissue (Frischknecht, Chang, Rasband, & Seidenbecher, 2014). It is a highly dynamic structure, which can be remodeled in an activity-dependent manner and can modulate neuronal activity and promote structural and functional plasticity. Recent evidence points to important roles of ECM in regulation of synaptic plasticity and behavioral responses to drugs of abuse, including ethanol (Lasek, 2016). For example, ethanol regulated the expression of a key ECM component, tissue plasminogen activator, in cultured astrocytes, which may result in re-organization of ECM and changes in

neuronal activity (Zhang, Kusumo, Sakharkar, Pandey, & Guizzetti, 2014). An increasing role of glial and endothelial cells in mechanisms of drug abuse has been highlighted in recent reviews (Crews et al., 2015; Eggleton & Abbruscato, 2014; Mayfield, Ferguson, & Harris, 2013).

In vitro studies of the effects of decitabine on neuronal populations provide somewhat conflicting results. On one hand, decitabine promoted neuronal survival in the models of hydrogen peroxide-induced death in cerebellar granule cells (Xin et al., 2015), mutant huntingtin-induced toxicity in cortical and striatal neurons (Pan et al., 2016), and degeneration of retinal photoreceptors (Farinelli et al., 2014). On the other hand, it decreased survival and induced apoptosis in DA-like cell cultures (Wang et al., 2013). The difference between the first three studies and the 2013 report appears to be that the former used primary neuronal postmitotic cultures, while the latter used immortalized cell lines still capable of dividing. These studies suggest that DNA methylation in non-dividing neurons promotes cell death and that decitabine has protective effects, likely, via DNA repair-assisted incorporation into DNA and downstream regulation of DNA methylation and gene expression. This research opens an intriguing possibility of using hypomethylating agents in treatment of neurodegenerative disorders. Loss of neurons is a hallmark of alcohol dependence (Crews et al., 2015), and this possibility can be extended to AUD to prevent neurodegeneration. These studies also highlight the importance of investigating other brain regions implicated in the regulation of alcohol consumption to extend the current findings that are limited to VTA.

Epigenetic mechanisms appear to play an important role in alcohol abuse and dependence in humans (Manzardo, Henkhaus, & Butler, 2012; Ponomarev, Wang, Zhang, Harris, & Mayfield, 2012; Taqi et al., 2011; Zhou, Yuan, Mash, & Goldman, 2011). Epigenetic compounds are attractive targets for drug development and drug repurposing because they are capable of correcting or neutralizing gene expression changes associated with disease. DNA hypomethylating agents change gene expression and downstream functions by inhibiting key methylation enzymes in the cell. Our study provides novel evidence for the central effects of decitabine, an FDA-approved hypomethylating drug, and the results point to the importance of both neuronal and non-neuronal mechanisms in the regulation of decitabine-induced reduction of ethanol intake and preference. Neurons have been traditionally targeted for drug development for brain disorders, while our data provide additional support to recent studies nominating glial and endothelial cells as targets for drug development for central conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work was supported by the National Institutes of Health (grant numbers: AA021462, AA020683, AA013520). The authors thank Courtney Bridges, Mendy Black, Jill Benavidez, and Adriana Da Costa for technical assistance.

References

- Abel T, Zukin RS. Epigenetic targets of HDAC inhibition in neurodegenerative and psychiatric disorders. *Current Opinion in Pharmacology*. 2008; 8(1):57–64. <http://doi.org/10.1016/j.coph.2007.12.002>. [PubMed: 18206423]
- Barbier E, Tapocik JD, Juergens N, Pitcairn C, Borich A, Schank JR, et al. DNA Methylation in the Medial Prefrontal Cortex Regulates Alcohol-Induced Behavior and Plasticity. *Journal of Neuroscience*. 2015; 35(15):6153–6164. <http://doi.org/10.1523/JNEUROSCI.4571-14.2015>. [PubMed: 25878287]
- Berridge KC, Robinson TE. What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain Research Brain Research Reviews*. 1998; 28(3):309–369. [PubMed: 9858756]
- Brodie MS, Appel SB. The effects of ethanol on dopaminergic neurons of the ventral tegmental area studied with intracellular recording in brain slices. *Alcoholism: Clinical and Experimental Research*. 1998; 22(1):236–244.
- Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics*. 2013; 14(1):128. <http://doi.org/10.1186/1471-2105-14-128>. [PubMed: 23586463]
- Cooper-Kuhn CM, Kuhn HG. Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. *Brain Research. Developmental Brain Research*. 2002; 134(1–2): 13–21. [PubMed: 11947933]
- Copeland RA, Olhava EJ, Scott MP. Targeting epigenetic enzymes for drug discovery. *Curr Opin Chem Biol*. 2010; 14(4):505–510. <http://doi.org/10.1016/j.cbpa.2010.06.174>. [PubMed: 20621549]
- Coppède F. The potential of epigenetic therapies in neurodegenerative diseases. *Frontiers in Genetics*. 2014; 5(243):220. <http://doi.org/10.3389/fgene.2014.00220>. [PubMed: 25071843]
- Crews FT, Sarkar DK, Qin L, Zou J, Boyadjieva N, Vetreno RP. Neuroimmune Function and the Consequences of Alcohol Exposure. *Alcohol Research: Current Reviews*. 2015; 37(2):331–341. 344–351. [PubMed: 26695754]
- Egleton RD, Abbruscato T. Drug abuse and the neurovascular unit. *Advances in Pharmacology (San Diego, Calif)*. 2014; 71:451–480. <http://doi.org/10.1016/bs.apha.2014.06.019>.
- Ewald B, Sampath D, Plunkett W. Nucleoside analogs: molecular mechanisms signaling cell death. *Oncogene*. 2008; 27(50):6522–6537. <http://doi.org/10.1038/onc.2008.316>. [PubMed: 18955977]
- Farinelli P, Perera A, Arango-Gonzalez B, Trifunovic D, Wagner M, Carell T, et al. DNA methylation and differential gene regulation in photoreceptor cell death. *Cell Death & Disease*. 2014; 5(12):e1558. <http://doi.org/10.1038/cddis.2014.512>. [PubMed: 25476906]
- Franklin, K., Paxinos, G. The mouse brain in stereotaxic coordinates. Elsevier Academic Press; New York: 2008.
- Frischknecht R, Chang KJ, Rasband MN, Seidenbecher CI. Neural ECM molecules in axonal and synaptic homeostatic plasticity. *Progress in Brain Research*. 2014; 214:81–100. <http://doi.org/10.1016/B978-0-444-63486-3.00004-9>. [PubMed: 25410354]
- Gessa GL, Muntoni F, Collu M, Vargiu L, Mereu G. Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area. *Brain Research*. 1985; 348(1):201–203. [PubMed: 2998561]
- Gonzales RA, Job MO, Doyon WM. The role of mesolimbic dopamine in the development and maintenance of ethanol reinforcement. *Pharmacology & Therapeutics*. 2004; 103(2):121–146. <http://doi.org/10.1016/j.pharmthera.2004.06.002>. [PubMed: 15369680]
- Hollenbach PW, Nguyen AN, Brady H, Williams M, Ning Y, Richard N, et al. A comparison of azacitidine and decitabine activities in acute myeloid leukemia cell lines. *PLoS ONE*. 2010; 5(2):e9001. <http://doi.org/10.1371/journal.pone.0009001>. [PubMed: 20126405]
- Jabbour E, Issa JP, Garcia-Manero G, Kantarjian H. Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies. *Cancer*. 2008; 112(11):2341–2351. <http://doi.org/10.1002/cncr.23463>. [PubMed: 18398832]
- Karahoca M, Momparler RL. Pharmacokinetic and pharmacodynamic analysis of 5-aza-2'-deoxycytidine (decitabine) in the design of its dose-schedule for cancer therapy. *Clinical Epigenetics*. 2013; 5(1):3. <http://doi.org/10.1186/1868-7083-5-3>. [PubMed: 23369223]

- Lasek AW. Effects of Ethanol on Brain Extracellular Matrix: Implications for Alcohol Use Disorder. *Alcoholism: Clinical and Experimental Research*. 2016; 40(10):2030–2042. <http://doi.org/10.1111/acer.13200>.
- Liang J, Yin G, Dong Y, Ma Z, Xiao Y, Ji T. 5-aza-2'-deoxycytidine suppresses the growth of human lung adenocarcinoma cells in nude mouse xenograft models and its effect on methylation status and expression of TFPI-2 gene. *Zhonghua Zhong Liu Za Zhi [Chinese Journal of Oncology]*. 2015; 37(7):485–489.
- Manzardo AM, Henkhaus RS, Butler MG. Global DNA promoter methylation in frontal cortex of alcoholics and controls. *Gene*. 2012; 498(1):5–12. <http://doi.org/10.1016/j.gene.2012.01.096>. [PubMed: 22353363]
- Marballi K, Genabai NK, Blednov YA, Harris RA, Ponomarev I. Alcohol consumption induces global gene expression changes in VTA dopaminergic neurons. *Genes, Brain, and Behavior*. 2016; 15(3): 318–326. <http://doi.org/10.1111/gbb.12266>.
- Mayfield J, Ferguson L, Harris RA. Neuroimmune signaling: a key component of alcohol abuse. *Current Opinion in Neurobiology*. 2013; 23(4):513–520. <http://doi.org/10.1016/j.conb.2013.01.024>. [PubMed: 23434064]
- Osterndorff-Kahanek E, Ponomarev I, Blednov YA, Harris RA. Gene expression in brain and liver produced by three different regimens of alcohol consumption in mice: comparison with immune activation. *PLoS ONE*. 2013; 8(3):e59870. <http://doi.org/10.1371/journal.pone.0059870>. [PubMed: 23555817]
- Pan Y, Daito T, Sasaki Y, Chung YH, Xing X, Pondugula S, et al. Inhibition of DNA Methyltransferases Blocks Mutant Huntingtin-Induced Neurotoxicity. *Nature Publishing Group*. 2016; 6:31022. <http://doi.org/10.1038/srep31022>.
- Pandey SC, Ugale R, Zhang H, Tang L, Prakash A. Brain chromatin remodeling: a novel mechanism of alcoholism. *J Neurosci*. 2008; 28(14):3729–3737. <http://doi.org/10.1523/JNEUROSCI.5731-07.2008>. [PubMed: 18385331]
- Ponomarev I. Epigenetic control of gene expression in the alcoholic brain. *Alcohol Research: Current Reviews*. 2013; 35(1):69–76. <http://doi.org/10.1164/rccm.201010-1579PP>. [PubMed: 24313166]
- Ponomarev I, Wang S, Zhang L, Harris RA, Mayfield RD. Gene Coexpression Networks in Human Brain Identify Epigenetic Modifications in Alcohol Dependence. *Journal of Neuroscience*. 2012; 32(5):1884–1897. <http://doi.org/10.1523/JNEUROSCI.3136-11.2012>. [PubMed: 22302827]
- Qiang M, Li JG, Denny AD, Yao JM, Lieu M, Zhang K, et al. Epigenetic mechanisms are involved in the regulation of ethanol consumption in mice. *International Journal of Neuropsychopharmacology*. 2015; 18(2):1–11. <http://doi.org/10.1093/ijnp/pyu072>.
- Rhodes JS, Best K, Belknap JK, Finn DA, Crabbe JC. Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiology & Behavior*. 2005; 84(1):53–63. <http://doi.org/10.1016/j.physbeh.2004.10.007>. [PubMed: 15642607]
- Sanchis-Segura C, Lopez-Atalaya JP, Barco A. Selective boosting of transcriptional and behavioral responses to drugs of abuse by histone deacetylase inhibition. *Neuropsychopharmacology*. 2009; 34(13):2642–2654. <http://doi.org/10.1038/npp.2009.125>. [PubMed: 19727068]
- Spanagel R. Alcoholism: a systems approach from molecular physiology to addictive behavior. *Physiological Reviews*. 2009; 89(2):649–705. <http://doi.org/10.1152/physrev.00013.2008>. [PubMed: 19342616]
- Taqi MM, Bazov I, Watanabe H, Sheedy D, Harper C, Alkass K, et al. Prodynorphin CpG-SNPs associated with alcohol dependence: elevated methylation in the brain of human alcoholics. *Addiction Biology*. 2011; 16(3):499–509. <http://doi.org/10.1111/j.1369-1600.2011.00323.x>. [PubMed: 21521424]
- Tulisiak CT, Harris RA, Ponomarev I. DNA modifications in models of alcohol use disorders. *Alcohol*. 2016; 0(0) <http://doi.org/10.1016/j.alcohol.2016.11.004>.
- Walker DM, Cates HM, Heller EA, Nestler EJ. Regulation of chromatin states by drugs of abuse. *Current Opinion in Neurobiology*. 2015; 30:112–121. <http://doi.org/10.1016/j.conb.2014.11.002>. [PubMed: 25486626]
- Wang Y, Wang X, Li R, Yang ZF, Wang YZ, Gong XL, et al. A DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine, exacerbates neurotoxicity and upregulates Parkinson's disease-related

- genes in dopaminergic neurons. *CNS Neuroscience & Therapeutics*. 2013; 19(3):183–190. <http://doi.org/10.1111/cns.12059>. [PubMed: 23441691]
- Warnault V, Darceq E, Levine A, Barak S, Ron D. Chromatin remodeling—a novel strategy to control excessive alcohol drinking. *Translational Psychiatry*. 2013; 3(2):e231. <http://doi.org/10.1038/tp.2013.4>. [PubMed: 23423140]
- Wilson VL, Jones PA, Momparler RL. Inhibition of DNA methylation in L1210 leukemic cells by 5-aza-2'-deoxycytidine as a possible mechanism of chemotherapeutic action. *Cancer Research*. 1983; 43(8):3493–3496. [PubMed: 6190553]
- Wolstenholme JT, Warner JA, Capparuccini MI, Archer KJ, Shelton KL, Miles MF. Genomic analysis of individual differences in ethanol drinking: evidence for non-genetic factors in C57BL/6 mice. *PLoS ONE*. 2011; 6(6):e21100. <http://doi.org/10.1371/journal.pone.0021100>. [PubMed: 21698166]
- Xin YJ, Yuan B, Yu B, Wang YQ, Wu JJ, Zhou WH, et al. Tet1-mediated DNA demethylation regulates neuronal cell death induced by oxidative stress. *Nature Publishing Group*. 2015; 5:7645. <http://doi.org/10.1038/srep07645>.
- Yim HJ, Gonzales RA. Ethanol-induced increases in dopamine extracellular concentration in rat nucleus accumbens are accounted for by increased release and not uptake inhibition. *Alcohol*. 2000; 22(2):107–115. [PubMed: 11113625]
- Zhang X, Kusumo H, Sakharkar AJ, Pandey SC, Guizzetti M. Regulation of DNA methylation by ethanol induces tissue plasminogen activator expression in astrocytes. *Journal of Neurochemistry*. 2014; 128(3):344–349. <http://doi.org/10.1111/jnc.12465>. [PubMed: 24117907]
- Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keefe S, et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci*. 2014; 34(36):11929–11947. <http://doi.org/10.1523/JNEUROSCI.1860-14.2014>. [PubMed: 25186741]
- Zhou Z, Yuan Q, Mash DC, Goldman D. Substance-specific and shared transcription and epigenetic changes in the human hippocampus chronically exposed to cocaine and alcohol. *Proc Natl Acad Sci U S A*. 2011; 108(16):6626–6631. <http://doi.org/10.1073/pnas.1018514108>. [PubMed: 21464311]

Highlights

- The DNA methyltransferase inhibitor decitabine reduces ethanol intake and preference.
- Transcriptional changes in the VTA are associated with decitabine actions.
- Decitabine changes sensitivity of VTA dopaminergic neurons to ethanol.
- Ethanol intake may be regulated by both neuronal and non-neuronal mechanisms in the VTA.

Fig. 1. Effects of SAHA (A) and decitabine (B) on ethanol intake in a limited-access DID procedure. Asterisks indicate a main effect of Treatment (***) ($p < 0.001$).

Fig. 2. Effects of SAHA and decitabine on ethanol intake (A) and preference (B) in a chronic intermittent EOD procedure. Drugs were administered 1 h before ethanol consumption on days 4, 6, 10, and 12, while vehicle was administered before day 8. Asterisks indicate statistically significant differences between the Decitabine and Vehicle groups (Bonferroni *post hoc* test, * = $p < 0.05$; ** = $p < 0.01$).

Fig. 3.

Effects of *in vivo* pretreatment with decitabine on firing rates of DA neurons *in vitro*. There was no group difference in basal firing rate (A). The Decitabine group showed a faster desensitization of ethanol-induced excitation: all data summarized at 20-sec intervals (B), and critical time points representing the peak of ethanol effect in the Decitabine group (1 min), the peak of ethanol effect in the Vehicle group (5 min), and returning to baseline for both groups (9 min) (C). Asterisks indicate statistically significant differences between the Decitabine and Vehicle groups (Bonferroni *post hoc* test, ** = $p < 0.01$).

Table 1

Functional group over-representation analysis of genes up-regulated in VTA of the decitabine-treated group compared to vehicle control. These changes represent molecular correlates of decitabine-induced reduction in ethanol intake and preference. All *p* values are significant at FDR = 5%. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Biological Domain	Over-represented Functional Group	<i>p</i> value	# of Genes
Cerebral Blood Circulation	angiogenesis (GO:0001525)	1.1E-19	38
	vasculogenesis (GO:0001570)	2.2E-08	13
	blood coagulation (GO:0007596)	2.8E-06	29
	blood circulation (GO:0008015)	5.2E-04	6
	response to hypoxia (GO:0001666)	6.6E-04	15
Extracellular Matrix	extracellular matrix organization (GO:0030198)	3.0E-12	35
	regulation of cell adhesion (GO:0030155)	2.0E-10	31
	extracellular matrix disassembly (GO:0022617)	1.2E-06	14
	Focal adhesion (KEGG:hsa04510)	2.8E-06	18
	positive regulation of cell motility (GO:2000147)	1.8E-06	22
Neuroimmune Functions	leukocyte migration (GO:0050900)	6.8E-07	20
	negative regulation of cytokine production (GO:0001818)	4.4E-05	15
	Leukocyte transendothelial migration (KEGG:hsa04670)	1.8E-04	11
	Cytokine-cytokine receptor interaction (KEGG:hsa04060)	2.0E-03	15
	regulation of inflammatory response (GO:0050727)	2.3E-03	14
Molecular Pathways	PI3K-Akt signaling pathway (KEGG:hsa04151)	8.8E-07	25
	Rap1 signaling pathway (KEGG:hsa04015)	1.9E-05	17
	Notch signaling pathway (KEGG:hsa04330)	1.4E-03	6