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## The Histone Deacetylase Inhibitor Suberoylanilide Hydroxamic Acid (SAHA) Alleviates Depression-Like Behavior and Normalizes Epigenetic Changes in the Hippocampus During Ethanol Withdrawal

Wei-Yang Chen<sup>a</sup>, Huaibo Zhang<sup>a,b</sup>, Eleonora Gatta<sup>a</sup>, Elizabeth J. Glover<sup>a</sup>, Subhash C. Pandey<sup>a,b</sup>, and Amy W. Lasek<sup>a,\*</sup>

<sup>a</sup>Center for Alcohol Research in Epigenetics, Department of Psychiatry, University of Illinois at Chicago, 1601 West Taylor Street, Chicago, Illinois 60612 USA

<sup>b</sup>Jesse Brown VA Medical Center, 820 South Damen Avenue, Chicago, Illinois 60612 USA

### Abstract

Withdrawal from chronic alcohol drinking can cause depression, leading to an inability to function in daily life and an increased risk for relapse to harmful drinking. Understanding the causes of alcohol withdrawal-related depression may lead to new therapeutic targets for treatment. Epigenetic factors have recently emerged as important contributors to both depression and alcohol use disorder (AUD). Specifically, acetylation of the N-terminal tails of histone proteins that package DNA into nucleosomes is altered in stress-induced models of depression and during alcohol withdrawal. The goal of this study was to examine depression-like behavior during alcohol withdrawal and associated changes in histone acetylation and expression of histone deacetylase 2 (HDAC2) in the hippocampus, a brain region critical for mood regulation and depression. Male Sprague-Dawley rats were treated with the Lieber-DeCarli ethanol liquid diet for 15 days and then underwent withdrawal. Rats were treated with the HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), during withdrawal and tested for depression-like behavior. In a separate group of rats, the hippocampus was analyzed for mRNA and protein expression of HDAC2 and levels of histone H3 lysine 9 acetylation (H3K9ac) during chronic ethanol exposure and withdrawal. Rats undergoing ethanol withdrawal exhibited depression-like behavior and had increased HDAC2 and decreased H3K9ac levels in specific structures of the hippocampus. Treatment with SAHA during withdrawal ameliorated depression-like behavior and normalized changes in hippocampal HDAC2 and H3K9ac levels. These results demonstrate that ethanol withdrawal causes an altered epigenetic state in the hippocampus. Treatment with an HDAC inhibitor can correct this state and alleviate

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\*Corresponding Author: Amy W. Lasek, Ph.D., Department of Psychiatry, University of Illinois at Chicago, 1601 West Taylor Street, MC 912, Chicago, IL 60612, Phone (312) 355-1593, FAX: (312) 996-7658, alasek@uic.edu.

#### Conflicts of Interest

No biomedical financial interests or potential conflicts of interest were reported by authors.

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depression-like symptoms developed during withdrawal. Targeting histone acetylation may be a novel strategy to reduce ethanol withdrawal-induced depression.

## Keywords

Alcohol withdrawal; depression; epigenetic; HDAC; Vorinostat; hippocampus

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## Introduction

Alcohol use disorder (AUD) is highly comorbid with depression (Boden & Fergusson, 2011). One reason for this comorbidity is that abstinence from chronic alcohol drinking can cause depression (Petit, Luminet, Cordovil de Sousa Uva, Monhonval, et al., 2017; Petit, Luminet, Cordovil de Sousa Uva, Zorbas, et al., 2017). The functional link between AUD and depression is supported by preclinical studies demonstrating increased depression-like behavior in rodents during alcohol withdrawal (Anton et al., 2017; Getachew, Hauser, Csoka, Taylor, & Tizabi, 2017; Kang, Li, Bekker, & Ye, 2018; Li et al., 2017; Pang, Renoir, Du, Lawrence, & Hannan, 2013; Roni & Rahman, 2017; Walker et al., 2010; Yawalkar, Changotra, & Gupta, 2018). Depression during abstinence from alcohol also contributes to relapse to alcohol drinking, thus perpetuating the cycle of addiction (Greenfield et al., 1998; Oliva et al., 2018). Depression and alcohol abuse negatively impact overall health, productivity, and quality of life. A better understanding of the brain mechanisms contributing to alcohol withdrawal-induced depression would be extremely helpful in the development of novel therapeutic approaches effective in reducing comorbid alcohol abuse and depression.

The hippocampus is an important region of the brain for modulating emotional states such as depression (Akil et al., 2018; Fanselow & Dong, 2010). Chronic alcohol consumption is associated with decreased hippocampal volume, as measured using magnetic resonance imaging in alcohol-dependent subjects compared with controls (Beresford et al., 2006). Notably, this is also observed in patients with major depressive disorder (Malykhin, Carter, Seres, & Coupland, 2010), suggesting a link between alcohol-induced changes in the hippocampus and depression. Numerous molecules, signal transduction pathways, and basic cellular processes in the hippocampus are causally linked with depression. These include alterations in the serotonergic system, brain-derived neurotrophic factor (BDNF) expression, cyclic AMP response element binding protein (CREB) activity, neurogenesis, and neuroinflammation (Kubera, Obuchowicz, Goehler, Brzeszcz, & Maes, 2011; Rogers, Renoir, & Hannan, 2017).

Epigenetic mechanisms, such as acetylation of the N-terminal tails of histone proteins that package DNA into nucleosomes, also play an important role in depression (Misztak, Panczyszyn-Trzewik, & Sowa-Kucma, 2018). Levels of histone acetylation and the histone deacetylases (HDACs) that remove acetyl groups from the histone tails and thus decrease chromatin accessibility, are altered in the hippocampus of mice and rats in various models of stress-induced depression (Covington, Vialou, LaPlant, Ohnishi, & Nestler, 2011; Ferland & Schrader, 2011; Han, Sung, Chung, & Kwon, 2014; Kv et al., 2018; Liu et al., 2014;

Tsankova et al., 2006). Of note, systemic or intra-hippocampal treatment of animals with HDAC inhibitors can reduce depression-like behavior (Covington et al., 2011; Gundersen & Blendy, 2009; Han et al., 2014; Liu et al., 2014; Yamawaki et al., 2012) and injection of MS-275 (Entinostat) directly into the hippocampus of mice subjected to chronic social defeat stress reversed the deficit in sucrose preference and increased social interaction and acetylated histone H3 levels in the hippocampus (Covington et al., 2011).

Similar to stress-induced models of depression, withdrawal from chronic alcohol exposure also has profound effects on histone acetylation and HDAC expression (Pandey, Ugale, Zhang, Tang, & Prakash, 2008; Simon-O'Brien et al., 2015; You, Zhang, Sakharkar, Teppen, & Pandey, 2014). Decreased histone H3 and H4 acetylation and elevated HDAC activity were observed in the amygdala of rats during withdrawal from chronic alcohol drinking, along with an associated increase in anxiety-like behavior (Pandey et al., 2008; You et al., 2014). Treatment of rats during ethanol withdrawal with the histone deacetylase inhibitor trichostatin A (TSA) attenuated anxiety-like behavior and restored normal levels of histone acetylation in the amygdala (Pandey et al., 2008; You et al., 2014). Additionally, in a rat model of dependence induced by ethanol vapor exposure, treatment with MS-275 decreased drinking only in ethanol-dependent rats and altered histone acetylation in various brain regions (Simon-O'Brien et al., 2015). In mice subjected to repeated ethanol injections and withdrawal, histone H3 lysine 9 acetylation (H3K9ac) was decreased and HDAC2 expression increased in the ventral tegmental area (Arora et al., 2013). Interestingly, adolescent alcohol exposure leads to an increase in HDAC activity and deficits in H3K9ac in rat hippocampus that are associated with decreased neurogenesis in adulthood. These changes were normalized by TSA treatment (Sakharkar et al., 2016). Together, these results indicate that alcohol withdrawal leads to dysregulated histone acetylation due to an increase in HDAC2 expression in several brain regions and that treatment with HDAC inhibitors can normalize this imbalance and alleviate negative affective states induced by alcohol withdrawal. However, histone acetylation and HDAC2 expression in the hippocampus and associated depression-like behavior during withdrawal after chronic ethanol exposure have not been examined.

In this study, we hypothesized that withdrawal from chronic alcohol exposure would result in depression-like behavior and lead to a corresponding decrease in histone acetylation associated with an increase in HDAC2 expression in the hippocampus. Therefore, we investigated depression-like behavior and levels of HDAC2 and H3K9ac in the hippocampus during alcohol withdrawal. We also tested if treatment with the HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA, also known as Vorinostat), would be effective in attenuating depression-like behavior induced by alcohol withdrawal and normalize H3K9ac levels and HDAC2 expression. Our results provide preclinical evidence that using an HDAC inhibitor in individuals with comorbid AUD and depression, particularly alcohol-induced depression, might be an effective novel therapeutic strategy to treat these disorders.

## Materials and methods

### Animals

Adult male Sprague-Dawley rats (postnatal day 75–85) weighing ~250 g were purchased from Harlan (Indianapolis, IN) and individually housed in a temperature-controlled room with a 12/12 hour light/dark cycle, with food and water provided ad libitum prior to beginning the chronic ethanol treatment protocol described below. All procedures were conducted according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

### Chronic ethanol treatment

Rats were fed with the nutritionally complete Lieber-DeCarli control liquid diet or ethanol liquid diet (Bio-Serv, Frenchtown, NJ) as their only source of food and fluid as previously described (Pandey et al., 2008). Rats were randomly assigned to three treatment groups: (i) control diet fed, (ii) ethanol diet fed (no withdrawal), and (iii) ethanol diet fed with a 24-hour withdrawal, referred to as the ethanol-withdrawn group. Briefly, rats were first fed 80 ml/day control diet for 3 days. The control group continued with control diet for 16 days, while the ethanol groups were gradually introduced to ethanol (1.8% to 8.1% within 7 days), and then maintained on 9% v/v ethanol diet for 15 days. Ethanol-withdrawn rats were switched to control liquid diet for 24 hours after removal of the ethanol liquid diet. One week before behavioral testing (described below), rats were handled for 3 minutes per day by the same individual to habituate them to handling. Rats were pair fed and their liquid diet intake and body weights were closely monitored. We have previously reported blood ethanol levels in the range of 172 to 198 mg % for ethanol diet fed (no withdrawal) rats, whereas the blood ethanol level after 24 hours of ethanol withdrawal was undetectable using this alcohol treatment paradigm (Pandey et al., 2008; You et al., 2014).

### Drug treatment

Rats in the control and ethanol-withdrawn groups were treated intraperitoneally (IP) with either 50 mg/kg SAHA (Selleck Chemicals, Houston, TX) in 2% DMSO, 40% PEG 300, 5% Propylene glycol, and 1% Tween 80 (vehicle) or vehicle, 2 hours before behavioral testing or euthanasia for tissue collection at 24 hours of withdrawal. All rats were euthanized by decapitation under isoflurane anesthesia.

### Sucrose splash test

Depression-like behavior was measured using the splash test as described by Marrocco et al. (Marrocco et al., 2014). A 10% sucrose solution was sprayed on the dorsal coat and abdomen of each rat in a clear cage to induce grooming behavior. The time the rat spent grooming and latency to groom was recorded for a period of 5 minutes after application of the sucrose solution. This test takes advantage of the natural propensity of the rats to groom, with a reduction in grooming and increased latency to groom indicating anhedonia and a lack of motivation for self-care. Decreased grooming time in the splash test is observed in rats after manipulations that induce multiple indices of depression-like behavior such as

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prenatal restraint stress, maternal separation, and chronic unpredictable mild stress (Hu et al., 2017; Marrocco et al., 2014; Masrour, Peeri, Azarbayjani, & Hosseini, 2018). Moreover, treatment with antidepressant medications such as fluoxetine normalizes grooming time in the splash test in mice and rats (Marrocco et al., 2014; Masrour et al., 2018; Santarelli et al., 2003; Surget et al., 2008; Yalcin, Belzung, & Surget, 2008).

### Sucrose preference test

Bottles containing the control liquid diet were removed from the home cage at 24 hours of withdrawal (of both ethanol-withdrawn and control rats) and replaced with two standard water bottles containing either a 0.5% sucrose solution or water. Bottles were weighed after 1 hour and sucrose preference was calculated as the volume of sucrose solution consumed divided by the total volume of fluid consumed x 100. Decreased sucrose preference is indicative of anhedonia, a defining symptom of major depressive disorder, and is normalized by chronic treatment with antidepressants (Dichter, Damiano, & Allen, 2012; Papp, Moryl, & Willner, 1996; Willner, Towell, Sampson, Sophokleous, & Muscat, 1987).

### In situ reverse transcription-polymerase chain reaction (RT-PCR)

*In situ* RT-PCR of *Hdac2* mRNA was performed on 40  $\mu\text{m}$  thick coronal brain sections, as described previously (Pandey et al., 2008; Zhang et al., 2010). Briefly, brain sections were treated with proteinase K and then digested with DNase. Sections were rinsed in PBS and transferred to PCR tubes containing 100  $\mu\text{l}$  of PCR reaction mixture (Applied Biosystems, Foster City, CA) to reverse transcribe for 1 h at 42°C with oligo d(T)<sub>16</sub> and reverse transcriptase (RT) enzyme. PCR was then performed as follows: 95°C for 5 minutes; 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, for a total of 25 cycles; 72°C for 7 minutes using *Taq* DNA polymerase enzyme, 100 pmol each of *Hdac2* forward and reverse primers (*Hdac2* forward: 5'-CGGTGGCTCAGTTGCTGGGG; *Hdac2* reverse: 5'-GGCCTCTGACTTCTTGCCGTGG) and 1 mM of each NTP (except that dTTP was replaced by digoxigenin (DIG)-11-dUTP) in 100  $\mu\text{l}$  reaction mixture. Sections were then mounted on slides and *Hdac2* mRNA-positive cells were detected using an alkaline phosphatase conjugated anti-DIG antibody with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolylphosphate p-toluidine (NBT-BCIP) as the substrate, yielding a purple reaction product (Roche Molecular Biochemical, Mannheim, Germany). The intensity of *Hdac2*-positive cell bodies in the CA1, CA3 and dentate gyrus (DG) region of the dorsal hippocampus (approximately -2.3 to -3.3 mm posterior to bregma) was obtained by outlining the cell body layer and measuring the intensity using NIH ImageJ software from each section of 2–3 brain sections from each rat. Values were averaged and presented as mean intensity/100  $\mu\text{m}^2$ .

### Gold immunolabeling of HDAC2 and H3K9ac

HDAC2 and acetylated H3K9ac proteins were measured in the dorsal hippocampus of the rat brain using gold immunolabeling (Pandey et al., 2008; Sakharkar et al., 2014). Free floating 20  $\mu\text{m}$  thick coronal sections were incubated with antibodies against HDAC2 (catalog number JM-3602-100, MBL International, Woburn, MA) and H3K9ac (catalog number 06-942, Millipore, Billerica, MA). After washing with PBS, the sections were incubated in gold particle-labeled goat anti-rabbit secondary antibodies (Nanoprobe,

Yaphank, NY) and were counter-stained using the silver enhancement kit (Ted Pella, Redding, CA). Gold immunolabeling was quantified using an image analysis system (Loats Associates, Westminster, MD) at 100× magnification. We first set up a threshold for counting by outlining an area without staining, and then several object fields in each section were used for counting immunogold particles. Mean counts for each group were calculated and results are presented as the mean ( $\pm$  SEM) of the number of immunogold particles/100  $\mu\text{m}^2$  area in the Cornu Ammonis (CA)1, CA3, and dentate gyrus (DG) regions of the hippocampus.

### Statistical analysis

All data are presented as the mean  $\pm$  SEM. Statistical testing was performed on the *in situ* RT-PCR and immunogold labeling data with a one-way analysis of variance (ANOVA) within each region of the hippocampus, followed by Tukey's multiple comparisons test. Behavioral data was analyzed using a two-way ANOVA followed by post hoc comparisons using Sidak's multiple comparisons tests. Statistical tests were performed using Prism software (GraphPad, San Diego, CA, version 7). A value of  $p < 0.05$  was considered to be significant.

## Results

### Withdrawal from chronic ethanol drinking causes depression-like behavior that is reversed by SAHA treatment

Withdrawal from chronic ethanol exposure is associated with a depression-like state that has been observed behaviorally in mice and rats (Anton et al., 2017; Getachew et al., 2017; Kang et al., 2018; Li et al., 2017; Pang et al., 2013; Roni & Rahman, 2017; Walker et al., 2010; Yawalkar et al., 2018). We examined depression-like behavior using the splash test in rats that were fed with a control or chronic ethanol Lieber DeCarli diet and underwent 24 hours of withdrawal. We also tested for the effect of the HDAC inhibitor SAHA, given acutely 2 hours prior to testing, since numerous studies have demonstrated that HDAC inhibitors, including SAHA, can reduce depression-like behavior in rats and mice (reviewed in (Misztak et al., 2018)). Rats withdrawn from chronic ethanol drinking groomed less and had increased latency to groom in the splash test, indicative of depression-like behavior, while treatment with SAHA normalized grooming time (Fig. 1A-B). A two-way ANOVA indicated a significant withdrawal by SAHA interaction in grooming time [ $F(1, 47) = 8.18$ ,  $p = 0.006$ ] and latency to groom [ $F(1, 47) = 5.86$ ,  $p = 0.019$ ], but no main effects of ethanol withdrawal [grooming time:  $F(1, 47) = 0.21$ ,  $p = 0.65$ ; latency:  $F(1, 47) = 0.51$ ,  $p = 0.48$ ] or SAHA treatment [grooming time:  $F(1, 47) = 0.00028$ ,  $P = 0.99$ ; latency:  $F(1, 47) = 2.0$ ,  $p = 0.16$ ]. Post-hoc multiple comparisons tests demonstrated that there was a nearly significant decrease in grooming time and increased latency to groom between control and ethanol-withdrawn rats within the vehicle-treated group (grooming time:  $p = 0.054$ ; latency:  $p = 0.072$ ), but no difference between control and ethanol-withdrawn rats within the SAHA-treated group (grooming time:  $p = 0.16$ ; latency:  $p = 0.39$ ). Latency to groom was highly variable, and SAHA-treated rats drinking control liquid diet exhibited an increased latency to groom compared with vehicle-treated controls, indicating that SAHA delays the initiation

of behavior in the control rats. Nonetheless, SAHA-treated rats drinking control diet did not significantly differ in total grooming time when compared to vehicle-treated controls.

We next confirmed depression-like behavior during withdrawal from chronic ethanol drinking using the sucrose preference test. Ethanol-withdrawn rats had decreased sucrose preference compared with control rats and treatment with SAHA increased preference for sucrose to control levels (Fig. 1C). A two-way ANOVA indicated a significant ethanol withdrawal by SAHA interaction in sucrose preference [ $F(1, 57) = 11.18, p = 0.0015$ ], but no main effects of ethanol withdrawal or SAHA treatment [withdrawal:  $F(1, 57) = 0.73, p = 0.398$ ; SAHA:  $F(1, 57) = 1.16, p = 0.287$ ]. Post-hoc multiple comparisons tests showed a significant difference between vehicle-treated control vs. ethanol-withdrawn rats ( $p = 0.028$ ) and vehicle-treated, ethanol-withdrawn rats vs. SAHA-treated, ethanol-withdrawn rats ( $p = 0.018$ ). These results indicate that depression-like behavior is observed in rats during withdrawal from chronic ethanol drinking and that acute treatment with SAHA during withdrawal attenuates the depression phenotype.

### **Increased Hdac2 mRNA expression in the hippocampus during chronic ethanol drinking and withdrawal**

We next examined the expression of *Hdac2* transcript in the hippocampus during withdrawal. *Hdac2* mRNA expression was higher in the CA1, CA3 and DG regions of the hippocampus during ethanol drinking and withdrawal by *in situ* RT-PCR (Fig. 2). There were significant treatment effects by one-way ANOVA in all three regions [CA1,  $F(2, 17) = 6.38, p = 0.0086$ ; CA3,  $F(2, 14) = 11.41, p = 0.0012$ ; DG,  $F(2, 12) = 16.14, p = 0.0004$ ]. Post-hoc multiple comparisons tests showed that *Hdac2* expression increased in the CA1 ( $p = 0.0072$ ), CA3 ( $p = 0.0012$ ), and DG ( $p = 0.0005$ ) during withdrawal when compared with the control group. Moreover, *Hdac2* expression increased in the CA3 ( $p = 0.012$ ) and DG ( $p = 0.0052$ ) regions of the ethanol-drinking group (no withdrawal) compared with the control group. These results indicate that chronic ethanol drinking and withdrawal result in increased expression of *Hdac2* mRNA in the hippocampus.

### **Increased HDAC2 protein in the hippocampus during withdrawal from chronic ethanol drinking is normalized by SAHA treatment**

To determine if the increase in *Hdac2* mRNA in the hippocampus during ethanol withdrawal results in an increase in HDAC2 protein, we next measured protein levels of HDAC2 in the hippocampus using the gold immunolabeling procedure. HDAC2 protein levels were significantly increased in the CA1, CA3, and DG regions of the hippocampus during withdrawal (Fig. 3). Notably, HDAC2 protein returned to control levels when rats were treated with SAHA during withdrawal (Fig. 3). One-way ANOVAs revealed significant effects of treatment in all three regions of the hippocampus [CA1,  $F(4, 20) = 19.29, p < 0.0001$ ; CA3,  $F(4, 20) = 16.65, p < 0.0001$ ; DG:  $F(4, 20) = 38.10, p < 0.0001$ ], with post-hoc multiple comparisons tests showing a significant increase in HDAC2 expression in rats treated with vehicle during withdrawal compared with vehicle-treated control diet rats ( $p < 0.0001$  for CA1, CA3, and DG), and a significant decrease in HDAC2 expression in rats treated with SAHA during withdrawal when compared with the withdrawal-vehicle group ( $p < 0.0001$  for CA1, CA3, and DG). There were no significant differences in HDAC2

expression between rats that had the control diet (both vehicle- and SAHA-treated) and rats treated with SAHA during withdrawal, indicating that HDAC2 protein levels returned to normal after SAHA treatment in the ethanol-withdrawn rats. These results indicate that, in addition to increased *Hdac2* transcript in the hippocampus during ethanol withdrawal, there is an associated increase in HDAC2 protein, which is normalized by SAHA treatment.

### Decreased H3K9ac in the hippocampus during withdrawal from chronic ethanol drinking is normalized by SAHA treatment

Increased HDAC2 expression in the hippocampus during withdrawal from chronic ethanol drinking is predicted to cause a corresponding decrease in histone acetylation, while inhibition of HDACs with SAHA would increase histone acetylation. We measured global H3K9ac in the hippocampus during ethanol withdrawal using gold immunolabeling. There was a significant reduction in H3K9ac immunogold particles in the CA3 region during ethanol withdrawal that returned to control levels after SAHA treatment (Fig. 4). A one-way ANOVA showed significant treatment effects [ $F(4, 20) = 18.47, p < 0.0001$ ], with post-hoc comparisons demonstrating a significant decrease in H3K9ac in withdrawal-vehicle rats compared with all other groups ( $p < 0.0001$ ). H3K9ac was not altered in the DG or CA1 regions during withdrawal [one-way ANOVA, DG:  $F(4, 20) = 0.23, p = 0.92$ ; CA1:  $F(4, 20) = 0.128, p = 0.97$ ]. Together, these results indicate that hippocampal CA3 H3K9ac levels are decreased during withdrawal from chronic ethanol exposure and that normal levels can be restored by treatment with the HDAC inhibitor SAHA.

## Discussion

Withdrawal from alcohol drinking causes negative mood states that contribute to relapse, including anxiety and depression. Several studies in rats and mice have shown that depression-like behavior is induced during ethanol withdrawal (Anton et al., 2017; Getachew et al., 2017; Kang et al., 2018; Li et al., 2017; Pang et al., 2013; Roni & Rahman, 2017; Walker et al., 2010; Yawalkar et al., 2018). In this study, we confirmed that rats exhibit depression-like behavior during withdrawal from an ethanol liquid diet using two behavioral measures and demonstrate that systemic treatment with the HDAC inhibitor SAHA during withdrawal alleviates depression-like behavior. In addition, we showed that ethanol withdrawal increases HDAC2 expression, with an associated decrease in H3K9ac in the hippocampus, alterations that are normalized by acute treatment with SAHA during ethanol withdrawal. This effect was apparent 2 hours following treatment with SAHA, which is consistent with previous studies demonstrating that acute treatment with the HDAC inhibitor TSA can reduce anxiety-like behavior, increase *Npy*, *Bdnf*, and *Arc* gene expression, and increase dendritic spine density in the central and medial nuclei of the amygdala in a similar time frame (Pandey et al., 2008; You et al., 2014). In addition, 2 hours after treatment with SAHA, H3K9ac and sensitivity to GABA in the VTA are increased (You et al., 2018). Collectively, these data indicate that SAHA can act fairly rapidly to alter both gene expression and behavior. Together, our results demonstrate that treatment with SAHA might be an effective strategy to reduce depression during alcohol withdrawal and identify an epigenetic change in the hippocampus as a potential molecular contributor to this condition.



Several behavioral tests have been used to measure depression-like behavior in rats and mice. These include the forced swim test (FST), sucrose preference test, learned helplessness test, and splash test (Akil et al., 2018). Studies of depression-like behavior during ethanol withdrawal in rats have employed the FST and found increased immobility time in animals during withdrawal (Anton et al., 2017; Getachew et al., 2017; Kang et al., 2018; Li et al., 2017; Walker et al., 2010; Yawalkar et al., 2018). In this study, we used the splash test because it is a straightforward measure of depression that takes advantage of the natural propensity of the animal to groom, with less grooming time indicating decreased motivation for self-care. This characteristic is similar in humans diagnosed with depression. In addition, the splash test does not require any prior habituation session, which is needed when performing the FST with rats (Slattery & Cryan, 2012), thus allowing the investigation of depression-like behavior after 24 hours of withdrawal without prior training during the intoxication period. Decreased grooming in the splash test is observed after chronic unpredictable mild stress, a well-validated model of depression (Hu et al., 2017; Isingrini et al., 2010; Surget et al., 2008; Yalcin et al., 2008). The splash test has also been pharmacologically validated using fluoxetine, with fluoxetine treatment increasing grooming time (Marrocco et al., 2014; Masrour et al., 2018; Santarelli et al., 2003; Surget et al., 2008; Yalcin et al., 2008). To our knowledge, this is the first time the splash test has been used to measure depression-like behavior during ethanol withdrawal. We propose that it is an easily employed measure of ethanol withdrawal-induced depression that does not cause undue stress to the animal. Importantly, our findings in the splash test were confirmed using another simple test, the sucrose preference test, which verified that ethanol withdrawal-induced depression-like behavior is ameliorated by SAHA treatment.

Our finding that treatment with SAHA during withdrawal alleviates depression-like behavior is consistent with the effect of SAHA in reducing depression in stress-induced models of depression. Systemic treatment of mice with SAHA during chronic mild unpredictable stress sessions reduced depression- and anxiety-like behavior as measured using the social interaction test, sucrose preference test, novelty-suppressed feeding, and FST (Uchida et al., 2011). Microinjection of SAHA into the nucleus accumbens reversed stress-induced social aversion and decreased immobility time in the FST (Covington et al., 2009). In a different model of depression involving repeated injections of corticosterone in mice, SAHA corrected several measures of anxiety and depression, including grooming time in the splash test, and normalized hypothalamic-pituitary-adrenal axis reactivity and inflammatory-related gene expression in the hippocampus (Kv et al., 2018). SAHA was also able to decrease depression-like behavior exhibited by mice with a knockout of the CREB-regulated transcription coactivator-1 (*Crtc1*) gene when mice were tested in a repeated open-space forced swim procedure, but not in the novelty-suppressed feeding test (Meylan, Halfon, Magistretti, & Cardinaux, 2016). Overall, SAHA appears to be an effective treatment for anxiety and depression in multiple animal models of these psychopathological conditions.

Previous work found that treatment with the HDAC inhibitor TSA alleviated ethanol withdrawal-induced anxiety, as measured using the light-dark box and elevated plus maze (Pandey et al., 2008; You et al., 2014). Our results with the splash and sucrose preference tests add another dimension to the negative affective state observed during ethanol withdrawal using the same treatment paradigm and suggest that these rats undergoing

withdrawal not only demonstrate anxiety-like behaviors but also show depression-like behaviors as indicated in the current study. The effect of SAHA on depression-like behavior during withdrawal is consistent with the effect of TSA on anxiety-like behavior. Both SAHA and TSA are pan- HDAC inhibitors, so the specific HDAC target of these compounds in decreasing anxiety and depression is not known. We demonstrate here that HDAC2 transcript and protein expression are higher in the hippocampus during ethanol withdrawal. Previous studies have found that the innately anxious and high alcohol-preferring P rats have increased HDAC2 in the amygdala, and infusion of an HDAC2 siRNA into the amygdala or treatment with TSA decreased anxiety-like and alcohol drinking behaviors (Moonat, Sakharkar, Zhang, Tang, & Pandey, 2013; Sakharkar et al., 2014). HDAC2 expression in the amygdala is also observed after binge alcohol exposure in rats (Lopez-Moreno et al., 2015) and in adulthood after adolescent alcohol exposure (Pandey, Sakharkar, Tang, & Zhang, 2015). Interestingly, treatment with TSA was able to attenuate adolescent alcohol exposure-induced anxiety-like and alcohol drinking behaviors in adulthood (Pandey et al., 2015). These results implicate HDAC2 as a putative target of SAHA involved in depression-like as well as anxiety-like behavior during withdrawal. We measured mRNA expression by qPCR of *Hdac1–10* in the hippocampus during withdrawal from chronic ethanol drinking and found that *Hdac6* was the only other HDAC family member increased during withdrawal (unpublished results). Since SAHA is a pan-HDAC inhibitor, it is possible that the effect of SAHA on reducing depression-like behavior during withdrawal could be due to inhibition of either HDAC6 or HDAC2. Both of these HDACs have been implicated in depression (Jochems et al., 2014; Uchida et al., 2011). Future work will explicitly address whether increased HDAC2, specifically in the hippocampus, is responsible for depression induced by ethanol withdrawal.

Associated with the increase in HDAC2 was a corresponding decrease in H3K9ac in the CA3 region of the hippocampus during alcohol withdrawal, and given that H3K9ac is an HDAC2 substrate, our results are consistent with higher levels of HDAC2 directly acting to deacetylate H3K9ac during ethanol withdrawal. Treatment with SAHA increased total levels of H3K9ac in the CA3 region of the hippocampus. Although we observed an overall increase in HDAC2 in the CA1, CA3, and DG regions of the hippocampus during ethanol withdrawal, the associated change in H3K9ac was only observed in the CA3 region. It is possible that H3K9ac was reduced in the CA1 and DG, but occurred at the level of individual gene promoters, which would not be detectable when measuring total H3K9ac. One intriguing finding is that treatment of control rats with SAHA did not increase total H3K9ac in the hippocampus. It is possible that HDAC2 activity is very low under control conditions and that the increase in HDAC2 during withdrawal provides more active HDAC2 for inhibition by SAHA. Indeed, Guan *et al* did not observe a significant increase in acetylated lysines in the hippocampus of control mice treated with SAHA (Guan et al., 2009), and treatment with another pan-HDAC inhibitor, TSA, also does not alter H3K9ac in the amygdala of control rats (Pandey et al., 2008; Sakharkar, Zhang, Tang, Shi, & Pandey, 2012; You et al., 2014).

A putative target gene modified by H3K9ac and HDAC2 that might be involved in depression during alcohol withdrawal is *Bdnf*. Numerous evidence supports the hypothesis that low levels of BDNF in the hippocampus are associated with depression (Bjorkholm &

Monteggia, 2016) and that *Bdnf* expression is regulated by H3K9ac through the histone acetyltransferase CREB binding protein (CBP) (Hing, Sathyaputri, & Potash, 2018) and HDAC2 (Guan et al., 2009). Decreased BDNF in the hippocampus is observed during alcohol withdrawal in rats, which is normalized by antidepressant treatment (Hauser, Getachew, Taylor, & Tizabi, 2011). In addition, adolescent rats exposed to a binge ethanol exposure protocol had decreased BDNF in the hippocampus and exhibited depression-like behavior during withdrawal, which was ameliorated by intra-hippocampal infusion of an agonist to the BDNF receptor TrkB (Briones & Woods, 2013). However, other genes involved in depression might also be regulated by histone acetylation during ethanol withdrawal. Increased inflammation-related genes such as cytokines and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) signaling molecules are also observed in the hippocampus during ethanol withdrawal (Doremus-Fitzwater et al., 2014; Gano, Doremus-Fitzwater, & Deak, 2016; He & Crews, 2008; Kane et al., 2014; Pascual, Balino, Aragon, & Guerri, 2015), and HDAC inhibitors reduce expression of neuroimmune genes in glial cells in culture and in the brain (Faraco et al., 2009; Kazantsev & Thompson, 2008; Patnala, Arumugam, Gupta, & Dheen, 2017). It is likely that multiple genes expressed in the hippocampus are under epigenetic control and contribute to the negative affective state during alcohol withdrawal. Investigation into these epigenetic mechanisms will no doubt lead to potential new therapeutic targets for the treatment of comorbid AUD and depression.

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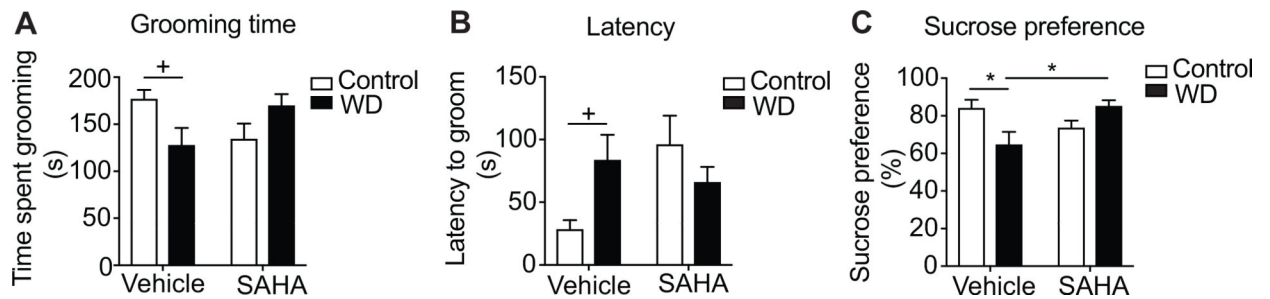
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### Highlights

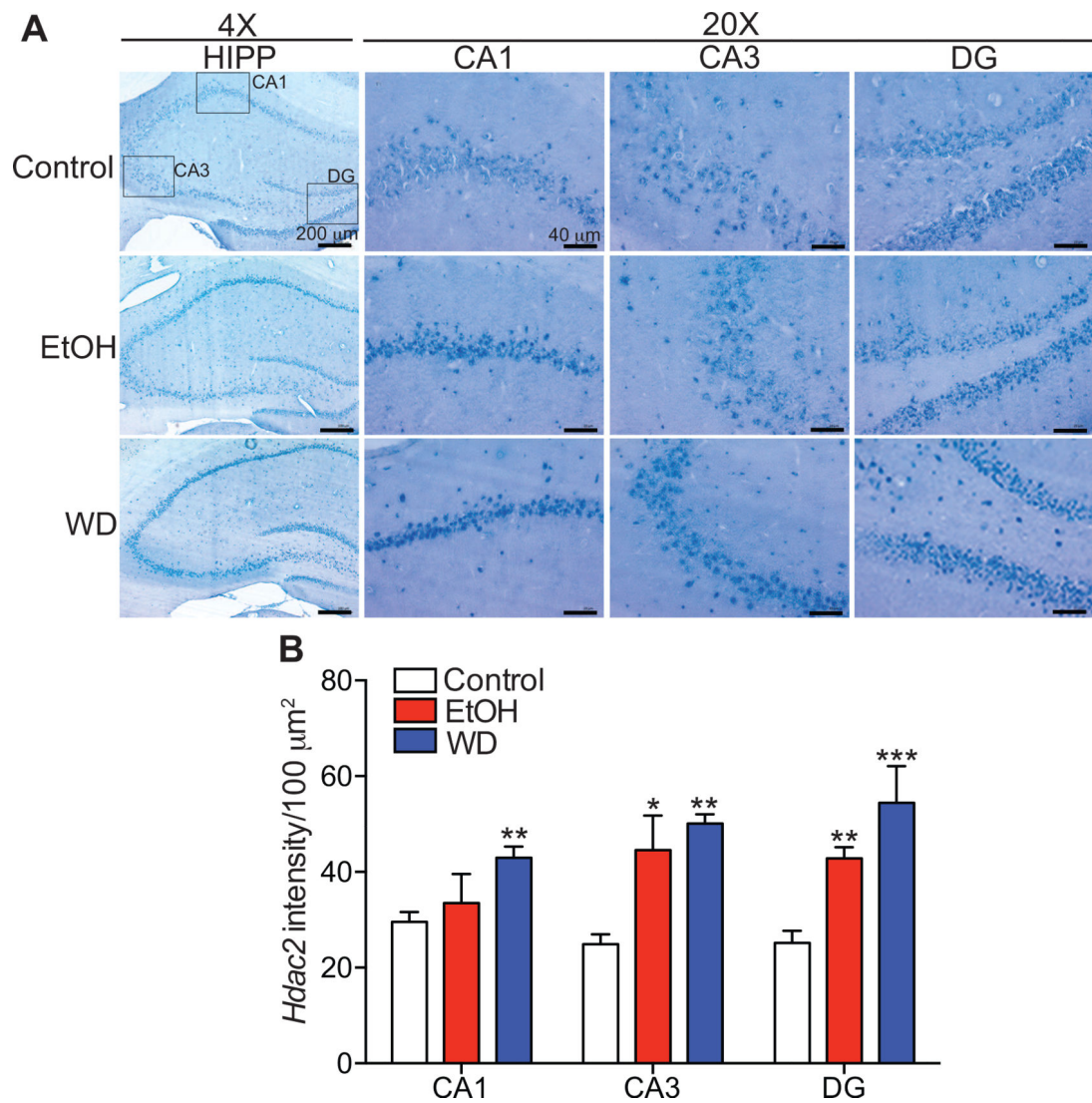
- SAHA treatment reduces depression-like behavior during ethanol withdrawal.
- HDAC2 expression increases in the hippocampus during ethanol withdrawal.
- Acetylated histone H3 decreases in the hippocampus during ethanol withdrawal.





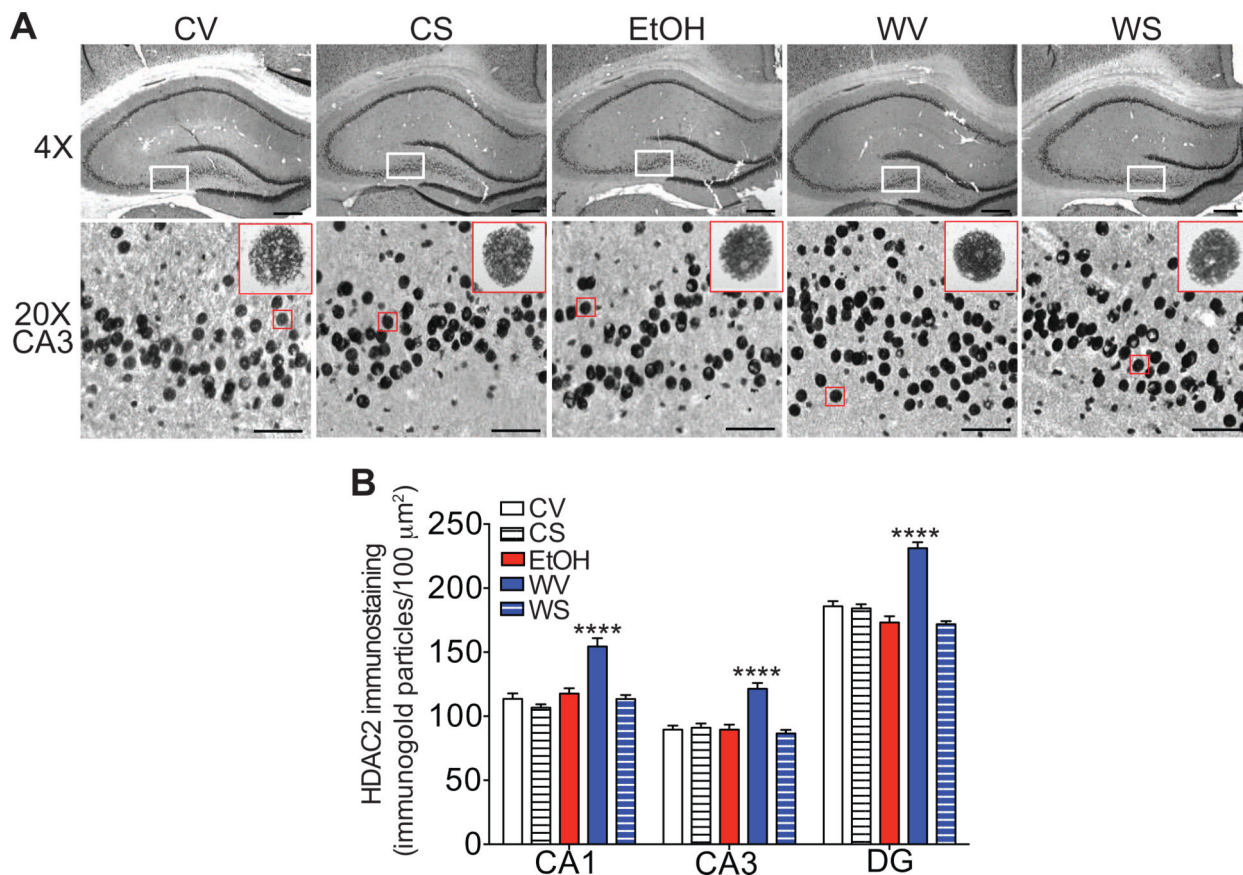
**Fig. 1. Withdrawal from chronic ethanol drinking results in depression-like behavior that is reversed by SAHA treatment.**

Rats drank control or ethanol liquid diet for 15 days. Ethanol diet was then removed and replaced with control diet for 24 hours prior to behavioral testing. SAHA (50 mg/kg, IP) or vehicle was administered 2 hours prior to testing. **A.** Time spent grooming over 5 minutes. Control/vehicle,  $n = 11$ ; withdrawal/vehicle,  $n = 13$ ; control/SAHA,  $n = 14$ ; withdrawal/SAHA,  $n = 13$ .  $^+p = 0.054$  by post-hoc Sidak's multiple comparisons test. **B.** Latency to groom.  $^+p = 0.072$ . **C.** Sucrose preference. Control/vehicle, withdrawal/vehicle, and withdrawal/SAHA,  $n = 15$ ; control/SAHA,  $n = 16$ ;  $*p < 0.05$ . Abbreviations: WD, ethanol withdrawal. Data is shown as means  $\pm$  SEM.



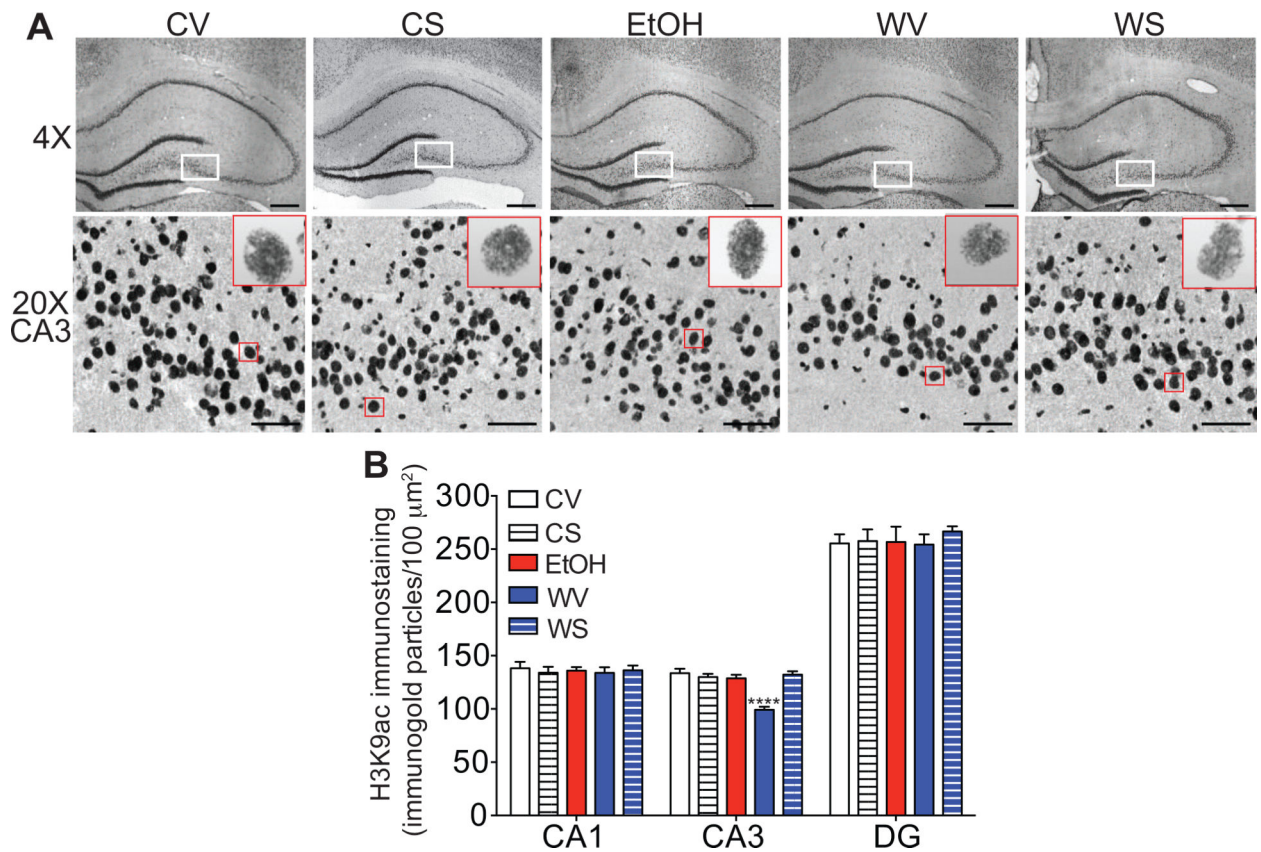
**Fig. 2. Increased *Hdac2* mRNA expression in the hippocampus during chronic ethanol drinking and withdrawal.**

*In situ* RT-PCR for *Hdac2* was performed on brain sections containing the hippocampus from rats that drank control or ethanol (EtOH) liquid diet and then underwent withdrawal (WD) from the EtOH diet for 24 hours. **A.** Representative images of the hippocampus (HIPP) in sections at 4× and 20× magnification with boxes in the 4× image showing the cornus ammonis (CA)1, CA3, and dentate gyrus (DG) regions that were analyzed at 20×. Scalebars: 4×, 200 μm; 20× 40 μm. **B.** Quantified intensity of *Hdac2* RT-PCR digoxigenin signal in each region of the hippocampus using NIH ImageJ software (mean ± SEM). CA1: control, n = 8; EtOH, n = 4; WD, n = 8; CA3: control, n = 6; EtOH, n = 5; WD, n = 6; DG: control, n = 6; EtOH, n = 6; WD, n = 3. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ , by post-hoc Tukey's test when compared with control group.



**Fig. 3. Increased HDAC2 protein in the hippocampus during withdrawal from chronic ethanol drinking is normalized by SAHA treatment.**

Immunohistochemistry using gold-labeled antibodies to HDAC2 was performed on brain sections containing the hippocampus from rats that drank control or ethanol (EtOH) liquid diet and then underwent withdrawal from the EtOH diet for 24 hours. Rats were also treated with vehicle or SAHA 2 hours prior to tissue collection. **A.** Top panels, representative images of the hippocampus at 4 $\times$  magnification. Scalebar: 200  $\mu\text{m}$ . White box outlines area shown at higher magnification in bottom panels. Bottom panels, representative images of the CA3 region of the hippocampus at 20 $\times$  magnification. Scalebar: 50  $\mu\text{m}$ . The red box outlines a representative magnified cell shown in the inset at high magnification (100 $\times$ ). **B.** Quantification of immunogold particles per 100  $\mu\text{m}^2$  area of each region. All data are shown as mean  $\pm$  SEM,  $n = 5$  rats per treatment group. \*\*\*\* $p < 0.0001$  when compared with all other groups by post-hoc Tukey's test. Abbreviations: CV, control-vehicle; CS, control-SAHA; EtOH, ethanol group with no withdrawal; WV, withdrawal-vehicle; WS, withdrawal-SAHA; CA, cornus ammonis; DG, dentate gyrus.



**Fig. 4. Decreased H3K9ac in the hippocampus during withdrawal from chronic ethanol drinking is normalized by SAHA treatment.**

Immunohistochemistry with gold-labeled antibodies to H3K9ac was performed on brain sections containing the hippocampus from rats that drank control or ethanol (EtOH) liquid diet and then underwent withdrawal from the liquid diet for 24 hours. Rats were also treated with vehicle or SAHA 2 hours prior to tissue collection. **A.** Top panels, representative images of the hippocampus at 4 $\times$  magnification. Scalebar: 200  $\mu\text{m}$ . White box outlines area shown at higher magnification in bottom panels. Bottom panels, representative images of the CA3 region of the hippocampus at 20 $\times$  magnification. Scalebar: 50  $\mu\text{m}$ . The red box outlines a representative magnified cell shown in the inset at high magnification (100 $\times$ ). **B.** Quantification of immunogold particles per 100  $\mu\text{m}^2$  area of each region. All data are shown as mean  $\pm$  SEM,  $n = 5$  rats per treatment group. \*\*\*\* $p < 0.0001$  when compared with all other groups by post-hoc Tukey's test. Abbreviations: CV, control-vehicle; CS, control-SAHA; EtOH, ethanol group with no withdrawal; WV, withdrawal-vehicle; WS, withdrawal-SAHA; CA, cornus ammonis; DG, dentate gyrus.