Regular Research Article

Essential Role of Histone Methyltransferase G9a in Rapid Tolerance to the Anxiolytic Effects of Ethanol

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Abstract

Background: Tolerance to ethanol-induced anxiolysis promotes alcohol intake, thus contributing to alcohol use disorder development. Recent studies implicate histone deacetylase-mediated histone H3K9 deacetylation in regulating neuropeptide Y expression during rapid ethanol tolerance to the anxiolytic effects of ethanol. Furthermore, the histone methyltransferase, G9a, and G9a-mediated H3K9 dimethylation (H3K9me2) have recently emerged as regulators of addiction and anxiety; however, their role in rapid ethanol tolerance is unknown. Therefore, we investigated the role of G9a-mediated H3K9me2 in neuropeptide Y expression during rapid ethanol tolerance.

Methods: Adult male rats were administered one injection of n-saline followed by single acute ethanol injection (1 g/kg) 24 hours later (ethanol group) or 2 injections (24 hours apart) of either n-saline (saline group) or ethanol (tolerance group). Anxiety-like behaviors and global and Npy-specific G9a and H3K9me2 levels in the amygdala were measured. Effects of G9a inhibitor (UNC0642) treatment on behavioral and epigenetic measures were also examined.

Results: Acute ethanol produced anxiolysis and decreased global H3K9me2 and G9a protein levels in the central and medial nucleus of the amygdala as well as decreased occupancy levels of H3K9me2 and G9a near a putative binding site for cAMP-response element binding protein on the Npy gene. Two identical doses of ethanol produced no behavioral or epigenetic changes relative to controls, indicating development of rapid ethanol tolerance. Interestingly, treatment with UNC0642, before the second ethanol dose reversed rapid ethanol tolerance, decreased global H3K9me2 and increased neuropeptide Y levels in the central and medial nucleus of the amygdala.

Conclusions: These results implicate amygdaloid G9a-mediated H3K9me2 mechanisms in regulating rapid tolerance to the anxiolytic effects of ethanol via neuropeptide Y expression regulation.

Keywords: alcohol use disorder, amygdala, anxiety, G9a, rapid ethanol tolerance
Significance Statement

Rapid tolerance to the anxiolytic effects of alcohol is crucial in facilitating AUD, and rapid ethanol tolerance (RET) is believed to be a reliable index of chronic tolerance. Amygdaloid structures play a critical role in negative emotional states in AUD, such as anxiety. We identified a novel epigenetic mechanism in the amygdala that modulates rapid ethanol tolerance. Our findings suggest G9a-mediated epigenetic mechanisms regulate anxiolytic NPY expression in RET and that the G9a inhibitor, UNC0642, alters Npy expression in the amygdala secondary to altered histone methylation (H3K9me2). Ultimately, this study suggests the importance of epigenetic mechanisms in the development of AUD and highlights the therapeutic potential of targeting G9a and epigenetic pathways in AUD.

Introduction

Alcohol tolerance is a key component of alcohol use disorder (AUD) development and diagnosis that occurs when increased doses of alcohol are required to produce a desired response (Tabakoff et al., 1986; American Psychiatric Association, 2013). Rapid ethanol tolerance (RET) is developed within 24 hours of alcohol exposure and has been recognized as a reliable index of chronic tolerance, which is characteristic of AUD (Khanna et al., 1991; Kalant, 1998). In humans and animals, alcohol exposure induces anxiolytic effects, and tolerance to this effect is believed to encourage increased drinking and thus facilitate development and diagnosis that occurs when increased doses of alcohol are required to produce a desired response (Tabakoff et al., 1986; American Psychiatric Association, 2013). Rapid ethanol tolerance (RET) is developed within 24 hours of alcohol exposure and has been recognized as a reliable index of chronic tolerance, which is characteristic of AUD (Khanna et al., 1991; Kalant, 1998). In humans and animals, alcohol exposure induces anxiolytic effects, and tolerance to this effect is believed to encourage increased drinking and thus facilitate development and diagnosis that occurs when increased doses of alcohol are required to produce a desired response (Tabakoff et al., 1986; American Psychiatric Association, 2013).

Methods

Animals

All experiments were conducted with adult male Sprague-Dawley rats in accordance with the National Institute of Health’s Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the University of Illinois at Chicago. Male Sprague-Dawley adult rats were group-housed (2–3 rats in each cage (size, 18-inch length × 10-inch width × 8-inch height)) under a 12-hour-light/-dark cycle and age-matched to ensure consistency among groups with ad libitum access to food and water. They were single-housed 24 hours before the start of ethanol or saline injections followed by behavioral testing as described below. All animals were anesthetized with isoflurane and killed by decapitation for biochemical studies or perfusion followed by decapitation for immunohistochemistry as earlier described by us (Sakharkar et al., 2012, 2014). Dissected brain tissues were stored at −80°C until use.

RET Model

To establish RET to the anxiolytic effects of ethanol, we used a previously reported paradigm by us (Sakharkar et al., 2012), wherein 2 n-saline (5 μL/g of body weight), 2 ethanol (1 g/kg from 20% w/v diluted in n-saline), or combinatorial i.p. injections are provided 24 hours apart. On the first day, the saline group and ethanol group received n-saline while the tolerance

modulating gene expression in concert (Zhang et al., 2015; Berkel and Pandey, 2017).

Recent studies have begun elucidating the role of the HMT G9a and its downstream dimethylation of histone H3 lysine 9 (H3K9me2) in addiction and anxiety mechanisms (Schaefer et al., 2009; Covington et al., 2011; Sun et al., 2012; Anderson et al., 2018). HMTs such as G9a can add methyl groups to histones, resulting in altered chromatin architecture and gene expression depending on the site of methylation and degree of valiance (Rea et al., 2000; Rice et al., 2003). G9a exhibits active specificity for H3K9 dimethylation (Tachibana et al., 2001) and is known to cooperate with DNA methyltransferases (Du et al., 2015), which are both repressive epigenetic marks (Kouzarides, 2007), suggesting G9a functions predominately as an inhibitor of gene expression. Currently, the role of G9a in NPY expression during development of RET is unknown. Therefore, we have used the well-established RET animal model to investigate the specific role of G9a and H3K9me2 in tolerance to the anxiolytic effects of ethanol via NPY expression regulation in the amygdaloid brain structures.
group received ethanol. After 24 hours, the saline group received n-saline while the ethanol group and tolerance group received ethanol. Behavioral testing was completed 1 hour after the second administration of either n-saline or ethanol (between 9:00 AM and 2:00 PM). Fresh right atrial blood from rats was collected at the time of brain collections and used to determine blood ethanol levels (mg/dL) using an AM-1 Analox Alcohol Analyzer (Analox Instruments, Lunenberg, MA).

**UNC0642 Treatment in RET Model**

To test the effects of UNC0642 (Sigma Aldrich, St. Louis, MO) on anxiety measures, we administered 2 i.p. 2.5-mg/kg doses of UNC0642 (dissolved in dimethyl sulfoxide [DMSO] at 5 mg/mL and diluted in phosphate-buffered saline [PBS] to 1:7) or vehicle (DMSO: PBS, 1:7 dilution) to a saline group and a tolerance group both at 6 hours and 23 hours after the first day of either n-saline or ethanol exposure (Table 1). The dose is based on a previously published study where pharmacokinetics and drug permeability of brain tissue was established (Liu et al., 2013). Behavior was tested 1 hour after the final injections of n-saline/ethanol and 2 hours after the last dose of UNC0642.

**Light Dark Box Exploration Test**

The light/dark box (LDB) exploration test was used for anxiety measures as reported earlier by us (Pandey et al., 2008; Sakharkar et al., 2012). Data were reported as mean ± SEM of the percentage of time spent in light or dark compartment of LDB. General activity was reported as mean ± SEM of total ambulation.

**Chromatin Immunoprecipitation Assay**

Amygdala tissue was processed using previously described chromatin immunoprecipitation (ChIP) protocol (Kyzar et al., 2017; Zhang et al., 2018). Samples were homogenized and formaldehyde fixed, then DNA was sheared via the Covaris M220 focused ultrasonicator (Covaris, Woburn, MA). Chromatin fragments were then immunoprecipitated using ChIP-grade antibodies directed to G9a (ab40542; Abcam, Cambridge, UK) or H3K9me2 (ab1220; Abcam), collected with A/G plus-agarose beads (Santa Cruz, Santa Cruz, CA), and boiled for 10 minutes in 10% Chelex solutions (Bio-Rad, Berkeley, CA), as described by other investigators (Nelson et al., 2006). DNA was then isolated for RT qPCR with appropriate primers (see Table 2) and SYBR Green master mix (Bio-Rad). Input DNA was used as normalizing factor for each sample. Data were analyzed using the \( \Delta \Delta C_{t} \) method.

**Gold Immunolabeling Histochemical Procedure**

Gold immunolabeling was performed as previously described (Moonat et al., 2013; Sakharkar et al., 2012, 2014). Coronal slices (20 µm thick) containing amygdala were processed with primary antibodies for G9a (1:200, ab31874, Abcam), H3K9me2 (1:500, 9753S, Cell Signaling, Beverly, MA), and NPY (1:500, 22940, Immunostar, Hudson, WI). Gold-conjugated anti-rabbit antibody (1:200, Nanoprobe, Yaphank, NY) was used as secondary. Protein levels were calculated using the Image Analyzer software with a light microscope by counting the number of immunogold-labeled particles per 100 µm² of bregma-matched amygdala tissue at high magnification (100×) after setting threshold in such a way that area without labeling should give zero count. The CeA, MeA, and basolateral amygdala regions (BLA) were evaluated per animal via the average of 9 object fields from 3 separate brain sections of each animal and reported as mean ± SEM for each experimental group.

**Data Analysis**

Behavioral and immunohistochemistry data were analyzed using 1-way ANOVA followed by posthoc comparisons using Tukey’s test. ChIP fold change of H3K9me2 and G9a occupancy was analyzed via the nonparametric Kruskal-Wallis test followed by posthoc Dunn’s test. Significance for all experiments was set at P < .05.

**RESULTS**

**RET and Amygdalar H3K9me2 and G9a**

First, we replicated our model of development of RET to the anxiolytic effects of ethanol in rats (Figure 1). As previously reported (Sakharkar et al., 2012), we observed that a single injection of ethanol (1 g/kg) induced anxiolysis (ethanol group), as evidenced by significantly (P < .001) reduced time spent in the dark compartment and increased time spent in the light compartment during LDB exploration relative to n-saline-exposed rats (saline group). Two equivalent doses (1 g/kg) administered 24 hours apart (tolerance group) produced no such change relative to the controls. Total ambulation was recorded during the LDB exploration and showed no change in general activity among groups (Figure 1A). There were no significant differences.

**Table 1. Timeline for UNC0642 Behavioral Study**

| Time   | Treatment                  |
|--------|----------------------------|
| 0 hours| Saline group               |
| 6 hours| Saline + UNC0642 group     |
|        | Ethanol group              |
|        | Tolerance group            |
|        | Tolerance + UNC0642 group  |
| 23 hours| Vehicle                   |
| 24 hours| Vehicle                   |
| 25 hours| Vehicle                   |
|        | Behavioral testing        |

**Table 2. Showing Primers Sequence for Various Locations Within Npy Gene as Shown in Figure 3A**

| Primer   | Sequence                                      |
|----------|-----------------------------------------------|
| Npy loc a| F: 5’ ACGCAGCGCACCTCATTTA3’                  |
|          | R: 5’ GGCTCGTGTAGTAGGCTTATTTA3’              |
| Npy loc b| F: 5’ GTTAGAGGGAGGTGCTTCTATG 3’              |
|          | R: 5’ ACATGCTGTCATTCGATCTGAT3’               |
| Npy loc c| F: 5’ AGTACGGTTCAGTAGTCCTCGAGCATAC3’        |
|          | R: 5’ GAAGCAGTCGCCCGAGGTATTTTT3’             |
| Npy loc d| F: 5’ CCAAGTCTGAGCGCTGTAGTAC3’               |
|          | R: 5’ AAGAACCAGCAGGGGATAG 3’                 |
| Npy loc e| F: 5’ CTTDAACCACCTGAGGCCATCTT 3’            |
|          | R: 5’ CAAACAAGCAGCCAAATC 3’                  |
in mean ± SEM (n = 7–8 rats in each group) blood ethanol levels (mg/dL) between the ethanol group and tolerance group (ethanol group, 99.10 ± 5.2; tolerance group, 101.3 ± 8.4). There was also no significant difference in the body weights (gm; mean ± SEM) between groups (saline group, 340 ± 5.1; ethanol group, 334 ± 5.4; tolerance group, 336 ± 2.4). These results suggest that rapid tolerance to the anxiolytic effects of ethanol is reproducible and represents a valid model for further investigation underlying epigenetic mechanisms.

We have previously shown that epigenetic regulation via H3K9 acetylation contributes to regulation of NPY expression in the amygdala and might be involved in the development of RET (Sakharkar et al., 2012). To determine the potential role of G9a-mediated histone dimethylation at the same lysine residue (H3K9me2) in tolerance to the anxiolytic effects of ethanol, we determined global changes in H3K9me2 and G9a protein levels in various amygdaloid structures in the RET model. Relative to the saline group, rats exposed to acute ethanol expressed significantly reduced H3K9me2 and G9a protein levels in the CeA (G9a, P < .01; H3K9me2, P < .001) and MeA (G9a, P < .01; H3K9me2, P < .05), but not the BLA (Figure 2A–D). In tolerance group rats administered 2 equivalent doses of ethanol (24 hours apart), however, there was no significant difference in H3K9me2 or G9a protein levels relative to controls, suggesting potential involvement of histone methylation similar to histone acetylation (Sakharkar et al., 2012) in cellular tolerance to the anxiolytic effects of ethanol.

H3K9me2 and G9a Occupancy of NPY Gene in the Amygdala

Previous studies have heavily implicated amygdalar NPY in anxiety and AUD regulation (Heilig et al., 1989; Pandey, 2003; Gilpin et al., 2015), and our laboratory has shown that epigenetic dysregulation of amygdalar NPY production regulates alcohol-drinking behavior (Sakharkar et al., 2014; Pandey et al., 2017) and RET to the anxiolytic effects of ethanol (Sakharkar et al., 2012). To examine the potential role of H3K9me2 epigenetic mechanisms in amygdalar NPY expression in RET, we employed a ChIP assay to investigate the occupancy levels of H3K9me2 at multiple locations (loc a–e) along the promoter and gene body of Npy (Figure 3A). A single ethanol exposure resulted in significantly (P < .05) decreased H3K9me2 levels at one site (loc e), which lies adjacent to a putative CREB-binding site, while the tolerance group exhibited no such change (Figure 3B). At other investigated locations (loc a–d), neither the ethanol group nor the tolerance group exhibited significant changes in H3K9me2 occupancy (Figure 3B). To affirm the role of G9a in the H3K9me2 changes, we examined the occupancy of G9a at loc e and found the ethanol group also exhibited reduced G9a occupancy (P < .05) with no change in the tolerance group relative to the saline control group (Figure 3C). The dissimilar inhibitory H3K9me2 and G9a occupancy between the ethanol and tolerance groups at a potential CREB-binding site in the Npy gene implicates epigenetic control of Npy transcription by G9a-mediated H3K9me2 in the amygdala during RET.

Effect of Systemic UNC0642 on Rapid Tolerance to the Anxiolytic Effects of Ethanol

To investigate G9a-mediated epigenetic mechanisms in rapid tolerance to the anxiolytic effects of ethanol, we used the LDB exploration test after treatment with the G9a inhibitor UNC0642 (Liu et al., 2013). We observed that subacute UNC0642 (2 doses of 2.5 mg/kg) induced significant (P < .05) anxiolytic effects in control rats (saline + UNC0642), relative to vehicle-treated controls (saline + vehicle) (Figure 4). Additionally, identical doses of UNC0642 treatment in a tolerance group (tolerance + UNC0642) reversed RET and resulted in significant (P < .05) anxiolytic-like effects relative to vehicle-treated tolerant rats (tolerance + vehicle) or vehicle-treated controls (saline + vehicle) (Figure 4). The anxiolytic effects produced in tolerant rats, reported as a decrease in time spent in the dark compartment (dark box) and increase in time spent in the light compartment (light box), were nearly identical to anxiolytic effects displayed after a single exposure to acute ethanol (ethanol + vehicle). General activity was also recorded during the LDB exploration test by monitoring total ambulation, which did not differ among groups (Figure 4). There were also no significant differences in mean ± SEM body weight (gm) among groups (saline + vehicle, 311 ± 20; saline + UNC0642, 315 ± 25; ethanol + vehicle, 311 ± 4.1; tolerance + vehicle, 314 ± 2.9; tolerance + UNC0642, 318 ± 3.1). These results suggest that G9a inhibitor treatment not only produced anxiolytic effects itself in control rats but was able to reverse the development of rapid tolerance to the anxiolytic effects of ethanol in rats.

Effect of Systemic UNC0642 on H3K9me2, G9a, and NPY Expression in the Amygdala During RET

To investigate the effect of the G9a inhibitor, UNC0642, on H3K9me2, G9a, and NPY protein levels in the amygdaloid
structures in the RET model, we performed gold immunolabeling to determine their protein expression after treatment with i.p. UNC0642 (2 doses of 2.5 mg/kg). As expected, acute ethanol (1 g/kg) exposure (ethanol + vehicle) significantly reduced protein levels of H3K9me2 and G9a while increasing NPY in the CeA (H3K9me2, P < .001; G9a, P < .001; NPY, P < .001) and MeA (H3K9me2, P < .01; G9a, P < .001; NPY, P < .05) while ethanol-tolerant rats (tolerance + vehicle) exhibited no such changes relative to controls (saline + vehicle) (Figures 5A.B and 6A.B). Subacute UNC0642 treatment administered before the second day of ethanol exposure in rats given 2 ethanol (1 g/kg) injections 24 hours apart (tolerance + UNC0642) resulted in no changes in G9a protein expression in any region of the amygdala; however, treatment significantly reduced levels of H3K9me2 as well significantly increased NPY protein in the CeA (H3K9me2, P < .001; NPY, P < .001) and MeA (H3K9me2, P < .001; NPY, P < .05) relative to controls (saline + vehicle) (Figures 5A,B and 6A,B). Interestingly, the UNC0642 treatment in control rats given 2 n-saline injections (saline + UNC0642) also significantly reduced H3K9me2 and increased NPY protein levels in the CeA (H3K9me2, P < .001; NPY, P < .05) and MeA (H3K9me2, P < .001; NPY, P < .05) relative to controls (saline + vehicle) (Figures 5A,B and 6A,B). These data suggest that G9a inhibition can decrease H3K9me2 levels and increase the NPY levels in the CeA and MeA that is associated with anxiolytic effects in rats, thereby reversing RET.

Figure 2. (a) Low-magnification representative microphotographs (scale bar = 50 µm) of G9a immunolabeling in the central (CeA), medial (MeA), and basolateral (BLA) amygdala of saline group, ethanol group, and tolerance group rats. (b) Bar diagrams showing changes in G9a protein expression in the CeA, MeA, and BLA of saline group, ethanol group, and tolerance group rats. Data are presented as mean ± SEM and derived from n = 5 rats in each group [**P < .01 significantly different from saline group as determined by ANOVA (F2, 12 = 11.1, P < .01 for G9a in CeA; F2, 12 = 12.0, P < .001 for G9a in MeA) followed by Tukey's test]. (c) Low-magnification representative microphotographs (scale bar = 50 µm) of H3K9 dimethylated (H3K9me2) protein immunolabeling in the CeA, MeA, and CeA of saline group, ethanol group, and tolerance group rats. Data are presented as mean ± SEM and derived from n = 7 rats in each group [*P < .05 and ***P < .001 significantly different from saline group as determined by ANOVA (F2, 18 = 15.5, P < .001 for H3K9me2 in CeA; F2, 18 = 5.7, P < .05 for H3K9me2 in MeA) followed by Tukey's test].
Figure 3. H3K9me2 and G9a occupancy along the neuropeptide Y (Npy) gene promoter and body during development of rapid ethanol tolerance (RET). (a) Npy gene map detailing examined locations (loc a–e), exon sequence sites, CpG islands, and the location of a putative cAMP response element binding protein (CREB) binding site. (b) Bar diagram showing fold change of amygdalar H3K9me2 occupancy in saline, ethanol, and tolerance rats at loc a–e via chromatin immunoprecipitation (ChIP) assay. Data are presented as mean ± SEM and derived from n = 5 to 6 rats [*P < .05 significantly different from saline group as determined by ANOVA on Ranks (H(2) = 7.2, P < .05) followed by Dunn’s test]. (c) Bar diagram showing fold change of amygdalar G9a occupancy in saline, ethanol, and tolerance rats at a putative CREB-binding site (loc e) using ChIP assay. Data are presented as mean ± SEM and derived from n = 7 to 8 rats in each group [*P < .05 significantly different from saline group as determined by ANOVA on Ranks (H(2) = 8.2, P < .05) followed by Dunn’s test].

Figure 4. Effect of G9a inhibitor, UNC0642, on rapid tolerance to the anxiolytic effects of ethanol in rats. Bar diagram showing light/dark box (LDB) exploration in saline, ethanol, and tolerance rats treated with vehicle or UNC0642 (2 doses of 2.5 mg/kg each between the first and second days of saline and/or ethanol exposure as described in Methods). Total ambulation indicates no difference in general activity. Data are presented as mean ± SEM and derived from n = 6 to 7 rats in each group [*P < .05, **P < .01 significantly different from saline + vehicle group as determined by ANOVA (F(3,19) = 9.9, P < .001) followed by Tukey’s test].
**Discussion**

The present study revealed that ethanol exposure produces changes in both global and Npy-specific G9a-mediated H3K9me2 in the amygdala that were not observed during RET to anxiolysis, suggesting that ethanol regulates chromatin remodeling and RET via G9a-mediated epigenetic mechanisms. The novel data presented here suggest that systemic administration of the G9a inhibitor, UNC0642, reversed rapid tolerance to the anxiolytic effects of ethanol. We believe UNC0642 altered chromatin architecture via inhibition of G9a-mediated H3K9me2 production and thereby permitted expression of anxiolytic NPY in the CeA and MeA (Figure 7). Furthermore, our findings suggest UNC0642 produced anxiolytic effects in ethanol-naive control rats through a similar or identical mechanism. Cumulatively, our findings suggest that G9a-mediated regulation of histone methylation (H3K9me2) within the CeA and MeA are potential therapeutic targets that might play a significant role in anxiety and AUD development.

Anxiety and AUD are believed to reciprocally facilitate one another, such as through self-medication of intrinsic anxiety, alcohol withdrawal-induced anxiety, or alcohol-induced dysfunction of stress response neurocircuitry (Pandey, 2003; Koob, 2009; Smith and Randall, 2012). The anxiolytic properties of alcohol are believed to encourage alcohol intake, and tolerance to these effects further increases alcohol consumption and likelihood of sustained neuroconnectivity dysfunction and alcohol dependence (Lipscomb et al., 1980; Koob and Volkow, 2010; Sakharov et al., 2012; Pandey et al., 2017). Tolerance can present either chronically (chronic ethanol tolerance) or within 8 to 24 hours of the first ethanol exposure (RET) (Kalant, 1998), and RET has been found to molecularly parallel chronic ethanol tolerance (Koob et al., 1987; Khanna et al., 1991, 1992; Pandey et al., 2017). In RET, mice or rats in the tolerance group are given equivalent doses of ethanol 24 hours apart and develop tolerance to anxiolytic effects of alcohol when compared with the ethanol group, which is exposed to 1 acute dose of ethanol (Debatin et al., 1980; Koob and Volkow, 2010; Pandey et al., 2017). In RET, mice or rats in the tolerance group are given equivalent doses of ethanol 24 hours apart and develop tolerance to anxiolytic effects of alcohol when compared with the ethanol group, which is exposed to 1 acute dose of ethanol (Debatin et al., 1980; Koob and Volkow, 2010; Pandey et al., 2017).

![Figure 5. Effect of the G9a inhibitor, UNC0642, on amygdalar H3K9me2 and G9a protein levels in a model of rapid ethanol tolerance (RET).](image-url)

(a) Low-magnification representative micrographs (scale bar = 50 µm) of H3K9me2 and G9a gold immunolabeling in the central nucleus of the amygdala (CeA) of saline, ethanol, and tolerance rats treated with vehicle or UNC0642 (2 doses of 2.5 mg/kg between the first and second days of saline and/or ethanol exposure). Box area within each image represents photograph of random one nucleus at high magnification (100×) showing immunogold particles. (b) Quantification of H3K9me2 and G9a protein levels in the CeA, medial (MeA), and basolateral (BLA) amygdala in saline, ethanol, and tolerance rats treated with vehicle or UNC0642. Data are presented as mean ± SEM and derived from n = 5 to 6 rats in each group. ***P < 0.001, and ****P < 0.001 significantly different from saline + vehicle group as determined by ANOVA (F4,20 = 17.7, P < 0.001 for H3K9me2 in CeA; F4,20 = 22.6, P < 0.001 for H3K9me2 in MeA; F4,22 = 15.7, P < 0.01 for G9a in CeA; F4,22 = 7.8, P < 0.001 for G9a in MeA) followed by Tukey’s test.
Previous work from our laboratory showed that acute ethanol exposure decreased HDAC activity and increased H3K9ac and NPY expression in the CeA and MeA of rats while an identical second ethanol exposure 24 hours later resulted in no difference in these measures compared with saline group controls (Sakharkar et al., 2012).

Figure 6. Effect of the G9a inhibitor, UNC0642, on amygdalar NPY protein levels in a model of rapid ethanol tolerance (RET). (a) Low-magnification representative micrographs (scale bar = 50 µm) of NPY gold immunolabeling in the central nucleus of the amygdala (CeA) of saline, ethanol, and tolerance rats treated with vehicle or UNC0642 (2 doses of 2.5 mg/kg between the first and second days of saline and/or ethanol exposure). Box area within each image represents photograph of random one nucleus at high magnification (100×) showing immunogold particles. (b) Quantification of NPY protein levels in the CeA, medial (MeA), and basolateral (BLA) amygdala in saline, ethanol, and tolerance rats treated with vehicle or UNC0642. Data are presented as mean ± SEM and derived from n = 5 to 6 rats in each group [*P < .05 and ***P < .001 significantly different from saline + vehicle group as determined by ANOVA (F4,24 = 20.0, P < .001 for NPY in CeA; F4,24 = 7.2, P < .001 for NPY in MeA) followed by Tukey’s test].

Figure 7. Model of proposed epigenetic mechanisms in the amygdala implicated in development of rapid ethanol tolerance (RET) to the anxiolytic effects of ethanol via neuropeptide Y (NPY) regulation. Acute ethanol exposure produced anxiolytic effects via inhibition of G9a-mediated dimethylation of histone H3 lysine 9 (H3K9me2), in addition to the previously established increase in H3K9 acetylation due to inhibition of histone deacetylases activity (Sakharkar et al., 2012), thereby altering chromatin architecture and permitting increased NPY expression in the amygdala. Furthermore, an identical dose of ethanol 24 hrs later results in tolerance to these cellular and behavioral effects. Interestingly, systemic inhibition of G9a via UNC0642 reverses rapid tolerance to the anxiolytic effects of ethanol most likely via a similar mechanism as that of acute ethanol. A = acetylation, M = methylation, TF = transcription factor.

and Barbosa, 2006; Sakharkar et al., 2012). Previous work from our laboratory showed that acute ethanol exposure decreased HDAC activity and increased H3K9ac and NPY expression in the CeA and MeA of rats while an identical second ethanol exposure 24 hours later resulted in no difference in these measures compared with saline group controls (Sakharkar et al., 2012).
Here, we extended these studies and found that a single ethanol exposure attenuated global and Npy-specific H3K9me2 and G9a levels and increased NPY levels in the CeA and MeA while animals administered an identical dose 24 hours later exhibited no such differences. Taken together, these results further suggest that acute ethanol opens chromatin via increased histone acetylation and decreased histone methylation due to inhibition of both HDACs and G9a and that these epigenetic marks appear to play an important role in RET (Figure 7).

NPY is a 36-amino acid peptide expressed in the amygdala, among other brain regions, to regulate behaviors such as appetite, anxiety, and alcohol intake (Thiele et al., 1998; Adam and Epel, 2007; Gilpin et al., 2015). The role of endogenous NPY signaling in regulating anxiolytic mechanisms and alcohol-drinking behaviors is well established; specifically, amygdalar NPY is recognized as a critical player (Thiele and Badia-Elder, 2003; Gilpin, 2012). Intracerebroventricular administration of NPY has reportedly attenuated ethanol intake in selectively bred alcohol-preferring rats with comorbid anxiety and high alcohol-drinking model strains (Badia-Elder et al., 2001, 2003) as well as anxiety-like behaviors in rat models for anxiety (Heilig et al., 1989). Furthermore, alcohol-preferring rats inherently express lower levels of NPY in the CeA and MeA compared with their nonpreferring counterparts (Suzuki et al., 2004; Pandey et al., 2005), and both ethanol intake and NPY infusion in the CeA are anxiolytic in alcohol-preferring rats (Pandey et al., 2005; Zhang et al., 2010). In fact, amygdalar overexpression or exogenous administration of NPY, particularly in the CeA, is anxiolytic and reduces drinking behaviors in anxious and alcohol-preferring animals (Primeaux et al., 2006; Zhang et al., 2010; Christiansen et al., 2014). Furthermore, NPY mutant animals exhibit higher alcohol preference and elevated sensitivity to withdrawal-associated anxiety (Sparta et al., 2007). Our findings reported here clearly indicate that NPY expression in the amygdala is increased by acute ethanol exposure and that changes in NPY expression via epigenetic mechanism contributes to cellular tolerance during RET (Figure 7).

Epigenetic manipulation of gene expression has emerged as a strong candidate for therapeutic targeting in psychiatric disorders, including AUD and anxiety (Nestler, 2014; Berkel and Pandey, 2017; Palmisano and Pandey, 2017). Dimethylation of H3K9 via G9a is one such mechanism known to remodel chromatin in an inhibitory fashion that attenuates gene expression and has been implicated in addictive mechanisms and alcohol-associated developmental disruptions. For instance, extensive exploration into the role of G9a in neurodevelopment and alcohol-induced disruptions has revealed that G9a-mediated mechanisms are critical and sensitive to the influence of alcohol (Epsztejn-Litman et al., 2008; Subbanna et al., 2013; Basavarajappa and Subbanna, 2016). In addition studies, morphine and cocaine exposure have been found to reduce G9a levels in the nucleus accumbens (Maze et al., 2010; Sun et al., 2012), and overexpression of G9a with associated increases in H3K9me2 in the nucleus accumbens increases sensitivity to drug reinforcement and drug-seeking behaviors (Anderson et al., 2018). Furthermore, G9a is also believed to modulate activation of the transcription factor, CAMP-response element-binding protein (CREB), via control of upstream brain-derived neurotrophic factor signaling (Covington et al., 2011; Anderson et al., 2018), both of which our laboratory and others have extensively linked to AUD and anxiety (Wand, 2005; Pandey et al., 2006; Warnault et al., 2016; Berkel and Pandey, 2017). Notably, the Npy gene is a recognized CREB target (McCling and Nestler, 2003; Pandey, 2003; Pandey et al., 2004), and our study showed that G9a-mediated H3K9me2 occupancy is reduced on the Npy gene near a putative CREB-binding site in response to acute ethanol exposure, suggesting acute ethanol exposure may be associated with altered chromatin architecture near this site that would promote transcriptional competence. Though few studies have investigated the relationship of H3K9me2 and G9a with anxiety, induced overexpression of G9a accompanied by increased H3K9me2 in the nucleus accumbens has been shown to be anxiogenic (Anderson et al., 2018), while conditional neuronal knockdown and chronic pharmacological inhibition of G9a each reduce H3K9me2 in the brain and produce anxiolytic-like effects (Schaefer et al., 2009; Wang et al., 2018). Here, we have expanded on these studies and systematically administered the G9a inhibitor, UNC0642, and found changes in G9a, H3K9me2, and NPY protein levels in the amygdala are associated with significant reductions in anxiety-like behaviors, including the reversal of tolerance to ethanol-induced anxiolysis. Interestingly, a recent study reported that while chronic UNC0642 administration via i.p. injection in mice reduced anxiety in a dose-dependent manner, a single injection failed to produce significant reductions in anxiety 30 minutes later (Wang et al., 2018). However, our findings show that subacute treatment of UNC0642 (2 doses of 2.5 mg/kg, 17 hours apart) is sufficient for an anxiolytic effect measured 2 hours after the last drug administration. Ultimately, we have established here that inhibition of G9a, specifically via UNC0642, possesses therapeutic potential in the treatment of AUD and anxiety via chromatin remodeling in the brain.

In conclusion, our findings highlight that G9a-mediated H3K9me2 in the amygdala neurocircuitry may orchestrate chromatin remodeling of the Npy gene, resulting in increased amygdalar NPY expression in response to acute ethanol exposure and normalized expression during RET. Furthermore, these findings established the therapeutic potential of G9a inhibition in reducing anxiety and reversing tolerance to the anxiolytic effects of ethanol. These findings, in conjunction with our previous findings regarding HDAC-mediated NPY expression regulation in the context of AUD and anxiety (Pandey et al., 2008; Sakharkar et al., 2012, 2014), further validate the emerging role of epigenetic regulation in AUD pathophysiology.

Funding
This work was supported by the National Institute on Alcohol Abuse and Alcoholism (grant nos. P50AA-022538, Center for Alcohol Research in Epigenetics; RO1AA-010005; U01AA-019971, U24AA-024605, Neurobiology of Adolescent Drinking in Adulthood project) and by the Department of Veterans Affairs (VA merit grant and Senior Research Career Scientist award) to S.C.P.

Acknowledgments
Studies presented here are part of the PhD thesis of T.D.B. for her MD/PhD degree in the Graduate College and College of Medicine, University of Illinois at Chicago. Some of these data were presented at the scientific meetings of the Society of Biological Psychiatry and Research Society on Alcoholism.

Interest Statement
None.

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