Published in final edited form as:

Alcohol Clin Exp Res. 2014 September ; 38(9): 2377–2386. doi:10.1111/acer.12502.

Histone Acetylation in the Nucleus Accumbens Shell Modulates Ethanol-Induced Locomotor Activity in DBA/2J Mice

Gretchen M. Sprow, Ph.D.[a], **Jennifer A. Rinker, Ph.D.**[a,b], and **Todd E. Thiele, Ph.D.**[a,b,*]

Gretchen M. Sprow: gretchen.sprow@duke.edu; Jennifer A. Rinker: jrinker@email.unc.edu; Todd E. Thiele: thiele@unc.edu

[a]Department of Psychology, University of North Carolina at Chapel Hill, Chapel Hill, NC

[b]Bowles Center for Alcohol Studies, University of North Carolina at Chapel Hill, Chapel Hill, NC

Abstract

Background—A growing body of literature suggests that epigenetic mechanisms, including histone acetylation, may play key roles in drug abuse and the development of addiction. Experiments in the present study were designed to investigate the role of histone acetylation in ethanol-induced locomotor sensitization.

Methods—Immunohistochemical, western blotting, and site-directed pharmacological techniques were used to explore the roles of histone acetylation at histone H3 (acH3K9) in both the expression of and acquisition of ethanol-induced locomotor sensitization. A commonly used sensitization protocol, in which animals were exposed to repeated injections of a low dose of ethanol while in their home cage, was used to examine this behavioral phenomenon. Additionally, site-directed administration of the histone deacetylase inhibitor (HDACi) Trichostatin A (TSA), in the absence of repeated ethanol injections, was used to examine the role of hyperacetylation in the nucleus accumbens shell in ethanol-induced locomotor sensitization.

Results—Sensitized mice displayed elevated acH3K9 immunoreactivity (IR) localized to the shell of the nucleus accumbens. This augmentation in acH3K9 IR was confirmed, in a separate experiment, using western blot analyses. Next, repeated intra-accumbal infusions of TSA, in the absence of repeated ethanol injections, were sufficient to induce an augmented locomotor response to a later injection of a low dose (2.0 g/kg, i.p.) of ethanol, indicative of cross-sensitization to this locomotor stimulation between TSA and ethanol. Finally, a local infusion of TSA into the shell of the accumbens was also associated with a significant increase in acH3K9 IR within this region.

Conclusions—Together, the present observations suggest that histone acetylation, particularly within the shell of the nucleus accumbens, is important for the development and expression of ethanol-induced locomotor sensitization.

Keywords

sensitization; ethanol; acetylation; locomotor; TSA

[*]Address correspondence to: Todd E. Thiele, Ph.D., Department of Psychology, University of North Carolina, Davie Hall, CB# 3270, Chapel Hill, NC 27599-3270, USA, Phone: 919-966-1519, Fax: 919-962-2537, thiele@unc.edu.

INTRODUCTION

Behavioral sensitization has long been investigated as an important mechanism that may explain the transition from drug use to drug dependence (Robinson and Berridge, 1993, Robinson and Berridge, 2001). Defined by the progressive augmentation of locomotor responses to a given dose of ethanol following repeated administration, ethanol-induced locomotor sensitization has been observed in both rodent and human populations (Lessov and Phillips, 1998, Newlin and Thomson, 1991, Fee et al., 2006). Indeed, repeated drug exposure is thought to usurp natural reward circuitry within the mesolimbic dopaminergic pathway, leading to a hypersensitized state (Pierce and Kalivas, 1997, White and Kalivas, 1998, Robinson and Berridge, 2003). While a number of proteins (Lee et al., 2013, Nona et al., 2013) have been suggested to govern the development of locomotor sensitization, much remains unknown about the mechanisms underlying this phenomenon. The current experiments were thus designed to further examine the neurobiological mechanisms underlying ethanol-induced locomotor sensitization.

An abundance of recent work has investigated the epigenetic mechanisms involved in the development and maintenance of drug abuse and addiction (Renthal and Nestler, 2008, Wong et al., 2010). Such mechanisms induce changes in gene expression through remodeling of chromatin structure via DNA methylation or post-translational histone modifications, including acetylation and methylation, resulting in observable phenotypic changes. An increase in the acetylation of core histones H3 and H4, in particular, has been linked with an increase in gene expression, while deacetylation of these histones has been linked with decreased gene expression (Eberharter and Becker, 2002). A number of drugs of abuse, including cocaine, amphetamine, and ethanol, have been shown to dynamically regulate histone modifications in animals (Sanchis-Segura et al., 2009, Kumar et al., 2005, Renthal and Nestler, 2008). In regards to ethanol in particular, withdrawal from chronic ethanol has been shown to increase histone deacetylase (HDAC) activity: these changes were correlated with both increased levels of anxiety-like behavior and reduced NPY expression in specific amygdaloid regions, suggestive of a relationship between histone acetylation and NPY expression (Pandey et al., 2008). Indeed, peripheral administration of Trichostatin A (TSA), a potent and selective class I and II HDAC inhibitor (HDACi), not only increased the amount of H3 and H4 acetylation seen in ethanol withdrawn rats following chronic ethanol exposure, but rescued withdrawal-induced deficits in NPY mRNA and protein expression in these animals. More recent data has also implicated ethanol-induced inhibition of HDAC activity as playing a role in the rapid tolerance to the anxiolytic effects of ethanol (Sakharkar et al., 2011). Further, intermittent ethanol exposure during adolescence has been linked with region-specific changes in histone acetylation, histone acetyltransferase activity, and histone dimethylation in mice (Pascual et al., 2012). Given these data, it is likely that histone acetylation plays critical roles in other neurobiological responses to ethanol, such as locomotor sensitization. Indeed, several studies have indicated that chromatin modulation plays a role in drug-induced behavioral sensitization (Schroeder et al., 2008). For example, peripheral administration of the Class I HDACi sodium butyrate has been shown to enhance cocaine-induced locomotor sensitization, with a similar effect on morphine- and ethanol-induced locomotor sensitization (Sanchis-Segura et al., 2009).

Recent work has also indicated that epigenetic mechanisms within the striatum may partially explain individual differences between mice resistant or susceptible to ethanol-induced locomotor sensitization (Botia et al., 2012). An important caveat to these and other studies, however, is that they traditionally utilize peripheral administration of HDACi. This begs the question, then, of how histone hyperacetylation within specific regions may regulate ethanol-induced locomotor sensitization.

As recent studies have shown conflicting results (i.e. either an augmentation of (Sanchis-Segura et al., 2009) or blunting of (Kalda et al., 2007, Moretti et al., 2011) a sensitized response to a drug of abuse), the present experiments were designed to further examine the roles of histone acetylation in both the development and expression of ethanol-induced locomotor sensitization, with a particular focus on the shell of the nucleus accumbens. First, we examined acetylation at histone 3 lysine 9 (acH3K9) using immunohistochemical analysis following ethanol-induced locomotor sensitization; we then sought to confirm observed changes using western blotting techniques. Given changes observed within the shell of the nucleus accumbens, we examined whether repeated intra-accumbal infusions of TSA, in the absence of repeated ethanol exposure, would be sufficient to induce a sensitized locomotor response to a later injection of ethanol. Taken together, results from these data provide further evidence that chromatin remodeling through histone acetylation, particularly within the shell of the nucleus accumbens, plays a role in the development and expression of ethanol-induced locomotor sensitization.

MATERIALS AND METHODS

Animals

Male DBA/2J mice (Jackson Laboratory, Bar Harbor, ME) were 6–8 weeks of age, weighed ~23 g at the beginning of each experiment, and were housed as previously described (Lowery-Gionta et al., 2012). These mice have previously been shown to exhibit robust sensitization to the locomotor stimulant effects of ethanol (Fee et al., 2007). All procedures were in accordance with the NIH guidelines and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Drugs

Ethanol solutions (15%, 20%, and 25%, w/v) were prepared using 0.9% saline and 100% (Experiment 1) or 95% (Experiments 2, 3 & 4) ethyl alcohol; mice received an intraperitoneal (i.p.) injection of 1.5, 2.0, or 2.5 g/kg ethanol during the sensitization protocol. Equivolume saline injections were used as an ethanol control. TSA (0.0, 1.0, 2.0, 4.0 mg/kg, i.p.; 0.0, 22 mM/0.5 μl intra-accumbal infusion; Sigma Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and diluted 1:5 with phosphate buffered saline (PBS; pH 7.4). DMSO diluted 1:5 with PBS was used as the vehicle control for the intra-accumbal TSA study. A cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg) was used for survival surgery procedures.

Ethanol-Induced Locomotor Sensitization

The sensitization protocol was based on work previously described (Fee et al., 2006, Fee et al., 2007). Briefly, animals were transported to the testing room at least 30 min prior to testing, removed from their home cages, given an i.p. injection of saline or ethanol, and immediately placed into the center of an open field (Harvard Apparatus, Inc., Holliston, MA). Locomotor activity was recorded for 20 min sessions (5 min data bins). All mice experienced 3 days of habituation to this procedure. On the initial test day, mice received an injection of ethanol or saline to establish baseline locomotor behavior. Animals then received homecage (HC) injections of either saline or ethanol, one injection per day for 7–10 consecutive days. On the final test day mice received an injection of ethanol or saline immediately preceding locomotor measurements.

Surgery and Infusion Procedures

Approximately 1 week after arrival, mice in Experiments 3 & 4 underwent surgery to implant bilateral 26-G guide cannulae aimed at the nucleus accumbens shell [NACSH; coordinates in reference to bregma, anterior-posterior (AP): + 1.1 mm; medial-lateral (ML) ± 0.5 mm; dorsal-ventral (DV): − 4.5 mm)] using the Angle II Sterotax (Leica Instruments). Animals were allowed to recover for approximately 1 week before experimental procedures were initiated. Vehicle or 22mM TSA was delivered bilaterally to the NACSH in a volume of 0.5 μl over a 2 min period. The injector was left in place for 1 min in order to allow drug diffusion. Cannulae placements were verified histologically at the end of the experiment. Data from animals in which cannulae did not target the NACSH (n=10) in Experiment 3 were analyzed separately to examine site-specificity of drug effects.

Perfusions, Brain Preparation, and Immunohistochemistry (IHC)

Mice were deeply anesthetized with a supra-anesthetic dose of ketamine and xylazine (approximately 3x the anesthetic dose) and transcardially perfused with 0.1M PBS followed by 4% paraformaldehyde in phosphate buffer using previously described perfusion and immunohistochemical procedures (Cubero et al., 2010, Navarro et al., 2008, Hayes et al., 2005). Briefly, brains were cut into 30 μm slices and acetylated histone expression was detected using primary rabbit anti-acH3K9 antibody (Millipore, Temecula, CA; 1:5000). Densitometric procedures were used to assess levels of acH3K9 immunoreactivity (acH3K9 IR) as we have previously described (Lowery-Gionta et al., 2012, Sparrow et al., 2012).

Western Blot Analysis

Tissue punches (1mm x 1mm cylindrical punch) from the nucleus accumbens (NAC), dorsolateral striatum (DLS), prefrontal cortex (PFC), and ventral tegmental area (VTA) were grossly dissected from frozen tissue and prepared for western blot analysis of acH3K9. Due to technical limitations, the entire NAC region was taken in one punch (i.e., the core and shell subregions were not separated). Bilateral punches were combined into one homogenate. Tissue was homogenized by manually pipetting and vortexing with 100 μl lysis buffer containing 320 mM sucrose, 1% SDS, 5 mM HEPES buffer, and Halt 100x Protease/ Phosphotase Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL). Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher

Scientific) and 5 μg of total protein was loaded onto 4–20% Tris-Glycine gels for electrophoresis (Life Technologies, Grand Island, NY). Samples were then transferred to PVDF membranes and blocked for 1 h in 1% BSA (Roche, Indianapolis, IN) or 5% non-fat milk at room temperature (RT). Blocked membranes were incubated for 2 h with primary antibodies (1:1000 acH3K9 or 1:5000 total H3, Abcam, Cambridge, MA) in 0.5% BSA or 5% non-fat milk at RT. Membranes were washed with tris-buffered saline with 0.1% Tween-20 (TBST) and incubated with HRP-conjugated anti-rabbit secondary antibody (Abcam) in 0.5% BSA or non-fat milk for 1.5 h at RT. Membranes were washed in TBST and immunostaining was detected following the manufacturer's instructions for enhanced chemiluminescence detection (Thermo Fisher Scientific). Blots were imaged via autoradiography and quantified using densitometric analysis with Image J software (Image J, National Institutes of Health, Bethesda, MD). The amount of acH3K9 in each lane was normalized to levels of total H3; data are presented as a ratio of acH3K9 to total H3 (% of control).

Experiment 1: Analysis of acH3K9 IR Following Ethanol-Induced Locomotor Sensitization

DBA/2J mice were divided into three groups (n = 12–14 per group), counterbalanced for locomotor activity following an initial injection of saline or 1.5 g/kg ethanol. Saline (SAL) animals received saline injections for the remainder of the experiment. Acute ethanol (ACUTE) animals received saline for each of 10 HC injections, and 1.5 g/kg ethanol on the first and final test days only. Sensitized (SEN) mice received injections of 2.5 g/kg ethanol for each of the 10 HC injections, and 1.5 g/kg EtOH on the first and final test days. Brains were collected for IHC analysis 48 or 96 hours after the final locomotor assessment; these time points were chosen to examine whether lasting changes in histone acetylation would be responsible for the persistent behavioral effects observed following this sensitization protocol. As no significant differences in IR were evident between these time points, the data were collapsed. See Figure 1A for a schematic overview of the timeline for Experiment 1.

Experiment 2: Western Blot Analysis of acH3K9 Following Ethanol-Induced Locomotor Sensitization

DBA/2J mice (n = 10–11 per group) underwent a sensitization protocol similar to that described above, except that ACUTE and SEN mice received an injection of 2.0 g/kg ethanol on the initial and final test days. This dose of ethanol was chosen to more closely match that used in other reports; further, as this dose of ethanol elicited sensitization to the locomotor stimulant effects of ethanol and analysis of acH3K9 confirmed changes detected in Experiment 1, this dose was also used for future experiments. Twenty-four hours after the final locomotor test session all mice were sacrificed and their brains flash-frozen for western blot analysis. See Figure 2A for a schematic overview of the timeline for Experiment 2.

Experiment 3: Effect of Repeated Intra-accumbal TSA on the Acquisition on Ethanol-Induced Locomotor Sensitization

DBA/2J mice (n = 7–8 per group) surgically implanted with bilateral cannulae aimed at the nucleus accumbens shell were divided into two groups based on locomotor activity following an initial injection of 2.0 g/kg ethanol. Mice then received 10 daily homecage

intra-accumbal infusions of 22mM TSA or vehicle; mice did not receive ethanol during this 10 day period. On the final test day, all animals received an injection of 2.0 g/kg ethanol immediately before being placed in the locomotor chamber. Two days later, all animals received an injection of saline immediately before a locomotor assessment to evaluate the possible effect of intra-accumbal TSA treatment on general activity levels. Two days later, all animals received an injection of 2.0 g/kg ethanol prior to a locomotor assessment to examine the long-lasting effects of repeated intra-accumbal TSA treatment. See Figure 3A for a schematic overview of the timeline for Experiment 3.

Experiment 4: Effect of Intra-accumbal TSA on acH3K9 IR

DBA/2J mice (n = 7–8 per group) were surgically implanted with bilateral cannulae aimed at the nucleus accumbens shell. After at least 5 days of recovery from surgery, mice were divided into two groups based on body weight. Half of the animals received a bilateral infusion of 22 mM TSA and the other half received a bilateral infusion of vehicle. Mice were returned to their home cages and left undisturbed for approximately 4 hours, after which mice were transcardially perfused and brains were processed for IHC analysis of acH3K9 IR. This time point was chosen because previous work has shown that site-directed administration of TSA into the hippocampus leads to peak acetylation of histone H3, as measured by western blotting procedures, approximately 4 hr after drug administration (Vecsey et al., 2007).

Data Analysis

Locomotor activity, immunoreactivity (IR) data, and western blot data were analyzed using analyses of variance (ANOVA). When significant differences were found, LSD *post-hoc* analyses were performed to examine specific between-groups differences. Repeated-measures ANOVAs were used to compare locomotor activity between initial and final locomotor testing sessions. For all data, significance was accepted at $p<0.05$ (two tailed). In cases where IR or western blot data was unclear or otherwise unquantifiable, samples were removed from the respective analysis, thus accounting for differences in degrees of freedom between comparable analyses.

RESULTS

As the most robust sensitization behavior typically emerged during the first 5 min of locomotor testing, most analyses focused on this time bin.

Experiment 1: Ethanol-induced locomotor sensitization induces region-specific augmentation of acH3K9-IR

Locomotor activity data from the first five minutes of the final test are presented in Figure 1B. A one-way ANOVA revealed a significant effect of group ($F(2,36)=9.820$, $p<0.001$), and post-hoc analysis confirmed that sensitized mice displayed a significant increase in locomotor activity during the final test session relative to both SAL ($p<0.001$) and ACUTE ($p<0.01$) mice.

Representative photomicrographs of acH3K9 IR within NACSH of saline treated (left) and sensitized (right) mice are shown in Figure 1C. acH3K9 IR in the nucleus accumbens shell (NACSH) differed by group ($F(2,33)=3.309$, $p=0.049$; Figure 1D). Post-hoc analysis confirmed that the SEN mice displayed significantly greater acH3K9 IR in the NACSH relative to both SAL ($p=0.05$) and ACUTE ($p=0.028$) mice; no group differences were detected in the nucleus accumbens core (NACC) ($F(2,33)=0.669$, $p=0.519$; Figure 1D). Additionally, no significant group differences in acH3K9 IR were detected in the dorsolateral or dorsomedial striatum (DLS; DMS), the basolateral amygdala (BLA), the central nucleus of the amygdala (CEA), the PFC, or the VTA. (See Table 1A for a summary of IHC data).

Experiment 2: Western blot analysis confirms changes in acH3K9 following sensitization

Locomotor activity from the first five minutes of the final test session is presented in Figure 2B. A one-way ANOVA of this locomotor activity on the final test day revealed a significant difference between groups ($F(2,30)=26.145$, $p<0.001$), and post-hoc tests confirmed that SEN mice displayed significantly augmented locomotor activity relative to both SAL and ACUTE mice ($p<0.001$). Analysis of western blot data from these animals revealed significant group differences in the expression of acH3K9 relative to total histone H3 within the NAC ($F(2,28)=6.174$, $p=0.006$; Figure 2C) and post-hoc tests confirmed that the SEN group displayed significantly elevated levels of acH3K9 relative to both SAL and ACUTE animals ($p<0.01$). No significant differences were detected elsewhere (see Table 1B for a summary of western blot data).

Experiment 3: Intra-accumbal TSA cross-sensitizes to the locomotor stimulant effects of a low-dose ethanol injection

After repeated intra-accumbal infusions of 22 mM TSA alone, in the absence of repeated ethanol injections, mice displayed a sesitized locomotor response following an injection of a low dose of ethanol. Figure 3B shows the location of cannulae hits (marked with a filled circle) and misses (marked with an X). Figure 3C shows that TSA-treated animals, following an injection of ethanol in the absense of an injection of TSA, exhibited significantly augmented locomotor activity during the first five minutes of the final test session relative to mice previously treated with repeated injections of vehicle ($t(13) = -0.2379$, $p=0.003$). Figure 3D shows the locomotor activity of both vehicle-treated and TSA-treated animals over the entire 20-min locomotor test session during all four test sessions (Initial Test, Final Test, Saline Retest, EtOH Retest). A repeated measures ANOVA performed on the data shown in Figure 4B revealed a significant drug x test day interaction ($F(3,39)=4.669$, $p=0.007$), as well as a between-subjects effect of treatment ($F(1,13)=5.747$, $p=0.032$) on the amount of locomotor activity displayed by these mice during the 20 min testing sessions. Post-hoc analyses confirmed that TSA-treated animals displayed significantly elevated locomotor activity on both the final ($p=0.012$) and ethanol retest ($p=0.009$) test days relative to the initial test day; no significant differences in locomotor activity between test days were detected in vehicle-treated animals. Importantly, TSA-treated mice did not show significantly elevated locomotor activity on the saline retest day relative to the initial test day, indicating that the repeated TSA treatment did not have an effect on general locomotor activity. Further, locomotor data from animals with bilateral

incorrect cannulae placement (that is, both cannulae in the vicinity of the nucleus accumbens shell, but not within the structure; see Figure 3B) were also analyzed. TSA-treated mice with cannulae outside of the NACSH did not differ in locomotor activity during the first five minutes of the final test session relative to vehicle-treated animals ($t(8)= -0.603$, $p=0.568$). Further, a repeated measures ANOVA over the four test sessions confirmed that there was no drug x test day interaction ($F(3,24)=0.559$; $p=0.647$), as well as no main effect of drug treatment ($F(1,6)=0.586$; $p=0.462$) in these animals.

Experiment 4: Site-directed infusion of TSA leads to augmented acH3K9 IR in the nucleus accumbens shell

Figure 4A shows the placement of cannulae that successfully targeted the shell of the nucleus accumbens (marked with filled circles). As the goal of this experiment was to examine changes in the expression patterns of acH3K9 IR following drug treatment, IHC analysis was performed on each cannula placement site individually, and any cannulae outside of the target region was removed from all analysis (data not shown). Figure 4B shows representative photomicrographs of acH3K9 IR from a vehicle-treated (left) and TSA-treated (right) animal. The quantification of acH3K9 IR the shell of the nucleus accumbens is shown in Figure 4C. Mice that received an infusion of TSA approximately 4 hours prior to showed significantly augmented acH3K9 IR relative to mice that that received an infusion of vehicle ($t(10)= -3.408$, $p=0.007$).

DISCUSSION

The goal of the present study was to further explore the role of chromatin remodeling in the modulation of ethanol-induced locomotor sensitization and to explore the possibility that TSA, a potent HDAC Class I and II inhibitor, may display cross-sensitization to the locomotor stimulant effect of ethanol. To this end, we used immunohistochemical and site-directed pharmacological techniques to first examine the role of histone acetylation in this behavioral phenomenon. Immunohistochemical analysis of acetylation at histone 3 lysine 9 (acH3K9) revealed a significant increase in acH3K9 within the nucleus accumbens shell in sensitized animals; this effect was region-specific, as no increases were detected in any other region examined, including the core of the nucleus accumbens. Further, western blot analysis of acH3K9 in a separate group of sensitized animals confirmed that only the nucleus accumbens displayed this increase, as no between-groups differences in acH3K9 were detected in any other region examined. Additionally, this increase in acetylation was specific to H3K9, as acetylation of H4K8 (another core histone protein often associated with transcriptional activation) remained unchanged by the repeated ethanol injections as part of the behavioral sensitization paradigm (see Supplemental Material Table S1).

Given this specific upregulation of acH3K9 within the shell of the nucleus accumbens following sensitization, we sought to examine whether hyperacetylation within this structure would be sufficient to elicit a cross-sensitized response to the locomotor stimulating effects of ethanol in the absence of repeated ethanol exposure. Indeed, this report presents the first evidence, to our knowledge, that the repeated induction of hyperacetylation specifically within the shell of the nucleus accumbens in the absence of ethanol is sufficient to induce a

sensitized locomotor response to a later peripheral injection of 2.0 g/kg ethanol. Importantly, this was not a side effect of the repeated infusion procedure, as mice that received repeated infusions of vehicle did not show this cross-sensitized response. Additionally, mice that received repeated infusions of TSA into neighboring regions did not show this sensitized effect, again suggesting a particular importance of increased histone acetylation the NACSH in modulating ethanol-induced locomotor sensitization. In order to confirm that the dose of TSA utilized in this protocol was indeed sufficient to alter levels of acH3K9 within the region of interest, a separate group of animals with cannulae targeting the NACSH received an infusion of TSA or vehicle; here, the dose of TSA (22 mM) used to elicit cross-sensitization to the locomotor stimulant effects of ethanol was shown to augment the level of acH3K9 IR detected within the shell of the nucleus accumbens. This is consistent with numerous studies demonstrating a rapid and large accumulation of acetylation on H3K9, H3K5, and H4K8, among others (Yoshida et al., 1990, Bartova et al., 2008).

These findings are particularly enlightening as they not only highlight a role for the ventral, rather than the dorsal, striatum in ethanol-induced locomotor sensitization as previously described (Sanchis-Segura et al., 2009), but also indicate that hyperacetylation within a particular subregion of the NAC may be particularly important in modulating this behavioral phenomenon. Indeed, the nucleus accumbens is a well-studied reward center involved in psychomotor sensitization (Robinson and Berridge, 2000, Abrahao et al., 2011, Pierce and Kalivas, 1997). The present data fall in line with previous showing that nucleus accumbens subregions differentially respond to drug-induced locomotor sensitization (Cadoni et al., 2008, Cadoni et al., 2000) and that rats demonstrating amphetamine-induced sensitization show an increase in dopamine firing specifically in neurons innervating the medial shell, but not the core, of the NAC (Lodge and Grace, 2011, Ikemoto, 2007). It is important to note that although there are clear structural differences between the subregions of the accumbens, evidence has also shown that there is a widespread network of intra-accumbal projection patterns (van Dongen et al., 2005). Thus given previous evidence for acH4 alterations in the core (Botia et al., 2012), as well as the current alterations in acH3K9 within the shell that are associated with ethanol-induced locomotor sensitization, it is likely that complex signaling pathways both within and extending from this region play a critical role in modulating this complex behavioral phenomenon.

Our findings also confirm the inability of low-dose repeated ethanol injections to induce changes in the expression of acetylated H3 within the dorsal striatum (Sanchis-Segura et al., 2009), and are particularly interesting in light of recent evidence showing that treatment with an HDACi under a similar sensitization protocol blocked the induction and blunted the expression of ethanol-induced locomotor sensitization (Legastelois et al., 2013). Importantly, this previous study used the HDACi sodium butyrate (NaB), while the current studies used TSA. These drugs fall into different structural classes (NaB is an aliphatic acid, while TSA a hydroxyamate) and have different mechanisms of action (Dokmanovic et al., 2007). Further, TSA has been shown to be a more potent and selective HDACi than NaB (Yoshida et al., 1990, Davie, 2003). Additionally, Legastelois et al. gave peripheral injections of NaB, and while peripheral administration of TSA shows no changes in behavioral sensitization (see Supplemental Material, Figures S1 & S2), global changes in acetylation could be obscuring the ability to observe changes similar to those reported here

following site-directed administration. Such divergent results, therefore, may be attributable to regional versus global changes in histone acetylation.

The role of chromatin remodeling in drug-induced sensitization has remained unclear despite an abundance of recent work, as treatment with several HDACi have been shown to both augment (Sanchis-Segura et al., 2009) or blunt (Kalda et al., 2007, Moretti et al., 2011) the expected sensitized response to several drugs of abuse, including ethanol. Thus the intent of the present study was to clarify these discrepancies, particularly in relation to ethanol-induced locomotor sensitization. Additional inconsistencies between this and previous studies may be related to a number of different factors including ethanol dose, treatment regimen, as well as species, strain and sex. Further, it is known that not only does ethanol itself exert differential effects on discrete brain regions (Pascual et al., 2009) but also that histone modifications suggested to underlie predispositions to anxiety and alcoholism (Moonat et al., 2013) are also very region-specific. Given these data, it is not surprising that site-directed HDACi treatment, such as that utilized in the present study, may confer different patterns of behavior than peripherally administered treatments (Sanchis-Segura et al., 2009, Legastelois et al., 2013). Additionally, it has been reported that HDAC inhibition by TSA can cause acetylation of non-histone proteins, such as transcriptional co-factors like p300 and CBP (Das and Kundu, 2005), and thus the off target effects of different HDAC inhibitors may represent an additional level of difficulty when comparing these studies and interpreting results.

Importantly, the present analyses measured gross alterations in acH3K9; thus we do not know which genes are directly influenced by the observed changes. Recent work has indicated a number of genes, including *dnmt1*, *esco2*, and *rps6ka5*, that may contribute to individual susceptibility to ethanol-induced behavioral sensitization (Botia et al., 2012). Further investigation is required to determine whether the changes in acH3K9 observed in the present study directly influence the expression of these and other epigenetically-regulated genes. Additionally, as a number of neuropeptides, including corticotrophin releasing factor and neuropeptide Y (Fee et al., 2007, Pastor et al., 2012, Hayes et al., 2012), and several neurotransmitter systems (Taylor et al., 2007, Robinson and Berridge, 2000, Kelly and Iversen, 1976, Vanderschuren and Kalivas, 2000) within the striatum play critical roles in drug-induced locomotor sensitization, determination of whether the regulation of genes underlying the expression of these neuropeptides and neurotransmitter systems is influenced by acH3K9 will be critical in understanding the development of ethanol-induced locomotor sensitization. This behavioral phenomenon is believed to be reflective of neuroadaptations underlying the transition to drug and alcohol dependence; thus investigation into these mechanisms may provide valuable information that will guide the development of novel pharmacological targets aimed at treating alcoholism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank the members of the Thiele laboratory for their support of the experiments described herein as well as helpful discussion of the manuscript. This work was supported by National Institute of Health grants AA019839 (GMS), AA021611 (JAR), AA013573 (TET), and AA015148 (TET).

Funding Sources: National Institute of Health grants AA019839 (GMS), AA021611 (JAR), AA013573 (TET), AA015148 (TET), and AA022048 (TET).

References

ABRAHAO KP, QUADROS IM, SOUZA-FORMIGONI ML. Nucleus accumbens dopamine D1 receptors regulate the expression of ethanol-induced behavioural sensitization. Int J Neuropsychopharmacol. 2011; 14:175–85. [PubMed: 20426882]

BARTOVA E, KREJCI J, HARNICAROVA A, GALIOVA G, KOZUBEK S. Histone modifications and nuclear architecture: a review. J Histochem Cytochem. 2008; 56:711–21. [PubMed: 18474937]

BOTIA B, LEGASTELOIS R, ALAUX-CANTIN S, NAASSILA M. Expression of ethanol-induced behavioral sensitization is associated with alteration of chromatin remodeling in mice. PLoS One. 2012; 7:e47527. [PubMed: 23110077]

CADONI C, SOLINAS M, DI CHIARA G. Psychostimulant sensitization: differential changes in accumbal shell and core dopamine. Eur J Pharmacol. 2000; 388:69–76. [PubMed: 10657548]

CADONI C, VALENTINI V, DI CHIARA G. Behavioral sensitization to delta 9-tetrahydrocannabinol and cross-sensitization with morphine: differential changes in accumbal shell and core dopamine transmission. J Neurochem. 2008; 106:1586–93. [PubMed: 18513369]

CUBERO I, NAVARRO M, CARVAJAL F, LERMA-CABRERA JM, THIELE TE. Ethanol-induced increase of agouti-related protein (AgRP) immunoreactivity in the arcuate nucleus of the hypothalamus of C57BL/6J, but not 129/SvJ, inbred mice. Alcohol Clin Exp Res. 2010; 34:693–701. [PubMed: 20102560]

DAS C, KUNDU TK. Transcriptional regulation by the acetylation of nonhistone proteins in humans -- a new target for therapeutics. IUBMB Life. 2005; 57:137–49. [PubMed: 16036576]

DAVIE JR. Inhibition of histone deacetylase activity by butyrate. J Nutr. 2003; 133:2485S–2493S. [PubMed: 12840228]

DOKMANOVIC M, CLARKE C, MARKS PA. Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res. 2007; 5:981–9. [PubMed: 17951399]

EBERHARTER A, BECKER PB. Histone acetylation: a switch between repressive and permissive chromatin. Second in review series on chromatin dynamics. EMBO Rep. 2002; 3:224–9. [PubMed: 11882541]

FEE JR, KNAPP DJ, SPARTA DR, BREESE GR, PICKER MJ, THIELE TE. Involvement of protein kinase A in ethanol-induced locomotor activity and sensitization. Neuroscience. 2006; 140:21–31. [PubMed: 16529875]

FEE JR, SPARTA DR, PICKER MJ, THIELE TE. Corticotropin releasing factor-1 receptor antagonist, CP-154,526, blocks the expression of ethanol-induced behavioral sensitization in DBA/2J mice. Neuroscience. 2007; 150:14–21. [PubMed: 17919825]

FRANKLIN, KBJ.; PAXINOS, G. The Mouse Brain in Stereotaxic Coordinates. San Diego, CA: Academic Press; 1997.

HAYES DM, FEE JR, MCCOWN TJ, KNAPP DJ, BREESE GR, CUBERO I, CARVAJAL F, LERMA-CABRERA JM, NAVARRO M, THIELE TE. Neuropeptide Y signaling modulates the expression of ethanol-induced behavioral sensitization in mice. Addict Biol. 2012

HAYES DM, KNAPP DJ, BREESE GR, THIELE TE. Comparison of basal NPY and CRF levels between the high ethanol drinking C57BL/6J and low ethanol drinking DBA/2J inbred mouse strains. Alcohol Clin Exp Res. 2005; 29:721–729. [PubMed: 15897715]

IKEMOTO S. Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. Brain Res Rev. 2007; 56:27–78. [PubMed: 17574681]

KALDA A, HEIDMETS LT, SHEN HY, ZHARKOVSKY A, CHEN JF. Histone deacetylase inhibitors modulates the induction and expression of amphetamine-induced behavioral sensitization partially through an associated learning of the environment in mice. Behav Brain Res. 2007; 181:76–84. [PubMed: 17477979]

KELLY PH, IVERSEN SD. Selective 6OHDA-induced destruction of mesolimbic dopamine neurons: abolition of psychostimulant-induced locomotor activity in rats. Eur J Pharmacol. 1976; 40:45–56. [PubMed: 1033072]

KUMAR A, CHOI KH, RENTHAL W, TSANKOVA NM, THEOBALD DE, TRUONG HT, RUSSO SJ, LAPLANT Q, SASAKI TS, WHISTLER KN, NEVE RL, SELF DW, NESTLER EJ. Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. Neuron. 2005; 48:303–14. [PubMed: 16242410]

LEE DK, OH JH, YANG JH, YOUN B, SHIM YB, SHIM I, WANG JQ, CHOE ES. Protein kinase G linked to dopamine D3 receptors in the dorsal striatum controls dopamine release, DeltaFosB expression and locomotor activity after repeated cocaine administration. Neurosci Lett. 2013; 541:120–5. [PubMed: 23428510]

LEGASTELOIS R, BOTIA B, NAASSILA M. Blockade of ethanol-induced behavioral sensitization by sodium butyrate: descriptive analysis of gene regulations in the striatum. Alcohol Clin Exp Res. 2013; 37:1143–53. [PubMed: 23488934]

LESSOV CN, PHILLIPS TJ. Duration of sensitization to the locomotor stimulant effects of ethanol in mice. Psychopharmacology. 1998; 135:374–382. [PubMed: 9539262]

LODGE DJ, GRACE AA. Divergent activation of ventromedial and ventrolateral dopamine systems in animal models of amphetamine sensitization and schizophrenia. Int J Neuropsychopharmacol. 2011:1–8.

LOWERY-GIONTA EG, NAVARRO M, LI C, PLEIL KE, RINKER JA, COX BR, SPROW GM, KASH TL, THIELE TE. Corticotropin releasing factor signaling in the central amygdala is recruited during binge-like ethanol consumption in C57BL/6J mice. J Neurosci. 2012; 32:3405–13. [PubMed: 22399763]

MOONAT S, SAKHARKAR AJ, ZHANG H, TANG L, PANDEY SC. Aberrant histone deacetylase2-mediated histone modifications and synaptic plasticity in the amygdala predisposes to anxiety and alcoholism. Biol Psychiatry. 2013; 73:763–73. [PubMed: 23485013]

MORETTI M, VALVASSORI SS, VARELA RB, FERREIRA CL, ROCHI N, BENEDET J, SCAINI G, KAPCZINSKI F, STRECK EL, ZUGNO AI, QUEVEDO J. Behavioral and neurochemical effects of sodium butyrate in an animal model of mania. Behav Pharmacol. 2011; 22:766–72. [PubMed: 21989497]

NAVARRO M, CUBERO I, KNAPP DJ, BREESE GR, THIELE TE. Decreased immunoreactivity of the melanocortin neuropeptide alpha-melanocyte-stimulating hormone (alpha-MSH) after chronic ethanol exposure in Sprague-Dawley rats. Alcohol Clin Exp Res. 2008; 32:266–76. [PubMed: 18162070]

NEWLIN DB, THOMSON JB. Chronic tolerance and sensitization to alcohol in sons of alcoholics. Alcohol Clin Exp Res. 1991; 15:399–405. [PubMed: 1877726]

NONA CN, GUIRGUIS S, NOBREGA JN. Susceptibility to ethanol sensitization is differentially associated with changes in pCREB, trkB and BDNF mRNA expression in the mouse brain. Behav Brain Res. 2013; 242:25–33. [PubMed: 23291223]

PANDEY SC, UGALE R, ZHANG H, TANG L, PRAKASH A. Brain chromatin remodeling: a novel mechanism of alcoholism. J Neurosci. 2008; 28:3729–37. [PubMed: 18385331]

PASCUAL M, BOIX J, FELIPO V, GUERRI C. Repeated alcohol administration during adolescence causes changes in the mesolimbic dopaminergic and glutamatergic systems and promotes alcohol intake in the adult rat. J Neurochem. 2009; 108:920–31. [PubMed: 19077056]

PASCUAL M, DO COUTO BR, ALFONSO-LOECHES S, AGUILAR MA, RODRIGUEZ-ARIAS M, GUERRI C. Changes in histone acetylation in the prefrontal cortex of ethanol-exposed adolescent rats are associated with ethanol-induced place conditioning. Neuropharmacology. 2012; 62:2309–19. [PubMed: 22349397]

PASTOR R, REED C, MEYER PJ, MCKINNON C, RYABININ AE, PHILLIPS TJ. Role of corticotropin-releasing factor and corticosterone in behavioral sensitization to ethanol. J Pharmacol Exp Ther. 2012; 341:455–63. [PubMed: 22333290]

PIERCE RC, KALIVAS PW. A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. Brain Res Brain Res Rev. 1997; 25:192–216. [PubMed: 9403138]

RENTHAL W, NESTLER EJ. Epigenetic mechanisms in drug addiction. Trends Mol Med. 2008; 14:341–50. [PubMed: 18635399]

ROBINSON TE, BERRIDGE KC. The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Brain Res Rev. 1993; 18:247–91. [PubMed: 8401595]

ROBINSON TE, BERRIDGE KC. The psychology and neurobiology of addiction: an incentive-sensitization view. Addiction. 2000; 95(Suppl 2):S91–117. [PubMed: 11002906]

ROBINSON TE, BERRIDGE KC. Incentive-sensitization and addiction. Addiction. 2001; 96:103–14. [PubMed: 11177523]

ROBINSON TE, BERRIDGE KC. Addiction. Annu Rev Psychol. 2003; 54:25–53. [PubMed: 12185211]

SAKHARKAR AJ, ZHANG H, TANG L, SHI G, PANDEY SC. Histone Deacetylases (HDAC)-Induced Histone Modifications in the Amygdala: A Role in Rapid Tolerance to the Anxiolytic Effects of Ethanol. Alcoholism: Clinical and Experimental Research. 2011

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; 34:2642–54. [PubMed: 19727068]

SCHROEDER FA, PENTA KL, MATEVOSSIAN A, JONES SR, KONRADI C, TAPPER AR, AKBARIAN S. Drug-Induced Activation of Dopamine D1 Receptor Signaling and Inhibition of Class I//II Histone Deacetylase Induce Chromatin Remodeling in Reward Circuitry and Modulate Cocaine-Related Behaviors. Neuropsychopharmacology. 2008; 33:2981–2992. [PubMed: 18288092]

SPARROW AM, LOWERY-GIONTA EG, PLEIL KE, LI C, SPROW GM, COX BR, RINKER JA, JIJON AM, PENA J, NAVARRO M, KASH TL, THIELE TE. Central neuropeptide Y modulates binge-like ethanol drinking in C57BL/6J mice via Y1 and Y2 receptors. Neuropsychopharmacology. 2012; 37:1409–21. [PubMed: 22218088]

TAYLOR JR, LYNCH WJ, SANCHEZ H, OLAUSSON P, NESTLER EJ, BIBB JA. Inhibition of Cdk5 in the nucleus accumbens enhances the locomotor-activating and incentive-motivational effects of cocaine. Proc Natl Acad Sci U S A. 2007; 104:4147–52. [PubMed: 17360491]

VAN DONGEN YC, DENIAU JM, PENNARTZ CM, GALIS-DE GRAAF Y, VOORN P, THIERRY AM, GROENEWEGEN HJ. Anatomical evidence for direct connections between the shell and core subregions of the rat nucleus accumbens. Neuroscience. 2005; 136:1049–71. [PubMed: 16226842]

VANDERSCHUREN LJ, KALIVAS PW. Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. Psychopharmacology (Berl). 2000; 151:99–120. [PubMed: 10972458]

VECSEY CG, HAWK JD, LATTAL KM, STEIN JM, FABIAN SA, ATTNER MA, CABRERA SM, MCDONOUGH CB, BRINDLE PK, ABEL T, WOOD MA. Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. J Neurosci. 2007; 27:6128–40. [PubMed: 17553985]

WHITE FJ, KALIVAS PW. Neuroadaptations involved in amphetamine and cocaine addiction. Drug Alcohol Depend. 1998; 51:141–53. [PubMed: 9716936]

WONG CC, MILL J, FERNANDES C. Drugs and addiction: an introduction to epigenetics. Addiction. 2010; 106:480–9. [PubMed: 21205049]

YOSHIDA M, KIJIMA M, AKITA M, BEPPU T. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem. 1990; 265:17174–9. [PubMed: 2211619]

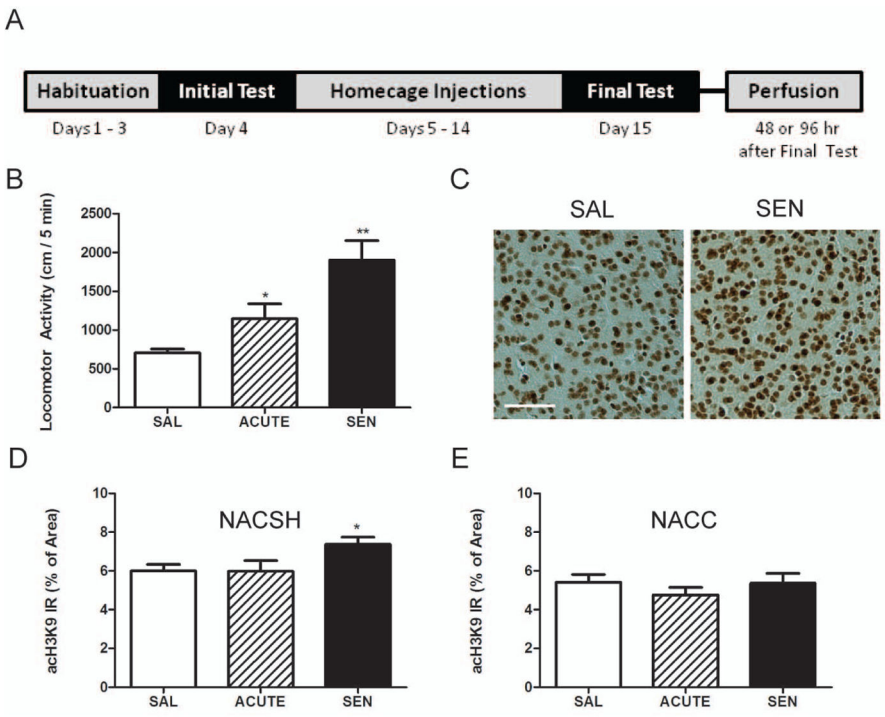

Figure 1. DBA/2J mice develop robust locomotor sensitization and exhibit region-specific differences in acH3K9 IR

(A) Timeline of Experiment 1. (B) Sensitized (SEN) mice exhibited significantly augmented locomotor activity in the first five minutes of the final test session of Experiment 1 relative to both saline-treated (SAL) and acute ethanol (ACUTE) mice, indicative of ethanol-induced locomotor sensitization. (C) Representative photomicrographs of acH3K9 IR in the shell of the nucleus accumbens of SAL (left) and SEN (right) mice. Images photographed with a 20x objective; scale bar = 50 μm. Quantification of acH3K9 immunoreactivity revealed that SEN mice displayed significantly augmented acH3K9 IR in the shell (D) but not the core (E) of the nucleus accumbens. All values are reported as means + SEM. *$p<0.05$ vs. SAL mice; **$p<0.01$ vs. SAL and ACUTE mice.

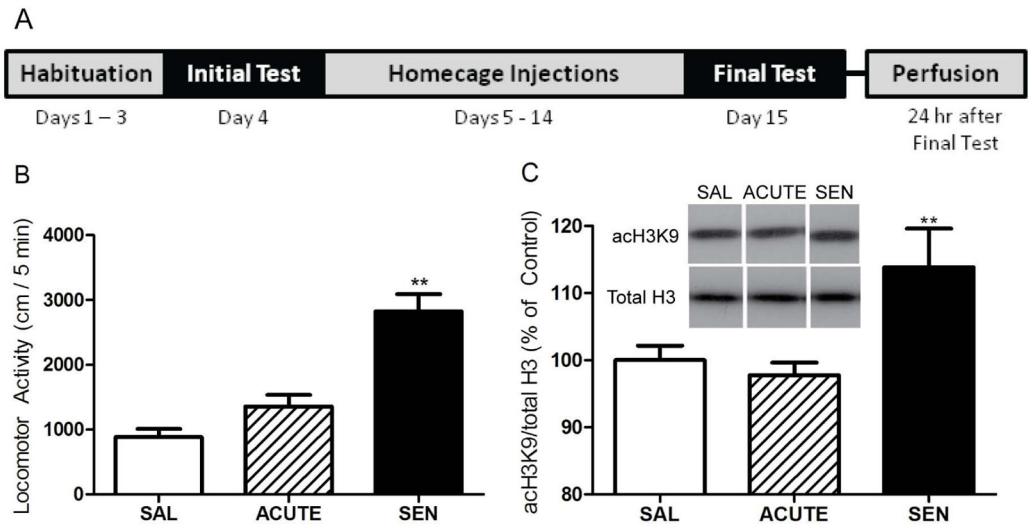

Figure 2. Western blot analysis confirms that sensitized mice display augmented acH3K9 IR in the nucleus accumbens

(A) Timeline of Experiment 2. (B) Sensitized (SEN) mice exhibited significantly augmented locomotor activity in the first five minutes of Experiment 2 relative to both saline-treated (SAL) and acute ethanol (ACUTE) mice. (C) Western blot (representative photomicrographs, inset) revealed that SEN mice displayed an augmented expression of acH3K9 (normalized to total H3, shown here as a percentage of SAL control), relative to both SAL and ACUTE mice, confirming the immunohistochemical results detected in Experiment 1. Western blot images taken from non-adjacent lanes from the same gel. All values are reported as mean + SEM; **p<0.01 vs. SAL and ACUTE.

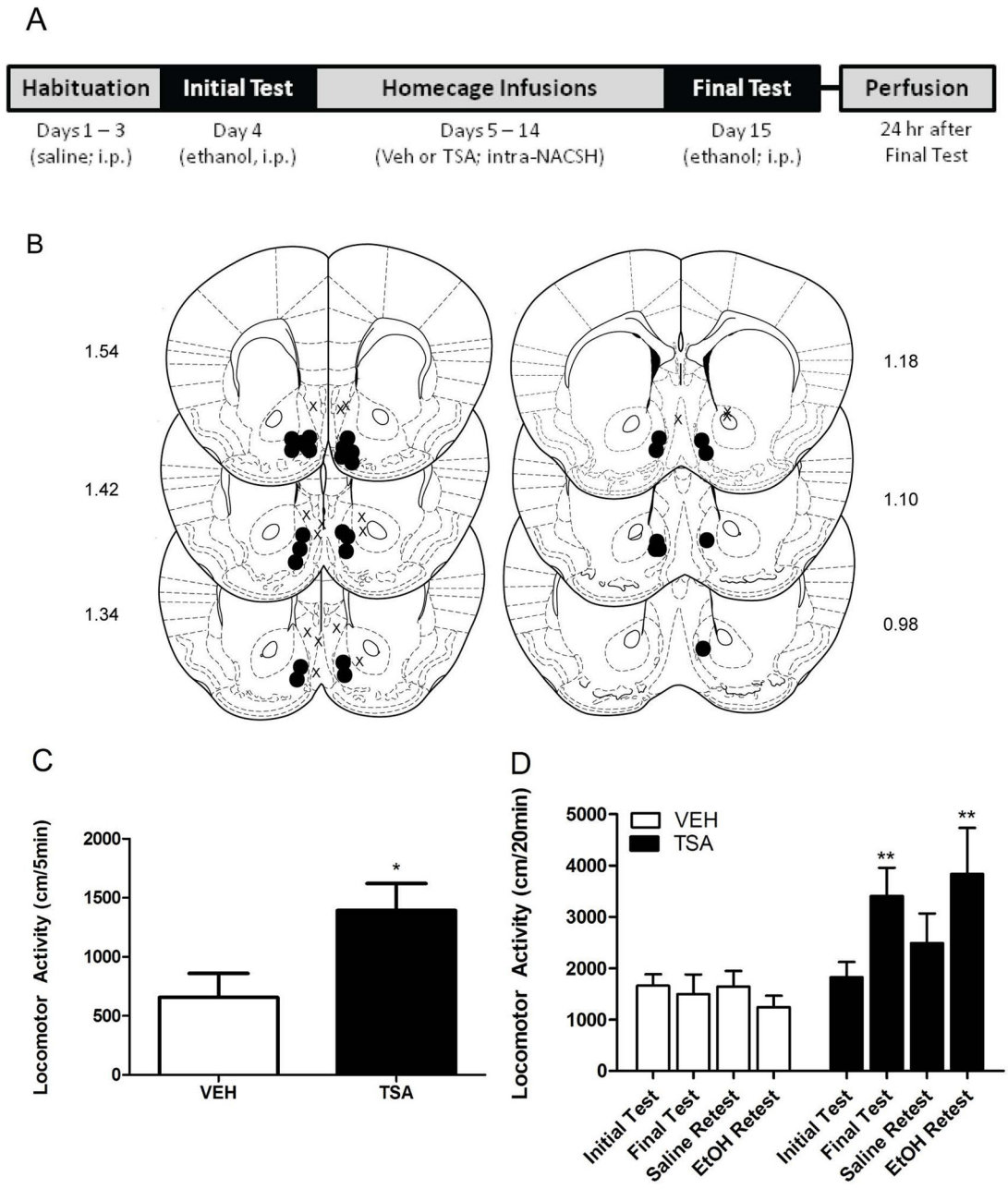

Figure 3. Repeated infusion of TSA into the shell of the nucleus accumbens is sufficient to induce cross-sensitization to the locomotor stimulant effects of ethanol

(A) Timeline of Experiment 3. (B) Representative schematic showing correct (filled circle) and incorrect (x) cannulae placements. Numbers indicate distance from bregma; coronal slice figures adapted from (Franklin and Paxinos, 1997). (C) Mice that received repeated infusions of 22 mM TSA into the shell of the nucleus accumbens displayed a significantly augmented locomotor response to a later injection of a low dose of ethanol (2.0 g/kg), particularly within the first five minutes of the locomotor testing session. (D) Analysis of 20 min locomotor activity over all four testing sessions revealed a treatment by test session interaction. Additionally, mice that received repeated intra-accumbal shell infusions of TSA

exhibited significantly augmented locomotor activity during the 20 min Final and EtOH Retest sessions relative to their Initial Test session; this augmentation of locomotor activity was not detected in vehicle-treated animals. Importantly, TSA-treated animals did not show elevated locomotor activity following an injection of saline. All data are shown as mean + SEM. *$p<0.05$ vs. Veh; **$p<0.05$ vs. initial test.

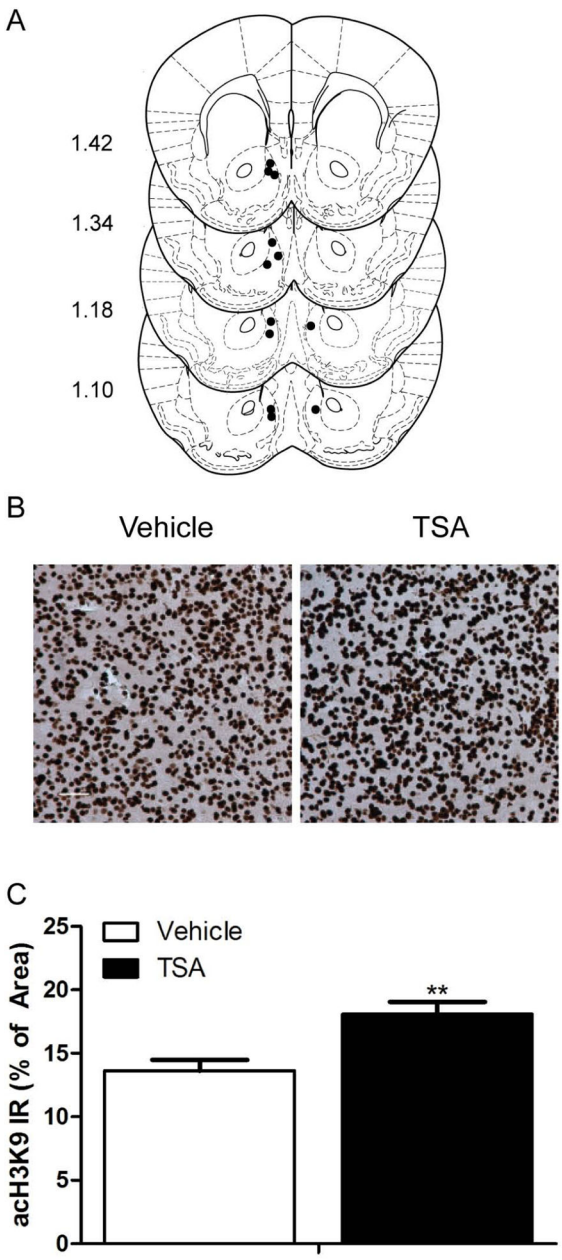

Figure 4. TSA infusions in the shell of the nucleus accumbens results in an increase in acH3K9 IR in the NACSH

(A) Representative schematic showing correct (filled circle) cannulae placements. Numbers Indicate distance of coronal segment from bregma; schematic is adapted from (Franklin and Paxinos, 1997). (B) Representative photomicrographs of acH3K9 IR in the NACSH from a vehicle-treated (left) and TSA-treated (right) mouse. Images photographed with a 20x objective; scale bar = 50 μm. (C) Quantification of acH3K9 immunoreactivity revealed that TSA-treated mice displayed significantly augmented acH3K9 IR in the shell of the nucleus accumbens relative to vehicle-treated mice. Values are reported as mean + SEM. **p<0.01 vs. Vehicle.

Table 1

Summary of IHC (A) and Western Blot (B) data following ethanol-induced locomotor sensitization (Experiments 1 & 2).

A		NACSH	NACC	DLS	DMS	BLA	CEA	PFC	VTA
IHC Analysis acH3K9-IR	Saline	6.006 ±0.334	5.430 ±0.389	7.603 ±0.412	5.978 ±0.454	3.651 ±0.833	5.004 ±0.625	5.144 ±0.411	4.727 ±0.539
	NS	5.984 ±0.547	4.759 ±0.396	5.845 ±0.637	4.463 ±0.511	3.329 ±0.446	4.077 ±0.658	4.223 ±0.643	4.580 ±0.905
	SEN	7.367 ±0.377	5.370 ±0.511	6.579 ±0.587	5.451 0.817	3.625 ±0.393	4.272 ±0.603	5.1141 ±0.556	4.899 ±0.670
	F-statistic	*3.309*	*0.669*	*2.235*	*1.369*	*0.106*	*0.573*	*0.867*	*0.051*
	P-value	*0.049*	*0.519*	*0.122*	*0.268*	*0.900*	*0.570*	*0.430*	*0.951*

B		NAC	STR	PFC	VTA
Western Blot Analysis H3K9-IR	Saline	100.0 ±1.814	100.0 ±5.831	100.0 ±7.023	100.0 ±16.228
	NS	102.014 ±2.387	101.2 ±7.476	100.22 ±4.563	71.6969 ±8.310
	SEN	112.754 ±3.246	89.598 ±7.074	112.15 ±4.889	84.170 ±12.177
	F-statistic	*7.239*	*0.843*	*1.464*	*1.286*
	P-value	*0.003*	*0.441*	*0.248*	*0.292*

Summary of IHC and western blotting data (Experiments 1 & 2). Data are listed as mean + SEM; bolded values indicate significant group differences ($p<0.05$). NS, non-sensitized; SEN, sensitized; NAC, nucleus accumbens; NACSH, nucleus accumbens shell; NACC, nucleus accumbens core; STR; striatum; DLS, dorsolateral striatum; DMS, dorsomedial striatum; BLA, basolateral amygdala; CEA, central nucleus of the amygdala; PFC, prefrontal cortex; VTA, ventral tegmental area; acH3, acetylated histone H3.