Ethanol-Induced Locomotor Sensitization: NPY Signaling and Histone Acetylation in the Nucleus Accumbens and Striatum of DBA/2J Mice

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ABSTRACT

Gretchen M. Sprow: Ethanol-Induced Locomotor Sensitization: NPY Signaling and Histone Acetylation in the Nucleus Accumbens and Striatum of DBA/2J Mice
(Under the direction of Dr. Todd E. Thiele)

Recent evidence indicates that neuropeptide Y (NPY) signaling modulates ethanol-induced locomotor sensitization. Additionally, a growing body of literature suggests that epigenetic mechanisms, including histone acetylation, may play important roles in drug addiction. In the present study, we used immunohistochemical techniques to investigate the expression of both NPY and the acetylation of histones H3 and H4 in the nucleus accumbens and striatum following the induction of locomotor sensitization. Mice sensitized to the locomotor stimulant effects of ethanol displayed increases in NPY in the dorsolateral striatum and increases in acetylated H3 in the nucleus accumbens shell. Interestingly, mice that received an acute injection of ethanol displayed decreases in NPY specific to the core of the nucleus accumbens. Finally, peripheral administration of Tricostatin A, a histone deacetylase inhibitor, augmented the acquisition, but not the expression, of locomotor sensitization. The present observations indicate involvement of NPY and histone acetylation in ethanol-induced locomotor sensitization.

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LIST OF ABBREVIATIONS

acH3	Acetylated histone 3
acH4	Acetylated histone 4
ANOVA	Analysis of variance
DLS	Dorsolateral striatum
DMS	Dorsomedial striatum
g/kg	Grams per kilogram
H3	Histone 3
H4	Histones 4
HC	Homecage
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
IR	Immunoreactivity
mg/kg	Milligrams per kilogram
NaBut	Sodium butyrate
NPY	Neuropeptide Y
TSA	Trichostatin A
VLS	Ventrolateral striatum
VMS	Ventromedial striatum

CHAPTER 1

INTRODUCTION

Behavioral sensitization has long been investigated as an important mechanism that may explain the transition to drug dependence (Robinson and Berridge 1993; Robinson and Berridge 2000; Robinson and Berridge 2001). 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). 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 (Newlin and Thomson 1991; Lessov and Phillips 1998). In the current experiments, we have attempted to clarify pieces of the mechanism underlying this striking phenomenon.

Neuropeptide Y (NPY) is a 36-amino acid neuromodulator prevalent throughout numerous brain structures, including regions of the mesolimbic dopaminergic pathway (Gray and Morley 1986; Dumont, Martel et al. 1992)**.** NPY has been implicated in a number of neurobiological responses to ethanol, including consumption, sedation, and self-administration (Thiele, Marsh et al. 1998; Thiele, Sparta et al. 2003; Thorsell, O'Dell et al. 2003). Levels of NPY are differentially influenced by acute and chronic ethanol exposure, as well as ethanol withdrawal (Kinoshita, Jessop et al. 2000; Roy and Pandey 2002; Thorsell, Slawecki et al. 2005). Additionally, data from our laboratory has suggested a link between central NPY signaling and ethanol-induced locomotor sensitization, as not only do NPY

knockout mice show reduced ethanol-induced behavioral sensitization relative to wild-type littermates, but blunted endogenous NPY signaling selectively in the nucleus accumbens core attenuates the expression of locomotor sensitization (Hayes, Fee et al. in press). Further, RIIβ-/- mice, previously shown to exhibit increased sensitivity to ethanol-induced locomotor activity and behavioral sensitization, exhibit sub-region-specific increases in NPY immunoreactivity (IR) in the nucleus accumbens and striatum (Fee, Knapp et al. 2006; Hayes, Fee et al. in press). As NPY is prevalent in many brain regions implicated in drug- and ethanol-induced locomotor sensitization, and has known involvement in a great number of neurobiological responses to ethanol, the role of NPY in ethanol-induced locomotor sensitization was further characterized in the present investigation.

Drug abuse research has traditionally focused on the role of genetics in drug and alcohol addiction. Recent work, however, has shifted to investigating epigenetic mechanisms that may underlie the development and maintenance of this devastating disorder (Renthal and Nestler 2008; Wong, Mill et al. 2010). Such mechanisms induce changes in phenotype and/or gene expression through remodeling of chromatin structure via DNA methylation or post-translational histone modifications, including acetylation and phosphorylation. While methylation is typically associated with a decrease in gene expression, increased acetylation of core histones H3 and H4, in particular, has been shown to induce a looser chromatin structure, leading to an increase in gene expression; deacetylation of these histones leads to a more compact structure, thereby decreasing gene expression (Eberharter and Becker 2002).

Drugs of abuse, including cocaine, amphetamine, and ethanol, have been shown to dynamically regulate histone modification in animals (Kumar, Choi et al. 2005; Renthal and Nestler 2008; Sanchis-Segura, Lopez-Atalaya et al. 2009). D2-like receptor antagonists

induce phosphoacetylation of histone H3 in striatal chromatin (Li, Guo et al. 2004) and repeated administration of ethanol during adolescence leads to changes in the acetylation of histones H3 and H4 in several regions, including the nucleus accumbens and striatum (Pascual, Boix et al. 2009). Further evidence suggests an epigenetic association between paternal ethanol exposure and decreased fetal weight in Sprague-Dawley rats (Dawn, Fadi et al. 2002). Elevated DNA methylation has also been found in human alcoholic patients, and class I alcohol dehydrogenase genes have been shown to be differentially regulated by both DNA methylation and histone deacetylation (Luke, Hui-Ju et al. 2006). Withdrawal from chronic ethanol has also 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 (Pandey, Ugale et al. 2008). Indeed, data from that study suggests a relationship between histone acetylation and NPY expression, as peripheral administration of Trichostatin A, an HDAC inhibitor (HDACi), not only increased the amount of H3 and H4 acetylation seen in ethanol withdrawn rats following chronic ethanol exposure, but also rescued withdrawal-induced deficits in NPY mRNA and protein expression (Pandey, Ugale et al. 2008). Given these data, it is possible that histone acetylation may play a role in other neurobiological responses to ethanol mediated by NPY.

Further evidence suggests that chromatin remodeling plays a key role in behavioral sensitization. Co-treatment with a D1/D5 agonist and the nonspecific HDACi sodium butyrate (NaBut) synergistically increased cocaine-induced locomotor sensitization (Schroeder, Penta et al. 2008). Additionally, acute cocaine is associated with acetylation of H4, while chronic cocaine is associated with changes in H3 acetylation associated with specific gene promoters in the striatum (Kumar, Choi et al. 2005). A dose of NaBut with no

intrinsic locomotor effects also nearly doubled the locomotor stimulant response to cocaine. Finally, a separate study confirmed that NaBut enhances cocaine-induced locomotor sensitization, and that a similar enhancement of activity is seen with morphine- and ethanol-induced locomotor sensitization. (Sanchis-Segura, Lopez-Atalaya et al. 2009).

Chromatin remodeling is affected by many drugs of abuse, including ethanol. Additionally, manipulation of histone acetylation using a potent HDACi has been shown to not only alter the expression of NPY, a neuropeptide implicated in ethanol-induced locomotor sensitization, but also to influence NPY-mediated behaviors following withdrawal from ethanol exposure. The goal of the present experiments was two-fold. The first experiment sought to examine expression patterns of both NPY protein and core histones H3 and H4 in key brain regions previously associated with drug-induced locomotor sensitization. Here, we found treatment-specific and region-specific alterations in both NPY and H3 immunoreactivity. The second and third experiments were designed to further investigate the role of histone acetylation in the expression and acquisition of ethanol-induced locomotor sensitization. Given the region-specific increase in histone acetylation following ethanol-induced behavioral sensitization in Experiment 1, we expected hyperacetylation to augment the expression and/or acquisition of sensitization; interestingly, treatment with the HDACi augmented the acquisition, but did not affect the expression, of ethanol-induced locomotor sensitization. Taken together, these results provide further evidence for the role of both NPY and chromatin remodeling through histone acetylation in ethanol-induced locomotor sensitization.

CHAPTER 2

METHODS

Animals

Male DBA/2J mice (see below for animal numbers) were 6-8 weeks of age and weighed approximately 22g at the beginning of the experiment. Mice were individually housed in polypropylene cages with corncob bedding and allowed to habituate to the environment for one week prior to study initiation. While in the home cage, animals had *ad libitum* access to food and water. The colony room was maintained at approximately 22° Celsius with a 12h:12h light:dark cycle. All procedures and protocols used in the present studies were in accordance with the National Institute of Heath guidelines and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Drugs

Ethanol solutions for injections (1.5, 2.0, 2.5 g/kg, i.p.) were prepared using 0.9% saline and 95% (Experiment 3) or 100% (Experiment 1 and 2) Ethyl Alcohol. Equivolume saline injections were used as an ethanol control in all experiments. Trichostatin A (TSA; 1.0, 2.0, 4.0 mg/kg, i.p.; Sigma Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and diluted (1:5) with phosphate buffered saline (PBS; pH 7.4). For TSA studies, DMSO diluted with PBS (1:5) was used as the vehicle control.

Ethanol-Induced Locomotor Sensitization

A sensitization protocol based on a previously described protocol was used for all experiments (Fee, Knapp et al. 2006; Fee, Sparta et al. 2007). Mice were tested during the

light phase of the light:dark cycle. Animals were transported to the testing room and habituated in their home cages for at least 30 minutes prior to testing. Fans in the testing room provided white noise. Mice were removed from their home cages, given an intraperitoneal (i.p) injection of ethanol or equivolume saline (see Tables 1A-1C for injection schedules) and immediately placed into the center of an open field (Harvard Apparatus, Inc., Holliston, MA). This apparatus, measuring 40.64cm by 40.64cm x 30.48cm and made of clear Plexiglas, recorded locomotor activity via photo beam breaks. Corncob bedding, the same used in home cages, was placed into the locomotor chamber to both aid in cleaning and prevent the buildup of odor; chambers were cleaned between each subject. Locomotor activity was recorded for 20 min sessions and broken down into five minute sections. To briefly summarize the injection schedule, on days 1-3, mice received an i.p. injection of saline and were placed into the open field to habituate them to both the injection procedure and the open field apparatus. On day 4 ("Initial Test"), mice received an i.p. injection of either ethanol or equivolume saline to serve as a measure of baseline locomotor behavior and a reference point for the development of sensitization. Animals then received homecage (HC) injections of saline or ethanol for either 7 or 10 consecutive days. On these days, mice were not removed from the animal colony: following injections, mice were returned to the HC and left undisturbed for 24hr. Mice were again brought to the locomotor testing room ("Final Test") for a final locomotor assessment following either an injection of ethanol or saline, in a procedure exactly like that of the Initial Test. Following the final test session, mice were returned to the HC until perfusion and brain collection.

Perfusions, Brain Preparation, and Immunohistochemistry (IHC)

Perfusion and immunohistochemistry procedures were based on those routinely used in our laboratory (Hayes, Knapp et al. 2005; Navarro, Cubero et al. 2008; Cubero, Navarro et al. 2010). After completion of each experiment, mice were injected with a cocktail of ketamine (117 mg/kg) and xylazine (7.92 mg/kg) and transcardially perfused with 0.1 mM PBS followed by 4% paraformaldehyde in phosphate buffer. Brains were collected and post-fixed in paraformaldehyde for 48 hours at 4°C. At this point they were transferred to either PBS, for immediate processing, or to a cryopreserve solution for long-term storage. Brains were cut using a vibratome into 40μm slices and stored in either PBS or cryopreserve until ready for IHC processing. Sections were transferred back into PBS 24 hours before each IHC assay and allowed to sit at room temperature. Sections were then transferred to PBS for 24 h before processing with NPY, acetylated H3 (lys9), or acetylated H4 (lys8) antibodies. After rinsing in fresh PBS 5 times (10 minutes each), tissue sections were blocked in 10% goat serum and 0.1% triton-X-100 in PBS for 1 hour. Sections were then transferred to fresh PBS containing primary antibody and agitated for 72 hours at 5°C. NPY expression was detected using primary rabbit anti-NPY (Peninsula Laboratories, LLC, San Carlos, CA; 1:1000). Acetylated H3 (acH3) expression was detected using primary rabbit anti-acH3 (Millipore, Temecula, CA; 1:5000). Acetylated H4 (acH4) expression was detected using primary rabbit anti-H4 antibody (Millipore, Temecula, CA; 1:1:000). As a control to determine if staining required the presence of the primary and secondary antibodies, some sections were run through the assay without either the primary antibody or the secondary antibody. In each assay described below, tissue processed without either the primary or the secondary antibody failed to show staining that was evident in tissue processed with the correct antibodies. After

the 72 hour incubation, tissue sections were rinsed 3 times and then processed with Vectastain Elite kits (Vector Laboratories, Burlingame, CA) as per the manufacturer's instructions for standard ABC/HRP/diaminobenzidine-based immunohistochemistry. The staining was visualized by reacting the sections with a 3,3′-diamino-benzidine tetrahydrochloride (DAB; Polysciences, Inc., Warrington, PA) reaction solution containing 0.05% DAB, 0.005% cobalt chloride, 0.007% nickel ammonium sulfate, and 0.006% hydrogen peroxide. All sections were mounted on glass slides, air-dried, and cover slipped for viewing. Digital images of NPY immunoreactivity (NPY-IR), acH3 immunoreactivity (acH3-IR), and acH4 immunoreactivity (acH4-IR) were obtained on a Nikon E400 microscope equipped with a Nikon Digital Sight DS-U1 digital camera run with Nikon-provided software. Densitometric procedures were used to assess levels of IR in predetermined brain regions of interest. Flat-field corrected digital pictures (8-bit grayscale) were taken using the Digital Sight DS-U1 camera. The density of staining was analyzed using Image J software (Image J, National Institute of Health, Bethesda, MD) by calculating the percent of the total area examined that showed staining relative to a subthreshold background. The size of the areas that were analyzed was the same between animals and groups. The subthreshold level for the images was set in such a way that any area without an experimenter-defined level of staining was given a value of zero. Anatomically matched pictures of the left and right sides of the brain were used to produce an average density for each brain region from each slice. In all cases, quantification of immunoreactivity data was conducted by an experimenter blind to group identity. For analysis, great care was taken to match sections through the same region of brain and at the same level using anatomic landmarks with the aid of a mouse stereotaxic atlas (Franklin 1997).

Experiment 1: Analysis of NPY-IR, acH3-IR, and acH4-IR Following Ethanol-Induced Locomotor Sensitization

This experiment was designed to examine the presence of acetylated histones H3 (acH3) and H4 (acH4) and NPY in key brain regions following acquisition of ethanol-induced locomotor sensitization. Male DBA/2J mice (n=40) underwent three days of habituation to the injection procedure and the open field. Mice were divided into three groups, counterbalanced for locomotor activity following the initial test day injection of saline or 1.5 g/kg EtOH. Saline (SAL) control animals received saline injections for the remainder of the experiment. Non-sensitized (NS) animals received saline for each of 10 HC injections, and 1.5 g/kg EtOH on the first and final test days. Thus these animals received a total of two ethanol injections, separated by 10 days, over the course of the experiment. Sensitized (SEN) mice received injections of 2.5 g/kg EtOH for each of the 10 HC injections, and 1.5 g/kg EtOH on the first and final test days (see Table 1A). These animals received a total of 12 ethanol injections over the course of the experiment. Sensitization was assessed as the change in locomotor activity on the Final Test relative to the Initial Test. Forty-eight or 96 hours after the final locomotor assessment, mice were sacrificed and brains collected for IHC analysis as outlined above. As no significant differences in IR were evident between these time points, all presented IHC data has been collapsed.

Experiment 2: Effect of TSA on the Expression of Ethanol-Induced Locomotor Sensitization

This experiment was designed to examine the effect of TSA, a potent HDAC inhibitor, on the expression of ethanol-induced locomotor sensitization. Male DBA/2J (n=40) mice underwent three days of habituation to the injection procedure and the open field. Mice

were divided into four groups, counterbalanced for locomotor activity during the initial test day injection of 2.0 g/kg EtOH. All animals received 2.5 g/kg EtOH for all 10 HC injections. Two hours before the final test day injection of 2.0 g/kg EtOH, animals received an injection of vehicle or 1.0, 2.0, or 4.0 mg/kg TSA (see Table 1B). The dose and timing of TSA administration in the present studies were based on previous work showing success in manipulating brain histone acetylation (Korzus, Rosenfeld et al. 2004; Pandey, Ugale et al. 2008). Sensitization was assessed as in Experiment 1.

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

This experiment was designed to examine the effect of TSA on the acquisition of ethanol-induced locomotor sensitization. Male DBA/2J mice (n=70) underwent three days of habituation to the injection procedure and the open field. Mice were divided into seven groups based on locomotor activity following an initial test day injection of either 2.0 g/kg EtOH or saline. Saline-treated control mice were pretreated with either vehicle (VEH+SAL) or TSA (TSA+SAL) two hours before each HC injection of saline. Non-sensitized mice were pretreated with either vehicle (VEH+NS) or TSA (TSA+NS) two hours before each HC injection of saline. Sensitized mice were pretreated with either vehicle (VEH+SEN) or TSA (TSA+SEN) two hours before each HC injection of ethanol. A non-injected group of mice was also included to control for the potential effects of stress due to repeated injections: locomotor activity of mice in the non-injected group was assessed on all habituation and test days, and all non-injected mice were weighed and handled at the same time as their injected counterparts on HC injection days.

For this experiment, mice received seven HC injections of saline or 2.5 g/kg EtOH. Sensitization was assessed as in Experiment 1 following a final test day injection of either saline or 2.0 g/kg EtOH. See Table 1C for schedule of injections. Importantly, neither vehicle nor TSA was injected preceding the final locomotor assessment: thus any increases in locomotor activity on the final test day should be due to changes resulting from the acquisition of sensitization.

Data Analysis

One-way analyses of variance (ANOVAs) were used to analyze immunoreactivity in Experiment 1 and group differences in locomotor activity on the final test day in Experiment 3; specific differences between groups were assessed using LSD post-hoc tests. Repeated measures ANOVAs with post-hoc tests were used to analyze group differences in locomotor sensitization in all experiments. Significance was accepted at $p<0.05$ (two-tailed).

CHAPTER 3

RESULTS

Experiment 1:

Locomotor activity from the first five minutes of the Initial and Final test sessions from Experiment 1 are presented in Figure 1. There were no differences in activity between the initial and final test sessions in either the saline-treated (SAL) or the non-sensitized (NS) mice, indicating these animals did not develop ethanol-induced locomotor sensitization. Repeated measure ANOVA revealed a significant group x day interaction [$F(2,36)=7.028$, $p=0.003$]. Post hoc tests showed that locomotor activity was significantly increased in the sensitized (SEN) mice on the final locomotor assessment compared to the initial locomotor assessment, indicating that the mice developed ethanol-induced locomotor sensitization. Both NS and SEN mice also showed significantly more locomotor behavior on the initial test day relative to the SAL mice, indicative of the stimulant-like properties of the low-dose of ethanol [$F(2,37)=4.24$, $p=0.02$]. As these behavioral data show the SEN mice achieved adequate ethanol-induced locomotor sensitization, immunohistochemical analyses were performed to examine differences in acH3-IR, acH4-IR, and NPY-IR between SAL, NS, and SEN mice.

acH3 Immunoreactivity

Representative photomicrographs and quantification of acH3 immunoreactivity (acH3-IR) in the nucleus accumbens are shown in Figure 2. As seen in Figure 2D, acH3 IR in the nucleus accumbens shell differed by group [$F(2,33)=3.309$, $p=0.049$]. Post-hoc analysis

revealed that the SEN mice displayed significantly greater acH3 IR in the nucleus accumbens shell relative to both SAL and NS mice. No group differences were seen in the nucleus accumbens core [F(2,33)=0.669, p=0.519; Figure 2C]. Additionally, there were no group differences in acH3-IR in the dorsolateral striatum (DLS) [F(2,35)=2.235, p=0.122], the dorsomedial striatum (DMS) [F(2,35)=1.369, p=0.268], the ventrolateral striatum (VMS) [F(2,35)=1.263, p=0.295], or the ventromedial striatum (VMS) [F(2,35)=1.486, p=0.240] (Figure 2E, 2F).

acH4 Immunoreactivity

Quantification of acH4-IR in the nucleus accumbens and striatum is shown in Figure 3. There were no group differences in the nucleus accumbens core [F(2,34)=0.034, p=0.967] or shell [F(2,34)=0.016, p=0.984]. Similarly, no group differences were seen in the DLS [F(3,37)=1.097, p=0.345], DMS [F(2,35)=1.729, p=0.192], VLS [F(2,35)=0.511, p=0.604] or VMS [F(2,35)=0.727, p=0.491].

NPY Immunoreactivity

Representative photomicrographs and quantification of NPY-IR in the nucleus accumbens and striatum are shown in Figure 4. As shown in Figure 4C, NS mice showed reduced NPY-IR in the core of the accumbens relative to both SAL and SEN mice [F(2,31)=6.087, p=0.006]. NPY-IR in the shell of the accumbens showed a non-significant trend in a similar direction, with post-hoc tests revealing that NS mice showed significantly reduced NPY-IR relative to SEN mice [F(2,32)=2.991, p=0.064; Figure 4D]. Interestingly, NPY-IR in the striatum showed a different expression pattern. As shown in Figure 4E, NPY-IR in the DLS differed by group [F(2,33)=3.531, p=0.041]. Post-hoc analysis revealed the SEN mice showed significantly elevated NPY-IR relative to NS, but not to SAL mice. There

were no group differences in NPY-IR expression in the DMS [F(2,33)=0.135, p=0.874], VLS [F(2,33)=0.040, p=0.960] and VMS [F(2,33)=0.477, p=0.625] (Figure 4E, 4F).

Immunoreactivity Summary

Immunoreactivity results are summarized in Table 2A and Table 2B. Briefly, mice that showed ethanol-induced locomotor sensitization showed an increase in acH3-IR specific to the nucleus accumbens shell and an increase in NPY-IR specific to the dorsolateral striatum. Additionally, non-sensitized animals given an acute injection of ethanol showed a decrease in NPY-IR in the nucleus accumbens core relative to both saline-treated and ethanol-sensitized mice. A trend in this direction was also seen in non-sensitized mice in the shell of the nucleus accumbens. No changes were seen in acH4-IR in any of the regions examined. These data indicate a potential role of histone acetylation and/or NPY in ethanol-induced locomotor sensitization.

Experiment 2:

The goal of Experiment 2 was to examine the effect of peripheral administration of TSA on the expression of ethanol-induced locomotor sensitization. For this experiment, all mice were sensitized to a low dose of ethanol and treated with either vehicle or one of three doses of TSA two hours prior to the final locomotor assessment. Locomotor data from this experiment is shown in Figure 5. No group differences in locomotor activity were detected on either the initial test day [F(3,29)=0.182, p=0.907] or the final test day [F(3,29)=1.818, p=0.166]. All groups of mice showed robust ethanol-induced locomotor sensitization: that is, all groups of mice showed significantly augmented locomotor activity on the final test day as compared to the initial test day. However, repeated measure ANOVA revealed no group x treatment interaction, indicating that the HDACi did not affect the degree of locomotor

sensitization [F(3,29)=1.496, p=0.237]. These results suggest that peripheral administration of an HDACi does not affect the expression of ethanol-induced locomotor sensitization, as all groups achieved similar degrees of locomotor sensitization.

Experiment 3:

As Experiment 2 showed that administration of TSA had no effect on the expression of ethanol-induced locomotor sensitization, the goal of Experiment 3 was to examine the effect of peripheral administration of TSA on the acquisition of ethanol-induced locomotor sensitization. For this experiment, mice received an injection of either vehicle or 2.0 mg/kg TSA two hours before each of seven consecutive daily HC injections of ethanol or equivolume saline. Locomotor data from this experiment is shown in Figure 6. Only mice that received repeated HC injections of 2.5 g/kg ethanol showed ethanol-induced locomotor sensitization, evidenced by augmented locomotor activity on the final test day relative to the initial test day. Repeated measures ANOVA revealed a group x test day interaction [F(6,58)=9.614, p=0.000]. Additionally, one-way ANOVA of final test day locomotor activity revealed significant group differences in locomotor activity [F(6,58)=14.090, p=0.000]. Post-hoc analysis revealed that both VEH-SEN and TSA-SEN mice showed significantly greater locomotor activity relative to all other groups. Importantly, LSD post-hoc analysis also indicated that VEH-SEN and TSA-SEN showed significantly different locomotor activity. That is, mice pretreated with TSA on each day of HC ethanol injections showed significantly greater locomotor activity on the final test day, when TSA was not on board, relative to mice pretreated with saline on each day of HC ethanol injections. Importantly, daily pretreatment with TSA affected neither general locomotor activity nor the locomotor stimulant response to an acute injection of low-dose ethanol, as measured in the

NS mice. Taken together, these data suggest that peripheral administration of TSA, administered prior to HC ethanol injections during the induction of locomotor sensitization, is sufficient to augment ethanol-induced locomotor sensitization when TSA is not on board.

CHAPTER 4

DISCUSSION

The goal of the present experiments was to examine NPY, acH3, and acH4 immunoreactivity and their involvement in ethanol-induced locomotor sensitization. First, NPY-IR, acH3-IR, and acH4-IR were assessed following the induction of sensitization. Immunohistochemical analysis revealed region-specific upregulation of striatal NPY-IR (Figure 4C) and accumbal acH3-IR (Figure 2C) in animals showing robust sensitization to the locomotor stimulant effects of ethanol. No changes were seen in acH4-IR in either region (Figure 3). Interestingly, mice that received an acute injection of ethanol showed a decrease in NPY-IR in the nucleus accumbens core (Figure 4E); a trend in the same direction was seen in the nucleus accumbens shell (Figure 4F).

Our findings confirm the inability of low-dose repeated ethanol to induce detectable changes in the expression of acetylated H3 within the striatum (Sanchis-Segura, Lopez-Atalaya et al. 2009). We extended our analysis to the nucleus accumbens, a well-studied reward center with likely involvement in psychomotor sensitization (Pierce and Kalivas 1997; Robinson and Berridge 2000; Abrahao, Quadros et al. 2011). Despite no detected change in acetylated H3 or H4 in the nucleus accumbens core, an increase in acetylated H3 was detected in the nucleus accumbens shell, a region known to receive dopaminergic input from the ventral tegmental area (Robbins and Everitt 1996; Ikemoto 2007). Indeed, recent evidence has also demonstrated that the rats demonstrating amphetamine-induced locomotor sensitization show an increase in dopamine neuron firing specifically in dopamine neurons

innervating the medial shell, but not the core, of the nucleus accumbens (Ikemoto 2007; Lodge and Grace 2011). Thus these data provide further evidence for the involvement of the nucleus accumbens shell in drug-induced locomotor sensitization. Additionally, as changes in H3 acetylation were detected in the shell of the accumbens, it is unlikely that the lack of changes detected in either the striatum or the nucleus accumbens core were due to the immunohistochemical technique utilized in the present experiment. However, the possibility remains that the technique was not a sensitive enough measure to detect smaller changes in acetylated histone expression in these regions, due to high baseline levels of histone acetylation in saline-treated control animals.

The present results differ from recent reports showing simultaneous changes in both histones H3 and H4 acetylation in response to acute ethanol exposure (Pandey, Ugale et al. 2008). Our results are not surprising, however, in light of drug abuse literature showing differential expression patterns of acetylated H3 and H4. For example, rats exposed to chronic toluene inhalation showed increased acH3-IR, but no changes in acH4-IR, within the accumbens (Sanchez-Serrano, Cruz et al. 2011). Additionally, genes influenced by acute cocaine exposure show hyperacetylation at histone H4, while genes influenced by chronic exposure show hyperacetylation at histone H3 (Kumar, Choi et al. 2005). Although speculative, perhaps the increases in accumbal H3 reflect the 'chronic' nature of repeated ethanol exposure, and a transient change in H4 in the non-sensitized ('acute' exposed) mice was not captured by our experimental protocol. Future studies will need to examine the temporal patterns of hyperacetylation to address this question.

In light of preliminary evidence from our laboratory showing attenuated ethanol-induced locomotor sensitization in both mice lacking normal NPY production as well as mice

with blunted endogenous NPY release specifically in the accumbens (Hayes, Fee et al. in press), the lack of changes in NPY protein expression in the nucleus accumbens following the induction of locomotor sensitization is somewhat surprising. However, differences in patterns of NPY-IR may be due to strain differences: while previous studies utilized RIIβ-/- mice maintained on a C57BL/6J background, the present study utilized DBA/2J mice. In the present study, sensitized mice showed an increase of NPY-IR specific to the dorsolateral region of the striatum, but no changes in the nucleus accumbens, relative to non-sensitized mice. Interestingly, non-sensitized mice showed decreases in NPY-IR in both sub-regions of the nucleus accumbens core, a finding consistent with the attenuated striatal NPY-IR seen in RIIβ-/- mice following an acute injection of 2.0 g/kg dose of ethanol (Hayes, Fee et al. in press) and with the observation that an acute injection of ethanol in rats can decrease NPY mRNA in the arcuate nucleus of the hypothalamus (Kinoshita, Jessop et al. 2000). Thus these data support the idea that NPY signaling in the nucleus accumbens may be important in the initial acquisition of ethanol-induced locomotor sensitization, but may not play a key role in the expression of said sensitization: perhaps lasting changes in striatal NPY (as seen in the dorsolateral striatum of sensitized animals in the present work) reflect neuroadaptive changes important to the expression of sensitization.

Recent work from the cocaine literature supports the finding that alterations in NPY expression in certain brain regions following drug exposure may be transient: though an upregulation of NPY (and corresponding upregulation of acetylated histone H3 bound to the NPY promoter) was found in the medial prefrontal cortex following one day of cocaine abstinence, expression of protein and acetylated histones returned to control levels within 10 days of abstinence (Freeman, Patel et al. 2008). In the present study, mice were sacrificed 48

and 96 hours after the final locomotor test to ensure the animals did not have ethanol on board and were not in acute ethanol withdrawal. As the nucleus accumbens has been proposed as a link between the limbic and motor circuits, and changes in accumbal NPY-IR expression appear specific to non-sensitized mice (that is, in response to an acute dose of ethanol), it is possible that changes in NPY expression in the nucleus accumbens are transient and may influence later, lasting changes in the striatum. Future studies are needed to examine the mechanisms that may underlie this transition.

Surprisingly, the present results do not reveal an overlap in the expression patterns of acH3-IR and NPY in either of the brain regions examined, despite previous evidence showing direct manipulation of amygdalar NPY expression by the HDACi TSA (Pandey, Ugale et al. 2008). In that study, ethanol withdrawal-induced deficits in NPY and acetylated histone expression in the amygdala were both rescued by peripheral administration of TSA. However, a number of differences in the current work may explain the lack of parallel findings, including species examined and ethanol regimen. While the previous work examined brain chemistry during withdrawal from an acute ethanol exposure in rats, the current work examined immunohistochemistry in mice, beyond acute withdrawal, following chronic ethanol exposure. Additionally, the effect of histone acetylation may differentially affect proteins in different brain regions: while previous work has examined the acH3-NPY relationship in response to ethanol in amygdalar regions, the current work focuses on the core and shell of the nucleus accumbens and the four quadrants of the striatum. Finally, the present investigation did not examine the exact gene location of acetylation, as the immunohistochemical approach used measured general levels of H3 and H4 acetylation in the regions of interest. Future studies will need to investigate the gene promoter regions

affected by ethanol-induced hypreacetylation. It may be that changes in histone acetylation seen following locomotor sensitization are either not related, or are indirectly related to the changes in NPY signaling implicated in modulating ethanol-induced locomotor sensitization.

As our laboratory has recently utilized both genetic and site-directed viral vector manipulations to examine the role of NPY signaling in modulating ethanol-induced locomotor sensitization (Hayes, Fee et al. in press), we focused the remainder of our investigation on the role of histone acetylation in this behavioral phenomenon. To this end, we peripherally administered a potent, reversible HDAC inhibitor, TSA, during two distinct phases of ethanol-induced locomotor sensitization. As inhibition of HDAC activity results in increased histone acetylation, Experiments 2 and 3 examined the effect of hypreacetylation on both the acquisition and expression of locomotor sensitization.

In Experiment 2, mice received an intraperitoneal injection of TSA two hours prior to the final locomotor assessment. As the neuroadaptations responsible for locomotor sensitization likely form during the regimen of repeated homecage injections, the goal of this experiment was to measure the role of global histone hyperacetylation on the expression of ethanol-induced locomotor sensitization. Locomotor data from this experiment showed no effect of the TSA administration on the expression of locomotor sensitization, as none of the groups that received pretreatment with TSA on the test day showed a difference in their activity level relative to mice pretreated with vehicle (Figure 5). Importantly, the middle dose used in this experiment (2 mg/kg) has been shown sufficient to both affect NPY-modulated anxiety-like behaviors during ethanol-withdrawal in rats (Pandey, Ugale et al. 2008) and augment locomotor behavior in mice when administered two hours before behavioral assays (Experiment 3). As we included a range of doses from 1.0 mg/kg to 4.0

mg/kg, it is unlikely that this lack of effect on the expression of ethanol-induced locomotor sensitization is due to an insufficient or inappropriate dose of Trichostatin A. Thus we conclude that peripheral administration of TSA is unable to further manipulate neuroadaptations involved in ethanol-induced locomotor sensitization after acquisition is complete.

In the final study, mice received peripheral administration of vehicle or TSA before each of seven consecutive daily homecage injections of saline or ethanol. The goal of this study was to induce global histone hyperacetylation during the induction, or acquisition, of ethanol-induced locomotor sensitization, the period of time when neuroadaptations underlying the augmented locomotor behavior are thought to form. Behavioral data from this experiment showed not only that all animals that received repeated homecage ethanol injections developed robust locomotor sensitization, but also that mice pretreated with TSA before each homecage ethanol injection showed greater locomotor activity on the final test day relative to mice pretreated with vehicle (Figure 6). Thus we conclude that hypreacetylation following peripheral administration of TSA is sufficient to strengthen the formation of neuroadaptations that contribute to ethanol-induced locomotor sensitization.

The present results both confirm and extend previous findings regarding the ability of NaBut to enhance ethanol-induced locomotor sensitization (Sanchis-Segura, Lopez-Atalaya et al. 2009). Here, we show that not only is pretreatment with a different HDACi, TSA, before HC injections of ethanol sufficient to augment the acquisition of ethanol-induced locomotor sensitization, but also that administration of TSA prior to the final locomotor assessment is insufficient to alter the expression of acquired sensitization. As sensitization to the psychomotor stimulant properties of drugs of abuse is highly persistent, likely reflecting

the strength of the underlying neuroadaptations, it may be that hyperacetylation of histones on the final locomotor test day is insufficient to further manipulate changes in neuronal plasticity (Paulson, Camp et al. 1991; Lessov and Phillips 1998). However, hyperacetylation of histones during the acquisition phase of sensitization, during which time neuroadaptations are forming and the system is in a more flexible state, may be sufficient to influence and potentially strengthen the neuroadaptations, thus increasing sensitization.

The present results extend current knowledge of the functional implications of histone acetylation, as we witnessed the ability of peripheral administration of an HDAC inhibitor to influence the acquisition of ethanol-induced locomotor sensitization. Unsurprisingly, changes in the expression of both NPY and histone acetylation, as measured with immunohistochemistry, appear to be treatment- and region-specific. Together, the current data provide evidence for a role of both NPY and histone acetylation in ethanol-induced locomotor sensitization and warrant further investigation. Future studies will examine potential downstream factors of histone acetylation that may influence ethanol-induced locomotor sensitization, including NPY. Additionally, site-directed infusions of TSA, specifically into sub-regions of the striatum and nucleus accumbens, will examine the effect of targeted histone hyperacetylation on the expression of NPY as well as the acquisition and expression of ethanol-induced locomotor sensitization. Together, these observations may lead to new pharmacological targets aimed at treating alcoholism.

Table 1A: Daily dosing schedule for ethanol-induced locomotor activity, Experiment 1. All injections given i.p.*

	Days 1-3	***Day 4***	***Days 5-14***	***Day 15***
Treatment	**Habituation: LC**	**Initial Test: LC**	**Conditioning: HC**	**Final Test: LC**
Saline control (SAL)	Saline	Saline	Saline	Saline
Non-sensitized (NS)	Saline	1.5 g/kg EtOH	Saline	1.5 g/kg EtOH
Sensitized (SEN)	Saline	1.5 g/kg EtOH	2.5 g/kg EtOH	1.5 g/kg EtOH

LC, locomotor chamber; HC, homecage; EtOH, ethanol
*Saline injections equivolume to EtOH injections

Table 1B: Daily dosing schedule for the effect of TSA on the expression of ethanol-induced locomotor sensitization, Experiment 2. All injections given i.p.*

	Days 1-3	***Day 4***	***Days 5-14***	***Day 15***
Treatment	**Habituation: LC**	**Initial Test: LC**	**Conditioning: HC**	**Final Test: LC**
Vehicle control (VEH)	Saline	2.0 g/kg EtOH	2.5 g/kg EtOH	Vehicle + 2.0g/kg EtOH
1 mg/kg TSA (1-TSA)	Saline	2.0 g/kg EtOH	2.5 g/kg EtOH	1 mg/kg TSA + 2.0 g/kg EtOH
2 mg/kg TSA (1-TSA)	Saline	2.0 g/kg EtOH	2.5 g/kg EtOH	2 mg/kg TSA + 2.0 g/kg EtOH
4 mg/kg TSA (4-TSA)	Saline	2.0 g/kg EtOH	2.5 g/kg EtOH	4 mg/kg TSA + 2.0 g/kg EtOH

LC, locomotor chamber; HC, homecage; VEH, vehicle; TSA, Trichostatin A
*Saline and vehicle injections equivolume to EtOH and TSA injections

Table 1C: Daily dosing schedule for the effect of TSA on the acquisition of ethanol-induced locomotor sensitization, Experiment 3. All injections given i.p.*

	Days 1-3	***Day 4***	***Days 5-11***	***Day 12***
Treatment	**Habituation: LC**	**Initial Test: LC**	**Conditioning: HC**	**Final Test: LC**
Non-injected Control	NONE	NONE	NONE	NONE
Vehicle + Saline (VEH+SAL)	Saline	Saline	Vehicle + Saline	Saline
TSA + Saline (TSA+SAL)	Saline	Saline	2.0 mg/kg TSA + Saline	Saline
Vehicle + Non-sensitized (VEH+NS)	Saline	Saline	Vehicle + Saline	2.0 g/kg EtOH
TSA + Non-sensitized (TSA-NS)	Saline	Saline	2.0 mg/kg TSA + Saline	2.0 g/kg EtOH
Vehicle + Sensitized (VEH+SEN)	Saline	2.0 g/kg EtOH	Vehicle + 2.5 g/kg EtOH	2.0 g/kg EtOH
TSA + Sensitized (TSA+SEN)	Saline	2.0 g/kg EtOH	2.0 mg/kg TSA + 2.5 g/kg EtOH	2.0 g/kg EtOH

LC, locomotor chamber; HC, homecage; NONE, no injection; VEH, vehicle; TSA, Trichostatin A

*Saline and vehicle injections equivolume EtOH and TSA injections

Table 2A: Summary of IR in ethanol-sensitized mice (Experiment 1)

	Nucleus Accumbens		Striatum			
	Core	**Shell**	**DL**	**DM**	**VL**	**VM**
NPY-IR	---	---	↑*	---	---	---
acH3-IR	---	↑	---	---	---	---
acH4-IR	---	---	---	---	---	---

*Relative to NS mice only

Table 2B: Summary of IR in ethanol non-sensitized mice (Experiment 1)

	Nucleus Accumbens		Striatum			
	Core	**Shell**	**DL**	**DM**	**VL**	**VM**
NPY-IR	↓	↓*	---	---	---	---
acH3-IR	---	---	---	---	---	---
acH4-IR	---	---	---	---	---	---

*Non-significant trend

Figure 1: Locomotor behavior over the first five minutes of the Initial and Final locomotor assessment sessions for Experiment 1. Mice were split into three groups: saline control (SAL), non-sensitized (NS), or sensitized (SEN). SAL and NS mice received 10 HC injections of saline, and SEN mice received 10 HC injections of 2.5 g/kg EtOH. SEN mice showed significantly augmented locomotor behavior in the first five minutes of the Final Test relative to the Initial Test. NS and SEN mice also showed significantly greater locomotor behavior relative to SAL mice on the Initial Test day, indicative of the locomotor stimulant properties of an acute injection of low-dose ethanol. All values are means + SEM. *p<0.05 vs. SAL, **p<.0.01 vs. Initial.

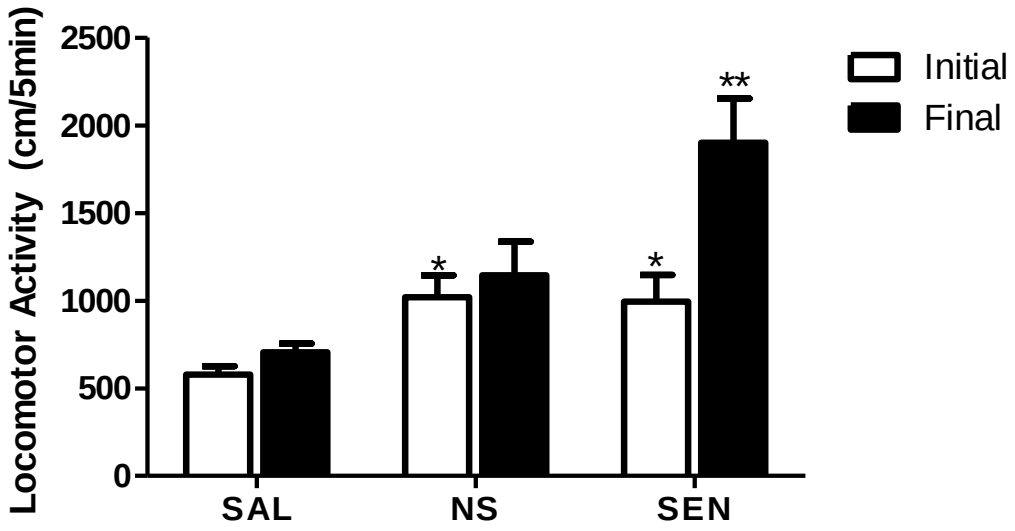


Figure 2: acH3 immunoreactivity in the nucleus accumbens and striatum. Representative photomicrographs are coronal brain slices taken from SAL (A) and SEN (B) mice. Quantification of the nucleus accumbens core (C) and shell (D) and dorsal (E) and ventral (F) striatum show region-specific increases in acH3-IR in SEN mice. DL = dorsolateral; DM=dorsomedial; VL=ventrolateral; VM=ventromedial. Images were photographed with 10x objectives and scale bar = 200µm. All values are mean + SEM. *p<0.05 vs. SAL.

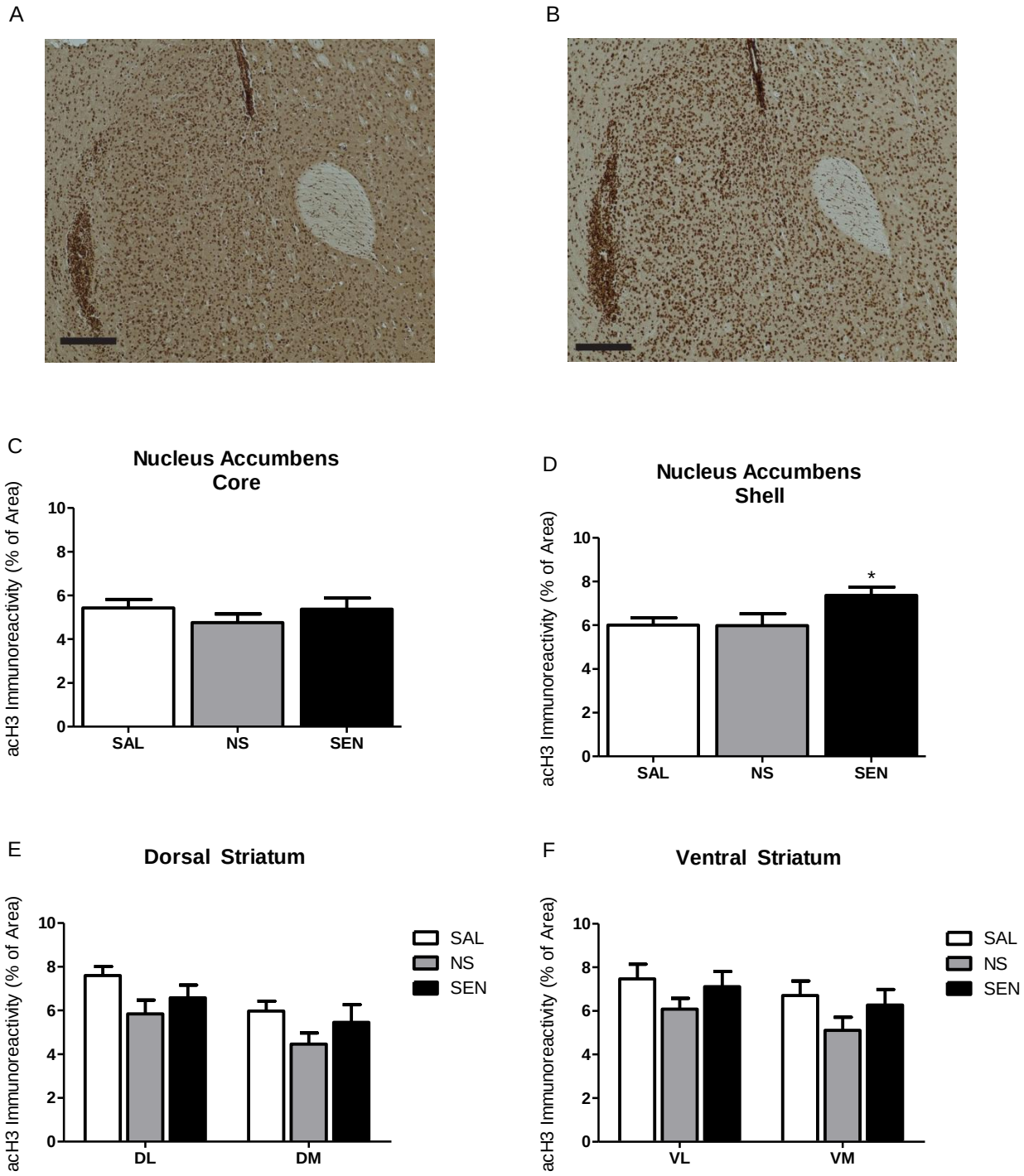


Figure 3: acH4 immunoreactivity in the nucleus accumbens core (A) and shell (B) and the dorsal (C) and ventral (D) striatum. All values are mean + SEM.

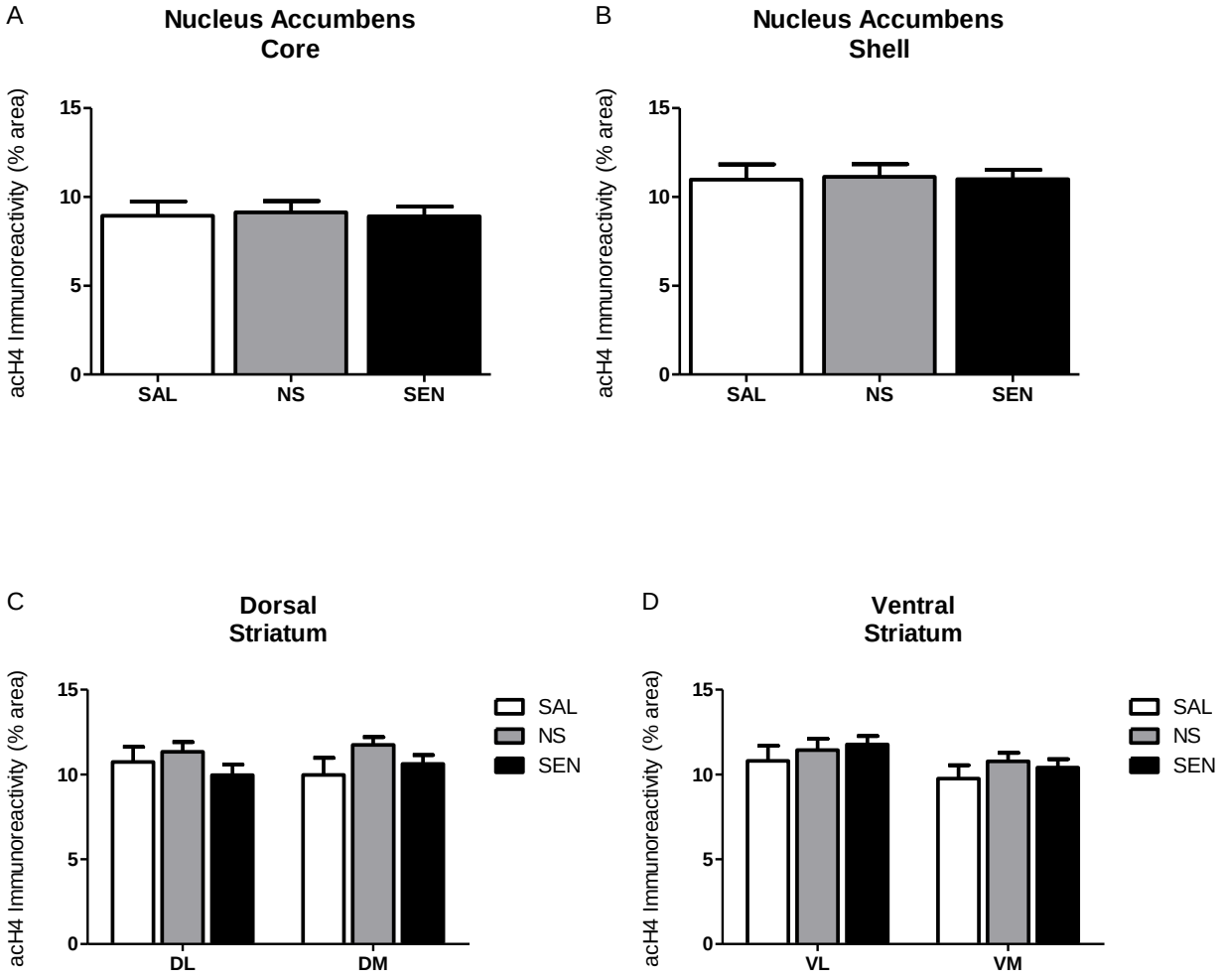


Figure 4: NPY immunoreactivity in the nucleus accumbens and striatum. Representative photomicrographs of the dorsolateral striatum are coronal brain slices taken from SAL (A) and SEN (B) mice. Quantification of the nucleus accumbens core (C) and shell (D) and dorsal (E) and ventral (F) striatum show region-specific increases in NPY-IR in SEN mice and region-specific decreases in NS mice. DL=dorsolateral; DM=dorsomedial; VL=ventrolateral; VM=ventromedial. Images were photographed with 20x objectives and scale bar =100µm. All values are mean + SEM. *p<0.05 vs. SAL, **p<0.05 vs. SAL and SEN.

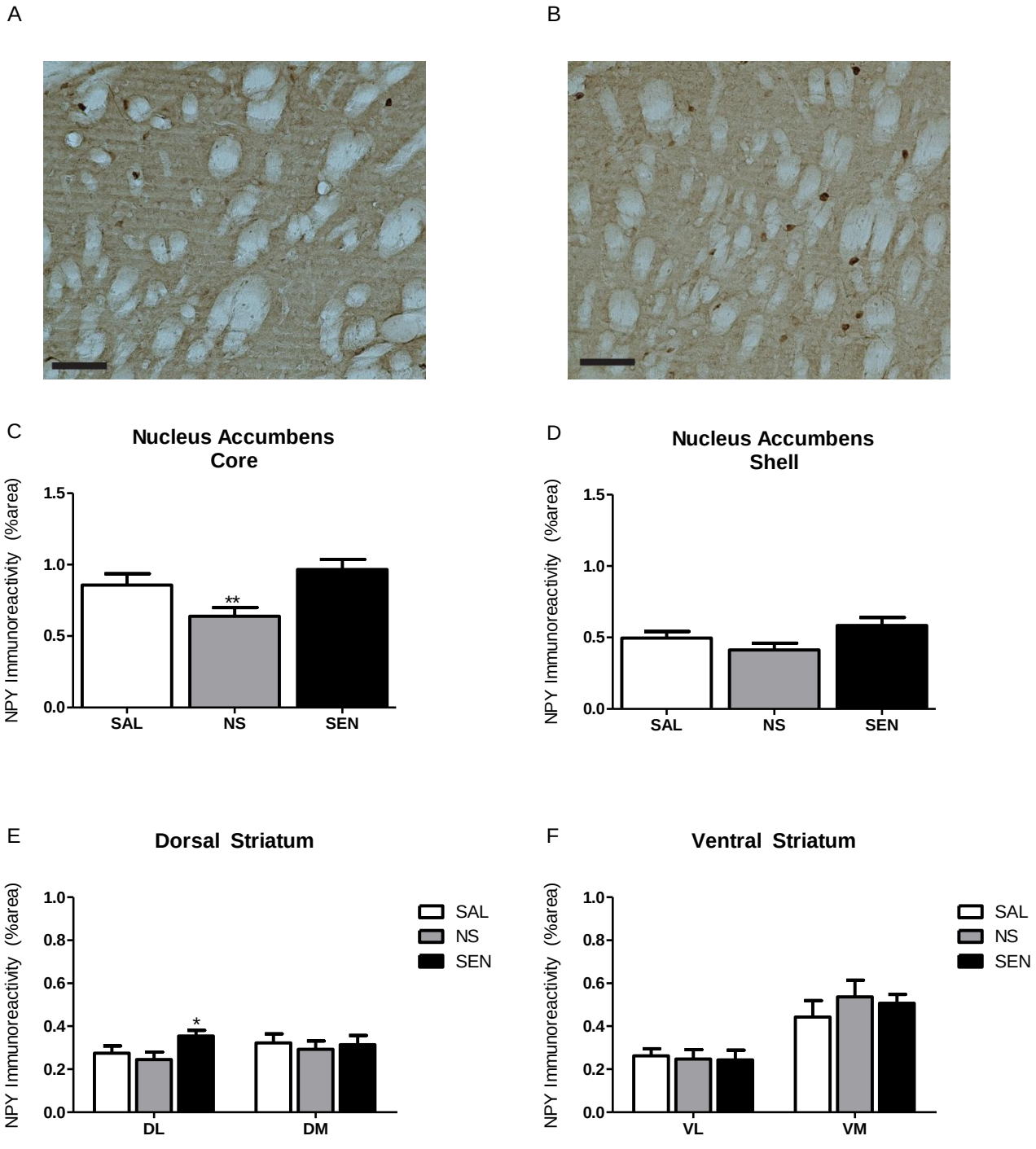


Figure 5: Locomotor behavior over the first five minutes of the Initial and Final locomotor assessment sessions for Experiment 2. All mice were sensitized using repeated homecage injections of 2.5 g/kg EtOH. Mice were split into four groups: vehicle (VEH), 1 mg/kg TSA (1-TSA), 2 mg/kg TSA (2-TSA), and 4 mg/kg TSA (4-TSA). Mice received an injection of vehicle or TSA two hours prior to the final locomotor assessment. All groups showed significant ethanol-induced locomotor sensitization. There were no group differences in degree of locomotor sensitization. All values are mean + SEM.

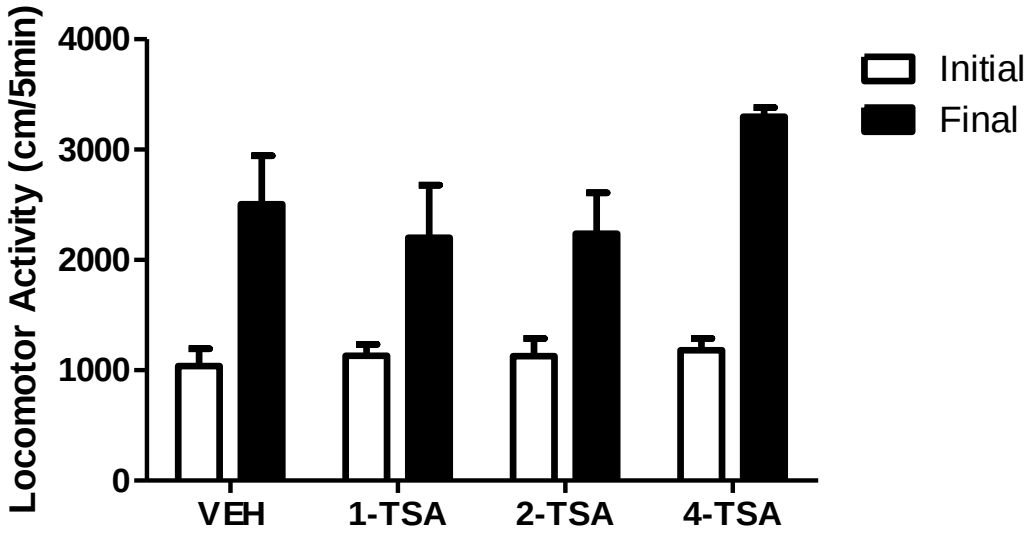
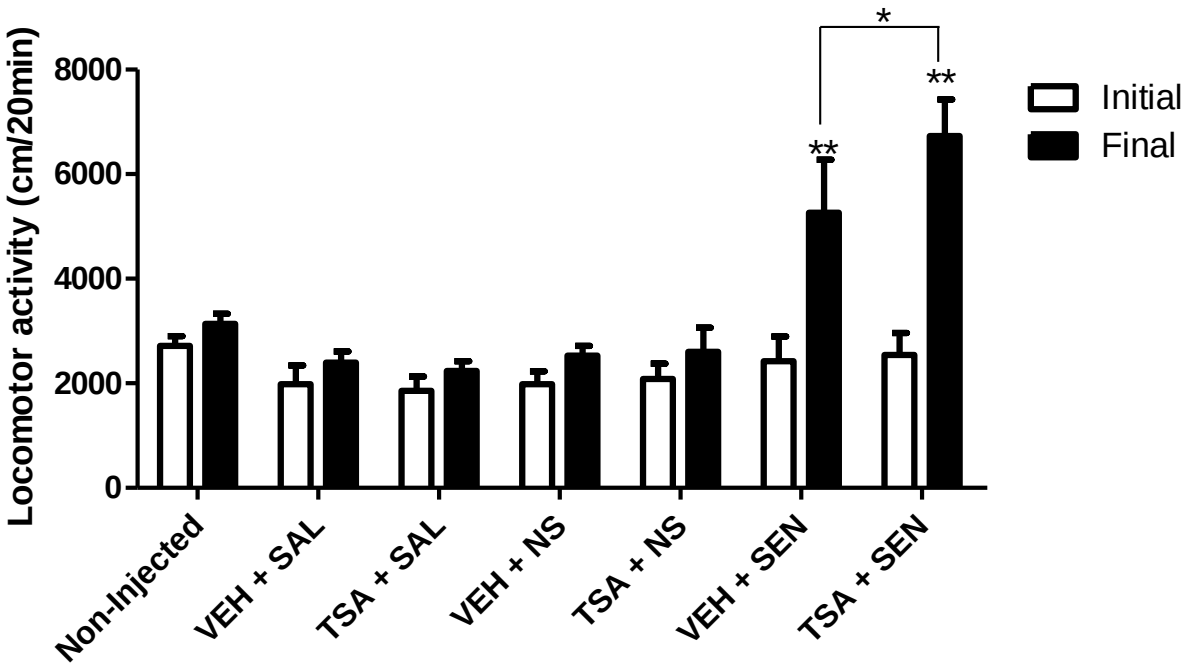


Figure 6: Locomotor behavior during 20-minute Initial and Final locomotor assessments for Experiment 3. Mice were split into seven groups: non-injected control (Non-injected), saline-treated mice pretreated with vehicle or TSA (VEH+SAL, TSA+SAL), non-sensitized mice pretreated with vehicle or TSA (VEH+NS, TSA+NS), and sensitized mice pretreated with vehicle or TSA (VEH+SEN, TSA+SEN). SAL and NS mice received 7 HC injections of saline; SAL mice received saline injection on the final test day, and NS mice received an injection of 2.0 g/kg EtOH on the final test day. SEN mice received 7 HC injections of 2.5 g/kg EtOH and an injection of 2.0 g/kg EtOH on the final test day. *p<0.05; **p<0.01 vs. initial.



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