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Aberrant HDAC2-Mediated Histone Modifications and Synaptic Plasticity in the Amygdala Predisposes to Anxiety and Alcoholism

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Abstract

Background—Epigenetic mechanisms have been implicated in psychiatric disorders, including alcohol dependence. However, the epigenetic basis and role of specific histone deacetylase (HDAC) isoforms in the genetic predisposition to anxiety and alcoholism is unknown.

Methods—We measured amygdaloid HDAC activity, levels of HDAC isoforms and histone H3 acetylation in selectively-bred alcohol-preferring (P) and -nonpreferring (NP) rats. We employed HDAC2 siRNA infusion into the central nucleus of amygdala (CeA) of P rats to determine the causal role of HDAC2 in anxiety-like and alcohol-drinking behaviors. Chromatin immunoprecipitation analysis was performed to examine the histone acetylation status of brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton associated protein (Arc) genes. Golgi-Cox staining was performed to measure dendritic spine density.

Results—We found that P rats innately display higher nuclear HDAC activity and HDAC2, but not HDAC 1, 3, 4, 5, and 6 protein levels, and lower acetylation of H3-K9, but not H3-K14, in the CeA and medial nucleus of amygdala (MeA) compared with NP rats. Acute ethanol exposure decreased amygdaloid HDAC activity and HDAC2 protein levels, increased global and gene (BDNF and Arc)-specific histone acetylation and attenuated anxiety-like behaviors in P rats, but had no effects in NP rats. HDAC2 knockdown in the CeA attenuated anxiety-like behaviors and voluntary alcohol, but not sucrose, consumption in P rats and increased histone acetylation of BDNF and Arc with a resultant increase in protein levels that correlated with increased dendritic spine density.

Conclusions—These novel data demonstrate the role of HDAC2-mediated epigenetic mechanisms in anxiety and alcoholism.

Keywords

Epigenetic; HDAC2; Amygdala; Anxiety and Alcoholism; BDNF; Dendritic spines

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Alcoholism is a multifactorial psychiatric disorder driven by underlying genetic and environmental factors (1, 2). Evidence from animal and human studies indicates that negative emotionality is clinically relevant in alcohol dependence, and anxiety sensitivity may serve as a risk factor for the development of alcohol use disorders (3–9). Anxiety may promote alcohol consumption due to its ability to reduce dysphoria, leading to self-medication (4–11). The alcohol-preferring (P) and -nonpreferring (NP) rat lines have been selectively-bred for higher and lower alcohol preference, respectively (12). P rats innately display anxiety-like behaviors compared to NP rats, thus providing a suitable animal model for human alcoholism with comorbid anxiety (13–17). The central nucleus of amygdala (CeA), a part of the extended amygdala, appears to serve as a major neuroanatomical substrate underlying the interaction between anxiety and alcoholism (5, 11).

Identifying the epigenetic mechanisms in specific neurocircuitries underlying anxiety and alcoholism is crucial to understand the regulation of molecular pathways leading to alcohol addiction. Covalent modifications of histones, such as acetylation, dynamically regulate gene transcription that is involved in the regulation of neuronal functions (18–23). Histone deacetylases (HDACs) are enzymes that deacetylate histone proteins, regulating chromatin structure. They are grouped into four classes based on cellular localization and functional regulation (23–25). Recent studies suggest aberrant regulation of class I and II HDACs may play a role in psychiatric disorders, including alcohol dependence, thereby serving as therapeutic targets for these disorders (18–20,26–33). Recently we showed that in an unselected stock of Sprague Dawley (SD) rats, withdrawal from chronic ethanol exposure resulted in anxiety-like behaviors that were associated with an increase in HDAC activity and a reduction in histone H3-K9 acetylation in the CeA and MeA (28). Treatment of ethanol-withdrawn rats with trichostatin A, an HDAC inhibitor, attenuated anxiety-like behaviors, and normalized HDAC activity and deficits in histone acetylation (28). We further identified the involvement of amygdaloid HDAC-mediated changes in histone acetylation in the development of rapid tolerance to the anxiolytic effects of ethanol in SD rats (27).

Epigenetic mechanisms have been implicated in the regulation of brain-derived neurotrophic factor (*BDNF*) gene expression and associated synaptic plasticity (26, 33, 34). *BDNF* signaling is involved in the regulation of synaptic plasticity via induction of activity-regulated cytoskeleton (*Arc*) associated protein expression (35–41). *BDNF* and *Arc* are also implicated to play a causative role in both anxiety and alcoholism (13, 42–47). Previously, we found that in comparison to NP rats, P rats have innately lower levels of *BDNF*, *Arc* and dendritic spine density in the CeA and medial nucleus of amygdala (MeA) which are increased by acute ethanol treatment (13, 45). However, the role of HDAC isoform-specific chromatin remodeling in the amygdala underlying the genetic predisposition to anxiety and alcoholism is currently unknown. We therefore investigated the role of amygdaloid HDAC2-mediated histone modifications in the regulation of synaptic plasticity associated events and the phenotypes of anxietylike and alcohol-drinking behaviors using P and NP rats.

Materials and Methods

Animals

Adult male P and NP rats (Indiana Alcohol Research Center at Indiana University, Indianapolis, IN) were housed in a temperature- and humidity-controlled facility under a 12 h light/dark cycle, with *ad libitum* food and water access. Age-matched rats weighing 330–400 g were used. Animal protocols were approved by the Institutional Animal Care and Use Committee.

Acute Ethanol Treatment

Rats were housed individually 1 day before acute ethanol exposure and injected intraperitoneally (IP) with ethanol (diluted to 20% w/v in *n*-saline; 1 g/kg) or *n*-saline alone. The procedure and ethanol dose are based on our and other studies in the field (13, 15, 27, 28). One hour following injection, anxiety-like behaviors were measured followed by anesthetization with pentobarbital (50 mg/kg). The rats were then either perfused for the collection of brains or directly decapitated and amygdaloid tissue containing predominantly CeA and MeA was punched out for neurochemical measures. Blood was collected to measure ethanol levels using the Analox Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Stereotaxic Cannulation Surgery and HDAC2 siRNA Infusion

For siRNA infusion experiments, rats were surgically implanted with CMA/11 guide cannulae targeting CeA (CMA Microdialysis, North Chelmsford, MA) using coordinates as described previously by us (14, 47, 48). Rats were housed individually after surgery and one week after recovery, bilateral infusions into the CeA with 0.5 μ L of HDAC2 siRNA, negative control siRNA or vehicle was performed using a microdialysis probe. siRNAs were dissolved in iFect solution (Neuromics, Edina, MN), a cationic lipid-based transfection solution, such that the final concentration was 2 μ g/ μ L. The sequence of the HDAC2 siRNA (Qiagen, Valencia, CA) was as follows: sense, 5'-CAAGUUUCUACGAUCAAUATT-3'; antisense 5'-UAUUGAUCGUAGAAACUUGAT-3'. In some cases, HDAC2 siRNA was tagged with a 3' AlexaFluor-488 fluorescent probe. The control siRNA used was AllStars Negative Control siRNA (Qiagen). Vehicle was prepared by mixing RNase-free water in iFect solution instead of siRNA. Anxiety-like behaviors were tested 16 hours after infusion, followed immediately by collection of brains for epigenetic measures.

Measurement of Anxiety-like Behaviors

Rats were tested for anxiety-like behaviors using the elevated plus maze (EPM) or light/dark box (LDB) exploration test as reported earlier by us (13, 27, 28).

HDAC2 siRNA Infusion and Voluntary Alcohol and Sucrose Consumption

Alcohol-naïve P rats were bilaterally cannulated and then ethanol or sucrose consumption was measured using the two-bottle free-choice paradigm as described previously (14, 48). Cannulated P rats were housed individually and habituated to drink water equally from two bottles. Rats were then given one bottle containing ethanol or sucrose solution and another bottle of water. Rats were given 7% ethanol for 3 days or 2% sucrose for 2 days, followed by 9% ethanol or 4% sucrose. The concentration of sucrose solution is based on studies in rats used for sucrose drinking behaviors (49, 50). Bilateral HDAC2 siRNA or vehicle infusions into CeA were performed following the third day of 9% ethanol or 4% sucrose consumption and intake was monitored for several days. The alcohol or sucrose intake was calculated as g/kg/day.

HDAC Activity, Immunohistochemistry, *in situ* RT-PCR and Golgi-Cox Staining

HDAC activity, immunohistochemistry, *in situ* RT-PCR and Golgi-Cox staining were performed as described previously (27, 28, 45–48). The nuclear and cytosolic fractions of the amygdala were used to measure HDAC activity by a colorimetric HDAC activity assay (Biovision Research, Mountain View, CA). Immunohistochemistry was performed using the following primary antibodies: anti-Arc and anti-BDNF (Santa Cruz Biotechnology, Santa Cruz, CA); anti-HDAC2, anti-HDAC4 (MBL International, Woburn, MA) and anti-HDAC (1, 3, 5, and 6) (BioVision, Milpitas, CA); anti-acetyl histone H3-K9 and H3-K14 (Millipore, Billerica, MA); anti-NeuN (Millipore). Gold particle-conjugated anti-rabbit IgG

(Nanoprobes, Yaphank, NY) and AlexaFluor-568-conjugated goat anti-mouse IgG (Invitrogen, Eugene, OR) were used as secondary antibodies for gold-immunolabeling (13, 28) and immunofluorescence (27, 48) labeling, respectively. *In situ* RT-PCR was performed as published previously (13, 45). The primers for HDAC2 (Integrated DNA Technologies, Coralville, IO) were: Forward, 5'-CGGTGGCTCAGTTGCTGGGG-3'; Reverse, 5'-GGCCTCTGACTTCTTGGCGTGG-3'. Golgi-Cox staining was performed according to the user manual for the FD Rapid Golgi stain TM Kit (FD Neuro Technologies, Baltimore, MD) and as published (13, 46).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed following manufacturer instructions for the ChIP-IT express kit (Active Motif, Carlsbad, CA). Amygdaloid tissue was fixed, homogenized, and subjected to DNA shearing and the amount of sample normalized to contain equivalent protein amounts. Chromatin was immunoprecipitated with anti-acetyl histone H3-K9/14 antibody (Millipore) or IgG (Active Motif). Associated DNA fragments were quantified using qPCR with the RT² Sybr Green Master Mix (Super Array Biosciences, Frederick, MD) with primers for BDNF exons I and IV, and Arc designed within or adjacent to the promoter region. The primer sequences were as follows: Arc, Forward, 5'-AGTGCTCTGGCGAGTAGTCC-3', Reverse, 5'-TCGGGACAGGCTAAGAACTC-3'; BDNF exon I, Forward, 5'-GCGCCCAAAGCCCACCTTCT-3', Reverse, 5'-GCGTCGGCTCCGTGCTTCTT-3'; BDNF exon IV, Forward, 5'-GTTCGCTAGGACTGGAAGTGG-3', Reverse, 5'-CCTCTGCCTCGAAATAGACAC-3'. Relative quantification of acetylated H3-associated genes in various groups was calculated by the $\Delta\Delta C_t$ method (51). The ΔC_t for each sample was calculated from their input DNA and then $\Delta\Delta C_t$ and fold changes were calculated batch-wise from ΔC_t of respective groups.

Statistical Analyses

Statistical differences between groups were evaluated by a one or two-way ANOVA test or Student's t-test. Two-way repeated measures ANOVA was performed for evaluation of alcohol and sucrose drinking behaviors. *Post hoc* comparisons were performed using Tukey's test. A $p < 0.05$ value was considered significant.

Results

Effects of Acute Ethanol Exposure on Anxiety Measures and HDAC Activity

P and NP rats were injected with saline or ethanol (1 g/kg; IP) and anxiety-like behaviors were measured 1hr after injections using the LDB exploration test [mean \pm SEM blood ethanol levels: P = 100.8 \pm 5.0 mg% (n=13); NP= 101.1 \pm 5.2 mg% (n=13)]. Similar to our previous findings (13) we found that at baseline, P rats spent significantly ($p < 0.001$) less time in the light compartment than NP rats. Acute ethanol treatment produced an anxiolytic effect in P, but not NP rats, as evidenced by a significant ($p < 0.001$) increase in the percentage of time spent in the light compartment (Figure 1A). The total number of ambulations did not differ significantly between the groups (data not shown).

We examined effects of acute ethanol exposure on HDAC activity and found that at baseline, compared to NP rats, P rats had significantly ($p < 0.001$) higher nuclear, but not cytosolic, amygdaloid HDAC activity. Acute ethanol significantly ($p < 0.001$) inhibited HDAC activity only in the nuclear fraction of P rats without any effect in NP rats (Figure 1B). These data suggest that the anxiety-like behaviors of P rats may be related to innately higher nuclear HDAC activity in the amygdala, and the anxiolytic effects of acute ethanol may be related to inhibition of nuclear HDAC activity.

Effects of Acute Ethanol on Amygdaloid HDAC Protein Levels

Since P and NP rats showed differences in nuclear HDAC activity in the amygdala at baseline and during acute ethanol treatment, we explored the possibility that class I HDAC proteins, such as HDAC 1, 2, and 3 which are mainly localized in the nucleus (25), may be differentially expressed in the amygdala of P and NP rats. We also studied the differences in protein levels of class II HDACs, such as HDAC 4, 5, and 6, which are mainly cytosolic (25). Differences in amygdaloid HDAC 2 and 4 protein levels in P and NP rats following acute ethanol exposure was also investigated.

We found that at baseline, P rats had significantly ($p < 0.001$) higher HDAC 2, but not HDAC 1, 3, 4, 5, and 6 protein levels, in the CeA and MeA compared to NP rats. The protein levels of all measured HDAC isoforms at baseline did not differ in the BLA of P and NP rats (Figure 2A, B, and Figure S1). Acute ethanol treatment resulted in a significant ($p < 0.001$) reduction in HDAC2, but not HDAC4 protein levels, in P, but not NP rats (Figure 2A, B). These data suggest that in P rats, higher HDAC2 protein levels may be responsible for higher nuclear HDAC activity and that acute ethanol may inhibit HDAC activity via reductions in HDAC2 protein levels in the CeA and MeA.

Effects of Acute Ethanol on Amygdaloid Histone H3 Acetylation

We also measured baseline histone acetylation status of H3-K9 and H3-K14 and found that acetylated H3-K9 but not H3-K14 protein levels were significantly ($p < 0.001$) lower in the CeA and MeA, but not BLA, of P in comparison to NP rats (Figure 2A, B, and Figure S1). Acute ethanol exposure resulted in a significant ($p < 0.001$) increase in acetylated H3-K9 protein levels in the CeA and MeA of P rats, but was not significantly altered in NP rats (Figure 2A, B). These data suggest that innately high levels of amygdaloid HDAC2 could be responsible for deficits in H3-K9 acetylation in the CeA and MeA of P when compared to NP rats. Also, anxiolytic effects of ethanol may be related to the normalization of deficits in H3-K9 acetylation in the CeA and MeA of P rats.

Effects of Acute Ethanol on the Levels of Acetylated Histone H3 of *BDNF* and *Arc* Genes

We performed ChIP analysis of amygdaloid tissues using an H3-K9/14 antibody and quantified acetylated histone H3 levels of *BDNF* exons I and IV, and *Arc* genes in P and NP rats treated with either *n*-saline or acute ethanol. We found that at baseline, P rats had significantly lower levels of acetylated histone H3 of *BDNF* exon IV ($p < 0.001$) and *Arc* ($p < 0.001$) as compared to NP rats. Acute ethanol treatment increased the levels of acetylated H3 of *BDNF* exon IV ($p < 0.01$) and *Arc* ($p < 0.001$) in P, but not NP rats (Figure 2C). No significant baseline differences or acute ethanol-induced changes were observed in the levels of acetylated histone H3 of *BDNF* exon I in the amygdala of P and NP rats (Figure 2C).

Effects of HDAC2 siRNA Infusion on Anxiety-like and Alcohol Drinking Behaviors

In order to determine the direct role of HDAC2 on anxiety-like and alcohol-drinking behaviors of P rats, we infused vehicle, HDAC2 siRNA, or negative control siRNA into the CeA of P rats. Using the LDB exploration test, we found that P rats treated with HDAC2 siRNA spent significantly ($p < 0.001$) more time in the light versus the dark compartment as compared to rats infused with either vehicle or control siRNA (Figure 3A). General activity as measured by total ambulations in the LDB apparatus was not significantly different among the groups (data not shown).

In a second batch of P rats, anxiety-like behaviors were measured by the EPM test. The HDAC2 siRNA group showed a significantly ($p < 0.001$) increased percentage of open arm entries and time spent in the open arms in comparison to controls (Figure 3B). The number

of total arm entries did not differ between groups, suggesting that the treatment had no effect on the general activity level of the rats.

We utilized the two-bottle free choice paradigm of voluntary ethanol and sucrose consumption to examine the effects of HDAC2 siRNA on drinking behaviors of P rats. P rats infused with HDAC2 siRNA consumed significantly ($p < 0.01-0.001$) less ethanol than vehicle-infused P rats, which lasted six days following a single infusion of HDAC2 siRNA (Figure 3C). However, HDAC2 siRNA infusion into the CeA had no effects on sucrose intake in P rats (Figure 3D). Total fluid intake did not significantly differ between the groups (Figure 3C, D). Taken together, these data indicate that HDAC2 siRNA infusion into the CeA attenuated anxiety-like and alcohol-drinking behaviors in P rats.

Effects of HDAC2 siRNA Infusion on HDAC2 Expression and Histone H3 Acetylation

To confirm whether the infusion of HDAC2 siRNA was effectively entering neurons, we used fluorescence-tagged HDAC2 siRNA and amygdaloid brain regions were examined using confocal microscopy. Co-localization of the fluorescence-tagged HDAC2 siRNA and neurons was analyzed via staining of a neuron-specific nuclear protein (NeuN). Qualitative analysis of the confocal images revealed that HDAC2 siRNA-linked fluorescence was restricted to the CeA and did not diffuse into surrounding areas of MeA or BLA and was also primarily co-localized with cells expressing NeuN, suggesting that the siRNA preferentially transfected the neuronal population in the CeA (Figure 4A).

To determine the functional effects of HDAC2 siRNA infusion, we measured HDAC2 mRNA and protein levels, and associated changes in histone H3-K9 acetylation in amygdaloid brain regions. In comparison to P rats infused with vehicle or negative control siRNA, HDAC2 siRNA infusion resulted in a significant ($p < 0.001$) reduction in HDAC2 mRNA and protein levels and a significant ($p < 0.001$) increase in levels of acetylated H3-K9 in the CeA, but not MeA or BLA (Figure 4B, C). These data indicate that HDAC2 siRNA infusion resulted in reductions in HDAC2 mRNA and protein levels thereby, increasing histone H3-K9 acetylation in the CeA of P rats.

Effects of HDAC2 siRNA Infusion on BDNF and Arc Levels and Acetylated Histone H3 Levels of *BDNF* and *Arc* Genes

We next examined whether HDAC2 could play a role in the regulation of BDNF and Arc expression. We found HDAC2 siRNA infused P rats showed significantly ($p < 0.001$) higher levels of BDNF and Arc protein in the CeA than rats infused with vehicle or negative control siRNA (Figure 5A, B). These data suggest an association between knockdown of HDAC2 expression and increased protein levels of BDNF and Arc.

We utilized the ChIP assay to determine whether there is a change in the acetylation level of histone H3 associated with the transcriptional control regions of the *BDNF* and *Arc* genes. To further determine if there is an exon-specific effect, we looked at *BDNF* exons I and IV. The results revealed that HDAC2 siRNA infusion increased ($p < 0.001$) acetylated histone H3 levels of *BDNF* exon IV and *Arc*, but not *BDNF* exon I (Figure 5C). This suggests that the increases in BDNF and Arc levels associated with HDAC2 knockdown in the CeA could be related to increased histone H3 acetylation of *BDNF* exon IV and *Arc* genes.

Effects of HDAC2 siRNA Infusion on Dendritic Spines in the CeA

We performed Golgi-Cox staining to quantify the dendritic spines of P rats to examine whether HDAC2 siRNA-infusion had a downstream effect on dendritic spines. Compared to vehicle-infused rats, HDAC2 siRNA infusion significantly increased ($p < 0.001$) the dendritic spine density of P rats in the CeA, but not MeA or BLA (Figure 6A, B). These data imply

that lower dendritic spine density in the CeA of P as compared to NP rats, as reported earlier by us (13), could be due to higher HDAC2 levels.

Discussion

Our data demonstrate that innate abnormalities in chromatin structure and related deficits in synaptic plasticity within specific circuitry of the amygdala could play a role in alcohol drinking and anxiety-like behaviors. At the baseline level, P rats have higher nuclear HDAC activity and higher HDAC2, but not HDAC 1, 3, 4, 5, or 6, protein levels and lower acetylation of histone H3-K9, but not H3-K14 in the CeA and MeA compared to NP rats. Acute ethanol exposure caused an inhibition of HDAC activity and a reduction in HDAC2 levels resulting in increased histone H3 acetylation of *BDNF* and *Arc* genes as well as increased dendritic spines (13) in the amygdaloid brain regions of P, but not NP rats, indicating a direct correlation between histone acetylation and synaptic regulation. Additionally, HDAC2 siRNA infusion into the CeA produced anxiolytic effects and attenuated voluntary alcohol, but not sucrose, consumption in P rats. This was associated with an increase in BDNF and Arc expression and dendritic spines, resulting most likely from increased histone H3 acetylation of *BDNF* exon IV and *Arc* promoters. These novel results suggest that higher HDAC2 expression in the CeA may be involved in chromatin and synaptic remodeling which might play a fundamental role in the genetic predisposition to alcoholism and anxiety (Figure 7).

Recent studies have shown that regulation of specific HDAC isoforms may play a role in brain function and disease (26, 32, 40, 52–58). Increased HDAC2 expression has been linked to depression, Alzheimer's and Huntington's disease, and pharmacological reduction of HDAC2 appears to correct the deficits associated with these disorders (32, 58, 59). Similarly, we found that innately higher HDAC2 expression in the amygdala appears to be responsible for anxietylike and alcohol drinking behaviors in P rats, whereas knockdown of HDAC2 expression corrects these behavioral phenotypes. The innate expression of other HDAC isoforms, such as HDAC 1, 3, 4, 5, and 6 did not differ in the amygdaloid structures of P and NP rats. Taken together, these studies specifically implicate HDAC2 in the molecular mechanisms of various psychiatric disorders including alcoholism.

Interestingly HDAC2, which is predominantly expressed in neurons (60), has also been found to play an important role in the dynamic regulation of synaptic plasticity and associated gene expression and memory formation (33). For example, neuron-specific overexpression of HDAC2, but not HDAC1, resulted in decreased hippocampal synaptic plasticity (dendritic spines and synapse number) and impaired memory formation whereas, HDAC2 deficiency increased dendritic spines and synapse number in the hippocampus and enhanced memory formation in mice (33). We previously reported a deficit in amygdaloid BDNF and Arc proteins, and lower dendritic spine density in the CeA and MeA of P compared to NP rats (13, 45). The *BDNF* gene shows complex expression patterns dictated in part by differential exon-specific regulation (61). Epigenetic regulation has been found to play a role in the regulation of BDNF exon-specific transcription (26, 34, 62–64). *BDNF* exons appear to play a significant role in the regulation of synaptic plasticity and have been shown to be dynamically regulated through histone acetylation (26, 63–66). Here we observed that ethanol-induced increases in BDNF and Arc expression as well as dendritic spines in P rats (13) could be related to the increase in acetylated histone H3 levels of *BDNF* exon IV and *Arc*, but not *BDNF* exon I. We also reported that the anxiolytic effects of acute ethanol treatment were associated with increases in BDNF, Arc and dendritic spines in the CeA and MeA, suggesting that the modulation of amygdaloid synaptic plasticity could be related to the anxiety-like behaviors of P rats (13).

As observed, higher expression of HDAC2 and lower acetylation of histone H3-K9, but not H3-K14 may be responsible for deficits in BDNF and Arc expression in the amygdaloid structures of P as compared with NP rats. Ethanol also inhibited HDAC activity, reduced HDAC2 protein levels and increased histone H3-K9 acetylation in the amygdaloid brain regions of P but not NP rats. These results are clearly suggestive of a specific role for CeA HDAC2-induced chromatin remodeling in mediating anxiety-like and alcohol drinking behaviors in a manner that could be related to downstream changes in BDNF-Arc signaling and dendritic spines. Our data also suggest that HDAC2-induced chromatin remodeling in the CeA and MeA, but not BLA may be involved in controlling baseline anxiety or anxiolytic effects of ethanol in P rats compared to NP rats. Recently, an optogenetic study indicated that manipulations of BLA projections in the CeA, but not the BLA somata, were able to control anxiety-like behaviors (67). Also, abnormal neuropeptide Y (NPY) and GABA_Aα₂-regulated systems in CeA have been implicated in anxiety-like and alcohol-drinking behaviors of P rats (14, 48, 68). Future studies are needed to evaluate the epigenetic regulation of these and other genes in the amygdaloid circuitry of P rats contributing to anxiety and alcoholism.

Acute ethanol sensitivity in P and NP rats has been well characterized (12, 69). For example, P rats are less sensitive to the ataxic, aversive, and sedative/hypnotic effects of ethanol and more sensitive to its stimulatory and anxiolytic effects as compared with NP rats (12–16, 69–71). The present study raises the question as to why amygdaloid HDAC2-induced histone modifications and anxiety-measures are not affected in NP rats but modulated in P and SD rats (27, 28) by ethanol (1g/kg) exposure. This finding could be suggestive of resilience or a ceiling effect in NP rats to this dose of ethanol and differences in innate anxiety-like behaviors and anti-anxiety responses of ethanol in P and NP rats may contribute to differences in alcohol intake between these lines of rats. It has been shown that P rats consume higher amounts (>5 g/kg/day) of alcohol compared to NP (<1.5 g/kg/day) rats (12, 14, 69, 72). Interestingly, infusion of HDAC2 siRNA into the CeA of P rats caused a drastic reduction in ethanol (from about 7g/kg/day to 1.4 g/kg/day) but not sucrose consumption, suggesting HDAC2-mediated regulation of motivational aspects of alcohol drinking behaviors. Furthermore, the anxiolytic effects of HDAC2 siRNA infusion into CeA and ethanol-induced HDAC2 inhibition in the CeA and MeA observed in this study support the notion that P rats consume alcohol to attenuate anxiety-like behaviors. We have shown previously that voluntary ethanol drinking and acute ethanol exposure increased cAMP responsive element-binding protein phosphorylation and expression of NPY and BDNF in the CeA and MeA of P, but not NP rats (13, 14, 48). Here, we provide additional evidence of the complex and dynamic regulation of molecular pathways by epigenetic mechanisms leading to alcoholism and anxiety.

Conclusion

The present study identifies a causal role of HDAC2-mediated histone modifications in the CeA in the regulation of anxiety-like and alcohol drinking behaviors in P rats (Figure 7) and suggests that HDAC2-specific inhibitors may serve as useful pharmacotherapeutic agents for the treatment of both alcoholism and anxiety.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Dr. Pandey reports grand rounds presentations at the School of Medicine North Carolina at Chapel Hill; Department of Pharmacology, Howard University, Washington, DC; Department of Neurobiology, University of Texas at Austin; and the Mayo clinic Rochester, MN. He also served as an expert on the research workshop on pathological gambling organized by the Ministry of Youth and Sports, Singapore. In addition, he served as a member on NIH study sections. Dr. Pandey also reports that a US patent application on a related topic (serial number 60/848237 filed on September 29th, 2006) is currently pending. All other authors reported no biomedical financial interests or potential conflicts of interest.

The data of the manuscript has been presented at both the Research Society on Alcoholism and the American College of Neuropsychopharmacology scientific meetings and is a part of the PhD dissertation of SM.

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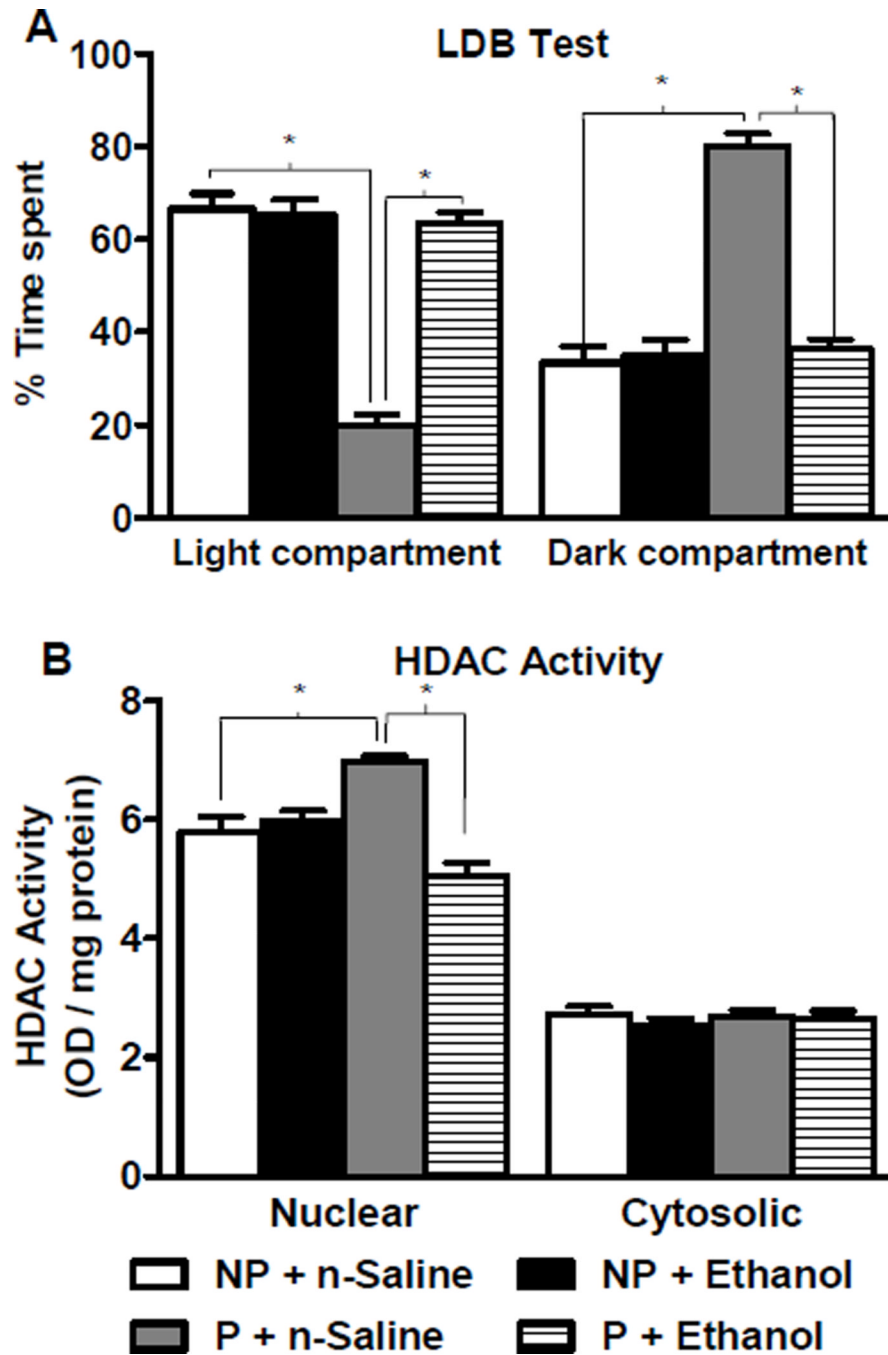


Figure 1. Effects of acute ethanol exposure on anxiety-like behaviors and HDAC activity in the amygdala of P and NP rats
A. The light/dark box (LDB) exploration test showed that alcohol-preferring (P) rats display innate anxiety-like behaviors in comparison to alcohol-nonpreferring (NP) rats. Acute ethanol treatment (1 g/kg) produced anxiolytic effects in P rats, but not NP rats. Values are represented as the mean \pm SEM of the percentage of time spent in each compartment averaged from 13 rats per group. *Significantly different from respective control groups (Two-way ANOVA; group \times treatment, $F_{1,48} = 58.6$, $p < 0.001$ followed by *post hoc* analysis by Tukey's test, $p < 0.001$).

B. The nuclear fraction, but not cytosolic fraction, of the amygdala from P rats displays higher histone deacetylase (HDAC) activity at baseline compared to NP rats. Acute ethanol treatment inhibited the nuclear HDAC activity in the amygdala of P rats, without any effect in NP rats. Values are represented as the mean \pm SEM of the optical density (OD) per mg of protein from 6 rats per group. *Significantly different from respective control groups (Nuclear HDAC activity, two-way ANOVA; group \times treatment, $F_{1, 20} = 29.6$, $p < 0.001$ followed by *post hoc* analysis by Tukey's test, $p < 0.001$).

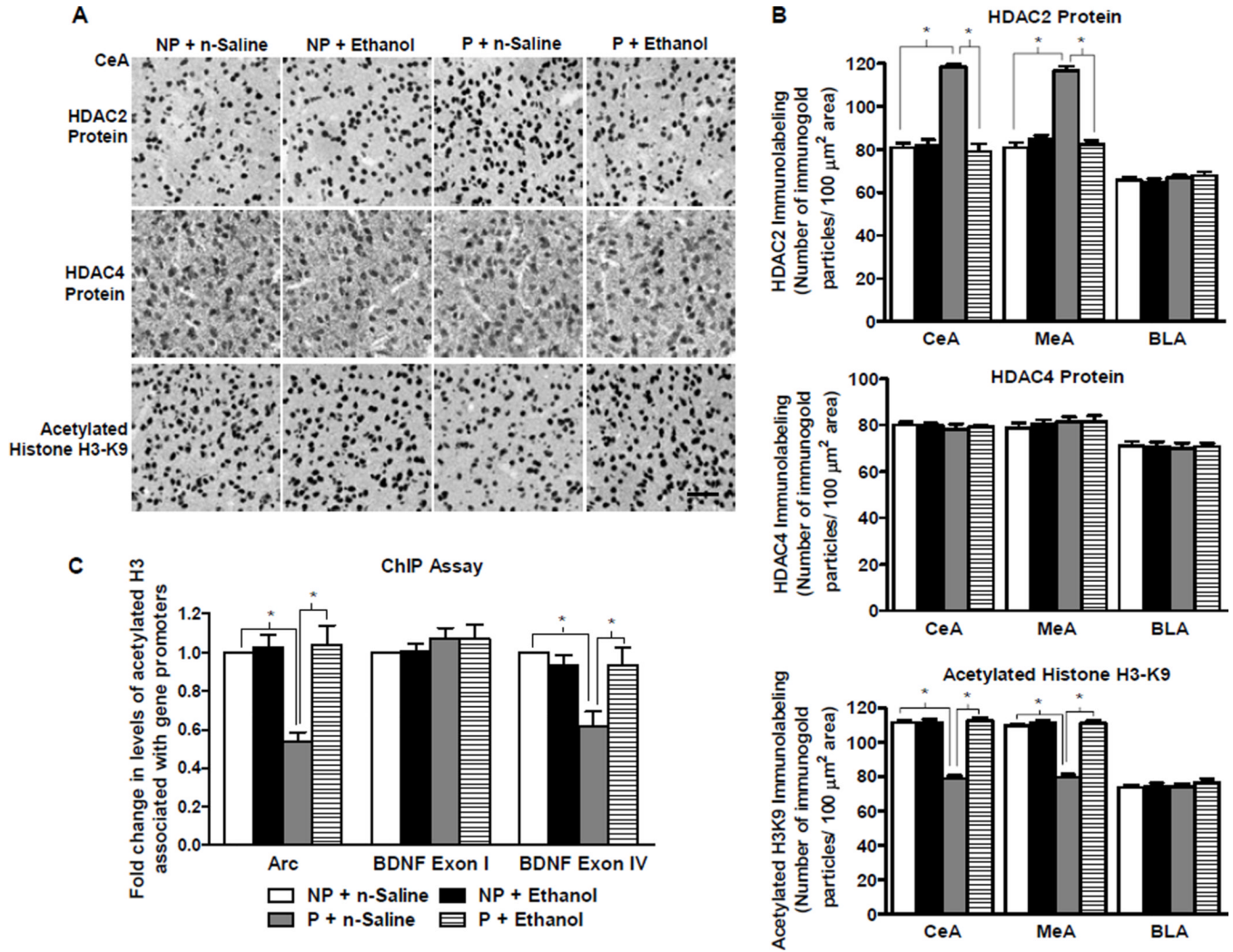


Figure 2. The effect of acute ethanol exposure on amygdaloid expression of histone deacetylases (HDAC) 2 and 4, and histone H3-K9 acetylation in P and NP rats

A. Representative low-magnification photomicrographs (Scale bar = 50 μm) of gold-immunolabeling of HDAC2, HDAC4, and H3-K9 acetylation in the central nucleus of amygdala (CeA) of alcohol-preferring (P) and -nonpreferring (NP) rats treated with either *n*-saline or ethanol (1 g/kg).

B. HDAC2 protein levels, but not HDAC4 protein levels, were innately higher in the CeA (Group \times treatment, $F_{1,20} = 64.8$, $p < 0.001$) and MeA (Group \times treatment, $F_{1,20} = 84.2$, $p < 0.001$), but not BLA, of P rats in comparison to NP rats. Acute ethanol exposure reduced HDAC2 expression, but did not modulate HDAC4 expression, in the CeA and MeA of P rats, but not NP rats. Histone H3-K9 acetylation was also significantly different between the groups in the CeA and MeA, but not BLA. Acetylated H3-K9 levels were lower in the CeA (Group \times treatment, $F_{1,20} = 104.7$, $p < 0.001$) and MeA (Group \times treatment, $F_{1,20} = 101.7$, $p < 0.001$), but not BLA, of P rats in comparison to NP rats at baseline. Treatment with acute ethanol increased levels of histone H3-K9 acetylation in the CeA and MeA of P rats, but not NP rats. Values are represented as the mean \pm SEM of the number of immunogold particles per 100 μm^2 area from 6 rats per group. *Significantly different from their respective control groups ($p < 0.001$; two-way ANOVA followed by *post hoc* analysis by Tukey's test).

C. Chromatin Immunoprecipitation (ChIP) analysis identified significant differences between the groups for acetylated histone H3-K9/14 associated BDNF exon IV and Arc, but not BDNF exon I (BDNF exon IV: group \times treatment, $F_{1, 20} = 8.6$, $p < 0.01$; Arc: group \times treatment $F_{1, 20} = 14.8$, $p < 0.001$). The levels of acetylated histone H3-associated gene promoter of BDNF exon IV and Arc, but not BDNF exon I, were lower in tissue homogenates composed primarily of CeA and MeA in P rats than in NP rats at baseline. Treatment with acute ethanol (1 g/kg) increased amygdaloid levels of acetylated histone H3 associated BDNF exon IV and Arc, but not BDNF exon I, in P rats, but not NP rats. Values are represented as the mean \pm SEM of the fold change of acetylated histone levels associated with the aforementioned genes normalized to the NP + n-saline group and derived from 6 rats per group. *Significantly different from their respective control groups ($p < 0.01$ – 0.001 ; two-way ANOVA followed by *post hoc* analysis by Tukey's test).

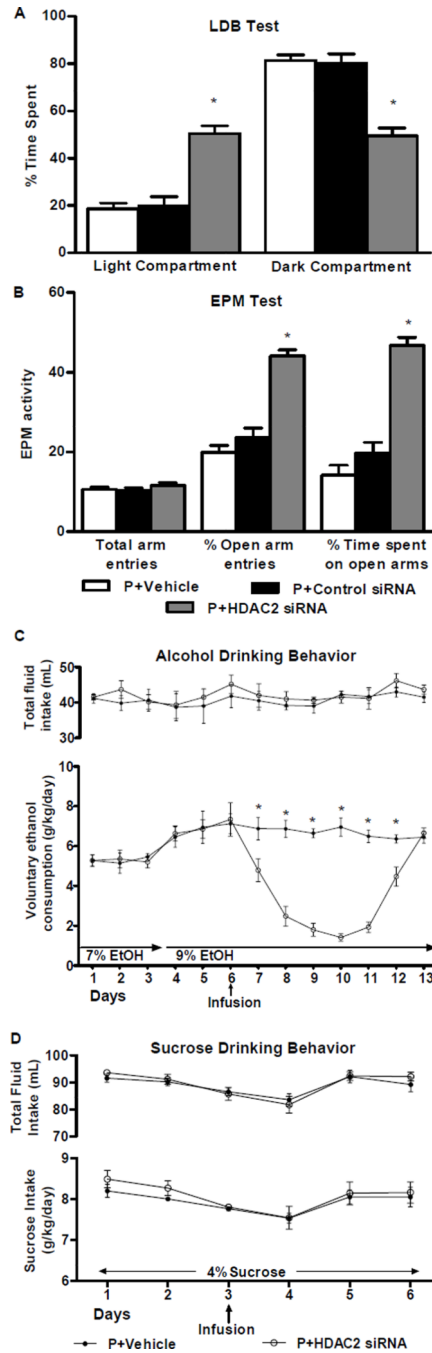


Figure 3. Effects of HDAC2 siRNA infusion into the CeA on anxiety-like behaviors and alcohol and sucrose intake in P rats

A. The light/dark box (LDB) exploration test showed that HDAC2 siRNA infusion attenuated anxiety-like behaviors of P rats in comparison to vehicle- or control siRNA-infused rats. The percentage of time spent in the light and dark compartments was significantly different among the treatment groups ($F_{2, 36} = 36.3, p < 0.001$). HDAC2 siRNA infused rats spent more time in the light compartment and less time in the dark compartment than vehicle- and control siRNA-infused rats. Values are represented as the mean \pm SEM of the percentage of time spent in each compartment averaged from 5–17 P rats per group.

*Significantly different from control groups ($p < 0.001$; one-way ANOVA followed by *post hoc* analysis by Tukey's test).

B. The elevated plus maze (EPM) exploration test also showed that HDAC2 siRNA infusion into the CeA resulted in a reduction in the anxiety-like behaviors of P rats. P rat performance on the EPM test was significantly different between the treatment groups in percentage of open arm entries ($F_{2,21} = 46.7, p < 0.001$) and the percentage of time spent in the open arms ($F_{2,21} = 52.2, p < 0.001$). P rats infused with HDAC2 siRNA showed a higher percentage of open arm entries and time spent in the open arms than those infused with vehicle or control siRNA. The number of total arm entries does not differ significantly between the groups, suggesting that there are no effects of HDAC2 siRNA infusion on the general activity of P rats. Values represent the mean \pm SEM of the percentage of open arm entries, percentage of time spent in the open arm, and number of total arm entries from 8 rats per group.

*Significantly different from control groups ($p < 0.001$; one-way ANOVA followed by *post hoc* analysis by Tukey's test).

C. Voluntary ethanol consumption as measured by the two-bottle free choice paradigm was reduced by infusion of HDAC2 siRNA, but not vehicle, into the CeA of P rats. Analysis by two-way repeated measures ANOVA identified a significant difference in the amount of ethanol consumed between the treatment groups overall and daily, as indicated by the group \times day interaction (Group: $F_{1,120} = 71.9, p < 0.001$; Group \times Day: $F_{12,120} = 13.8, p < 0.001$). P rats were given access to water and 7% ethanol followed by water and 9% ethanol.

Following the sixth day of ethanol access, P rats received infusion of vehicle or HDAC2 siRNA and consumption of water and 9% ethanol were monitored for several days. Total fluid intake did not significantly differ between the groups. Values are represented as the mean \pm SEM (6 P rats per group) of the ethanol consumption (g/kg/day) or total fluid intake (mL) plotted daily. *Significantly different between the groups ($p < 0.01$ – 0.001 ; *post hoc* analysis of the group by day interaction by Tukey's test).

D. Voluntary sucrose consumption as measured by the two-bottle free choice paradigm was unaltered by the infusion of HDAC2 siRNA and vehicle into the CeA of P rats. Analysis by two-way repeated measures ANOVA indicated no significant differences between groups. P rats were given access to water and sucrose solution as described in the methods section. Following the third day of 4% sucrose intake, P rats received infusion of vehicle or HDAC2 siRNA and consumption of water and sucrose solution were monitored for several days. Total fluid intake did not significantly differ between the groups. Values are represented as the mean \pm SEM (5 P rats per group) of the sucrose intake (g/kg/day) or total fluid intake (mL) plotted daily.

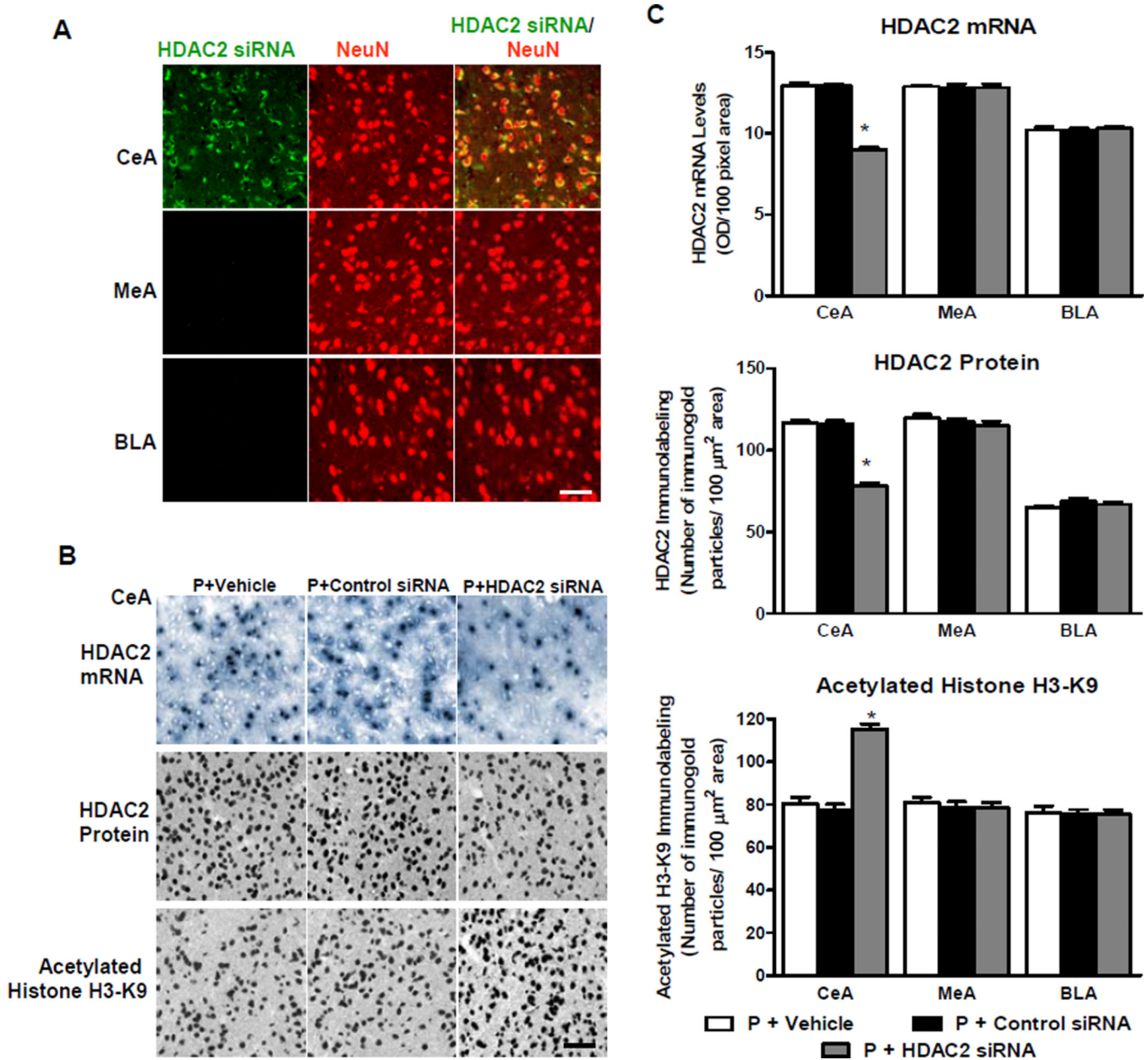


Figure 4. Effects of HDAC2 siRNA infusion into the CeA on mRNA and protein levels of HDAC2 and on histone H3-K9 acetylation in amygdaloid structures of P rats

A. Representative confocal photomicrographs reveal that HDAC2 siRNA penetrates neurons as evidenced by co-localization of fluorescence-tagged HDAC2 siRNA and neuron-specific nuclear protein (NeuN) in CeA, but not MeA or BLA. Scale bar = 50 μm.

B. Representative low-magnification photomicrographs illustrating cells of *in situ* RT-PCR for HDAC2 mRNA and immunohistochemical staining (gold immunolabeling) for HDAC2 protein and acetylated histone H3-K9 in CeA. In comparison to vehicle and control siRNA infusion, HDAC2 siRNA infusion reduced HDAC2 mRNA and protein levels, and increased acetylated histone H3-K9 protein levels in the CeA. Scale bar = 50 μm.

C. HDAC2 mRNA and protein, and histone H3-K9 acetylation levels, were significantly different between the treatment groups in the CeA, but not MeA or BLA (HDAC2 mRNA: CeA, $F_{2, 12} = 161.0$, $p < 0.001$; HDAC2 protein: CeA, $F_{2, 17} = 127.0$, $p < 0.001$; Acetylated

histone H3-K9: CeA, $F_{2, 17} = 70.0$, $p < 0.001$). HDAC2 mRNA and protein levels were lower in the CeA, but not MeA or BLA, of P rats infused with HDAC2 siRNA than those infused with vehicle or control siRNA. These results confirm that HDAC2 siRNA infusion causes a significant reduction in HDAC2 mRNA levels in the CeA. HDAC2 siRNA infusion into CeA also increased the levels of histone H3-K9 acetylation in the CeA, but not MeA or BLA. Values are represented as the mean \pm SEM derived from 5–7 P rats per group.

*Significantly different from control groups ($p < 0.001$; one-way ANOVA followed by *post hoc* analysis by Tukey's test).

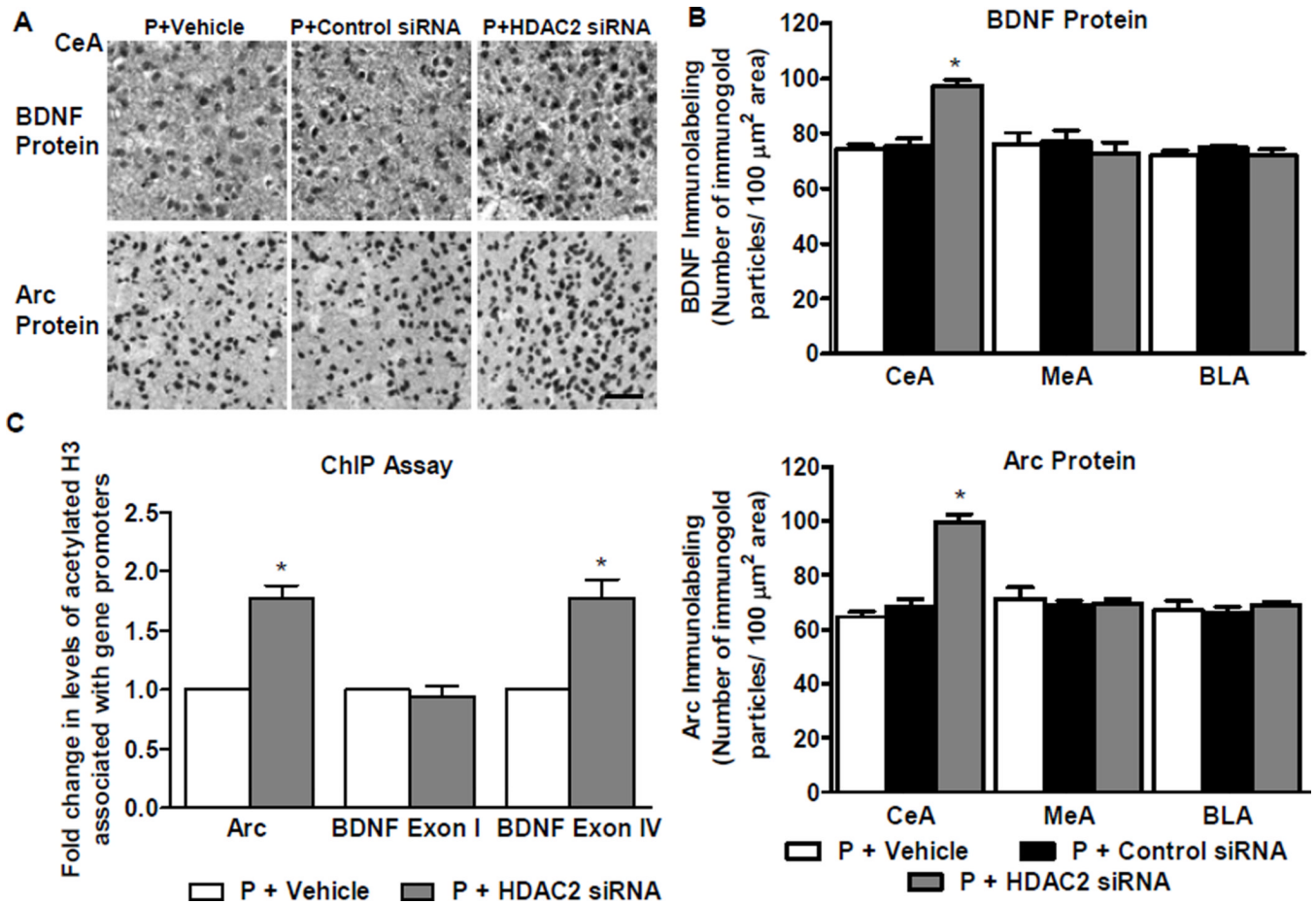


Figure 5. Effects of HDAC2 siRNA infusion into the CeA on protein levels of brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated (Arc) protein and on acetylated histone H3 levels of *BDNF* exons I, IV and *Arc* genes in the amygdala of P rats

A. Representative low-magnification photomicrographs showing immunohistochemical staining (gold immunolabeling) for BDNF and Arc protein in CeA. Scale bar = 50 μm .

B. Analysis of BDNF and Arc protein levels identified a significant difference between the vehicle-, HDAC2 siRNA-, and control siRNA-infused P rat groups in the CeA, but not MeA or BLA (BDNF: CeA, $F_{2,11} = 34.5$, $p < 0.001$; Arc: CeA, $F_{2,14} = 57.8$, $p < 0.001$). HDAC2 siRNA infusion into CeA resulted in an increase of BDNF and Arc protein in the CeA, but not MeA or BLA, of P rats in comparison to those infused with vehicle or control siRNA. Values (number of the immunogold particles per 100 μm^2 area) are represented as the mean \pm SEM and derived from 4–6 rats per group. *Significantly different from control groups ($p < 0.001$; one-way ANOVA followed by *post hoc* analysis by Tukey's test).

C. ChIP analysis revealed that HDAC2 siRNA infusion increased the levels of acetylated histone H3-K9&14 of BDNF exon IV and Arc, but not BDNF exon I. Values represent mean \pm SEM derived from 6 P rats per group. *Significantly different from vehicle-infused P rats ($p < 0.001$; Student's *t*-test).

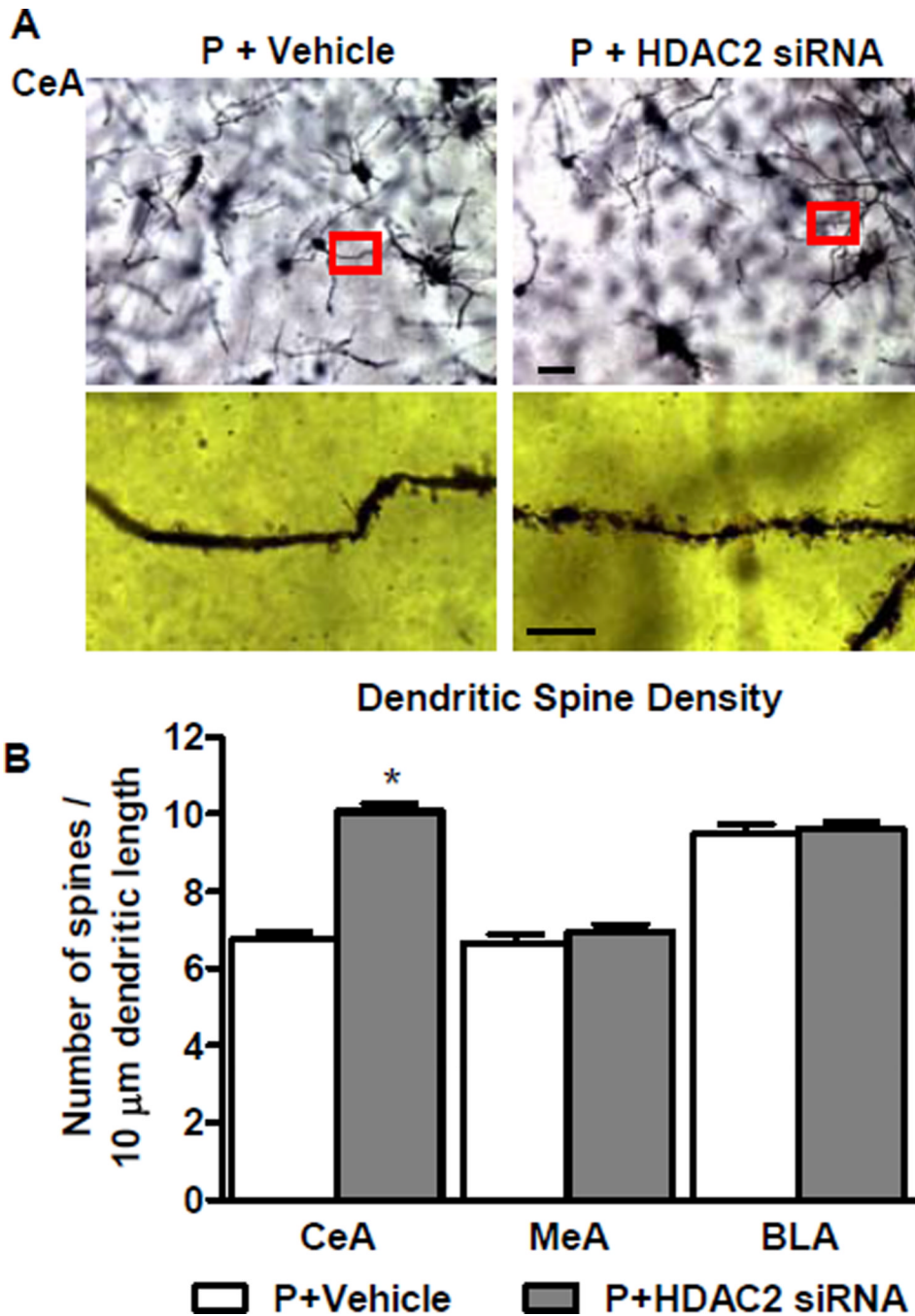


Figure 6. Effects of HDAC2 siRNA infusion into the CeA on dendritic spine density in the amygdaloid structures of P rats
A. Representative low-magnification photomicrographs (Scale bar = 50 μ m) showing Golgi-impregnated neurons in the CeA of P rats infused with HDAC2 siRNA or vehicle. The boxed areas of the low magnification photographs are shown at high magnification (Scale bar = 10 μ m) in adjacent photomicrographs showing dendritic spines.
B. HDAC2 siRNA infusion into the CeA of P rats resulted in increased dendritic spine density in the CeA, but not MeA or BLA in comparison to vehicle-infused rats. Values (number of dendritic spines per 10 μ m of dendritic length) are represented as the mean \pm

SEM derived from 6 rats per group. *Significantly different from control groups ($p < 0.001$; Student's t-test).

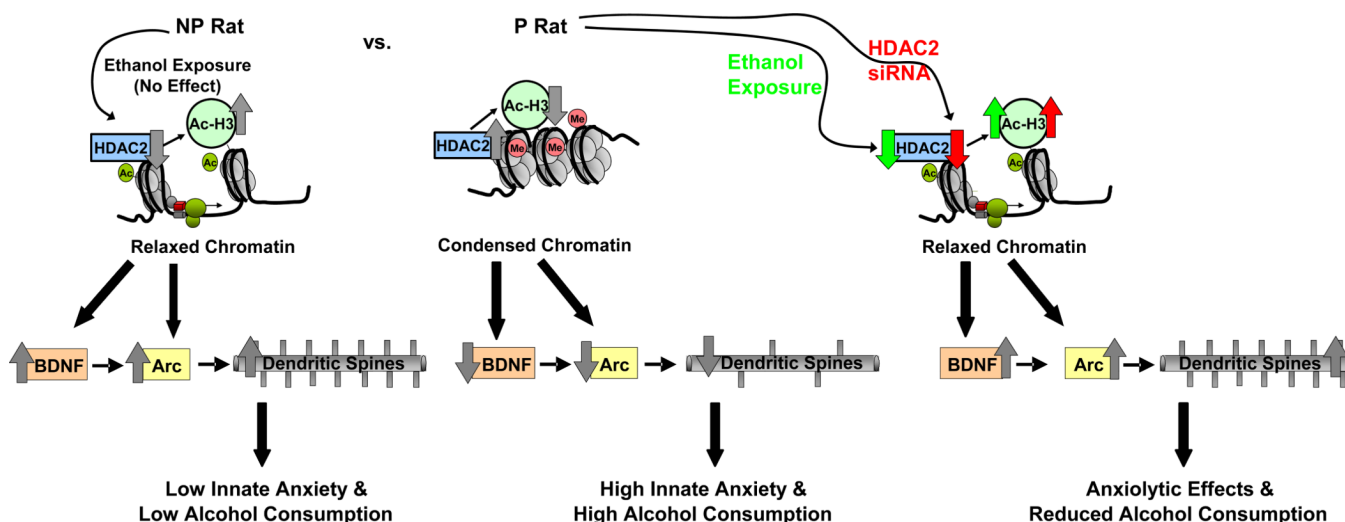


Figure 7.

Hypothetical model of baseline differences between P and NP rats, and the effect of ethanol treatment or HDAC2 siRNA infusion into CeA with regards to chromatin structure, synaptic proteins and dendritic spines, and anxiety-like and alcohol drinking behaviors. In comparison to alcohol-nonpreferring (NP) rats, alcohol-preferring (P) rats have higher levels of histone deacetylase isoform 2 (HDAC2) in the central (CeA) and medial amygdala (MeA) resulting in deficits in histone H3-K9 acetylation. The innately higher HDAC2 levels are associated with lower histone H3 acetylation of the synaptic genes, brain-derived neurotrophic factor (BDNF) and activity-regulated cytoskeleton-associated (Arc) protein in the amygdala. This suggests that HDAC2-induced chromatin condensation, due to lower histone H3 acetylation levels, results in reduced BDNF and Arc expression and lower dendritic spine density (13) in the CeA and MeA which may play a role in anxiety-like and alcohol drinking behaviors of P rats as compared to NP rats. In P rats, the anxiolytic effects of acute ethanol exposure are associated with a reduction in HDAC2 levels in the CeA and MeA which result in increased histone H3 acetylation and relaxation of chromatin of BDNF and Arc genes. The resulting upregulation of protein levels of these genes may be responsible for the increased dendritic spine density in the CeA and MeA. Knockdown of HDAC2 via infusion of HDAC2 siRNA into the CeA results in similar effects on chromatin and synaptic remodeling, and decreases the anxiety-like and alcohol drinking behaviors of P rats. These results implicate a crucial role for HDAC2-induced chromatin and synaptic remodeling in the CeA in the regulation of anxiety-like and alcohol drinking behaviors. Ac, acetylation; Me, methylation; Ac-H3, histone H3 acetylation.