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Role of Growth Arrest and DNA Damage-Inducible, Beta (GADD45b) in Alcohol Drinking Behaviors

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Abstract

Background—The contribution of epigenetic factors, such as histone acetylation and DNA methylation, to the regulation of alcohol drinking behavior has been increasingly recognized over the last several years. GADD45b is a protein demonstrated to be involved in DNA demethylation at neurotrophic factor gene promoters, including at Brain-derived neurotrophic factor (*Bdnf*) which has been highly implicated in alcohol drinking behavior.

Methods—DNA methyltransferase-1 (*Dnmt1*), *3a*, and *3b*, and *Gadd45a*, *b*, and *g* mRNA were measured in the nucleus accumbens (NAc) and ventral tegmental areas (VTA) of high ethanol consuming C57BL/6J (C57) and low alcohol consuming DBA/2J (DBA) mice using qRT-PCR. In the NAc GADD45b protein was measured via immunohistochemistry and *Bdnf9a* mRNA using in situ PCR. *Bdnf9a* promoter histone H3 acetylated at lysines 9 and 14 (H3K9,K14ac) was measured using chromatin immunoprecipitation, and 5-methylcytosine (5MC) and 5-hydroxymethylcytosine (5HMC) using methylated DNA immunoprecipitation. Alcohol drinking behavior was evaluated in *Gadd45b* haplodeficient (+/−) and null mice (−/−) utilizing drinking in the dark (DID) and 2-bottle free-choice paradigms.

Results—C57 mice had lower levels of *Gadd45b* and *g* mRNA and GADD45b protein in the NAc relative to the DBA strain. C57 mice had lower NAc shell *Bdnf9a* mRNA levels, *Bdnf9a* promoter H3K9,K14ac, and higher *Bdnf9a* promoter 5HMC and 5MC. Acute ethanol increased GADD45b protein, *Bdnf9a* mRNA, and histone acetylation and decreased 5HMC in C57 mice. *Gadd45b* +/− mice displayed higher drinking behavior relative to wild-type littermates in both DID and 2-bottle free-choice paradigms.

Conclusions—These data indicate the importance of the DNA demethylation pathway and its interactions with histone post-translational modifications in alcohol drinking behavior. Further, we

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suggest that lower DNA demethylation protein GADD45b levels may affect *Bdnf* expression possibly leading to altered alcohol drinking behavior.

Keywords

GADD45b; histone; DNA methylation; *Bdnf*; alcoholism; nucleus accumbens

Introduction

Epigenetic pathways as possible explanatory mechanisms for addiction have gained increasing support over the last several years as they are able to account for both the heritable (genetic or epigenetic) and environmental factors widely believed to underlie these illnesses (Renthal and Nestler, 2009; Krishnan et al., 2014). However, among the drugs of abuse epigenetic mechanisms are uniquely pertinent to alcohol in that ethanol affects gene expression not only by altering neurotransmission on the cell surface, but can freely traverse all biological membranes including permeating the nucleus where it can directly alter epigenetic machinery (Pandey et al., 2008a; Warnault et al., 2013; Sakharkar et al., 2012; Zhong et al., 2010). Moreover, systemic inhibition of DNA methyltransferase (DNMT) activity decreases alcohol drinking and seeking behaviors in rodents (Warnault et al., 2013).

Histone post-translational modifications and DNA methylation are two important aspects of epigenetic gene regulation. Histone H3 acetylation generally induces an open chromatin state allowing for the binding of transcription factors, while DNMT-catalyzed methylation at the 5′ carbon of cytosine bases (5-methylcytosine (5MC)) reduces DNA access (Gavin and Floreani, 2014; Szyf, 2010). 5MC can be further processed into another stable cytosine modification, 5-hydroxymethylcytosine (5HMC). The effects of 5HMC on gene expression are complex and still under investigation with roles likely largely dependent on whether it precedes a guanine or adenine and its location relative to the gene transcription start site (TSS) (i.e., proximal to the promoter or in the gene body) (Kinde et al., 2015). 5HMC or 5MC are thought to be deaminated by a cytidine deaminase forming 5-hydroxymethyluracil (5HMU) or uracil. 5HMU or uracil is then believed to be removed by a DNA glycosylase. Although not enzymes themselves, Growth arrest and DNA Damage-inducible (GADD45) proteins (GADD45a, GADD45b, and GADD45g) are cornerstones of the DNA demethylation process in that they are capable of recruiting cytidine deaminases and DNA glycosylases to genomic loci (Cortellino et al., 2011; Guo et al., 2011; He et al., 2011).

Human and animal studies indicate dysregulation in both histone acetylation and DNA methylation in alcoholism. In humans with alcoholism, upregulation of histone methyltransferases SET domain containing 1A (SETD1A) and mixed-lineage leukemia 4 (MLL4) in the frontal cortex and basolateral nucleus of the amygdala, and decreased DNMT1 in the frontal cortex and basolateral and central nuclei of the amygdala have been reported (Ponomarev et al., 2012), while in psychotic subjects alcohol abuse appears to increase the cytidine deaminase Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC3C) mRNA in the parietal cortex (Guidotti et al., 2012). In rodents it was found that DNMT1 is increased in the nucleus accumbens (NAc) of mice undergoing cycles of excessive alcohol intake and withdrawal (Warnault et al., 2013). One

hour after an acute ethanol injection there is a decrease in histone deacetylase (HDAC) activity and increase in histone H3 and H4 acetylation in rat amygdaloid brain regions. On the other hand, after an intermittent access paradigm or alcohol withdrawal there are decreases in histone acetylation (Pandey et al., 2008a; Sakharkar et al., 2012; Warnault et al., 2013).

The Brain-derived neurotrophic factor (*Bdnf*) gene is a target of epigenetic machinery, and its dysregulation has been highly implicated in alcoholism (Ma et al., 2009; Chen et al., 2003; Martinowich et al., 2003; McGough et al., 2004; Jeanblanc et al., 2009). Lower BDNF levels in rodent models is associated with increased ethanol consumption (Hensler et al., 2003; McGough et al., 2004; Yan et al., 2005; Jeanblanc et al., 2006; Moonat et al., 2011). The role of BDNF in alcohol drinking behavior is further supported by the fact that BDNF is an ethanol-responsive gene in humans and rodents (Pandey et al., 2008b; Joe et al., 2007; Huang et al., 2011). Acute ethanol, whether administered intraperitoneal (i.p.) or voluntarily consumed, increases *Bdnf* mRNA levels in the mouse dorsal striatum (McGough et al., 2004). While these studies indicate that ethanol increases BDNF expression leading to reduced ethanol consumption the mechanisms behind this response remain to be elucidated.

In the current study we examined baseline and ethanol-induced changes in epigenetic *Bdnf* regulation by comparing the high-drinking C57BL/6J (C57) and low-drinking DBA/2J (DBA) strains as well as mice with a deficiency in a DNA demethylating protein known to demethylate *Bdnf*, GADD45b (Misra and Pandey, 2003; Shelton and Grant, 2002; Kerns et al., 2005; Belknap et al., 1977; Belknap et al., 1993; Gupta et al., 2005; Ma et al., 2009; Gavin et al., 2012). We measured baseline and acute ethanol-induced changes in *Gadd45* gene expression and the effects on *Bdnf* promoter 5MC, 5HMC, and histone H3 acetylation (H3K9,14ac) in these two strains. Subsequently, we used *Gadd45b* haploinsufficient and total knockout mice to further examine the direct role of GADD45b in ethanol drinking behaviors.

Methods & Materials

C57BL/6J vs. DBA/2J procedures

All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Male 12–16 week-old C57BL/6J (C57) and DBA/2J (DBA) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were group housed in a temperature-controlled room with a 12/12 hr light/dark cycle, with food and water provided ad lib. Mice received a single injection of 2 g/kg ethanol or normal saline i.p. and after 1 hr were injected with pentobarbital 50 mg/kg. Under sedation mice were either immediately decapitated and brain tissues were dissected and quickly frozen, or perfused with normal saline followed by 4% paraformaldehyde (PFA) solution prepared in 0.1 M phosphate buffer (pH 7.4). All brains were frozen and kept at −80 °C until further use. Blood was collected to measure ethanol levels using an Analox alcohol analyzer (Analox Instruments; Lunenburg, MA).

Breeding and genotyping of *Gadd45b* haploinsufficent and knockout mice

Gadd45b knock-out mice were obtained from the Liebermann laboratory at Temple University (Gupta et al., 2005) and backcrossed at least 5 generations to mice of a C57BL/6J genetic background. Wild-type, knockout and heterozygous mice were generated from heterozygous breeding pairs. Male mice (12–16 weeks old) were used in all experiments. Genotyping was performed by PCR. The DNA samples isolated from tail tips were used for PCR amplifications [95°C for 10 min; then 40 cycles of 95°C for 30 sec, 60°C for 1 min, and 72°C for 30 sec] to identify the wild-type (253 bp band), heterozygous (165 + 253 bp bands), and knockout (165 bp band) mice. The primers used were as follows: *Gadd45b* 1: 5′-TCAGGCAAGAGGAGACTGAGA-3′; *Gadd45b* 2: 5′-CAAGCGATCTGTCTTGCTCA-3′; PGK promoter: 5′-TAAAGCGCATGCTCCAGACT-3′.

Two bottle free-choice procedure

Twenty-four hour continuous access ethanol preference measurements were conducted using the two bottle free-choice paradigm (Thiele et al., 2000; Misra and Pandey, 2003). Singly housed mice were provided with two water bottles for two weeks with ad libitum access to food and water; a duration sufficient to habituate mice to drink equally from both bottles. Bottle positions were changed daily to avoid a positional habit. After two weeks one water bottle was replaced with a bottle containing ethanol with the position of the ethanol and water bottles being switched daily. Concentrations of ethanol were increased every 3 days from 3% to 6% to 9%, and finally 12% for the final 3 days. Consumption of ethanol and water was measured daily at 10AM and fresh water and ethanol (3, 6, 9, or 12%) solution in water were provided every day. The mean ethanol intake in g/kg, and water intake were calculated.

Drinking-in-the-Dark procedure

Drinking-in-the-Dark (DID) was performed as documented in previous publications (Rhodes et al., 2005). Mice were singly housed and allowed to acclimate to the reverse light-dark cycle for 2 weeks with ad libitum access to food and water. The light cycle was from 8:00PM to 8:00AM and the dark cycle from 8:00AM to 8:00 PM. Three hours after the beginning of the dark cycle (11:00AM), water bottles in home cages were replaced with bottles containing 20% ethanol in tap water. These bottles remained in place for 2 hrs (removed at 1:00PM) for the first 3 days. The experimental procedures on Days 4 and 5 were the same as those for Days 1, 2, and 3, except the ethanol containing bottles remained in place for 4 hrs (removed at 3:00PM). Ethanol consumption was assessed daily; average daily ethanol consumption across all genotypes was 5.58 g of ethanol per kg body weight (SEM=0.24).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated using TRIZOL reagent (Life Technologies; Carlsbad, CA), and DNA removed using DNA-free™ DNA Removal Kit (Life Technologies; Carlsbad, CA). Target gene expression was normalized to Hypoxanthine Phosphoribosyltransferase 1 (*Hprt1*) (see Table 1 for primer sequences). For qRT–PCR, cDNA samples were analyzed

using PikoReal Real-Time PCR (Thermo Fisher; Waltham, MA) and Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Fisher; Waltham, MA). Primers were designed to span at least one intron–exon boundary, with the exception of *Bdnf9a* which lacks an intervening intron. The following cycling conditions were used: 10 min at 95 °C then 40 cycles at 95°C for 30 s, 60 °C for 1 min, and 72°C for 30 s.

GADD45b immunolabeling

Protein levels of GADD45b were determined by the gold-immunolabeling histochemical procedure as described previously (Pandey et al., 2004). Mice were anesthetized, perfused intracardially with normal saline (40 ml), and then 100 ml of 4% ice-cold paraformaldehyde fixative. Brains were removed and placed in fixative for 20 hr at 4°C. After fixation, brains were incubated in 10%, 20% then 30% sucrose (prepared in 0.1 M phosphate buffer, pH 7.4). Twenty micron sections were used for gold immunolabelling. Anti-GADD45b antibody (Aviva Systems Biology; San Diego, CA) in a 1:300 dilution in 1% BSA in 0.01 M PBS was incubated overnight at room temperature followed by incubation with conjugated anti-rabbit secondary antibody (1:200 dilution in 1% BSA in 0.01 M PBS) for 1 hr at room temperature. The quantification of gold-immunolabeled protein was performed using an Image Analysis System connected to a light microscope that calculated the staining intensity based on the number of immunogold particles per 100 μm^2 area of a defined brain structure at high magnification (100×). Immunogold particles in the defined brain structures of three adjacent brain sections of each mouse were counted then averaged for each mouse.

In situ RT-PCR for *Bdnf9a* mRNA measurements

Mouse brain sections were used to determine mRNA levels using in situ RT-PCR, as reported previously (Pandey et al., 2004). Floating brain sections (40 μm thickness) were treated with proteinase K (1 μg/ml in 1× PBS containing 0.05% Triton X-100) for 15 min at 37°C and then subjected to DNase digestion. After the sections were washed with 1× PBS, they were transferred to PCR tubes containing 100 μl of PCR mixture (Applied Biosystems, Foster city, CA) and reverse transcribed for 1 hr at 42°C with reverse transcriptase in the presence of oligo-dT. In the negative sections, reverse transcriptase enzyme was not added. The PCR was performed with Taq DNA polymerase enzyme and 36–38 pmol of each *Bdnf9a* primers (primers 5′-GCAGCTGGAGTGGATCAGTAA-3′ and 5′-TGGTCATCACTCTTCTCACCTG-3′) and 1 mM each of NTP and dTTP, except that the dTTP was replaced by digoxigenin (DIG)-11-dUTP PCR conditions for *Bdnf9a*: 95°C for 5 min; 95°C for 30 sec; 60°C for 30 sec; 72°C for 30 sec (total of 28 cycles), and then 72°C for 7 min]. After PCR, sections were mounted on slides, and *Bdnf9a*-positive cell bodies were detected using alkaline phosphatase-conjugated anti-DIG Ab and subsequent staining of the complex with the specific substrate nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate (Roche Molecular Biochemical; Mannheim, Germany). The optical density (OD) of *Bdnf9a* positive cell bodies was calculated with an Image analyzer (Loats Associates; Westminster, MD). The OD of the negative brain sections was subtracted from the positive brain sections. The OD of positive cell bodies in the defined brain structures of three adjacent brain sections of each mouse was calculated, and values were averaged for each mouse. The results are represented as the mean OD per 100 pixel area.

Methylated DNA Immunoprecipitation (MeDIP) or hydroxymethylated MeDIP (hMeDIP)

MeDIP and hMeDIP were performed as previously published by our group (Gavin et al., 2015; Gavin et al., 2012). Tissue samples were sonicated to 200–500 bp segments in SDS lysis buffer. DNA was extracted using proteinase K 37°C overnight followed by phenol-chloroform. Two micrograms of DNA were resuspended in 250μL of ChIP dilution buffer, and incubated with 5 μL of 5-methylcytosine (5MC) monoclonal mouse (Diagenode; Denville, NJ) or 2 μL of rabbit polyclonal 5-hydroxymethylcytosine (Active Motif; Carlsbad, CA) antibodies overnight on a rotator at 4°C. Antibody-DNA complexes were then precipitated using Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology; Dallas, TX) for 3 hrs on a rotator at 4°C. Following low salt, high salt, LiCl, and TE washes DNA was eluted from beads using elution buffer at 67°C for 2 hrs. DNA was then extracted using proteinase K 37°C overnight followed by phenol-chloroform. Samples were resuspended in nuclease-free water and measured via qRT-PCR relative to input DNA. IP samples were normalized to input. Values presented are calculated as percent input.

Chromatin Immunoprecipitation (ChIP)

ChIP experiments were performed as previously published by our group (Gavin et al., 2009). Cross-linking was performed by adding a final concentration of 1.5% formaldehyde to tissue homogenized in RPMI media (Life Technologies; Carlsbad, CA) for 15 minutes. Cross-linking was quenched using a final concentration of 130mM of glycine. Cells were washed with PBS in the presence of protease inhibitors then sonicated to 200–500 bp segments in SDS lysis buffer. Sonicated chromatin was resuspended at 1:10 in ChIP dilution buffer. IP samples were pre-cleared with 20 μL Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology; Dallas, TX) for 2 hrs on a rotator at 4°C. Subsequently, samples were incubated overnight with rabbit polyclonal anti-H3K9,K14ac antibody (EMD Millipore; Kankakee, IL) on a rotator at 4°C. Antibody-DNA complexes were precipitated using Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology; Dallas, TX) for 3 hrs on a rotator at 4°C. Following low salt, high salt, lithium chloride, and TE washes complexes were eluted from beads at 67°C for 2 hrs using elution buffer then IP and input (33% of IP by volume) samples were uncrosslinked using a final concentration of 0.2 M NaCl. DNA was then extracted using proteinase K at 37 °C overnight followed by phenol-chloroform extraction and resuspended in water. Samples were measured using qPCR. IP samples were normalized to input. Values presented are calculated as percent input.

Statistics

Data were analyzed with Student’s t-test or one-way analysis of variance (ANOVA) with post hoc Bonferroni tests as appropriate. Ethanol consumption data (both two-bottle choice and DID) were analyzed using two-way repeated measures (RM) ANOVA for genotype and duration of administration for DID or genotype and time for two-bottle free choice. Post hoc comparisons for two-way ANOVA were performed using Bonferroni. All statistical analyses were performed using GraphPad Prism (version 15.0 for Windows) (Graphpad; La Jolla, CA). All statistical tests were two sided.

Results

C57 mice have lower GADD45b mRNA and protein expression in the nucleus accumbens (NAc) compared to the DBA strain

We compared mRNA expression encoding key components of DNA demethylation, the *Gadd45* genes (*Gadd45a*, *b*, and *g*). We found that relative to DBA mice C57 mice had lower mRNA expression of *Gadd45b* (t_{16} = 2.618, p = 0.02) and *Gadd45g* (t_{16} = 2.672, p = 0.02) in the NAc (Figure 1A). By comparison, a similar difference was not found in the ventral tegmental area (VTA) (Figure 1C). We did not find a difference in the mRNA expression levels of the DNA methylating enzymes, *Dnmt1*, *3a*, and *3b* in the entire NAc (Figure 1B). The lower level of NAc *Gadd45b* mRNA expression we found translated to lower GADD45b protein levels in the NAc shell (ANOVA F ($_{3,19}$)= 24.30, Bonferroni post hoc test, P < 0.0001), but not NAc core of normal saline treated mice. Moreover, an acute i.p. injection of ethanol 2 g/kg increased GADD45b protein expression in the NAc shell of C57 mice, but did not affect expression in DBA mice relative to saline treated controls (Figure 2). This was despite equivalent blood alcohol concentrations achieved in the two strains (C57 Mean±SEM 179.2±6.2 mg/dl vs. DBA Mean±SEM 193.8±9.1 mg/dl, n = 12 per group).

Lower *Bdnf9a* expression in the NAc shell of C57 mice relative to DBA

Considering the regionally lower levels in GADD45b protein expression we examined mRNA expression of a known target of GADD45b, *Bdnf9a*. We found significantly lower *Bdnf9a* mRNA expression in the NAc shell but not core of normal-saline treated C57 mice relative to DBA (ANOVA F($_{3,19}$)= 23.04, Bonferroni post hoc test, P < 0.0001). This difference between the strains was reduced by acute ethanol treatment (Figure 3).

Epigenetic differences in the NAc of C57 vs. DBA mice at *Bdnf9a*

GADD45b has been shown to be involved in demethylating the *Bdnf9a* promoter (Ma et al., 2009; Gavin et al., 2015). We measured 5-hydroxymethylcytosine (5HMC) and 5-methylcytosine (5MC) at the *Bdnf9a* promoter in C57 and DBA mice. We found a significant increase in both 5HMC (ANOVA F($_{3,28}$) = 4.660, Bonferroni post hoc test, P = 0.01) and 5MC (ANOVA F($_{3,30}$) = 4.628, Bonferroni post hoc test, P = 0.01) in normal saline treated C57 mice relative to DBA. Moreover, acute ethanol treatment reduced the 5HMC difference between the strains. On the other hand, 5MC remained significantly elevated in the C57 strain relative to DBA following ethanol treatment (Figure 4A).

In addition, to measuring DNA methylation we measured histone H3 acetylation (H3K9,14ac). This was in part based on a previous publication from our group in which it was noted that C57 mice have lower levels of CREB protein and its activated form, phosphorylated CREB (pCREB), which is involved in histone acetylation in the NAc, compared to DBA mice (Misra and Pandey, 2003). In accord with the lower CREB and pCREB levels we found lower H3K9,14ac at the *Bdnf9a* promoter in normal saline treated C57 mice relative to the DBA strain which was no longer significantly different from DBA following acute ethanol (ANOVA F($_{3,30}$) = 4.286, Bonferroni post hoc test, P = 0.01) (Figure 4B).

Baseline *Gadd45* mRNA expression differences in *Gadd45b* deficient mice

We measured *Gadd45* mRNA transcripts in the entire NAc of *Gadd45b* +/+, *Gadd45b* +/−, and *Gadd45b* −/−. We found no change in *Gadd45a* or *Gadd45g* in *Gadd45b* +/− mice relative to *Gadd45b*+/+ at baseline (ANOVA $F(_{2,15}) = 7.793$, Bonferroni post hoc test, $P = 0.01$). Predictably, *Gadd45b* was reduced in *Gadd45b* +/− and *Gadd45* −/− mice relative wild-type control (ANOVA $F(_{2,15}) = 63.42$, Bonferroni post hoc test, $P < 0.0001$) (Figure 5A).

GADD45b deficiency alters ethanol drinking behaviors

In order to clarify the role of GADD45b in ethanol drinking behaviors we assessed ethanol intake using the drinking in the dark (DID) paradigm and 2-bottle free choice paradigms of *Gadd45b* haploinsufficient (*Gadd45b* +/−) and complete knockout animals (*Gadd45b* −/−), which had been backcrossed into a C57 genetic background.

In the DID paradigm, during the 4 hr drinking sessions on days 4 and 5, *Gadd45b* +/−, but not *Gadd45b* −/− mice, drank significantly more ethanol than *Gadd45b* +/+ controls (two-way RM ANOVA, effect of genotype: $F_{2,52} = 3.367$, $P = 0.04$; duration: $F_{1,52} = 111.5$, $P < 0.0001$; interaction: $F_{2,27} = 4.191$, $P = 0.02$) (Figure 5B).

In the 2-bottle free choice paradigm mice were provided with two water bottles for two weeks. Subsequently, we provided mice with one bottle of 3% ethanol and a bottle of water for 3 days, then 6% ethanol for the next 3 days, 9% for 3 days, and finally 12% ethanol. The locations of the water and ethanol bottles were switched daily. Similar to the DID paradigm we found an increase in ethanol consumption in the *Gadd45b* +/− mice relative to the *Gadd45b*+/+ wild-type control on the 12% ethanol concentration days, which was not found in the total knockout *Gadd45b* −/− (two-way RM ANOVA, effect of genotype: $F_{2,252} = 31.81$, $P < 0.0001$; time: $F_{11,252} = 136.4$, $P < 0.0001$; interaction, $F_{22,252} = 1.929$, $P = 0.009$) (Figure 5C). No significant differences were found in water consumption during the 12% ethanol consuming days (Table 2).

Discussion

In the current study we used the high-alcohol consuming C57BL/6J (C57) and low consuming DBA/2J (DBA) mouse strains to study potential pathways leading to increased ethanol consumption based on numerous reports documenting the higher ethanol consumption of C57 mice compared to DBA in a variety of drinking paradigms (Yoneyama et al., 2008; Belknap et al., 1993; Rhodes et al., 2005). We found lower *Gadd45b* and *Gadd45g* mRNA expression in the entire NAc and GADD45b protein in the NAc shell of C57 mice compared to DBA, but no differences in *Dnmt* expression and no VTA *Gadd45* gene expression differences. As perhaps a downstream consequence of lower NAc GADD45b and g we also found lower *Bdnf9a* mRNA expression in the NAc shell. GADD45b has been documented to play an important role in DNA demethylation at the *Bdnf9a* promoter, thereby having a positive modulatory function (Gavin et al., 2012; Ma et al., 2009; Gavin et al., 2015). In agreement with this reported role we found higher 5HMC and 5MC at the *Bdnf9a* promoter in the NAc of C57 mice as compared with DBA. In

conjunction with higher 5HMC and 5MC at the *Bdnf9a* promoter there was lower H3K9,14ac in C57 vs. DBA mice.

The role of ethanol as an HDAC inhibitor has been well-documented. Acute ethanol has been reported to increase histone acetylation in the brain likely in part through its HDAC inhibitory activity, including in the NAc (Pandey et al., 2008a; Sakharkar et al., 2012; Botia et al., 2012). We also found that acute ethanol injection increased NAc histone acetylation and reduced 5HMC at the *Bdnf* gene in the NAc of C57 mice without producing any effects in DBA mice. We recently reported in primary neuron cultures 5HMC is highly responsive to exogenous stimuli (Gavin et al., 2015). Further, the ability of an HDAC inhibitor, in this case acute ethanol, to induce DNA demethylation has been previously documented (Detich et al., 2003; Dong et al., 2007).

Over the last two decades studies have implicated BDNF in the survival and function of adult dopamine neurons, learning, memory, synaptic plasticity and regulation of dendritic morphology (Russo et al., 2009). In humans, BDNF polymorphisms have been associated with vulnerability to alcohol dependence and relapse following treatment. Decreased plasma BDNF levels have been observed in alcoholics, particularly those with a family history of alcoholism (Joe et al., 2007; Zanardini et al., 2011). Additionally, gene microarrays in humans indicate BDNF as an ethanol-responsive gene (Davis, 2008). In rodent models, microarray data indicate differential effects of acute ethanol on *Bdnf* signaling in the NAc between C57 and DBA strains (Kerns et al., 2005). Further, i.p. injection or self-administration of ethanol increases BDNF expression (Jeanblanc et al., 2009; Pandey et al., 2008b). Moreover, *Bdnf* haploinsufficient mice (*Bdnf*+/−) exhibit a preference for voluntary ethanol consumption (Jeanblanc et al., 2009), and alcohol preferring rats have innately lower levels of BDNF in the bed nucleus of the stria terminalis and in the central extended and medial amygdala as compared with alcohol non-preferring rats (Prakash et al., 2008). On the other hand, BDNF infusion decreases ethanol intake (Jeanblanc et al., 2009; Pandey et al., 2006). Even among C57 mice, BDNF expression in the NAc is inversely correlated with alcohol-drinking behavior (Wolstenholme et al., 2011). Taken together, these data indicate the importance of BDNF in alcohol use disorders as a potential contributor through both the reward and anxiety circuity.

Considering the lower GADD45b protein levels in the higher ethanol consuming C57 strain, we examined drinking behavior in *Gadd45b* +/− and *Gadd45b* −/− mice compared to wild-type control to directly implicate GADD45b in alcohol drinking behaviors. Interestingly, *Gadd45b* +/− mice but not *Gadd45b* −/− consumed more ethanol in both DID and 2-bottle free choice paradigms compared to wild-type controls. Although it is true that some genetic differences are additive (i.e., the null mutant is more different from wild-type control than the heterozygote) other genetic differences produce phenotypes in which there is dominance or overdominance. Overdominance (the haploinsufficient genotype showing a more robust phenotype than either the null mutant or wild-type) is commonly found in the alcohol literature. On a multi-genic level, F1 hybrid mouse strains produced from breeding inbred strains often display increased drinking behavior compared to either parent strain (Blednov et al., 2010). Gene specific examples exist as well. Transcription factor Lmo3 null mice have been reported to be no different than their wild-type littermates, while Lmo3 partial

knockdowns drink more (Savarese et al., 2014; Lasek et al., 2011). Similarly, dopamine transporter, GABA transporter, and synaptic vesicle protein Rab3A haploinsufficient mice voluntarily consume more ethanol compared to their wild-type and homozygous null littermates (Savelieva et al., 2002; Cai et al., 2006; Kapfhamer et al., 2008). Further, all three Gadd45 proteins share partial sequence homology and Gadd45a, like Gadd45b, has also been shown to be involved in DNA demethylation (Gavin et al., 2012; Jung et al., 2007; Barreto et al., 2007). Therefore, it is possible that there is redundancy in aspects of their functions. Nonetheless, these data along with the findings of lower expression of *Gadd45g* and *b* in the NAc of C57 mice suggests the involvement of *Gadd45b* in the regulation of alcohol drinking behaviors.

Gadd45b −/− mice have been shown to have deficits in dendritic arborization and neurogenesis following electroconvulsive shock (Ma et al., 2009). Behaviorally, *Gadd45b* −/− mice have been reported to have both deficits and enhanced hippocampus dependent learning, and no differences in amygdala-dependent learning or increased measures of anxiety (Leach et al., 2012; Sultan et al., 2012). The current study is the first to our knowledge to examine *Gadd45b* in the reward pathway and to demonstrate its potential role in alcohol addiction.

Several studies indicate that ethanol treatment alters DNA methylation homeostasis. In cultured astrocytes prolonged ethanol treatment reduces DNMT activity, while intermittent alcohol treatment of mice increases NAc DNMT1 protein levels (Warnault et al., 2013; Zhang et al., 2014). In human alcoholics DNMT1 expression is reduced in the basolateral and central nuclei of the amygdala and the cortex (Ponomarev et al., 2012). Our own data indicate increased NAc DNA methylation at the *Bdnf9a* gene promoter and lower levels of a DNA demethylating protein, GADD45b, predisposed to higher ethanol drinking behavior. Taken together it appears that an imbalance of DNA methylating and demethylating factors, favoring increased DNA methylation, may predispose to increased alcohol drinking behavior, while alcohol reverses this imbalance (Figure 6).

Here, we demonstrate that a deficiency in GADD45b in the NAc altered the epigenetic milieu at the *Bdnf9a* promoter, thereby affecting *Bdnf* expression. Lower GADD45b levels corresponded to increased alcohol drinking behavior whether measured by DID or 2-bottle free choice paradigm. We reported earlier that epigenetic regulation of *Bdnf* expression in the amygdala regulates alcohol drinking behaviors (Moonat et al., 2013). Based on the results of present studies, we hypothesize that an epigenetically-mediated reduction in *Bdnf* predispose to higher ethanol consumption. This study implicates DNA methylation dynamics in the NAc as important variables leading to increased alcohol consumption.

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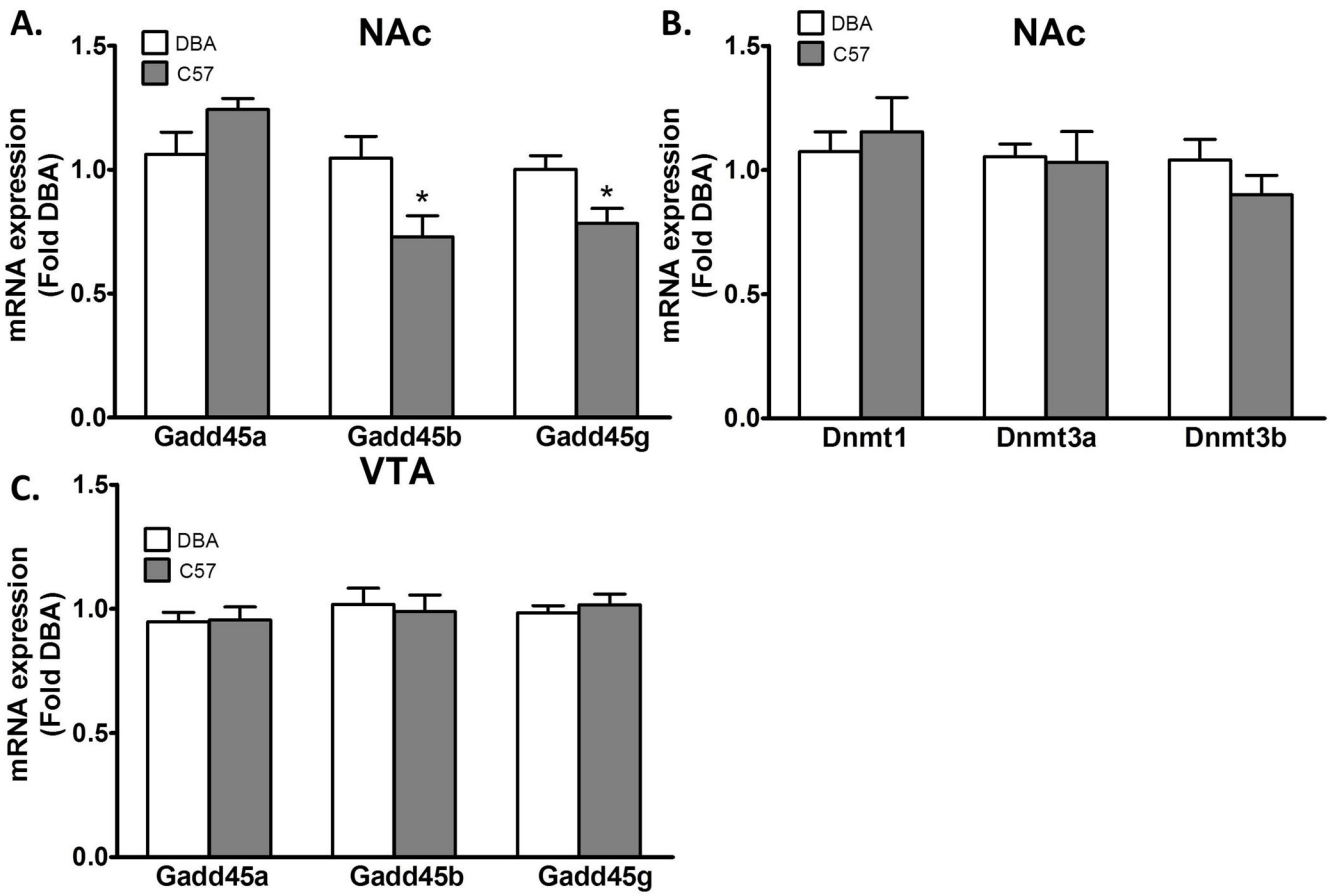

Figure 1. Baseline differences in *Gadd45* gene expression in the entire nucleus accumbens (NAc) in C57BL/6J (C57) mice compared to DBA/2J (DBA)

A. *Gadd45b* and *Gadd45g* mRNA expression is lower in the NAc of C57 mice (n = 9) compared to the DBA strain (n = 9). **B.** We find no significant differences in *Dnmt1*, *3a*, or *3b* mRNA expression in the NAc in these strains. **C.** We also find no differences in *Gadd45* gene expression in the VTA in C57 and DBA mice. Values are mean±SEMs. *p<0.05.

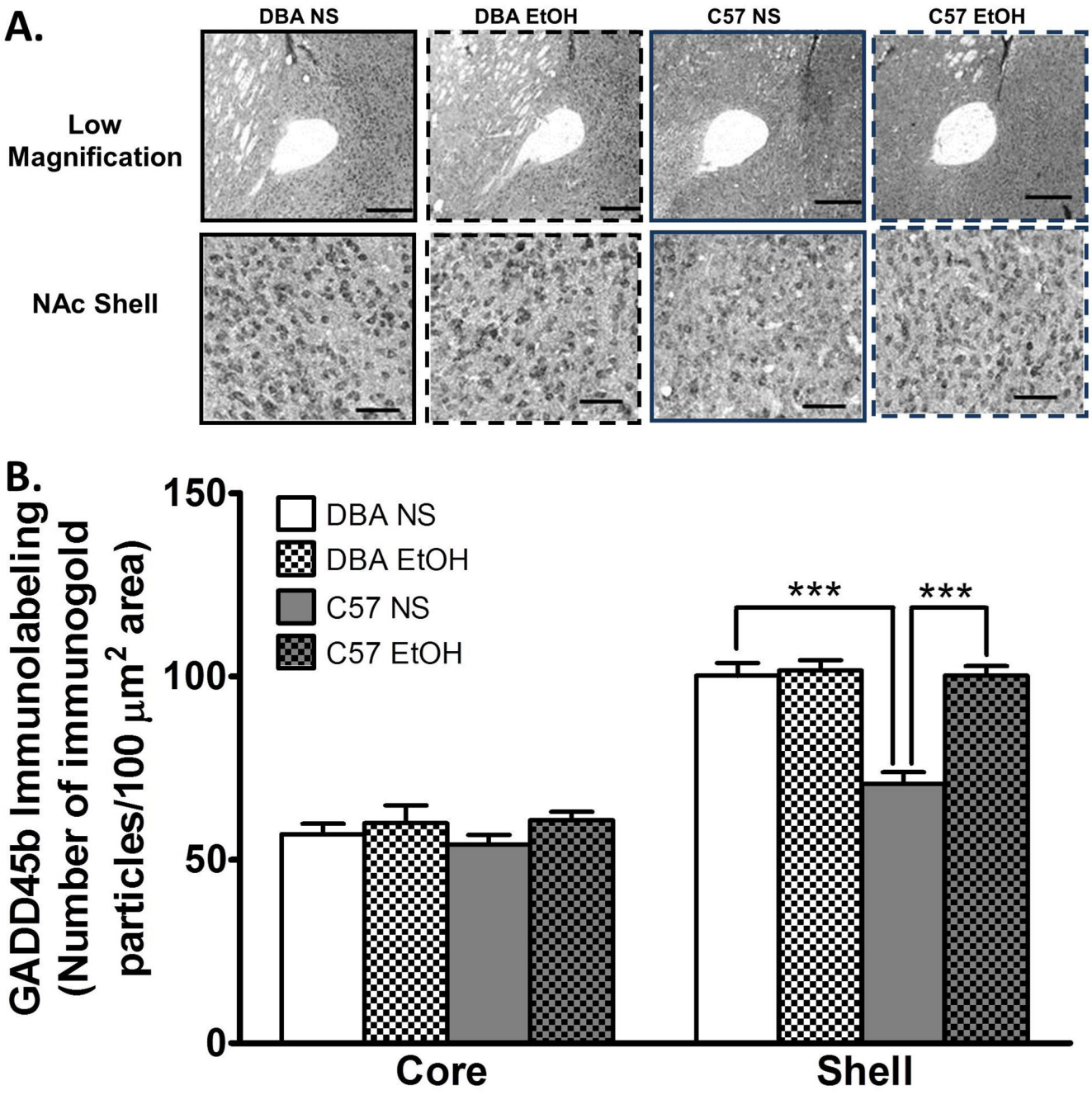

Figure 2. Lower GADD45b protein expression in the C57BL/6J (C57) nucleus accumbens (NAc) shell compared to DBA/2J (DBA) mice is reversed by acute ethanol

A. Representative photomicrographs at low magnification(scale bar = 200 μm) and higher magnification (scale bar = 50 μm) of GADD45b gold-immunolabeling in the core and shell of the NAc of C57 and DBA mice treated with normal saline (NS) or ethanol 2 g/kg after 1 h (EtOH). **B.** GADD45b protein expression is lower in the NAc shell of C57 mice compared to DBA in NS treated mice. EtOH increased GADD45b in C57 mice (n = 5 per group). Values are mean±SEMs. ***p<0.001.

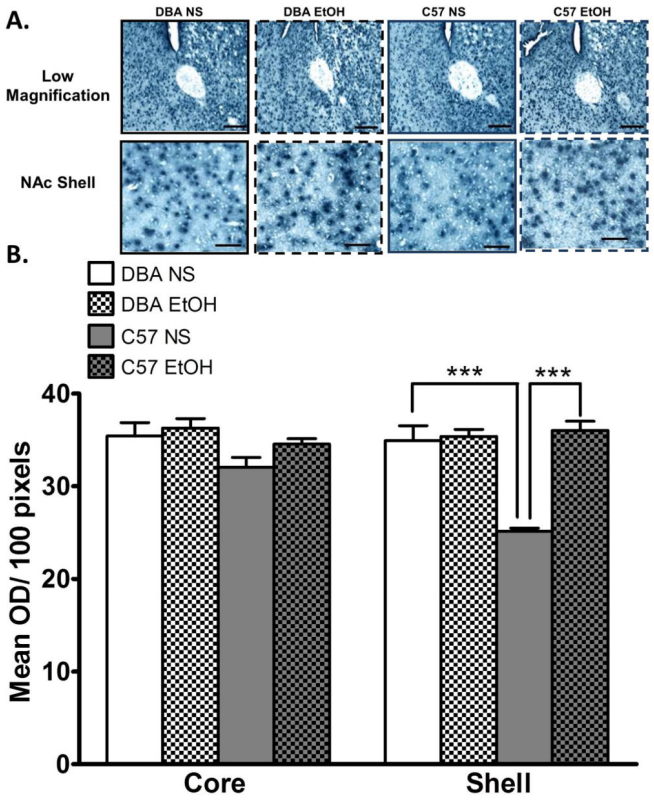

Figure 3. Lower *Bdnf9a* mRNA expression in the C57BL/6J (C57) nucleus accumbens (NAc) shell compared to DBA/2J (DBA) mice is reversed by acute ethanol

A. Representative photomicrographs at low magnification (scale bar = 200 μm) and higher magnification (scale bar = 50 μm) of *Bdnf9a* mRNA in the core and shell of the NAc of C57 and DBA mice treated with normal saline (NS) or ethanol 2 g/kg after 1 h (EtOH). **B.** *Bdnf9a* mRNA expression is lower in the NAc shell of C57 mice compared to DBA in NS treated mice. EtOH increased *Bdnf9a* in C57 mice (n = 5 per group). Values are mean ±SEMs. ***p<0.001.

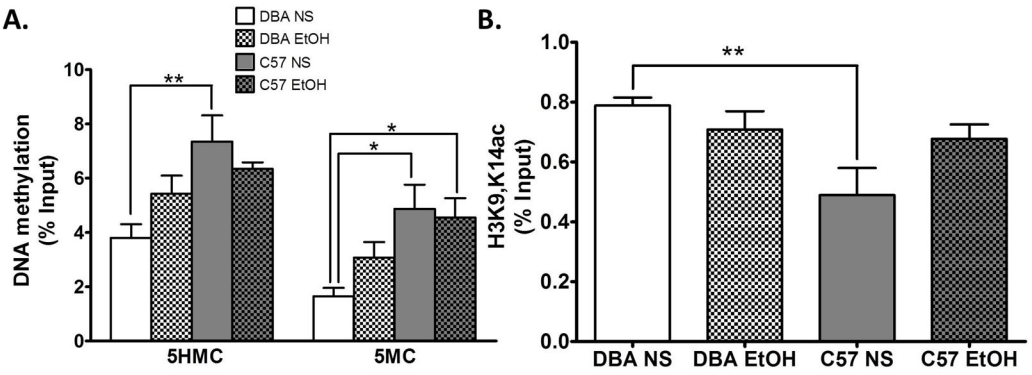

Figure 4. Increased 5-hydroxymethylcytosine (5HMC) and 5-methylcytosine (5MC) and lower histone H3 acetylation (H3K9,14ac) in the C57BL/6J (C57) entire nucleus accumbens (NAc) compared to DBA/2J (DBA) mice is reversed by acute ethanol

A. We measured 5HMC and 5MC at the *Bdnf9a* promoter via hydroxymethylayted (hMeDIP) and methylated DNA immunoprecipitation (MeDIP). We find that C57 mice have increased levels of 5HMC and 5MC at the *Bdnf9a* promoter in the NAc compared to DBA mice. One hour after an i.p. administration of ethanol 2 g/kg (EtOH) the 5HMC difference between C57 and DBA mice is no longer significant (n = 6–8 per group). **B.** H3K9,14ac was measured at the *Bdnf9a* promoter via chromatin immunoprecipitation (ChIP). We find that C57 mice have lower levels of H3K9,14ac at the *Bdnf9a* promoter in the NAc compared to DBA mice. One hour after an i.p. administration of EtOH 2 g/kg H3K9,14ac levels no longer differ between C57 and DBA mice (n = 7–8 per group). Values are mean±SEMs. *p<0.05, **p<0.01.

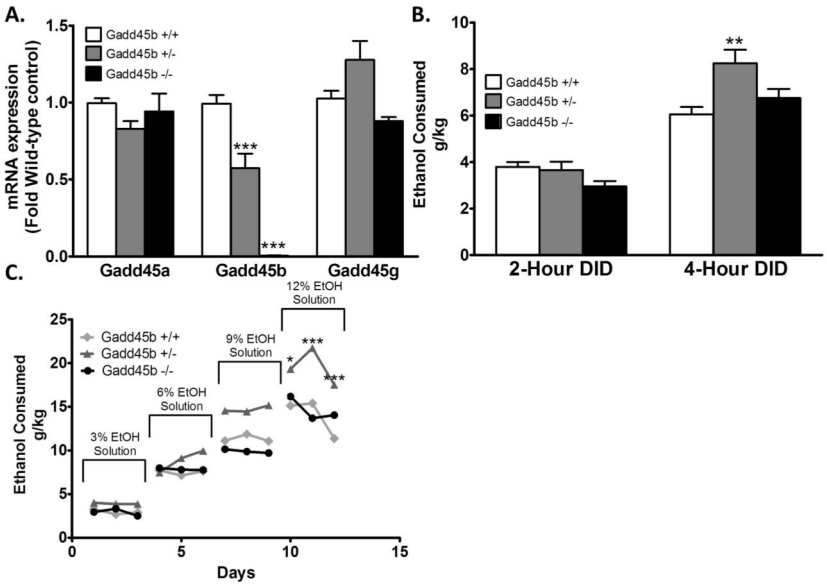

Figure 5. Increased binge-like and 2-bottle free-choice ethanol consumption in *Gadd45b* haploinsufficient (+/−) mice

A. In the entire NAc there is no significant difference in *Gadd45a* or *Gadd45g* expression between wild-type (*Gadd45b* +/+) and haploinsufficient (*Gadd45b* +/−) or complete knockout (*Gadd45b* −/−) mice. Predictably, *Gadd45b* mRNA expression is lower in *Gadd45b* +/− mice and absent in *Gadd45b* −/− mice (n = 5–6). **B.** Mean ethanol consumption in g/kg during first three days of 2-h drinking-in-the-dark (DID) sessions and last two days of 4-h DID sessions. *Gadd45b* +/− mice (n = 10) drink significantly more ethanol during the 4-day sessions than wild-type (+/+) (n = 11) or knockout mice (−/−) (n = 9). **C.** In a two-bottle free-choice paradigm *Gadd45b* +/− mice consume more ethanol than either *Gadd45b* +/+ or *Gadd45b* −/− at 12% ethanol (n = 8 per group). Values are mean ±SEMs. *p<0.05, **p<0.01; ***p<0.001.

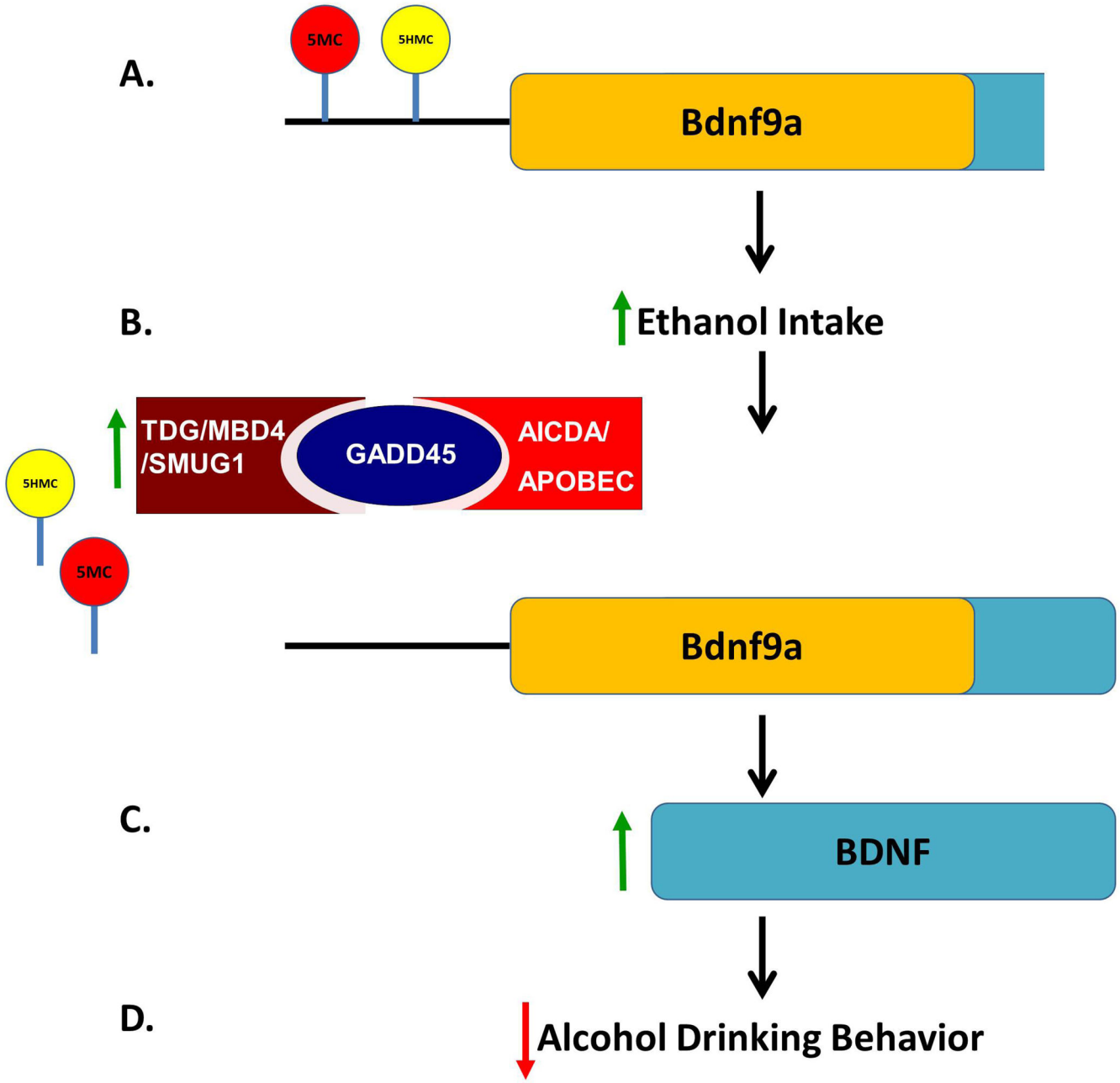

Figure 6. Hypothetical pathway of ethanol-induced *Bdnf* induction through DNA demethylation

A. The *Bdnf9a* gene promoter is both methylated (5MC) and hydroxymethylated (5HMC) leading to less expression. A deficiency in BDNF may contribute to increased alcohol consumption. **B.** Acute alcohol induces the DNA demethylation machinery including GADD45b which leads to the removal of 5HMC and 5MC. **C.** The removal of methylated cytosines allows for increased *Bdnf9a* mRNA and consequently peptide expression. **D.** Increased BDNF possibly reduces further ethanol drinking.

Table 1

Primer sequences used in this study.

mRNA Expression Primers		
Genes	**5′ primer**	**3′ primer**
Bdnf9a	GCAGCTGGAGTGGATCAGTAA	TGGTCATCACTCTTCTCACCTG
Dnmt1	AACCATCACCGTGCGAGACA	AGGGTGCTGAATTGCCTGGA
Dnmt3a	AGGAAGACCCCTGGAACTGCT	ACGTCCCCGACGTACATGATCT
Dnmt3b	GCGTCAGTACCCCATCAGTT	CACGAGGTCACCTATTCCAAA
Gadd45a	TGCGAGAACGACATCAACAT	TCCCGGCAAAAACAAATAAG
Gadd45b	GTTCTGCTGCGACAATGACA	TTGGCTTTTCCAGGAATCTG
Gadd45g	ATGACTCTGGAAGAAGTCCGT	CAGGGTCCACATTCAGGACT
Hprt1	GCCGAGGATTTGGAAAAAGT	ACAGAGGGCCACAATGTGAT
Methylated DNA Immunoprecipitation Primers		
Genes	**5′ primer**	**3′ primer**
Bdnf9a −171 to +32	CATGAGACCGGGCAAGTC	CCTTGGGAGGAATGTGTGAT

Table 2

	Gadd45b +/+ (n=8)	Gadd45b +/− (n=8)	Gadd45b −/− (n=8)	Sig.
Weight (kg)	0.029±0.001	0.028±0.001	0.028±0.001	NS
Total Fluid Intake (mL/kg)	179.0±8.67	215.9±11.6	179.4±10.9	NS
Water Intake (mL/kg)	58.0±10.0	50.5±9.5	54.9±10.4	NS
Percent Water Intake	31.8±4.5	23.1±4.6	29.4±5.6	NS
Percent Ethanol Intake	68.2±4.5	76.9±4.6	70.6±5.6	NS