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Histone Deacetylase Gene Expression Following Binge Alcohol Consumption in Rats and Humans

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Background: Alcohol binge drinking is one of the most common patterns of excessive alcohol use and recent data would suggest that histone deacetylases (*HDACs*) gene expression profiling could be useful as a biomarker for psychiatric disorders.

Methods: This study aimed to characterize the gene expression patterns of *Hdac 1–11* in samples of rat peripheral blood, liver, heart, prefrontal cortex, and amygdala following repeated binge alcohol consumption and to determine the parallelism of *Hdac* gene expression between rats and humans in peripheral blood. To accomplish this goal, we examined *Hdac* gene expression following 1, 4, or 8 alcohol binges (3 g/kg, orally) in the rat, in patients who were admitted to the hospital emergency department for acute alcohol intoxication, and in rats trained in daily operant alcohol self-administration.

Results: We primarily found that acute alcohol binging reduced gene expression (*Hdac1–10*) in the peripheral blood of alcohol-naïve rats and that this effect was attenuated following repeated alcohol binges. There was also a reduction of *Hdac* gene expression in the liver (*Hdac2,4,5*), whereas there was increased expression in the heart (*Hdac1,7,8*) and amygdala (*Hdac1,2,5*). Additionally, increased blood alcohol concentrations were measured in rat blood at 1 to 4 hours following repeated alcohol binging, and the only group that developed hepatic steotosis (fatty liver) were those animals exposed to 8 alcohol binge events. Finally, both binge consumption of alcohol in humans and daily operant alcohol self-administration in rats increased *Hdac* gene expression in peripheral blood.

Conclusions: Our results suggest that increases in *HDAC* gene expression within the peripheral blood are associated with chronic alcohol consumption, whereas *HDAC* gene expression is reduced following initial exposure to alcohol.

Key Words: Histone Deacetylases, Gene Expression, Alcohol Binge, Human and Rat, Translational Research.

BINGE ALCOHOL CONSUMPTION is one of the most common patterns of excessive alcohol use (Centers for Disease Control and Prevention, 2014) and can be defined as a drinking pattern that increases the blood alcohol concentration (BAC) by up to 80 mg/dl or more. This generally requires the consumption of at least 4 or 5 drinks (for women and men, respectively) within a span of 2 hours (Crabbe et al., 2011; Fillmore and Jude, 2011; NIAAA, 2015). Binge drinking is associated with many immediate consequences, including intentional and nonintentional injuries, death from overdose, and additional long-term consequences if repeated over time (Crabbe et al., 2011). Chronic binge drinking leads to liver and heart disease, abnormal electrophysiological signals within the brain, and psychological impairment (López-Caneda et al., 2013; Maurage et al., 2012; Parada et al., 2012). Therefore, it would be useful if clinicians had the ability to reliably assess the progression of alcoholism through biomarker analysis, which in addition to identifying individuals suffering from alcohol abuse, would further allow the efficacy of pharmacological interventions to be monitored (Litten et al., 2010).

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Recently, several biomarkers for alcohol-related phenotypes have been identified, many of which are associated with the enzymes utilized during alcohol metabolism and the concentration of their resultant metabolites throughout different bodily tissues and fluids (for reviews, see Bühler et al., 2015; Litten et al., 2010). A relatively new approach of detecting novel biomarkers utilizes the analysis of gene expression patterns within the peripheral blood, integrating genomics, and environmental influence. Such analyses have been accomplished in studies researching a variety of different neuropsychiatric and neurological diseases, such as Alzheimer's disease, Parkinson's (Booij et al., 2011; Masliah et al., 2013), and very recently schizophrenia and major depressive disorder (Guidotti et al., 2014; Redei et al., 2014). Early preclinical studies evaluating mouse and rat brain tissues revealed that binge consumption of alcohol altered gene expression. For example, it has been demonstrated that the expression of select neurotransmitter receptor genes is decreased following alcohol exposure (Coleman et al., 2011), particularly in cholinergic, GABAergic, and peptide genes. Conversely, the expression of genes involved in intracellular signaling pathways in the extended amygdala and prefrontal cortex is increased (McBride et al., 2014; Vetreno and Crews, 2012). While these studies have produced promising results, the alteration of gene expression patterns within the peripheral blood following alcohol binging remains poorly characterized, even in the cases of genes that control gene expression (i.e., epigenetic genes).

Along with DNA methylation, histone deacetylation is one of the main epigenetic mechanisms that controls gene silencing. In mammals, histone deacetylation is catalyzed by 4 classes of histone deacetylases (HDACs) (Egger et al., 2004). Eighteen human HDACs have been identified thus far and are grouped into 4 classes: Class I (comprising HDACs 1, 2, 3, and 8), Class IIA (comprising HDACs 4, 5, 7, and 9), Class IIB (HDACs 6 and 10), Class III (Sirtuins 1 to 7), and Class IV (HDAC11) (Lombardi et al., 2011). Each HDAC class has a unique bodily tissue distribution. Class I HDACs are ubiquitously distributed; Class II and IV HDACs are located mainly within the heart, skeletal muscle, brain, liver, and kidney; and Class III Sirtuins are expressed either ubiquitously or in a wide range of tissues (Dokmanovic et al., 2007; Nogueiras et al., 2012). Recent studies have shown that *HDAC* gene expression is altered following alcohol exposure both in vivo and in vitro, although the results are heterogeneous. For example, alcohol has been shown to induce *HDAC1* and *HDAC3* gene expression in human SK-N-MC cells (Agudelo et al., 2012), to inhibit *Hdac1* and *Hdac4* gene expression in a model of rat hippocampal-entorhinal cortex brain slice cultures (Zou and Crews, 2014), and to decrease *Hdac11* gene expression in mouse striatum tissue (Botia et al., 2012). These results strongly suggest that it would be helpful to conduct translational studies examining *HDAC* gene expression patterns in both humans and animal models following the administration of alcohol to determine the parallelism among species, tissues, and treatments.

In this context, this study aimed to first characterize *Hdac* gene expression in the peripheral blood, liver, heart, prefrontal cortex, and amygdala following repeated alcohol binging in the rat. The next aim was to determine the correlation of *Hdac* gene expression among these tissues and finally to examine the parallelism of *Hdac* gene expression between rats and humans in peripheral blood in vivo. The brain structures chosen for analysis have been shown to play a predominant role in alcohol related behaviors (Gilpin et al., 2015; Goldstein and Volkow, 2011; Stephens and Duka, 2008; Vilpoux et al., 2009), while the liver and heart are known to be associated with alcohol-induced injury. Analysis of the peripheral blood was included due to its accessibility for biomarker tests.

MATERIALS AND METHODS

Animal Studies

Subjects. Forty-eight male Wistar rats were used for the binge-like drinking protocol, and 20 were used for the operant alcohol self-administration experiment. These animals were purchased from Harlan Laboratories (Barcelona, Spain), weighed 275 to 325 g at the start of the experiments, and housed in groups of 4 per cage in a temperature- and humidity-controlled environment (21 ± 1°C) on a 12-hour reverse light/dark cycle (lights off at 08:00 hour). Binge alcohol treatments and operant self-administration sessions were performed during the dark phase, as rats are nocturnal. Food and water were available ad libitum during the experimental procedures except in select conditions as specified below. All research was conducted in strict adherence with the European Directive 2010/63/EU on the protection of animals used for scientific purposes.

Binge Alcohol Treatment and Experimental Design. To habituate the animals to intragastric (i.g.) fluid delivery and to reduce nonspecific stress responses prior to alcohol administration, rats were administered tap water (i.g.) once per week for 2 weeks. The animals were then assigned into 1 of the following groups: the control group (administered water) and the alcohol binge groups, comprising 1 alcohol binge, 4 alcohol binges, and 8 alcohol binges (see Fig. 1). Binge alcohol treatment was performed once per week on Thursdays. Alcohol was orally administered through gavage (i.g.) at a dose of 3 g/kg using a 25% alcohol solution in tap water at a volume of 15 ml/kg. We used this dose of alcohol to obtain com-

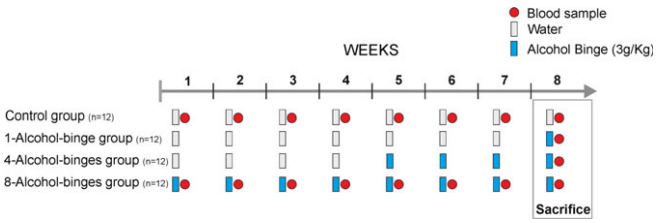

Fig. 1. Schematic representation of the experimental design. Red dots represent blood collected following cuts made to the tail veins of rats. White and blue rectangles represent cohorts of animals that were administered either water or alcohol and sham cut. Animal sacrifice was performed at 2 hours postalcohol binge to increase similarity in alcohol blood concentrations measured between rat and human subjects.

parable BACs to those found in humans after alcohol binge (approximately 240 mg/dl from our human samples). Control animals received the same volume of liquid as above but were given tap water alone. The rats were deprived of food for 12 hours prior to the i.g. treatment to normalize the absorption of alcohol among the animals (avoiding the presence or absence of food into their stomach).

Blood samples were collected for analysis of BACs from the tail caudal vein at 60, 120, 180, and 240 minutes following alcohol binge (see Fig. 1). To avoid cutting the rat tail 4 times, half of the animals (i.e., $n = 6$) were used to collect the blood at 60 and 180 minutes and the other half at 120 and 240 minutes. In the eighth week, post-binge blood samples were collected only at the 120-minute time point, as all animals were then sacrificed following blood collection. The other experimental groups were treated in the same way every week, and the blood samples of the group that was administered water only were used as a control. In the eighth week, for the analysis of gene expression, all animal cohorts were sacrificed by decapitation at 120 s following binge alcohol treatment, and the blood, liver, heart, and brain (prefrontal and amygdala) tissues were immediately dissected on ice and frozen on dry ice at −80°C. Frozen tissue samples were stored for subsequent histology and gene expression analysis.

Operant Alcohol Self-Administration. To habituate rats to operant alcohol self-administration, we followed the protocol described by Echeverry-Alzate and colleagues (2014). Briefly, operant alcohol sessions were conducted in 20 modular chambers (Med Associates Inc., St. Albans, VT). The chambers were equipped with 2 retractable levers on either side of a drinking reservoir positioned in the center of the front panel of the chamber. The levers were counterbalanced to respond as the active lever (delivering 0.1 ml either 10% alcohol v/v or 0.02% saccharin w/v) or as the inactive lever. For the entirety of the study, each operant alcohol session lasted 30 minutes under a fixed ratio 1 schedule for 7 days a week.

To analyze gene expression in the animal cohorts described above, peripheral blood samples were collected from the tail vein at 60 s before and 60 minutes after the alcohol self-administration session on the fourth day of 10% alcohol availability. An additional cohort of rats ($n = 10$) only had access to saccharin (0.02% saccharin) and did not consume any alcohol during the study. This cohort served as a control group for assay calibration purposes in polymerase chain reaction (PCR) experiments.

Alcohol Analysis. To determine BACs following binge alcohol treatment, 250 μl of blood was collected from the rat tail vein at 60, 120, 180, and 240 minutes posttreatment using a capillary tube (Microvette CB 300 K2E; Sarstedt AG & Co., Nümbrecht, Germany) that contained EDTA dipotassium salt. Whole blood samples were centrifuged for 15 minutes at 1,500×g using a refrigerated centrifuge, and the plasma was stored at −80°C for further use. The alcohol concentration was measured using the EnzyChrom alcohol assay kit following the protocol recommended by the manufacturer (Bioassay Systems, Hayward, CA). All measurements were performed in duplicate.

Histological Study of the Liver. As stated above, animals were sacrificed by rapid decapitation, after which livers were immediately harvested and frozen. Each liver sample was divided in half to provide samples for both histology and gene expression analysis. Liver samples were cryosectioned using a LEICA CM3050 cryostat (Leica Microsistemas, Barcelona, Spain) to produce 10-μm thick sections and stained with Oil Red O to visualize neutral lipid content according to the recommendations of Koopman and colleagues (2001). An Oil Red O stock solution consisting of 0.5 g Oil Red O powder and 100 ml of 60% triethyl-phosphate was prepared. Oil Red O was obtained from Sigma-Aldrich (Steinnhem, Germany). Liver sections were fixed in 4% formaldehyde (Merck, Darmstadt, Germany) for 1 hour at room temperature. During this time and prior to staining, a 36% triethyl-phosphate working solution containing 12 ml Oil Red O stock solution and 8 ml deionized water was prepared and filtered. Following fixation in paraformaldehyde, slides were rinsed 3 times in water to remove excess paraformaldehyde and then immersed in filtered Oil Red O solution for 30 minutes. Immediately thereafter, slides were rinsed with running tap water and mounted in glycerine jelly. Prior to being mounted, a subset of sections was counterstained using Gill's hematoxylin.

Lipid droplets were quantified using high-resolution digital microphotographs that were taken with the 40× objective of a Zeiss Axiophot microscope (Oberkochen, Germany) under equivalent conditions of light and brightness and contrast. Densitometry measurements of select regions of each image were obtained for quantification purposes using the analysis software ImageJ 1.383 (NIH, Bethesda, MD). Three separate areas surrounding the central vein from 4 random tissue sections per animal were evaluated. Analysis included conversion of the color image into a binary image and subsequent measurement of the optical density (OD) from each of 3 different areas of equal size. The basal OD was obtained from the average of 3 separate OD values, which were then averaged for each section. Four sections were averaged for each animal. Six to 8 animals were evaluated per group.

Real-Time Quantitative PCR Experiments for Rats. Real-time quantitative PCR, which has been described as one of the most powerful tools to quantify gene expression (Schmittgen and Livak, 2008), was performed using a LightCycler 480-II machine (Roche, Barcelona, Spain) with SYBR Green Real-Time qPCR master mix (Applied Biosystems, Warrington, UK) and specific primers kept at a concentration of 200 nm (see Table S1 for rat primers). The melting curve analyses showed only a single clear peak, and the size of the PCR products were confirmed by agarose gel electrophoresis. A 10-fold dilution series of the template was used to amplify each gene to validate the efficiency of each assay and to confirm that the amplification efficiencies of the target and reference genes were comparable (indicated by a near-zero slope value for both the target and reference genes). The 18S ribosomal RNA gene (18S) was used as an internal control for normalization. The control group of rats that were administered only water was used to calibrate the assay for analysis of the cohort of rats that underwent binge alcohol consumption, whereas the cohort of rats that drank only saccharin was used to calibrate the assay for the cohort that was offered operant self-administration of alcohol. The $2\Delta C_t$ method was used to analyze the expression data (Schmittgen and Livak, 2008). Total RNA was isolated from brain, liver, and heart samples using Tripure Isolation Reagent (Roche) and was stored at −80°C. Total RNA was isolated from whole blood using Trizol LS Reagent (Life Technologies, Carlsbad, CA). One microgram of total RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche).

Human Study

Subjects. Our clinical study was performed on 20 patients who were admitted to the Emergency Department of the University Hospital of Salamanca (Spain) with moderate to severe acute alcohol intoxication. Acute alcohol intoxication was defined by clinical signs (e.g., slurred speech, ataxia, impaired reasoning, confusion, or disorientation), blood alcohol levels >1 g/l, and the consumption of at least 5 standard drinks (50 g) in men or 4 (40 g) in women during the 6 hours prior to hospital admission. Toxicological urinary analysis was also performed, and patients

Table 1. Characteristics of Patients ($n = 20$) with Acute Alcohol Intoxication

Age (years)	20.4 (0.92)
Female gender (%)	10 (50.0)
Blood ethanol content (g/l)	2.39 (0.12)
Aspartate aminotransferase levels (IU/l)	23.44 (2.52)
Alanine aminotransferase levels (IU/l)	15.72 (0.99)
Alkaline phosphatase levels (IU/l)	73.67 (7.92)
γ- glutamyl transpeptidase levels (IU/l)	17.33 (1.41)
White blood cell count/μl	8,550.53 (497.85)
Reported weekend drinking ($n = 17$) (%)*	14 (82.4)

IU, international units.

*Three patients refused to answer the questionnaire regarding drinking patterns.

Quantitative variables are presented as the mean (SEM) and qualitative variables are presented as absolute frequencies (percentage).

were excluded from our study if either clinical data or patient history demonstrated the consumption of illegal drugs (apart from cannabis). Additional exclusionary criteria included chronic or acute illness (aside from symptoms produced by acute alcohol intoxication) and the use of medication. The clinical, epidemiological, and analytical characteristics of our patient cohort are shown in Table 1. Twenty-two healthy controls (8 male and 14 female) with an average age of 23.2 years (SEM $\pm$ 0.45) were also included in the study. Controls were carefully selected to include subjects who did not consume alcohol apart from light sporadic drinking and who reported no alcohol consumption during the 72 hours leading up to the study and no binge drinking episodes during the 3 months prior. These patients were subject to the same exclusionary criteria as those who binged on alcohol and had normal hematological and plasma biochemical parameters. This study was approved by the Ethics Committee of the University Hospital of Salamanca and written informed consent was obtained from each participant. Blood samples were obtained from patients upon admission for use in standard care as well as for research purposes, and the samples analyzed in this study were only used after patients could understand the details of the study adequately enough to provide informed consent for inclusion.

Real-Time Quantitative PCR Analysis of Human Blood Samples. Whole blood samples (2.5 ml) from each individual were drawn into PAXgene Blood RNA tubes (Qiagen, Valencia, CA). Samples were kept at room temperature for at least 2 hours following collection, and stored for long-term access at −20°C. Total RNA was isolated from each sample using the PAXgene Blood miRNA Kit (Qiagen) according to manufacturer's instructions, and processed samples were stored at −80°C. The concentration and purity of extracted RNA samples were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Further processing of RNA samples was identical to that described in the animal PCR study section described above, except for the use of human-specific primer sets (see Table S2 for human primers).

Statistical Analysis

SPSS statistical software (version 20.0) for Windows (Chicago, IL) was used to perform all statistical analysis. Significant analysis of variance (ANOVA) results were followed by Tukey's post hoc tests. The data obtained from experiment 1 were analyzed using 1-way ANOVA within each tissue (Figs 2–4), and correlations were determined by Pearson's correlations analysis (Fig. 5). Data from experiment 2 were analyzed using a 2-way mixed ANOVA (week × hour) (Fig. 6) and a 2-sided independent Student's *t*-test (Fig. 7*E*). Data from experiment 3 were analyzed using a 2-way ANOVA (gene × treatment) (Figs 8 and 9). After confirming the significance of our primary findings using ANOVA, a significance level of $p < 0.05$ was applied to all remaining statistical analyses.

RESULTS

Experiment 1. Hdac *Gene Expression in Blood, Liver, Heart, Prefrontal Cortex, and Amygdala Following Repeated Binge Alcohol Consumption in Rats*

The objectives of this experiment were 2-fold. The first was to quantify the effect of repeated binge alcohol consumption (3 g/kg) on the gene expression of Class I, IIA, IIB, and IV *Hdacs* in samples taken from peripheral blood, liver, heart, prefrontal cortex, and amygdala tissues. The second was to assess the potential correlative relationships of *Hdac* gene expression among these tissues.

As shown in Figs 2–4, which depict the relative fold changes in *Hdac* expression, results varied according to tissue type and to the number of alcohol binge episodes experienced by the subject. One of the more consistent results to emerge from this experiment was the observation that, except for Class IV (*Hdac11*), there was a reduction of *Hdac* gene expression in peripheral blood collected from the tail vein following the initial alcohol binge (e.g., Class I *Hdacs*: 1-way ANOVA blood *Hdac1*, $F(3, 34) = 3.22$, $p < 0.05$; *Hdac2*, $F(3, 32) = 8.09$, $p < 0.01$; *Hdac3*, $F(3, 34) = 4.91$, $p < 0.01$; *Hdac8*, $F(3, 33) = 3.62$, $p < 0.05$). With the exception of *Hdac4* and *5* (Fig. 3), this reduction in gene expression was generally lost following successive alcohol binge episodes. Additional significant results obtained from these assays include a reduction of *Hdac* gene expression in the liver (*Hdac2, 4,* and *5*) and an increase in *Hdac* expression in the heart (*Hdac1, 7,* and *8*) and amygdala (*Hdac1, 2,* and *5*). Also, *Hdac8* increased its expression in the amygdala but only after 8 alcohol binges.

We next evaluated whether *Hdac* gene expression patterns among varying tissues correlated with the details of binge alcohol consumption. To investigate this question, we utilized 2^{-C_t} values according to the protocol described by Schmittgen and Livak (2008). Figure 5 shows the corresponding matrix of correlations, in which only statistically significant results ($p < 0.05$) are presented. We generally observed that the most frequent positive correlative association was among values obtained from amygdala and heart tissue samples, regardless of the conditions of binge alcohol consumption (e.g., Pearson correlations from the control group, *Hdac1* $r = 0.65$, $p < 0.05$; *Hdac2* $r = 0.79$, $p < 0.01$; *Hdac3* $r = 0.81$, $p < 0.01$; *Hdac4* $r = 0.70$, $p < 0.05$). Additionally, we detected that the most frequent negative association was between values measured from prefrontal cortex and amygdala tissue samples (e.g., Pearson correlations 8 alcohol binge group, *Hdac4* $r = 0.75$, $p < 0.05$; *Hdac7* $r = 0.74$, $p < 0.05$). Another finding was that while the highest number of positive correlations was produced

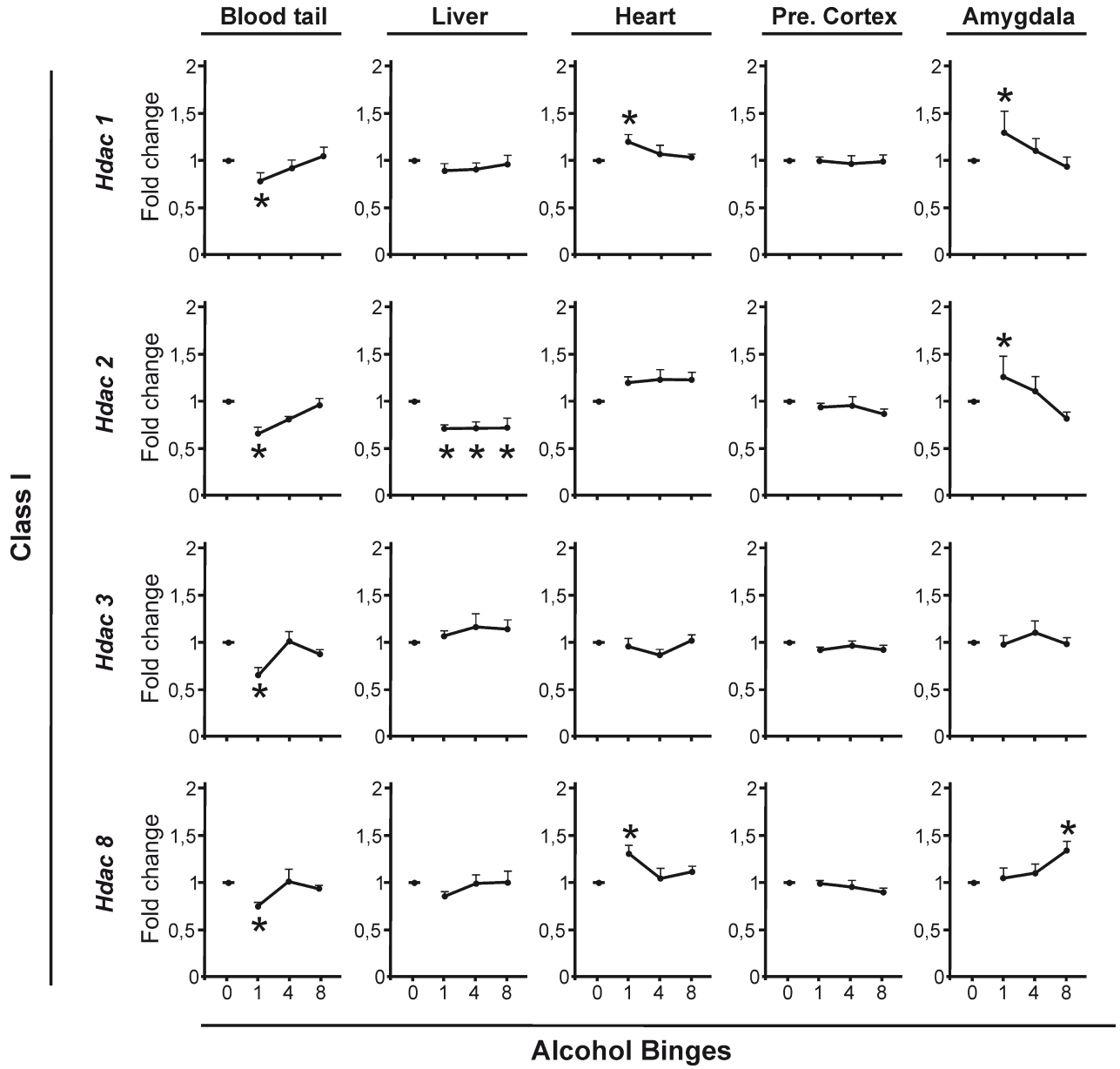

Fig. 2. Effects of 1, 4 or 8 alcohol binges on Class I histone deacetylase (*Hdac*) gene expression in rat peripheral blood, liver, heart, prefrontal cortex, and amygdala. Data represent the mean ± SEM (n = 10 to 12 animals per group) and the relative fold change obtained using the $2\Delta C_t$ method (see Materials and Methods section). *$p < 0.05$ compared with the control group (no alcohol binge).

within the control group, the highest number of negative correlations came from the cohort exposed to 8 alcohol binges. These observations were in spite of the fact that there was no observed linear relationship between alcohol binge consumption and *Hdac* gene expression across different tissues.

Experiment 2. Determination of Blood Alcohol Levels and Histological Analysis of the Rat Liver

The purpose of this experiment was to determine the blood alcohol levels of rats following repeated binge alcohol consumption and to explore the extent of hepatic steatosis (fatty liver) within these animals. Figure 6 shows that BAC peaked during the first hour following consumption and decreased progressively thereafter. Additionally, chronic binge drinking led to increased blood alcohol levels independent of the time point under evaluation (2-way mixed ANOVA: week $F(6, 108) = 13.27$, $p < 0.001$; hour $F(3, 18) = 15.46$, $p < 0.001$; interaction $F(18, 108) = 0.60$, NS).

The effects that 8 alcohol binge episodes produced on the liver are shown in Fig. 7. Steatosis (fat deposition) was observed in hepatocytes. Panels *A–D* depict representative images stained with either Red Oil O or Red Oil O and Hematoxylin, and Panel *E* provides the OD quantification values of the lipid droplets from Oil Red O staining

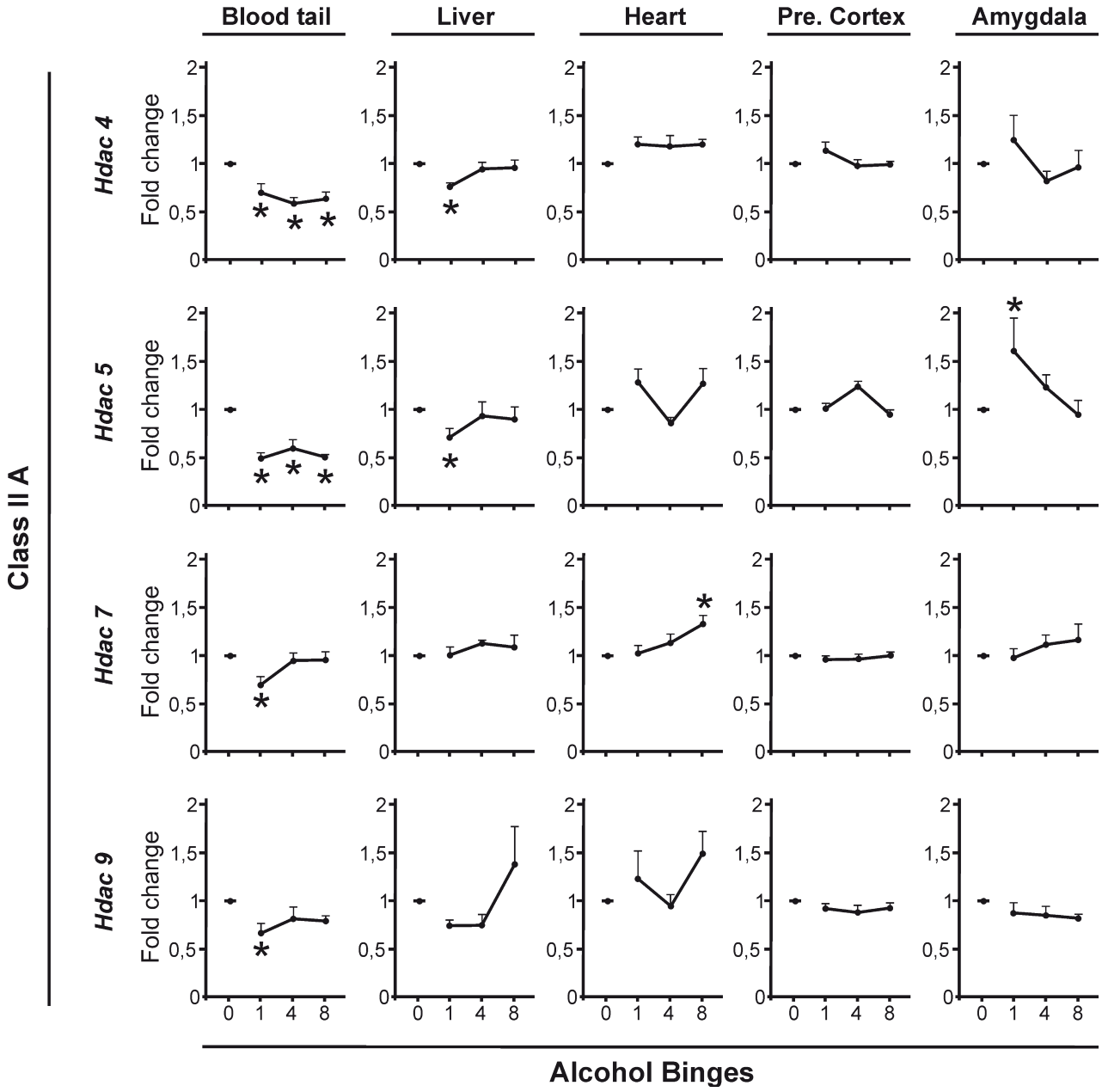

Fig. 3. Effects of 1, 4 or 8 alcohol binges on Class IIA histone deacetylase (*Hdac*) gene expression in rat peripheral blood, liver, heart, prefrontal cortex, and amygdala. Please see Fig. 2 Legend for details. *$p < 0.05$ compared with the control group (no alcohol binge).

($p < 0.005$, 2-sided Student's *t*-test). No effects were observed in animal cohorts exposed to either 1 or 4 alcohol binges (data not shown).

Experiment 3. Comparison of HDAC *Gene Expression Profiles from Peripheral Blood Samples Collected from Human and Rat Subjects*

The third experiment was designed to explore if there was a parallelism between rat and human *HDAC* gene expression in peripheral blood following binge alcohol consumption. In contrast with the acute decrease of *Hdac* gene expression observed in rat tissue samples, human tissue samples demonstrated an increase in human expression (except for *HDAC*9 and 10), as shown in Fig. 8. Since there were no significant differences between genders in *HDAC* gene expression the data were analyzed collectively. ANOVA analysis revealed that average *HDAC* gene expression was significantly higher in subjects that were binge drinking alcohol versus the control group and that there were no differences among *HDAC* species or their interactions (2-way ANOVA: treatment $F(1, 440) = 80.29$, $p < 0.001$; gene $F(10, 440) = 1.45$, NS; interaction $F(10, 440) = 1.46$, NS). Based on the above results, we conducted a complementary study using operant

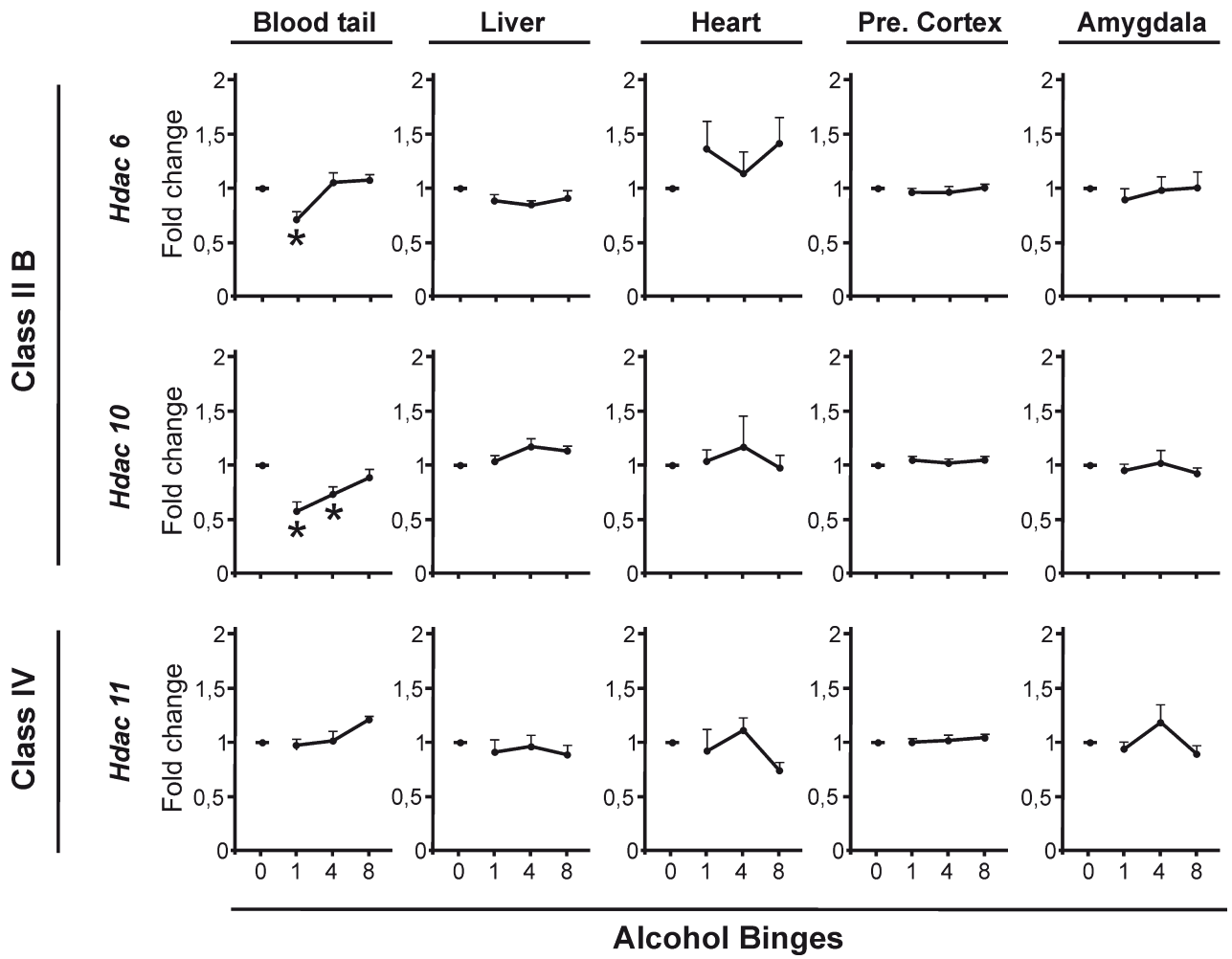

Fig. 4. Effects of 1, 4, or 8 alcohol binges on Class IIB and IV histone deacetylase (*Hdac*) gene expression in rat peripheral blood, liver, heart, prefrontal cortex, and amygdala. Please see Fig. 2 Legend for details. *$p < 0.05$ compared with the control group (no alcohol binge).

alcohol self-administration in rats. This allowed us to determine whether *Hdac* gene expression is linked to repeated exposure to alcohol rather than merely to alcohol binging. Figure 9 shows that repeated operant alcohol self-administration led to a general increase in *Hdac* gene expression, which mimicked more closely the increase observed in human samples. This effect was observed at time points both 60 minutes before and/or 60 minutes after alcohol self-administration (*Before*: 2-way ANOVA: treatment $F(1, 197) = 17.18$, $p < 0.001$; gene $F(10, 197) = 0.69$, NS; interaction $F(10, 197) = 0.68$, NS)/(*After*: 2-way ANOVA: treatment $F(1, 185) = 31.08$, $p < 0.001$; gene $F(10, 185) = 0.75$, NS; interaction $F(10, 185) = 0.74$, NS).

DISCUSSION

The aim of this work was to characterize *Hdac* gene expression in a sampling of tissues and the correlations of such in an animal model of binge alcohol consumption and to investigate whether these results could be replicated in humans. Our hope was that this study would facilitate the use of *HDAC* gene expression as a potential biomarker for alcohol abuse and alcohol-induced bodily damage.

Our first major finding was that acute alcohol binging led to a reduction of *Hdac* gene expression in rat peripheral blood and that this response became attenuated following repeated alcohol binges. The obtained values in *Hdac* gene expression in rat tissues were in agreement with previous studies (i.e., Finegersh and Homanics, 2014; Sarkar et al., 2014). As tissue samples were collected at the early time point of just 2 hours following the alcohol binge, it is clear that changes to *Hdac* gene expression occur rapidly and can be reliably detected using our methodology. These results are in agreement with previous studies. For example, Kirpich and colleagues (2012) have reported the down-regulation of select *Hdacs* in the mouse liver as early as 4 hours after 3 consecutive alcohol binges. Furthermore, Botia and colleagues (2012) demonstrated a reduction in the expression of *Hdac11* in the mouse striatum as early as 30 minutes after acute alcohol exposure. Even some studies have repeatedly proven that the activity of *Hdacs* is reduced in the amygdala 1 hour after

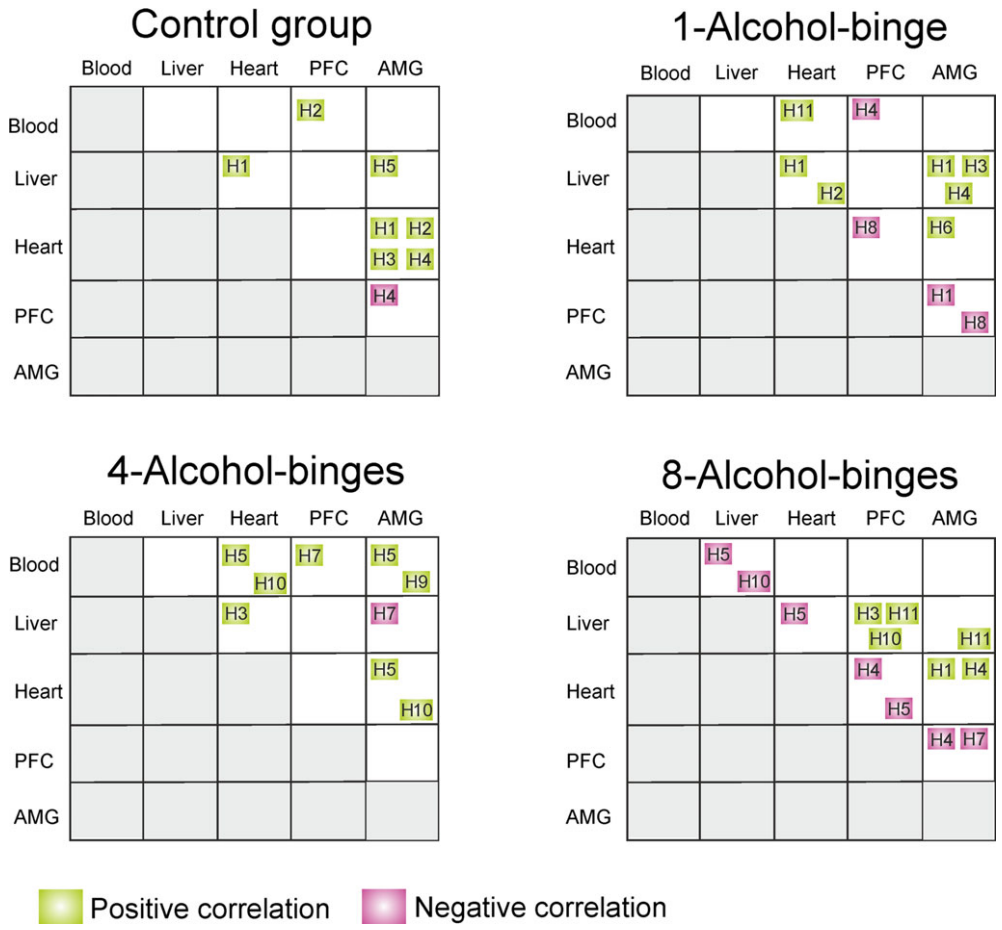

Fig. 5. Correlations among histone deacetylase (*Hdac*) gene expression in rat peripheral blood, liver, heart, prefrontal cortex (PFC), and amygdala (AMG) following variable alcohol binge conditions. Only correlations with significance lower than $p < 0.05$ are shown (green represents positive correlations, purple represents negative; $n = 10$ to 12 animals per group).

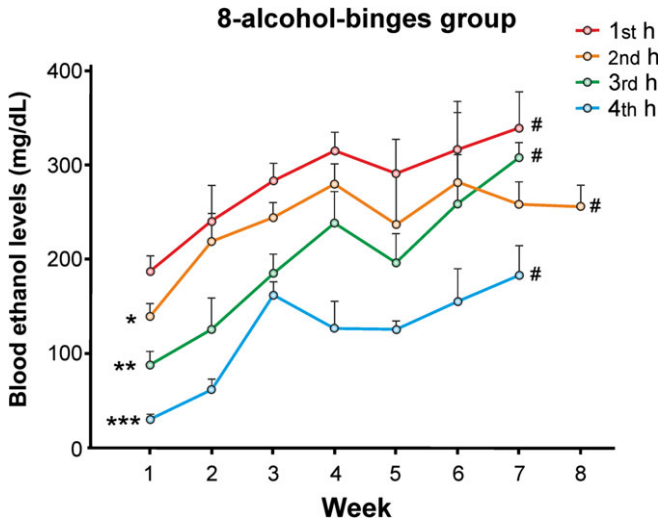

Fig. 6. Blood alcohol concentrations (BACs) over a 4-hour period following a weekly alcohol binge (3 g/kg) in rats. In the eighth week, blood samples were only collected at the 120-minute time point following binge alcohol treatment, as animals were sacrificed at this time point. Values represent the mean of BACs ± SEM ($n = 6$ animals per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the first hour. #$p < 0.001$ compared with the first week (first alcohol binge).

alcohol exposure (Pandey et al., 2008, 2015; Sakharkar et al., 2014), including in selectively bred alcohol-preferring rats (Moonat et al., 2013). Nevertheless, our results show that *Hdac* gene expression in rat peripheral blood undergoes degrees of tolerance as a consequence of repeated alcohol binges. For example, statistically significant differences disappeared in the expression of Class I and IIB *Hdacs*, whereas values measured for Class IIA gene expression (*Hdac4* and *5*) remained consistent. Additionally, a reduction of *Hdac* gene expression (*Hdac4* and *5*) was observed in the rat liver, while significant increases in gene expression (*Hdac1* and *2*) were found in the heart and amygdala following alcohol binging. No significant differences were noted in the prefrontal cortex. The discrepancy of *Hdac* gene expression among varying tissue types might be explained by the fact that different Hdac variants possess different patterns of tissue distribution. For example, class I Hdacs are ubiquitously distributed throughout the body, whereas Class IIA and IV Hdacs are found within the heart, brain, and skeletal muscle. Class IIB Hdacs primarily localize to the cytoplasm of liver and kidney cells (Dokmanovic et al., 2007). *Hdac11*, a Class IV Hdac, was the only gene included in our

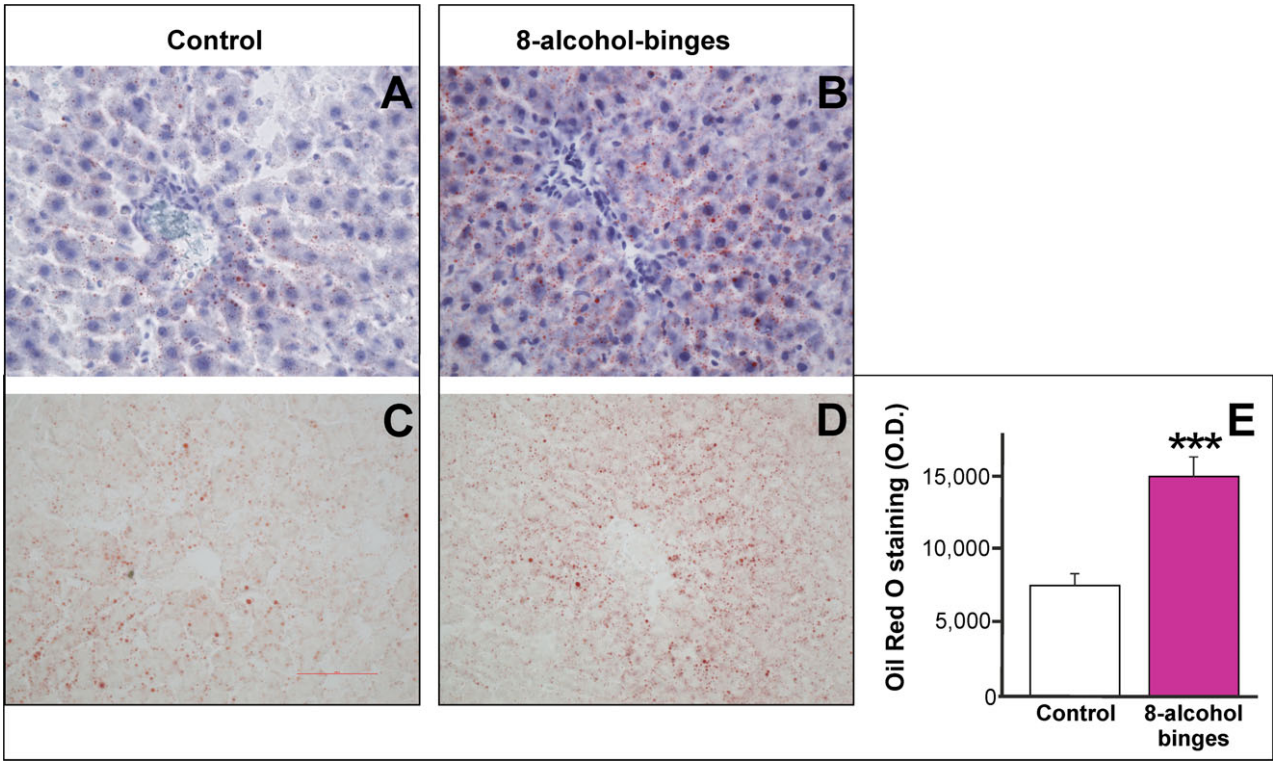

Fig. 7. Upper panels depict 20× representative microphotographs of Oil Red O-Hematoxylin stained liver sections obtained from the control group of rats (**A**), and the group exposed to 8 alcohol binges (**B**). Lower panels depict Oil Red O staining of lipid droplets of the control group (**C**), the group exposed to 8 alcohol binges (**D**), and their corresponding quantification analysis (**E**). Values represent the mean ± SEM (n = 6 to 8 animals per group). ***$p < 0.005$ compared with the control group. OD, optical density.

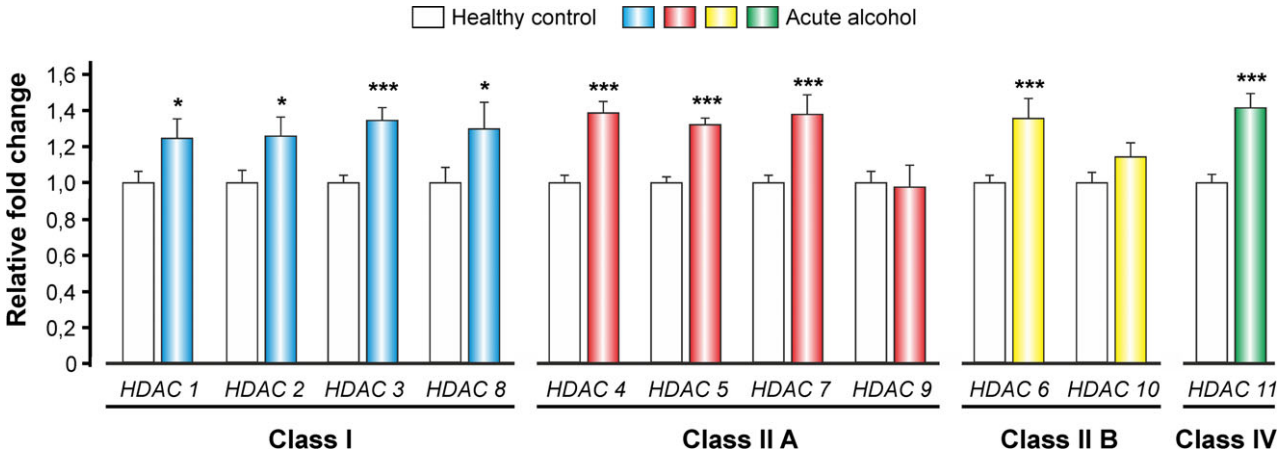

Fig. 8. Effect of acute alcohol intoxication (mean ± SEM blood alcohol level = 239 ± 0.13 mg/dl) on histone deacetylase (*HDAC*) gene expression in human peripheral blood. Data represent the mean ± SEM (n = 20 to 22 patients per group) and the relative fold change obtained using the $2\Delta C_t$ method. *$p < 0.05$, ***$p < 0.005$ compared with the control group.

study whose expression was not reduced within the peripheral blood following acute alcohol binging. This was likely due to the comparatively low concentration of mRNA measured for this *Hdac* in rat blood. For example, as a rough estimation based on PCR amplification data obtained from the control group, *Hdac11* was 1.024-fold and 128-fold less expressed in peripheral blood than *Hdac7* and *Hdac10*, respectively.

We hypothesized that there would be significant correlations in *Hdac* gene expression patterns between the peripheral blood and other examined tissues and that these correlations would be modulated by the number of alcohol binge episodes to which a given subject was exposed; however, this hypothesis was not verified. Instead, we found that the more frequent positive correlation was between the amygdala and the heart, the more frequent negative

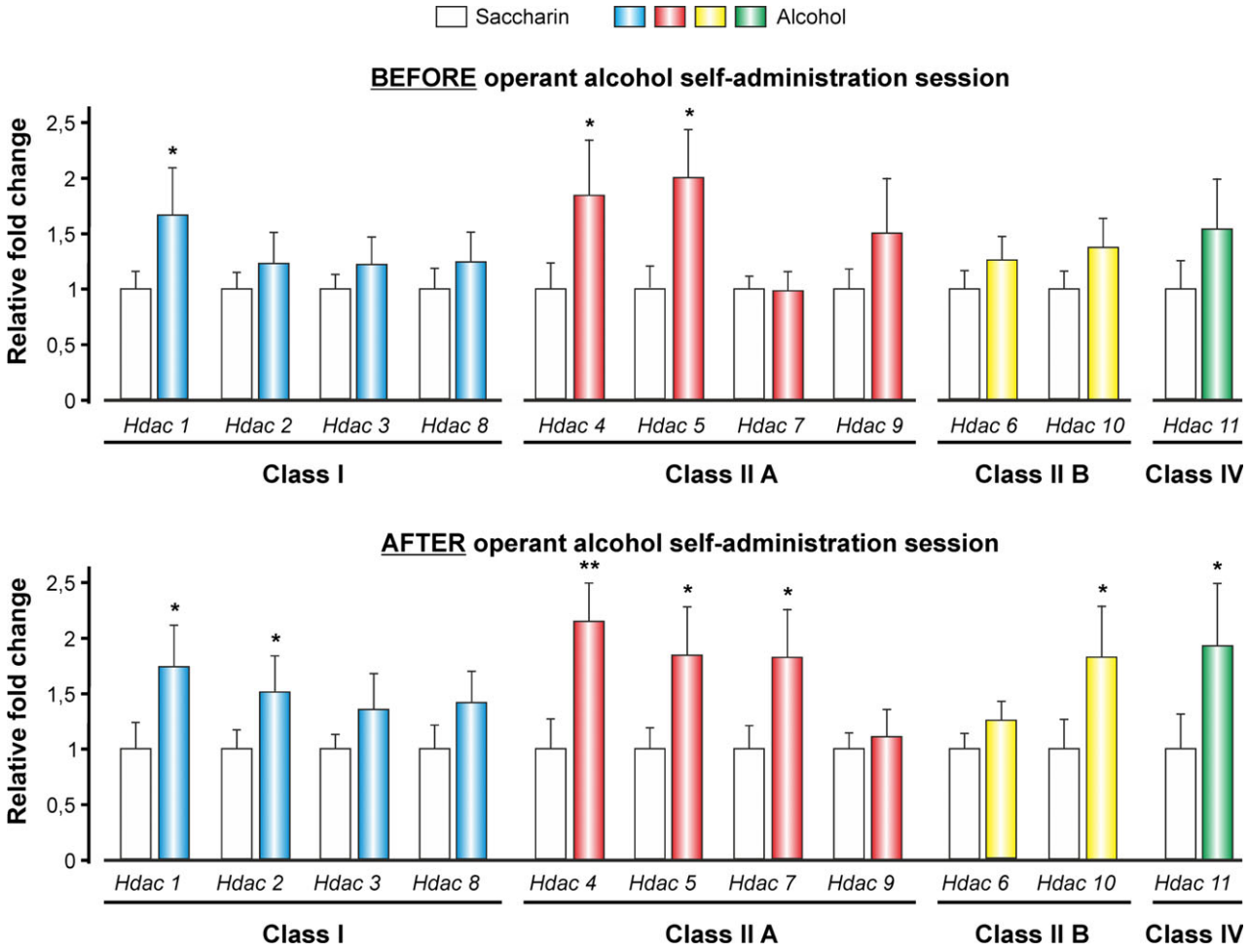

Fig. 9. Effects of operant alcohol self-administration on histone deacetylase (*Hdac*) gene expression in rat peripheral blood at 60 minutes before (upper panel) and 60 minutes after (lower panel) operant alcohol self-administration. Data represent the mean ± SEM ($n = 10$ animals per group) and the relative fold change obtained using the $2\Delta C_t$ method. *$p < 0.05$, **$p < 0.01$ compared with the control group. Please note that a saccharin group was used as control.

correlation between the prefrontal cortex and the amygdala; further, the highest number of negative correlations was within the cohort exposed to 8 alcohol binges. This last finding indicates that as the number of alcohol binge episodes increases, *Hdac* gene expression becomes more variable among different tissues (i.e., increases in gene expression were observed in select types of tissue, while decreases in gene expression were observed in others).

We examined BACs, as well as alcohol-induced fatty liver (steatosis). As was expected, a reduction in BAC was observed as sample collection was delayed from 1 to 4 hours. However, as each alcohol binge treatment occurred only once per week, the significant increases in BACs following repeated, intermittent alcohol binging was unexpected. We would expect the total clearance of alcohol from the rat in between alcohol binge treatments. It is notable, however, that other authors have reported similar results. For instance, Maier and colleagues (1995) administered sufficient alcohol to rats (5 g/kg) to result in a state of daily intoxication, and found that the peak of averaged BACs was higher on the final day of alcohol treatment (18th day). Future studies should examine the biochemical basis for the cumulative nature of BAC following repeated and intermittent alcohol administration. On the other hand, hepatic steatosis only developed in animals subjected to 8 episodes of alcohol binging, despite the fact that there was no direct relationship between alcohol consumption and *Hdac* gene expression in the liver for this cohort and that the liver was the second tissue in which we found a reduction in gene expression. These findings contrast those published by Kirpich and colleagues (2012, 2013) in which a down-regulation of hepatic *Hdac 1, 7, 9, 10, 11* and an up-regulation of *Hdac3* were found to be directly associated with alcohol binge treatment. One possible explanation for this discrepancy is the use of a markedly different protocol, which essentially focused on alcohol-induced hepatic steotosis. Furthermore, in contrast with our dosing schedule of 3 g/kg administered once per week, Kirpich and colleagues (2012, 2013) administered alcohol at a dose of 4.5 g/kg 3 times, at 12-hour intervals. While this dosing schedule agrees with the recent review of Math-

ews and colleagues (2014) regarding animal models of alcoholic liver injury, which claims that the most commonly used doses for the study of alcohol-induced liver injury are 4 to 6 g/kg, we instead chose to use a dose of 3 g/kg to obtain comparable BACs to those found in humans following the binge alcohol consumption (239 ± 0.13 mg/dl from our human samples).

To the best of our knowledge, this is the first study to assess *HDAC* gene expression in human peripheral blood following an alcohol binge. The obtained values in *HDAC* gene expression in human blood were in agreement with previous reports (i.e., Hobara et al., 2010). Overall, we found *HDAC* expression to be increased in response to alcohol, with the exception of the Class IIA *HDAC9* and the Class IIB *HDAC10*. At the present time there are only 2 published studies that have evaluated the effects of alcohol on *HDAC* gene expression in humans. Both of these also found increased *HDAC* expression, though it should be noted that these studies were performed in vitro (Agudelo et al., 2011, 2012). The increase of *HDAC* gene expression that was measured in human peripheral blood samples following an acute alcohol binge contrasts with the reduction of gene expression observed in rat peripheral blood under the same conditions. Our first hypothesis to explain this discrepancy centered around the acute nature of the alcohol binge: in the case of an alcohol-naïve rat, the first binge actually represented the animal's first exposure to high quantities of alcohol, whereas the majority of patients presenting with acute alcohol intoxication reported regular alcohol consumption during the weekend. Considering the high rate of underreporting of alcohol use (Boniface et al., 2014), it is very likely that these patients engaged in repeated episodes of binge drinking in the past. To empirically verify our hypothesis, we performed an additional experiment in which rats were allowed operant alcohol self-administration, wherein the animals drank low to moderate doses of alcohol daily and reached blood alcohol levels between 10 and 40 mg/dl (approximately 25 to 60 responses were recorded). In peripheral blood samples obtained from this cohort of rats, we found that the expression of select *Hdacs* increased more similarly to what was observed for humans. Such increases in *Hdac* expression were slightly more pronounced 60 minutes after alcohol self-administration versus 60 minutes before. Collectively, these data suggest that variability in *HDAC* gene expression in response to alcohol exposure is linked to past alcohol use. For example, while initial exposure to alcohol leads to an acute decrease of *HDAC* gene expression in peripheral blood, repeated exposure to alcohol (either in binge form or moderate intake) either does not affect or increases *HDAC* gene expression. A limitation of this operant self-administration study was that the saccharin group was used as the control group (calibrator) as far as the animals do not work to obtain water.

Several researchers of psychiatric conditions have proposed the monitoring of *HDAC* gene expression within human peripheral blood to serve as a biomarker of alcohol-related behaviors. For example, Hobara and colleagues (2010) found that variability in the expression of the same 11 *HDACs* that were investigated here (*HDAC1–11*) was associated with the pathophysiology of mood disorders (essentially a decrease in gene expression was indicative of a remissive state of depression). Taken together, these results indicate that *HDAC* gene expression profiling of peripheral blood may serve as a potential biomarker for many psychiatric conditions. Here, we have performed a set of translational experiments that provide valuable insights into how *HDAC* gene expression changes in response to alcohol exposure in rats and humans and provide methodology that allows the reliable detection of these changes.

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CONFLICT OF INTEREST

None.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Details of the rat primers used for quantitative real-time PCR of each gene.

Table S2. Details of the human primers used for quantitative real-time PCR of each gene.