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Research Report

The histone deacetylase (HDAC) inhibitor valproic acid reduces ethanol consumption and ethanol-conditioned place preference in rats

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ABSTRACT

Recent evidence suggests that epigenetic mechanisms such as chromatin modification (specifically histone acetylation) may play a crucial role in the development of addictive behavior. However, little is known about the role of epigenetic modifications in the rewarding properties of ethanol. In the current study, we studied the effects of systemic injection of the histone deacetylase (HDAC) inhibitor, valproic acid (VPA) on ethanol consumption and ethanol-elicited conditioned place preference (CPP). The effect of VPA (300 mg/kg) on voluntary ethanol intake and preference was assessed using continuous two-bottle choice procedure with escalating concentrations of alcohol (2.5–20% *v/v* escalating over 4 weeks). Taste sensitivity was studies using saccharin (sweet; 0.03% and 0.06%) and quinine (bitter; 20 μM and 40 μM) tastants solutions. Ethanol conditioned reward was investigated using an unbiased CPP model. Blood ethanol concentration (BEC) was also measured. Compared to vehicle, VPA-injected rats displayed significantly lower preference and consumption of ethanol in a two-bottle choice paradigm, with no significant difference observed with saccharin and quinine. More importantly, 0.5 g/kg ethanol-induced-CPP acquisition was blocked following VPA administration. Finally, vehicle- and VPA-treated mice had similar BECs. Taken together, our results implicated HDAC inhibition in the behavioral and reinforcement-related effects of alcohol and raise the question of whether specific drugs that target HDAC could potentially help to tackle alcoholism in humans.

1. Introduction

Excessive alcohol drinking and alcoholism are major health concern in western societies; however there is still lack of effective treatments. Alcohol addiction is a multifactorial disease that is regulated by multiple mechanisms including chromatin modifications. Although epigenetic mechanisms, such as DNA methylation and histone modifications

Abbreviations: BEC, Blood ethanol concentration; CPP, Conditioned place preference; HDAC, Histone deacetylase; NAcc, Nucleus accumbens; TSA:, Trichostatin A; VPA, Valproic Acid

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(acetylation/deacetylation, methylation, and phosphorylation), are known to regulate gene transcription during addictive processes [for review see Maze and Nestler (2011); Wong et al. (2011)], the exact role of HDAC inhibition has not been examined in ethanol addiction.

Depending on their structural homology to yeast deacetylases, expression pattern, and catalytic mechanisms, eighteen HDACs were divided into four groups: class I HDACs (HDAC1-3 and 8) are associated with yeast RPD3; class II HDACs (HDAC4-7 and 9, 10) share homology with the yeast HDAC1; class III HDACs (SIRT1-7) are related to yeast Sir2 and class IV HDAC (HDAC11) exhibits features of both class I and class II HDACs (deRuijter et al., 2003; Feng et al., 2014; Marks, 2010). Class I, II, IV HDACs are zinc dependent as they are found within a conserved catalytic domain containing a zinc cation. In contrast, class III HDACs require cofactor NAD^+ for their catalytic activity and, therefore, are not responsive to compounds that inhibit zinc dependent deacetylases (Feng et al., 2014). Inhibition of HDAC activity-induced histone hyperacetylation resulted in genetic transcriptional activation.

Multiple studies have shown that the regulation of gene expression is highly involved in the pathophysiology of psychiatric diseases including anxiety (Archer et al., 2013; Hodes, 2013; Narayan and Dragunow, 2010), depression (Archer et al., 2013; Dalton et al., 2014), schizophrenia (Misiak et al., 2013; Svrakic et al., 2013) and addiction (Crepaldi and Riccio, 2009; Malvaez et al., 2009; McQuown and Wood, 2010). In fact it has been shown that systemic injection of HDAC inhibitors butyric acid (630 mg/kg, i.p.) or VPA (175 mg/kg, i.p.) significantly potentiated the amphetamine-induced behavioral sensitization in mice (Kalda et al., 2007). In contrast, the microinjection of VPA and butyric acid in the ventricle, amygdala, striatum, and prefrontal inhibited the HDAC activity and blocked m-amphetamine-induced hyperactivity (Arent et al., 2011). Similarly, it has been reported that HDAC inhibitors reversed and prevented d-amphetamine-induced behavioral effects (Steckert et al., 2013; Stertz et al., 2014).

Other studies have shown that the administration of the HDAC inhibitor, butyric acid enhances morphine-induced locomotor sensitization and CPP with no effect on the development of morphine tolerance and dependence (Malvaez et al., 2009) and similar effects were observed for cocaine-induced behaviors (Malvaez et al., 2009). In the same line, it has been demonstrated that HDAC inhibition during extinction consolidation facilitated extinction of cocaine-induced CPP more quickly and to a greater extent than did vehicle-treated C57BL/6 mice (Malvaez et al., 2010; Raybuck et al., 2013). As for cocaine-CPP acquisition, it has been reported that pretreatment with HDAC inhibitor sodium butyrate, potentiated cocaine-induced CPP (Hui et al., 2010; Raybuck et al., 2013). Taken together, it appears that histone modifications may be an important mechanism that underlies motor- and conditioned-effects of psychostimulants.

In relation with ethanol addiction, Sakharkar et al. (2012) have shown that a single ethanol exposure (1 g/kg; i.p.) was able to inhibit amygdaloid HDAC activity, and increase histone acetylation in the central nucleus of amygdala (CeA) and medial nucleus of amygdala (MeA) of rat. The same group also reported that, compared to the non-preferring (NP), the selectively bred alcohol-preferring (P) rats display higher nuclear HDAC activity in the CeA and MeA (Moonat et al., 2013). Importantly, acute ethanol exposure decreased amygdaloid HDAC activity and attenuated anxiety-like behaviors in P rats but not in NP rats (Moonat et al., 2013). Also, using a model of rat hippocampal-entorhinal cortex brain slice cultures, Zou and colleagues have shown that ethanol exposure triggered the translocation of HDACs proteins from nuclear to cytosolic fractions. This translocation was accompanied by a decrease of HDACs mRNA expression leading to increased acetylated high-mobility group box 1 (HMGB1) protein release into the culture media (Zou and Crews, 2014). Although these data demonstrate the potential role of HDAC-mediated epigenetic mechanisms in alcoholism in rodents, most of the studies have been focused on the effects of ethanol exposure on HDAc activity. Evidence showed that VPA increased γ-aminobutyric acid (GABA) transmission (Loscher, 2002) as the non-competitive $GABA_A$ receptor antagonist Picrotoxin, blocked the anxiolytic- and panicolytic-like properties of VPA (Dombrowski et al., 2006; Liljequist and Engel, 1984; Vellucci and Webster, 1984). Therefore, a GABAergic mechanism could mediate VPA effects on ethanol-related behaviors.

Although the emerging literature demonstrated the efficiency of HDAC inhibitors in reducing ethanol-related behaviors, some issues remain unresolved. First, several reports have shown that HDAC inhibitors reduced-ethanol-CPP. However, these studies often have examined a single dose of drug. Second, few studies have examined the effects of HDAC inhibition on voluntary ethanol intake using the two-bottle choice drinking paradigm. This is crucial because clinical application of alcohol-intake reducing drugs could have the unexpected effect of altering peripheral ethanol metabolism.

The following experiments examine the dose-effects of the HDAC inhibitor VPA on voluntary ethanol intake and the initial formation of a context-drug memory in ethanol-induced CPP.

2. Results

2.1. *VPA decreased ethanol intake and preference*

We evaluated voluntary oral ethanol intake by allowing rats' continuous access to two drinking bottles; one containing water and the other containing an ascending range of ethanol concentrations. Fig. 1A represents ethanol consumption over the 6-day access period to 2.5%, 5%, 10% and 20% in vehicle- and VPA-injected rats ($n=14$ for each group). A two-way ANOVA revealed a significant effect of concentration ($F(3104)=86.599$, $p<0.001$) as the ethanol consumption increased when the concentration of ethanol offered increased. In addition, there was a significant effect of treatment ($F(1104)=61.966$, $p<0.001$) as the VPA-treated rats consumed less amounts ethanol as their controls from 5%, 10% and 20% solutions. More importantly, the ANOVA of treatment × concentration interaction was significant ($F(3104)=8.646$, $p<0.001$). As expected, ethanol preference results generally paralleled the consumption differences. Therefore, and as depicted in Fig. 1B, the two-way ANOVA revealed a significant effect of concentration on ethanol preference as ethanol was absolutely preferred over water ($F(3104)=20.390$, $p<0.001$). Similarly, there was significant effect of treatment as the VPA

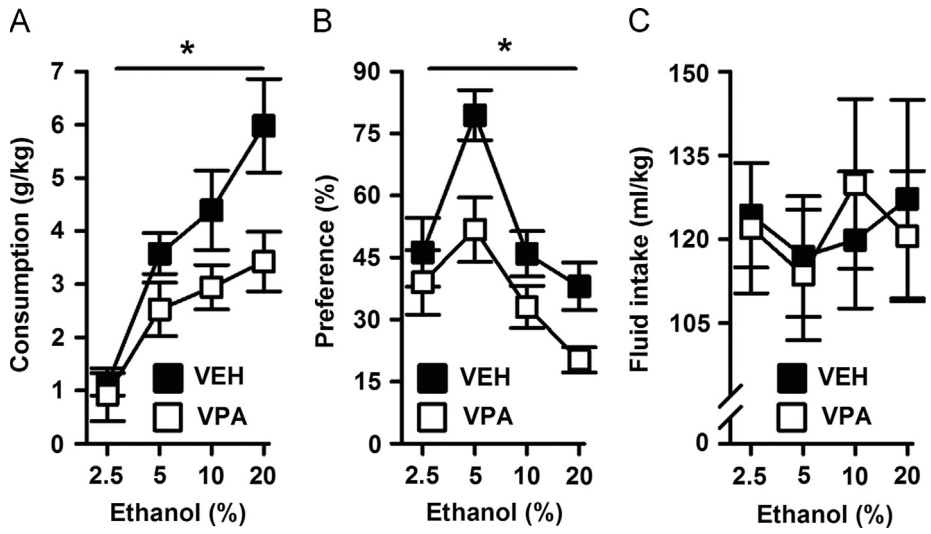

Fig. 1 – Effect of vehicle and VPA (300 mg/kg) on ethanol (2.5%, 5%, 10% and 20%) consumption and preference. (A) Ethanol consumption calculated as grams of alcohol consumed per kilogram of body weight in male Wistar rats. (B) Ethanol preference expressed as ethanol consumed/total fluid consumed and (C) Average total fluid (water+ethanol) intake. Data are expressed as mean ± SEM. *$p<0.001$; vehicle ($n=14$) and VPA ($n=14$).

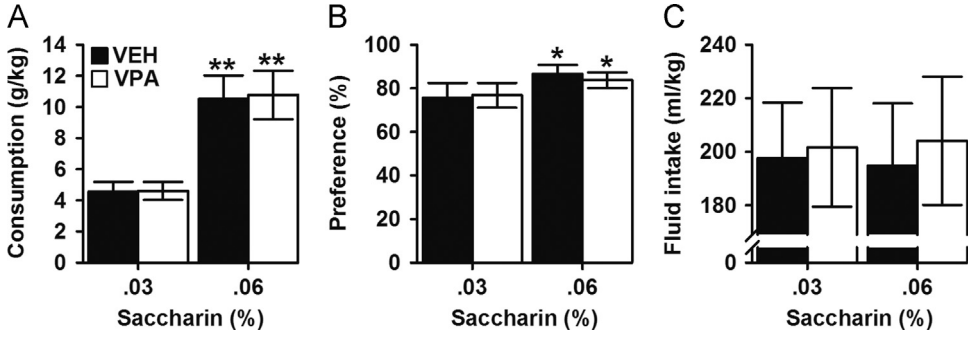

Fig. 2 – Effect of vehicle and VPA (300 mg/kg) on saccharin (0.03% and 0.06%) consumption and preference. (A) Saccharin consumption calculated as grams of alcohol consumed per kilogram of body weight in male Wistar rats. (B) Saccharin preference expressed as saccharin consumed/total fluid consumed and (C) Average total fluid (water+saccharin) intake. Data are expressed as mean ± SEM. *$p<0.05$; **$p<0.001$; vehicle ($n=14$) and VPA ($n=14$).

(300 mg/kg) significantly reduced ethanol preference injected rats preferred across alcohol concentrations ($F(1104)=23.210$, $p<0.001$). However, ethanol concentration × drug treatment interaction did not reach significance ($F(3104)=1.630$, $p=0.187$). For total fluid consumption, no significant main effects of treatment ($F(3104)=0.014$, $p=0.059$), and ethanol concentration ($F(3104)=0.539$, $p=0.418$) were observed. In addition, the interaction between the two factors was not significant ($F(3104)=0.651$, $p=0.584$) (Fig. 1C).

2.2. *VPA did not affect tastants intake and preference in rats*

To assess whether the decreased ethanol consumption and preference are unrelated to the pharmacological effects of ethanol, the same rats were tested for consumption of nonalcoholic tastants. Results have shown that both vehicle and VPA groups had equal intake of saccharin (sweet) solutions at both concentrations. In detail, the two-way ANOVA analysis revealed a main effect of concentration ($F(1,52)=36.367$, $p<0.001$) on saccharin drinking as the amount saccharin consumed by both vehicle and VPA-injected rats increased as the concentration of saccharin offered was increased. In contrast, no significant main effects of treatment ($F(1,52)=0.025$, $p=0.874$), and concentration x treatment interaction ($F(1,52)=0.012$, $p=0.914$) were observed (Fig. 2A). As expected, there was no difference between the two groups in terms of saccharin consumption (main effect of treatment: $F(1,52)=0.042$, $p=0.839$) (treatment x concentration interaction: $F(1,52)=0.243$, $p=0.624$). However, the ANOVA revealed a significant concentration effect ($F(1,52)=4.893$, $p=0.031$), which reflected a general tendency towards higher intake of more concentrated saccharin solutions (Fig. 2B). In line with the above, total fluid intake (from both bottles) did not differ between the two groups regardless of the solution available in the second bottle: main effect of treatment ($F_{(1,52)}=0.148$, $p=0.702$); main effect of concentration: ($F_{(1,52)}=0.000$, $p=0.990$) and the interaction between treatment and concentration ($F_{(1,52)}=0.024$, $p=0.878$) (Fig. 2C).

In the quinine intake experiment, the ANOVA indicated that there was a significant effect of concentration ($F_{(1,52)}=68.589$, $p<0.001$) on quinine consumption (Fig. 3A). This latter observation reflected the fact that quinine intake increased as its concentration increased. However, the ANOVA showed no effect of treatment on quinine consumption ($F_{(1,52)}=1.416$, $p=0.239$). The treatment × concentration interaction ($F_{(1,52)}=1.639$, $p=0.206$) was not significant. As expected, there were no between-group differences in terms of quinine preference for the two concentrations. In fact and as depicted in Fig. 3B, the effect of treatment ($F(1,52)=0.028$, $p=0.868$); concentration ($F(1,52)=0.375$, $p=0.543$); and the interaction between the two factors ($F(1,52)=0.000$, $p=0.985$) were not significant. This observation reflected the fact that quinine preference did not change with concentration. Finally, for total fluid intake, there was no significant effect of treatment ($F(1,52)=1.075$, $p=0.305$); no significant effect of quinine concentration ($F(1,52)=0.102$, $p=0.750$); and no significant interaction between treatment and concentration ($F(1,52)=0.041$, $p=0.841$) (Fig. 3C). These results suggest that VPA-induced reduction of alcohol intake and preference does not correlate with alteration in taste sensitivity or ethanol pharmacokinetics.

2.3. VPA dose dependently decreased ethanol intake and preference in rats

After establishment of stable patterns of drinking during the last 3–4 days (data not shown), rats ($n=7$–9) were distributed to various groups such that their mean ethanol consumption was normalized. Animals received saline and VPA (150, 300, or 600 mg/kg) ~30 min prior to 5% ethanol and water access at the beginning of the dark phase. Ethanol (g/kg) and water (ml/kg) consumption were recorded over 24 h at predetermined time point (24-h post injection). As depicted in Fig. 4A, one way ANOVA revealed a significant main effect of VPA treatment on ethanol consumption ($F(3,27)=21.683$, $p<0.001$). Further analysis revealed that VPA (300 mg/kg) significantly reduced ethanol intake post 24-h treatment (44% of control; $p=0.001$) while 600 mg/kg dose significantly reduced ethanol intake post 24-h treatment (67% of control; $p<0.001$). No significant effect on ethanol consumption was observed with low dose of VPA (150 mg/kg) ($p=1.000$).

Regarding percent ethanol preference, the main effect of VPA treatment was found significant ($F(3,27)=45.098$; $p<0.000$) (Fig. 4B). Further analysis with a *post-hoc* test revealed that higher doses of VPA (300 or 600 mg/kg) reduced ethanol preference significantly (37% and 65% of control, respectively) ($p<0.000$). Although, low dose of VPA (150 mg/kg) showed a modest reduction in ethanol preference (5%), this effect was not significantly different from control ($p=1.000$). Finally, regarding total fluid intake, the main effect of VPA treatment was not significant at any dose across 24-h post injection ($F(3,27)=0.198$, $p=0.897$) (Fig. 4C).

2.4. VPA reduced ethanol-elicited CPP acquisition

To further test whether inhibition of the HDAC alters ethanol's behavioral effects, we tested rats for ethanol-induced CPP. As shown in Fig. 5A, the two-way ANOVA revealed that both vehicle and VPA rats showed no initial preference for any of the compartments. Thus, preconditioning was not affected by the two-between subject factors: main effect of conditioning ($F(1,44)=1.482$, $p=0.230$); main effect of treatment ($F(1,44)=1.635$, $p=0.208$). However, both groups developed a significant preference for the ethanol-paired chamber when conditioned with ethanol: main effect of conditioning ($F(1,44)=98.407$, $p<0.001$); main effect of treatment ($F(1,44)=6.468$, $p=0.015$). More importantly the interaction between ethanol conditioning and drug treatment was also significant ($F(1,44)=8.494$, $p=0.006$). These results suggest that ethanol-induced CPP is affected by blockade of HDAC in these rats.

2.5. VPA did not affect blood ethanol concentration (BEC) in response to acute ethanol injection

The last key question of the current study was whether HDAC inhibition, using VPA, causes primary disturbances in ethanol metabolism. Therefore, we measured BECs (mg/dl) in response to acute ethanol exposure (3 g/kg, i.p.) in vehicle ($n=6$) and VPA ($n=6$) rats. Results, depicted in Fig. 5B revealed no group differences in BECs reached in the vehicle and VPA-treated animals in response to administration of 3 g/kg doses of ethanol. Thus, the one-way ANOVA repeated measures analysis revealed that in vehicle- and VPA-treated animals, BEC decreased significantly over time ($F(2,20)=13.663$, $p<0.001$).

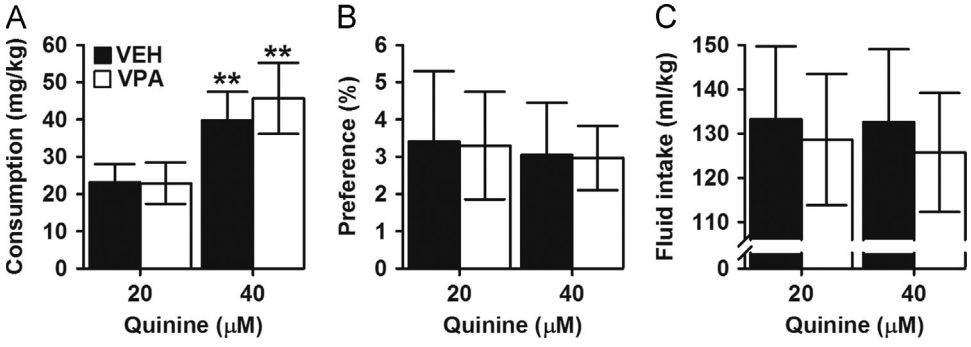

Fig. 3 – Effect of vehicle and VPA (300 mg/kg) on quinine (20 and 40 μM) consumption and preference. (A) Quinine consumption calculated as milligrams of alcohol consumed per kilogram of body weight in male Wistar rats. (B) Quinine preference expressed as quinine consumed/total fluid consumed and (C) Average total fluid (water+quinine) intake. Data are expressed as mean ± SEM. $^{**}p<0.005$; vehicle ($n=14$) and VPA ($n=14$).

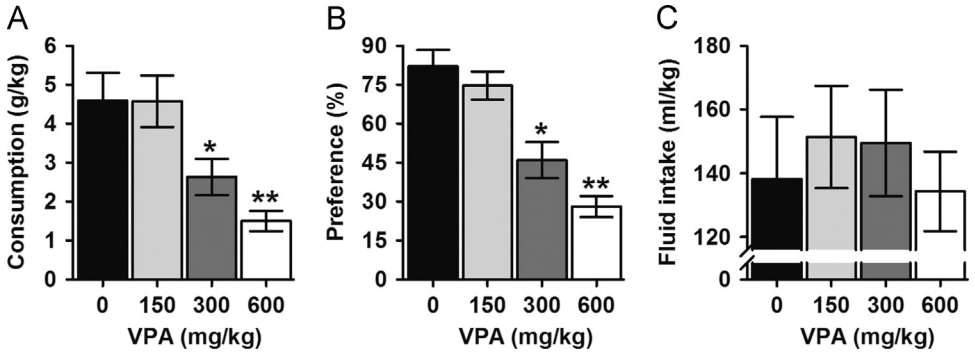

Fig. 4 – Effect of VPA (0, 150, 300 and 600 mg/kg) on ethanol (5%) consumption and preference. (A) Ethanol consumption calculated as grams of alcohol consumed per kilogram of body weight in male Wistar rats. (B) Ethanol preference expressed as ethanol consumed / total fluid consumed and (C) Average total fluid (water+ethanol) intake. Data are expressed as mean ± SEM. *$p<0.01$; **$p<0.001$; 0 mg/kg ($n=7$); 150 mg/kg ($n=8$); 300 mg/kg ($n=9$) and 600 mg/kg ($n=7$).

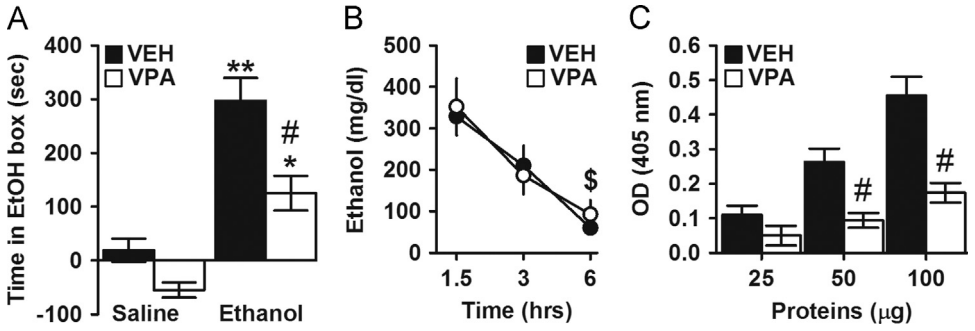

Fig. 5 – Effect of vehicle and VPA (300 mg/kg) on ethanol elicited conditioned place preference, blood ethanol concentration and HDAC enzymatic activity. (A) Ethanol-elicited place preference test expressed as mean times ± SEM (s) spent on the EtOH paired box (Test minus habituation). Male Wistar in the conditioning subgroups had previously received pairings of vehicle or VPA before saline or EtOH (0.5 g/kg; i.p.). *$p<0.05$ and **$p<0.01$ *vs.* saline conditioning; #$p<0.05$ *vs.* vehicle. VEH-Saline ($n=12$); VPA-Saline ($n=12$); VEH-Ethanol ($n=12$); VPA-Ethanol ($n=12$). (B) Blood alcohol concentrations 1.5, 3 and 6 hours after an acute injection of 3 g/kg ethanol. $n=6$ in each group. \$$p<0.001$ *vs.* 1.5 h. (C) Using 25, 50 and 100 μg protein extracts, VPA inhibited HDAC activity in the NAcc. $n=7$ in each group. #$p<0.05$ *vs.* vehicle.

However, the rate of alcohol metabolism, as measured by the slope of the line, was not significantly different between the two groups; main effect of drug: ($F(1,10)=0.157$, $p=0.700$). Interestingly, the interaction between drug and time was not significant ($F(2,20)=0.180$, $p=0.836$). Taken together, our findings suggest that VPA treatment does not alter ethanol metabolism. Consequently, one can rule out the existence of metabolic disturbances following HDC inhibition in adult rats.

2.6. *VPA markedly reduced HDAC activity in protein extracts-derived from the Nucleus Accumbens (NAcc)*

We next quantitated the HDAC inhibitory property of VPA in NAcc protein extracts using a commercially available kit (see the Methods section for additional details). Data described in Fig. 5C demonstrate a significant inhibition of HDAC activity by VPA. In fact, the two-way ANOVA revealed that in vehicle- and VPA-treated animals, HDAC activity increased significantly when the amount of proteins tested increased; main effect of concentration: ($F(2,36)=22.801$, $p<0.001$). In addition, the rate of HDAC activity as measured by the slope of the lines was significantly different between the two groups; main effect of drug: ($F(1,36)=35.625$, $p<0.000$). More importantly, the interaction between drug and concentration was found significant ($F(2,36)=5.017$, $p=0.012$). Post hoc evaluations indicated a significant inhibition of HDAC activity by VPA in protein extracts from both 50 μg (~65%; $p=0.003$) and 100 μg (~62%; $p=0.001$) samples. At lower protein concentration, VPA also inhibited total HDAC activity, albeit to a lesser extent (~55%; $p=0.150$). Together, these data demonstrate that the reduced alcohol intake and ethanol-CPP acquisition observed following VPA administration could be explained with a decreased deacetylating efficiency of HDACs at least in the NAcc.

3. Discussion

In the present study, we tested the hypothesis that HDAC activity is involved in the regulation of two-bottle choice voluntary ethanol intake as well as ethanol-induced CPP. Saccharin and quinine solutions were used as natural rewarding and aversive stimuli, respectively. The first major finding is that HDAC inhibition, using VPA, produced a significant reduction of ethanol drinking behavior without affecting saccharin or quinine consumption in rats suggesting that the decreased alcohol consumption of the VPA-injected rats may be related to

the pharmacological effects of alcohol. More importantly, the second major finding is that HDAC inhibition blocked ethanol-induced CPP acquisition. In agreement with our results, Sakharkar et al. (2014) examined the effects of trichostatin A (TSA; 2 mg/kg) on the alcohol intake in alcohol-preferring (P) and -non-preferring (NP) rats during the last three days of 9% alcohol intake and reported that HDAC inhibitor treatment significantly attenuated the alcohol-consumption in P but not in NP rats. Similarly, Warnault et al. (2013) reported that systemic administration of the FDA-approved HDAC inhibitor, SAHA, inhibited the motivation of rats to seek alcohol in a binge-like alcohol drinking paradigm where mice had intermittent access to a single bottle of 20% alcohol. However and in contrast to our results, Wolstenholme et al. (2011) recently showed that TSA (TSA, 2 mg/kg, i.p.) significantly increased ethanol intake at three and four weeks following administration while vehicle treated animals showed no significant change. The discrepancies in our findings compared with the results of Wolstenholme et al. can be explained by the different conditions used to determine voluntary alcohol intake. Whereas Wolstenholme et al. (2011) used intermittent access (4 consecutive drinking sessions followed by 4 days of abstinence repeated 4 times) to 10% ethanol for only 18 hours per day followed by 6 h access to water, in our study alcohol concentration was gradually increased with 6 days of free access for each concentration. We hypothesize that our standard two-bottle choice drinking procedure may reduce the initial taste aversion that laboratory animals could display to alcohol as well as eliminate the contribution of neophobia or olfactory and gustatory factors. Therefore, methodological issues, such as animal species (Wistar rats versus C57BL/6NCrl mice), and the ethanol access procedure could underlie the seemingly discrepant findings. However, it is clear that future studies are required to further dissect the reasons for the different observations with HDAC inhibitors on voluntary ethanol consumption and preference. Therefore, the impact of HDAC function on ethanol-related behaviors is currently under investigation in our laboratory using stereotaxic injections of viral vectors in specific brain regions. Finally, a taste sensitivity analysis of saccharin and quinine revealed that VPA and saline-treated rats consumed comparable amounts of saccharin and quinine. Also, the preference ratios for these tastants were comparable between the two groups of rats. Alcohol is different from other drugs of abuse as it must be absorbed via the oral cavity and therefore it was of high interest to control for taste sensitivity. To this aim, saline and VPA-injected rats had access to different concentrations of both a sweet (saccharin) and bitter (quinine) solution. These solutions were chosen because they are devoid of any pharmacological or caloric effect and detect taste neophobia (Bahi and Dreyer, 2014; Bahi et al., 2013b; Crabbe et al., 1996). Results have shown that there were no differences in consumption or preference between the saline and VPA-treated animals. These findings suggest that HDAC-function inhibited rats have comparable oral perception and that any changes in ethanol intake in preference do not result of any deficit in taste-processing following VPA-injection, at least on the Wistar genetic background.

To test the involvement of the chromatin architecture opening in drug reward, rats were submitted to ethanol-induced CPP test. Because the procedure is based on conditioned reward in which the animal itself regulates exposure to the drug-associated cues during expression it is considered in great validity to modulate drug abuse in humans. Using an unbiased CPP procedure, we found that VPA reduced the ethanol-CPP acquisition. More importantly, the effect was not observed when rats were conditioned with saline alone, suggesting that the HDAC inhibitor actually reduced the reinforcing properties of ethanol. The fact that the inhibition of HDAC activity by VPA had no negative effect on saline-induced-CPP strongly suggests that the compound did not produce anhedonia, which would have explained a lack of motivation. Our findings are in agreement with previous reports indicating that HDAC inhibition suppresses behaviors related to drugs of abuse. For example, it has been shown that the HDAC inhibitors, TSA and phenylbutyrate, reduced the cocaine reinforcing properties, the motivation of rats for cocaine and cocaine-seeking behavior induced by the combination of a cocaine injection together with the exposure to a light cue previously associated with cocaine taking (Host et al., 2011; Romieu et al., 2011, 2008). Similarly, and using a nicotine-CPP protocol, Pastor and co-workers have also shown that the HDAC inhibitor phenylbutyrate dramatically reduced the preference for nicotine, without altering the intrinsic aversive properties of the drug (Pastor et al., 2011). More importantly, our results are in line with a recent report showing that ethanol-induced behavioral sensitization was dose-dependently prevented or reversed by the HDAC inhibitor sodium butyrate (200–600 mg/kg) (Legastelois et al., 2013). The present findings together with existing literature demonstrate that HDAC inhibition can modulate CPP conditioning, but the brain regions in which VPA may be activating gene expression to drive these expressing effects are not yet clear. It should be emphasized that a number of reports have used pharmacological and genetic approaches to demonstrate the importance of the NAcc in CPP, specifically that VPA increased histone acetylation in the prefrontal cortex and NAcc-shell (Montalvo-Ortiz et al., 2013). Therefore, there is a crucial role for these reward structures in the epigenetic modulation of ethanol-cue learning. However, the role of histone acetylation in the many structures that support CPP is still largely unknown. Further investigation will be necessary to determine how regional histone acetylation modulates expression of CPP.

The observed behavioral effects of VPA could be related to increased GABA transmission (Loscher, 2002). Most importantly, the $GABA_B$ receptor agonist baclofen reduced the reinforcing effects of abused drugs in animal models under multiple experimental procedures [for review, see (Cousins et al., 2002)]. In fact, human studies have demonstrated that baclofen is effective treating alcohol-dependent individuals (Addolorato and Leggio, 2010). In pre-clinical studies using laboratory animals, it has been shown that baclofen reduced ethanol consumption in both mice (Orru et al., 2012; Tanchuck et al., 2011) and rats (Janak and Michael Gill, 2003). In addition, the $GABA_A$ receptor agonist, with selectivity for the extrasynaptic δ-subunit, gaboxadol dose-dependently decreased ethanol intake as measured with limited-access 2-bottle choice and operant self-administration paradigms (Ramaker et al., 2012). Very recently, Arora and colleagues have shown that in brain slices from ethanol-withdrawn mice incubated with the HDAC inhibitors vorinostat

or TSA for 2 h, the hyposensitivity of putative dopaminergic ventral tegmental area neurons to GABA was significantly attenuated (Arora et al., 2013). The authors concluded that "inhibition of HDACs can reverse ethanol-induced neuroadaptational changes in reward circuitry". Taken together, these results suggest that the anti-alcohol effects of VPA are, in part, related to GABAergic neurotransmission most probably through the $GABA_A$ receptors subtypes.

The present results, together with existing literature demonstrate that enhancement of histone acetylation via inhibition of HDACs can modulate voluntary alcohol intake and ethanol-induced learning. Since VPA was used in the present study, we cannot exclude that the phenotypes we observed result from different members of the HDAC families. Future studies aiming at silencing specific HDACs in local brain regions of adult animals, using viral-mediated gene transfer technology, will help better tease apart the role of HDAC inhibition and chromatin acetylation in the behavioral manifestations of ethanol dependence.

Bearing these caveats in mind, the current findings provide promising evidence that the modulation of HADC activities can represent a successful approach to selectively tackle the motivational drive of subjects to drink ethanol.

4. Experimental Procedures

4.1. Animals

Adult male Wistar rats obtained from the central breeding facility of the College of Medicine and Health Sciences (United Arab emirates University) were single housed at room temperature (~22 °C) with a 12/12 h light/dark cycle with the light off at 6 pm. Rats had free access to tap water and rodent chow diet obtained from the National Feed and Flour Production and Marketing Company LLC (Abu Dhabi, UAE), Bedding was produced locally and autoclaved before use. The local Ethics committee approved the research's procedures (protocol number: A26/12).

4.2. Drugs

Ethanol solutions (2.5%, 5%, 10% and 20%, v/v) were prepared from absolute ethyl alcohol (Panreac Quimica SAU, Barcelona, Spain) and diluted using tap water. For taste sensitivity, saccharin sodium salt dihydrate (0.03% and 0.06%; w/v) and quinine hemisulfate (20 and 40 μM; w/v) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in tap water. For ethanol-induced CPP, 0.5 g/kg alcohol was diluted in 0.9% sodium chloride and sterile water (10%; v/v). Valporic acid (VPA) purchased from Sigma-Aldrich (St. Louis, MO, USA) was dissolved in 0.9% sodium chloride and sterile water and administered intraperitoneally (i.p.) at a volume of 1 ml/kg.

4.3. Two-bottle choice drinking procedure: 24-hour ethanol access

Table 1 presents a general design of the study. Voluntary oral ethanol intake and preference were examined using a two-bottle choice drinking procedure as described previously (Bahi and Dreyer, 2013, 2014; Bahi et al., 2012, 2013a, 2013b). In brief, rats were housed individually and given access to two bottles containing tap water for 6 days. Animals were then given access to two bottles, one containing ethanol in tap water and the other containing tap water alone. During the course of the ethanol exposure period, ethanol concentration was increased gradually from 2.5% to 20% (2.5%, 5%, 10%, and 20%) with 6 days of access at each concentration. Each day, the rats were injected and placed back into their home cages while the bottles were weighed. Fluid levels were recorded at the beginning and end of 24 h fluid access periods. The position (left or right) of each solution was alternated daily to control for side preference. Ethanol intake data were calculated and expressed as grams of alcohol consumed per kilogram of body weight per day (g/kg/day). The ethanol preference was calculated as volume of ethanol consumed per total volume of water plus ethanol consumed. Total fluid intake was calculated and expressed as volume of water plus ethanol consumed per kilogram of body weight per day (ml/kg/day). For VPA dose curve response, rats received saline and VPA (150, 300, or 600 mg/kg) ~30 min prior to 5% ethanol and water access at the beginning of the dark phase. Ethanol (g/kg) and water (ml/kg) consumption were recorded over 24 h at predetermined time point (24 h post injection).

Table 1 – Summary of the experimental procedure used in the two-bottle choice test.

Fluids	Days
Baseline water drinking [a]	6
2.5% ethanol *vs.* water [b]	6
5% ethanol *vs.* water [b]	6
10% ethanol *vs.* water [b]	6
20% ethanol *vs.* water [b]	6
Washout/water [a]	7
0.03% saccharin *vs.* water [b]	6
0.06% saccharin *vs.* water [b]	6
Washout/water [a]	7
20 μM quinine *vs.* water [b]	6
40 μM quinine *vs.* water [b]	6

[a] Tap water was available in both drinking bottles.
[b] In all the two-bottle tests, the drinking bottles were rotated daily to prevent position preference.

4.4. Two-bottle choice drinking procedure: 24-h tastant access

A week after ethanol consumption test, the same rats were tested for saccharin and quinine consumption and preference. Therefore, vehicle- and VPA-injected rats (300 mg/kg) were used to determine if these two groups have similar taste preference for sweet and bitter tastes. Taste-preference testing was conducted as for ethanol consumption testing and as previously reported (Bahi and Dreyer, 2013, 2014; Bahi et al., 2012, 2013a, 2013b). Briefly, rats were serially offered two concentrations of saccharin (0.03% and 0.06%; w/v) and quinine (20 and 40 μM; w/v), each versus tap water. Lower concentrations were offered first for each tastant, each

concentration was offered for 6 consecutive days and the position of each solution was alternated daily to control for side preference.

4.5. Ethanol-induced conditioned place preference

An unbiased CPP test in rats was used to test the effect of HDAC inhibition of ethanol-induced CPP acquisition as described previously (Bahi, 2013; Bahi and Dreyer, 2012; Bahi et al., 2013a, 2013b). In brief, the testing apparatus consisted of two $30 \times 30\ cm^2$ compartments with distinct visual and tactile cues. The two compartments were separated by a 10 cm guillotine door that provided access to both of the conditioning compartments. On the first day of testing (*habituation session*), all rats received an i.p. injection of saline and were given access to both conditioning compartments for 30 min and the amount of time spent in each compartment was manually scored. Over the next 5 days (*conditioning sessions*), animals received alternating i.p. injections (two injections per day) of either saline or ethanol (0.5 g/kg; 10% solution) immediately before placement within either the black-walled or the white-walled conditioning compartment for 30 min. Saline- and ethanol-paired environments were counterbalanced within each group. This pretreatment - treatment combination created four test groups: [saline–saline conditioning: VEH ($n=12$), VPA ($n=12$)] and [saline–ethanol conditioning: VEH ($n=12$), VPA ($n=12$)]. On the final (*test session*) day, animals were placed between and given access to both compartments for 30 min and the amount of time spent in each compartment was manually scored.

4.6. Blood ethanol concentrations (BECs) in response to acute ethanol following VPA injection

To estimate whether acute VPA administration altered ethanol metabolism, we avoided the induction of confounding effects on the interpretation of behavioral data by giving separate sets of VPA (300 mg/kg) and vehicle pre-treated adult rats i.p. injections of 3 g/kg (i.p.; 20% *v/v* in isotonic saline) (vehicle: $n=6$; VPA: $n=6$) doses of ethanol, and blood samples were collected 1.5, 3 and 6 h later to assess BECs. For this purpose, approximately 50 μl of blood was collected from the tail vein of each rat; the samples were centrifuged, and 10 μl of plasma from each sample was analyzed for BECs, which were measured in mg/dl (BioVision Research Products, CA, USA) as described previously (Bahi, 2013; Bahi et al., 2013a). This study assured that differences found in voluntary alcohol intake and ethanol-induced-CPP between experimental conditions were not primarily associated with group differences in peripheral ethanol metabolism.

4.7. Histone deacetylase activity assay

After completion of the CPP experiments, HDAC activity form vehicle- and 300 mg/kg VPA-injected rats were measured by means of kit assay from BioVision Inc. (Milpitas, CA, USA) according to the manufacturer's instructions. In brief, animals were killed by rapid decapitation, brains were removed and Nucleus Accumbens (NAcc) were dissected out and homogenized (1:10, *w/v*) in the assay buffer containing (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate) supplemented with protease inhibitor cocktail (1:100, *v/v*) from Sigma-Aldrich. The samples (20–100 μg of protein extract) were diluted to 85 μl (final volume) of ddH20 in each well. Then, 10 μl of the 10X HDAC Assay Buffer were added to each well before adding 5 μl of the HDAC colorimetric substrate. The plate was incubated at 37 °C for 1.5 hour and the reaction was terminated by adding 10 μl of Lysine Developer. The plate was incubated for an additional 30 min at 37 °C and the color developed was read at 405 nm using an ELISA plate reader. The HDAC activity was expressed as the relative absorbance value per μg protein sample.

4.8. Statistical analysis

For statistical comparisons, the software package IBM SPSS Statistics 20 was used. Data were expressed as means ± SEM. The effects of VPA on ethanol and tastants consumption and preference were analyzed using a mixed repeated-measure analysis of variance (ANOVA) with drug (vehicle or VPA) and ethanol or tastant concentration as the between-subjects factor and time as the within-subject factor. The effect of VPA on the ethanol-induced CPP was analyzed using two-way ANOVA with repeated measure with drug and conditioning (ethanol or saline) as the between-subject factor and time (Pre- and post-conditioning) as the within-subject factor. The effect of VPA on BECs was analyzed using one-way ANOVA repeated measure with drug (vehicle or VPA) as the between-subject factor and time as the within-subject factor. The effect of VPA on HDAC activity was analyzed using two-way ANOVA with drug and protein concentration as the between-subject factors. In case of a significant main effect, post hoc comparisons were performed with Bonferroni's test. The criterion for statistical significance was set at $p \leq 0.05$.

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Disclosure/conflict of interest

The authors have no financial interests that might be perceived to influence the results or the discussion reported in this article.

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