Effects of histone deacetylase inhibitors on amygdaloid histone acetylation and neuropeptide Y expression: a role in anxiety-like and alcohol-drinking behaviours

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Abstract
Recent studies have demonstrated the involvement of epigenetic mechanisms in psychiatric disorders, including alcoholism. Here, we investigated the effects of histone deacetylase (HDAC) inhibitor, trichostatin A (TSA) on amygdaloid HDAC-induced histone deacetylation and neuropeptide Y (NPY) expression and on anxiety-like and alcohol-drinking behaviours in alcohol-preferring (P) and –non-preferring (NP) rats. It was found that P rats displayed higher anxiety-like and alcohol-drinking behaviours, higher amygdaloid nuclear, but not cytosolic, HDAC activity, which was associated with increased HDAC2 protein levels and deficits in histone acetylation and NPY expression in the central (CeA) and medial nucleus of amygdala (MeA), as compared to NP rats. TSA treatment attenuated the anxiety-like and alcohol-drinking behaviours, with concomitant reductions in amygdaloid nuclear, but not cytosolic HDAC activity, and HDAC2, but not HDAC4, protein levels in the CeA and MeA of P rats, without effect in NP rats. TSA treatment also increased global histone acetylation (H3-K9 and H4-K8) and NPY expression in the CeA and MeA of P, but not in NP rats. Histone H3 acetylation within the NPY promoter was also innately lower in the amygdala of P rats compared with NP rats; which was normalized by TSA treatment. Voluntary ethanol intake in P, but not NP rats, produced anxiolytic effects and decreased the HDAC2 levels and increased histone acetylation in the CeA and MeA. These results suggest that higher HDAC2 expression-related deficits in histone acetylation may be involved in lower NPY expression in the amygdala of P rats, and operative in controlling anxiety-like and alcohol-drinking behaviours.

Introduction
Both genetic and environmental factors play important roles in the development of alcoholism (Cloninger, 1987; Heath et al., 1997; Enoch and Goldman, 2001). Anxiety disorders have been found to be potential risk factors for the development of alcohol-use disorders, often co-morbid together (Schuckit and Hesselbrock, 1994; Kushner et al., 2000; Robinson et al., 2009). Acute alcohol exposure produces anxiolytic effects, whereas withdrawal after protracted exposure leads to the development of anxiety-like behaviours (Koob, 2003; Pandey, 2003; Moberg and Curtin, 2009). Several animal models have been developed to study the genetic and neurobiological basis of alcoholism (McBride and Li, 1998). One such animal model is the selectively bred alcohol-prefering (P) and non-prefering (NP) rats who display higher and lower alcohol preference, respectively (Li et al., 1993; Murphy et al., 2002). In addition, P rats also display heightened anxiety-like behaviours as compared to NP rats (Stewart et al., 1993; Pandey et al., 2005; Gilpin et al., 2011; Moonat et al., 2011). Studies have implicated the role of neuropeptide Y (NPY), corticotrophin releasing factor (CRF) and brain-derived neurotrophic factor (BDNF), in alcoholism and co-morbid anxiety disorders (Koob, 2003; Pandey et al., 2003; Heilig and Koob, 2007; Jeanblanc et al., 2009; Zhang et al., 2010; Moonat et al., 2011). Furthermore, deficits in NPY levels have been reported in various brain regions, including the amygdala, of P rats compared with NP rats (Ehlers et al., 1998; Hwang et al., 1999, 2004a; Suzuki et al., 2004; Pandey et al., 2005). Interestingly, when NPY was infused i.c.v.
or directly into the central nucleus of amygdala (CeA) of P rats, it significantly attenuated anxiety-like and alcohol-drinking behaviours, suggesting the involvement of NPY in these behaviours (Badia-Elder et al., 2001; Pandey et al., 2005; Zhang et al., 2010; Gilpin et al., 2011).

Epigenetic mechanisms, such as histone acetylation and DNA methylation, dynamically regulate gene transcription and are implicated in the aetiologies of psychiatric disorders including addictive behaviours (Abel and Zukin, 2008; Pandey et al., 2008; Robison and Nestler, 2011; Romieu et al., 2011; Sakhkhar et al., 2012). Histone deacetylases (HDAC) remove acetyl groups from histone tails condensing the chromatin state that regulates gene transcription (Jenuwein and Allis, 2001; de Ruijter et al., 2003; Thiagalingam et al., 2003; Abel and Zukin, 2008). HDAC-induced histone modifications have been shown to be involved in the pathophysiology of neurodegenerative and psychiatric disorders leading to the emergence of HDAC inhibitors as promising therapeutic agents (Tsankova et al., 2007; Renthal and Nestler, 2008; Grayson et al., 2010). The amygdaloid brain circuitry, particularly CeA has been shown to serve as a neuroanatomical substrate for negative reinforcement mechanisms associated with development of alcoholism (Koob, 2003; Koob and Volkow, 2010). We have recently identified a role for amygdaloid HDAC-induced histone deacetylation in rapid tolerance to the anxiolytic effects of ethanol (Sakharkar et al., 2012) and development of anxiety-like behaviours during withdrawal after chronic ethanol exposure (Pandey et al., 2008) in Sprague-Dawley rats. More recently, it was found that protein levels of HDAC2, but not HDAC1, 3, 4, 5 isoforms, were innately higher in the CeA and medial nucleus of amygdala (MeA) of P as compared with NP rats. This was associated with deficits in histone H3 acetylation (H3-K9) but not H3-K14 acetylation and lower dendritic spines in the CeA and MeA, and in regulating anxiety-like and alcohol-drinking behaviours in P rats (Sakharkar et al., 2012).

We therefore investigated the effects of histone acetylation and DNA methylation, dynamically regulate gene transcription and are implicated in the aetiologies of psychiatric disorders including addictive behaviours (Abel and Zukin, 2008; Pandey et al., 2008; Robison and Nestler, 2011; Romieu et al., 2011; Sakhkhar et al., 2012). Histone deacetylases (HDAC) remove acetyl groups from histone tails condensing the chromatin state that regulates gene transcription (Jenuwein and Allis, 2001; de Ruijter et al., 2003; Thiagalingam et al., 2003; Abel and Zukin, 2008). HDAC-induced histone modifications have been shown to be involved in the pathophysiology of neurodegenerative and psychiatric disorders leading to the emergence of HDAC inhibitors as promising therapeutic agents (Tsankova et al., 2007; Renthal and Nestler, 2008; Grayson et al., 2010). The amygdaloid brain circuitry, particularly CeA has been shown to serve as a neuroanatomical substrate for negative reinforcement mechanisms associated with development of alcoholism (Koob, 2003; Koob and Volkow, 2010). We have recently identified a role for amygdaloid HDAC-induced histone deacetylation in rapid tolerance to the anxiolytic effects of ethanol (Sakharkar et al., 2012) and development of anxiety-like behaviours during withdrawal after chronic ethanol exposure (Pandey et al., 2008) in Sprague-Dawley rats. More recently, it was found that protein levels of HDAC2, but not HDAC1, 3, 4, 5 isoforms, were innately higher in the CeA and medial nucleus of amygdala (MeA) of P as compared with NP rats. This was associated with deficits in histone H3 acetylation (H3-K9) but not H3-K14 acetylation and lower dendritic spines in the CeA and MeA, and in regulating anxiety-like and alcohol-drinking behaviours in P rats (Sakharkar et al., 2012).

However, the effects of HDAC inhibitors on HDAC2-induced histone deacetylation globally, specifically within the NPY gene promoter, and subsequent effects on NPY expression in the amygdala of P and NP rats in relation to anxiety-like and alcohol-drinking behaviours are still unclear.

We therefore investigated the effects of trichostatin A (TSA), a pan HDAC inhibitor, on the expression of HDAC2 and 4 isoforms and HDAC activity, histone acetylation (global and NPY gene-specific), as well as on NPY gene expression in the amygdala of P and NP rats. We measured the protein levels of HDAC2 and HDAC4 isoforms, as representatives of class I and class II HDACs with nuclear and cytosolic expression patterns (Konsoula and Barile, 2012). We also examined the effects of TSA on anxiety-like and alcohol-drinking behaviours in P and NP rats. We recently have shown that acute ethanol decreases HDAC2 expression and increases histone acetylation (H3-K9) in the amygdaloid structure of P rats, but not in NP rats (Moonat et al., 2013). However, the effects of long-term voluntary alcohol consumption on HDAC2 isoform expression and histone acetylation are currently unknown. We therefore examined the effects of voluntary long-term ethanol consumption on anxiety-like behaviours, HDAC2 expression and related histone (H3-K9 & H4-K8) acetylation levels in the amygdala of P and NP rats.

**Method**

**Animals**

Adult male alcohol naïve P and NP rats were received from the Alcohol Research Centre (Indiana University, USA). Animals weighing between 290–360 g were used in all experiments and randomly assigned to various experimental groups. Animals were housed in a temperature-, light-, and humidity-controlled facility under a 12 h normal light/dark cycle, with food and water ad libitum. All experiments were performed in accordance with the National Institute of Health’s ‘Guidelines for the Care and Use of Laboratory Animals’ and were approved by the Institutional Animal Care and Use Committee.

**Effect of TSA on anxiety-like behaviours and epigenetic changes in the amygdala of P and NP rats**

Alcohol naïve P and NP rats were injected i.p. with either vehicle (5 ml/kg; DMSO diluted in PBS at 1:5 dilution; [P (Vehicle) or NP (Vehicle)], or TSA [2 mg/kg; P (TSA) or NP (TSA)] once daily for three days. TSA was prepared as described in our previous publications (Pandey et al., 2008; Sakhkhar et al., 2012). Two hours after the last TSA injection, P and NP rats were subjected to the behavioural measurements during the light phase of a normal light/dark cycle using the elevated plus-maze (EPM; n=13) or the light/dark box (LDB) exploration (n=12–13) tests as described below. Immediately after the behavioural measurements, rats were anesthetized with pentobarbital (50 mg/kg, i.p.), and brains were collected for the epigenetic measures described below. The dose of TSA was based on our previous publications indicating that this dose is effective in attenuating anxiety-like behaviours and correcting the deficits in histone acetylation and NPY expression in the amygdala during ethanol withdrawal after chronic ethanol exposure in rats (Pandey et al., 2008; You et al., 2013).

**Effect of TSA on alcohol-drinking behaviours in P and NP rats**

Ethanol consumption of alcohol-naïve P and NP rats was measured using the two-bottle free choice paradigm as described previously (Pandey et al., 2005; Zhang et al., 2010; Moonat et al., 2013). After habituation to drink water equally from two bottles, P and NP rats were given one bottle of water and another bottle of 7% ethanol.
(V/V diluted in tap water) for three days, followed by 9% ethanol for seven days and referred as P(W/E) and NP(W/E) rats. During the last three days of 9% ethanol drinking, these rats were i.p. injected daily (between 17:00-18:00 hr) with either vehicle and referred to as [P(W/E+ Vehicle); (n=7)] and [NP(W/E+ Vehicle); (n=6)] or TSA (2 mg/kg) and referred to as [P(W/E+TSA); (n=7)] and [NP(W/E+TSA); (n=6)]. Ethanol intake was calculated as g/kg/d.

The two-bottle free choice paradigm was employed as described above to examine the effects of voluntary ethanol drinking on the anxiety-like behaviours (n=7–9) and HDAC2 expression and histone acetylation in the amygdala of P and NP rats. Control P and NP rats were given the water in both the bottles [P (W/W) and NP (W/W)] whereas; ethanol-drinking groups received one bottle of water and the other of ethanol [P (W/E) and NP (W/E)]. After drinking of 7% ethanol for three days and 9% ethanol for seven days, animals were subjected to anxiety measurements (without ethanol withdrawal) using the LDB test. These rats were immediately anesthetized with pentobarbital and then perfused for immunohistochemistry as described by us previously (Zhang et al., 2010; Moonat et al., 2011). In all experiments, blood was collected to determine blood ethanol levels using the Analox alcohol analyser (Analox Instruments, USA) prior to perfusion and brain dissection.

**LDB exploration test**

The LDB exploration test procedure was performed as described previously by us (Pandey et al., 2008; Sakhrkark et al., 2012; Moonat et al., 2013). In brief, the rats were allowed to acclimate in the procedure room for 5 min prior to placement into the dark compartment facing away from the opening that connects to the light compartment of the light/dark box apparatus. The activity of the rats in each compartment (equipped with infrared beam sensors) was assessed by a computer for the measurement of time spent and ambulation during the 5-min test session. The results were represented as mean± S.E.M of the number of immunogold particles/100 μm² area for each amygdaloid brain region.

**EPM exploration test**

The EPM test was performed as previously described (File, 1993; Pandey et al., 2008; Sakhrkark et al., 2012). Rats were acclimated to the procedure room for 5 min prior to measurement of anxiety-like behaviours in the EPM apparatus. The exploration activity of the rats in terms of number of entries and the time spent on the open or closed arms was recorded for the 5-min test session. The results are represented in terms of number of entries and the time spent on the open arms, and the number of closed arm entries (±S.E.M).

**HDAC activity in the amygdala**

The nuclear and cytosolic fractions from the amygdaloid and cortex tissues were prepared using a nuclear extraction kit (Sigma, USA) and HDAC activity were measured, as described by us previously (Pandey et al., 2008; Sakhrkark et al., 2012; Moonat et al., 2013). HDAC activity was assayed using the colorimetric HDAC activity assay kit (BioVision Research, USA) and the results are represented as the mean optical density (OD)/mg protein.

**Gold immunolabelling of HDAC2, HDAC4, acetylated histones (H3-K9 and H4-K8) and neuropeptide Y**

The gold-immunolabelling histochemical procedure described previously (Pandey et al., 2008; Sakhrkark et al., 2012; Moonat et al., 2013) was employed using antibodies against HDAC2 (1:100 dilution, MBL International, USA) and HDAC4 (1:100 dilution, MBL International, USA), acetylated histone H3-K9 (1:500 dilution, Millipore, USA), acetylated histone H4-K8 (1:500 dilution, Millipore, USA) and NPY (1:500 dilution, Immunostar, USA). Gold immunolabelling was quantified using an image analysis system (Loats Associates, USA) at 100× magnification and the results were represented as mean±S.E.M of the number of immunogold particles/100 μm² area for each amygdaloid brain region.

**In situ RT-PCR for neuropeptide Y mRNA**

NPY mRNA levels were determined using in situ RT-PCR as previously described (Pandey et al., 2008; Zhang et al., 2010) using the following primers for NPY (Primers 5'-TAGTAAACAAACGAATGGG-3' and 5'-AGGATGAGATGAGATGTTG-3'). Following PCR cycling, sections were mounted on slides, incubated with alkaline phosphatase-conjugated anti-DIG antibody (1:200 dilution), and stained with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (Roche Diagnostics, USA). NPY mRNA levels were quantified by calculation of optical density using Image Analyser and the results were represented as mean±S.E.M of the OD/100 pixels of area.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assay was performed using ChIP-IT express kit (Active Motif, USA) using antibodies against anti-acetylated histone H3-K9/14 antibody (Millipore, USA) as described by us previously (Moonat et al., 2013). Following immunoprecipitation, DNA fragments were isolated and were quantified using real-time PCR (SYBR green) using primers designed within the promoter region for NPY and GAPDH. The primer sequences were as follows: NPY, Forward-5'-AGTAGTGCTCCAGTGGCATGAGT-3'; Reverse-5'-GAAGCAGTCGCAAGTTT-3'; GAPDH, Forward-5'-TCCCCGTTCTCCTGAGCT-3'; Reverse-5'-CCAGACCCGAAACCAGAA. The levels of acetylated
histone H3-K9/14 within the NPY gene promoter in the amygdala of vehicle- or TSA-treated P and NP rats was calculated using the \( \Delta \Delta \) method (Schmittgen and Livak, 2008). The \( c(t) \) value of NPY was corrected with \( c(t) \) value of GAPDH of respective group. The \( \Delta \Delta c(t) \) values were calculated for each group by subtracting from the \( \Delta c(t) \) of NP (Vehicle) group and the respective fold changes were calculated as \( 2^{-\Delta \Delta c(t)} \).

**Confocal microscopy for the localisation of HDAC2 in neurons (NeuN) and astrocytes (GFAP) in amygdala**

The double immunofluorescence staining, as previously described by us (Zhang et al., 2010; Sakharkar et al., 2012), was performed using the antibodies against HDAC2, NeuN (Millipore, USA), or GFAP (Millipore, USA). The neuronal or astroglial co-localisation with HDAC2, NeuN (Millipore, USA), or GFAP (Millipore, USA) was examined using confocal microscopy.

**Statistical analyses**

The differences between the groups were evaluated by a one-way or two-way analysis of variance (ANOVA) followed by post-hoc comparisons using Tukey’s test. A value of \( p<0.05 \) was considered to be significant.

**Results**

**Effects of TSA on the anxiety-like behaviour in P and NP rats**

In agreement with previous reports from our lab, (Pandey et al., 2005; Moonat et al., 2011, 2013), P rats were found to display anxious-like behaviours as compared to NP rats, as measured by the LDB (Fig. 1a) and EPM (Fig. 1b) exploration tests. As compared to the NP rats, P rats spent significantly more time \( p<0.001 \) in the dark compartment and less time in the light compartment of LDB. Similarly, P rats also spent less time in the open arms \( p<0.001 \) with concomitant less per cent of open arm entries \( p<0.001 \) in the EPM test compared to NP rats (Fig. 1b). We also observed that TSA treatment produced anxiolytic effects in P, but not in NP rats. It was found that TSA treatment significantly decreased \( p<0.001 \) the time spent in the dark compartment by the P rats as compared to the vehicle-treated P rats, with concomitant increase \( p<0.001 \) in time spent in the light compartment (Fig. 1a). Likewise, TSA-treated P rats showed more entries and also spent more time in the open arms of the EPM, as compared to the vehicle-treated P rats (Fig. 1b). However, TSA treatment did not affect the behaviour measures in NP rats in either apparatus. In addition, TSA treatment did not produce any change in general activity of P and NP rats, as measured by total ambulations in LDB \( \text{Mean}\pm\text{S.E.M of total ambulations} \ (n=12-13); \text{P(Vehicle)}=596\pm32.2, \text{P(TSA)}=592\pm26.5 \), NP\((\text{Vehicle})=571\pm34.7, \text{NP} \ (\text{TSA})=523\pm33.5\) and total closed arm entries in the EPM test (Fig. 1b).

**Effects of TSA on alcohol-drinking behaviour in P and NP rats**

As described previously by us (Pandey et al., 2005; Zhang et al., 2010) and others (Li et al., 1993; Gilpin et al., 2008), P rats have an innately higher alcohol preference as compared to NP rats (Fig. 1c). We examined the effects of TSA on the alcohol intake in P and NP rats during the last three days of 9% alcohol intake and found that TSA treatment significantly attenuated \( p<0.001 \) the alcohol-drinking in P but not in NP rats (Fig. 1c). The alcohol drinking pattern observed in TSA-treated P rats \( \text{P(W/E+TSA) group} \) significantly decreased to 2.72±0.12 g/kg/day 9% ethanol for the last three days during TSA treatment, as compared to vehicle-treated P rats \( \text{P(W/E+Vehicle) group} \) (6.85±0.13 g/kg/day)]. Blood alcohol levels \( \text{Mean}\pm\text{S.E.M} \) in P (W/E+Vehicle) group was 173±5.3 mg% and in P (W/E+TSA) group was 75±2.7 mg%.

TSA treatment did not affect the alcohol-drinking pattern in the NP rats. Similarly, no significant differences were observed in the blood ethanol levels of the NP rats treated with either vehicle or TSA \( \text{NP (W/E+Vehicle) group} \) (34±3.1 and NP (W/E+TSA) group: 35±2.0). The values of blood ethanol levels in P and NP rats (vehicle group) reported here are consistent with several other reports in the literature (McBride and Li, 1998; Pandey et al., 2005; Bell et al., 2006; Gilpin et al., 2008; Zhang et al., 2010). Regression analysis indicated a significant \( p<0.0001 \) positive correlation between last day of 9% ethanol intake and blood ethanol levels in P and NP rats treated with vehicle (Fig 1d). In addition, a significant \( p<0.0001 \) positive correlation was also observed between last day of 9% ethanol intake with blood ethanol levels in P rats treated with vehicle or TSA (Fig. 1e). No significant difference in the total fluid intake was observed between P and NP rats treated with either vehicle or TSA (data not shown). These results suggest that TSA is able to attenuate ethanol intake in P, but not in NP rats.

**Effects of TSA on the HDAC activity and HDAC protein expression**

HDAC activity was measured in the nuclear and cytosolic fractions of the amygdala and cortex of P and NP rats \( n=6 \), treated with or without TSA (Fig. 2a). As reported by us earlier (Moonat et al., 2013), here we also observed significantly higher \( p<0.01 \) baseline levels of nuclear, but not cytosolic HDAC activity in the amygdala of P rats as compared to NP rats. In addition, it was found that TSA treatment inhibited \( p<0.001 \) the nuclear, but not cytosolic, HDAC activity in the amygdala of P rats (Fig. 2a); however, TSA had no effect on nuclear or cytosolic HDAC activity in the amygdala of NP rats.
No baseline differences were found in both nuclear and cytosolic HDAC activities in the cortex of P and NP rats. TSA had no effect on the HDAC activity in either nuclear or cytosolic fractions of cortex in P or NP rats (Fig. 2a).

We next examined whether decreased nuclear HDAC activity by TSA may be related to changes in the expression of specific HDAC isoforms in the amygdala. We employed the gold immunolabelling procedure to
Fig. 2. (a) Bar diagrams showing the effects of TSA on the HDAC activity [optical density (OD)/mg protein] in the nuclear and cytosolic fractions of the amygdala and cortex. Values are the mean±s.e.m of 6 rats per group. *Significantly (p<0.01–0.001) different from other groups [ANOVA (F3,20 = 10.4, p<0.001) followed by Tukey’s test]. (b) Representative low-magnification photomicrographs of HDAC2 and HDAC4 gold-immunolabelling in central nucleus of amygdala (CeA) of P and NP rats treated with either vehicle [P (Vehicle) and NP (Vehicle)] or TSA [P(TSA) and NP(TSA)] (Scale bar=50 μM). (c) Bar diagrams showing the changes in the protein levels of HDAC2 and HDAC4 in various amygdaloid (CeA, MeA, and BLA) structures of P and NP rats treated with either vehicle or TSA. Values are the mean±s.e.m of 5 rats per group. *Significantly (p<0.001) different from other groups [ANOVA (HDAC2: CeA, F3,16 = 340.7, p<0.001; MeA, F3,16 = 256.8, p<0.001) followed by Tukey’s test]. (d) Representative photomicrographs showing immunofluorescence staining of HDAC2 (green) or NeuN (red), and GFAP (red) in the cells of the central nucleus of amygdala (CeA). The yellow colour represents localisation of HDAC2 in NeuN-positive and GFAP-positive cells. HDAC2 are predominantly expressed in NeuN-positive cells. Scale bar=50 μM.
measure the protein levels of HDAC2 and HDAC4 isoforms. We found that the HDAC2 protein levels in the CeA and MeA, but not BLA, of the P rats were significantly higher (p<0.001) than in the NP rats (Fig. 2b, c). Interestingly, TSA treatment significantly decreased (p<0.001) the HDAC2 protein levels in CeA and MeA of the P rats, but not in NP rats. However, neither baseline differences were observed in the HDAC4 levels, nor did TSA affected HDAC4 protein expression in the amygdala of P and NP rats (Fig. 2b, c). These results suggest that TSA is able to inhibit the nuclear HDAC activity most likely due to a decrease in the HDAC2 protein expression in the amygdala of P, but not in NP rats.

**Double-immunofluorescence labelling of HDAC2 with NeuN and GFAP**

Double-immunofluorescence labelling was performed in order to determine neuronal or astrocytic pattern of localisation of HDAC2 in the amygdala of the P and NP rats (n=3). We observed that the HDAC2 was predominantly localised in the neurons, since it was highly co-localised in the NeuN-positive cells in the CeA of P and NP rats (Fig. 2d). Only few GFAP positive cells were expressed with HDAC2 as shown in Fig. 2d. A similar pattern of localisation was also observed in the MeA and BLA of P and NP rats (data not shown).

**Effects of TSA on the histone acetylation globally (H3-K9 and H4-K8 levels) and at the NPY gene promoter**

We have shown earlier that baseline H3-K9 acetylation, but not H3-K14, was lower in the CeA and MeA of P rats compared to NP rats (Moonat et al., 2013). We extended these studies and examined the effects of TSA on the histone H3-K9 and H4-K8 acetylation in the amygdala of P and NP rats (n=6). Acetylation levels of H3-K9 and H4-K8 in the CeA and MeA, but not in the BLA, of P rats was significantly lower (p<0.001) as compared to that in the NP rats (Fig. 3a, b). TSA treatment significantly (p<0.001) increased H3-K9 and H4-K8 acetylation levels in the CeA and MeA of P rats without modulating in NP rats.

In order to examine the histone acetylation levels specifically at the promoter of the NPY gene in the amygdala of P and NP rats (n=7) with and without TSA treatment, we performed ChIP assay using the antibody against acetylated histone H3-K9/14 (Fig. 3c). We found significantly (p<0.001) lower levels of histone H3 acetylation (H3-K9/14) within the NPY gene promoter in the amygdala of the P rats, as compared to NP rats (Fig. 3a, b); moreover, the TSA treatment corrected H3 acetylation deficits (p<0.001) (Fig. 3c). However, similar to global acetylated histone H3 levels, acetylation of the H3-K9/14 at the NPY gene promoter was not changed in the amygdala of NP rats after the TSA treatment.

**Effects of TSA on the neuropeptide Y expression**

To establish the link between the histone acetylation of NPY gene promoter with NPY expression, we examined the NPY protein and mRNA levels in the amygdala of P and NP rats (n=6) treated with or without TSA (Fig. 4a, b). In conjunction with our previous findings (Pandey et al., 2005), NPY protein and mRNA levels are significantly lower (p<0.001) in the CeA and MeA, but not the BLA of P rats, as compared to NP rats. Here, we found that TSA treatment was able to normalise the deficits in NPY expression in CeA and MeA of the P rats without producing any change in NP rats (Fig. 4a, b). The results of histone acetylation at the promoter of the NPY gene tightly correlate with the NPY expression levels, in terms of baseline differences and also after the TSA treatment, suggestive of a role for histone acetylation in the regulation of NPY gene expression in the amygdala of P rats.

**Effect of voluntary alcohol consumption on anxiety-like behaviours, HDAC2 and acetylated H3-K9 and H4-K8 protein levels**

As previously reported by us (Pandey et al., 2005; Moonat et al., 2011, 2013) and as described above, P rats display innate anxiety-like behaviours as compared to NP rats (Fig. 5a), which were significantly (p<0.001) reduced following ethanol drinking as measured by the LDB test (Fig. 5a). P rats consuming high amounts of ethanol spent significantly less time in the dark compartment and more time in the light compartment as compared to the control P rats (drinking water only). The small amounts of alcohol voluntarily consumed by the NP rats did not affect LDB measures (Fig. 5a).

Voluntary ethanol consumption did not produce any significant change in general activity (as measured by the total ambulations) of P and NP rats [Mean±S.E.M total ambulations (n=7–9); P (W/W)=466±51.4, P (W/E)=493±52.3, NP (W/W)=533±78.1, NP (W/E)=579±21.3].

These results suggest that voluntary ethanol intake reduced the anxiety-like behaviours in P rats. These results are similar to previous studies of voluntary ethanol drinking-induced attenuation of anxiety-like behaviours measured by the EPM test in P rats (Pandey et al., 2005).

We also examined the effects of voluntary ethanol intake (without ethanol withdrawal) on HDAC2 protein levels and acetylation of histones H3-K9 and H4-K8 (Fig. 5b). As described above and reported earlier (Moonat et al., 2013), we have observed higher (p<0.001) baseline protein levels of HDAC2 and deficits in the histone H3-K9 and H4-K8 acetylation in the CeA and MeA, but not the BLA of the P rats, as compared to NP rats. However, voluntary ethanol drinking significantly reduced (p<0.001) the HDAC2 protein levels and concomitantly increased (p<0.05–0.001) histone acetylation (H3-K9 and H4-K8) in the CeA and MeA of the
Fig. 3. (a) Representative low-magnification photomicrographs of acetylated histones H3-K9 or H4-K8 gold-immunolabelling in central nucleus of amygdala (CeA) of P and NP rats treated with either vehicle [P (Vehicle) and NP(Vehicle)] or TSA [P(TSA) and NP(TSA)] (Scale bar=50μm). (b) Bar diagrams showing the changes in the acetylation levels of histones H3-K9 and H4-K8 in various amygdaloid (CeA, MeA, and BLA) structures of P and NP rats treated with either vehicle or TSA. Values are the mean±S.E.M of 6 rats per group. *Significantly (p<0.001) different from other groups [ANOVA (acetylated H3-K9: CeA, F(3,20)=84.1, p<0.001; MeA, F(3,20)=48.3, p<0.001 and acetylated H4-K8: CeA, F(3,20)=40.7, p<0.001; MeA, F(3,20)=55.9, p<0.001) followed by Tukey’s test]. (c) Bar diagram showing the fold changes in the histone acetylation (H3-K9/14) at the promoter of the NPY gene in the amygdala of P and NP rats as measured by the chromatin immunoprecipitation assay followed by quantitative real-time PCR. Values are the mean±S.E.M of 7 rats per group. *Significantly (p<0.001) different from other group [ANOVA (F(3, 24)=16.1, p<0.001) followed by Tukey’s test].
However, voluntary lower ethanol consumption neither affected the HDAC2 levels, nor did it change the histone acetylation in the amygdala of NP rats (Fig. 5b).

Discussion

This study showed that the HDAC inhibitor TSA was able to attenuate both anxiety-like and alcohol-drinking behaviours of P rats and correct the deficits in histone acetylation and NPY expression due to inhibition of nuclear HDAC activity in the amygdala of P rats, as compared to NP rats. We believe TSA was able to produce these changes via decreasing the protein levels of HDAC2 in the CeA and MeA of P rats. Furthermore, decreases in HDAC2 protein levels and nuclear HDAC activity after TSA treatment was associated with an increase in the global and NPY gene specific histone acetylation in the amygdala of P rats. These results implicate HDAC2-mediated histone deacetylation as a potential epigenetic mechanism for the regulation of NPY in the amygdala of P rats. These epigenetic events in the CeA and MeA of P rats may play an important role in controlling anxiety-like and excessive alcohol-drinking behaviours that could be treated by HDAC inhibitors.

Recently, the acetylation state of histones has emerged as a potential epigenetic mechanism in coordinating the effects of alcohol in the brain as well as related behaviours (Wang et al., 2007; Pandey et al., 2008; Pascual et al., 2012; Sakharkar et al., 2012; Arora et al., 2013; Moonat et al., 2013; Warnault et al., 2013). We recently reported that intrinsically higher levels of HDAC2 in the CeA and MeA of P rats was reciprocally correlated with deficits in histone H3 acetylation levels of synaptic plasticity-related genes,
e.g. BDNF and activity-regulated cytoskeleton associated (Arc) protein, and specific knock-down of HDAC2 expression in the CeA of P rats by siRNA infusion corrected these deficits and attenuated anxiety-like and alcohol-drinking behaviours (Moonat et al., 2013). Here, we extended these studies and found that TSA treatment inhibited nuclear HDAC activity, decreased HDAC2 expression with a concomitant increase in histone H3 acetylation of the NPY gene leading to the up-regulation of NPY expression and attenuation of anxiety-like behaviours and excessive alcohol intake in P rats without modulating these measures in NP rats. We have previously shown that acute ethanol exposure of an unselected stock of rats (Sprague-Dawley) inhibited HDAC activity and increased histone H3-K9 and H4-K8 acetylation, which were well correlated with enhanced NPY expression in the CeA and MeA and its anxiolytic-like effects (Pandey et al., 2008). Conversely, the withdrawal from chronic ethanol exposure resulted in increased HDAC activity, decreased acetylated histone H3-K9 and H4-K8 levels and NPY expression, further leading to anxiety-like behaviours (Pandey et al., 2008). Interestingly, treatment with TSA reversed these biochemical and behavioural effects of ethanol withdrawal. TSA treatment similar to control Sprague-Dawley rats (Pandey et al., 2008; You et al., 2013) does not produce anxiolytic-like effects and changes in histone acetylation in the amygdala of NP rats. In yet another study, we found that the development of rapid ethanol tolerance (RET) to the anxiolytic effects of acute ethanol exposure was associated with decreased sensitivity of HDAC following acute ethanol exposure, because the TSA treatment normalised the decreased response of HDAC to the ethanol and subsequently reversed the development of RET (Sakharkar et al., 2012). Recently, Warnault et al. (2013) have reported that treatment with a variety of pan
HDAC inhibitors, i.e. suberoylanilide hydroxamic acid (SAHA), MS275 and TSA, is able to attenuate alcohol-drinking behaviours in rats and mice. Together, these studies highlight the importance of HDAC-driven epigenetic mechanisms in alcohol preference, tolerance and dependence in several preclinical models.

HDACs have been classified in four different classes (HDAC I-IV) depending on their cellular localization, mechanisms of actions and functions. While class I HDACs (HDAC 1-3, 8) are mostly nuclear, class II HDACs (HDAC 4-7,9,10) are accessible in cytosol and/or the nucleus. HDAC 11 is a class IV HDAC and prominently localised in the nucleus (de Ruijter et al., 2003; Thiagalingam et al., 2003). In our previous study, we found that up-regulation of nuclear HDAC activity is due to higher expression of the HDAC2 isoforms but not HDAC1, 3, 4 and 5, a finding which was associated with lower histone H3-K9 but not H3-K14 acetylation in the amygdala of P rats as compared to NP rats (Moonat et al., 2013). Here, we further confirmed that innately higher expression of the HDAC2 isoform in P rats might underlie the elevated nuclear HDAC activity in the amygdala as compared with NP rats. Because we did not see changes in HDAC4, or in cytosolic HDAC activity by TSA treatment, this further suggests that higher HDAC2 expression might be contributing to the lower global histone acetylation and H3 acetylation at the NPY promoter in the P rats, as compared to NP rats. This notion was further supported by the observation that TSA treatment inhibited HDAC activity and down-regulated HDAC2 expression, thereby correcting the deficits in histone acetylation globally, and also at the NPY promoter in attenuating the anxiety-like and alcohol-prefering behaviours in P rats. Interestingly, we also found that the chronic voluntary ethanol drinking by P rats decreased the HDAC2 expression and increased histone acetylation in the CeA and MeA with a parallel decline in the anxiety-like behaviours. Similarly, acute i.p. treatment with ethanol (1 g/kg) produced anxiolytic effects and decreased HDAC2 levels and increased histone H3-K9 acetylation in the CeA and MeA of P rats but not in NP rats (Moonat et al., 2013). It is important to point out that TSA may have several other cellular effects such as acetylation of non-histone proteins (Spange et al., 2009). However, TSA (2 mg/kg) is effective in increasing histone acetylation under experimental conditions where HDAC2 or HDAC activity is increased in the amygdala, such as in P rats or ethanol-withdrawn Sprague-Dawley rats (Pandey et al., 2008). A similar dose of TSA is not effective in altering anxiety measures or histone acetylation in the amygdala of NP or control Sprague-Dawley rats. These results suggest the possibility that the dose of TSA used here has a high affinity for HDAC2 in the brain regions where they were highly expressed such as the CeA and MeA of P, but not NP, rats and is therefore able to modulate HDAC activity and histone acetylation in the amygdala of P, but not in NP rats. Taken together, these studies clearly suggest that an innately higher expression of HDAC2 in the CeA and MeA of P rats plays a major role in the anxiety-like and excessive alcohol-drinking behaviours of P rats.

Recent research advocates the role of different HDAC isoforms in the actions of other drugs of abuse and psychiatric disorders (Tsankova et al., 2006; Ding et al., 2008; Covington et al., 2009; Guan et al., 2009; Kilgore et al., 2010; McQuown et al., 2011; Fukada et al., 2012). Guan et al. (2009) reported that HDAC2, but not HDAC1, is involved in regulating synaptic plasticity via altering the gene expression, which is believed to be crucial for dendritic spine density, synapse formation and associated learning and memory behaviour. Similarly, alterations in HDAC2 were recently demonstrated to be an underlying cause of epigenetic blockade in transcription of genes vital for synaptic plasticity in two mouse models of neurodegeneration and in patients with Alzheimer's disease (Gräff et al., 2012). These studies have shown that the inhibition of HDAC2 by pharmacological manipulation (e.g. SAHA) or by short-hairpin-RNA-mediated knockdown reversed the behavioural impacts by modulating underlying gene transcription. Here, we observed that HDAC2 is predominantly expressed in the neuronal cells of the amygdala of P and NP rats, which is similar to the neuronal cell-type specific HDAC2 expression in the rat brain (Broido et al., 2007). Interestingly, as reported earlier, the HDAC2 knockdown in the CeA of P rats increased the levels of synthetically active proteins i.e. BDNF and Arc, and is associated with an increase in dendritic spine density (Moonat et al., 2013). Taken together, these studies suggest a role of specific HDAC isoforms in articulating diverse gene transcriptions underlying synaptic plasticity of brain disorders including alcoholism.

For several years, NPY in the amygdala has been implicated in the mechanisms of anxiety disorders and alcoholism, using various animal models and treatment paradigms (Pandey, 2003; Thiele and Badia-Elder, 2003; Heilig, 2004; Spence et al., 2009). The deficits in NPY levels in the amygdala of P rats as compared to NP rats have been reported (Hwang et al., 1999, 2004a; Suzuki et al., 2004). Our previous studies in P rats have shown that a deficiency in NPY in the CeA may be involved in regulating anxiety-like and excessive alcohol-drinking behaviours of P rats (Pandey et al., 2005; Zhang et al., 2010). Besides NPY, other important players such as CRF, BDNF and Arc levels have been shown to be lower in the CeA and MeA of P rats as compared with NP rats (Hwang et al., 2004b; Moonat et al., 2011). Here, we extended these studies and provided evidence that the deficits in histone acetylation due to higher expression of HDAC2 may be epigenetically regulating the expression of NPY in the CeA and MeA of P rats as compared with NP rats. Interestingly, innately higher expression of HDAC2 in the CeA and MeA of P rats was down-regulated by voluntary ethanol consumption,
concurrently increasing histone acetylation, which is similar to our previous findings of acute ethanol exposure in P rats (Moonat et al., 2013). Voluntary ethanol consumption and acute ethanol injections in NP rats were not able to modify HDAC activity, HDAC2 levels and histone acetylation in the amygdala. Interestingly, acute ethanol (1 g/kg) and TSA injections produced anxiolytic-like effects in several behavioural tests of anxiety in P, but not in NP rats (Pandey et al., 2005; Moonat et al., 2011, 2013). The lack of behavioural and epigenetic effects of these treatments may be related to a ‘ceiling effect’ in NP rats. As discussed above, TSA treatment purged down the HDAC2 levels, inhibited HDAC activity, augmented global and NPY gene specific histone acetylation as well as NPY expression, and these events were correlated with the reduction in anxiety-like and alcohol-preferring behaviour of P rats as compared with NP rats. This is further supported by the fact that the i.c.v or CeA infusion of NPY attenuated the alcohol-drinking and anxiety-like behaviours in the P rats (Badia-Elder et al., 2001; Pandey et al., 2005; Zhang et al., 2010; Gilpin et al., 2011). The data collected here suggests that epigenetic regulation of NPY expression in the amygdala contributes to anxiety-like and alcohol-drinking behaviours of P rats.

In conclusion, the present study highlights that higher expression of HDAC2 may drive the restricted state of chromatin within the NPY gene causing decreased expression in the CeA and MeA of P rats as compared with NP rats. Since aberrant chromatin remodelling in amygdaloid structures may be corrected by treatment with HDAC inhibitors or specific knockdown of HDAC2 in the CeA (Moonat et al., 2013) resulting in the attenuation of anxiety-like and alcohol-drinking behaviours, we suggest that inhibition of HDAC2 may serve as a potential therapeutic target for developing the future treatment of anxiety and alcoholism.

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Conflict of Interest
SCP reports that a US patent application on a related topic (serial number 60/848237 filed on September 29th, 2006) is currently pending. All other authors reported no biomedical financial interests or potential conflicts of interest.

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