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Histone deacetylase inhibition decreases preference without affecting aversion for nicotine

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Abstract

Epigenetic mechanisms have recently been shown to be involved in the long-term effects of drugs of abuse. A well described epigenetic mechanism modulating transcriptional activity consists in the binding to DNA of methyl-CpG binding proteins, such as MeCP2, recruiting histone deacetylases (HDACs). Nicotine causes long-term changes in the brain, but little is known concerning the mechanisms involved in nicotine-preference. Using a nicotine-conditioned place preference protocol, we demonstrate here that the histone deacetylase inhibitor phenylbutyrate was able to dramatically reduce the preference for nicotine, without altering the aversive properties of the drug. We measured immunohistochemically the acetylation of lysine-9 of histone H3, and the expression of phosphorylated cAMP-response element-binding protein, HDAC2 and methyl-CpG-binding protein 2 in the striatum and prefrontal cortex of rats displaying nicotine-preference or aversion and treated with phenylbutyrate. We show that, at the dose administered, the inhibitor was effective in inhibiting HDAC activity. The data suggest that phosphorylated cAMP-response element-binding protein participates in the establishment of conditioned place preference, but not in the reduction of nicotine-preference in response to phenylbutyrate. Moreover, striatal expression of HDAC2 in response to phenylbutyrate mirrored the behavioral effects of the inhibitor, suggesting that HDAC2 is involved in promoting synaptic plasticity underlying the preference for nicotine.

Keywords: conditioned-place preference, HDAC2, MeCP2, nicotine, phenylbutyrate.

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Nicotine, the primary psychoactive component of tobacco smoke, is believed to be responsible for the development and maintenance of tobacco dependence. It acts on nicotinic acetylcholine receptors that cooperate with other neurotransmitter systems to modulate synaptic plasticity (Dajas-Bailador and Wonnacott 2004). By binding to nicotinic receptors in the ventral tegmental area (VTA), nicotine stimulates the activity of dopaminergic neurons that project to the nucleus accumbens (NAc), frontal cortex and associated limbic structures (Mansvelder and McGehee 2000; Laviolette and van der Kooy 2004). The NAc is well characterized as playing a crucial role in the reward circuit. By maintaining a close relationship with other structures such as the VTA or the frontal cortex, it is central in the establishment of neurobiological plasticity related to addiction, including tobacco and nicotine dependence (Brunzell *et al.* 2009; Zhang *et al.* 2009; Brody *et al.* 2010). Few exposures to nicotine are sufficient to produce long-lasting alterations in the mesolimbic system that probably underlies early steps of nicotine dependence (Radcliffe *et al.* 1999 Mansvelder *et al.* 2002).

Current therapeutic interventions for quitting smoking are not quite satisfactory. Improvement of therapeutics necessitates a better understanding of the mechanisms that underlie the addictive properties of nicotine (Vaszar *et al.* 2002). Unfortunately, not much is known about the mechanisms initiated by nicotine-induced activation of the mesolimbic pathway that would ultimately be responsible for long-lasting neuroadaptations (Barik and Wonnacott 2009). Such

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Abbreviations used: CPA, conditioned place aversion; CPP, conditioned place preference; CPu, dorsal striatum; CREB, cAMP-response element-binding protein; H3-K9Ac, histone H3 lysine-9 acetylation; HDAC, histone deacetylase; MeCP2, methyl-CpG-binding protein 2; NAc, nucleus accumbens; PBS, phosphate-buffered saline; pCREB, phosphorylated CREB; PFC, prefrontal cortex; PhB, phenylbutyrate; VTA, ventral tegmental area.

adaptations most probably require genome-wide alterations in gene transcription (Renthal and Nestler 2009). We recently reported that nicotine-induced preference and reinstatement in rats require an enhanced phosphorylation state of cAMP-response element-binding protein (CREB) (Pascual *et al.* 2009; see also Walters *et al.* 2005; Brunzell *et al.* 2009). Phospho-CREB (pCREB) binds the CREB-binding protein, a transcriptional coactivator which possesses histone acetyltransferase activity (Goodman and Smolik 2000; Kalkhoven 2004). CREB-binding protein together with histone deacetylases (HDACs) regulate CREB activity through histone modifications in response to a variety of signaling molecules (Michael *et al.* 2000; Ryan *et al.* 2006). In general, histone hyper-acetylation is associated with DNA relaxation and elevated transcriptional activity (Shahbazian and Grunstein 2007).

On the other hand, gene regulation in response to repeated cocaine administration has been shown to induce long-term cellular alterations, which are partially under the control of HDACs (Cassel *et al.* 2006; Renthal and Nestler 2009). HDACs are recruited by a complex that includes the transcriptional repressor MeCP2 (methyl-CpG-binding protein 2) bound to methylated DNA. Gene silencing brought about by MeCP2 can be reversed by HDAC inhibitors (Jones *et al.* 1998; Dobosy and Selker 2001). The use of HDAC inhibitors has therefore rapidly emerged as a powerful tool to study the role of histone acetylation in transcription regulation (MacDonald and Roskams 2009). Previous reports have shown that administration of an HDAC inhibitor either facilitates the extinction of cocaine-induced conditioned place preference (CPP; Malvaez *et al.* 2010) or increases morphine preference (Sanchis-Segura *et al.* 2009). Moreover, HDAC inhibition has been shown to improve memory and synaptic plasticity (Fischer *et al.* 2007; Vecsey *et al.* 2007; Guan *et al.* 2009). Since memory for nicotine-associated cues are highly resistant to extinction, contributing to the high rate of relapse among tobacco addicts (Kelley 2004; Hyman 2005; Pascual *et al.* 2009), HDACs are likely involved in nicotine-induced long-term behavioral effects.

In the present study, we evaluated whether the HDAC inhibitor phenylbutyrate (PhB) would modify the nicotine-induced place preference or place aversion. Changes in histone H3 lysine-9 acetylation (H3-K9Ac), CREB phosphorylation, HDAC2 and MeCP2 gene expression were investigated in parallel.

Material and methods

Animals

Male Sprague–Dawley rats weighing 100–140 g (30–35 days old) were housed by groups of four on a 12 h light/dark cycle with access to food and water *ad libitum*. The housing conditions and animal care were consistent with those specified in the *Guide for the care and use of laboratory animals*. All procedures were performed during the light part of the diurnal cycle. Rats were handled for 5 days prior to behavioral conditioning. They were killed 3 h after the CPP or conditioned place aversion (CPA) experiment. For the behavioral experiments groups of 11 and 5 animals were used for nicotine- and control CPP experiment, respectively; six and five animals were used for nicotine- and control CPA experiments, respectively. For the study of PhB effect, groups of 19 and 12 animals were used for nicotine- and control CPP experiment, respectively; six and five animals were used for nicotine- and control CPA experiments, respectively. For the immunochemistry experiments were used five animals per group select at random from the behavioral groups.

Drugs

Rats undergoing CPP or CPA were injected subcutaneously (s.c.) with 0.21 mg/kg nicotine (Sigma-Aldrich, St Louis, MO, USA) for CPP, and with 2 mg/kg for CPA in a volume of 1 mL/kg body weight. An equal volume of phosphate-buffered saline (PBS) was injected for the control condition. Indicated doses are based on the molecular weight of the freebase. For treatment with the HDAC inhibitor, animals were i.p. injected each conditioning day with PBS or with 100 mg/kg PhB (sodium 4-phenylbutyrate, Sigma-Aldrich) 30 min prior to PBS or nicotine injection.

Biased place conditioning

Place conditioning was performed in home-made boxes divided into two equally sized compartments (30 × 25 × 30 cm) that were separated with a door allowing access to either side of the box. The door was closed during conditioning days. The two compartments had different visual, tactile and olfactory cues: one compartment had horizontally striped black and white walls and a wire mesh floor above pine shavings; the other compartment had vertically striped black and white walls and a bar-grid floor above cedar shavings. During the habituation period, animals were handled twice a day for 5 days and were injected s.c. with PBS to habituate them to the injections. We used a biased protocol to establish CPP (Tzschentke 1998, 2007; Le Foll and Goldberg 2005), since biased assignment procedures are more effective when nicotine is used to induce preference. This is not the case for cocaine or morphine for which an unbiased protocol is more effective (Calcagnetti and Schechter 1994; Brunzell *et al.* 2009; Pascual *et al.* 2009).

Pre-conditioning phase

On day 1 after habituation, animals were injected with PBS and placed in the box with the door open, which allowed them to roam freely from side to side for 10 min, and the time spent in each compartment was recorded. Balanced groups of animals showing approximately equal bias were constituted from the data.

Conditioning phase

On conditioning days, the two compartments were separated by the partition (door closed). Animals were injected twice a day, in the morning with PBS and in the afternoon with PBS or nicotine. Control group was given PBS in both compartment and drug groups received nicotine in one compartment and PBS in the other

compartment. For CPP, nicotine was given in the initially non-preferred compartment and for CPA, in the initially preferred compartment. Conditioning sessions of 20 min were carried on for four consecutive days.

Test phase

On day 6, animals were tested after they were given a PBS injection. They were allowed to roam freely between the two compartments for 10 min with the door open. Time spent in each compartment was recorded.

Data analysis

The time spent in each compartment was converted into a preference/avoidance coefficient {Coefficient (%) = [(time spent in initially non-preferred compartment – time spent in initially preferred compartment)/(time spent in initially non-preferred compartment + time spent in initially preferred compartment)] × 100}. Positive values of the coefficient indicate a preference for the drug-paired compartment or CPP, while negative values indicate an aversion to the drug-paired compartment or CPA. Significant effects between groups were determined by analyzing conditioning chambers as a within-subject measure (nicotine paired vs. PBS paired), using one-way ANOVA, followed by Student–Newman–Keuls *post hoc* tests, when required. Data are expressed as mean ± SEM, and significance was set at $p \leq 0.05$.

Antibodies

Following rabbit polyclonal antibodies were used: anti-acetylated histone H3-K9 antibody (Abcam, Cambridge, UK) diluted 1 : 1000; anti-MeCP2 antibody (Upstate, Millipore, Billerica, MA, USA) diluted 1 : 600; anti-pCREB antibody (Cell Signaling, Danvers, MA, USA) diluted 1 : 800 and anti-HDAC2 antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) diluted 1 : 500. Antibody binding was detected with secondary biotinylated horse anti-rabbit IgG.

Immunohistochemistry

Three hours after the CPP test, animals were anesthetized and perfused with 4% paraformaldehyde. Brains were removed and 50-µm thick coronal sections were prepared. HDAC2, H3-K9Ac, pCREB and MeCP2 immunostaining was performed as previously described (Cassel *et al.* 2004; Pascual *et al.* 2009). Briefly, sections were incubated overnight at 4°C with primary antibodies. Sections were then incubated successively with biotinylated secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA, USA) for 2 h at ~25°C, and with an avidin-biotin-peroxydase complex (Vectastain Elite ABC Kit Universal, Vector Labs, Burlingame, CA, USA). Antibody labeling was detected using 3,3′-diaminobenzidine and H_2O_2. Slices were dehydrated and coverslipped with mounting medium (Vector Labs). Numbers of 3,3′-diaminobenzidine-stained cells were determined using a light microscope (Olympus, Center Valley, PA, USA) and the optical dissector principle (Gundersen *et al.* 1988; Cogesshal and Lekan 1996) for comparison between nicotine and vehicle groups. Quantification of immunolabeling for each antibody was performed in several brain structures using Image-Pro Plus (Media Cybernetics Inc., Bethesda, MD, USA) by an investigator blind to the identity of the samples. Appropriate areas were digitally imaged and quantification was subjected to a stringent criterion (Miller and Marshall 2005; Pascual *et al.* 2009) according to the staining intensity. For each animal, immunopositive cells were counted in both hemispheres on five to six sections. Counts were averaged in squares of 0.5 mm^2 drawn randomly in the prefrontal cortex (PFC), NAc core and shell, dorsal striatum (CPu) and VTA, and the counts were averaged for each immunopositive cell type per millimeter square. For quantification studies, statistical analyses were performed using one-way ANOVA (number of positive cells as factor of variation), followed by Student–Newman–Keuls *post hoc* tests, when required.

Results

Effect of PhB on nicotine-induced CPP and CPA

To examine the effects of histone deacetylase (HDAC) inhibition on the development of nicotine-induced place preference or aversion, we trained rats in a well established biased CPP or CPA protocol. The results clearly show that 0.21 mg/kg nicotine given once a day for 4 days was sufficient to induce an important CPP (Fig. 1). We also treated animals with PhB at a dose which was shown to

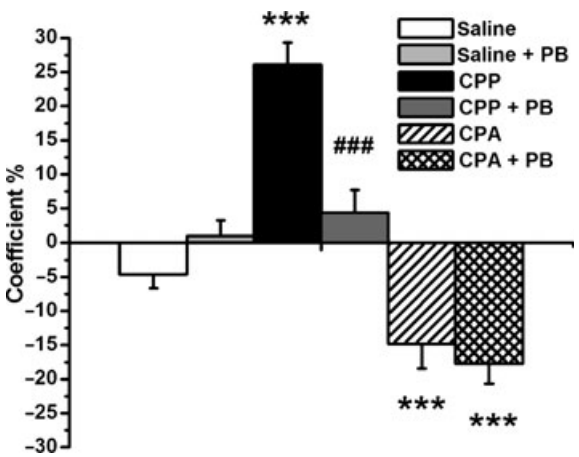

Fig. 1 Effect of PhB on nicotine-induced CPP and CPA in rats. CPP and CPA experiments were carried on as described in Material and methods. Rats were injected s.c. with 0.21 mg/kg nicotine for CPP, and with 2 mg/kg for CPA. An equal volume of PBS was injected for the control condition. Animals were i.p. injected each conditioning day with PBS or with 100 mg/kg PhB 30 min before PBS or nicotine injection, as indicated. Bar graphs indicate preference coefficients (positive values) or avoidance coefficients (negative values) for nicotine. Since nicotine was administrated in the initially non-preferred compartment for CPP, value for the coefficient is negative in the PBS control group. Values found in the CPA group are negative after pairing to the initially preferred compartment, showing aversion for nicotine at the higher dosage. Results are expressed as mean ± SEM for each group. $^{***}p < 0.001$ comparison with the control group, $^{###}p < 0.001$ comparison with the corresponding nicotine-treated group. ANOVA followed by Student–Newman–Keuls *post hoc* test.

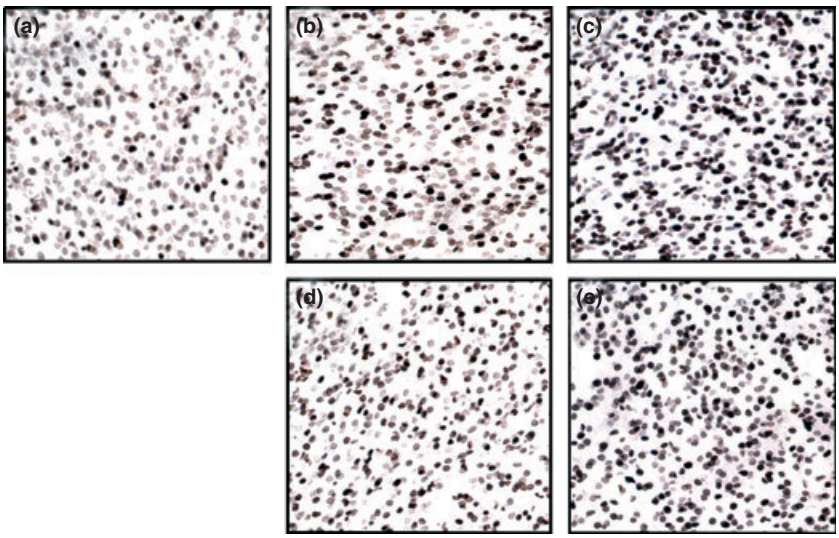

Fig. 2 Photomicrographs illustrating immunoreactivity of acetylated histone H3 at K9 in NAc core of the different behavioral groups. The micrographs show representative acetylated histone H3-K9 immunostaining in NAc core area from control (a), CPP (b), CPP + PhB (c), CPA (d), and CPA + PhB (e) groups of animals. Scale bar, 40 μm. CPP, conditioned place preference; CPA, conditioned place aversion; PhB, phenylbutyrate.

inhibit HDACs in other behavioral paradigm (Romieu *et al.* 2008).When 100 mg/kg PhB was injected 30 min before each conditioning session, the preference for nicotine was abolished (Fig. 1). In contrast, while 2 mg/kg nicotine given during the 4 days of conditioning sessions induced a clear CPA as previously reported, the administration of the same PhB dosage 30 min prior to each conditioning session had no significant effect on the aversive properties of nicotine (Fig. 1).

H3-K9Ac levels in mesolimbic structures of the different behavioral groups

Figure 2 illustrates H3-K9Ac immunoreactivity in NAc core of control, CPP and CPA animals, treated with the HDAC inhibitor PhB. Immunostaining was found to be exclusively nuclear, exhibiting various levels of intensity, as expected for a nuclear protein. A clear increase in the number of positive cells can be observed mainly in the CPP group, but also in the CPA group, which was further enhanced by the PhB treatment.

Quantitative analysis of the number of H3-K9Ac-immunoreactive cells in the CPu, NAc core and shell and PFC of groups of rats treated as indicated above is summarized in Fig. 3. The quantification was subjected to a stringent criterion described in Materials and Methods. One-way ANOVA indicated significant differences between groups (CPu $F_{4,152} = 70.0$, $p < 0.001$; NAc core $F_{4,164} = 97.9$, $p < 0.001$; NAc shell $F_{4,146} = 53.0$, $p < 0.001$ and PFC

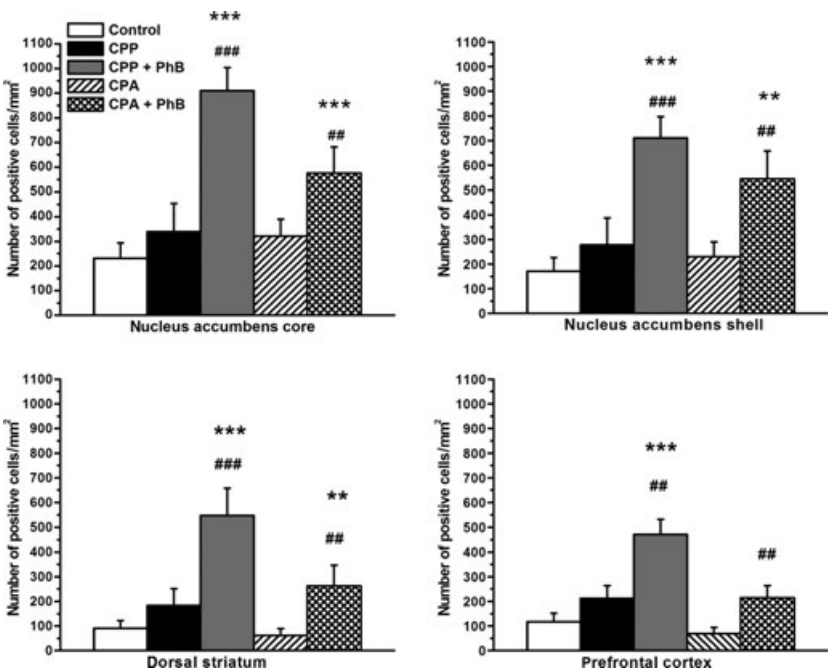

Fig. 3 Quantification of acetylated histone H3-K9-positive cells in mesolimbic structures. Bar graphs indicate the number of H3-K9-immunopositive cells/mm^2 in Nac core and shell, CPu and PFC of the various experimental groups of rats. Results are expressed as mean ± SEM for each group. $^{**}p < 0.01$, and $^{***}p < 0.001$, comparison with control group. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, comparison CPP or CPA with the corresponding PhB-treated group. ANOVA followed by Student–Newman–Keuls *post hoc* test.

$F_{4,137} = 98.6$, $p = 0.001$). In comparison to control rats, immunoreactivity in caudate nucleus and in the two subregions of NAc was statistically different in both CPP + PhB and CPA + PhB groups of rats. The CPP + PhB group exhibited significantly enhanced labeling when compared to the CPP group in all four structures examined, including the PFC.

Immunohistochemical studies showing pCREB, HDAC2 and MeCP2 labeling in NAc core

Figure 4 illustrates pCREB, HDAC2 and MeCP2 immunoreactivity found in the NAc core of control, CPP and CPA animals treated with the HDAC inhibitor PhB. All the markers showed a clear staining and distribution. Immunostaining of pCREB, HDAC2 and MeCP2 was restricted to the nuclear compartment, in agreement with the proteins interacting directly or indirectly with DNA. The number of pCREB-positive cells was clearly increased in CPP and CPP + PhB groups of rats, but not in the CPA group. HDAC2 labeling was increased in CPP and CPA groups, but not in the CPP + PhB group. Finally, the number of MeCP2-positive cells was increased in CPP and CPA groups and it seems that the PhB treatment further increased MeCP2 labeling in both cases.

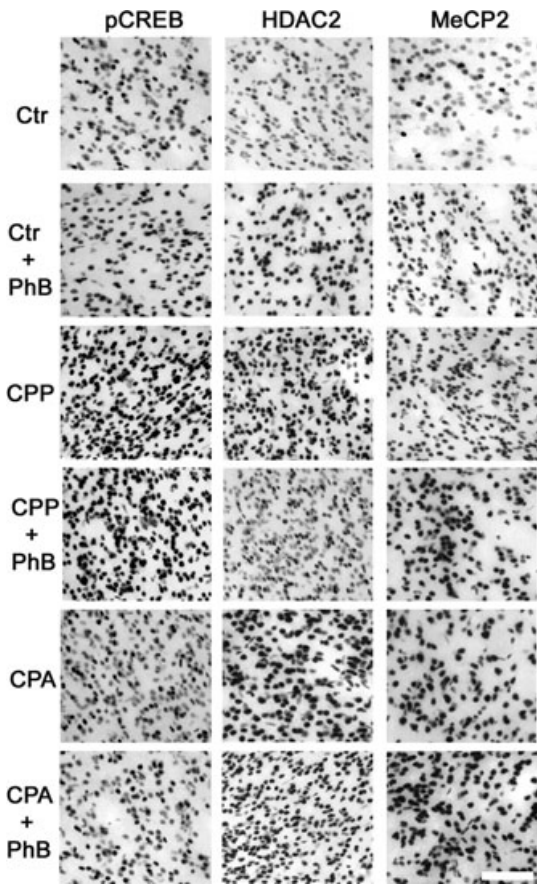

Fig. 4 Photomicrographs showing pCREB, HDAC2 and MeCP2 immunoreactivity in NAc core of the different behavioral groups. The micrographs show representative pCREB-, HDAC2- and MeCP2-positive immunostaining in the NAc core area from control (Ctr), control + PhB (Ctr + PhB), CPP, CPP + PhB, CPA, and CPA + PhB groups of animals. Scale bar, 50 μm.

Phospho-CREB levels in dopaminergic brain areas

Figure 5 shows the quantitative analysis of pCREB-positive cells expressed in several dopaminergic brain areas of rats that had been subjected to CPP and CPA and treated with PhB, as indicated in legend to Fig. 1. The areas examined were the CPu, NAc core and shell, PFC and VTA. Significant differences across experimental groups could be established (CPu: $F_{4,22} = 10.2$, $p < 0.0001$; NAc core: $F_{4,22} = 6.45$, $p < 0.0021$; NAc shell: $F_{4,23} = 13.5$, $p < 0.0001$; PFC: $F_{4,25} = 13.1$, $p < 0.0001$; VTA (data not shown): $F_{4,22} = 18.9$, $p < 0.0001$). In the NAc core and shell, numbers of pCREB-positive cells in CPP and CPP + PhB groups were significantly different from those expressed in control animals. In the CPu, difference in labeling was observed between control and CPP, CPP + PhB or CPA + PhB groups of rats ($p < 0.001$, $p < 0.05$ and $p < 0.05$, respectively). In the PFC, the number of pCREB-positive cells in CPP and CPP + PhB groups was different from that found in the control group ($p < 0.001$ and $p < 0.05$, respectively). A significant decrease was noticed in response to the PhB treatment, when compared to the CPP group ($p < 0.05$). In the VTA (data not shown), the CPP, CPP + PhB and CPA + PhB groups displayed significant differences in the number of immunoreactive cells when compared to control ($p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively). Importantly, the CPA + PhB group showed a significant difference in labeling when compared to CPA animals ($p < 0.05$). The CPA group expressed a lower level of pCREB positive cells in all the structures examined, when compared to the CPP group.

HDAC2 expression in dopaminergic brain areas

Figure 6 summarizes the quantitative analysis of the number of HDAC2-immunoreactive cells in NAc core and shell, CPu and PFC of groups of rats treated as indicated above. One-way ANOVA indicated significant differences between groups (CPu: $F_{5,27} = 8.90$, $p < 0.0001$; NAc core: $F_{5,27} = 8.57$, $p < 0.0001$; NAc shell: $F_{5,31} = 9.24$, $p < 0.0001$ and PFC: $F_{5,29} = 0.88$, $p = 0.50$). In comparison to control rats, number of immunoreactive cells in the NAc core was statistically different in the CPP, CPA and CPA + PhB groups of rats ($p < 0.01$). The CPP + PhB group exhibited significantly less labeling when compared to the CPP group of rats ($p < 0.05$). Labeling in the CPu was very similar to that observed in the NAc shell. No significant difference was found between the various behavioral groups in the PFC.

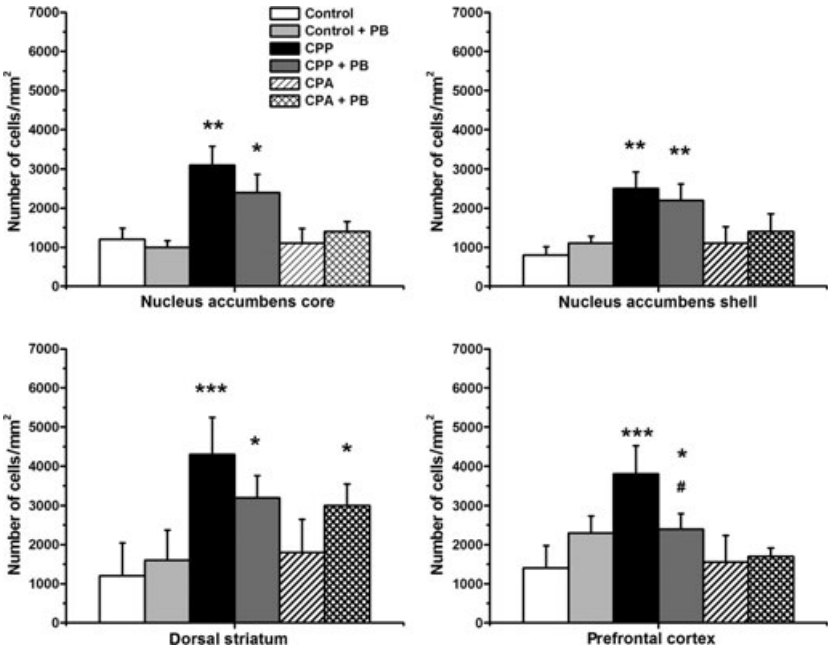

Fig. 5 Quantification of pCREB-positive cells in dopaminergic brain structures. Bar graphs indicate the number of pCREB-immunopositive cells/mm^2 in Nac core and shell, CPu, and PFC of the various experimental groups of rats. Results are expressed as mean ± SEM for each group. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, comparison with control group. $^{\#}p < 0.05$, comparison CPP or CPA with the corresponding PhB-treated group. ANOVA followed by Student–Newman–Keuls *post hoc* test.

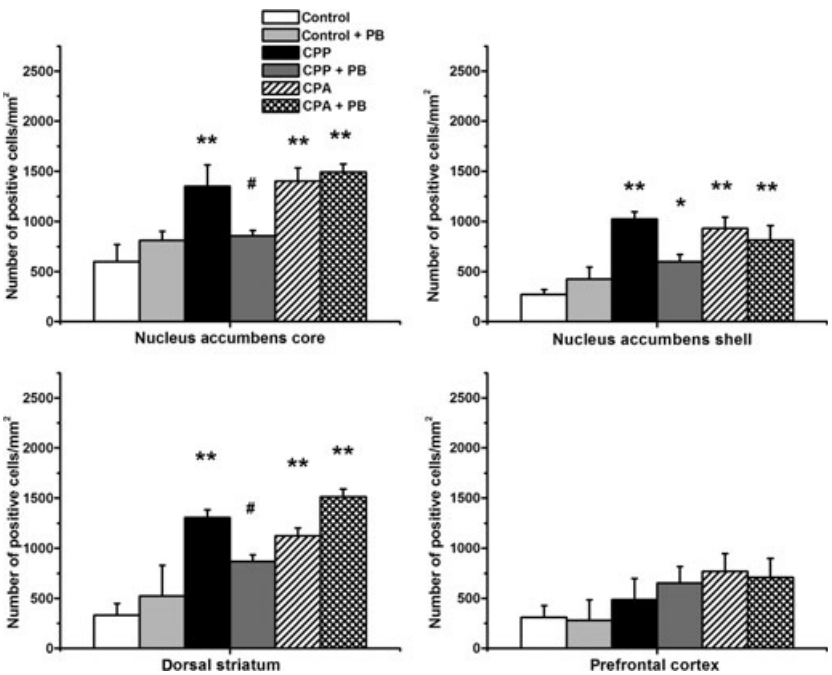

Fig. 6 Quantification of HDAC2-immunoreactive cells in dopaminergic brain structures. Bar graphs indicate the number of HDAC2-immunopositive cells/mm^2 in NAc core and shell, CPu and PFC of the various experimental groups. Results are expressed as mean ± SEM. $^{**}p < 0.01$, comparison with control group. $^{\#}p < 0.05$, comparison between CPP and CPP + PhB groups. ANOVA followed by Student–Newman–Keuls *post hoc* test. $^*p < 0.05$, $^{**}p < 0.01$, comparison with control group.

MeCP2 expression in dopaminergic brain areas

Figure 7 shows the quantitative analysis of the number of MeCP2-immunopositive cells in NAc core and shell, CPu and PFC of the same groups of rats. One-way ANOVA indicated significant differences between groups (NAc core: $F_{5,32} = 10.0$, $p < 0.0001$; NAc shell: $F_{5,29} = 13.2$, $p < 0.0001$; CPu: $F_{5,25} = 10.4$, $p < 0.0007$; and PFC: $F_{5,29} = 1.56$, $p = 0.20$). In the NAc core, animals from the CPP, CPP + PhB, CPA and CPA + PhB groups displayed different levels of MeCP2-immunoreactive cells when compared to the control group ($p < 0.001$, $p < 0.001$, $p < 0.05$, and $p < 0.01$, respectively). Similar results were observed in the NAc shell. In the CPu, number of immunopositive cells in the CPP, CPA and CPA + PhB groups was significantly different when compared to control group ($p < 0.01$, $p < 0.001$ and $p < 0.01$, respectively). A statistically significant difference in MeCP2 expression was also found between the CPP and the CPP + PhB groups ($p < 0.05$). It

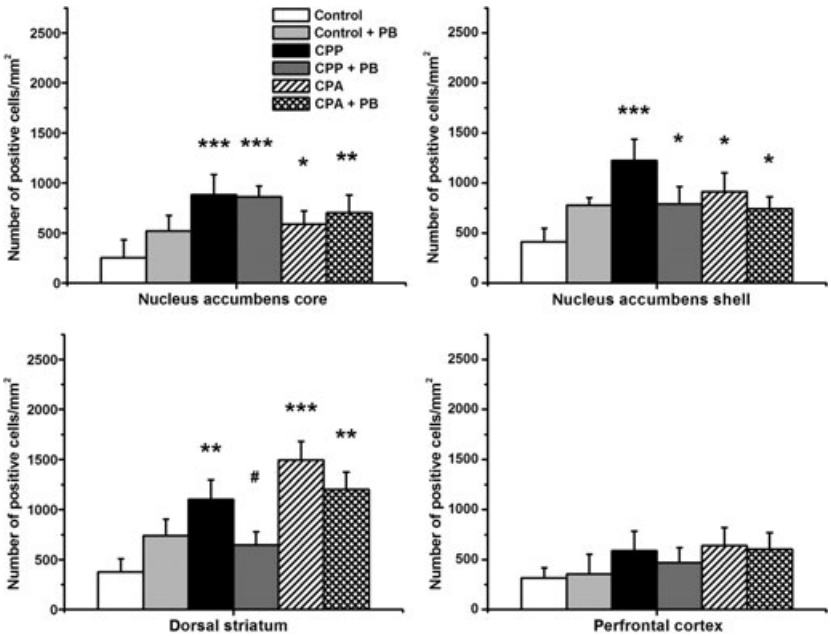

Fig. 7 Quantification of MeCP2-immunoreactive cells in dopaminergic brain structures. Bar graphs indicate the number of MeCP2-immunopositive cells/mm^2 in NAc core and shell, CPu and PFC of the various experimental groups. Results are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, comparison with control group. $^{\#}p < 0.05$, comparison between CPP and CPP + PhB groups. ANOVA followed by Student–Newman–Keuls *post hoc* test.

is noteworthy that animals of the CPA group showed no difference in MeCP2 expression in the three structures examined, when compared to the CPP group of animals. No significant difference could be found between the various behavioral groups in the PFC.

Discussion

In the present study, we evaluated the effect of an HDAC inhibitor, phenylbutyrate, on the acquisition of nicotine-conditioned place preference in rats. CPP is a classical conditioning test that is usually employed to measure preference for drugs of abuse (Le Foll and Goldberg 2005; Tzschentke 2007; Pascual *et al.* 2009). It is shown here that inhibition of HDACs was sufficient to reduce the place preference for nicotine. The fact that the HDAC inhibitor PhB dramatically reduced CPP suggests that nicotine-associated cues are under the control of neurobiological mechanisms that involve histone acetylation processes. Administration of higher doses of nicotine is known to induce place aversion (Le Foll and Goldberg 2005; Pascual *et al.* 2009), which was not found to be affected by the administration of the same dosage of PhB, suggesting that the mechanism underlying drug preference differs somewhat from that underlying drug aversion. When considering the concept that drug addiction shares commonality with learning and memory processes, the question arises whether general learning would similarly be impaired by HDAC inhibitors. In fact, studies rather indicate that HDAC inhibition does improve learning and memory performances (Bredy and Barad 2008; Malvaez *et al.* 2010). This apparent divergence may arise because the learning component, although required for the animal to associate the drug with its environment, plays only a minor role during CPP expression. This is attested by the fact that place preference is dependent on the drug dosage; nicotine in particular has to be injected at a precise dosage in order to establish place preference. CPP expression is for the most part based on a motivational aspect, which probably represents the major target of HDAC inhibitors (among the various components elicited by drugs of abuse), as was previously demonstrated by experiments using the self-administration paradigm. Furthermore, we have shown that HDAC inhibition affects cocaine self-administration but not sucrose self-administration, which indicates that HDAC inhibitors play a much subtler role than just affecting the common reward pathway (Romieu *et al.* 2008). The observation we made in the present study, in which we show that PhB inhibits preference but not aversion for nicotine represents an additional argument for HDAC inhibitors affecting primarily the motivational component of nicotine, and not its learning component.

In order to assure that in our experimental conditions, the HDAC inhibitor indeed increased histone acetylation, we first measured immunohistochemically the acetylation level of lysine-9 of histone H3. The increased levels of H3-K9Ac found in response to PhB in several brain areas demonstrate that the dosage used for the inhibitor was sufficient to inhibit HDAC activity. This PhB dose was previously reported to be effective in another behavioral paradigm (Romieu *et al.* 2008; Host *et al.* 2009).

To further evaluate the effect of PhB on appetitive and aversive conditions, we also followed some markers of gene transcription, such as pCREB, HDAC2 and MeCP2 proteins

in mesolimbic dopaminergic brain areas of rats that underwent the behavioral tests. The data showing pCREB induction by nicotine confirm a previous observation, i.e. that the transcription factor CREB is highly phosphorylated 3 h after CPP, but not after CPA (Pascual *et al.* 2009). Furthermore, it is noteworthy that, with the exception of the PFC, the number of cells expressing activated pCREB in animals displaying CPP was not modified by the administration of the HDAC inhibitor. The latter observation suggests that, while pCREB is necessary to establish CPP as previously shown (Pascual *et al.* 2009), it apparently plays no essential role in the reduction of CPP in response to the HDAC inhibitor 3 h after conditioning. This might occur because pCREB and HDAC2 operate independently, or because CREB activation is positioned upstream of HDAC2 (Franklin and Mansuy 2010). In both cases, the inhibitor would affect HDAC activity and consequently the processes controlled by histone acetylation, without affecting pCREB levels. Alternatively, events regulated by active CREB may be modulated by the dephosphorylating enzyme protein phosphatase PP1, which is well known to be under the control of epigenetic mechanisms involving HDAC activity (Canettieri *et al.* 2003).

In the aversive conditioning test, pCREB level was not modified during the acquisition of CPA and PhB had no effect on pCREB level in NAc and PFC, strongly suggesting that the transcription factor is not involved in the aversive properties of nicotine, at least 3 h after conditioning. We only observed an increase of pCREB expression in the dorsal striatum of the CPA + PhB group compared to the CPA group. Given that pCREB is increased in fear or aversive memories, one can only speculate about the fact that we did not detect any increase in pCREB in the CPA group. A possible explanation for this is that the amygdala, which plays a central role in tone-shock association in fear memories, is not directly involved in the association between nicotine and cue during CPP (Rodrigues *et al.* 2004; Mamiya *et al.* 2009; Pascual *et al.* 2009; Ciocchi *et al.* 2010). Taken together, our data suggest that the aversive properties of nicotine concern distinct structures and substrates from those processing fear conditioning memory.

We also measured immunohistochemically the expression of HDAC2 in the same groups of animals. HDAC2 was chosen because the enzyme is highly expressed in the mesolimbic pathway (Cassel *et al.* 2006; Broide *et al.* 2007). The data show that nicotine-induced CPP and CPA were associated with an increase in HDAC2 expression. This is in accordance with previous reports in which we showed that repeated cocaine treatment induced HDAC2 expression in PFC and striatum (Cassel *et al.* 2006; Host *et al.* 2009). The fact that striatal HDAC2 expression was increased in both appetitive and aversive conditions strongly suggests that silencing of some genes is required in order to establish a conditioning, and is in line with reports showing the involvement of HDAC2 in long-term plasticity (Grissom and Lubin 2009; Guan *et al.* 2009). Interestingly, while treatment with PhB had no effect on the control condition, it considerably reduced the number of HDAC2 immunopositive cells in the striatal subregions of rats from the CPP group, but not from the CPA group. Hence, it appears that HDAC2 expression in response to PhB was correlated with the behavioral changes induced by the inhibitor: they were reduced in CPP and remained unchanged in CPA. Together, the findings strengthen the concept of HDAC2 being involved in learning processes: not only is HDAC2 required for conditioning, it is also down-regulated when drug-seeking behavior is reduced.

Histone deacetylase 2 is part of a complex composed also of Sin3A and MeCP2 bound principally to methylated DNA, with HDAC activity conferring transcriptional silencing to the corresponding genes (Yang and Seto 2008). The binding of MeCP2 to DNA can be relieved by the inhibition of HDAC, since this causes histone modifications that allow transcriptional activation (Buchwald *et al.*, 2009). We therefore measured MeCP2 protein expression in our groups of rats. Again, as was the case when rats were treated with cocaine (Cassel *et al.* 2006), we found that nicotine administration increased the number of cells expressing MeCP2 throughout the striatum. Treatment with PhB was found to reduce MeCP2 expression only in the dorsal striatum, when compared to the CPP group. No statistically significant effect was found in the NAc. As for HDAC2 expression, MeCP2 expression was not different between the CPA and CPA + PhB groups of rats.

Expression of pCREB, as well as of HDAC2 and MeCP2 proteins was up-regulated in the NAc (core and shell) and in the dorsal striatum of animals from the CPP group. In response to the HDAC inhibitor, HDAC2 expression was reduced in dorsal striatum and NAc core, and MeCP2 expression was only reduced in dorsal striatum. This observation confirms that during CPP expression, the dorsal striatum, and the NAc core in some respect, contribute mostly to nicotine preference, in contrast to previous studies in which the NAc shell was given a central role in nicotine CPP (Sellings *et al.* 2008; Brunzell *et al.* 2009). This indicates that, while early effects of various drugs converge on the NAc, the dorsal striatum becomes strongly involved in the neuronal plasticity underlying nicotine reward. Taken together, our data convincingly demonstrate that HDAC inhibition is able to modulate drug-seeking behavior. They suggest that histone deacetylation, particularly that ensured by HDAC2, plays some prominent role in establishing the synaptic plasticity underlying addictive processes.

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References

Barik J. and Wonnacott S. (2009) Molecular and cellular mechanisms of action of nicotine in the CNS. *Handb Exp Pharmacol* **192**, 173–207. *Nicotine Psychopharmacology.* J. E. Henningfield *et al.* (eds.), Springer-Verlag, Berlin, Heidelberg.

Bredy T. W. and Barad M. (2008) The histone deacetylase inhibitor valproic acid enhances acquisition, extinction and reconsolidation of conditioned fear. *Learn Mem.* **15**, 39–45.

Brody A. L., London E. D., Olmstead R. E. *et al.* (2010) Smoking-induced change in intrasynaptic dopamine concentration: effect of treatment for Tobacco Dependence. *Psychiatry Res.* **183**, 218–224.

Broide R. S., Redwine J. M., Aftahi N., Young W., Bloom F. E. and Winrow C. J. (2007) Distribution of histone deacetylases 1–11 in the rat brain. *J. Mol. Neurosci.* **31**, 47–58.

Brunzell D. H., Mineur Y. S., Neve R. L. and Picciotto M. R. (2009) Nucleus accumbens CREB activity is necessary for nicotine conditioned place preference. *Neuropsychopharmacology* **34**, 1993–2001.

Buchwad M., Krämer O. H. and Heinzel T. (2009) HDACi–targets beyond chromatin. *Cancer Lett.* **280**, 160–167.

Calcagnetti D. J. and Schechter M. D. (1994) Nicotine place preference using the biased method of conditioning. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **18**, 925–933.

Canettieri G., Morantte I., Guzmán E., Asahara H., Herzig S., Anderson S. D., Yates 3rd J. R. and Montminy M. (2003) Attenuation of a phosphorylation-dependent activator by an HDAC-PP1 complex. *Nat. Struct. Biol.* **10**, 175–181.

Cassel S., Revel M. O., Kelche C. and Zwiller J. (2004) Expression of the methyl-CpG-binding protein MeCP2 in rat brain. An ontogenetic study. *Neurobiol. Dis.* **15**, 206–211.

Cassel S., Carouge D., Gensburger C., Anglard P., Burgun C., Dietrich J. B., Aunis D. and Zwiller J. (2006) Fluoxetine and cocaine induce the epigenetic factors MeCP2 and MBD1 in adult rat brain. *Mol. Pharmacol.* **70**, 487–492.

Ciocchi S., Herry C., Grenier F. *et al.* (2010) Encoding of conditioned fear in central amygdala inhibitory circuits. *Nature* **468**, 7–82.

Cogesshal R. E. and Lekan H. A. (1996) Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J. Comp. Neurol.* **364**, 6–15.

Dajas-Bailador F. and Wonnacott S. (2004) Nicotinic acetylcholine receptors and the regulation of neuronal signalling. *Trends Pharmacol. Sci.* **25**, 317–324.

Dobosy J. R. and Selker E. U. (2001) Emerging connections between DNA methylation and histone acetylation. *Cell. Mol. Life Sci.* **58**, 721–727.

Fischer A., Sananbenesi F., Wang X., Dobbin M. and Tsai L. H. (2007) Recovery of learning and memory associated with chromatin remodelling. *Nature* **447**, 178–182.

Franklin T. B. and Mansuy I. M. (2010) The prevalence of epigenetic mechanisms in the regulation of cognitive functions and behaviour. *Curr. Opin. Neurobiol.* **20**, 441–449.

Goodman R. H. and Smolik S. (2000) CBP/p300 in cell growth, transformation, and development. *Genes Dev.* **14**, 1553–1577.

Grissom N. M. and Lubin F. D. (2009) The dynamics of HDAC activity on memory formation. *Cell Sci.* **6**, 44–48.

Guan J. S., Haggarty S. J. and Giacometti E. *et al.* (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature* **459**, 55–60.

Gundersen H. J., Bagger P. and Bendtsen T. F. *et al.* (1988) The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *Acta Pathol. Microbiol. Immunol. Scand.* **96**, 857–881.

Host L., Dietrich J. B., Carouge D., Aunis D. and Zwiller J. (2009) Cocaine self-administration alters the expression of chromatin-remodelling proteins; modulation by histone deacetylase inhibition. *J. Psychopharmacol.* [Epub ahead of print]

Hyman S. E. (2005) Addiction: a disease of learning and memory. *Am. J. Psychiatry* **162**, 1414–1422.

Jones P. L., Veenstra G. J., Wade P. A., Vermaak D., Kass S. U., Landsberger N., Strouboulis J. and Wolffe A. P. (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* **19**, 187–191.

Kalkhoven E. (2004) CBP and p300: HATs for different occasions. *Biochem. Pharmacol.* **68**, 1145–1155.

Kelley A. E. (2004) Memory and addiction: shared neural circuitry and molecular mechanisms. *Neuron* **44**, 161–179.

Laviolette S. R. and van der Kooy D. (2004) The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. *Nat. Rev. Neurosci.* **5**, 55–65.

Le Foll B. and Goldberg S. R. (2005) Nicotine induces conditioned place preferences over a large range of doses in rats. *Psychopharmacology (Berl)* **178**, 481–492.

MacDonald J. L. and Roskams A. J. (2009) Epigenetic regulation of nervous system development by DNA methylation and histone deacetylation. *Prog. Neurobiol.* **88**, 170–183.

Malvaez M., Sanchis-Segura C., Vo D., Lattal K. M. and Wood M. A. (2010) Modulation of chromatin modification facilitates extinction of cocaine-induced conditioned place preference. *Biol. Psychiatry* **67**, 36–43.

Mamiya N., Fukushima H., Suzuki A., Matsuyama Z., Homma S., Frankland P. W. and Kida S. (2009) Brain region-specific gene expression activation required for reconsolidation and extinction of contextual fear memory. *J. Neurosci.* **29**, 402–413.

Mansvelder H. D. and McGehee D. S. (2000) Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron* **27**, 349–357.

Mansvelder H. D., Keath J. R. and McGehee D. S. (2002) Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. *Neuron* **33**, 905–919.

Michael L. F., Asahara H., Shulman A. I., KrausW Lee. and Montminy M. (2000) The phosphorylation status of a cyclic AMP-responsive activator is modulated via a chromatin-dependent mechanism. *Mol. Cell. Biol.* **20**, 1596–1603.

Miller C. A. and Marshall J. F. (2005) Molecular substrates for retrieval and reconsolidation of cocaine-associated contextual memory. *Neuron* **47**, 873–884.

Pascual M. M., Pastor V. and Bernabeu R. O. (2009) Nicotine-conditioned place preference induced CREB phosphorylation and Fos expression in the adult rat brain. *Psychopharmacology (Berl)* **207**, 57–71.

Radcliffe K. A., Fisher J. L., Gray R. and Dani J. A. (1999) Nicotinic modulation of glutamate and GABA synaptic transmission of hippocampal neurons. *Ann. N Y Acad. Sci.* **868**, 591–610.

Renthal W. and Nestler E. J. (2009) Histone acetylation in drug addiction. *Semin. Cell Dev. Biol.* **20**, 387–394.

Rodrigues S. M., Schafe G. E. and LeDoux J. E. (2004) Molecular mechanisms underlying emotional learning and memory in the lateral amygdala. *Neuron* **44**, 75–91.

Romieu P., Host L., Gobaille S., Sandner G., Aunis D. and Zwiller J. (2008) Histone deacetylase inhibitors decrease cocaine but not sucrose self-administration in rats. *J. Neurosci.* **28**, 9342–9348.

Ryan C. M., Harries J. C., Kindle K. B., Collins H. M. and Heery D. M. (2006) Functional interaction of CREB binding protein (CBP) with nuclear transport proteins and modulation by HDAC inhibitors. *Cell Cycle* **5**, 2146–2152.

Sanchis-Segura C., Lopez-Atalaya J. P. and Barco A. (2009) Selective boosting of transcriptional and behavioral responses to drugs of abuse by histone deacetylase inhibition. *Neuropsychopharmacology* **34**, 2642–2654.

Sellings L. H., Baharnouri G., McQuade L. E. and Clarke P. B. (2008) Rewarding and aversive effects of nicotine are segregated within the nucleus accumbens. *Eur. J. Neurosci.* **28**, 342–352.

Shahbazian M. D. and Grunstein M. (2007) Functions of site-specific histone acetylation and deacetylation. *Annu. Rev. Biochem.* **76**, 75–100.

Tzschentke T. M. (1998) Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. *Prog. Neurobiol.* **56**, 613–672.

Tzschentke T. M. (2007) Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. *Addict Biol.* **12**, 227–462.

Vaszar L. T., Sarinas P. S. and Lillington G. A. (2002) Achieving tobacco cessation: current status, current problems, future possibilities. *Respiration* **69**, 381–384.

Vecsey C. G., Hawk J. D. and Lattal K. M. *et al.* (2007) Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB:CBP-dependent transcriptional activation. *J. Neurosci.* **27**, 6128–6140.

Walters C. L., Cleck J. N., Kuo Y. C. and Blendy J. A. (2005) Mu-opioid receptor and CREB activation are required for nicotine reward. *Neuron* **46**, 933–943.

Yang X. J. and Seto E. (2008) The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. *Nat. Rev. Mol. Cell Biol.* **9**, 206–218.

Zhang T., Zhang L., Liang Y., Siapas A. G., Zhou F. M. and Dani J. A. (2009) Dopamine signaling differences in the nucleus accumbens and dorsal striatum exploited by nicotine. *J. Neurosci.* **29**, 4035–4043.