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Pre-Exposure to Nicotine with Nocturnal Abstinence Induces Epigenetic Changes that Potentiate Nicotine Preference

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Abstract

Prior exposure to drugs of abuse may facilitate addiction. It has been described that pre-exposure to nicotine can increase or, contrarily, prevent conditioned place preference (CPP). Here, we evaluated the effect of nicotine pre-exposure on CPP performance using an original protocol mimicking smokers' behaviour in zebrafish. We simulated nicotine withdrawal at sleep time by exposing zebrafish to nicotine during daylight but not at night (D/N) for 14 days and then performed nicotine-CPP in zebrafish. D/N-nicotine-treated zebrafish obtained the highest CPP score, whereas zebrafish pre-exposed continuously to nicotine did not show nicotine-CPP. Evaluation of locomotor activity, seeking and anxiety-like behaviours supported the CPP findings. Nicotinic receptor subunit gene expression showed significant increases in the brain of zebrafish exposed to nicotine. Zebrafish exposed to D/N-nicotine showed further increases of α6- and α7-subunit expression after CPP establishment. Inhibition of histone acetylation by phenylbutyrate prevented nicotine-CPP. Transcriptional expression of epigenetic enzymes controlling histone acetylation/deacetylation and DNA methylation/demethylation was widely modified in brain portions containing reward areas of zebrafish exposed to D/N-nicotine after CPP. Zebrafish exposed to D/N-nicotine showed high levels of acetylated histone 3 and pCREB immunoreactivity differentially found in nuclei of the dopaminergic reward circuit in zebrafish homologous to the ventral tegmental area, nucleus accumbens and dorsal habenula. Our findings demonstrated that repetitive abstinent periods are risky factors for drug abuse that potentiate nicotine–environment associations and seeking. Brain modifications can persist long after nicotine use and are likely due to changes in the transcriptional expression of enzymes regulating drug reward-related gene expression via epigenetic modifications.

Keywords Nicotine · Conditioned place preference · Epigenetics · Addiction · Zebrafish

Introduction

Epidemiology and clinical research have extensively demonstrated that variations in predisposition to drug abuse among individuals are influenced by previous drug use [1]. Individual sensitivity to the reinforcing effects of nicotine is an important factor to develop drug consumption [2, 3]. One method to evaluate the rewarding properties of nicotine is the conditioned place preference (CPP), a classical conditioning in which an animal is taught to associate two stimuli, an environment with a drug and after several associations a single exposure to the environment that generates a response of seeking for the drug. It has been suggested that only rats that were exposed to nicotine for 7 days prior to conditioning were able to establish CPP [4]. On the other hand, it has been reported that rats which consumed more nicotine in drinking water in their home cage for 6 weeks developed low nicotine-CPP scores [5]. Moreover, other study described that forced nicotine oral delivery for 7 weeks did not alter nicotine preference in rats [6]. We have previously demonstrated that a single exposure to nicotine predicts the acquisition of nicotine-CPP in rats [3]. Other studies indicated that prior exposure to nicotine has a predictive significance for nicotine addiction; however, conclusions are ambiguous. It is also relevant to consider that withdrawal between doses is an important factor for establishing addiction [7]. Withdrawal can trigger craving and relapse. Smokers' withdrawal generally occurs every night at

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sleep time. Nicotine half-life in blood is around 2–3 h, and smokers usually burn on average one cigarette per hour [8, 9]. It has been reported that intermittent injections of nicotine to mice four times daily for 14 days produce a somatic abstinence syndrome after discontinuing treatment [10]. Repeated exposure to psychostimulants induces two major mechanisms of epigenetic regulation in the brain—histone post-translational modifications [11] and DNA methylation [12, 13]. Histone modifications that regulate gene expression are partially controlled by histone acetyltransferases (HATs) and deacetylases (HDACs). In this regard, repeated nicotine administration provokes long-term neuronal modifications based on gene expression regulation, which was demonstrated to be partially controlled by HDACs [14–16]. Previous reports showed that HDAC inhibitors facilitate the extinction of cocaine-induced CPP [17] and decrease nicotine reward in rodents [14]. Acetylation of histone 3 at lysine 9 (H3K9-Ac) is also modified by nicotine [14, 18]. DNA methyltransferase 1 (DNMT1) catalyzes covalent binding of methyl groups to specific CpG structures in DNA [19]. Nicotine treatment induces DNA methylation of several genes that have been implicated in drug abuse. Chronic administration of nicotine down-regulates the expression of DNMT1 in the striatum [20] and acute treatments with nicotine decreases methylation of H3 in promoter regions of the methyltransferase gene [21].

Zebrafish (*Danio rerio*) has been proposed as an alternative to mammalian models in several fields, including neuroscience [22, 23]. Zebrafish present all structures and functions of the reward pathway, including epigenetic regulation and all the components of the epigenetic machinery described so far [24–26]. In zebrafish, nicotine induces CPP [27–29] and treatment with the HDAC inhibitor phenylbutyrate (PhB) abrogates nicotine-CPP [29].

In this study, we aimed to mimic nicotine consumption in humans reproducing high and sustained nicotine levels during daytime, as in the case of heavy smokers, followed by abstinence periods when smokers are asleep at night. To this aim, we designed a protocol in which we exposed zebrafish to nicotine continuously during daytime but not during the night for 14 days (D/N-nicotine group). We also designed a group of zebrafish that was exposed to nicotine 24 h a day for 14 days (chronic nicotine group).

Considering our protocol of D/N pre-exposure to nicotine, the zebrafish was a more adequate experimental model for our study purposes. First, long-term or acute exposure to nicotine can be performed with minimal perturbation to the animal behaviour with no multiple injections or osmotic pumps necessary. Secondly, zebrafish can establish a high score and reproducible nicotine-CPP and allow to quantify several parameters determining locomotor activity, anxiety and seeking behaviours in three-dimensional space instead of the usual two-dimensional space used in rodents. The fact that zebrafish are diurnal animals, like humans, is also relevant to our study because zebrafish present daylight activity and sleep at night, meaning that their metabolic rate and physiology regulated by circadian oscillators are in accordance with the time of the day when nicotine is administered (daylight). This is not the case with rats because they are asleep during daytime and their motor activity and metabolic rate are higher at night. Therefore, nocturnal animals will receive nicotine in an inverted phase with respect to their circadian rhythm of activity or will receive nicotine at night, which is at odds with human behaviour and external time of the day. This seems less adequate to our aim of mimicking human behaviour. Furthermore, visual perception is much better during daylight for zebrafish and humans, which have cone-dominated retinas. In contrast, rodents have poor daylight visual acuity and a rod-dominated visual system. In this regard, we have demonstrated that nicotine improves perception and attention in zebrafish [16] as it has been described in humans [30].

We focused in evaluating at behavioural and molecular levels whether pre-exposure to nicotine during daytime, but not at night, has an incidence in the establishment of nicotine-CPP in zebrafish. Moreover, considering our previous findings in rats and zebrafish [14, 29], the effect of a histone acetylation inhibitor treatment during nicotine-conditioning on the expression of relevant genes and proteins in structures of the reward pathway was also evaluated.

Material and Methods

Animal Maintenance

Adult zebrafish (*Danio rerio*, Singapore strain; six to nine months old) were obtained from a local farmer (La Plata, Argentina) [28, 29]. Zebrafish were maintained according to standard methods [31].They were kept in a 120 L tank with a constant 14–10 h light/dark cycle at 26–28 °C, with aquatic plants and stone floor, and fed twice a day with *Artemia* sp. and dry food. All fish were given at least 14 days to acclimatize to the laboratory facility. Afterwards, the animals were moved to the behavioural room and housed in experimental housing tanks (12 × 20 × 15 cm) with 6 animals per tank. Experimental housing tanks were placed in the same room close to CPP tanks. Zebrafish manipulation carried out in this study was described in detail in a book chapter [32]. All animal work was carried out following approval from the University of Buenos Aires Research Ethics Committee. Care was taken to minimize the number of animals used in this work in accordance with the ARRIVE guidelines from the National Centre for the Replacement, Refinement & Reduction of Animals in Research (http://www.nc3rs.org.uk/page.asp?id=1357). Animals were euthanized using terminal anesthesia with tricaine methanesulfonate (MS-222, Sigma-Aldrich).

Drug

Nicotine (nicotine hydrogen tartrate salt, Sigma-Aldrich) was dissolved in system water to produce a 30 μM solution according to our previous studies [16, 28, 29]. Drug concentrations were calculated by weight of the salt. The HDAC inhibitor sodium 4-phenylbutyrate (PhB, Sigma-Aldrich) was dissolved in tank water to obtain a 15 μM solution according to our previous publications [16, 29]. We have previously tested different doses of nicotine that induce positive CPP scores in zebrafish [28]. The dose of nicotine used in this study was selected because it induces high and reproducible CPP scores and is the lowest dose we tested which is far of provoking aversive effects on CPP or any other apparent effect on zebrafish behaviour during CPP [16, 28, 29]. The PhB dose used in this work was extrapolated from the dose previously used in rats in our laboratory and was selected because it provoked inhibitory effects on CPP without causing aversive effects during conditioning to nicotine [14]. It has been demonstrated that PhB is a less potent but highly selective inhibitor for class I and IIa HDACs compared to trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), which show a higher potency but less selectivity [33].

Behavioural Studies

Pre-Exposure to Nicotine Procedure

All zebrafish were separated into the following three pretreatment groups: saline, chronic or intermittent nicotine, in which nicotine was delivered during daylight (D/N). The saline group was exposed to system water while the chronic group was constantly exposed to 30 μM nicotine for 14 days in experimental housing tanks. The D/N group was exposed to 30 μM nicotine during the light cycle (14 h) but not at night (10 h) for 14 days. Water in housing tanks (with or without drug) was changed every two days. Next, each group of zebrafish was divided into the following two subgroups: (1) nicotine-CPP subgroup which consisted of pretreated zebrafish (saline, chronic and D/N) that were selected to initiate the nicotine-CPP protocol and (2) pretreatment subgroup which consisted of pretreated zebrafish from the three groups that were euthanized 18 h after the 14-day exposure period.

Conditioned Place Preference Procedure

CPP was carried out essentially as previously described [28, 34]. The CPP tank has exactly the same characteristics than the tank described in our previous study [34]. Distinct visual cues divided the experimental tank into the following two halves: one half was coloured light-brown and the other half coloured white with six black spots placed at the bottom of the tank. The water level was kept at 12 cm from the bottom of the tank to minimize stress. Briefly, CPP consisted of the following three sessions: (1) *Pretest*. On day one, each fish was tested for baseline place preference over a 10 min period. The preferred compartment was defined as the compartment in which a fish spent most of the time during the pretest. (2) *Conditioning*. One day after pretest, zebrafish were randomly assigned to one of the treatment groups. Each zebrafish of the nicotine-paired group was restricted first to the preferred side (light-brown side) for 20 min and then to the white side, where it was exposed to 30 μM nicotine for 20 min. Zebrafish of the saline solution-treated group (saline-CPP) were handled in the same manner than zebrafish in the nicotine group but without exposure to nicotine. Conditioning was performed for three consecutive days. (3) *Test*. On the fifth day, CPP was tested for each zebrafish in a drug-free environment as it was performed in the pretest. The percentage of time spent on each side of the tank was recorded for 10 min (test session duration). Changes in place preference were determined by a score [(score % = percentage of the time spent in the non-preferred side during test − percentage of the time spent in the non-preferred side during pretest). Behaviour was recorded and videos were analyzed with Noldus Ethovision XT7 software (Noldus Information Technology, The Netherlands; http://www.noldus.com).

Behavioural Analysis Two cameras were used to record videos triggered at the same time, one from the front and the other from the top. The top camera was placed approximately 1.0 m above and the front camera at 0.8 m from the CPP tank. Behavioural data were recorded and analyzed during the last 10 min, after 5 min habituation to the tank, during pretest and test sessions of the CPP task. Zebrafish motor activity and anxiety were measured by analyzing the following parameters: total time spent, number of entries, distance swum in the nicotine-paired side and time swimming in the upper zone of the tank. For the behavioural parameter details, see the Supplementary methods and [28]. The resulting records were analyzed by direct observation and with Noldus Ethovision XT7 software. Time spent and number of entries to the nicotine-paired side are important parameters that complement CPP findings. Distance swum in the nicotine-paired compartment and time swimming in the upper zone of the tank give complementary information about the behavioural effect of nicotine exposure and can be used to determine the anxiety of the fish [35, 36].

Molecular Analysis

Reverse Transcription, Polymerase Chain Reaction, Quantitative Real-Time PCR Data Analysis

Zebrafish from all groups were euthanized after the pretreatments or CPP task. Pools of three brains were homogenized

for RNA extraction using TRIzol-chloroform method. Before homogenization, the olfactory bulb, cerebellum, rhombencephalon and most of the optic tectum were removed from each brain. Three independent samples were examined for gene targeting. Reverse transcription–polymerase chain reaction (RT-PCR) and quantitative qPCR were run from each experimental group with the primers showed in Table 1. For details, see the Supplementary methods and Pisera-Fuster et al. [34].

Immunohistochemistry

After 24 h from the CPP test, zebrafish were euthanized with anesthetic and brains were removed and fixed for 24 h in cold AFA (90% ethanol, 5% formalin, 5% glacial acetic acid). Then, 30-μm-thick coronal cryosections were prepared. Antibodies were directed against acetylated histone H3-K9 (rabbit polyclonal antibody, 1:1000; Abcam, UK) and pCREB (rabbit monoclonal antibody, 1:600, Cell Signaling, MA, USA). For a detailed description of H3-K9Ac and pCREB immunostaining, see the Supplementary methods. Briefly, sections were incubated overnight at 4 °C with primary antibodies. Then, sections were incubated with a biotinylated secondary antibody (Jackson ImmunoResearch Labs, USA) for 2 h at 25 °C and with avidin–biotin–peroxidase complex (Vectastain Elite ABC Kit Universal, Vector Labs, USA). Antibody detection was performed by using 3,3′-diaminobenzidine and H_2O_2. Slices were dehydrated and coverslipped with mounting medium (Vector Labs). The number of positive-stained cells was determined using a light microscope (Olympus, Center Valley, PA, USA) and the optical dissector principle [37, 38] for comparison between control and treatment groups. Quantification of immunolabelling for each antibody was performed in several brain structures using Image-Pro Plus (Media Cybernetics Inc., USA). Appropriate areas were digitally imaged, and quantification was subjected to a stringent criterion according to the staining intensity [39, 40]. For each animal, immunopositive cells were counted on five to six sections. Counts were averaged in squares of 100 μm^2 drawn randomly in the posterior tuberal nucleus (PTN), dorsal habenula (dHb), nucleus interpeduncularis (IPN), dorsal and ventral nucleus of the ventral telencephalic area (Vd/Vv) and tectum opticum (TeO). PTN, dHb, IPN and Vd/Vv were selected because they encompass the dopaminergic mesolimbic reward pathway in the zebrafish brain, and TeO is an associative sensitive polymodal brain structure similar to the cerebral cortex with associative polymodal function in mammals.

Statistical Analysis

CPP score and behavioural data, such as total time spent, number of entries, total distance swum in the nicotine-paired side and time swimming in the upper zone of the tank, were analyzed using two-way ANOVA (group × test, with behaviour as the repeated measure). All ANOVA tests were followed by Bonferroni's post hoc comparisons. For cell number quantification studies, statistical analyses were performed using two-way ANOVA (pre-exposure × treatment, with number of positive cells as the repeated measure) followed by Bonferroni's post hoc tests. Data were presented as the mean ± SEM, and significance was set at $p < 0.05$. All data analyses were computed using StatView 5.0.1 software.

Table 1 Sequence of primers designed to study mRNA levels in the zebrafish brain

Gene	Sense	Antisense
β-Actin (ENSDART00000054987)	TCCCAAAGCCAACAGAGAGAAG	GTCACACCATCACCAGAGTCC
α7 (ENSDART00000051931)	CCGACATCACAGGATACATTGC	GGTAGACGGAATGAGAGGTTCT
α6 (ENSDART00000031546)	TGTCTGACCCTGTTACTGTGG	CATCAAACTCTGCTGGTGACC
β2 (ENSDART00000143043)	TGGAGCCCAGAAGAGTTTGATG	CTCCAATGCTGTCGTCTCCTAT
dnmt1 (ENSDART00000021977)	GGAGGCAGTGGCAGAAGTAA	CCATGTTCTCATCATCCTCAG
egr1 (ENSDART00000054460)	AACGCCACAGCACCTGAAG	TCTGAAAGCGTATCTCCAGCA
gadd45α (ENSDART00000063996)	CATTCTGGTCACGGTTCCA	CTGGAAGGTTGATAATGGGCA
hdac1 (ENSDART00000051799)	AGTACCACAGTGACGACTACA	CCCCTACGTTAAATCTCTGCAT
mecp2 (ENSDART00000040672)	GACGTCTACCTTATCAACCCAG	CGTGAAGTCAAAGTCATTGGG
pitx3 (ENSDART00000102538)	GAATGTAAGCCCGCTGTCCT	GGCCATGTTCATGGAAGGGA
sirt1 (ENSDART00000098209)	TTCAGTGCCACGGGTCTTTT	GGACACCTGGGACAATGAGG
tet1 (ENSDART00000109642)	TCTCATCAACACCCCCTCCA	GCCAGATCCAAGGTGAGTGT

Primers were designed by using Beacon Designer Software (Premier Biosoft International, Palo Alto, CA) from exonic sequences of the zebrafish genome reported in the Ensembl database. Selected primer pairs were specific for the gene of interest and did not hybridize with other sequences

Results

Effect of Pretreatments on Nicotine-CPP in Zebrafish

Conditioned Place Preference

Figure 1 shows nicotine-CPP scores of zebrafish from the three different pre-exposure groups. Two-way ANOVA revealed significant effects of pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p < 0.0001$). Zebrafish of the nicotine-CPP group without previous exposure to nicotine showed a significantly positive score ($p < 0.001$, saline pretreatment-nicotine CPP). Animals chronically pre-exposed to nicotine for 14 days did not show CPP. Zebrafish pre-exposed to D/N-nicotine showed significantly higher scores than saline pre-exposed animals in the nicotine-CPP task ($p < 0.05$) and saline pre-exposed zebrafish in the saline-CPP task ($p < 0.001$). Therefore, zebrafish pre-exposed to D/N-nicotine showed an enhanced nicotine-induced reward response whereas a chronic pretreatment with nicotine prevented nicotine-induced place preference.

Behavioural Analysis of Zebrafish During the Nicotine-CPP Task

Figure 2a shows the total time spent by zebrafish in the nicotine-paired side during CPP. Two-way ANOVA revealed significant effects of pre-exposure ($p = 0.0001$), CPP ($p = 0.0094$) and pre-exposure × CPP interaction ($p < 0.0001$).

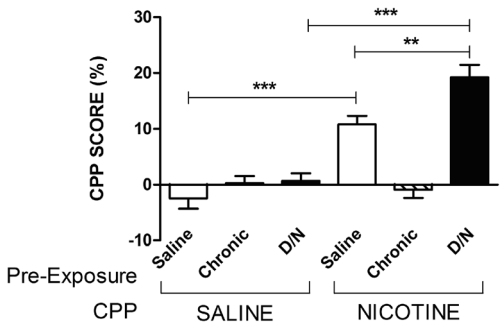


Fig. 1 A nicotine-CPP task was performed with zebrafish that had been previously exposed to nicotine. Zebrafish were pre-exposed constantly (chronic) or intermittently (D/N) to 30 μM nicotine for 14 days and then submitted to a nicotine-CPP or control saline solution-CPP task. The D/N group was exposed to nicotine during the light cycle (14 h) but at night (10 h) nicotine was withdrawn. Other group of zebrafish was exposed to saline solution for 14 days and then it was separated into two groups which were tested in the nicotine- or saline solution-CPP task. Conditioning with nicotine (30 μM) or saline solution was carried out during three days and zebrafish were tested in pretest and test sessions of this task. CPP score was calculated as % of time spent on drug-paired side after nicotine exposure (test) minus "baseline" % of time spent on drug-paired side before nicotine exposure (pretest) over a 300 s time period. Each bar represents mean ± SEM ($n = 8$–9 zebrafish per group). $^{**}p < 0.01$ and $^{***}p < 0.001$ by Bonferroni after two-way ANOVA

Zebrafish in the saline-CPP task showed no significant differences among pretreatment groups. Saline-pretreated zebrafish showed a significant increase of this parameter between pretest and test sessions in the nicotine-CPP task ($p < 0.001$), which supports positive CPP scores found in this group. Zebrafish in the group treated chronically with nicotine showed no significant differences in this parameter in the CPP task, which is consistent with the absence of nicotine-induced CPP. Zebrafish in the group treated with D/N-nicotine showed the biggest difference in this behaviour between pretest and test sessions of the nicotine-CPP ($p < 0.001$; see also Fig. S2), which supports the highest CPP score obtained for this group.

Figure 2b shows a significant reduction in the number of entries to the white side in the test than in the pretest session in the saline-CPP task. Two-way ANOVA revealed significant effects of pre-exposure ($p = 0.0005$), CPP ($p = 0.8219$) and pre-exposure × CPP interaction ($p < 0.0005$). Likewise, in the nicotine-CPP task, chronically nicotine-pretreated zebrafish showed a significant decrease in the number of entries to the nicotine-paired side (white side) in the test session. In contrast, the D/N-nicotine-pretreated zebrafish showed a significant increase in this parameter in the test compared to the pretest session ($p < 0.01$). Zebrafish in the saline-pretreated group did not show significant differences in this motor activity during nicotine-CPP between pretest and test sessions. These findings indicate that the number of times zebrafish entered to the nicotine-paired side positively correlated with high nicotine-CPP scores, suggesting zebrafish with high scores also displayed nicotine-induced seeking behaviour.

The distance swum by zebrafish from saline - and D/N-nicotine-pretreated groups in the nicotine-paired side was significantly enhanced in the test compared to the pretest session ($p < 0.05$). Two-way ANOVA revealed significant effects of pre-exposure ($p < 0.0001$), CPP ($p = 0.0517$) and pre-exposure × CPP interaction ($p = 0.0390$). Zebrafish pre-exposed to chronic nicotine showed the longest distance swum but did not show differences between pretest and test sessions (Fig. 2c).

Figure 2d shows time during which zebrafish swam in the upper zone of the tank in pretest and test sessions of the CPP task. Zebrafish pre-exposed to D/N-nicotine showed the highest values in this parameter during nicotine-CPP test ($p < 0.001$). Zebrafish chronically pretreated with nicotine showed the lowest values in this parameter, indicating zebrafish remained longer periods swimming at deeper positions than other groups of zebrafish during pretest and test sessions of the CPP task. This suggests that chronic nicotine-treated zebrafish developed an anxiety-like behaviour. The two last behavioural parameters confirm the effect of D/N-nicotine pre-exposure on CPP strengthening suggesting intermittent pre-exposure to nicotine affects the

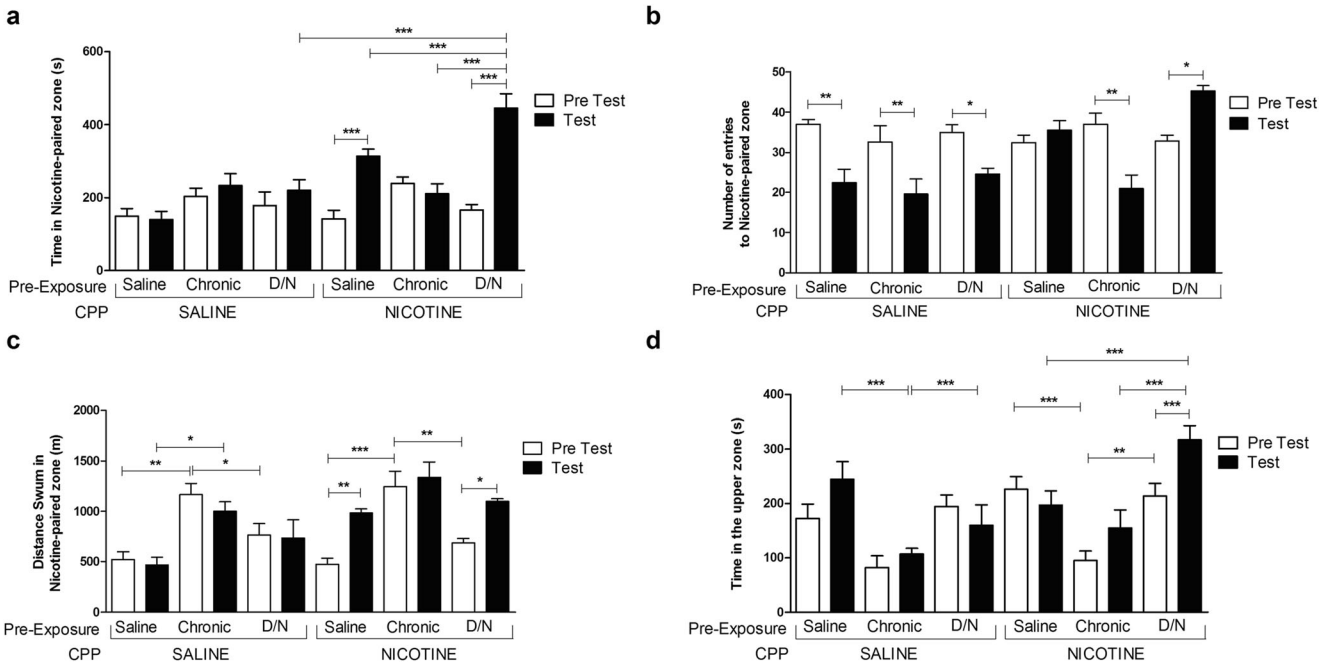


Fig. 2 Different behavioural parameters in zebrafish were analyzed during the CPP task. Different groups of zebrafish performed nicotine- or saline solution-CPP, and their behaviour was evaluated during pretest (white bars) and test (dark bars) sessions of conditioning. Before the CPP task, zebrafish were pre-exposed for 14 days to saline solution (vehicle), chronic nicotine (chronic: 30 μM nicotine delivered 24 h a day) and D/N-nicotine (D/N: 30 μM nicotine delivered for 14 h (at daylight) and withdrawn for 10 h every night). Nicotine was dissolved in the fish tank water in the white compartment, which was the non-preferred side of the tank by zebrafish during pretest. Graph in (**a**) depicts total time spent in the drug-paired side. Graph in (**b**) shows the number of entries to the white compartment. Graph in (**c**) depicts the distance swum in the white compartment. Graph in (**d**) shows time zebrafish spent swimming in the upper zone of the tank during pretest and test sessions. Each bar represents mean ± SEM ($n = 8$–9 zebrafish per group). $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ by Bonferroni after two-way ANOVA

reinforcing properties of nicotine without significantly modifying anxiety.

Effect of the HDAC Inhibitor PhB on Nicotine-CPP in Zebrafish Previously Exposed to Nicotine

Considering the robust increase in nicotine-CPP scores observed in the D/N-nicotine pretreated group, we next evaluated the effect of an HDAC inhibitor PhB, which according to our previous studies prevents nicotine-CPP in rats and zebrafish. Two-way ANOVA revealed significant effects of pre-exposure ($p = 0.0110$) and CPP ($p < 0.0001$) but not of pre-exposure × CPP interaction ($p = 0.1022$).

Figure 3 shows nicotine-CPP score in zebrafish exposed to the HDAC inhibitor PhB, during conditioning. Zebrafish chronically pre-exposed to nicotine for 14 days were omitted from this study because they did not establish CPP. Zebrafish that were pre-exposed to D/N-nicotine for 14 days and performed the nicotine-CPP task showed a significant increase in the CPP score compared with saline pre-exposed animals that also established nicotine-CPP ($p < 0.01$). The HDAC inhibitor, showed no effect when it was administered in the absence of nicotine during saline-CPP. When PhB was co-administered with nicotine during three days of conditioning, CPP score was reduced to zero in zebrafish pretreated with saline and D/N-nicotine, indicating that the HDAC inhibitor prevented the associative learning that links nicotine with the place in which it was administered.

Behavioural Characterization of Zebrafish Exposed to the HDAC Inhibitor PhB During Nicotine-CPP

Two-way ANOVA revealed no significant effects of pre-exposure ($p = 0.7913$), CPP ($p = 0.0219$) or pre-exposure × CPP interaction ($p = 0.3910$) in the total time spent by any group of zebrafish in the nicotine-paired side in pretest and test sessions when zebrafish were exposed to both nicotine and PhB during three days of conditioning (Fig. 4a). This parameter was in agreement with score values of zero in these experimental groups of zebrafish as shown in Fig. 3.

Next, the effect of the HDAC inhibitor on nicotine-CPP was further evaluated by analyzing the number of entries to the drug-paired side before (pretest) and after (test) conditioning (Fig. 4b). Two-way ANOVA revealed no significant effects of pre-exposure ($p = 0.3180$), CPP ($p = 0.9965$) or pre-exposure × CPP interaction ($p = 0.9777$), which was also consistent with the absence of nicotine-induced CPP establishment in the presence of the inhibitor.

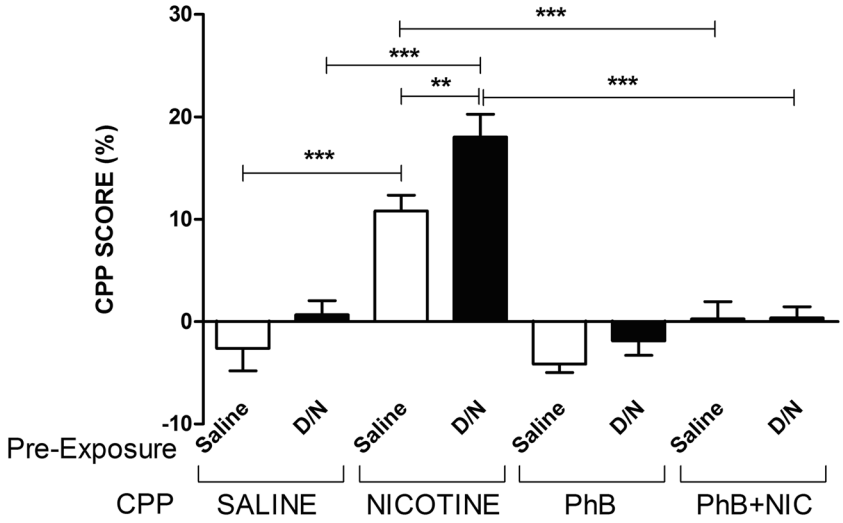


Fig. 3 Effect of the histone deacetylase inhibitor phenylbutyrate (PhB) on nicotine-induced-CPP performed by zebrafish pre-exposed to nicotine. Zebrafish were pre-exposed intermittently to 30 μM nicotine (D/N) or saline for 14 days and then submitted to a nicotine- or control saline solution-CPP task. The D/N group was exposed to nicotine during the light cycle (14 h) and at night (10 h) nicotine was withdrawn. Conditioning was carried out during three days with 30 μM nicotine, saline solution (vehicle), 30 μM nicotine plus 15 μM PhB, or saline solution plus 15 μM PhB diluted in the fish tank water. Drugs were delivered in the white compartment that was the non-preferred side of the tank by zebrafish during the pretest. Zebrafish were tested in pretest and test sessions of the CPP task. CPP score was calculated as % of time spent on drug-paired side after nicotine exposure (test) minus "baseline" % of time spent on drug-paired side before nicotine exposure (pretest) over a 300 s time period. Graph bars indicate the CPP score in saline and D/N-nicotine pre-exposed zebrafish during saline-CPP and nicotine-CPP with or without the HDAC inhibitor PhB. Each bar represents mean ± SEM ($n = 7$–9 zebrafish per group). $^{**}p < 0.01$ and $^{***}p < 0.001$ by Bonferroni after two-way ANOVA

Moreover, the total distance swum by zebrafish during pretest and test sessions was also evaluated (Fig. 4c). Two-way ANOVA showed no significant effects of pre-exposure ($p = 0.1773$), CPP ($p = 0.0907$) or pre-exposure × CPP interaction ($p = 0.5990$). Finally, the time zebrafish swam in the upper zone of the tank was measured during pretest and test sessions of the CPP task in the presence of the HDAC inhibitor. No significant changes were observed among experimental groups of zebrafish (Fig. 4d). These findings suggest that the lack of nicotine-induced CPP in the presence of the HDAC inhibitor failed to stimulate locomotor activity—which could indicate a seeking for drug response after reward establishment—and reduce anxiety-like behaviours as it was observed between pretest and test sessions when nicotine reward was strongly established (positive CPP score) as shown in Fig. 2c and d.

Expression Levels of nAChR Subunit and Epigenetic Enzyme mRNA in the Brain of Adult Zebrafish Exposed to Nicotine for 14 Days

In order to evaluate the effect of pre-exposure to nicotine at a molecular level, genetic marker expression was evaluated after pre-treatments with nicotine for 14 days (Fig. 5). We have used portions of the brain that include the areas of the zebrafish dopaminergic system from the subpallium to the basal diencephalon (see the "Material and Methods" section). These areas are homologous to mammalian brain areas of the reward dopaminergic pathway, which are involved in virtually the same functions in all vertebrate animals studied so far. Nicotine was administered continuously or intermittently—during daylight only—as indicated for all pre-exposure experiments, and animals were euthanized right after these treatments. Levels of all nicotinic acetylcholine receptor (nAChR) subunit mRNAs analyzed were significantly increased by both chronic and intermittent exposure to nicotine (one-way ANOVA: α7: $F_{2,17} = 73.07$, $p < 0.0001$; α6: $F_{2,17} = 147.1$; β2: $F_{2,17} = 97.83$, $p < 0.0001$, $p < 0.001$; Fig. 5a–c), but chronic nicotine effect was more potent. Next, we measured relative levels of histone deacetylase 1 (HDAC1) mRNA. HDACs catalyze acetyl group removal from lysine residues in histones and non-histone proteins, causing epigenetic transcriptional repression. Levels of HDAC1 mRNA were increased in the brain of zebrafish exposed to chronic and D/N nicotine compared to saline treatment ($F_{2,17} = 62.32$, $p < 0.001$; Fig. 5d). We next determined relative levels of NAD-dependent deacetylase sirtuin-1 (SIRT1) mRNA that also regulates gene expression silencing via an epigenetic mechanism. Relative levels of SIRT1 mRNA showed a significant increase in D/N nicotine-treated zebrafish but a decrease in chronic nicotine-treated animals ($F_{2,17} = 83.23$, $p < 0.0001$, $p < 0.01$ and $p < 0.001$; Fig. 5e). We have also measured relative levels of lysine acetyltransferase CREB-binding protein (CBP) mRNA, which showed enhanced values in the D/N nicotine-treated zebrafish ($F_{2,17} = 43.36$, $p < 0.001$; Fig. 5f). There are no previous reports analyzing

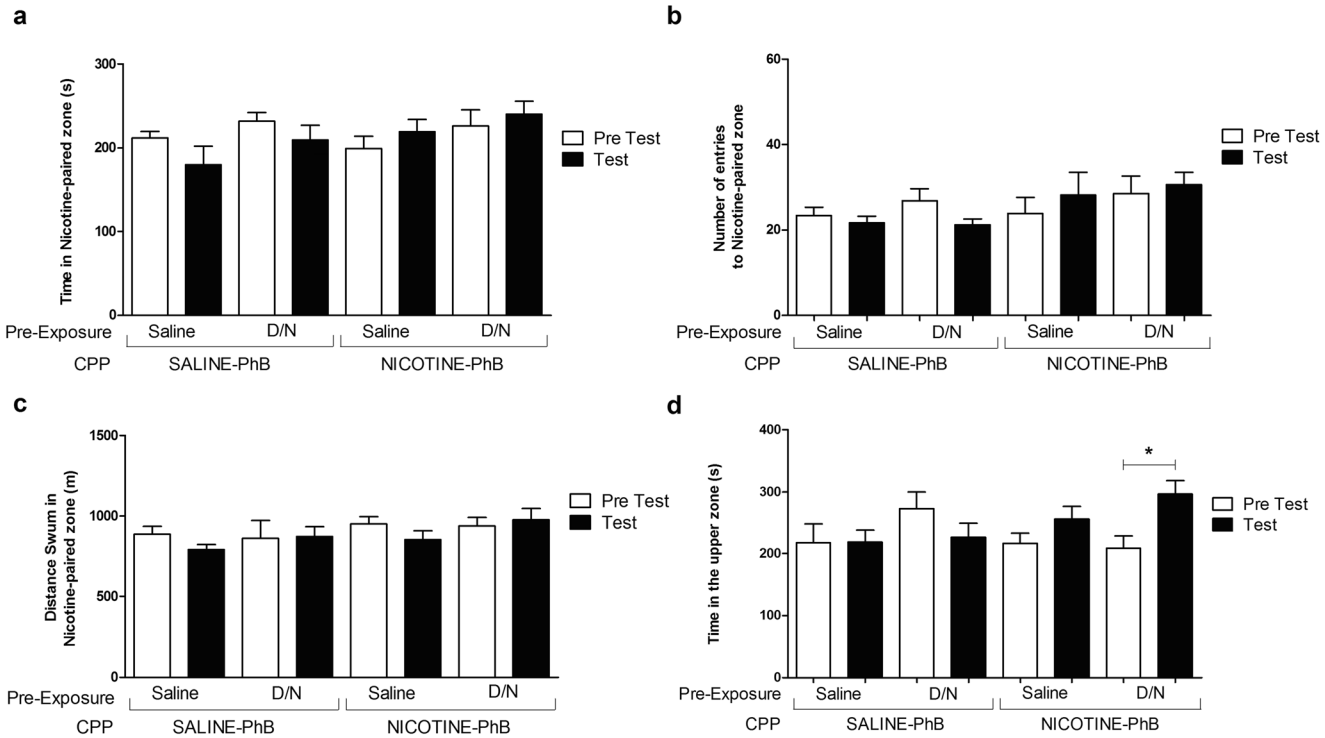


Fig. 4 Behavioural analysis was performed in zebrafish exposed to the histone deacetylase inhibitor phenylbutyrate (PhB) during nicotine-CPP. Different behavioural parameters were examined during pretest (white bars) and test (dark bars) sessions of the saline- and nicotine-CPP task in the presence or absence of PhB. Before CPP, zebrafish were exposed intermittently to 30 μM nicotine (D/N) or saline solution (vehicle) for 14 days. The D/N group was exposed to nicotine during the light cycle for 14 h and nicotine was withdrawn at night for 10 h. Conditioning was carried out during three days with saline solution, saline solution plus 15 μM PhB, 30 μM nicotine, or 30 μM nicotine plus 15 μM PhB were diluted in the fish tank water. The white compartment was the non-preferred side of the tank by zebrafish during pretest. Nicotine, PhB, or both drugs were delivered in the white compartment during saline- or nicotine-CPP. Graph in (**a**) shows the total time spent by zebrafish in the white compartment. Graph in (**b**) depicts the number of entries to the white compartment. Graph in (**c**) shows the distance swum by zebrafish in the white compartment. Graph in (**d**) shows time zebrafish spent swimming in the upper zone of the tank during pretest and test sessions. Each bar represents mean ± SEM ($n = 7$–9 zebrafish per group). *$p < 0.05$ by Bonferroni after two-way ANOVA

DNA methylation catalyzed by DNMT1 and DNMT3ab in the brain of zebrafish exposed to nicotine. We quantified relative levels of DNMT1 mRNA and found that they were reduced in chronic nicotine-treated zebrafish but enhanced in D/N nicotine-treated animals ($F_{2,17} = 361.4$, $p < 0.001$; Fig. 5g). Contrarily to DNMT1, relative levels of DNMT3ab mRNA showed increased values in chronic nicotine-treated animals and reduced levels in D/N nicotine-treated zebrafish ($F_{2,17} = 183.2$, $p < 0.001$; Fig. 5h). Next, we measured gene expression levels of two enzymes that regulate DNA demethylation in the zebrafish brain containing the reward pathway structures. Methylcytosine dioxygenase TET1 enzyme (TET1) catalyzes 5-methylcytosine hydroxylation which promotes demethylation of DNA in the central nervous system [41]. Levels of TET1 mRNA were significantly increased by both chronic- and D/N-nicotine treatments ($F_{2,17} = 230.3$, $p < 0.001$; Fig. 5i). Gadd45 regulates the final step of DNA demethylation using as substrates 5-hydroxymethylcytosines [42]. Levels of Gadd45 mRNA were significantly enhanced only by the intermittent D/N-nicotine treatment ($F_{2,17} = 224.4$, $p < 0.001$; Fig. 5j). Next, we quantified relative levels of methyl-CpG-binding protein 2 (MeCP2) mRNA which encodes a protein that associates to chromatin inducing and repressing gene transcription. MeCP2 mRNA displayed increased levels in the brain of chronic and D/N-nicotine-exposed zebrafish ($F_{2,17} = 224.1$, $p < 0.001$). The chronic treatment with nicotine caused the strongest induction in the expression of MeCP2 gene ($p < 0.001$; Fig. 5k). Subsequently, we examined levels of PITX3 mRNA, a transcription factor expressed only in midbrain dopamine containing neurons. This gene expression is important for differentiation and maintenance of mesencephalic dopaminergic neurons, including neurons of the reward mesolimbic pathway. Immediate early gene *egr1* expression is involved in maintaining the dopaminergic phenotype in rodents and colocalize with tyrosine hydroxylase expression in dopaminergic neurons in the brain of zebrafish [43]. We found basal expression levels of transcription factor PITX3 and EGR1 mRNA in the zebrafish brain with no differences among saline and nicotine treatments (Fig. 5l,m). Data presented in Fig. 5 indicate that continuous or intermittent nicotine exposure for 14 days increased relative levels of all nAChR subunit mRNA analyzed. Levels

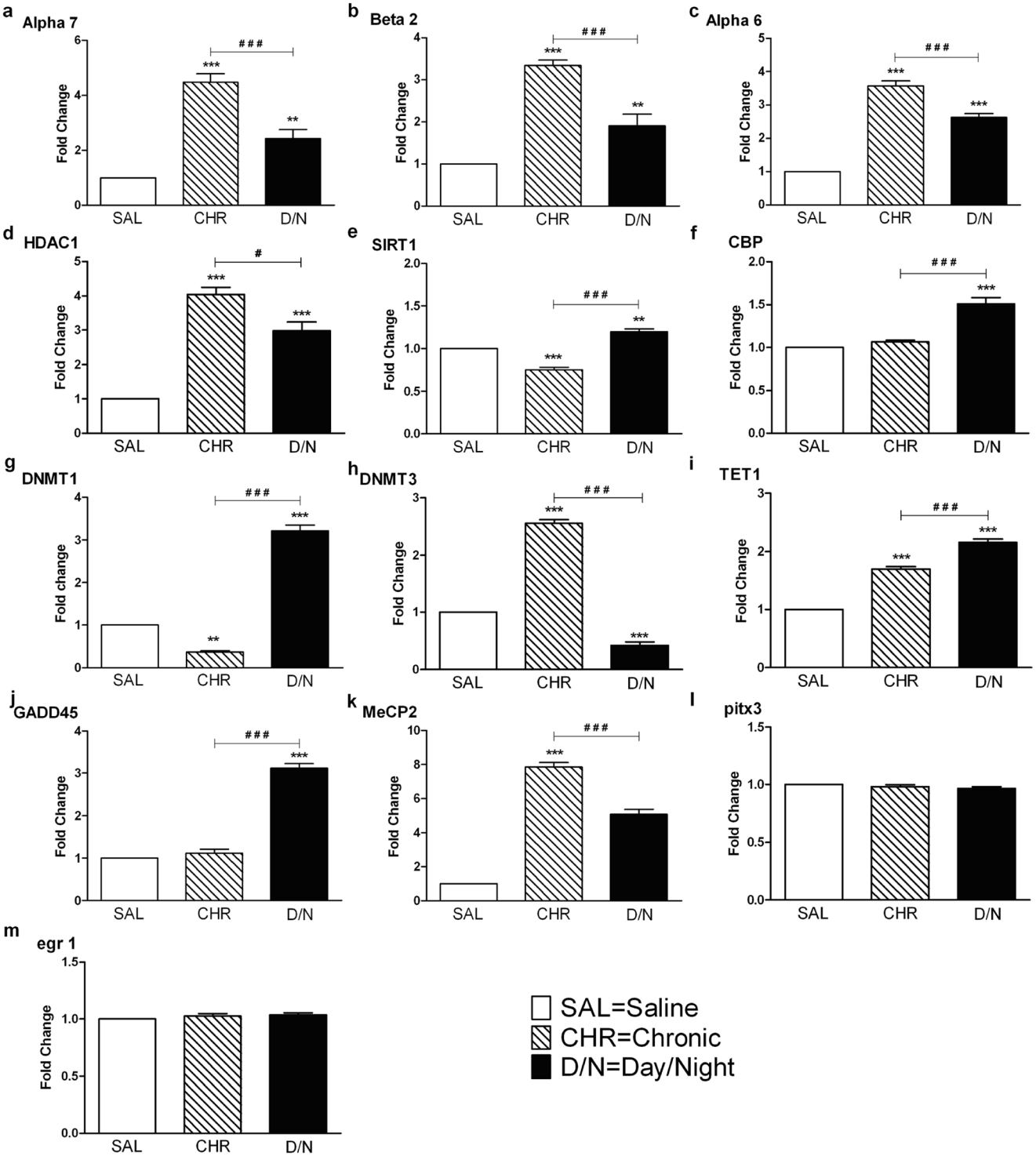


Fig. 5 Effect of pre-exposure to nicotine—delivered chronically or following a day/night schedule—on the expression of nicotinic receptor subunits and epigenetic factors in portions of zebrafish brain containing reward pathway structures. Zebrafish were exposed for 14 days to saline solution (vehicle) or nicotine diluted in the fish tank water and delivered constantly 24 h a day (chronic) or intermittently during daylight hours for 14 h and withdrawn for 10 h at night (D/N). Levels of different genetic marker mRNA were assessed by RT-qPCR. Graphs show the relative levels (fold change) of α7 (**a**), β2 (**b**) and α6 (**c**) nicotinic receptor subunit mRNA. The epigenetic factors analyzed by this method comprised enzymes controlling histone acetylation and DNA methylation. Regarding histone acetylation, relative expression of HDAC1 (**d**), SIRT1 (**e**), and CBP (**f**) genes was quantified. To evaluate transcriptional expression of enzymes involved in DNA methylation and demethylation, levels of DNMT1 (**g**), DNMT3 (**h**), TET1 (**i**), Gadd45 (**j**) and MeCP2 (**k**) mRNA were determined. Relative levels of transcription factor PITX3 (**l**) and EGR1 (**m**) mRNA were also assessed. D/N, day/night. Data are depicted as mean ± SEM ($n = 7$–9 zebrafish per group). *$p < 0.05$ and ***$p < 0.001$ by Bonferroni after ANOVA

of histone acetyltransferase CBP mRNA were enhanced in D/N-nicotine-treated zebrafish whereas levels of HDAC mRNA showed significant increases in both groups of nicotine-treated animals suggesting a higher level of deacetylation in chronic nicotine-treated zebrafish and a dynamic cycle of acetylation/deacetylation in zebrafish exposed to D/N-nicotine. In this study, we found that DNMT1 was increased in D/N-nicotine exposed zebrafish whereas DNMT3ab was increased in the group treated continuously with nicotine. DNMT3 participates in *de novo* methylation of DNA while DNMT1 promotes maintenance of DNA methylated states [44]. These findings suggest the intensification of a DNA methylation maintenance mechanism in zebrafish exposed to a D/N-nicotine treatment. DNA demethylating enzymes were highly expressed in D/N-nicotine pre-exposed zebrafish. This also suggests a dynamic process of methylation and demethylation in D/N-nicotine pre-exposed animals.

Expression Levels of nAchR Subunit and Epigenetic Enzyme mRNA in the Brain of Adult Zebrafish Exposed to Nicotine for 14 Days and After Performing a Nicotine-CPP Task

We first analyzed relative levels of nAChR α7-, β2- and α6-subunit mRNA in zebrafish brain portion containing structures of the reward pathway (Figs. 6a,b and S1a) because we have previously demonstrated that these nAChR subunits were transcriptionally upregulated after zebrafish performed a CPP task induced by nicotine. Two-way ANOVA showed significant effects in α7-subunit: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p = 0.0001$); α6-subunit: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p = 0.0004$); and β2-subunit: pre-exposure ($p < 0.0001$), CPP ($p = 0.4941$) and pre-exposure × CPP interaction ($p = 0.9503$). Zebrafish from the chronic nicotine group after CPP showed significant changes in the three subunits analyzed compared to the saline-pretreated group ($p < 0.001$). The relative expression level of α7- and α6-subunit mRNA in D/N nicotine-pretreated groups was significantly enhanced in the nicotine-CPP group ($p < 0.001$) whereas β2 subunit showed no significant changes (Fig. S1a). When zebrafish were conditioned in the presence of the HDAC inhibitor PhB, levels of α7- and α6-subunit mRNA showed a significant decrease in saline- and D/N-nicotine-pretreated animals that performed the nicotine-CPP task ($p < 0.01$) (Fig. 6a,b).

These findings suggest that the HDAC inhibitor prevented nicotine effect during conditioning on nAchR subunit transcriptional expression upregulation.

Next, we analyzed relative levels of HDAC1 mRNA. ANOVA revealed changes in pre-exposure ($p < 0.0001$), CPP ($p = 0.0004$) and pre-exposure × CPP interaction ($p = 0.0007$). Levels of HDAC1 mRNA were significantly increased in saline- and D/N-nicotine-pretreated zebrafish that performed nicotine-CPP compared to groups that performed the CPP task without nicotine (saline-CPP) ($p < 0.001$) (Fig. 6c). Treatment with the HDAC inhibitor during CPP decreased HDAC1 transcriptional expression in all groups of animals examined ($p < 0.001$). Interestingly, levels of HDAC1 mRNA were significantly reduced in the brain of zebrafish with chronic nicotine-pretreatment after the nicotine-CPP task ($p < 0.001$). Nicotine-CPP also provoked a significant increase in the transcriptional expression of another histone deacetylase SIRT1 in zebrafish brain from saline- and D/N-nicotine-pretreated groups (ANOVA: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p = 0.0009$)). Treatment with the HDAC inhibitor during CPP inhibited SIRT1 transcriptional expression (Fig. S1b). Relative levels of histone acetyltransferase CBP mRNA were significantly reduced in all the groups when zebrafish were exposed to nicotine during the CPP task (Fig. 6d; $p < 0.001$). In contrast, treatment with the HDAC inhibitor caused a significant increase in the transcriptional expression of CBP ($p < 0.001$).

The transcriptional expression of enzymes involved in DNA methylation was also analyzed. Relative levels of DNMT1 mRNA were significantly enhanced in saline- and D/N-nicotine-pretreated zebrafish after the nicotine-CPP task (Fig. 6e; $p < 0.05$ and $p < 0.001$, respectively) (two-way ANOVA: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p < 0.0001$)). Relative levels of DNMT1 mRNA were strongly induced by treatment with the HDAC inhibitor during nicotine-CPP in D/N-nicotine- and saline-pretreated zebrafish ($p < 0.001$). Chronic nicotine-pretreated animals showed very low levels of DNMT1 mRNA that were not modified by CPP. Levels of DNMT3 mRNA significantly increased in the brain of chronic nicotine-pretreated zebrafish, and maximal levels of this enzyme mRNA were observed in zebrafish that performed the nicotine-CPP task (Fig. S1c; $p < 0.001$). Two-way ANOVA: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p < 0.0001$). As aforementioned, DNA demethylation involves TET1 and Gadd45 activity. Zebrafish that were pretreated with saline and D/N-nicotine and performed nicotine-CPP tasks in either the presence or absence of the HDAC inhibitor PhB showed enhanced relative levels of TET1 mRNA (Fig. 6f; two-way ANOVA: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p = 0.0005$)). In contrast, levels of TET1 mRNA were reduced in animals that received a chronic treatment with nicotine and performed the nicotine-CPP task. Levels of Gadd45 mRNA showed a significant increase in zebrafish intermittently treated with D/N-nicotine and after nicotine-CPP performed with or without the HDAC inhibitor or saline-CPP performed with the HDAC inhibitor (Fig. S1d; $p < 0.001$). Two-way ANOVA: pre-exposure ($p < 0.0001$),

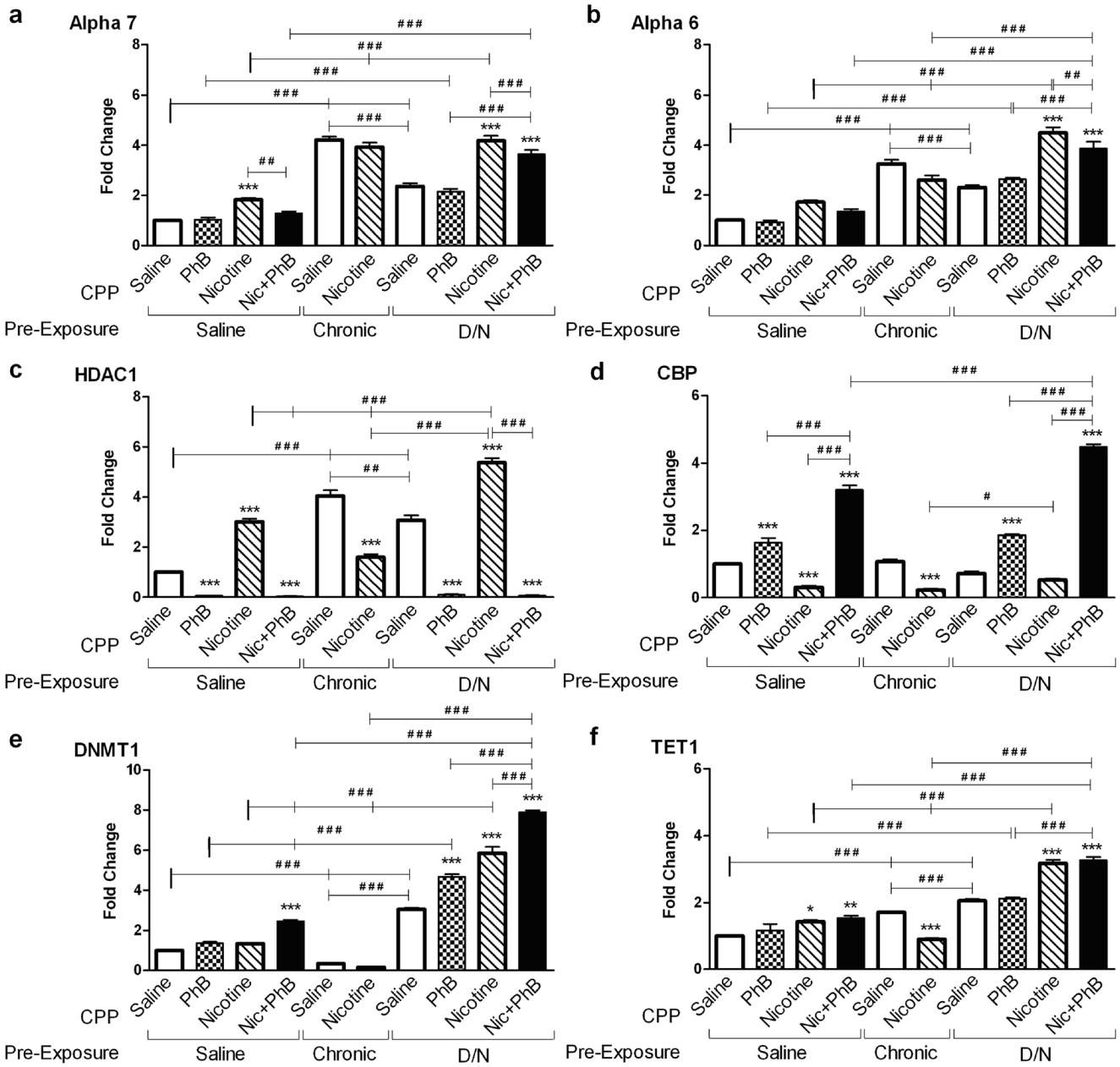


Fig. 6 Levels of nicotinic receptor subunit and epigenetic enzyme mRNA were identified by RT-qPCR in zebrafish brain portions containing structures of the reward pathway after nicotine pre-exposure followed by nicotine-CPP. Each graph represents the relative level (fold change) of expression for each type of mRNA tested in the different experimental groups. Experimental groups consisted in zebrafish pretreated with saline solution, chronic, or intermittent nicotine (D/N) delivered for 14 days. During pre-exposure, nicotine was delivered in the fish tank water for 24 h a day (chronic group) o for 14 h during daylight only (D/N group). After pre-exposure, zebrafish from the saline or D/N groups performed saline- or nicotine-CPP in the presence or absence of the histone acetylase inhibitor phenylbutyrate (PhB). Zebrafish from the chronic nicotine pre-exposed group performed saline- or nicotine-CPP without PhB treatment. Relative levels of mRNA for the different genes were measured and quantified by RT-qPCR (see the "Material and Methods" section). Relative transcriptional expression of nicotinic receptor α7- and α6-subunit, enzymes involved in acetylation of histone tails such as HDAC1 and CBP, and enzymes involved in methylation and demethylation of DNA such as DNMT1 and TET1, was assessed in the zebrafish brain. D/N, day/night. Data were expressed as mean ± SD from three independent experiments ($n = 7$–9 zebrafish per group). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ in comparison with Sal-SAL group (intra pre-exposed group). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, comparison among statistically different pretreated and CPP groups

CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p < 0.0001$). Transcriptional levels of MeCP2 significantly increased in chronic nicotine-pretreated groups and only after saline- or nicotine-CPP in D/N-nicotine-pretreated zebrafish (two-way ANOVA: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction

($p < 0.0001$)). Treatment with the HDAC inhibitor PhB reduced the expression of MeCP2 mRNA in all the experimental conditions assessed (Fig. S1e; $p < 0.001$).

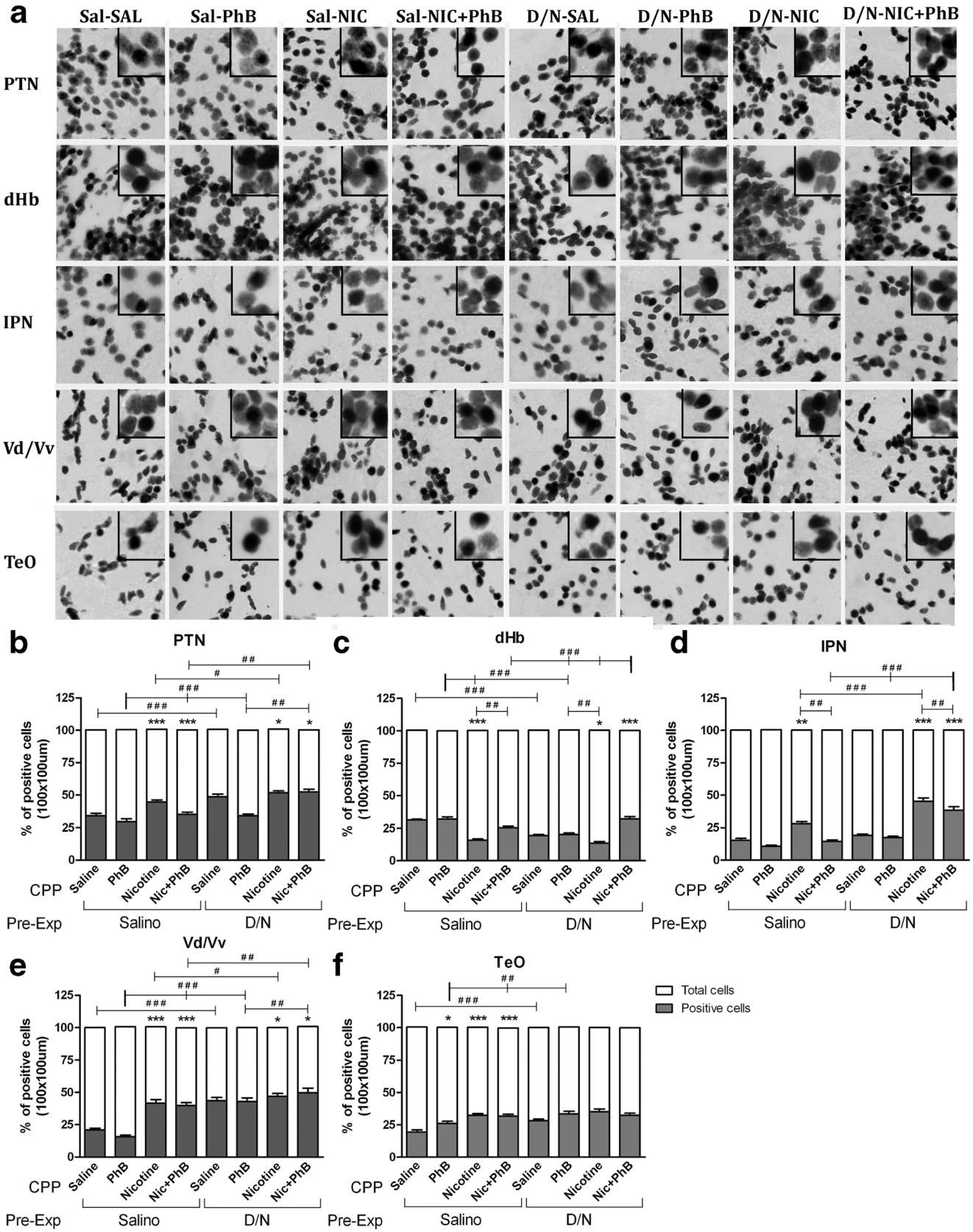
Finally, we evaluated the transcriptional expression of transcription factors PITX3 and EGR1 (Fig. S1f,g). Enhanced levels of PITX3 mRNA were observed after nicotine-CPP, with or without treatment with the HDAC inhibitor, in zebrafish brains pre-exposed to saline- and D/N-nicotine (Fig. S1f; $p < 0.001$). Levels of EGR1 mRNA showed a significant increase after nicotine-CPP, with or without treatment with the HDAC inhibitor, in zebrafish brains pre-exposed to D/N-nicotine (Fig. S1g; $p < 0.001$; $p < 0.01$). Nicotine-CPP decreased gene expression of both transcription factors in zebrafish brains pre-exposed to chronic nicotine (Fig. S1f,g; $p < 0.001$).

In these studies, we observed that nAChR examined were up-regulated by a treatment with nicotine for 14 days. However, only α6- and α7-subunits were further increased by nicotine induced-CPP. Moreover, nicotine-CPP increased the transcriptional expression of enzymes involved in histone acetylation principally in the brain of zebrafish that were pre-exposed to D/N-nicotine, whereas treatment with the histone deacetylase inhibitor PhB increased the expression of acetyltransferases. Enzymes involved in DNA methylation (CBP) and demethylation (TET1 and Gadd45) were increased by nicotine-CPP and the HDAC inhibitor principally in the brain of the D/N-nicotine pre-exposed zebrafish.

Number of Cells Expressing pCREB and H_3K_9Ac in Reward Pathway Structures of the Zebrafish Brain

Figures 7 and 8 depict immunoreactivity for pCREB and H3K9Ac detected in cells of different nuclei of the dopaminergic mesolimbic reward pathway in zebrafish. Particularly cells that showed higher contents of immunoreactivity than other cells were detected by a noticeable increase in staining density (Figs. 7a and 8a). Staining density for pCREB or H3K9Ac was considered significantly enhanced when it was above 50% of the level of staining ranging from 0 to 100% detected in all cells observed in the whole surface of a particular nucleus. Cells meeting this condition were counted as pCREB- or H3K9Ac-positive cells (see the "Material and Methods" section). As shown in Fig. 7, in the posterior tuberal nucleus (PTN), the number of pCREB-positive cells showed significant increases in all groups of zebrafish that were exposed to nicotine during conditioning, D/N-nicotine pretreatment or both protocols in either presence or absence of the HDAC inhibitor PhB (Fig. 7b; $p < 0.001$). Two-way ANOVA: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p = 0.1180$). In contrast, pCREB-positive cell number was significantly decreased in the dorsal habenula (dHb) in zebrafish that performed the nicotine-CPP task and was previously exposed to saline solution or D/N-nicotine (Fig. 7c). Treatment with the HDAC inhibitor during nicotine-CPP significantly increased pCREB content in cells of the dHb in zebrafish pre-exposed to D/N-nicotine and saline solution ($p < 0.001$). Two-way ANOVA: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p = 0.0007$). In the interpeduncular nucleus (IPN), nicotine-CPP caused a significant enhancement of pCREB-positive cells that was potentiated by D/N-nicotine pre-exposure (Fig. 7d) whereas HDAC inhibition with PhB significantly reduced pCREB content in IPN cells. In zebrafish pre-exposed to saline solution, the HDAC inhibitor abrogated nicotine-CPP effect on CREB phosphorylation in IPN cells ($p < 0.01$). Two-way ANOVA: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p < 0.0001$). Figure 7e shows that D/N-nicotine pretreatment and nicotine-CPP increased pCREB content in a major number of cells in the dorsal and ventral nucleus of the ventral telencephalic area (Vd/Vv). Two-way ANOVA: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p = 0.1062$). Treatment with the HDAC inhibitor did not affect cell content of pCREB in the Vd/Vv area. In the tectum opticum (TeO), the number of pCREB-positive cells was significantly increased by any protocol of exposure to nicotine tested (CPP and D/N pretreatment). Treatment with the HDAC inhibitor also had a weak effect on pCREB induction in TeO cells (Fig. 7f). Two-way ANOVA: pre-exposure

Fig. 7 Detection and quantification of pCREB-immunoreactive cells in four areas composing the reward pathway of the zebrafish brain. ▶ Photomicrographs show pCREB-positive cells in the posterior tuberal nucleus (PTN), dorsal habenula (dHb), nucleus interpeduncularis (IPN), dorsal and ventral nucleus of the ventral telencephalic area (Vd/Vv) and tectum opticum (TeO). Zebrafish were pre-exposed to saline or an intermittent delivery of nicotine during daylight only for 14 h (D/N). After pre-exposure, zebrafish performed a CPP task with saline only (sal-CPP) or with nicotine, which was delivered in the non-preferred side of the tank by zebrafish during pretest (nicotine-CPP). A treatment with the histone deacetylase inhibitor phenylbutyrate (PhB) was administered during the three days of the CPP protocol performed with saline solution or nicotine. D/N, day/night. Group nomenclature: Sal-SAL, saline-pretreated and saline-CPP; Sal-PhB, saline-pretreated and PhB treatment during saline-CPP; Sal-NIC, saline-pretreated and nicotine-CPP; Sal-NIC + PhB, saline-pretreated and PhB treatment during nicotine-CPP; D/N-SAL, nicotine-pretreated and saline-CPP; D/N-PhB, nicotine-pretreated and PhB treatment during saline-CPP; D/N-NIC, nicotine-pretreated and nicotine-CPP; D/N-NIC + PhB, nicotine-pretreated and PhB treatment during nicotine-CPP. Bar graphs indicate the percent of pCREB-positive cells under a stringent criterion (black bar) from the total percent (100%) of stained cells. pCREB-positive cells were considered the ones that showed at least a 50% increase in stain density over basal density from all stained cells on the surface of the particular structure analyzed (see the "Material and Methods" and "Results" sections). CPP, conditioned place preference; Pre-exp, pre-exposure. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ in comparison with Sal-SAL group (intra pre-exposed group). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, comparison among statistically different pretreated and CPP groups. The number of pCREB-positive cells was assessed with two-way ANOVA followed by Bonferroni test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Sal-SAL
Sal-PhB
Sal-NIC
Sal-NIC+PhB
D/N-SAL
D/N-PhB
D/N-NIC
D/N-NIC+PhB
PTN
dHb
IPN
Vd/Vv
TeO
b
PTN
c
dHb
d
IPN
e
Vd/Vv
f
TeO
% of positive cells (100x100um)
CPP
Saline
PhB
Nicotine
Nic+PhB
Pre-Exp
Salino
D/N
Total cells
Positive cells

Fig. 8 Detection and quantification of immunoreactive cells for histone 3 acetylated in lysine 9 (H3K9Ac) in the PTN, dHb, IPN, Vd/Vv and TeO of the zebrafish brain. Group nomenclature: Sal-SAL, saline-pretreated and saline-CPP; Sal-PhB, saline-pretreated and PhB treatment in saline-CPP; Sal-NIC, saline-pretreated and nicotine-CPP; Sal-NIC + PhB, saline-pretreated and PhB treatment during nicotine-CPP; D/N-SAL, nicotine-pretreated and saline-CPP; D/N-PhB, nicotine-pretreated and PhB treatment during saline-CPP; D/N-NIC, nicotine-pretreated and nicotine-CPP; D/N-NIC + PhB, nicotine-pretreated and PhB treatment during nicotine-CPP. Bar graphs indicate the percent of H3K9Ac-positive cells under a stringent criterion (black bar) from the total percent (100%) of stained cells. H3K9Ac-positive cells were considered the ones that showed at least a 50% increase in stain density over basal density from all stained cells on the surface of the particular structure analyzed (see the "Material and Methods" and "Results" sections). PTN, posterior tuberal nucleus; dHb, dorsal habenula; IPN, nucleus interpeduncularis; Vd/Vv, dorsal and ventral nucleus of the ventral telencephalic area; TeO, tectum opticum; PhB, histone deacetylase inhibitor phenylbutyrate. CPP, conditioned place preference; Pre-exp, pre-exposure. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, comparison with Sal-SAL group (intra pre-exposed group). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, comparison among statistically different groups pre-treated and after CPP. The number of positive cells was assessed with two-way ANOVA followed by Bonferroni test

($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p = 0.0348$).

Nicotine before and during CPP generally increased pCREB content in almost all areas analyzed suggesting cell activation in nuclei of the reward pathway and TeO induced by nicotine and CPP establishment. In contrast, pCREB level reduction induced by nicotine and CPP and pCREB level induction caused by the HDAC inhibitor suggests a cell activity inhibition in the dHb caused by nicotine-CPP establishment.

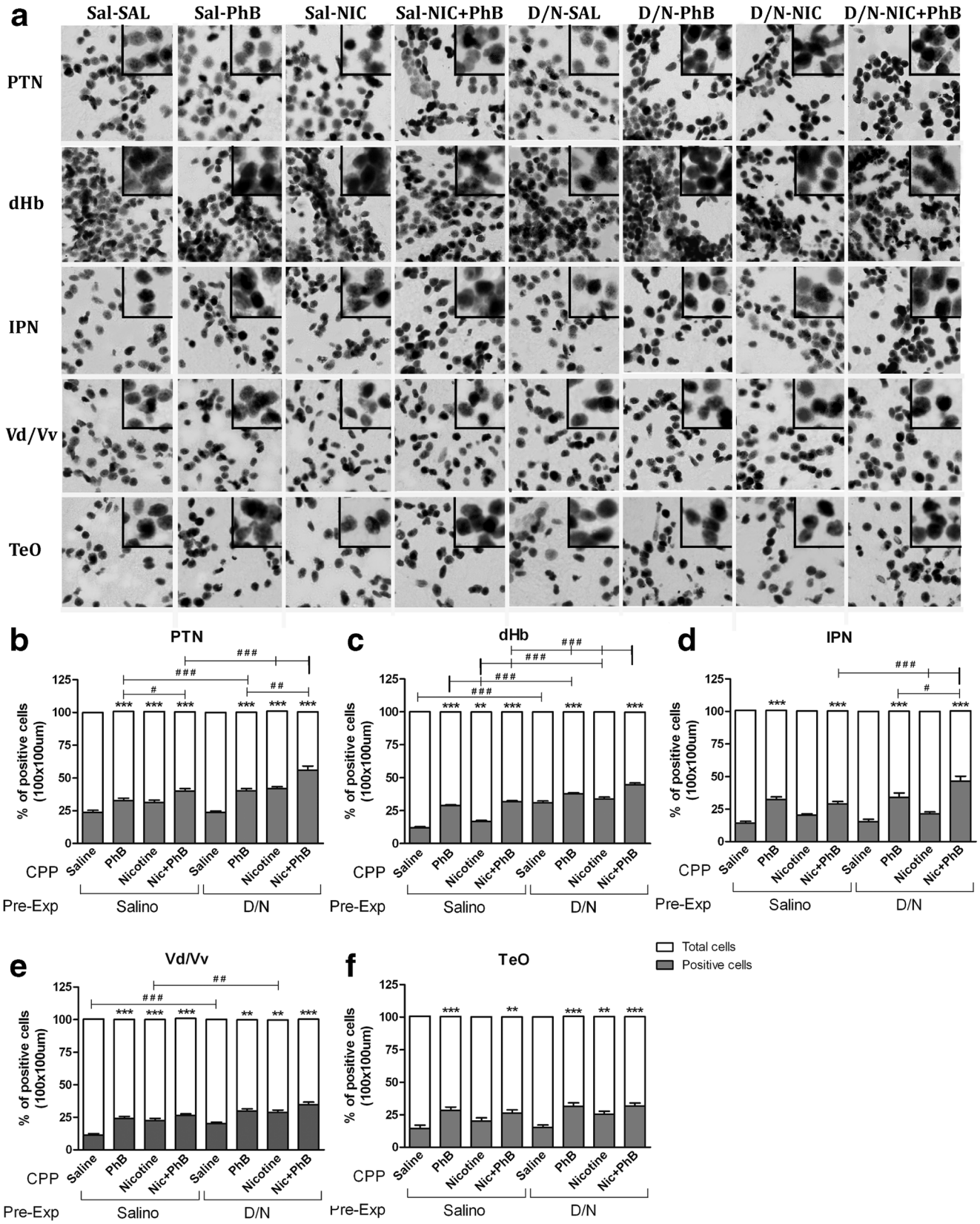
Figure 8 depicts the number of H3K9Ac-immunoreactive cells in nuclei that encompass the mesolimbic pathway PTN, dHb, IPN, and Vd/Vv, and in the TeO of the zebrafish brain. In the PTN pre-exposure to intermittent D/N-nicotine potentiated HDAC inhibitor and nicotine-CPP effects on the number of cells containing higher levels of H3K9Ac (Fig. 8b). Two-way ANOVA: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p = 0.0065$). The number of H3K9Ac-immunoreactive cells in the dHb and IPN showed the same tendency. Zebrafish exposed to the HDAC inhibitor PhB during nicotine-CPP showed a significant but slight increase in the number of cells with a higher content of H3K9Ac whereas nicotine-CPP alone did not affect H3K9Ac levels in dHb and IPN cells (Fig. 8c,d). Two-way ANOVA: pre-exposure ($p < 0.0001$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p < 0.0001$). H3K9Ac content in TeO cells showed a similar profile to the one found in IPN cells with the exception that D/N-nicotine exposure before nicotine-CPP caused a further enhancement of H3K9Ac cell content in the absence of the HDAC inhibitor (Fig. 8b,f). Two-way ANOVA: IPN: pre-exposure ($p = 0.0007$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p = 0.0080$) and TeO: pre-exposure ($p = 0.0192$), CPP ($p < 0.0001$) and pre-exposure × CPP interaction ($p = 0.6913$). Finally, pre-exposure to intermittent D/N-nicotine potentiated HDAC inhibitor and nicotine-CPP effects on the number of cells containing higher levels of H3K9Ac in Vd/Vv cells displaying virtually the same pattern found in PTN cells (Fig. 8e). In all structures analyzed, treatment with the HDAC inhibitor PhB during CPP increased the number of cells containing higher levels of H3K9Ac relative to basal levels of H3 acetylated in lysine 9. Moreover, pre-exposure to D/N-nicotine followed by a robust nicotine-CPP establishment generally potentiated H3K9 acetylation levels in the majority of nuclei examined.

Discussion

Prior exposure to drugs of abuse may regulate the reinforcing effect of the drug in later consumptions. Pretreatments with nicotine have usually involved one to four injections per day or the use of osmotic pumps to deliver the drug in rodents [45], and these studies have not been conclusive regarding the effect of nicotine pretreatment on nicotine-CPP [3, 4, 6, 32]. Therefore, we decided to evaluate an intermittent D/N nicotine pre-exposure protocol in zebrafish, using this animal model for the reasons exposed in the "Introduction" section. We have demonstrated that exposure to nicotine for 14 days during daytime only increases the rewarding properties of nicotine. In contrast, zebrafish pre-exposed to nicotine for 14 days in a continuous (chronic) way prevented nicotine-CPP establishment. Considering that smokers burn one cigarette every 1 or 2 h and nicotine is removed from blood every 2 to 3 h [46, 47], our D/N-nicotine protocol could mimic smokers' pattern of nicotine exposure. The use of zebrafish in our experimental approach was was key since nicotine was dissolved in tank water with minimal disturbance for the animal. Moreover, for diurnal animals like zebrafish and humans, nicotine during daytime following by abstinence periods at sleep time might work as a *Zeitgeber*, synchronizing high nicotine levels with high metabolic rates, increased attention and perception and locomotor activity whereas low nicotine levels are synchronized with low metabolic rates and motor activity as well as decreased or lack of awareness.

Effect of Different Pretreatments with Nicotine on Nicotine-CPP Task Performance

Zebrafish from the D/N group showed a nicotine-CPP score that was 78% higher than the score obtained from zebrafish pretreated with saline solution. Other parameters assessing locomotor activity, such as the number of entries to and total time spent in the drug-paired side of the tank, corroborated the rewarding properties of nicotine. Moreover, distance swum by zebrafish and time swimming in the upper zone of the tank before and after the CPP task were invariant between control and D/N-nicotine-treated groups suggesting intermittent delivery of nicotine did not influence locomotor activity and no significant anxious-like behaviours were developed [16].


a
Sal-SAL
Sal-PhB
Sal-NIC
Sal-NIC+PhB
D/N-SAL
D/N-PhB
D/N-NIC
D/N-NIC+PhB
PTN
dHb
IPN
Vd/Vv
TeO
b
PTN
c
dHb
d
IPN
e
Vd/Vv
f
TeO
% of positive cells (100x100um)
CPP
Saline
PhB
Nicotine
Nic+PhB
Pre-Exp
Salino
D/N
Total cells
Positive cells

Zebrafish chronically pretreated with nicotine showed an increased pretest locomotor activity and a reduced time in the upper zone of the tank, which indicates an anxiety-like behaviour probably caused by acute withdrawal that did not occur in the intermittent D/N-nicotine-pretreated zebrafish already habituated to periodical abstinent periods. This correlated with the inability of zebrafish pre-exposed to chronic nicotine to associate nicotine with CPP tank environmental cues. It has been suggested that continuous exposure to drugs without withdrawal fails to induce addictive behaviours [48]. Our findings suggest that zebrafish continuously pre-exposed to nicotine developed anxiety-like behaviours that may prevent CPP establishment. In contrast, intermittent abstinence periods were a critical factor for increasing the rewarding properties of nicotine.

Treatment with the HDAC inhibitor PhB abrogated nicotine-CPP and affected the other behavioural parameters assessed, such as total time spent and number of entries to the drug-paired side. In contrast, treatment with PhB did not affect the total distance swum by zebrafish (locomotor activity that is not directly related to drug seeking) and the time swimming in the upper zone of the tank (locomotor activity associated to anxiety-like behaviours). This suggests the HDAC inhibitor mainly influences neural mechanisms underlying nicotine preference in zebrafish. In fact, HDAC inhibition principally influences motivational aspects of drug addiction as it has been previously demonstrated by using CPP and self-administration in rodents [3, 49].

The Effect of Pretreatments with Nicotine, With or Without CPP, Was Analyzed by Quantifying the Relative Expression of Genes of Nicotinic Receptor Subunits and Enzymes that Regulate Epigenetic Mechanisms

Nicotine induces up-regulation of nAChR subunits [28, 50], which was observed for the three subunits principally in the chronic- but also in D/N-nicotine pretreated groups here examined, demonstrating chronic and intermittent exposure to nicotine increases the transcriptional expression of α7-, α6- and ß2-nAChR subunits. Furthermore, chronic nicotine provoked a significantly higher transcriptional expression of all these subunits compared with the intermittent D/N-nicotine treatment. Nicotine administered during the CPP task further induced α7- and α6-nAChR subunit gene expression when zebrafish were pre-exposed to D/N-nicotine. This increase was slightly diminished by treatment with the HDAC inhibitor PhB during nicotine-CPP. However, a further increase in the transcriptional expression of α7- and α6-nAChR subunits was not observed during the nicotine-CPP task when zebrafish were pre-exposed to chronic nicotine. The HDAC inhibitor PhB that prevented nicotine-CPP was unable to abolish nicotine effect on α7- and α6-nAChR subunit transcriptional expression. These findings suggest that the observed gene induction of nAChR subunits is not due to the rewarding properties of nicotine effect on brain circuits—i.e., the occurrence of the associative learning between nicotine and place cues during the CPP task—but to a direct action of nicotine, during both pretreatment and conditioning, on α7- and α6-nAChR subunit transcriptional expression in cerebral neurons.

Furthermore, our findings showed that pretreatments with nicotine induced long-term changes in the transcriptional expression of epigenetic factors.

As aforementioned, HDAC activity plays a central role in facilitating the seeking for nicotine triggered by environmental cues [14, 29]. The intermittent D/N pretreatment with nicotine likely contributes to this process by inducing HDAC1 and histone acetyltransferase CBP transcriptional expression. SIRT1—which also catalyzes histone deacetylation—controls exploratory drive by activating transcription of the gene encoding the MAO-A, a key enzyme in monoamine degradation [51]. In our study, mesolimbic areas of zebrafish pretreated chronically with nicotine showed reduced levels of SIRT1 mRNA compared to the same brain areas of zebrafish pretreated with D/N nicotine or saline solution after performing the nicotine-CPP task. These findings indicate that SIRT1 transcriptional expression correlates with nicotine reward.

HDAC1, Sin3A and MeCP2 form a complex that binds to methylated DNA provoking transcriptional silencing of specific genes [52]. Binding of MeCP2 to DNA is affected by HDAC activity inhibition with PhB, which maintains histone acetylation facilitating gene transcription [53]. Long-term treatment with nicotine induced HDAC1 and MeCP2 expression suggesting a repressor effect on gene expression related to CPP induction. During the CPP task, treatment with the histone acetylase inhibitor PhB reduced both MeCP2 and HDAC1 expression, suggesting MeCP2 expression is in part under transcriptional control of HDAC1 in zebrafish as it has been described in rodents [16, 54].

Intermittent D/N exposure to nicotine induced transcriptional expression of DNMT1. In contrast, DNMT3 expression was inhibited by this treatment but increased by chronic pretreatment with nicotine. This suggests that constant and intermittent pre-exposure to nicotine can differentially regulate DNA methylation of different groups of genes. DNMT1 is a methyltransferase that promotes conservative DNA methylation to guarantee the transmission of epigenetic patterns when DNA replication occurs [44]. Chronic nicotine might promote de novo methylation of genes via DNMT3 whereas intermittent D/N-nicotine might induce conservative methylation of genes via DNMT1 [55]. Moreover, according to our findings DNMT1 induction might be implicated in promoting the rewarding properties of nicotine whereas DNMT3 transcriptional upregulation might reduce nicotine reward in the mesolimbic pathway. Nonetheless, pharmacological HDAC

inhibition with PhB provoked further increases of DNMT1 expression while inhibited nicotine-CPP. This might indicate that upregulation of DNMT1 is not sufficient or that its overexpression might be detrimental for CPP induction because it must be compensated by HDAC1 activity—which was inhibited by PhB during the CPP task.

Enzymes from TET family catalyze the conversion of 5-mC to 5-hmC [56] and Gadd45 family removes 5hm-C from DNA. Transcriptional expression of these enzymes that promote DNA demethylation was increased by two-week exposure to D/N-nicotine. In contrast, their transcriptional expression was reduced by chronic exposure to nicotine after the CPP task. Gadd45 transcriptional expression was upregulated by the HDAC inhibitor PhB and its increment did not correlate with nicotine-CPP establishment. TET1 expression was potentiated by nicotine-CPP while it was not changed by the HDAC inhibitor treatment suggesting its down-regulation is not critical to prevent nicotine-CPP. Nevertheless, the facilitating effect of D/N nicotine pretreatment on nicotine-CPP might require upregulation of both enzymes, Gadd45 and TET1, counterbalanced by HDAC1 activity. Taken together, these findings suggest that demethylation is a dynamic process involved in nicotine reward that limits over-methylation due to DNMT overexpression. Moreover, both DNMT1 and Gadd45 transcriptional expression, but not TET1 expression, are negatively regulated by HDAC1 activity.

Effect of Pretreatments Plus Nicotine-CPP and the HDAC Inhibitor PhB on H3K9 Acetylation

Further analysis indicated that H_3K_9Ac protein levels were increased in D/N nicotine pre-exposed animals after nicotine-CPP in the PTN, Vd/Vv and TeO. The PTN and Vd/Vv are nuclei of the dopaminergic mesolimbic circuit in zebrafish, which are homologous to the ventral tegmental area (VTA) and nucleus accumbens (Nacc), respectively. The TeO, a complex structure with sensory polymodal function, from zebrafish pretreated with saline solution that performed nicotine-CPP showed no changes in H_3K_9Ac protein levels. Intermittent pre-exposure to nicotine for two weeks caused an increase in H_3K_9 acetylation in this structure. On the other hand, H_3K_9 acetylation in the dHb was enhanced in nicotine-treated zebrafish during either the pre-exposure phase or CPP task. The dHb-IPN pathway plays an important role in modulating VTA and Nacc activity during reward [57]. Nicotine treatment increased H_3K_9Ac levels in all structures analyzed. As expected, a systemic treatment with the HDAC inhibitor PhB during conditioning also enhanced H_3K_9 acetylation in all the cerebral areas studied here. Interestingly, zebrafish pre-exposed to D/N-nicotine that performed the nicotine-CPP task in presence of the HDAC inhibitor showed the highest levels of H3K9Ac, which seems to be detrimental to establish nicotine-CPP. Taken together, these findings suggest that a broad, non-specific and static level of acetylation is detrimental for mesolimbic circuit changes necessary for the establishment of conditioning place preference to nicotine, in which zebrafish brain must learn to associate spatial cues with nicotine reward. In opposition, a specific and malleable increase in H_3K_9 acetylation, which is balanced by HDAC activity (as in the absence of the HDAC inhibitor) in several but not all mesolimbic structures, seems to promote mechanisms for nicotine reward.

Effect of D/N Pretreatment with Nicotine on pCREB Protein Levels in Neurons of the Mesolimbic Structures

We have previously demonstrated that pCREB expression increases in the reward structures of the rat brain after nicotine-CPP and that HDAC inhibition reduced CREB phosphorylation [16]. Here, we found similar results in zebrafish, with the exception of dHb, where treatment with nicotine decreased pCREB levels. Treatment with the HDAC inhibitor PhB, with or without nicotine, during conditioning showed no or minimal effect on pCREB levels in the majority of the mesolimbic areas studied, with the exception of the IPN and dHb. The dHb–IPN axis is critical in addiction, mood and anxiety regulation and is a major cholinergic pathway in the brain [57]. In zebrafish, silencing of the dHb–IPN pathway disrupts previously learned associations [58]. Learning associations between environment cues and drugs is a key neural mechanism involved in CPP. Our findings suggest that the dHb–IPN pathway participates in nicotine-CPP establishment probably modulating interconnected structures of the reward pathway, such as the VTA and Nacc [59]. Moreover, it has been suggested that habenula activation regulates anxiety in zebrafish [59]. So, the fact that pCREB levels were reduced in dHb cells in zebrafish pre-exposed to saline solution and D/N-nicotine after performing nicotine-CPP might be considered as molecular evidence—together with the behavioural evidence—that no or very low anxiety was generated in these zebrafish. HDAC inhibition with PhB might modulate this pathway by activating dHb neurons and inhibiting the IPN, as shown here via pCREB level analysis [60].

In conclusion, our findings support the observation which indicates that previous history with drugs of abuse increases the activity of the mesolimbic pathway and the induction of neuroplasticity, via epigenetically regulated changes in gene expression, involved in the rewarding properties of drugs of abuse. Our study also showed that a pattern of pre-exposure to drugs separated by intermittent withdrawal periods, with the drug administered during the phase of the day where motor activity, metabolic rate and attention are elevated, is a very important risk factor to take into account, together with other factors, such as the type of drug and individual vulnerability.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no competing financial interests.

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