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Evaluation of the rewarding properties of nicotine and caffeine by implementation of a five-choice conditioned place preference task in zebrafish

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ABSTRACT

The rewarding properties of drugs in zebrafish can be studied using the conditioned place preference (CPP) paradigm. Most devices that have been used for CPP consist of two-half tanks with or without a central chamber. Here we evaluated the rewarding effects of nicotine and caffeine using a tank with five arms distributed radially from a central chamber that we have denoted Fish Tank Radial Maze (FTRM). Zebrafish were trained to associate nicotine or caffeine with a coloured arm. In testing sessions to assess CPP induction, between two and five different arms were available to explore. We found that when offering the two arms, one of them associated to the drug mediating conditioning for 14 days, zebrafish showed nicotine-induced CPP but not caffeine-induced CPP. When zebrafish had the option to explore drug-paired arms together with new coloured arms as putative distractors, the nicotine-CPP strength was maintained for at least three days. The presence of novel environments induced caffeine-CPP, which was still positive after three days of testing sessions. Complementary behavioural data supported these findings. Nicotine-CPP was prevented by the histone deacetylase inhibitor phenylbutyrate administered during conditioning; however, there were no effects on caffeine-CPP. The specific acetylation of lysine 9 in histone 3 (H3-K9) was increased in nicotine-conditioned zebrafish brains. This study suggests that novel environmental cues facilitate drug-environment associations, and hence, the use of drugs of abuse.

1. Introduction

Legal drugs such as alcohol, nicotine, and caffeine are widely used and have a high impact on society (World Health Organization, 2007). Nicotine and alcohol are among the drugs of abuse with the highest relapse rates (Lancaster et al., 2006). Caffeine does not meet the criteria necessary to elicit substance abuse according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), although it is the most commonly used drug in the world, with a mild reinforcing effect in humans (American Psychiatric Association, 2013; Griffiths and Mumford, 1995).

The relapsing behaviour associated with drugs of abuse in humans, rodents, fish, insects, and nematodes is thought to be caused by repeated activation of the brain's reward circuit with these drugs (Darland and Dowling, 2001; Mohn et al., 2004; Ninkovic and Bally-Cuif, 2006; Wolf and Heberlein, 2003). Zebrafish behaviour has become an increasingly important asset in biomedical research for studying the physiology of addiction (Kily et al., 2008; Stewart et al., 2011).

CPP is a paradigm based on classical conditioning used to measure the rewarding properties of a drug (Collier et al., 2014; Tzschentke, 1998). We have demonstrated that zebrafish can establish positive nicotine-induced CPP with significantly higher preference scores than rats (Kedikian et al., 2013; Pascual et al., 2009). Recent studies in zebrafish have shown that caffeine produces a weak CPP at higher doses than the ones used to investigate memory (Collier et al., 2014; Tran et al., 2017).

Individual vulnerability in response to psychostimulants has been found to be predicted by the animal's locomotor response to novelty (Allen et al., 2007; Mandt et al., 2008; Pastor et al., 2013). It has also been suggested that sensitivity to nicotine depends on the innate response to novel environments (Redolat et al., 2009). In fact, conditioned locomotor effects of nicotine were increased by previous exposure to a novel environment (Coolon and Cain, 2009).

Concerning novel environmental cues, several studies have demonstrated that zebrafish show innate colour preference, but data regarding which colour zebrafish prefer or reject is inconsistent (Avdesh et al., 2012; Colwill et al., 2005; Oliveira et al., 2015; Peeters et al., 2016). We have found that zebrafish also had an innate preference for exploring coloured objects (red and green over yellow and blue

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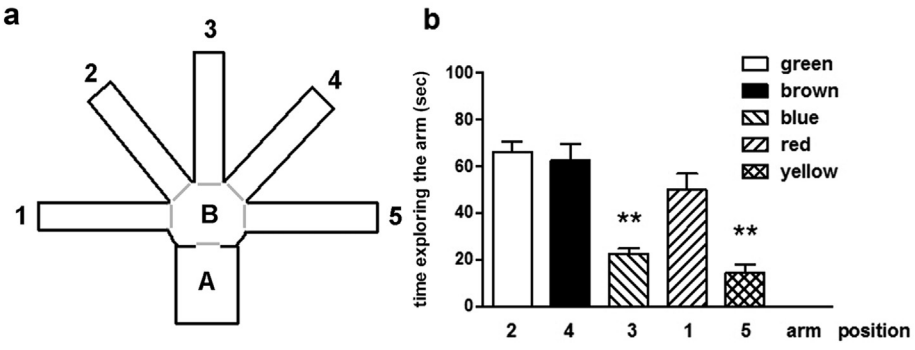

Fig. 1. a) Design of the conditioning tank. The fish tank radial maze (FTRM) is composed of an initial chamber for habituation (A), a central area (hall B) and 5 rectangular arms as shown in the scheme (arms 1–5). A gate placed in chamber A could be opened automatically without disturbing the fish. Gates at each arm were manually closed or opened before performing the test sessions. When zebrafish were placed in chamber A, the gate A was closed and 5 min later it was opened to let zebrafish to explore the maze. The tank was made of non-reflecting acrylic. For geometrical details such as compartment size and water level see Methods. b) Initial colour preference. The graph shows the average time (sec) spent by zebrafish in each coloured arm (1–5) over a 5 min period after habituation. Data are presented as mean ± SEM. n = 30 zebrafish. $^{**}p < 0.01$ compared to green, brown and red arms.

objects). Interestingly, nicotine was shown to modify both colour-based object recognition and innate preferences (Faillace et al., 2017).

On the other hand, the use of histone deacetylase (HDAC) inhibitors has rapidly emerged as a powerful tool to study the role of histone acetylation in the regulation of transcription (Cassel et al., 2006; Renthal and Nestler, 2009). It was demonstrated that HDAC inhibition alters long-lasting brain processes and executive tasks such as long-term memory (Hawk et al., 2011; Morris et al., 2010). Inhibiting HDAC activity with phenylbutyrate (PhB) induces chromatin relaxation which favours gene transcription. We have demonstrated that PhB reduces nicotine-induced CPP in rats (Pastor et al., 2011). Moreover, it was suggested that nicotine partially inhibits HDAC activity in mice (Levine et al., 2011). HDAC activity inhibitor effects on gene transcription for different processes have been studied in larvae and adult zebrafish (Dozawa et al., 2014; Faillace et al., 2017; Kim et al., 2012; Li et al., 2016). In adult zebrafish, we have demonstrated that PhB modifies long-term object recognition and also the innate preference for coloured objects (Faillace et al., 2017). However, HDAC activity inhibitor effects have not been examined on drug-induced CPP in zebrafish.

It has also been described that the presence of histone 3 acetylated in lysine 9 (H3K9Ac) is generally observed in regions of the nucleosome of active transcription. On the other hand, histone 3 trimethylated at lysine 9 (H3K9me3) has been correlated with transcriptional repression (Parker et al., 2015).

To date, novelty effects have not been previously tested on nicotine- or caffeine-environment associations in zebrafish. Therefore, the aim of this work was to study the effects of introducing novel environmental cues during the drug-seeking time window on the CPP induced by nicotine and caffeine. To this end, we designed a new behavioural task that we have denoted Fish Tank Radial Maze (FTRM), which combined "conventional" CPP and a five-choice serial reaction time task (5-CSRTT). We first evaluated whether exploration of one or several novel coloured environments during CPP expression could influence the rewarding properties of nicotine and caffeine. To examine the transcriptional regulation status, the levels of H3K9Ac and H3K9me3 were determined by Western blot in the brains of zebrafish that had been conditioned to nicotine and caffeine. The effects of PhB treatments in fish water during conditioning on nicotine- and caffeine-induced CPP, tested with or without novel environments, were also analysed.

2. Methods

2.1. Animals and maintenance

Adult zebrafish (*Danio rerio*, Singapore strain; six to nine months old) were obtained from a local farmer (La Plata, Buenos Aires, Argentina) (Battista et al., 2009; Faillace et al., 2017; Kedikian et al., 2013). Zebrafish were maintained according to standard methods (Faillace et al., 2017; Kedikian et al., 2013; Westerfield, 2007). After the acclimatization period (14 days), animals were moved to the behavioural room, housed in 12 l tanks at a maximum density of 12 animals per tank in order to reduce stress. The Committee on Animal Research of the University of Buenos Aires (based on the regulation specified in the Guide for the Care and Use of Laboratory Animals) approved all protocols for the use, housing, and care of experimental animals. For Western blot analysis, all animals were euthanized 16 h after the CPP test using tricaine.

2.2. Drugs and treatments

Nicotine (nicotine hydrogen tartrate salt, Sigma-Aldrich, St. Louis, USA) was dissolved in system water to produce a 15 mg/l (30 μM) solution (Faillace et al., 2017; Kedikian et al., 2013). Drug concentration was calculated from the weight of the salt. Caffeine (Sigma-Aldrich) was dissolved in system water and 50 mg/l was selected based in previous studies (Collier et al., 2014; Khor et al., 2013) and our preliminary studies in which 50, 100, and 150 mg/l were tested (data not shown). The HDAC inhibitor (PhB; Sigma-Aldrich) was dissolved in system water. We used 15 μM based on our previous and other authors' results (Faillace et al., 2017; Pastor et al., 2013; Romieu et al., 2008). Drug solutions were prepared fresh daily.

2.3. Five arm place preference device for CPP studies

Fig. 1a shows the design of the five arm FTRM. The radial maze consisted of a start chamber "A" that opened up toa central area (diameter 16 cm) that in turn connected to five rectangular hallways (arms) (30 cm (length) × 8 cm (width) × 12 cm (height)). The walls and floor of the radial maze arms were internally covered with custom made cardboard pieces of different colours, cut in rectangles to fit the arm walls and floor, which were then sealed in plastic coatings (in a commercial store). One coloured rectangle was fitted on the arm floor and three other pieces were fixed to the lateral and end walls of the arm with small transparent plastic clips that did not reach the water level. Arms 2 and 4 (see Fig. 1a) were covered with brown and green coatings, arm 3 was coated with blue rectangular pieces, and arms 1 and 5 were coated with red and yellow rectangles, respectively. Arm entries and chamber A exit were provided with sliding doors (Fig. 1a). Arm 2 and 4 covers were switched between experiments (in half of the experiments arm 4 (or 2) was coloured green and in the other half was coloured brown). The same criterion was applied to arms 1 and 5 (red and yellow) while arm 3 was always blue. We selected two equally preferred colours to pair with caffeine and nicotine (green and brown, respectively). For novelty, we selected blue, which zebrafish tend to avoid, yellow, which is also avoided but to a lesser degree, and red, which is attractive to zebrafish (Fig. 1b; Faillace et al., 2017). The entire maze was filled to a 10 cm depth with system water to avoid stress (Kedikian et al., 2013).

The preference was expressed as time (sec) that each zebrafish spent exploring each arm under all experimental conditions.

2.4. Procedure

2.4.1. Habituation

Each zebrafish was placed in the start chamber A (Fig. 1a) and after 2 min (habituation) the door was opened. The animal was then allowed to explore the entire maze (central chamber and each of the five arms which were coloured in white) for 5 min. The white colour was selected to avoid associations between colours selected for conditioning tests and arms. The experiments started the day after habituation.

2.4.2. Experiment 1: place preference in the FTRM task with novelty

2.4.2.1. Experiment 1, group 1: nicotine and caffeine CPP. One day after habituation, the zebrafish were placed in start chamber A and after 2 min the door was opened to let the zebrafish explore the central chamber and arms 2 and 4 only (pre-test session). Arms 2 and 4 were coloured green and brown, respectively. The time exploring each one of the open arms over a 5 min period was recorded. The following day, the procedure was similar but the colours were counterbalanced (arm 4 was the green one). Data recorded from these two days after habituation determined the baseline preference. The conditioning session started on the third day. During the conditioning day, each fish in the nicotine group was confined first to the green arm for 20 min (without drug) and then to the brown arm with nicotine for another period of 20 min. Each animal in the caffeine group was restrained in the brown arm for 20 min (without drug) and then was confined to the green arm with caffeine for a 20 min-interval. During conditioning, zebrafish from the control (saline solution) group were restrained in the green and then the brown arm for 20 min without drugs. This procedure was repeated for 14 days (14 trials). The colour of the arms was counterbalanced to avoid associations between the place paired with the drug and external cues. One day after conditioning, each zebrafish was tested for place preference in a drug-free environment. Zebrafish were allowed to swim freely to the central area and the conditioned arms (green and brown arms), and the time spent in each arm was determined over a 5 min period (test session). The results were expressed as: time (sec) spent in the nicotine- or caffeine-paired arm vs. time (sec) spent in the non-paired arm during the test session.

2.4.2.2. Experiment 1, group 2: nicotine and caffeine CPP with novelty. After habituation, we determined the baseline preference and performed the 14 days of conditioning trials, as described for experiment 1, group 1. One day after conditioning, animals were tested for place preference. The procedure was similar to the one used with group 1; however, a novel arm (blue coloured arm in position 3) was available to explore in addition to conditioned arms 2 and 4. In this test session, we recorded the time each zebrafish explored the three available arms associated with the brown, green, or novel blue environments, over a 5 min period. In order to evaluate if the colour of the new arm was a determinant, since we evaluated only one arm with a non-preferred colour, we performed the same experiment but with the new arm coloured in red (a preferred colour).

2.4.2.3. Experiment 1, group 3: nicotine and caffeine CPP with three novelty options. Procedures for habituation, pre-test (baseline preference), and conditioning were the same as the ones described for group 1. One day after conditioning, the animals were tested for place preference. In the test session, individual zebrafish were allowed to freely explore five arms (two previously known arms, one of them associated with drugs (arm 2 or 4) and three novel arms 1, 3 and 5). Arm 3 was coloured blue like in group 2, whereas arms 1 and 5 were coloured red and yellow, respectively. When the gate separating the start compartment (A) and the central area (B) was opened, zebrafish were allowed to freely swim to the central area and the five arms. The time spent in each arm was determined over a 5 min period (test session).

2.4.3. Experiment 2: establishing place preference for nicotine and caffeine in the FTRM by evaluating preference in three consecutive test sessions

Considering the findings of experiment 1, we performed a new series of experiments in which a group of zebrafish were habituated and conditioned in the same manner as experiment 1; however, testing for preference was performed over three successive days instead of on only one day. On the first day after conditioning, zebrafish were tested for nicotine or caffeine preference by exploring brown and green arms only, as was performed with group 1. On the second day after conditioning, the animals were tested again with the novel blue arm also available for exploration. On the third day after conditioning, the same group of zebrafish were tested and at this time all arms were opened: the two conditioned arms (green and brown), one familiar arm unpaired with drugs (blue), and two novel arms (red and yellow). The elapsed time spent exploring the five arms was recorded and analysed using the tracking software, over a 5 min period.

2.4.4. Experiment 3: place preference for nicotine and caffeine in the FTRM task

To evaluate the possibility of synergic effects of nicotine and caffeine we performed experiments in which zebrafish were exposed to nicotine and caffeine on the same conditioning day. Habituation and baseline preference procedures were performed in a similar way to the protocols used in experiment 1 group 1, in which only arms 2 and 4 were opened. In contrast to experiment 1 group 1, during each conditioning day, zebrafish were exposed to caffeine in the green arm (20 min) in the morning, and 5 h later, in the afternoon they were exposed to nicotine in the brown arm (20 min). This procedure was repeated for 14 days (14 trials). The colour of arms was counterbalanced to avoid associations with external stimuli. After conditioning, zebrafish were tested for preference in a drug-free environment. The time spent exploring the brown and green arms was recorded and analysed, as previously described. Control groups were confined for 20 min to each arm (green and brown) without drugs, during all conditioning days, and tested in exactly the same way as the experimental groups.

2.4.5. CPP in the biased two-half tank

All experimental procedures were similar to the ones described in our previous studies (Kedikian et al., 2013). Briefly, the CPP consisted of pre-test, conditioning (three days), and test sessions. During conditioning, zebrafish were exposed to nicotine (15 and 100 mg/l) or caffeine (50 and 100 mg/l) over a 20 min period in the white side of the tank. The white side is the non-preferred side for naïve zebrafish, whereas the preferred side is the brown side of the tank, as was determined in pre-test sessions. In test sessions, place preference changes were determined by a score (score (%) = percentage of time spent in the non-preferred side during the test - percentage of time spent in the non-preferred side during the pre-test).

2.4.6. HDAC inhibition effect on the FTRM task

The histone deacetylase inhibitor PhB (Sigma-Aldrich) was dissolved at 15 μM in the tank water, filling the arms of the tank. We utilised the lowest dose with pharmacological effect that had been found to be effective in previous studies (Faillace et al., 2017; Pastor et al., 2013; Romieu et al., 2008). The effect of PhB was examined in a protocol similar to the procedure performed in experiment 1 (groups 1, 2, and 3). The drug was applied alone (PhB control) or mixed with nicotine or caffeine (PhB-nicotine or PhB-caffeine groups). For control saline solution-treated animals, the drug tank contained system water without drugs.

2.4.7. Behavioural analysis

A camera connected to a computer was placed approximately 1.2 m above the maze tank (the FTRM and the two-half tank). During pre-test and test sessions, zebrafish behaviour was recorded and videos were analysed first by direct observation and then with Noldus Ethovision

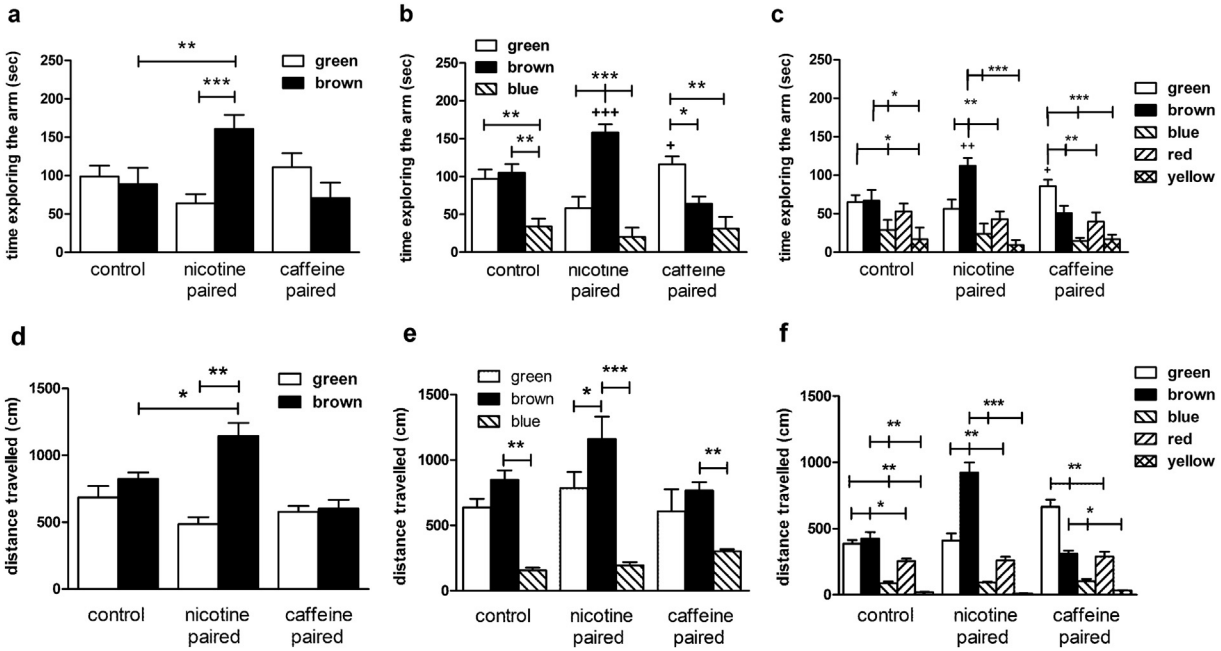

Fig. 2. Conditioned place preference using the FTRM. Conditioning was carried out during 14 consecutive days in the FTRM array. Control group: individual zebrafish were confined for 20 min to the green (or brown) arm and then to the brown (or green) arm without drugs. Nicotine-paired group: individual zebrafish were conditioned with nicotine in the brown arm for 20 min and then confined over an equivalent period to the green arm without drugs. Caffeine-paired group: zebrafish were conditioned with caffeine in the green arm for 20 min and then confined for 20 min to the brown arm without drugs. Conditioned zebrafish were divided in three groups and tested in three different environments. a) Test session for group 1: time (sec) spent in the green and brown arms (familiar environments only) over a 5 min period after 14 days of conditioning. b) Test session for group 2: time (sec) spent in the green, brown and novel blue arms over a 5 min period after 14 days of conditioning. c) Test session for group 3: time (sec) spent in the green, brown and novel blue, red, and yellow arms over a 5 min period after 14 days of conditioning. d) Distance travelled (cm) in the green and brown arms during the test session for group 1. e) Distance travelled (cm) in the green, brown and blue arms during the test session for group 2. f) Distance travelled (cm) in the green, brown, blue, red and yellow arms during the test session for group 3. Data are presented as mean ± SEM. Control n = 6 zebrafish per group; nicotine- and caffeine-paired groups; n = 10–12 zebrafish per group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ versus control comparing arms of the same colour (brown arm for nicotine and green arm for caffeine).

XT7 software (Noldus Information Technology, Netherlands, http://www.noldus.com). In all the environments, except for the one coloured in blue, the tracking system was able to follow the zebrafish without further setup adjustments. For the blue arm, bright and contrast were adjusted to follow the fish during arm exploration. The analysis of videos included the following measurements: time the fish remained in each arm; number of entries to the drug-paired arm; latency to enter the drug-paired arm; and number of entries, distance travelled, and time spent in the distal zone (zone 2) of each arm (see Fig. 3).

2.4.8. Western blot assay

2.4.8.1. Histone extraction. Histone extractions were performed as in Levenson et al. (2004). Briefly, brain tissue was homogenized (the olfactory bulb, cerebellum, rhombencephalon, and most of the optic tectum were removed from each brain) in ice-chilled buffer with protease inhibitors. Tissue homogenates were centrifuged at 1000 × *g* for 10 min and the pellet (nuclear fraction) was resuspended in 1 ml of 0.4 N H_2SO_4 for 30 min (acid extraction). The supernatant was transferred to a fresh tube, and proteins were precipitated with trichloroacetic acid for 30 min. The resulting purified proteins were resuspended in 10 mM Tris-HCl buffer (pH = 8) and stored at −80 °C.

2.4.8.2. Western blot. Proteins of different size were separated in polyacrylamide gel electrophoresis under reducing conditions (SDS/PAGE). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes for 2 h at 100 V at 4 °C. Immunoblots were performed by incubating membranes with antibodies against H3 (1:1200), H3K9ac (1:1000), and H3K9-me3 (1:1500) (Upstate Biotechnology, EMD Millipore, Billerica, MA). Detection of immunolabelled histone proteins was enhanced via electrogenerated chemiluminescence (ECL; GE Healthcare; SuperSignal, Pierce) and digitalized using a G-Box (Syngene). Band density was determined with Gel Pro Analyzer 6.0 software (Media Cybernetics).

2.5. Statistical analysis

Behavioural data, such as time spent in the arms (sec), latency to enter to the arm, and number of entries to the arm, were analysed using one-way ANOVA (group × test, with test as the repeated measure) and two-way ANOVA (with days as a repeated measure). All ANOVA statistics were followed by Scheffé post hoc comparisons for behavioural analysis. Western blot data were analysed using one way ANOVA followed by Dunnett's test. Data are presented as mean ± SEM, and significance was set at $p < 0.05$. Analyses were performed using Stat View 5.0.1 software (SAS Institute, Gary, NC, USA).

3. Results

3.1. The radial water maze as a novel behavioural task to study drug place preference in adult zebrafish

3.1.1. Experiment 1: nicotine and caffeine CPP in zebrafish using the FTRM task

We used the experimental protocol described for experiment 1, groups 1, 2, and 3 in Methods section for the evaluation of nicotine and caffeine place preference using the FTRM. In agreement with previous findings, we found no significant time differences in naïve preferences when zebrafish explored green, brown, and red environments (Fig. 1b). Blue and yellow environments were less visited or preferred ($p < 0.01$) by zebrafish. Brown was associated with nicotine, as in our previous studies using a two-half CPP tank (Kedikian et al., 2013) and green with caffeine, another preferred colour of similar significance for zebrafish.

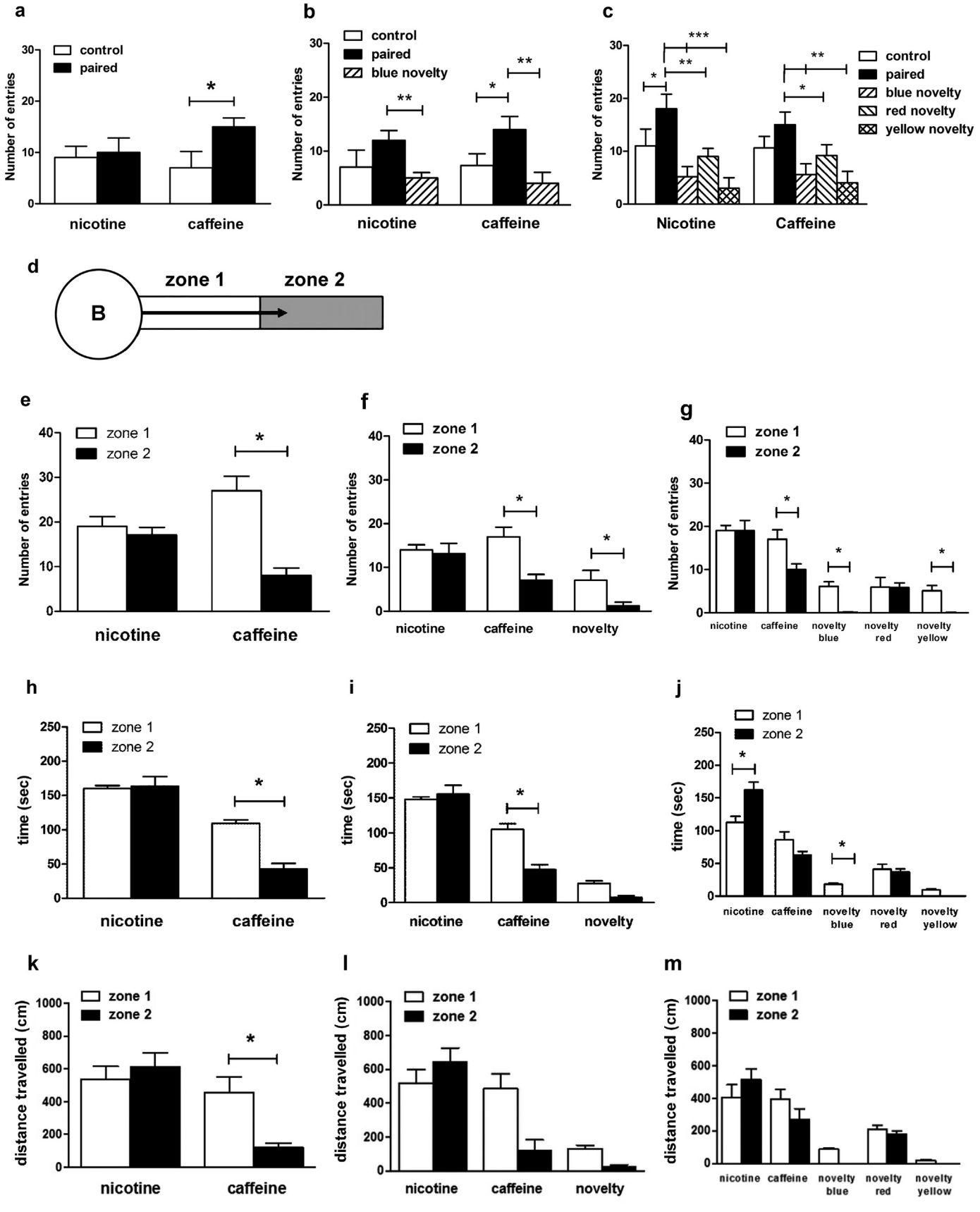

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Fig. 3. Behavioural analysis of the CPP performed in the FTRM tested in three different environments as described in Methods (Experiment 1: groups 1–3). Graphs a-c shows the average number of entries to each arm. a) Test session for Group1: the average number of entries tested with the conditioned brown and green arms only. b) Test session for Group 2: the average number of entries tested with the conditioned arms together with a novel blue arm (3 arms). c) Test session for Group 3: the average number of entries tested with the conditioned arms plus 3 novel arms of the radial maze (5 arms). The diagram in d) shows a portion of the central area of the maze (B), the zone 1 of each arm, and the zone 2 which is the outer region including the tip of the arm. Graphs e-g show the number of entries to the zones 1 and 2, graphs h-j depict the time spent by zebrafish in both zones of each arm, and graphs k-m show the distance travelled in each zone. Data are presented as mean ± SEM. Control group: n = 6 zebrafish per group, nicotine- and caffeine-paired groups: n = 9–11 zebrafish per group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

For novelty we decided to use blue, red, and yellow coloured arms.

3.1.1.1. Experiment 1, group 1: nicotine but not caffeine induced CPP using the FTRM task. In this experiment, zebrafish were allowed to explore the central area (B) and green and brown arms (arms 2 and 4). One-way ANOVA revealed significant differences among the groups ($F_{2,38} = 4.689$, $p < 0.001$). Fig. 2a clearly shows that nicotine ($p < 0.001$) but not caffeine ($p = 0.087$) induced CPP in the FTRM. Nicotine-pairing analysis showed a significant increase in the period of time that zebrafish remained swimming in the arm associated with nicotine. This significant difference was observed when this period was compared with the time spent by nicotine-conditioned zebrafish in the unpaired green arm, or with the time spent by zebrafish in the control group in the brown arm ($p < 0.01$). Control animals showed a similar preference for both arms (Fig. 2a, control) which confirms preliminary data evaluating naive (baseline) preferences (Fig. 1b).

3.1.1.2. Experiment 1, group 2: novelty effects on the preference for nicotine or caffeine using the FTRM task. Exposing animals to a novel environment prior to CPP increased the rewarding properties of cocaine and nicotine in rodents (Allen et al., 2007; Pastor et al., 2013); however, the effect of a novel environment "during" test sessions in experimental animals has not yet been reported. ANOVA revealed significant differences between the control and experimental groups in experiment 1, group 2 ($F_{2,35} = 24.29$, $p < 0.0001$). Fig. 2b shows that the novel blue arm, available for exploring during test sessions only, was significantly less visited than the conditioned arms ($p < 0.01$). This figure also shows that nicotine preference was maintained even when it was possible for zebrafish to explore a novel environment (compare Fig. 2b, green: 64 ± 13 s, brown: 161 ± 19 s, with Fig. 2a, green: 58.1 ± 21 s, brown: 158.1 ± 16 s). Interestingly, novelty (or aversive novelty) induced preference for the environment paired with caffeine (green arm) compared to the brown arm ($p < 0.05$) and the new blue arm ($p < 0.01$). To evaluate if an innate aversive coloured new environment (blue) maintained or induced the rewarding properties of nicotine or caffeine, respectively, a red arm was introduced as a novel stimulus in position 3 of the maze. The findings for nicotine- or caffeine-induced CPP were similar, but the red arm showed higher values compared to the blue arm (red arm in caffeine-CPP: 54.4 s; red arm in nicotine-CPP: 61.6 s; blue arm in caffeine-CPP: 31.2 s; blue arm in nicotine-CPP: 20 s). Furthermore, latency to enter the red arm was shorter than the latency to enter the blue arm (data not shown).

3.1.1.3. Experiment 1, group 3: three new environments to explore during the CPP test did not change the preference for nicotine and induced place preference to caffeine. We next evaluated the rewarding properties of nicotine or caffeine when zebrafish were able to explore the entire five-arm maze during test sessions. ANOVA revealed significant differences among the control and experimental groups ($F_{2,35} = 36.54$, $p < 0.0001$). Fig. 2c shows that, as expected, control zebrafish had a significantly reduced preference for exploring or remaining in the novel blue and yellow arms ($p < 0.05$). In contrast, they showed a similar propensity to explore the new red or the familiar green and brown arms, which reflects the innate colour preference regardless of novelty or familiarity. In the nicotine-paired group, zebrafish spent more time exploring the brown arm than all the other arms ($p < 0.001$ and $p < 0.01$), and spent more time exploring the brown arm ($p < 0.01$) when compared to the brown arm in control group. On the other hand, the caffeine-paired group also spent more time exploring the green arm compared to all the other arms ($p < 0.01$ and $p < 0.001$) and compared to the green arm not previously associated with caffeine (control group; $p < 0.05$).

3.1.2. Behavioural analysis of place preference in zebrafish using the FTRM task

3.1.2.1. Number of entries and latency to enter the drug-paired arm. The number of entries to each arm was quantified in the different groups of experiment 1. Fig. 3a (group 1) shows a significant increase in the number of entries to the arm paired with caffeine ($p < 0.05$) but not to the arm paired with nicotine. When a blue arm was available to visit during test sessions (Fig. 3b, group 2), the novel arm was less visited compared to the familiar, drug-paired, and unpaired arms ($F_{2,35} = 12.45$, $p < 0.01$). The number of entries to the nicotine-paired brown arm was significantly higher than the number of entries to the new blue arm ($p < 0.05$). The caffeine-paired arm was more frequently visited than the control ($p < 0.05$) and the blue arm ($p < 0.05$). A significant increase in the number of entries to the drug-paired arms was also observed when zebrafish were allowed to visit the five arms ($F_{4,35} = 35.78$, $p < 0.0001$) (Fig. 3c, group 3). The arm paired with nicotine was more frequently visited than the three new arms or the control arm (red and green, $p < 0.01$; blue and yellow, $p < 0.001$). When zebrafish were conditioned with caffeine, a significant increase in the number of entries to the caffeine-paired arm was found compared with the control ($p < 0.05$), and blue and yellow arms ($p < 0.01$); however, no significant differences were observed with the red arm.

To better characterise the behaviour within each arm, we analysed whether each zebrafish explored the entire arm or if they just performed a quick visit to the first portion of the arm connected to the central area. Thus, we quantified the number of entries, the distance travelled, and the time spent in zone 1 and zone 2 of each arm paired with nicotine or caffeine (Fig. 3d). Group 1 (Fig. 3e, h, and k) showed a higher number of entries to the caffeine-paired arm compared to the number of entries to the nicotine-paired arm. However, animals conditioned to nicotine spent more time exploring the arm (Fig. 3h). The distance travelled showed a significant reduction only in zone 2 of the caffeine-paired arm ($p < 0.05$) (Fig. 3k). Virtually all nicotine-conditioned zebrafish explored zone 2. The caffeine-conditioned group exhibited a significantly reduced period exploring zone 2.

Group 2 showed that zone 2 of the caffeine-paired and the new blue arm were less explored in relation to the number of entries to these arms (Fig. 3f and i). Like in group 1, most of the zebrafish went all the way to zone 2 when they entered the nicotine-paired arm. This was not the case when nicotine-conditioned animals entered the control unpaired or novel arms. The distance travelled for zebrafish in group 2 was reduced only in zone 2 of the caffeine-paired arm ($p < 0.05$) (Fig. 3l). Fig. 3g, j, and m show the findings obtained for group 3, which were similar to what was observed for zebrafish in group 2 for nicotine- and caffeine-paired arms. Interestingly, the number of entries, the time spent, and the distance travelled in zone 2 for the blue and yellow arms was zero. In contrast, zone 2 of the novel red arm was explored when zebrafish entered this arm.

Next we measured the latency to enter each arm in the three experimental groups. Fig. S1a (group 1) shows that zebrafish conditioned with nicotine exhibited a significantly reduced latency to enter the

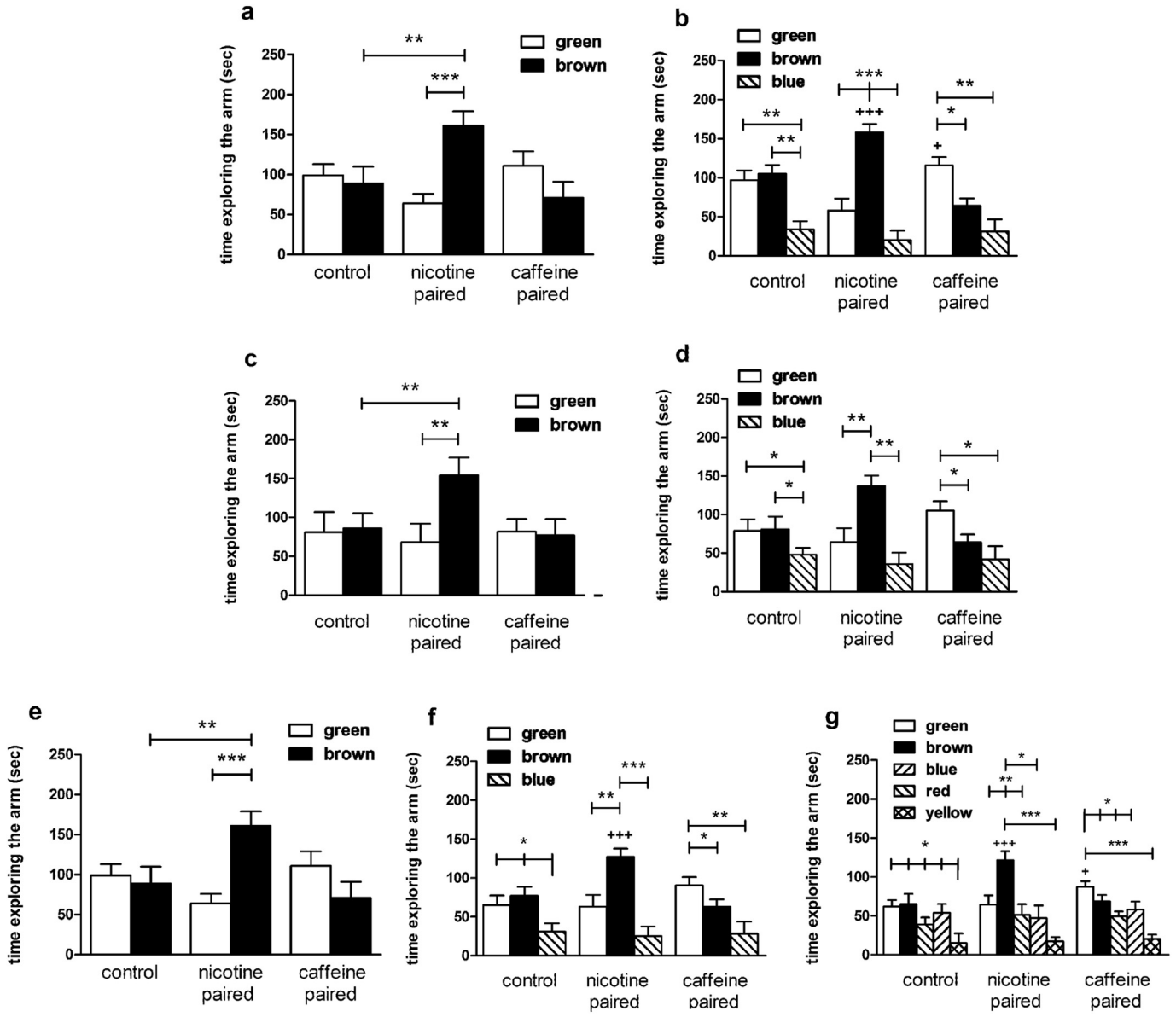

Fig. 4. Conditioned place preference tested for two or three consecutive days using the FTRM. Conditioning was carried out as was described Fig. 2 legend. Control and drug-conditioned zebrafish were divided in separate groups and tested for CPP in different conditions. Figures a-d: CPP was tested in the same environmental conditions through two days for zebrafish in group 1 or group 2. a) Test session for group 1: time spent (sec) in the green (caffeine-paired or nicotine-unpaired) and brown (nicotine-paired or caffeine-unpaired) arms over a 5 min period after 14 days of conditioning. b) Test session for group 2: time spent (sec) in the green, brown and novel blue arms during the test session over a 5 min period after 14 days of conditioning. c) Time spent (sec) exploring the green and brown arms on the second day of CPP after the first day of CPP described in a). d) Time spent (sec) in the nicotine-paired, caffeine-paired and the blue arm on the second day of CPP after the first day of CPP described in b).

Plots e–g show the same control and drug-conditioned groups of zebrafish tested for CPP in changing environments through three days after conditioning. e) Time spent (sec) exploring the green and brown arms during the first CPP test session on the day after conditioning. f) Time spent (sec) in the green, brown, and blue (novel) arms during the second day of CPP after the first day of CPP described in e). g) Time spent (sec) in the green, blue (drug-unpaired familiar arm), brown, red (novel preferred) and yellow (novel avoided) arms on the third day of CPP after conditioning. Data are presented as mean ± SEM. Control group: n = 6–7 zebrafish per group, nicotine- and caffeine-paired groups: n = 8–10 zebrafish per group. *p < 0.05, **p < 0.01 and ***p < 0.001. $^{+}$p < 0.05, $^{++}$p < 0.01 and $^{+++}$p < 0.001 versus the control group of zebrafish with the corresponding colour of the drug-associated arm (green arm for caffeine and brown arm for nicotine).

brown arm (nicotine-paired) compared to the green arm ($p < 0.01$). Caffeine-conditioned zebrafish also showed a reduction in the latency to enter the green arm (caffeine-paired) compared to the brown arm ($p < 0.05$). In Fig. S1b (group 2) it is evident that zebrafish showed a higher latency to enter the novel blue arm compared to the brown and green arms, in both drug-conditioned groups ($F_{2,35} = 54.02, p < 0.01$). Nicotine- and caffeine-conditioned zebrafish also showed reduced latencies for exploring the respective drug-paired arms (Fig. S1b). When conditioned zebrafish were free to explore the entire maze (Fig. S1c, group 3), latencies for exploring the blue and yellow arms were significantly higher ($F_{4,45} = 71.08, p < 0.0001$) compared to the latency to explore the arm paired with nicotine ($p < 0.001$) or caffeine ($p < 0.01$). Latencies for exploring the red arm showed intermediate values between drug-paired and blue or yellow arms ($p < 0.01$; $p < 0.05$).

3.2. Experiment 2

3.2.1. Analysis of place preference to nicotine or caffeine tested over two or three consecutive days

In order to better understand the effect of novelty during the CPP test, zebrafish were tested in similar conditions to experiment 1, group

1 (Fig. 4a), and were tested again the following day (Fig. 4c). The experiments showed a second day of positive CPP for nicotine. Other groups of zebrafish were tested for CPP on the first and second day after conditioning to caffeine and nicotine, with the conditioned arms and the novel blue arm (Figs. 4b and d). Nicotine- and caffeine-CPP scores were significantly higher than the scores obtained in the other two arms ($p < 0.01$ and $p < 0.05$, respectively).

Considering the previous results, we decided to go forward and further evaluate the preference for nicotine and caffeine by testing the animals for three consecutive days. On the first day of CPP, time spent in each arm was similar to the time spent by zebrafish exploring each arm in experiment 1, group 1 (Fig. 4e). The next day, the same group of zebrafish were tested for a second day of CPP in the presence of a new blue arm that was available for exploration (Fig. 4f). Nicotine-conditioned animals spent more time in the brown arm compared to the green and blue arms ($F_{2,75} = 6.5$, $p < 0.001$; $p < 0.01$, and $p < 0.001$, respectively). On the second day of CPP, the caffeine-conditioned zebrafish spent more time in the green arm (caffeine-paired) compared to the time spent in the brown and blue arms ($p < 0.05$ and $p < 0.01$, respectively). However, the time spent exploring the green arm in the caffeine paired group was not significantly different from the time naïve zebrafish explored the green arm in the control group ($p = 0.12$). On the third day of CPP, red and yellow arms were also opened (Fig. 4g; $F_{4,75} = 7.75$, $p < 0{,}001$). In the control groups, the yellow arm was significantly less explored than the other arms ($p < 0.05$). The red (novel) and blue (familiar) arms were explored over a more extensive period than the yellow arm (novel). The nicotine-paired group showed similar behaviour to that observed during the previous testing days. Caffeine-paired zebrafish explored the caffeine-paired arm for longer than the brown, red, and blue arms ($p < 0.05$), and the yellow arm ($p < 0.001$).

In order to determine the possible persistence of CPP in the drug-paired arm over the three days of testing, a two-way ANOVA was performed. The ANOVA indicated a significant effect of treatment condition ($F_{2,75} = 5.198$, $p < 0.0417$), a significant effect of green versus brown arm ($F_{2,75} = 4.717$, $p < 0.0308$), and a significant interaction between these variables ($F_{2,75} = 20.22$, $p < 0.0001$), demonstrating the persistence of CPP during the three consecutive days of testing.

3.2.2. Behavioural analysis of place preference during successive testing days in zebrafish

3.2.2.1. Number of entries to the drug-paired arm in CPP test sessions performed over three consecutive days

Fig. S2a shows that the number of entries to the arm paired with caffeine was significantly increased compared to control (brown arm in this case) on the first testing day ($p < 0.05$). In contrast, the group of zebrafish conditioned to nicotine did not show differences in this behavioural parameter. On the second testing day, the new blue arm was less frequently visited than the drug-paired arms (Fig. S2b). The nicotine-paired arm was significantly more visited than the new blue arm ($p < 0.05$). The caffeine-paired arm was significantly more visited than the control ($p < 0.05$) and the new blue arm ($p < 0.05$). On the third day of testing, a significant increase in the number of entries to drug-paired arms was observed (Fig. S2c). The arm paired with nicotine was more frequently visited than the arms not paired to drugs (vs. the red arm: $p < 0.05$; vs. the blue and yellow arms: $p < 0.01$). When the zebrafish were conditioned with caffeine, a significant increase in the number of entries to the caffeine-paired arm was found when it was compared with the control, blue and yellow arms ($p < 0.05$). However, no differences were observed when the number of entries to the caffeine-paired arm was compared with the number of entries to the red arm.

3.3. Experiment 3: analysis of CPP in zebrafish conditioned with caffeine and nicotine using the FTRM

We next evaluated the place preference associated with a drug (nicotine or caffeine) using a similar protocol to experiment 1 group 1,except that zebrafish were exposed to caffeine in the green arm and 5 h later to nicotine in the brown arm, over a 14-day period of conditioning (see Methods for details). Some groups of zebrafish were exposed first to nicotine and 5 h later to caffeine each conditioning day, and exactly the same results were observed. Zebrafish in the control group did not show a preference for either the green or brown arms (Fig. S3a, control). When the time spent by zebrafish in the green and brown arms was measured over a 5 min period after conditioning with both drugs, a significant increase ($F_{3,23} = 22.04$, $p < 0.0001$) in the time spent in the brown arm was observed when compared with the time spent in the green arm ($p < 0.001$).

To evaluate differences compared to the regular CPP tank, we performed experiment 3 using the two-half tank task. We first carried out separate CPP tasks for caffeine and nicotine. ANOVA revealed significant differences among the groups ($F_{3,23} = 14.96$, $p < 0.001$). Nicotine induced a positive CPP ($p < 0.01$); however, caffeine did not cause significant changes in place preference (Fig. S3b). Next, another group of zebrafish were conditioned with caffeine in the morning and nicotine in the afternoon. In test sessions, the time spent by zebrafish, over a 5 min period, in the drug-paired side was significantly increased, not only when compared to control unpaired arms ($p < 0.001$), but also when compared to CPP to nicotine alone ($p < 0.05$) or caffeine alone ($p < 0.01$).

3.4. Analysis of nicotine and caffeine CPP in zebrafish using a two-half tank

Next, we compared the results obtained using the FTRM task with the two-half CPP (Kedikian et al., 2013; Ninkovic and Bally-Cuif, 2006). Fig. S4a shows the results expressed as CPP scores (%) ($F_{4,24} = 11.90$, $p < 0.0001$) and the period spent in the drug-paired side ($F_{4,19} = 11.00$, $p < 0.001$) (Fig. S4b). CPP scores are the most common way to express values in the two-half tank CPP test. The amount of time in seconds is also depicted to more directly compare these findings with the results obtained with the FTRM, because scores cannot be calculated in experiments involving novelty. Fig. S4a and b show that 15 mg/l nicotine induced a clear CPP ($p < 0.01$) whereas 100 mg/l nicotine induced conditioned place aversion (CPA) ($p < 0.01$). Both 50 mg/l and 100 mg/l caffeine did not induce any significant effect on side preference, as compared to control groups.

3.5. Validation of the FTRM task at the pharmacological level by evaluating the effect of PhB on CPP in zebrafish using the FTRM task

Considering our previous results showing that the HDAC inhibitor PhB decreased nicotine-CPP in rats, and our more recent findings indicating that PhB significantly modified innate object preference in zebrafish (Faillace et al., 2017; Pastor et al., 2011), we evaluated this compound effect in the FTRM task. To this end, we used two protocols similar to the ones used in experiment 1 and experiment 2, because we sought to evaluate whether PhB could affect nicotine or caffeine place preference, as well as the effect of novelty on CPP. ANOVA revealed significant differences among groups ($F_{4,41} = 25.773$, $p < 0.0001$). Fig. 5a shows no significant differences among the experimental groups, indicating that the treatment with PhB inhibited CPP to nicotine (compare Figs. 5a and 2a). When the rewarding properties of nicotine or caffeine were tested with a novel environment (Fig. 5b), no nicotine-CPP was observed. Interestingly, the time spent by zebrafish in the caffeine-paired arm was significantly longer than the time spent by

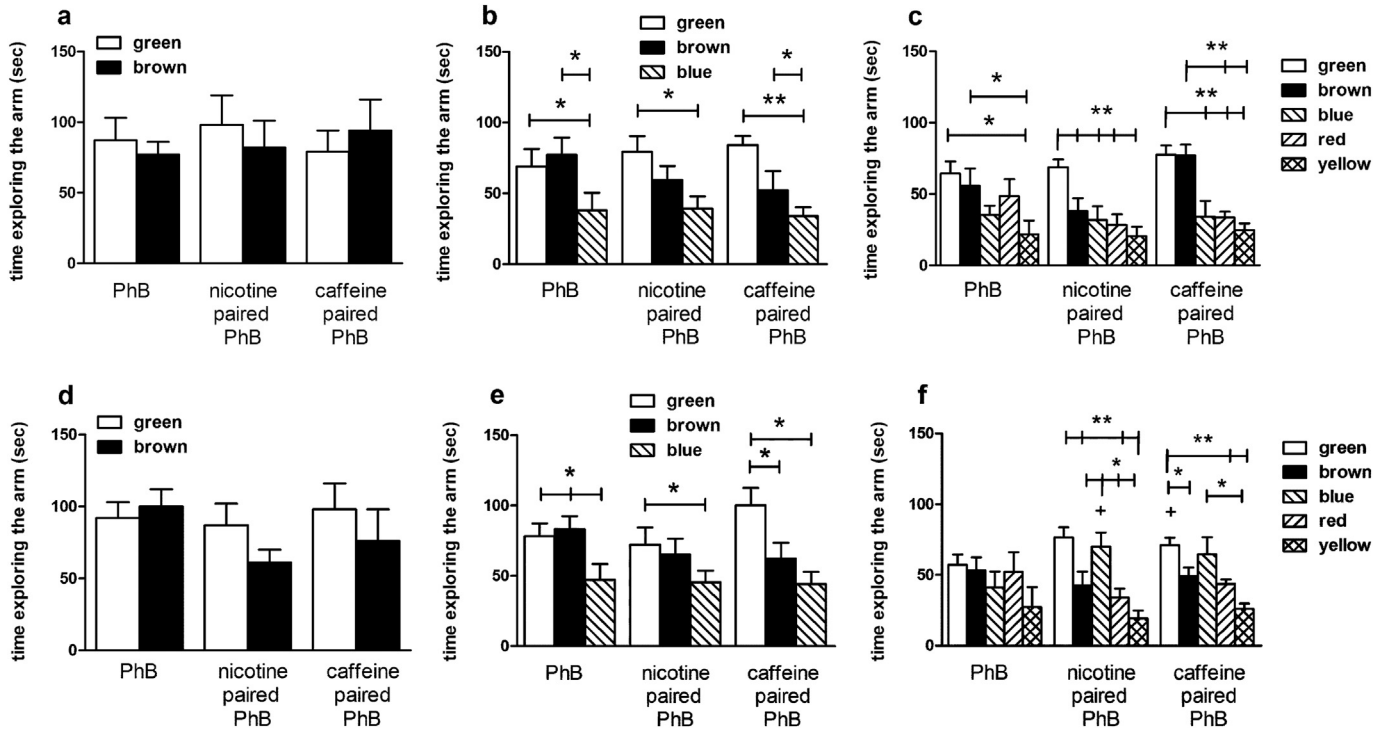

Fig. 5. Effect of Phenylbutyrate (PhB) on nicotine- and caffeine-induced CPP using the FTRM task. The procedure was similar to the one described in Fig. 2 (a–c) and Fig. 4 (d–f) except that 15 μM of PhB (histone deacetylase activity inhibitor) was added during conditioning alone (PhB-treated group), together with nicotine (Nicotine-conditioned PhB-treated group) or with caffeine (caffeine-conditioned PhB-treated group) to the tank water.

PhB (control): the inhibitor was dissolved into the water contained within the brown and green arms of the maze. Nicotine-conditioned PhB: 15 mg/l nicotine + PhB were dissolved into the water filling the brown arm whereas the green arm contained PhB only. Caffeine-conditioned PhB: a solution containing 50 mg/l caffeine + PhB was added into the green arm whereas the brown arm contained PhB only. a) Time spent (sec) in the green and brown arms over a 5 min period the day after conditioning (14 days); the other arms of the maze were blocked for exploration. b) Time spent (sec) in the green, brown and blue arms over a 5 min period the day after conditioning. c) Time spent (sec) by zebrafish in the green, blue, brown, red and yellow arms over a 5 min period the day after conditioning. d–f) Time spent (sec) in each arm during the CPP test through three consecutive days. All the groups of zebrafish were exposed during conditioning (14 days) to PhB. The first day of CPP the green and brown arms were opened. The second day of CPP the blue arm was also available. The third day of CPP the 5 arms of the maze were opened. Data are presented as mean ± SEM. Control group: n = 6 zebrafish per group, nicotine- and caffeine-paired groups: n = 8–10 zebrafish per group. *p < 0.05 and **$p < 0.01$. $^{+++}$p < 0.01 and $^{+}$p < 0.05 versus the same coloured arm in the control group.

zebrafish in the control brown or novel blue arm (Fig. 5b), as was observed without PhB (Fig. 2b). Exploration of the blue arm was not affected by PhB. In the testing environment of group 3, PhB caused a significant decrease in the exploration of the yellow arm compared to the green and brown arms (Fig. 5c; $p < 0.05$). The nicotine-conditioned group treated with PhB spent more time exploring the green arm than the blue, red, yellow, or brown (nicotine-paired) arms ($p < 0.01$). On the other hand, the caffeine-conditioned group treated with PhB spent more time exploring the caffeine-paired and control brown arms than the blue, red, or yellow arms ($p < 0.01$).

When the effect of PhB was evaluated over three days of CPP sessions, ANOVA revealed significant differences among the groups ($F_{4,45} = 37.588$, $p < 0.0001$). Fig. 5d shows similar results to Fig. 5a, indicating that the treatment with PhB prevented CPP to nicotine, normally observed without PhB, on the first day of testing in the FTRM. Likewise, when the rewarding properties of nicotine or caffeine were tested with the novel blue arm on the second day of CPP (Fig. 5e), no nicotine-CPP was observed. In contrast, CPP to caffeine was induced by the presence of the novel environment (Fig. 5e), as was found without PhB (Fig. 4b). On the third day of CPP, when zebrafish were tested with the five arms of the maze opened, PhB had no effect on the control groups (Fig. 5f). The nicotine-conditioned group treated with PhB spent more time exploring the blue and green arms compared to the red, yellow, or brown (nicotine-paired) arms ($p < 0.05$). The caffeine-conditioned group treated with PhB spent more time exploring the caffeine-paired arm than the drug-unpaired arms ($p < 0.05$ and $p < 0.01$) or the corresponding control group ($p < 0.05$).

3.6. Protein levels of H3-K9Ac and H3-K9me3 in zebrafish brain after nicotine or caffeine CPP in the FTRM

Considering the findings obtained with the PhB treatment, we examined the protein levels of H3-K9Ac and H3-K9me3 using Western blot in zebrafish brain portions containing structures of the reward pathway. The presence and levels of these modified forms of H3 were assessed in zebrafish of experimental group 1 (Fig. 6). One-way ANOVA indicated significant differences among groups when H3-K9Ac relative levels were quantified ($F_{5,23} = 9.54$, $p < 0.0001$). A significant increase in the level of H3-K9Ac was observed in the nicotine-conditioned group ($p < 0.01$) compared with the saline solution-treated control group. The levels of H3-K9Ac in nicotine-conditioned zebrafish brains were significantly higher than in the caffeine-conditioned group ($p < 0.01$) (Fig. 6c). In group 2, where a novel environment was introduced in the test session, H3-K9Ac levels in the nicotine-paired group were also significantly higher than the levels found in the control or caffeine-paired groups ($p < 0.05$). When the levels of H3-K9me3 (Fig. 6d) were analysed, no significant differences were found in the zebrafish from group 1 ($F_{5,23} = 2.69$, $p = 0.0549$). In contrast, in the brains excised from zebrafish in group 2, a significant increase in the levels of H3-K9me3 was observed in the caffeine-conditioned group compared to the nicotine-conditioned group ($p < 0.05$).

4. Discussion

The present study demonstrated that nicotine-conditioned zebrafish spent a significantly increased amount of time in the drug-paired

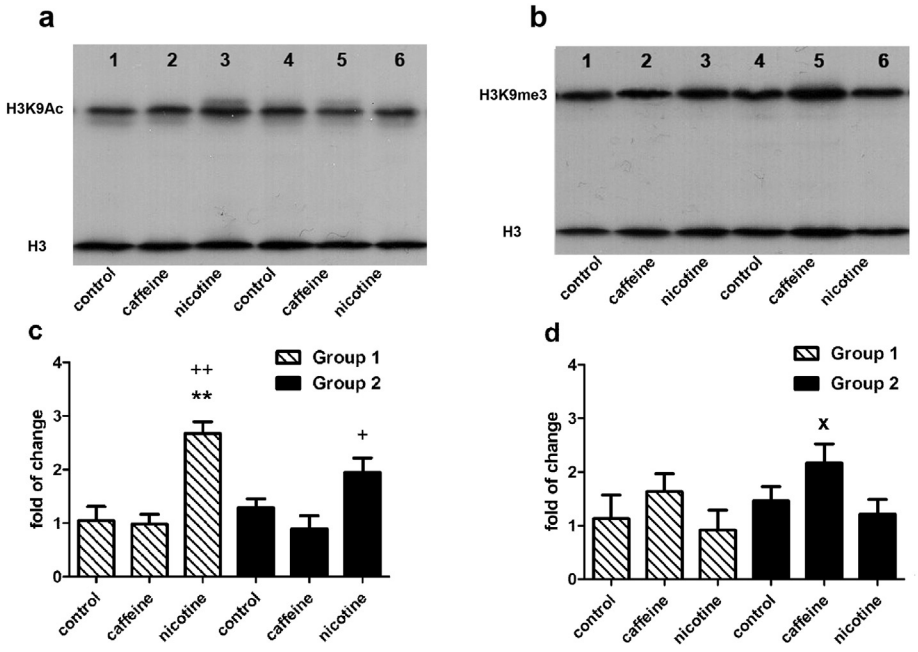

Fig. 6. Expression level of H3K9Ac (a) and H3K9-3me (b) proteins by Western blot in zebrafish brain homogenates. Protein bands were detected with specific primary antibodies against H3K9Ac (a) and H3K9-3me (b). During conditioning the brown and green arms of the radial maze were associated with nicotine or caffeine, respectively during 14 days. The day after conditioning, zebrafish were tested for preference with the conditioned arms (drug-paired and drug-unpaired arms) only (group 1) or with the conditioned arms plus a novel blue arm (group 2). For further details see protocols for Experiment 1, groups 1 and 2 in Methods. Graphs c) and d) show the densitometric analysis of the protein bands detected as indicated in a) and b). Values were expressed as a ratio relative to the control group of zebrafish (conditioned to the place without drugs and tested in the same way as the experimental groups 1 and 2); therefore, the control value equals 1. N = 6 zebrafish per group. $^{**}p < 0.01$ compared to the control group of the same experimental group 1 or 2.
$^{+}p < 0.05$ and $^{++}p < 0.01$ compared to caffeine-paired of the same experimental group. $^{x}p < 0.05$ compared to nicotine-paired of the same experimental group.

environment during CPP testing in the FTRM. The choices made by Zebrafish were not significantly modified by the presence of novel stimuli, such as one or more arms of different colours, suggesting that the nicotine-environment association was stable after at least 17 days. This preference suggests that zebrafish attribute a positive valence to the presence of the drug in the specific environment. Nicotine induced CPP in the FTRM, under all testing conditions, whereas caffeine did not. In basal testing conditions, caffeine-conditioned zebrafish showed a non-significant tendency to stay in the drug paired arm over a longer period of time during the testing sessions, although caffeine-CPP at the dose examined was only significantly positive when novel stimuli were introduced during the CPP test.

The aims of the current study were threefold: (1) to validate the use of the FTRM task in zebrafish for the evaluation of CPP to nicotine and caffeine; (2) to evaluate CPP strength by introducing novel stimuli as disturbing or choice disrupting factors during CPP expression; and (3) to examine the hypothesis that histone deacetylase activity inhibitors, such as PhB, prevent nicotine-CPP in this complex task in a similar matter to in our previous studies with classical CPP tasks (Faillace et al., 2017; Kedikian et al., 2013).

We designed a new task which combined “conventional” CPP and 5-CSRTT; this required a novel apparatus to evaluate the robustness of the drug-environment associations and the possible behavioural changes induced by novelty during drug seeking behaviour (test sessions). In this sensitive time window, environmental changes can improve or decrease drug-environment associations.

Therefore, we designed the FTRM experiments to simultaneously evaluate: 1) the rewarding properties of drugs of abuse based on the recognition of different coloured places in zebrafish; 2) the effect of novelty as a distractor during the test in which conditioned zebrafish were able to explore new arms of different colours; and 3) the choices of zebrafish in relation to reward and memory (novel or familiar stimuli during testing).

To test whether the distribution and size of the chambers (radial arms in the FTRM) influenced the conditioning, we also performed nicotine and caffeine CPP using a two-half tank. The results showed that CPP was established with the same concentration of nicotine used in the FTRM. Nevertheless, the time spent in the drug-paired area was lower in the FTRM than in the side paired with nicotine in the two-half tank task (161 ± 23 s and 198 ± 29 s over a 300 s testing period, respectively). These findings indicate that zebrafish spent more time exploring the whole FTRM tank, probably due to its topology, whereas in the two-half tank, the exploration area is reduced to two equally sized chambers. We showed that higher doses of nicotine can induce CPA in zebrafish (Fig. S4a), as has been previously demonstrated in rats (Pastor et al., 2011).

We observed that zebrafish conditioned to caffeine in the FTRM showed an increased number of entries to the caffeine-paired arm. This effect was not observed in zebrafish conditioned with nicotine (Fig. 3a). To further evaluate this behaviour, we digitally divided each arm into two halves: zone 1 and zone 2 (Fig. 3d). Considering the time spent and distance travelled in each zone, caffeine-conditioned zebrafish spent significantly less time exploring the farthest zone of the arm (Fig. 3h and k), which together with the increase in the number of entries, could suggest “anxiety like” behaviours (Maximino et al., 2011). In contrast, the majority of nicotine-conditioned zebrafish explored the whole length of the arm, which suggests a drug seeking behaviour. Moreover, a significantly lower latency to explore the nicotine-paired arm and a higher latency to explore the caffeine-paired arm, relative to the control groups, also indicates that nicotine-conditioned zebrafish behaviour could be classified as exhibiting anticipatory activity (Parker et al., 2015), whereas caffeine-conditioned zebrafish showed anxious behaviours during the test session.

Previous studies have demonstrated that pre-exposure to a novel environment can influence CPP (Arenas et al., 2016; Cachat et al., 2010; Pastor et al., 2013); however, there are no reports on the effect of novelty during CPP testing. Reinforcing and rewarding properties of drugs are evoked in the CPP test; thus, this is a critical and labile time window for long-term establishment of conditioning. In this study, we detected an effect of novelty on drug preference. Novelty could be considered a stressful situation for animals (Zorrilla et al., 2014). The novel environment slightly decreased nicotine-CPP on the second day of the CPP test (161.43 ± 18.55 s vs. 154.33 ± 23.72 s, Fig. 4a and c, and 158.41 ± 10.67 s vs. 13744 ± 13.54 s, Fig. 4b and d). However, during the second day of CPP, the blue arm was familiar to the fish and likely less distressing.

On the contrary, the distressing novel environment significantly induced caffeine-CPP, and hence, the reinforcing properties of caffeine which were not established by conditioning itself in the FTRM. Moreover, when zebrafish were tested with the blue arm on the second day of CPP, their preference for the caffeine-paired arm was also induced (Fig. 4b and d).

To assess whether it was only the innate aversive new environment (blue) that modified the rewarding properties of the drugs, a novel red arm (preferred) was available to explore during test sessions, replacing the blue arm (see Results section). CPP to nicotine and caffeine was established, although latencies to enter the novel red arm were lower and zebrafish explored the red arm over a longer period of time than the blue arm. These findings indicate that novelty particularly affected the rewarding properties of caffeine in the FTRM, and it is possible that stress added the component to transform a seeking tendency to a significant preference (Zorrilla et al., 2014).

Our observations indicate that the blue arms were less visited than the green, red, or brown arms in the FTRM. Nonetheless, when the blue arm was no longer a new environment, on the second and third day of CPP testing (compare Fig. 4d, g), the blue arm was explored for a longer period of time, suggesting that novelty could be more aversive than colour *per sé*. These findings also suggest that memory consolidation is implicated in the behavioural change of zebrafish toward the blue arm. Furthermore, PhB-treated zebrafish explored the blue arm for longer periods of time when they had been exposed to this arm the day before (compare Fig. 5c and f). These findings, together with previous studies (Faillace et al., 2017; Ganai et al., 2016), suggest that PhB enhanced memory consolidation perhaps by increasing attention. It might also be that PhB caused a reduction in place aversion in zebrafish (compare Fig. 4b with Fig. 5b and e).

The robustness of the nicotine-CPP established after 14 days of conditioning in the FTRM was further challenged when zebrafish were tested over three consecutive days, in increasingly disturbing environments. After two or three days of testing in a drug-free environment, under the same or different environmental conditions (with or without novel stimuli), nicotine-conditioned zebrafish barely modified their preference for the place associated with nicotine. Interestingly, caffeine-CPP was also induced by novelty on the second day of the CPP test, which endured through the third day of CPP testing in the presence of several putative disturbing stimuli (Fig. 4e–g). This suggests that once CPP has been established in zebrafish, it is more difficult to extinguish, as has been demonstrated for nicotine-CPP in rats (Pascual et al., 2009).

In order to evaluate the possibility of an interaction between nicotine and caffeine, we designed experiments where both drugs were administered at different intervals during the same conditioning day, and were paired with different environments. They were not administered together since, at the relatively low concentrations assayed in this work, caffeine and nicotine together induced seizure activity in zebrafish (data not shown). Zebrafish spent more time exploring the nicotine-paired arm compared to the caffeine-paired arm after the same group of zebrafish had been conditioned with both drugs in the green (caffeine in the morning) and the brown (nicotine in the afternoon) arms for 14 days. The findings in the FTRM task confirm that the rewarding properties of nicotine are much stronger than the rewarding properties of caffeine. Interestingly, the crosstalk between caffeine and nicotine during conditioning, even though they were associated with different environments, potentiated the rewarding properties of nicotine. A similar potentiation was observed using the two-half CPP tank. Apparently, caffeine increased the rewarding properties of nicotine, but not the opposite. Administration of caffeine with nicotine had no effect on plasma levels of nicotine and its metabolite cotinine (Gasior et al., 2002), but caffeine can increase acetylcholine neurotransmission in various brain areas (Acquas et al., 2002). In this work, after 14 days of nicotine exposure, up-regulation of the nicotinic receptors probably occurred; this could be a possible mechanism by which caffeine (and perhaps other drugs) potentiates nicotine-CPP. It has been previously observed that caffeine increases the effect of cocaine in rats (Prieto et al., 2015).

Of note, PhB decreased the rewarding properties of nicotine in the three conditions assessed, without affecting caffeine preference in the FTRM task. Thus, it appears that histone deacetylase activity is involved in the rewarding effects of nicotine, but not of caffeine. We have previously demonstrated that PhB decreases preference for nicotine in a CPP model in rats, whereas HDAC2 plays an important role in this behaviour (Pastor et al., 2011). PhB also reduces the reinstatement of cocaine-seeking behaviour and self-administration in rats (Romieu et al., 2008, 2011). On the other hand, treatment with caffeine decreased the number of HDAC-immunopositive cells in the rat brain (Machado-Filho et al., 2014). Considering the notion that drug addiction shares commonality with learning and memory processes, the question arises whether general learning would be similarly impaired by HDAC inhibitors. However, it has been demonstrated that sodium butyrate (another HDAC inhibitor) facilitates aversive memory consolidation in rodents (Bieszczad et al., 2015; Blank et al., 2015; Levenson et al., 2004). This apparent divergence may arise because the learning component, although required for the animal to associate the drug with its environment, may not play a primary role during CPP expression. Alternatively, it may also indicate that HDAC inhibitors principally facilitate aversive but not appetitive learning, as was observed in rats (Lattal et al., 2007). Another possibility is that CPP expression is principally based on the motivational aspect, which probably represents the major target of HDAC inhibitors (Pastor et al., 2011), as was previously demonstrated in experiments using the self-administration paradigm (Romieu et al., 2008).

Furthermore, H3K9Ac levels were increased only in nicotine-conditioned zebrafish brains. This finding, taken together with the behavioural data, indicates that histone acetylation plays a crucial role in the induction of the rewarding properties of nicotine at the brain level, as has been demonstrated in rats. This suggests conserved processes in vertebrates (Pastor et al., 2011). Interestingly, H3K9me3 levels were significantly increased only in group 2 (tested with novelty) caffeine-conditioned zebrafish. The change in histone-methylation could be a consequence of novelty-drug associations favouring the establishment of caffeine-CPP. In fact, de novo methylation takes approximately 8–12 h (Baker-Andresen et al., 2013; Zhou et al., 2014), and zebrafish were euthanized 16 h after testing. Previous studies have demonstrated that H3K9me3 can suppress transcription through a mechanism involving histone deacetylation (Stewart et al., 2005). Because we observed the lowest level of H3K9Ac in group 2 caffeine-conditioned zebrafish, the increase in H3K9me3 levels might be associated with a decrease in H3K9Ac levels. However, a deeper analysis of histone acetylation and histone and DNA methylation will be necessary to understand the complex phenomenon observed here at the molecular level.

5. Conclusion

In this study, using a new behavioural paradigm in zebrafish (FTRM), we investigated whether the introduction of novelty during the expression of CPP affects the rewarding properties of nicotine and caffeine. Our experiments introducing novel stimuli (up to three simultaneously) during the expression of the seeking behaviour, after conditioning, suggest that new or unexpected situations in environments that were previously associated with drugs can potentiate the rewarding pathway in the brain. However, these effects depend on the drug of abuse evaluated and the kind of stimuli introduced. Our results indicate that the effect of drugs with weaker rewarding properties, like caffeine, can be more easily potentiated by the introduction of environmental changes during CPP expression. Our findings also indicate that the introduction of disturbing novel stimuli are not able to disrupt the establishment of nicotine-CPP in zebrafish, indicating that nicotine rewarding properties and nicotine-environment associations are extremely robust. Histone acetylation and methylation appear to play an important role in these processes, depending on the rewarding properties of the drug. Inhibitors of HDAC are promising pharmacological compounds for treatments to reduce drug reinforcing behaviours.

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Author contributions

1. Conceived and designed the experiments: ROB.
2. Performed the experiments: MPF, APF, ROB.
3. Analysed the data: MPF, ROB.
4. Contributed reagents/materials/analysis tools: ROB.
5. Wrote the paper: MPF, ROB.

Conflict of interest

The authors did not report any conflict of interest.

Ethical statement

1) This material has not been published in whole or in part elsewhere;
2) The manuscript is not currently being considered for publication in another Journal;
3) All authors have been personally and actively involved in substantive work leading to the manuscript and are responsible for its content.

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