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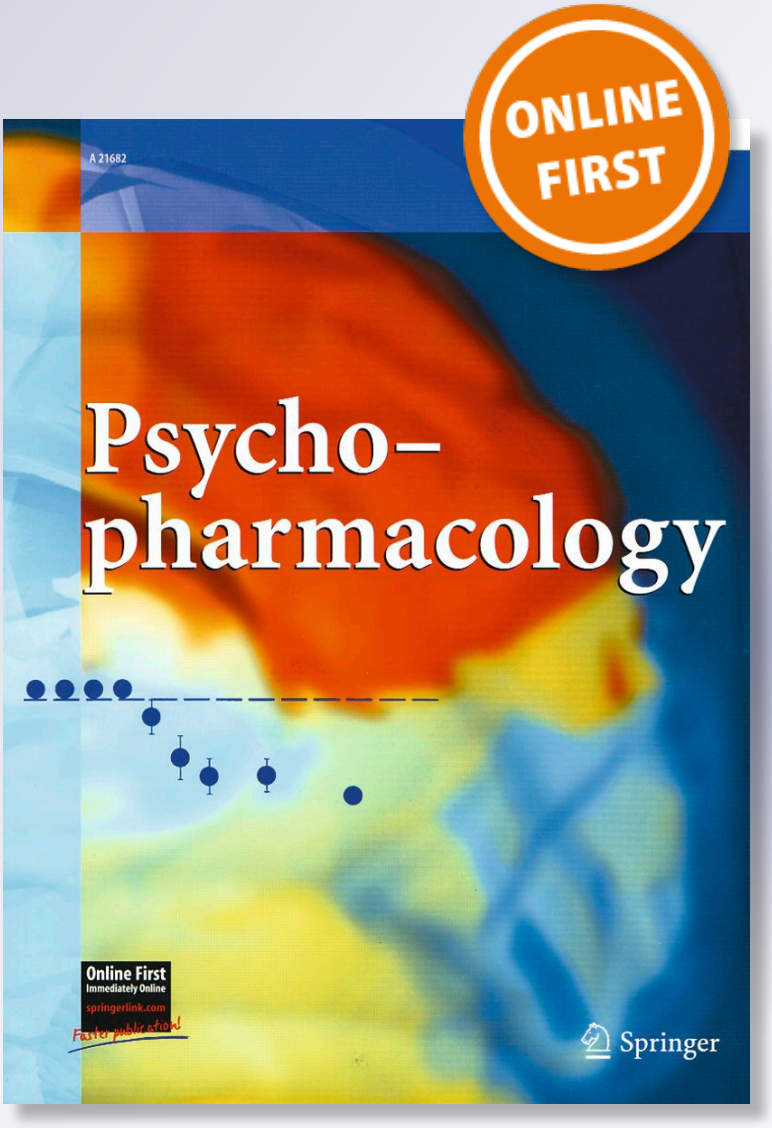

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ORIGINAL INVESTIGATION

Short- and long-term effects of nicotine and the histone deacetylase inhibitor phenylbutyrate on novel object recognition in zebrafish

MP Faillace[1] · A Pisera-Fuster[1] · MP Medrano[1] · AC Bejarano[1] · RO Bernabeu[1]

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Abstract
Rationale Zebrafish have a sophisticated color- and shape-sensitive visual system, so we examined color cue-based novel object recognition in zebrafish. We evaluated preference in the absence or presence of drugs that affect attention and memory retention in rodents: nicotine and the histone deacetylase inhibitor (HDACi) phenylbutyrate (PhB).
Objectives The objective of this study was to evaluate whether nicotine and PhB affect innate preferences of zebrafish for familiar and novel objects after short- and long-retention intervals.
Methods We developed modified object recognition (OR) tasks using neutral novel and familiar objects in different colors. We also tested objects which differed with respect to the exploratory behavior they elicited from naïve zebrafish.
Results Zebrafish showed an innate preference for exploring red or green objects rather than yellow or blue objects. Zebrafish were better at discriminating color changes than changes in object shape or size. Nicotine significantly enhanced or changed short-term innate novel object preference whereas PhB had similar effects when preference was assessed 24 h after training. Analysis of other zebrafish behaviors corroborated these results.
Conclusions Zebrafish were innately reluctant or prone to explore colored novel objects, so drug effects on innate preference for objects can be evaluated changing the color of objects with a simple geometry. Zebrafish exhibited recognition memory for novel objects with similar innate significance. Interestingly, nicotine and PhB significantly modified innate object preference.

Keywords Zebrafish behavior · Nicotine · Histone deacetylase inhibitor · Object recognition · Attention: perception

Introduction

Being able to discriminate objects in the environment has benefits when it comes to feeding, mate choice, defensive behavior, orientation, and survival (Engeszer et al. 2004). Interaction with environmental stimuli requires the organism to assess the stimuli, including whether or not they have been encountered before (Bilotta et al. 2005; Engeszer et al. 2007). Rodents have an innate tendency to approach and explore novel objects, i.e., objects with no innate or learned significance for them. Memory for objects, places, and events plays a critical role in how an organism experiences its environment. Novel object recognition (OR) tasks have been extensively used to study non-aversive memory in rodents (Bevins and Besheer 2006; Gaskin et al. 2010; Mathiasen and DiCamillo 2010; Winters et al. 2010). Zebrafish (*Danio rerio*) have become increasingly popular as subjects for research into learning and memory processes, and the species has been proposed as an alternative to mammalian models (Colwill et al. 2005; Lieschke and Currie 2007; Stewart et al. 2010; Gerlai 2011; Karnik and Gerlai 2012; Kalueff et al. 2013). Like rodents, zebrafish can distinguish between novel and familiar objects and so the species is recognized as a suitable model for studying non-aversive memory mechanisms (Levin et al. 2006; Spence and

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Smith 2008). Objects of different colors are particularly useful for OR tasks because zebrafish have very good color perception. Color vision in the zebrafish relies on four types of cone photoreceptor cells: UV- and blue-single cones as well as red- and green-double cones (Robinson et al. 1993). Humans have three types of cones and lack UV light sensitivity, whereas rodents have only two types of cone cells which significantly reduces their color perception capacity.

Color preference in zebrafish has been evaluated using foods of different colors and colored environments, and zebrafish showed a preference for red environments independently of the color of the food (Spence and Smith 2008). Zebrafish in a conditioned place preference (CPP) task spent more time in compartments with red and green backgrounds relative to yellow backgrounds and showed a strong aversion to compartments with blue backgrounds (Avdesh et al. 2012). In contrast, Colwill et al. (2005) found that blue and purple environments were preferred by zebrafish. A recently published study based on a modified CPP task reported that zebrafish exhibited a preference for blue and green environments and an aversion to yellow and red environments (Oliveira et al. 2015).

Additional studies have demonstrated that acute nicotine treatments enhance acquisition, consolidation, and retrieval of object memories in rats (Puma et al. 1999). There is also good evidence that in zebrafish activation of nicotinic receptors enhances discrimination learning (Levin et al. 2006) and CPP acquisition (Kily et al. 2008; Kedikian et al. 2013) which suggests that nicotine treatments may have therapeutic value in cognitive impairment disorders (Olincy and Stevens 2007). A recent study based on a modified OR task showed that nicotine increased attention and enhanced object discrimination performance (Braida et al. 2014). It has also been proposed that in zebrafish nicotine can improve recognition of complex objects (May et al. 2016).

Histone deacetylation results in heterochromatin formation and downregulation of gene expression. Memory consolidation and learning in rodents is inhibited by deacetylation of histones by histone deacetylases (HDACs) (Korzus et al. 2004; Bredy et al. 2008; Peleg et al. 2010). So preventing HDAC activity with inhibitors such as phenylbutyrate (PhB) is crucial to long-term memory (Wood et al. 2006; Morris et al. 2010; Roozendaal et al. 2010; Hawk et al. 2011). Likewise, we have demonstrated that acetylation of histones is necessary to establish nicotine-induced CPP in rats (Pastor et al. 2011). Correspondingly, OR and object location performance were enhanced by histone deacetylase inhibitors (HDACi) (Stefanko et al. 2009; Haettig et al. 2011; Hawk et al. 2011). Until now, HDACi have only been used in zebrafish to evaluate the effects of histone deacetylase on developmental morphogenesis (Kim et al. 2012).

We evaluated zebrafishes' innate color preferences in our experimental conditions as earlier studies have produced inconsistent results. Previous studies had not investigated short- and long-term novel object preference (Braida et al. 2014; Oliveira et al. 2015), so we carried out a series of experiments aimed to evaluate the effects of nicotine and PhB on performance on OR tasks with two memory retention intervals. Moreover, as it has been demonstrated that drugs of abuse such as cocaine can influence visual perception in zebrafish (Darland and Dowling 2001), we also investigated whether nicotine and PhB could modify zebrafishes' innate perception of novel objects. Our results suggested that the exploratory behavior of zebrafish is determined by innate preference rather than by novelty. Nicotine and PhB can modify innate object preference by increasing the salience of the environmental cues.

Methods

Animals and maintenance

We obtained adult zebrafish (*D. rerio*, Singapore strain; aged approximately 6 to 6 months), a wild-type-derived stock from a local farmer (La Plata, Buenos Aires, Argentina; Kedikian et al. 2013). Zebrafish were maintained according to standard methods (Westerfield 2007). They were kept at a density of 100 per tank (filled with 120 L of fish tank water) with a constant 14–10 h light-dark cycle and a temperature of 26–28 °C. The tanks had aquatic plants and stone floors (enriched environment). Water in the tanks was continuously filtered with an external canister filter (Eheim ECCO Pro 130, Germany). Zebrafish were fed twice a day with *Artemia* sp. and dry food. All zebrafish were experimentally naïve and were acclimatized to the laboratory facility for at least 30 days. After the acclimatization period, the animals were moved to the behavioral room and housed in 12-L tanks with stone floors and filtered water and at a maximum density of 12 animals per tank in order to reduce stress. All the behavioral assays took place during the light phase, between 09:00 and 16:00 h. The Committee on Animal Research of the University of Buenos Aires approved all protocols for the use, housing, and care of experimental animals.

Drugs and treatments

Nicotine (nicotine hydrogen tartrate salt, Sigma, St. Louis, USA) was dissolved in filtered water from the fish tanks to produce a 15-mg/L solution. We have demonstrated that 15 mg/L is the lowest nicotine dose that had rewarding effects in zebrafish, which were not enhanced by higher doses (30 or 50 mg/L). We have also observed that 50 mg/L nicotine solutions can generate unwanted motor effects that inhibit CPP (Kedikian et al. 2013). The HDACi 4-phenylbutiric acid (PhB; Aldrich, St. Louis, USA) was also dissolved in the fish tank water. We used three concentrations of PhB (15, 25, and 35 μM) based on our previous results and other authors' results in rats (Romieu et al. 2008; Pastor et al. 2013). The three concentrations produced similar results so all subsequent experiments were performed with 15 μM PhB, the

lowest pharmacologically effective dose in our studies. We have also observed that high doses of PhB can induce seizure-like activity.

Nicotine and PhB solutions were prepared fresh daily and administered by immersing the zebrafish in the solutions in a special tank (drug tank) for 10 min immediately after training sessions. The drug tank was 20 × 10 × 15 cm in size, and the same procedure was carried out with control animals, using a drug-free tank with identical characteristics. The pharmacokinetic and pharmacodynamic properties of these drugs, including clearance, are unknown in this species. Based on previous studies, we assumed that a 10-min period of drug exposure would allow the drug to act on the fish brain at a relatively constant concentration level. Other groups have used 5-min exposures to various drugs in beakers before or immediately after a behavioral task (Levin et al. 2007; Brennan et al. 2011), but in our experience, nicotine-induced locomotor activity in zebrafish stabilizes after 10 min (Kedikian et al. 2013). We did not use beakers to expose the zebrafish to the experimental drugs because being in restricted volumes can increase stress and cause non-specific effects; this has been observed in the context of nicotine administration (Levin and Chen 2004).

Novel object preference or recognition test

Two hundred zebrafish from the housing tank were separated into four groups: control, nicotine, PhB, and nicotine + PhB. All experimental groups experienced the following phases: (1) habituation to the testing tank (3 days); (2) training session: subjects were exposed to two identical objects; (3) exposure to drug solutions or free-drug water; and (4) testing session: after a delay of 1.5 or 24 h (retention interval), animals were placed back in the testing tank, which contained one of the original objects (familiar object) and a novel object.

All trials were videotaped using an HD digital camera placed 1.2 m above tanks and connected to a computer. All videos were analyzed using the Noldus EthoVision XT7 software (Noldus Information Technology, The Netherlands). Before training, every zebrafish was habituated to the testing tank (25 cm side × 25 cm side × 20 cm height) in the absence of objects for 5 min twice a day (5-h interval between habituation sessions) over three consecutive days (Bevins and Besheer 2006; Kily et al. 2008). On the fourth day, the training phase, animals were exposed to two identical cubes for 5 or 10 min. The objects were placed on the floor of the experimental tank. Just before the testing session, one of the original objects was replaced with a novel object differing in color, shape, or size from the training objects. The amount of time a zebrafish spent exploring one object was measured as the total time a subject's whole body remained within the exploration area delimited on the tank floor. The exploration area was defined based on the objects' size (Oliveira et al. 2015). The objects had sides of 3 cm length, and so the exploration area was defined as a 9 × 9-cm area centered on the object; the exploration area thus amounted to 6.4% (total of 12.8% for both objects) of the tank floor area (Fig. 6e). We examined the interactions of zebrafish with red, green, yellow, and blue cubes (side = 3 cm), red and yellow balls (diameter = 3 cm), and red and yellow towers (side = 1.5 cm, height = 8 cm). We used balls and cubes of similar dimensions to enable us to evaluate visual discrimination of shape. The towers were included to enable us to evaluate discrimination of visual changes in size as they were taller than the cubes. Preference percentages were calculated as follows: [time of exploration of novel object / (time of exploration of original object + time of exploration of novel object)] × 100 (Balderas et al. 2008; Haettig et al. 2011). A 50% preference indicates no preference for one object over the comparison object (chance: dashed lines in the graphs). Preference scores higher than 50% indicate a relative preference for the novel object, and scores lower than 50% indicate a relative aversion to the novel object. Mean exploration time was calculated, and the preference percentages of the groups were compared.

The locations of the object and the tank were counterbalanced. For half the testing sessions, the novel object was placed on the right side, and for the rest of the sessions, it was located on the left side of the tank. The tank was rotated 180° to avoid behavior being influenced by the context surrounding the exploration area.

Visual discrimination test

To determine whether the color cues selected for behavioral testing were equally perceived by zebrafish, we used the visual discrimination test described by Li and Dowling (1997). The test was carried out in an apparatus with a rotating drum that has been used to study reflexive escape in a variant of the optokinetic reflex test (Lau et al. 2011). Briefly, the apparatus consisted of an immobilized cylindrical tank (15 cm diameter, filled with 2 L of fish tank water) with transparent walls, surrounded by a rotating drum covered with white paper. A colored segment (5 × 5 cm) marked on the external drum was used as a threatening object. An opaque cylinder (5 cm diameter) was placed in the center of the tank to prevent the zebrafish swimming directly from one side of the tank to the other. The drum rotated at 10 rpm. If a subject sees the approaching colored segment (encounter), it typically displays one of two behaviors: it turns away to hide behind the central post (avoidance) or continues its path and thus dodges the stimulus (escape). Zebrafish were habituated to the tank for 2 min, and then red or yellow segments were attached to the external drum (Darland and Dowling 2001). The number of encounters, escape, and avoidance responses was quantified manually and using the tracking system described above.

Determination of other behavioral parameters

The behavioral parameter "distance swum," which is a measure of locomotor activity, was recorded as the total distance

traveled by zebrafish over 10-min periods. "Exploration latency" was measured as the time in seconds taken for a subject to begin exploring an object at the start of a session. "Exploration frequency" was measured as the number of full body entries to demarcated zones around objects over 5-min periods. The behavioral parameters were recorded separately for all experimental conditions (preference for object color; size + shape; color + shape, color + size or, color + size + shape) and groups (saline-, PhB-, nicotine-, and PhB + nicotine-treated zebrafish) during training and testing sessions. Subjects were exposed to the pharmacological treatments for 10 min immediately after training, and then test sessions were performed 1.5 or 24 h after training.

Statistics

We analyzed the time spent in delimited areas including the familiar or the novel object using ANOVA with the following factors: discrimination cue (color; size; shape), drug treatment (nicotine; PhB; nicotine + PhB; control), and retention interval (1.5; 24 h). Object preference was analyzed by ANOVA for multiple comparisons followed by post hoc comparisons using the Scheffé test. All data are expressed as mean ± SEM. Statistical analysis was done using the software StatView 5.0.1 (SAS Institute, Gary, NC, USA).

Results

Visual cues for object pre-existent preferences and object recognition tasks

Analysis of color and shape preference

The average time zebrafish spent exploring two identical objects during the training session differed according to the color of the object ($F_{7,40}$ = 43.331, $p < 0.01$); see Fig. 1. Zebrafish spent longer interacting with red objects (cubes 130.23 s ± 4.41; balls 114.525 s ± 3.94) and green objects (cubes 100.33 s ± 6.14; balls 90.65 s ± 7.32) than with yellow objects (cubes 43.52 s ± 4.94; balls 41.23 s ± 6.09) or blue objects (cubes 20.33 s ± 6.03; balls 21.56 s ± 5.94). Blue and yellow objects elicited significantly less exploratory activity than red or green objects ($p < 0.01$ and $p < 0.001$, respectively).

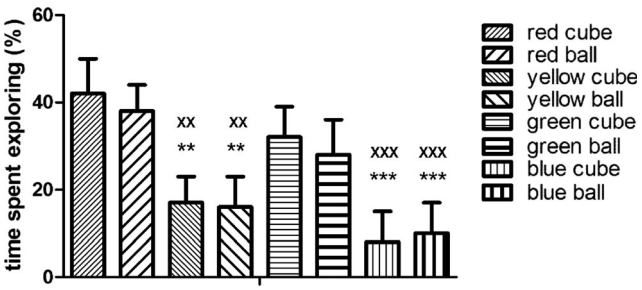


Fig. 1 Innate preferences of zebrafish for novel objects were assessed with objects of different color, shape, and size.Zebrafish were exposed individually to two identical objects for 5 min in the experimental tank. Preferences were assessed with ANOVA followed by Scheffé test. $^{**}p < 0.01$ (yellow vs. red objects), $^{***}p < 0.001$ (blue vs. red objects), $^{xx}p < 0.01$ (yellow vs. green objects), and $^{xxx}p < 0.01$ (blue vs. green objects)

Novel object recognition with color cue and objects of similar innate preference

Figure 2a shows preference scores for innately preferred red and green objects, and Fig. 2b depicts preference scores for two non-preferred blue and yellow objects in control, PhB-, nicotine-, and nicotine + PhB-treated animals ($F_{7,38}$ = 21.45, p = 0.043). When zebrafish were exposed to two identical red cubes and one of the red cubes was replaced with a green cube after a delay of 1.5 or 24 h, the control group showed no preference (p = 0.08). In contrast, zebrafish previously exposed to nicotine or PhB showed a preference for exploring the novel object at 1.5 or 24 h after training, respectively ($p < 0.05$). When a novel yellow cube replaced a blue cube, zebrafish spent longer exploring the novel object. A further increase in preference for novel yellow objects was found in the PhB-treated group of zebrafish 24 h after training. In contrast, nicotine decreased the preference for novel yellow objects at 1.5 and 24 h after training ($p < 0.05$).

Total exploration time in a preference test based on color, shape, and size cues

Our aim was to evaluate innate preference to novel objects during sessions lasting 5 or 10 min. Figure 3 shows that zebrafish displayed an innate preference for red objects relative to yellow objects regardless of whether the test involved cubes, balls, or towers ($F_{11,70}$ = 76.628, $p < 0.001$). Exploration durations for cubes, balls, and towers respectively were as follows: red objects 142.23 s ± 4.41, 138.525 s ± 3.94, and 129.22 s ± 5.01; yellow objects 61.32 s ± 7.94, 51.23 s ± 7.89, and 39.43 s ± 6.17. In subsequent sessions, we analyzed zebrafishes' exploratory behavior during a 5-min session in order to reduce habituation to the objects.

Analysis of novel object preference in zebrafish

Color preference Figure 4 depicts preference scores for novel red or yellow objects as determined in testing sessions. ANOVA revealed significant differences ($F_{31,80}$ = 7.857, $p < 0.001$) considering color cues, treatments, and retention intervals (1.5 and 24 h) as analysis factors. When we used a pair of cubes in the training session and one of the cubes was replaced with an identical cube in the test session, preference scores were close to 50% indicating no preference (Fig. 4a, b).

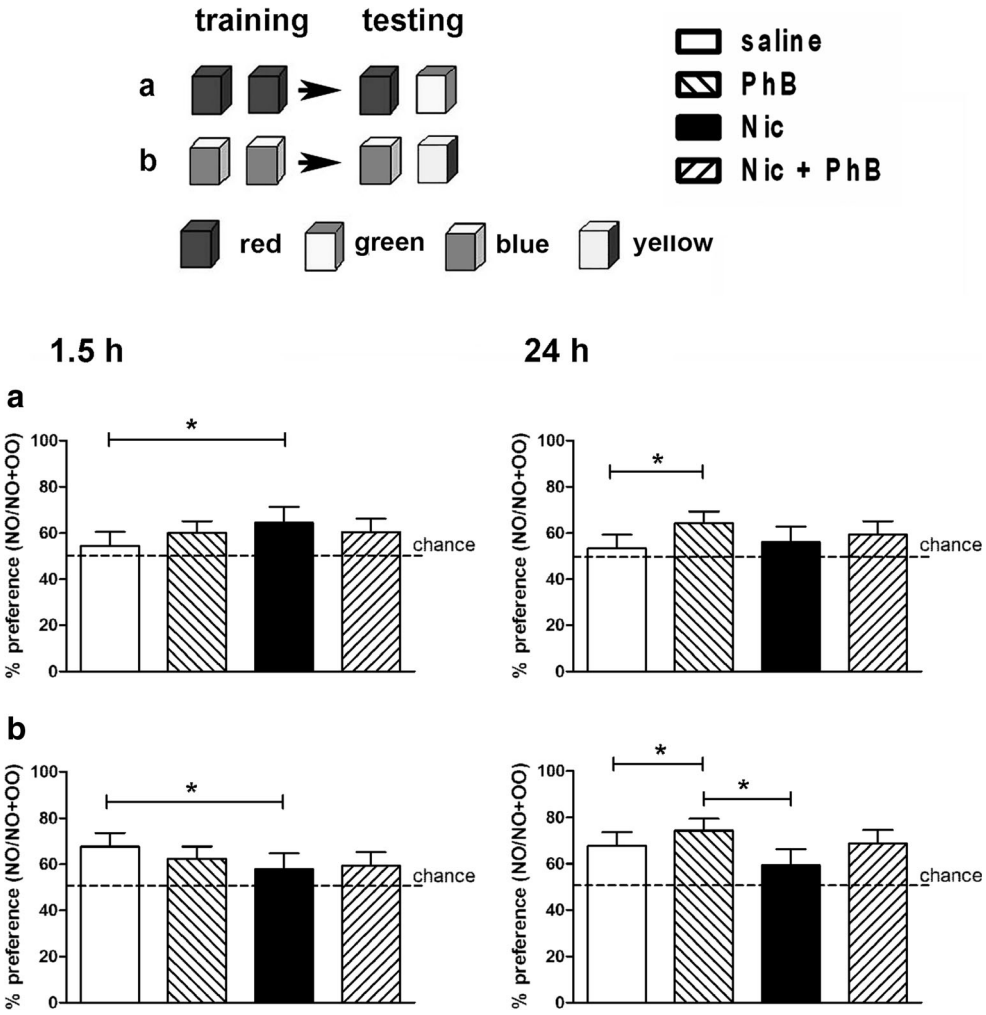


Fig. 2 Novel object recognition based on color cues of similar innate visual significance for zebrafish. Zebrafish were trained individually and then immersed for 10 min in tanks containing drug-free fish tank water (saline), 15 mg/l nicotine (Nic), 15 μM phenylbutyrate (PhB), or 15 mg/l nicotine plus 15 μM PhB (Nic + PhB). Drugs were dissolved in fish tank water. Testing sessions were performed after a delay of 1.5 h (*Graphs a* and *b* on the *left*) or 24 h (*graphs a* and *b* on the *right*). The *graphs* depict percentage (%) preference for green and red (*a*) or yellow and blue objects (*b*). *Dashed lines* (chance) indicate 50% preference. *NO* novel object, *OO* original object. Exploratory preferences were assessed with ANOVA followed by Scheffé test. *$p < 0.05$

As expected, neither nicotine nor PhB influenced scores when identical objects were paired.

When a yellow cube replaced a red cube in the test session (Fig. 4c), the control group had a mean preference score of less than 40%, indicating a preference for the familiar cube. When animals were tested after a 1.5-h retention interval, the nicotine and nicotine + PhB treatments further reduced preference for the novel object ($p < 0.05$) whereas PhB did not affect naïve preferences. In contrast, when subjects were tested 24 h after training, PhB or PhB + nicotine treatment further reduced the preference for the novel object ($p < 0.001$) whereas nicotine treatment did not affect preference. Figure 4d shows preference when a red cube replaced one of the familiar yellow cubes. When tested 1.5 h after trainingm nicotine-treated zebrafish showed an increased preference for the novel object ($p < 0.01$ and $p < 0.05$). PhB and PhB + nicotine treatments did not affect preference. With a 24-h retention interval, both PhB and PhB + nicotine treatments further enhanced preference for the novel red cube ($p < 0.01$).

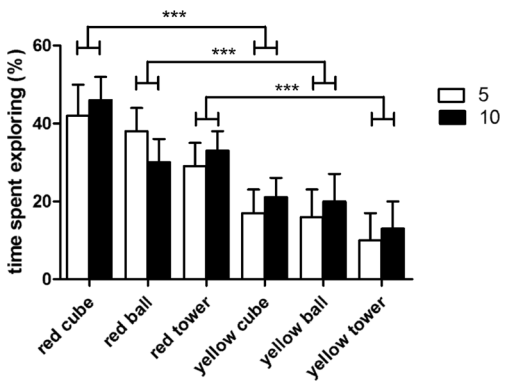


Fig. 3 The percentage of time zebrafish spent exploring different pairs of identical objects. *White* and *black bars* indicate exploration intervals of 5 (*5*) and 10 (*10*) minutes, respectively. Data are presented as mean ± SEM; n = 9–10 animals per group. Preferences were assessed using ANOVA followed by Scheffé test. ***$p < 0.001$

Shape and size preference ANOVA revealed significant differences ($F_{31,80} = 5.543$, $p < 0.05$) when shape or size cues, treatments, and testing interval were considered as analysis factors. To determine whether object shape influenced novel object preference, zebrafish were first familiarized with an identical pair of cubes during training sessions, and then in

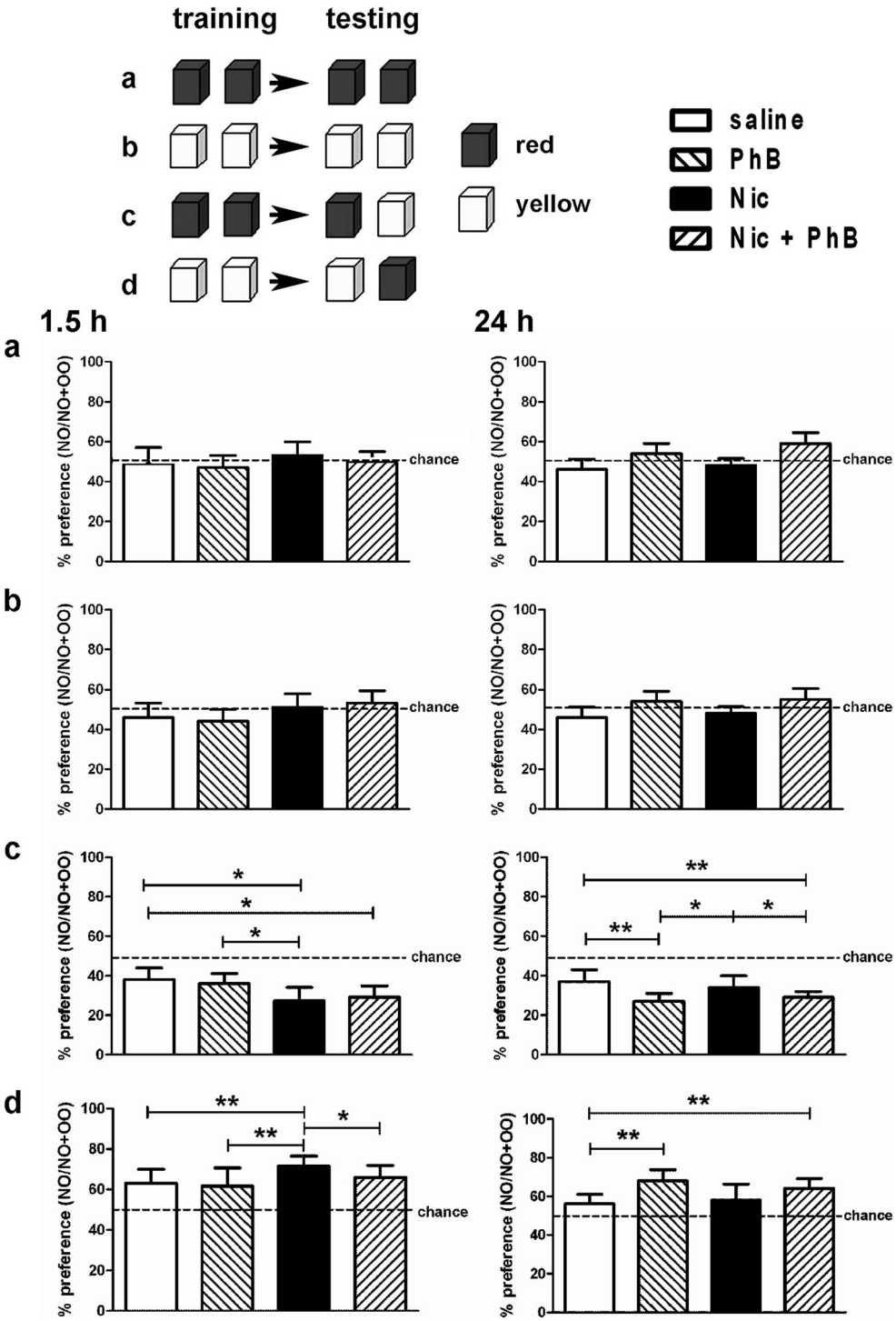


Fig. 4 Novel object preference when comparing objects for which there are different innate preferences. Zebrafish were trained individually and then immersed for 10 min in tanks containing drug-free fish tank water (saline), 15 mg/l nicotine (Nic), 15 μM phenylbutyrate (PhB), or 15 mg/l nicotine plus 15 μM PhB (Nic + PhB). Drugs were dissolved in fish tank water. Testing sessions were performed after a delay of 1.5 h (*graphs* on the *left*) or 24 h (*graphs* on the *right*). *Dashed lines* in all *graphs* (chance) indicate 50% preference. *NO* novel object, *OO* original object. Data are depicted as mean ± SEM; $n = 10–12$ animals per group. Preferences were assessed using ANOVA followed by Scheffé test. *$p < 0.05$; **$p < 0.01$

test sessions, one of the cubes was replaced with a ball of the same color as the training cubes (Fig. 5). When novel objects with changes in shape only were evaluated at the short- and long-retention intervals, there was no preference for the objects (Fig. 5a, b). In the test session designed to evaluate whether changes in object size affected exploratory behaviors, we replaced one of the training cubes with a tower of the same color. Notably, when a red cube was replaced with a red tower, we observed a nicotine-induced decrease in preference (30%) for the tower with a 1.5-h retention interval ($p < 0.05$). A similar result was observed in the PhB-treated group with a 24-h retention interval ($p < 0.05$) (Fig. 5c). A similar pattern of results was observed when a yellow cube was replaced with a yellow tower (Fig. 5d).

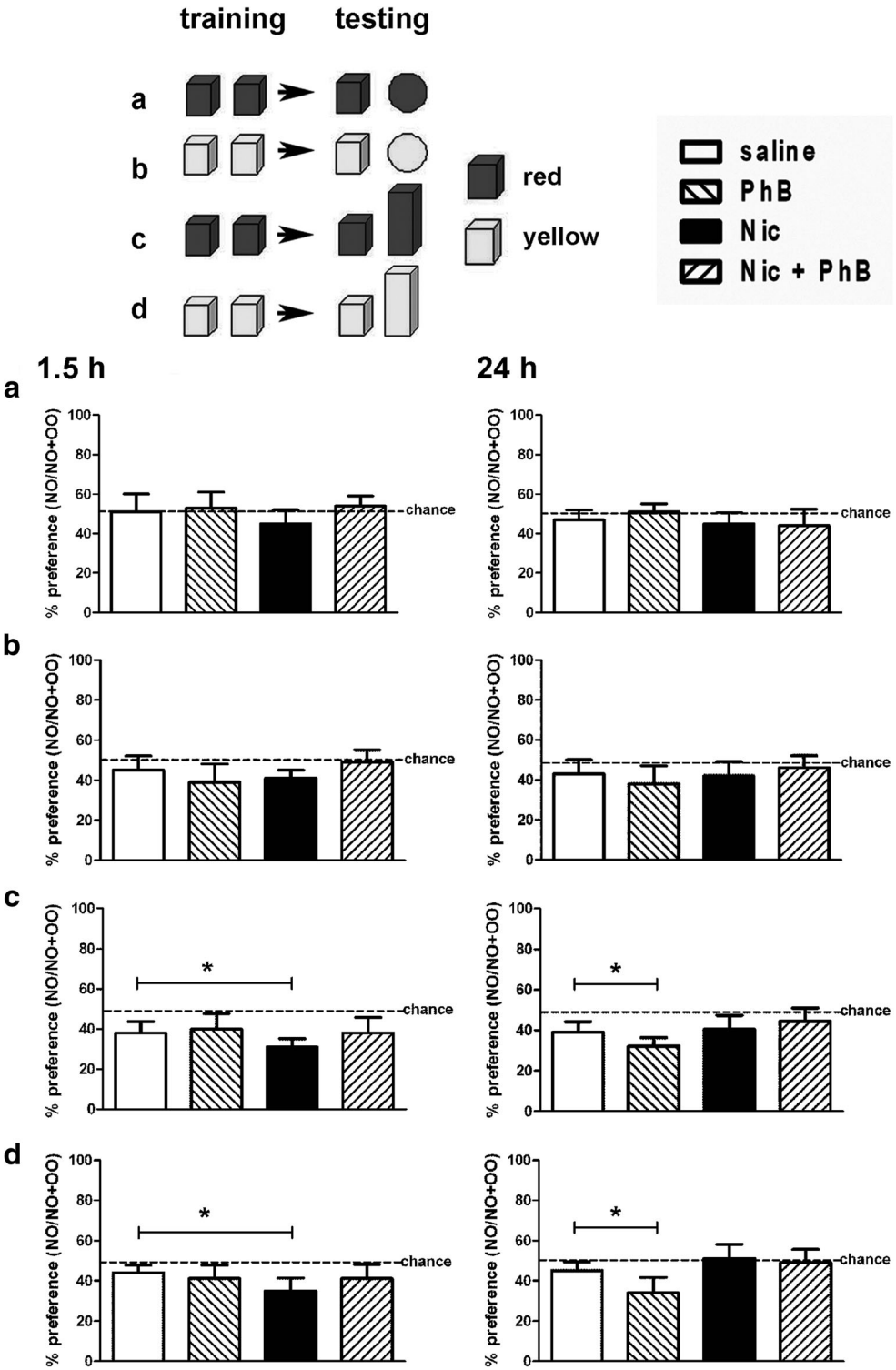
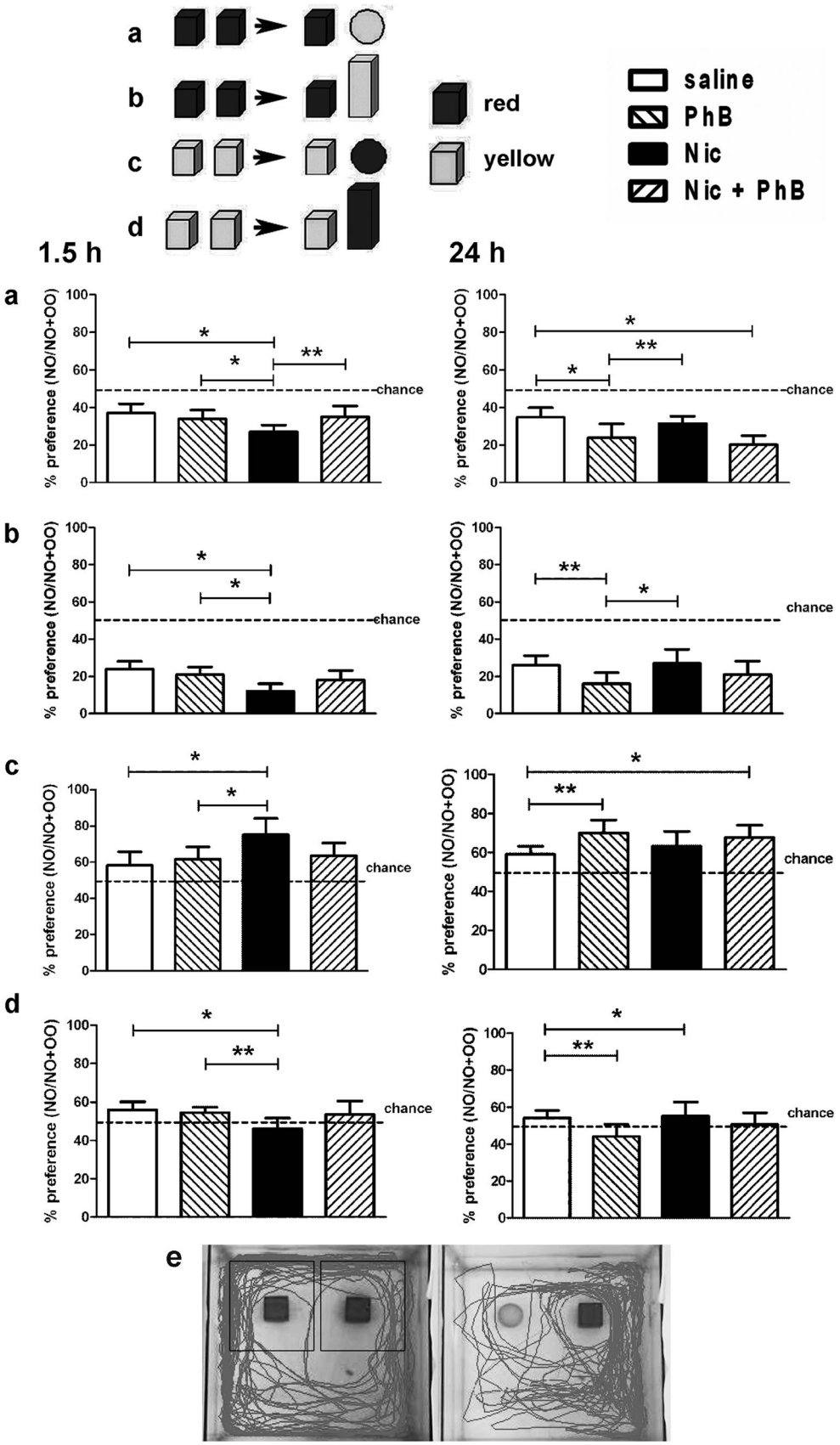


Fig. 5 Novel object preference based on changes in object size or shape. Zebrafish were trained individually and then immersed for 10 min in tanks containing drug-free fish tank water (saline), 15 mg/l nicotine (Nic), 15 μM phenylbutyrate (PhB), or 15 mg/l nicotine plus 15 μM PhB (Nic + PhB). Drugs were dissolved in fish tank water. *Dashed lines* in all *graphs* (chance) indicate 50% preference. *NO* novel object, *OO* original object. Data are depicted as mean ± SEM; $n = 10–12$ animals per group. Preferences were assessed using ANOVA followed by Scheffé test. $^{*}p < 0.05$

Compound preferences: color size and color shape ANOVA revealed significant differences ($F_{31,80} = 9.382$, $p < 0.0001$) considering color shape and color size cues, treatments, and retention interval as analysis factors. During training, zebrafish were exposed to two red cubes, and during testing, one of the cubes was replaced with a yellow ball (Fig. 6a). With a 1.5-h retention interval, the nicotine-treated group showed a lower mean preference score than all other groups ($p < 0.05$ and $p < 0.01$). Twenty-four hours after training, both the PhB- and PhB + nicotine-treated groups showed a further diminished preference for the novel yellow object ($p < 0.05$ and $p < 0.01$, respectively). When a red cube was



◀ **Fig. 6** Novel object preference based on changes in object size, shape, and color. Zebrafish were trained individually and then immersed for 10 min in tanks containing drug-free fish tank water (saline), 15 mg/l nicotine (Nic), 15 μM phenylbutyrate (PhB), or 15 mg/l nicotine plus 15 μM PhB (Nic + PhB). Drugs were dissolved in fish tank water. **e** Zebrafish trajectory recorded during the test session with the conditions depicted in *graph a* (saline tank *left*, nicotine tank *right*). The tank on the *left* shows delimited exploration zones. *Dashed lines* (chance) indicate 50% preference. *NO* novel object, *OO* original object. Data are presented as mean ± SEM; $n = 12–14$ animals per group. Preferences were assessed using ANOVA followed by Scheffé test. *$p < 0.05$ and **$p < 0.01$

replaced with a yellow tower, nicotine and PhB treatments reduced the preference for the novel object ($p < 0.01$; $p < 0.05$) with 1.5- and 24-h retention intervals, respectively (Fig. 6b). We also carried out experiments in which zebrafish explored two yellow cubes, and then a red ball was introduced in the test session. With a 1.5-h retention interval, the nicotine-treated group showed an increased preference for the novel red ball ($p < 0.05$; Fig. 6c). As expected, with a 24-h retention interval, the PhB- and PhB + nicotine-treated groups showed higher preferences for the red ball ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 6c). However, when a yellow cube was replaced with a red tower, nicotine-treated zebrafish preferred the yellow cube 1.5 h after training whereas control and PhB-treated animals tended to prefer the novel red tower ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 6d). When testing was carried out 24 h after training, PhB-treated zebrafish spent less time exploring the novel red tower than the familiar yellow cube, whereas control and nicotine-treated zebrafish preferred the red tower ($p < 0.01$; $p < 0.05$).

Visual discrimination analysis

Table 1 displays descriptive data for encounters and behavioral responses (escapes or avoidances) when zebrafish were exposed to a red or a yellow segment attached to the interior wall of a rotating drum. The colored segment suddenly appeared against the white background in the visual field of the swimming zebrafish (see "Methods" section for a full description of this procedure). The number of encounters is the number of times a zebrafish faced a segment. Yellow and red segments always provoked a behavioral response indicating that the colored segment was perceived on 100% of exposures, but yellow segments were more likely to elicit avoidance responses ($p < 0.05$).

Analysis of other behavioral parameters

Distance swum

We measured the distance traveled during training or testing sessions in order to evaluate whether treatments changed the locomotor activity of zebrafish and as an indirect index of anxiety. When testing was carried out 1.5 h after training, nicotine increased distance swum in all the experimental conditions, except when a red tower replaced a red cube ($p < 0.05$), and in this case, the trend was in the same direction (Supplementary Table 1, size + shape, 3rd row). When testing was carried out 24 h after training, none of the treatments affected distance swum (Supplementary Table 1, 24-h test session columns).

Latency

Delay in exploring an object for the first time was evaluated in training and testing sessions. In naïve zebrafish, the exploration latency for novel red objects was shorter than the exploration latency for novel yellow objects (Supplementary Table 2, training column). When the novel object on the OR task was yellow and the familiar object red, the novel object exploration latency was longer. In contrast, when the novel object was red and the familiar object yellow, zebrafish showed a shorter exploration latency for the novel object (Supplementary Table 2, color rows), and this effect was enhanced by nicotine with a 1.5-h retention interval and by PhB treatment with a 24-h retention interval ($p < 0.01$). We did not observe significant differences in the exploration latency when changes in size and shape were assessed in the test session (Supplementary Table 2, shape + size rows). When a novel either yellow ball or tower was presented with a familiar red cube, the exploration latency was longer regardless of size. The latencies were similar in testing and training sessions (Supplementary Table 2, color + shape + size rows).

Table 1 Visual discrimination task used to evaluate color sensitivity in zebrafish

Visual cue	No. of encounters	No. of escape responses	No. of avoidance responses	% of escape responses	% of avoidance responses
Red segment	13.25 ± 1.94	10.62 ± 1.39	2.62 ± 0.36	80.19 ± 12.36	19.81 ± 1.82
Yellow segment	14.12 ± 1.71	10.60 ± 1.37	3.52 ± 0.70	75.21 ± 8.90	24.94 ± 1.26*

The table displays the number and percentage (%) of encounters and avoidance or escape responses displayed by zebrafish when exposed to threatening visual stimuli consisting of a red or a yellow segment (see "Methods" section for details). Data are presented as mean ± SEM; $n = 10–12$ animals per group. Differences were assessed using ANOVA followed by Scheffé test

*$p < 0.05$

Frequency

Frequency was calculated as the number of times a subject's whole body entered one of the delimited zones around the objects. When a yellow cube replaced a familiar red cube, a significant decrease in frequency was observed, as shown in Supplementary Table 3. The opposite occurred when a red cube replaced a familiar yellow cube (color rows). Nicotine significantly increased visiting frequency for both objects when they were the same color, which is consistent with the observed increase in locomotor activity (Supplementary Table 1). When tested 24 h after training, subjects displayed their innate tendency to visit red objects more often. Changes in shape and size did not induce changes in frequency (Supplementary Table 3, shape + size rows). However, when tested 1.5 h after training, nicotine-treated animals visited a familiar red cube less often than the novel red ball it was paired with. When a 24-h retention interval was used and a familiar yellow cube was replaced with a yellow tower, zebrafish from all groups explored the cube more frequently than the tower and more frequently than during training. When changes in shape and size were combined with variations in color (Supplementary Table 3, color + shape + size rows), significant changes in frequency were observed. Again, when the familiar object was a red cube, significantly fewer visits were made to the novel yellow object. In contrast, when the familiar object was a yellow cube, more visits were made to the novel red object, regardless of shape and size. The pattern of behavior was observed in control subjects and generally potentiated by drug treatments. Notwithstanding this general potentiation, at short- and long-retention intervals, nicotine and PhB significantly reduced the frequency of visits to novel taller objects (towers), respectively, regardless of color.

Discussion

Innate color, shape, and size preferences of zebrafish

In this study, we analyzed behavior during training sessions to determine zebrafishes' innate exploratory preferences with respect to objects of four different colors, two shapes, and two sizes. We found that zebrafish are more sensitive to changes in color than changes in shape or size when objects are presented in their visual field. We observed that zebrafish explored red and green objects more frequently than yellow or blue objects. A color discrimination test showed that zebrafish were similarly good at detecting red and yellow cues, but that yellow objects elicited more aversive responses. Considering size and shape, zebrafish spent more time exploring cubes or balls of the same size than towers.

OR task with color cues in zebrafish

OR tasks have only recently been used with zebrafish (Mussulini et al. 2013; Braida et al. 2014). We examined recognition memory, which is the ability to recognize previously encountered objects. It has been reported that when in a group, zebrafish do not exhibit neophobia (Stewart et al. 2011; Kalueff et al. 2013); however, a recent report indicated that individual zebrafish do exhibit neophobia when exposed to novel objects (May et al. 2016). We were able to select innately preferred or non-preferred colors for the novel object recognition test, which can be used as appetitive or aversive stimuli. Our results indicated that zebrafishes' relative preference for exploring a novel object depends mainly on its color. Furthermore, we showed that zebrafish can discriminate simple objects based on color cues when tested after a delay of 24 h. An earlier study (Lucon-Xiccato and Dadda 2014) indicated that zebrafish always preferred a novel object, independently of shape and color (a pink ball and a dark-yellow prism), and those subjects could discriminate between novel and familiar objects even after a delay of 24 h. This discrepancy may be due to differences between the studies with respect to tank design, size difference between the objects and zebrafish—which was bigger in our study—and the brightness of objects. In Lucon-Xiccato and Dadda's (2014) study, the objects were attached to a glass pipette and introduced to the tank from the surface, which probably captured the zebrafishes' attention and induced exploratory responses. Our study also differs from earlier studies in that we presented pairs of simple geometrical objects that were related in shape and size, in order to make it difficult for the subjects to discriminate the objects. Oliveira et al. (2015) selected the color of objects based on zebrafish preference for blue and green environments, a result that conflicts with other studies that found that blue and yellow environments were aversive (Spence and Smith 2008; Avdesh et al. 2012). We found that red and green objects were preferred to yellow and blue objects. Zebrafish behavior could be influenced by the conditions in which they are housed; in our study, they were housed in an enriched environment, which can increase exploratory behavior and attention to environmental cues (Parker et al. 2012). Furthermore, the brightness and color intensity of the objects and the illumination conditions in the behavioral room (dim illumination in our study) can influence visual contrast, color perception, and behavior. Moreover, our results indicated that zebrafish failed to differentiate a red cube from a similar-sized red ball.

Effects of nicotine and the HDAC inhibitor PhB on novel object preference tasks

We examined the effects of nicotine and PhB on performance on object preference tasks because in rats and zebrafish, nicotine and PhB can modify innate environmental preferences in CPP tasks (Pastor et al. 2011; Kedikian et al. 2013). Additionally, our group

and others have demonstrated the rewarding effects of nicotine in zebrafish (Ninkovic and Bally-Cuif 2006; Kily et al. 2008; Kedikian et al. 2013). Our experiments are the first to evaluate the effects of PhB on object exploration and other behavioral parameters in adult zebrafish.

Effects of nicotine and PhB on a novel object recognition task based on color cues of similar innate significance for zebrafish

When red and green objects were paired in test sessions, nicotine induced an exploratory preference for the novel green object, which was not observed in the control group. This behavioral shift suggests that a short-term memory process was involved. When blue and yellow objects were paired, the control group showed an exploratory preference for the novel object, indicating that they were able to recognize the familiar from the novel object (Fig. 2b). When testing was carried out after a short-retention interval, nicotine treatment abolished the differences in preference.

Some neuropsychiatric diseases, such as depression and attention deficits, are accompanied by cognitive impairments. HDAC inhibitors show great promise as a treatment for these disorders. Our results in zebrafish demonstrated that PhB affected object recognition after a 24-h delay, indicating long-term effectiveness. When animals were tested after a 24-h retention interval, PhB increased exploration of novel green objects (relative to familiar red objects; Fig. 2a), which indicates memory for the familiar object. Furthermore, PhB treatment enhanced the preference for exploring novel yellow objects rather than familiar blue objects (Fig. 2b).

Effects of nicotine and PhB on novel object preference using pairs of objects with opposite innate significance for zebrafish

Overall, innate attraction or aversion for color cues, i.e., approach or avoidance behaviors, was potentiated by nicotine and PhB. The ability to distinguish between big and small objects has survival value, because big fish eat smaller ones. In this regard, it has been observed that zebrafish do not spend long in close proximity to zones containing large objects (Lucon-Xiccato and Dadda 2014). However, zebrafish failed to differentiate a familiar cube from a novel cube that was twice as big (Oliveira et al. 2015). Concordantly, in our study, the control group failed to distinguish between a cube and a tower that was almost twice as high. As an increase in height of the novel object was perceived only when zebrafish were under the effects of nicotine or PhB, it seems likely that these drugs enhanced zebrafishes' ability to differentiate objects based on size.

When animals were presented with pairs of objects that differed in terms of both color and size or color and shape, novel yellow towers induced the strongest avoidance response and novel red balls the strongest approach response (Figs. 3 and 6). When a yellow cube was paired with a novel red tower, zebrafish in the control group showed a mild preference for exploring the tower whereas nicotine and PhB changed the subject's behavior from approach to avoidance responses to novel taller objects regardless of color preferences.

The delayed effect of PhB could be due to its pharmacodynamic properties, as PhB needs to enter the cell nucleus to inhibit HDAC enzymes. Moreover, the physiological consequences of inhibiting histone deacetylation can only be observed after several hours. PhB potentiates object recognition in rodents (Stefanko et al. 2009; Hawk et al. 2011), and our results suggest that HDAC inhibitors can also stimulate attention and improve object recognition and discrimination in zebrafish. As nicotine has a mild inhibitory effect on HDAC activity (Levine et al. 2011), we evaluated the combined effect of these drugs. Largely, zebrafish treated with both nicotine and PhB behaved similarly to those treated with nicotine or PhB alone.

Does novel object preference or aversion depend on memory retention?

A preference for exploring either novel or familiar objects which are of similar innate significance for zebrafish implies recognition memory. Preference scores above or below 50% can be attributed to memory for the objects presented in training (May et al. 2016). The experiments depicted in Fig. 2 suggest that nicotine and PhB can induce object recognition, which may indicate that a memory process is involved, in contexts in which it is not observed in the absence of these drugs. In contrast, the results observed when paired objects were of different innate significance for zebrafish suggest that nicotine and PhB either potentiate or change innate behaviors by significantly improving perception of the visual landscape; these effects do not necessarily imply memory for objects.

Additional behavioral parameters

Analysis of total distance swum suggested that nicotine increases locomotor activity for 90 min, and this effect could account for the nicotine-induced enhancement of exploratory bias. However, this effect could not account for nicotine-induced reductions in the frequency of visits or significant decrements in the time spent exploring one of the testing objects. An increment in the total distance swum could not account either for nicotine-induced inversion of the innate pattern of preferences. On the other hand, latency to first exploration of objects during training and test sessions was virtually unchanged by drug treatments. Red objects generated curiosity and attraction, which was reflected in shorter latencies, whereas yellow objects provoked longer latencies. Analysis of exploration frequency helped to pinpoint the effects of nicotine and PhB on object recognition because it was affected similarly to exploratory

preference. Nicotine and PhB further decreased the exploration frequency for novel towers relative to familiar cubes of the same color. More importantly, drug treatments significantly decreased the frequency of visits to novel red towers and increased the frequency of visits to familiar yellow cubes; i.e., drug effects inverted the pattern of behavior of the control group. The nicotine-induced increment of the frequency to explore a novel red ball may also imply recognition memory given that red cubes and balls are of similar innate significance for zebrafish.

In conclusion, the results presented here highlight the importance of evaluating animals' innate behavior towards novel objects. For instance, the experimenter may consider that objects of different color are of neutral significance for zebrafish. However, humans do not perceive color in the same way as zebrafish, which have a tetrachromatic visual system, and so zebrafishes' perceptual experiences of the colors humans call red, green, yellow, and blue likely may be completely different. We have also observed that zebrafish may or may not remember a familiar object and its context, but they will not explore a novel object because it elicits an avoidance response or is intrinsically unattractive. Likewise, they may prefer to explore the novel object because it is more attractive than the familiar object. Preference for exploring the familiar or novel object on a recognition test only implies memory if the two objects elicit similar innate responses. However, preference scores above or below 50% do indicate the ability to discriminate between the objects. Relatively low doses of nicotine and cocaine improve zebrafishes' ability to detect small differences between objects, enhance memory for objects, and modify zebrafish behavior. In our study, nicotine and HDACi with short- and long-retention intervals, respectively, did alter visual perception of the environment and object salience in zebrafish, as cocaine has been shown to do (Darland and Dowling 2001).

Zebrafish are diurnal and perceive a wide spectrum of visible light including UV light; therefore, as we have evidenced in this study, color cues can be used to investigate appetitive and aversive innate responses. This study illustrates the use of a viable, useful method of evaluating preference in zebrafish and investigating the effect of drugs of abuse or epigenetic mechanisms on perception and cognitive processes.

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References

Avdesh A, Martin-Iverson MT, Mondal A, Chen M, Askraba S, Morgan N, Lardelli M, Groth DM, Verdile G, Martins RN (2012) Evaluation of color preference in zebrafish for learning and memory. J Alzheimers Dis 28:459–469

Balderas I, Rodriguez-Ortiz CJ, Salgado-Tonda P, Chavez-Hurtado J, McGaugh JL, Bermudez-Rattoni F (2008) The consolidation of object and context recognition memory involve different regions of the temporal lobe. Learn Mem 15:618–624

Bevins RA, Besheer J (2006) Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory. Nat Protoc 1:1306–1311

Bilotta J, Risner ML, Davis EC, Haggbloom SJ (2005) Assessing appetitive choice discrimination learning in zebrafish. Zebrafish 2:259–268

Braida D, Ponzoni L, Martucci R, Sala M (2014) A new model to study visual attention in zebrafish. Prog Neuro-Psychopharmacol Biol Psychiatry 55:80–86

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

Brennan CH, Parmar A, Kily LKM, Ananthathevan A, Doshi A, Patel S (2011) Conditioned place preference models of drug dependence and relapse to drug seeking: studies with nicotine and ethanol. In: Kalueff and Cachat (ed) Zebrafish models in neurobehavioral research, Neuromethods 52, Humana Press, New Orleans, USA pp 163–180

Colwill RM, Raymond MP, Ferreira L, Escudero H (2005) Visual discrimination learning in zebrafish (*Danio rerio*). Behav Process 70:19–31

Darland T, Dowling JE (2001) Behavioral screening for cocaine sensitivity in mutagenized zebrafish. Proc Natl Acad Sci U S A 98:11691–11696

Engeszer RE, Da Barbiano LA, Ryan MJ, Parichy DM (2007) Timing and plasticity of shoaling behaviour in the zebrafish, *Danio rerio*. Anim Behav 74:1269–1275

Engeszer RE, Ryan MJ, Parichy DM (2004) Learned social preference in zebrafish. Curr Biol 14:881–884

Gaskin S, Tardif M, Cole E, Piterkin P, Kayello L, Mumby DG (2010) Object familiarization and novel-object preference in rats. Behav Process 83:61–71

Gerlai R (2011) Associative learning in zebrafish (*Danio rerio*). Methods Cell Biol 101:249–270

Haettig J, Stefanko DP, Multani ML, Figueroa DX, McQuown SC, Wood MA (2011) HDAC inhibition modulates hippocampus-dependent long-term memory for object location in a CBP-dependent manner. Learn Mem 18:71–79

Hawk JD, Florian C, Abel T (2011) Post-training intrahippocampal inhibition of class I histone deacetylases enhances long-term object-location memory. Learn Mem 18:367–370

Kalueff AV, Gebhardt M, Stewart AM, Cachat JM, Brimmer M, Chawla JS, Craddock C, Kyzar EJ, Roth A, Landsman S, Gaikwad S, Robinson K, Baatrup E, Tierney K, Shamchuk A, Norton W, Miller N, Nicolson T, Braubach O, Gilman CP, Pittman J, Rosemberg DB, Gerlai R, Echevarria D, Lamb E, Neuhauss SC, Weng W, Bally-Cuif L, Schneider H, Zebrafish Neuroscience Research Consortium (2013) Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. Zebrafish 10:70–86

Karnik I, Gerlai R (2012) Can zebrafish learn spatial tasks? An empirical analysis of place and single CS-US associative learning. Behav Brain Res 233:415–421

Kedikian X, Faillace MP, Bernabeu R (2013) Behavioral and molecular analysis of nicotine-conditioned place preference in zebrafish. PLoS One 8:e69453

Kily LJ, Cowe YC, Hussain O, Patel S, McElwaine S, Cotter FE, Brennan CH (2008) Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways. J Exp Biol 211:1623–1634

Kim YS, Kim MJ, Koo TH, Kim JD, Koun S, Ham HJ, Lee YM, Rhee M, Yeo SY, Huh TL (2012) Histone deacetylase is required for the activation of Wnt/β-catenin signaling crucial for heart valve formation in zebrafish embryos. Biochem Biophys Res Commun 423:140–146

Korzus E, Rosenfeld MG, Mayford M (2004) CBP histone acetyltransferase activity is a critical component of memory consolidation. Neuron 42:961–972

Lau BY, Mathur P, Gould GG, Guo S (2011) Identification of a brain center whose activity discriminates a choice behavior in zebrafish. Proc Natl Acad Sci U S A 108:2581–2586

Levin ED, Bencan Z, Cerutti DT (2007) Anxiolytic effects of nicotine in zebrafish. Physiol Behav 90:54–58

Levin ED, Chen E (2004) Nicotinic involvement in memory function in zebrafish. Neurotoxicol Teratol 26:731–735

Levin ED, Limpuangthip J, Rachakonda T, Peterson M (2006) Timing of nicotine effects on learning in zebrafish. Psychopharmacology 184: 547–552

Levine A, Huang Y, Drisaldi B, Griffin EA Jr, Pollak DD, Xu S, Yin D, Schaffran C, Kandel DB, Kandel ER (2011) Molecular mechanism for a gateway drug: epigenetic changes initiated by nicotine prime gene expression by cocaine. Sci Transl Med 3:107–109

Li L, Dowling JE (1997) A dominant form of inherited retinal degeneration caused by a non-photoreceptor cell-specific mutation. Proc Natl Acad Sci U S A 94:11645–11650

Lieschke GJ, Currie PD (2007) Animal models of human disease: zebrafish swim into view. Nat Rev Genet 8:353–367

Lucon-Xiccato T, Dadda M (2014) Assessing memory in zebrafish using the one-trial test. Behav Process 106:1–4

Mathiasen JR, DiCamillo A (2010) Novel object recognition in the rat: a facile assay for cognitive function. Curr Protoc Pharmacol Chapter 5 Unit 5.59:1–15

May Z, Morrill A, Holcombe A, Johnston T, Gallup J, Fouad K, Schalomon M, Hamilton TJ (2016) Object recognition memory in zebrafish. Behav Brain Res 296:199–210

Morris MJ, Karra AS, Monteggia LM (2010) Histone deacetylases govern cellular mechanisms underlying behavioral and synaptic plasticity in the developing and adult brain. Behav Pharmacol 21:409–419

Mussulini BH, Leite CE, Zenki KC, Moro L, Baggio S, Rico EP, Rosemberg DB, Dias RD, Souza TM, Calcagnotto ME, Campos MM, Battastini AM, de Oliveira DL (2013) Seizures induced by pentylenetetrazole in the adult zebrafish: a detailed behavioral characterization. PLoS One 8:e54515

Ninkovic J, Bally-Cuif L (2006) The zebrafish as a model system for assessing the reinforcing properties of drugs of abuse. Methods 39:262–274

Olincy A, Stevens KE (2007) Treating schizophrenia symptoms with an alpha7 nicotinic agonist, from mice to men. Biochem Pharmacol 74: 1192–1201

Oliveira J, Silveira M, Chacon D, Luchiari A (2015) The zebrafish world of colors and shapes: preference and discrimination. Zebrafish 12:166–173

Parker MO, Gaviria J, Haigh A, Millington ME, Brown VJ, Combe FJ, Brennan CH (2012) Discrimination reversal and attentional sets in zebrafish (Danio rerio). Behav Brain Res 232:264–268

Pastor V, Andrés ME, Bernabeu RO (2013) The effect of previous exposure to nicotine on nicotine place preference. Psychopharmacology 226:551–560

Pastor V, Host L, Zwiller J, Bernabeu R (2011) Histone deacetylase inhibition decreases preference without affecting aversion for nicotine. J Neurochem 116:636–645

Peleg S, Sananbenesi F, Zovoilis A, Burkhardt S, Bahari-Javan S, Agis-Balboa RC, Cota P, Wittnam JL, Gogol-Doering A, Opitz L, Salinas-Riester G, Dettenhofer M, Kang H, Farinelli L, Chen W, Fischer A (2010) Altered histone acetylation is associated with age-dependent memory impairment in mice. Science 328:753–756

Puma C, Deschaux O, Molimard R, Bizot JC (1999) Nicotine improves memory in an object recognition task in rats. Eur Neuropsychopharmacol 9: 323–327

Robinson J, Schmitt EA, Harosi FI, Reece RJ, Dowling JE (1993) Zebrafish ultraviolet visual pigment: absorption spectrum, sequence, and localization. Proc Natl Acad Sci U S A 90:6009–6012

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

Roozendaal B, Hernandez A, Cabrera SM, Hagewoud R, Malvaez M, Stefanko DP, Haettig J, Wood MA (2010) Membrane-associated glucocorticoid activity is necessary for modulation of long-term memory via chromatin modification. J Neurosci 30:5037–5046

Spence R, Smith C (2008) Innate and learned colour preference in the zebrafish, *Danio rerio*. Ethology 114:582–588

Stefanko DP, Barrett RM, Ly AR, Reolon GK, Wood MA (2009) Modulation of long-term memory for object recognition via HDAC inhibition. Proc Natl Acad Sci USA 106:9447-52

Stewart A, Wong K, Cachat J, Gaikwad S, Kyzar E, Wu N, Hart P, Piet V, Utterback E, Elegante M, Tien D, Kalueff AV (2011) Zebrafish models to study drug abuse-related phenotypes. Rev Neurosci 22:95–105

Stewart A, Wu N, Cachat J, Hart P, Gaikwad S, Wong K, Utterback E, Gilder T, Kyzar E, Newman A, Carlos D, Chang K, Hook M, Rhymes C, Caffery M, Greenberg M, Zadina J, Kalueff AV (2010) Pharmacological modulation of anxiety-like phenotypes in adult zebrafish behavioral models. Prog Neuro-Psychopharmacol Biol Psychiatry 35:1421–1431

Westerfield M (2007) The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). 5th ed, Eugene: University of Oregon Press.

Winters M, Brauer M, Setton EM, Teschke K (2010) Built environment influences on healthy transportation choices: bicycling versus driving. Journal of Urban Health 87:969–993

Wood MA, Hawk JD, Abel T (2006) Combinatorial chromatin modifications and memory storage: a code for memory? Learn Mem 13:241–244