

## Zebrafish: An Animal Model to Study Nicotinic Drugs on Spatial Memory and Visual Attention

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### Abstract

Neuronal nicotinic acetylcholine receptors (nAChRs) are involved in learning and memory in both humans and animals. For their physical characteristics, including small size, easiness to grow, and robustness of the species, zebrafish (*Danio rerio*) is rapidly becoming a popular model in bio-behavioral studies. Zebrafish are also easy to manipulate for researchers who are not practical users of traditional animal models. Here we describe two cognitive tasks which are sensitive to nicotinic drugs in a similar manner as rodents. Spatial memory is studied using a T-maze apparatus, where animals choose between two arms one of which contains a reservoir that offers a favorable habitat. Each fish receives two training trials at an interval of 24 h. The difference between the running time taken to reach the reservoir (and stay for at least 20 s) obtained during the first and the second trial is a measure of memory of the spatial location of reward. Visual attention is studied using a virtual object recognition test (VORT) where two geometrical 2D virtual shapes are presented stationary on two iPod screens. Shape recognition is scored in terms of exploration time whenever the zebrafish approach to the iPod area and direct their heads towards the shapes. To elucidate the involvement of nicotinic subtype receptors on memory, different selective nAChRs compounds (agonists and antagonists) are given through intraperitoneal (i.p.) route. All the compounds are tested also on swimming behavior to ascertain their possible interference with motor function. Here, we propose zebrafish as a useful tool to rapidly screen new nicotinic compounds active on cognitive disorders.

**Key words** Teleost, Learning and memory, Cholinergic system, Nicotinic subtype receptors, Spatial memory, Visual attention, Cognitive disorders, Nicotinic partial agonist

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### 1 Introduction

The cholinergic system plays a fundamental role in learning and memory of mammalian and nonmammalian vertebrates and invertebrates. Cognitive deficit is a feature of multiple brain disorders such as Alzheimer's disease (AD), autism spectrum disorders (ASD), and schizophrenia [1–4]. In particular for patients suffering from AD, spatial cognition is strongly impaired [1–3], while clear attention problems have been described in children affected by ASD and in many neuropsychiatric disorders [4–6].

In AD patients both muscarinic and nicotinic acetylcholine receptors, or nAChRs, levels have been found to be reduced [7, 8] and patients in early stages of AD showed a reduced nAChR density in cortex and hippocampus [9]. Consequently, considerable research into cholinergic cognition enhancers has been carried out [10].

Zebrafish, due to their complex nervous system and having robust cognitive abilities, are gaining popularity as complementary model for neurobehavioral research. Notably, learning and memory capabilities of teleosts are complex as those of mammals and birds sharing homologous neural mechanisms [11]. The zebrafish cholinergic system is generally similar to that of other vertebrates having muscarinic [12] and the full set of nicotinic receptors [13].

Zebrafish perform well in some conditioning cognitive tasks such as appetitive choice discrimination [14], shuttle box active appetitive and choice discrimination [14, 15], and one-trial avoidance task [16].

Spatial learning and attentional memory are particularly important since their impairment is the hallmark of prevalent human neurodegenerative diseases [17]. Interestingly, fish are able to use the information provided by the geometric attributes of the surrounding for spatial navigation to reach the goal location by learning its position relative to the landmarks by using spatial information [18].

Spatial learning in zebrafish has been well characterized in the past years by using either an y-maze apparatus, in which different geometric forms were placed on the external maze walls [18, 19], or a T-maze where animals choose a correct arm on the basis of different stimuli such as the sight of conspecific, food [20, 21], a favorite color [22], or a favorable environment [23]. Aversive stimuli often used are mild shock [24] or a water soluble that smells or tastes bad [19].

Disorders of attention may underline cognitive dysfunctions associated with neurodegenerative and psychiatric disorders [25, 26]. Even if a robust literature is present for tasks assessing attention in rodents (for review, see ref. [27]) tasks on zebrafish to assess sustained attention are not available except the three-choice appetitive visual choice discrimination which however not effectively measures sustained attention [14, 28].

In an attempt to maximize the value of zebrafish as an animal model to study attention, we applied a modified version of novel object recognition task named virtual object recognition task (VORT). This test evaluates the animal's attention elicited by the presentation of novel stimuli, where virtual stationary geometric 2D shapes are presented on iPod screens [29].

This chapter provides a detailed description of how assess spatial memory through the T-maze and visual attention using VORT. Furthermore a third procedure is described regarding the evaluation of swimming activity, an important parameter to validate the pharmacological effects of nicotinic compounds on memory.

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## 2 Equipment, Materials, and Setup

### 2.1 Animals

Although various outbred or inbred or genetically modified zebrafish may be used to assess spatial and visual attention memory, care has to be given to the anxious state of fish. An increased anxiety can interfere with the tasks since the fish can freeze or jump decreasing their swimming. For example, some strains have been described to be highly anxious such as Nadia, long fin variant, and leopard color variant [30].

Adult short-finned wild-type zebrafish of heterogeneous genetic background can be easily obtained by local aquarium supply stores. Adult zebrafish (from 90 days to 2 years) can be used. Males and females are identified as previously reported [31], and both can be used for cognitive tasks. Males are longer, slimmer, and more yellow especially on the belly while females are plumper and more silvery.

Behavioral testing takes place during the light phase between 09:00 a.m. and 14:00 p.m. Tank water consists of deionized water and sea salts (0.6 g/10 l of water; Instant Ocean, Aquarium Systems, Sarrebourg, France). Approximately 30 adult fish are maintained in 96 l home tanks (75 cm long, 32 cm wide and 40 cm high) provided with constant filtration and aeration. Animals are acclimatized for at least 2 weeks before the start of experiments. Fish are fed twice a day with brine shrimp and flake tropical fish food. Zebrafish are maintained at approximately 28.5 °C on a 14:10-h light–dark cycle.

### 2.2 Drug Administration

The common routes of administration used in rodents can also be applied in zebrafish. The intraperitoneal (i.p.) route is the easiest way to deliver drugs. First of all zebrafish body weight must be measured as previously described [32, 33] Briefly, fish are gently removed from their tank using a net and placed in a container containing tank water, positioned on a digital balance. The weight of the container plus the fish minus the weight of the container before the fish is added, is determined calculating the mean of three consecutive measurements. For i.p. injection, fish are previously anesthetized with ice as previously described and placed in a supine position (Fig. 1). Briefly, a cut (10–15 mm deep) on a sponge (20 mm) is done. Each fish is put in a tank containing water and ice and a thermometer. When the temperature reaches 17 °C, the fish typically will spread its pectoral fins horizontally, gasp, and have rapid operculum movements. As the temperature drops, the fish will swim more slowly and finally stop swimming. The fish is ready for injection when it does not react to being handled with cold fingers, gently transfer the fish to the trough of the sponge. The fish are positioned with the abdomen up and the gills in the trough. The injection is made in the abdominal cavity using an



## Intraperitoneal injection

**Fig. 1** Illustration of the intraperitoneal injection in the abdominal cavity of zebrafish

Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) (for details see Fig. 1). No more than the tip of the needle is inserted into the abdomen of each fish, to prevent damage of internal organs. After injection, each fish is immediately transferred back to its warm water (about 28 °C) tank for recovery. The volume of administered drugs depends on the fish's weight (2  $\mu\text{l/g}$ ). The dosage and pretreatment time can vary, depending on the drug and the strain sensitivity. For example, for nicotine the concentration ranges from 0.0002 to 0.4  $\mu\text{g}/2 \mu\text{l}$ .

Importantly, all experimental procedures must be conducted in accordance with National and Institutional Guidelines for the care and use of Laboratory Animals. All efforts must be done to minimize the number of animals used and their discomfort.

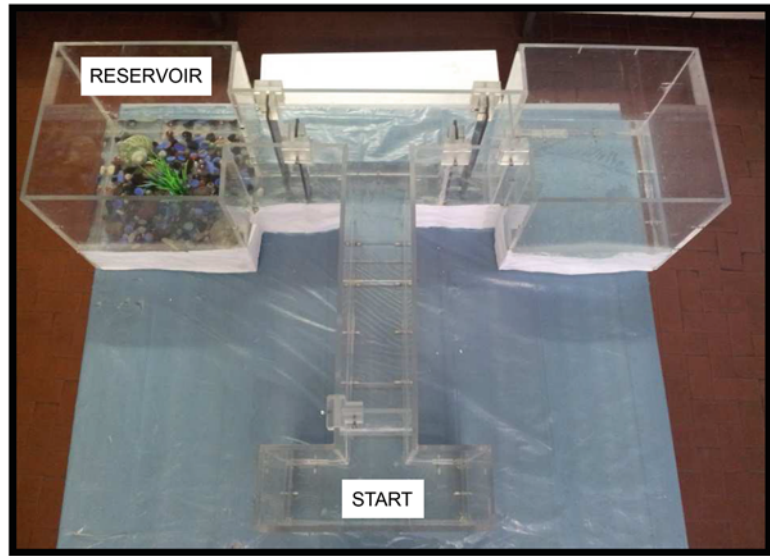
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## 3 Adopted Techniques

### 3.1 T-Maze

#### 3.1.1 Apparatus

A transparent Plexiglas T-maze (filled with tank water at a level of 10 cm) is used (Fig. 2). The apparatus includes a starting zone (30 cm  $\times$  10 cm) separated from the rest of the maze by a transparent removable door. Behind the partition, there is a long (50 cm  $\times$  10 cm) arm and two short (20 cm  $\times$  10 cm) arms, which lead to the removable deep water chambers (30 cm  $\times$  30 cm). One of two chambers, used as reservoir, contains artificial grass, shells, stones, and colored marbles that offered a favorable habitat for the fish. Two removable opaque partitions (4.5 cm  $\times$  30 cm) are put, in a staggered way, at the beginning of each short arm, to prevent viewing of the two chambers.



T-MAZE APPARATUS

**Fig. 2** Illustration of the T-maze apparatus for testing spatial learning in zebrafish (reproduced from Ref. [23]) with permission of Springer

### 3.1.2 Procedure

To minimize procedural novelty stress, the fish first undergo two habituation trials of 1 h every day for 3 days, which also serve to reduce handling stress according to Gaikwad et al. [34]. Each subject receives two training trials of exposure in the T-maze. During each trial, each fish is placed in the start box for 5 min with its door closed. Then, the start box door is raised and lowered after the fish has exited. Ten minutes are allowed to reach the reservoir or the other chamber. Fifty percent of the fish within each group has the reservoir to the left, and the other 50% to the right. For each subject the location remains the same through the experiment. The running time taken to reach the reservoir and stay for at least 20 s is recorded by an experimenter blind of pharmacological treatments. After 20 s, each fish returns to its home tank. A second session can be done to the same fish either at 3 or 24 h later. The interval will be chosen on the basis of the different pharmacological treatments. If nicotinic enhancer drugs must be tested the optimal interval is 24 h since fish show a poor performance. A session of 3 h is enough to show a good performance. Thus nicotinic antagonists can be tested at this interval. The obtained results can be expressed as running time (s) during each session or as difference between the running time taken to reach the reservoir and stay for at least 20 s between the first and the second trial.

### 3.1.3 Time Required

In order to minimize stress due to the novel procedure, acclimation to the maze requires 3 days in which fish undergo two daily habituation trials of 1 h each. During these trials, the fish (in a group of 12–16

each) are allowed to freely explore the entire maze. To minimize acute social isolation stress, zebrafish groups are only gradually reduced in size during the experiment according to Levin and Chen [35] starting for example with 16 fish per group on day 1, 8 fish per group on day 2, 4 fish per group on day 3, and individual fish from day 4. Each fish is submitted to two 10-min-session on day 4, with an inter trial time of 3 or 24 h. A total of 4–5 days is required to evaluate spatial memory of a very high number of fish.

### **3.2 Visual Attention (VORT)**

#### **3.2.1 Apparatus**

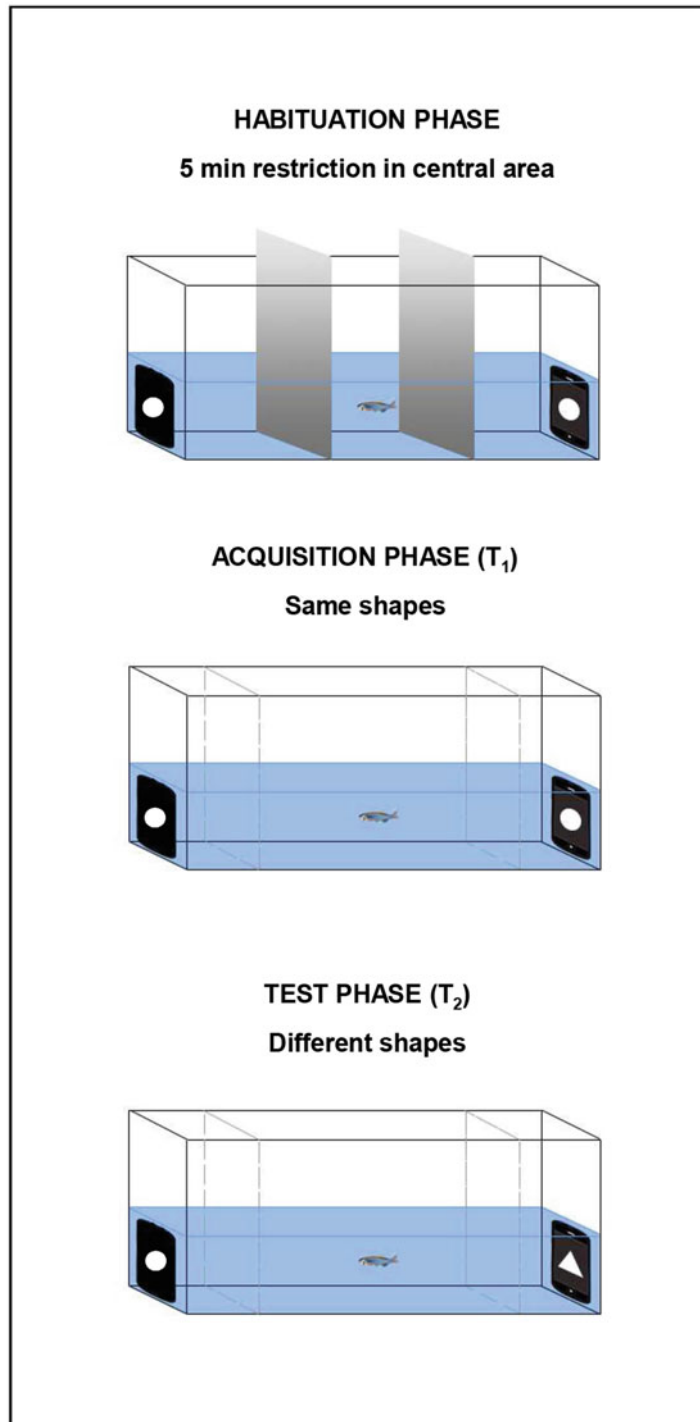
A rectangular transparent Plexiglas tank (70 cm long×30 cm high×10 cm wide) is filled with tank water at a level of 10 cm (Fig. 3). A central area of 20 cm is obtained inserting two opaque barriers to visually isolate the two stimuli areas where two identical white geometrical shapes, on a black background, are shown on two iPod 3.5-in. widescreen displays, located externally to the opposite 10 cm wide walls.

#### **3.2.2 Procedure**

After a week of habituation, as above described for T-maze, each fish is restricted in the central area for 5 min. After the barriers are gently removed, each animal is subjected to a 10 min familiarization trial ( $T_1$ ), during which two identical white geometrical shapes are shown on two iPod screens. After  $T_1$  each fish returns to its home tank. Then during  $T_2$  after different time delays (from 5 min to 96 h) each fish is put again in the central area. One of the two identical static familiar shapes is replaced with a novel one for 10 min. The shapes are simple geometric shapes (square, triangle, circle, cross, etc.) with equal surface ( $2.5 \text{ cm}^2$ ). The shapes are looped on a 3rd generation iPod Touch (Apple) through iTunes for the duration of the experiment (320 pixels horizontal axis and 480 pixels vertical axis). The luminosity of the screens is constant across the two screens and testing sessions. Attention must be paid to counterbalance the choice of the shapes and to randomly pair the discriminated shapes within every time delay. Shape recognition is manually scored with a stopwatch by an experimenter blind to the treatment. Whenever each zebrafish approaches to the iPod area (10 cm) and directs its head toward the shape, exploration time is recorded. Data are expressed as discrimination index [(time spent exploring novel shape – time exploring familiar shape)/(time spent exploring novel shape + time exploring familiar shape)].

#### **3.2.3 Time Required**

A week of habituation, as above described for T-maze, is required to decrease the anxiety due to the novel environment. Zebrafish are subjected to a familiarization trial for 10 min, during which two identical shapes are presented. Then, after different delays (from 5 min to 96 h) a novel shape recognition trial of 10 min, is given. Thus, the total time required is dependent on the delay length.



**Fig. 3** Illustration of VORT apparatus with different experimental phases





**Swimming tank**

**Fig. 4** Illustration of swimming behavior apparatus (a) and of zebrafish intraperitoneal injection (b)

### **3.3 Swimming Behavior**

To ascertain that the obtained results are effective to improve memory and not due to change in general activity, it is fundamental to verify swimming activity by recording the total number of crossed lines.

#### **3.3.1 Apparatus**

Fish are acclimated for 1 week to a transparent observation chamber (20 cm long  $\times$  10 cm wide  $\times$  15 cm high) (Fig. 4) containing home tank water filled at a level of 12 cm to the novel tank. The floor of the chamber is virtually divided into ten equal-sized 2 cm  $\times$  10 cm rectangles drawn on a sheet located under the floor.

#### **3.3.2 Procedure**

Using a time sampling procedure, swimming activity is monitored by counting the number of lines crossed in a 30 s observation period every 5 min, for a total of six observation bins over 30 min [36]. The mean of the six observation bins is calculated.

#### **3.3.3 Time Required**

In addition to 1 week of acclimation to the observation tank, in which each fish is daily put for 1 h a day, the time required to do the experiment is 30 min for each fish.

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## **4 Drug Treatment**

Nicotinic drugs can improve or impair learning and memory. Nicotine and its partial agonists improve cognitive function depending on the dose. Nicotine bi-tartrate is used in a range of doses between 0.2 and 200  $\mu\text{g}/\text{kg}$  of body weight while cytisine (CYT) between 0.01 and 100  $\mu\text{g}/\text{kg}$  and given i.p. 10 min before the first training trial in the T-maze or 20 min before  $T_1$  phase in



VORT. NIC can be also active if injected 10 min before  $T_1$  phase (pilot studies). Nicotine effects can be antagonized by nonselective antagonists like scopolamine (SCOP) (25  $\mu\text{g}/\text{kg}$ ) or mecamylamine (MEC) (100  $\mu\text{g}/\text{kg}$ ). Both the  $\alpha_4\beta_2$  and the  $\alpha_7$  subtype receptor have received a great deal of attention as important drug targets for cognitive enhancement [37–42]. To study the role of different nAChR subtypes, some selective drugs are available as methyllycaconitine with high affinity for  $\alpha_7$  subtype,  $\alpha$ -conotoxin (MII) with high affinity for  $\alpha_6$  subtype and Dihydro- $\beta$ -erythroidine (dH $\beta$ E) with high affinity for  $\alpha_4\beta_2$  subtype. The range to be used is between 1 and 100  $\mu\text{g}/\text{kg}$  based on previous study [23]. All the antagonists, used in the T-maze task, are given i.p. 10 min before the maximal active dose of NIC (20  $\mu\text{g}/\text{kg}$ ). Using VORT, SCOP (25  $\mu\text{g}/\text{kg}$ ) is given 20 min before  $T_1$  phase, while MEC (100  $\mu\text{g}/\text{kg}$ ) 30 min before. Vehicle group receives one or two injections of sterile saline (2  $\mu\text{l}/\text{g}$ ). All these drugs can be purchased from Sigma-Aldrich (St. Louis, MO, USA) and can be dissolved in saline. All the solutions are prepared fresh and the pH is about 7.2. Generally, at least ten animals per dose are used and each fish can be used only once. Experiments are to be carried out by experimenters blind to treatment.

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## 5 Data Analysis

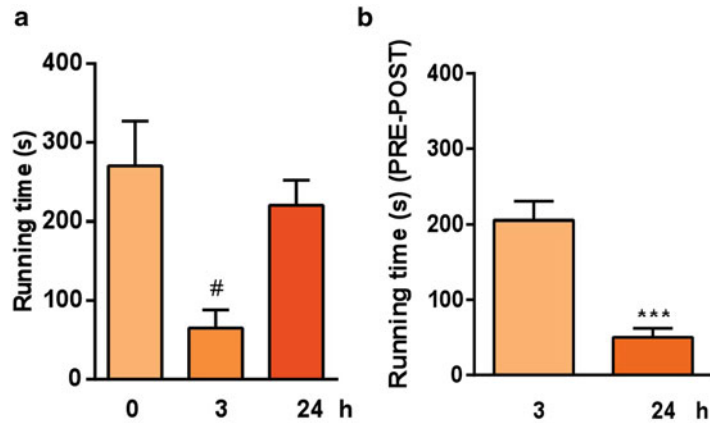
Data are expressed as mean  $\pm$  SEM. To analyze different groups, one-way analysis of variance (ANOVA) for multiple comparisons followed by an appropriate post hoc test, is suggested. In the T-maze task, running time obtained with different dosages of nicotinic compounds can be analyzed by linear regression lines. Since all nicotinic agonists show a U-shape dose–response curve, it is possible to calculate the  $ED_{50}$  only for the ascending linear portion of the curve. Comparisons between two groups can be done with Student's  $t$  test. Data from fish receiving vehicle at two different time intervals can be pooled after making sure that there is no statistical difference between the two groups. The level of significance is taken as  $P \leq 0.05$ .

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## 6 Typical Results

### 6.1 T-Maze

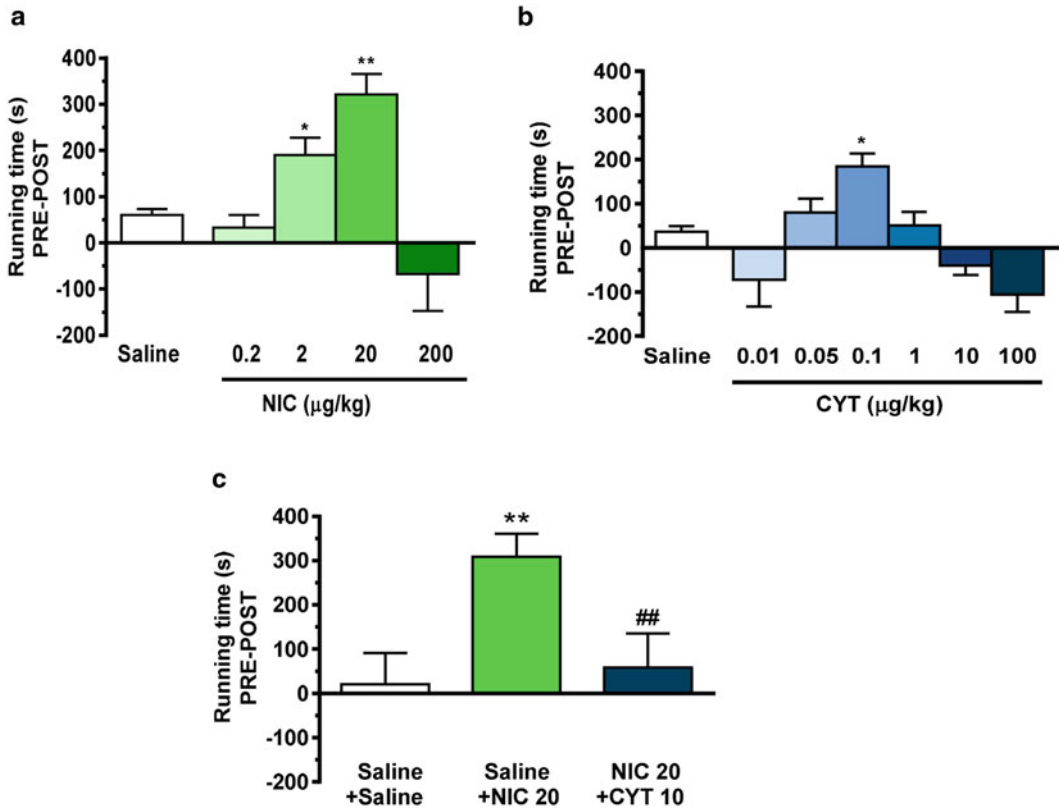
A typical result of short-finned wild-type zebrafish of heterogeneous background performance is reported in Fig. 5 where the cognitive ability can be expressed either in terms of running time to reach the reservoir (a) or in different pre-training running time minus post-training either at three or 24 h (b). Basally, zebrafish take about 270 s to find the reservoir. After 3 h a significant reduction is observed. However, if animals are tested after 24 h no difference from baseline is shown.



**Fig. 5** Cognitive ability in a T-maze can be expressed in terms of running time to reach the reservoir (a) and in terms of difference of pre-training (running time) (PRE) (at 0 h) minus post-training running time (POST) (at 3 or 24 h) (b) (See Ref. [23]). Performance is improved at 3 but impaired at 24 h. # $P < 0.05$  vs. the remaining groups, Tukey's test; \*\*\* $P < 0.0001$  vs. 3 h (Student's *t* test)

As expected, NIC effect on running time, expressed as difference of pre training time (basal) minus post-training time (at 24 h), follows a biphasic effect (increasing at low doses: 2–20 and decreasing at high: 200  $\mu\text{g}/\text{kg}$ ) (Fig. 6a). The same profile, but at different dosages (increasing at low dose: 0.1 and decreasing at high: 10–100  $\mu\text{g}/\text{kg}$ ), can be obtained using a typical partial agonist, like CYT (Fig. 6b). The calculated  $\text{ED}_{50}$  ( $\mu\text{g}/\text{kg}$ ) on ascending part of the trend is: 1.4 for NIC and 0.045 for CYT. The biphasic effect has been previously found in both zebrafish given nicotine dissolved in the water [42, 43] and mammals [44]. It is interesting to note that even if partial agonist CYT shows an improving effect in the T-maze, if used at a high dose, which per se is inactive, completely blocks NIC-induced improvement (Fig. 6c), confirming that CYT is a nicotinic partial agonist. It can be explained by the fact that CYT inhibits NIC-induced dopamine release [43], which plays an important role in zebrafish cognition [44].

Muscarinic and nicotinic blockers are known, per se, to impair different forms of memory in animals and to reduce NIC-induced memory improvement [45]. A typical experiment using SCOP and MEC, in the T-maze, is reported in Fig. 7. As expected, an amnesic effect, per se, is obtained better with SCOP than MEC (data not shown). This is not a surprising result since the effect of MEC appears to be related to the difficulty of the test [42]. SCOP and MEC blocked the improvement of memory induced by NIC reducing the difference of running time in comparison with saline group. Interestingly, the selective nicotinic antagonists, MLA and dH $\beta$ E, which per se have amnesic effects (data not shown), significantly blocked NIC pro cognitive effect. MII, which per se has slight but



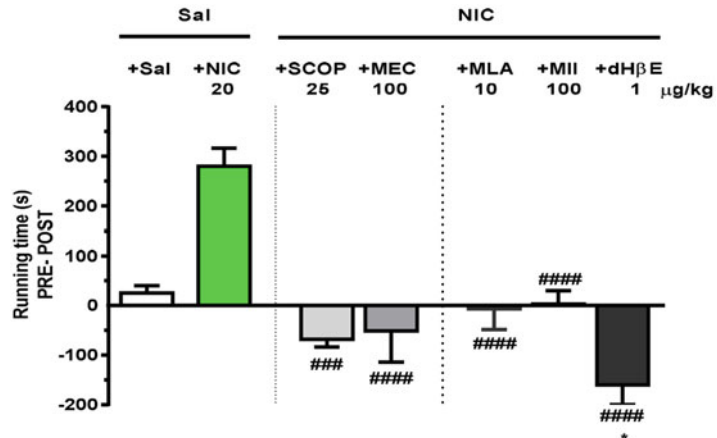
**Fig. 6** Nicotine (NIC) (a) and cytisine (CYT) (b) increase spatial memory performance following an inverted U-shaped dose–response curve. NIC performance is significantly reduced by pretreatment (10 min before) with CYT at a dose which per se does not affect cognitive ability. \* $P < 0.05$ , \*\* $P < 0.01$  compared to corresponding saline group; ## $P < 0.01$  compared to corresponding Sal + NIC group (See Ref. [23])

not significant enhancing effects (data not shown), blocks NIC-induced effect at a high dose. dH $\beta$ E is more active than MLA or MII in blocking NIC-effect suggesting a major role of the  $\alpha 4\beta 2$  subtype receptor in NIC-induced cognitive enhancement.

These results support the use of the T-maze as a tool for a rapid screening of the effect of new nicotinic partial agonists in zebrafish.

## 6.2 VORT

A number of different geometrical shapes have been tested for their ability to be discriminated by fish (Fig. 8a) where some shapes are easily discriminated, during T<sub>2</sub>, when simultaneously presented, and others are not. Before doing the experiment, researchers must check the ability of zebrafish to discriminate each pair of shapes. Thus, the mean exploration time for the familiar and novel shape during T<sub>2</sub> is significantly increased only when highly discriminated shapes are presented (Fig. 8b). High discriminated shapes lead to a good discrimination index while poorly discriminated lead to a very low discrimination index (Fig. 8c).



**Fig. 7** Treatment with different nonselective or selective nAChRs subtype receptor antagonists, given 10 min before nicotine (NIC) significantly block NIC-induced pro-cognitive effect. ### $P < 0.001$ , #### $P < 0.0001$  compared to corresponding Saline (Sal) + NIC group; \* $P < 0.05$  compared to corresponding Sal group (See Ref. [23])

Another important parameter is the choice of the inter-trial delay. During  $T_1$  phase, zebrafish spend a similar time to explore two identical shapes but starting from 5 min to 24 h inter-trial delay they spend a significant increase of time to explore the novel shape during  $T_2$  (Fig. 9, left). Consequently a good discrimination index, at the above delays, is observed while after 96 h a dramatic decrease of this parameter is shown (Fig. 9, right).

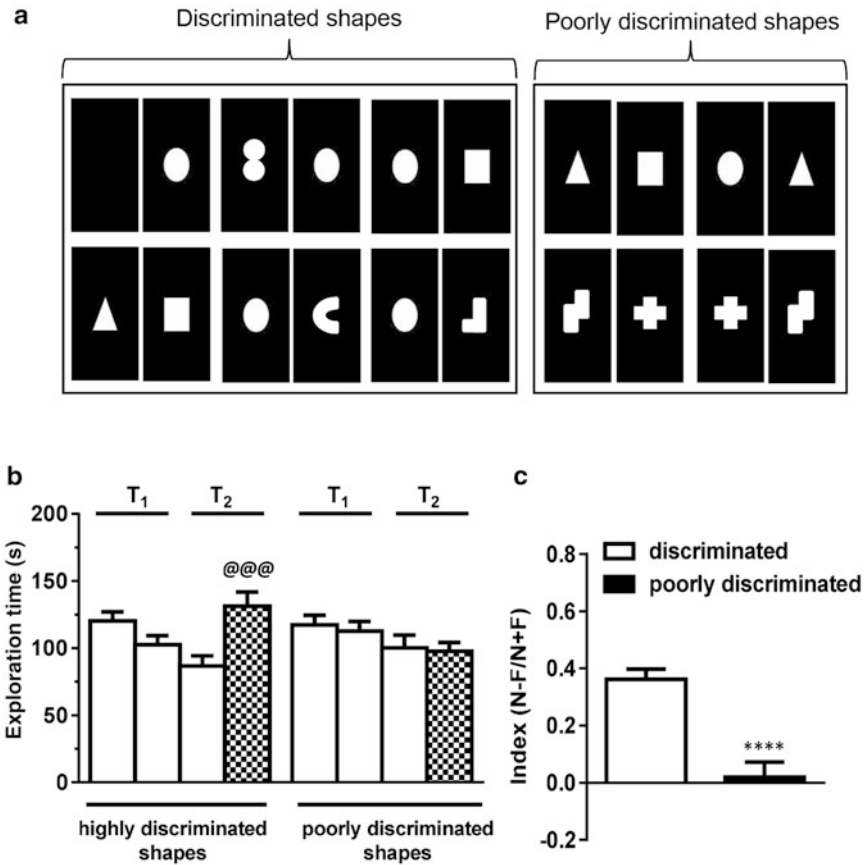
Memory performance can be ameliorated by treatment with NIC using only shapes that are difficult to be discriminated (Fig. 10, left) or worsened by SCOP/MEC using highly discriminated shapes (Fig. 10, right). The use of amnesic drugs like SCOP and MEC can help to investigate the enhancing memory effect of nicotinic drugs.

### 6.3 Swimming Behavior

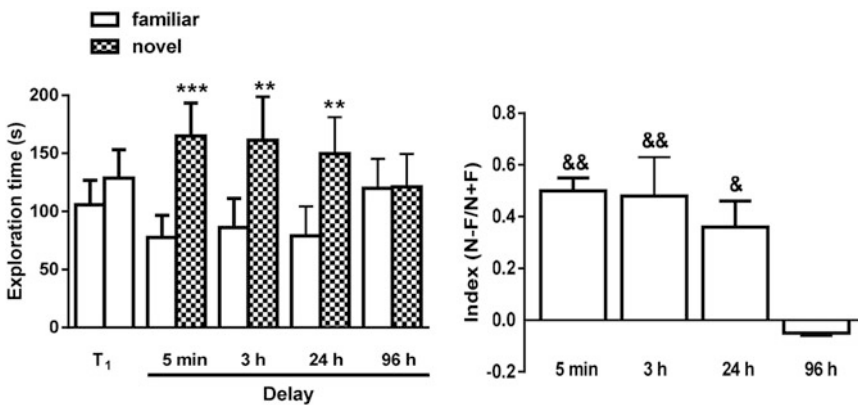
It is important to note that nicotinic compounds can alter motor function. Generally, nicotine can be stimulant at certain doses. Our employed doses are devoid of any significant effect on swimming behavior as the number of crossed lines does not differ from saline group (Fig. 11) confirming a selective effect on memory.

## 7 General Experimental Variables

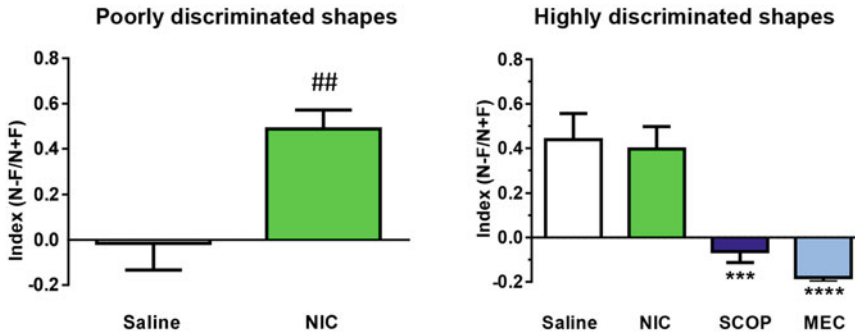
1. To decrease the variability due to manual recording, video recording system is recommended. A high-resolution Canon MV900 camera equipped with optical zoom is suggested with the possibility to transfer recordings to a PC, using the editing software supplied with the camera.



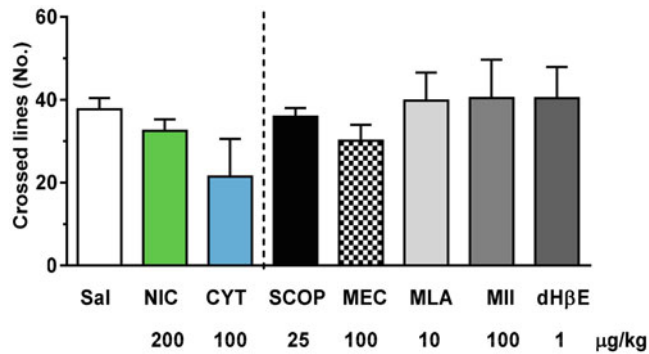
**Fig. 8** Using highly discriminated shapes a significant increase of mean exploration time (a) and of discrimination index (b). In contrast, poorly discriminated shapes lead to a significant decrease of both parameters. Examples of different pairs of shapes used in VORT are shown in panel (c) (reproduced from Ref. [29] with permission of Elsevier)



**Fig. 9** Performance evaluated in VORT at increasing time delays using highly discriminated shapes. An increase of mean exploration time to the novel shape from 5 min to 24 h (left) and a good discrimination index (right) is shown. At 96 h there is a worsened performance.  $**P < 0.01$ ,  $***P < 0.001$  as compared to corresponding familiar exploration time;  $&P < 0.05$ ,  $&&P < 0.01$  as compared to 96 h group (Tukey's test). (Reproduced from Ref. [29] with permission of Elsevier)



**Fig. 10** Nicotine (NIC, 20  $\mu\text{g}/\text{kg}$ ) injection significantly increases the discrimination index of poorly discriminated shapes (*left*) while it does not affect the discrimination index of highly discriminated shapes (*right*). Treatment with scopolamine (SCOP) (25  $\mu\text{g}/\text{kg}$ ) or mecamlamine (MEC, 100  $\mu\text{g}/\text{kg}$ ) injected 20 or 30 min before T1, respectively, reduces cognitive performance.  $\&\&P < 0.01$  as compared to corresponding saline group (Student's *t* test);  $***P < 0.001$ ,  $****P < 0.0001$  as compared to corresponding Saline and NIC groups (Tukey's test). (Reproduced from Ref. [29] with permission of Elsevier)



**Fig. 11** Treatment with saline (Sal), Nicotine (NIC), Cytisine (CYT) and different nonselective (Scopolamine, SCOP, and Mecamlamine, MEC) or selective antagonists (MLA, MII, or dH $\beta$ E) do not affect swimming behavior evaluated by counting the number of crossings in a 30-s observation period every 5 min over 30 min. (Reproduced from Ref. [23] with permission of Springer)

2. Zebrafish are known to be anxious fish [34]. Thus, the 2 weeks of acclimation can be prolonged until to a month to make zebrafish less anxious. Handling during injection may generate an anxious state. Researchers have to be quick and gently handle the fish.
3. The age of zebrafish may vary the results. Young zebrafish (from 1 to 3 months) are very small and thus the perception of the environment can be altered. Aged fish may slowly swim, resulting in an altered performance.
4. The apparatus needs a homogeneous light over the tank. Penumbra zones can alter the swimming of fish.

5. Water temperature must be controlled with a thermometer. Cold water can affect swimming behavior (freezing).
6. Drugs can also be dissolved in the water tank but in this case the amount of drug each fish receives is less precise. For nicotine, each fish is immersed in a beaker containing 50 ml of water for 3 min and then placed singly into a holding tank without nicotine for the interval between exposure and testing [43]. Water in the beaker is changed for each fish.

### **7.1 T-Maze**

1. The T-maze protocol is based on previous findings using similar apparatus but different cue stimuli to motivate zebrafish to choose the correct arm. Alternatively to a favorable habitat, the researcher can use a deeper habitat [21], food as reinforcer [20], particular color (red better than blue) [47], the sight of conspecifics [19], aversive stimuli like a mild shock [24], or a water soluble that smells or tastes bad [19]. Researchers who decide to use different stimuli with T-maze have to pay attention to some variables. For example if food is used zebrafish need a habituation to the bait for 3–5 days to avoid food neophobia before starting the experiment [34]. If colors are used as stimuli, pay attention that zebrafish have a preference for red and also yellow but avoid blue [22].
2. Initially, fish take an average about 250 s to find the reservoir; however, individuals can vary their performance. The initial time appears to be dependent on the stress levels of the fish. A small amount of fish never leaves the start zone or the long arm of the maze. In this case they have to be removed from data analysis. Fish which are very fast to reach the reservoir, probably for their initial anxious state, have to be removed from data analysis.
3. The acquisition learning can be also obtained in the same zebrafish trained to progressive intervals (3, 12, and 24 h). In this case animals progressively improve their performance decreasing their latency of about 60%.

### **7.2 VORT**

1. Researchers have to check different pairings of shapes delivered from the two iPods to establish which shapes are discriminated and which are not by their zebrafish. This is important before starting experiments with nicotinic drugs.
2. Drawing a line on the two walls of VORT apparatus at 10 cm from the iPod areas can help the experimenter to better score the time spent close to the iPods.

### **7.3 Swimming Activity**

1. Ten rectangles, which divide the floor of the observation chamber, can be varied [36]. If more lines are included, more activity can be better measured. The lines can be put also on the walls of the tank.



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## 8 General Troubleshooting

Several practical recommendations reported here may help the researchers to obtain more reliable and reproducible behavioral data.

1. To avoid social isolation stress, the animals have to return to their tanks after each time delay and housed in their home tanks in groups of 15 as described by [48]. A simple marking procedure to recognize the fish is the subcutaneous injection of a color dye as suggested by [49] that may alleviate this problem. The procedure allows to successfully mark zebrafish and distinguish them for a period of more than 30 days, which is sufficiently long for most behavioral paradigms developed for this species. In addition, the injection-based marking does not significantly alter social interaction, as defined by the frequency of agonistic behaviors within shoals.
2. Blind fish or with poor sight cannot be used. The visual acuity generally increases throughout the first year of development and then tails off a bit at 15 months of age [50].
3. Researchers can more accurately measure the amount of time to reach the reservoir or the time spent close to the novel shape or the crossed lines in the swimming activity using a video camera.
4. The choice of time interval to test nicotinic drugs is important. To study memory facilitating effects zebrafish must be impaired. A time of 24 h or more from the first training trial is the best time for T-maze or the choice of poorly discriminated shapes for VORT. In contrast, to evaluate if drugs impair memory, a high cognitive performance is needed. Thus, a short interval from the first training trial (1–3 h) in the T-maze or the use of highly discriminated shapes in VORT is warranted.
5. A limitation to study zebrafish with nicotinic compounds is the lack of information on drug absorption and metabolism rate. However, at least for nicotine, it is possible to measure its concentration in the brain after injection using liquid chromatography–tandem mass spectrometry as previously described [51].
6. There is a high degree of sequence identity to rats and human orthologs of nAChR [12], supporting the use of zebrafish to test the effect of nicotinic compounds. However, there is not a wide availability of selective antagonists for zebrafish. Binding studies can help to establish their affinity to nAChR subtypes.
7. It is important to pay attention to treat each fish correctly, without piercing it. In this case, animals must be discharged.

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