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Y-Maze memory task in zebrafish (*Danio rerio*): The role of glutamatergic and cholinergic systems on the acquisition and consolidation periods

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ABSTRACT

The interest in the behavioral features of zebrafish has significantly increased over the past two decades. However, most available protocols have used longer training periods and have been based on reinforcement/reward or avoidance. The Y-Maze memory task has the advantage of using a simple and rapid training session, but it has not been established in zebrafish. Here, we have characterized this task for zebrafish, with the addition of pharmacological interventions in the acquisition and consolidation memory phases. The results show that zebrafish spend more time in the novel arm than in the other arms of the Y-Maze, both in response to novelty and spatial memory training-test intervals (TTIs). We have also studied the involvement of the glutamatergic and cholinergic systems with pre- and post-training treatments with the NMDA receptor antagonist MK-801 (20 μM) and the cholinergic blocker scopolamine (200 μM). After 1 h of TTI, pre-training MK-801 and scopolamine-treated fish reduced their exploration of the novel arm when compared to the control group, with no changes in their locomotor activity. Post-training of MK-801 treatment also impaired their Y-Maze performance, while post-training of any scopolamine treatment failed to affect novel arm exploration. In conclusion, the Y-Maze memory task can be reliably used for zebrafish, providing a new, rapid, and preference/avoidance independent task for the study of memory in this teleost. In addition, our results highlight the implication of the glutamatergic and cholinergic systems in the memory of zebrafish.

1. Introduction

Zebrafish, a small tropical freshwater teleost, has emerged as a promising model organism for experimental studies in different biomedical areas. The large offspring, transparent embryos with external development and the easy maintenance of several animals in captivity are clear advantages of this animal model in numerous research areas (Nasevicius & Ekker, 2001), including toxicology (Spitsbergen & Kent, 2003), neurological diseases (Guo, 2004), drug addiction (Ninkovic & Bally-Cuif, 2006), and aging (Gerhard, 2007; Gerhard & Cheng, 2002). In the last decade, zebrafish have been gaining popularity in behavioral brain research (Sison & Gerlai, 2010). For instance, zebrafish performed well in several conditioning memory tasks, such as olfactory (Braubach, Wood, Gadbois, Fine, & Croll, 2009), shuttle box active appetitive (Pather & Gerlai, 2009) and appetitive choice discrimination (Bilotta, Risner, Davis, & Haggbloom, 2005). Moreover, this small vertebrate showed acquisition in one trial avoidance task (Blank, Guerim, Cordeiro, & Vianna, 2009) and achieved good performance in alternation memory tasks and plus maze non-spatial and spatial associative learning tasks (Al-Imari & Gerlai, 2008; Sison & Gerlai, 2010, 2011). All this data clearly demonstrates the cognitive and mnemonic capabilities of zebrafish. The characterization of zebrafish cognition is an important goal, and the identification of conserved cognitive patterns and their underlying mechanisms reinforces their potential for translational science with the unique opportunity of combining advanced genetics (Goldsmith, 2004; Guo, 2004; Salas et al., 2006; Xu, Scott-Scheiern, Kempker, & Simons, 2007). Moreover, behavioral studies are crucial in the development of disease models that affect the central nervous system and allow the evaluation of toxic or neuroprotective agents on cognition.

Classical neurotransmitter systems involved in learning and memory, such as the glutamatergic (Todd, Slatter, & Ali, 2004) and the cholinergic systems (Behra et al., 2002; Clemente et al.,

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2004) have already been identified in zebrafish. Molecular and pharmacological evidence shows that the glutamatergic activation of plastic synapses is critical to long-term memory formation (Blank et al., 2009) and can give rise to new or enlarged dendritic spines, which may constitute the main structural basis of some memory types (for review see Morgado-Bernal, 2011). Also, the disruption of cholinergic neurotransmission by targeted lesions, toxins, drugs, aging or disease induces impairments in a range of functions, including perception (Erskine et al., 2004), attention (Robbins et al., 1989), learning and memory (Kopelman, 1986), emotion (Kamboj & Curran, 2006), and sleep (Kim & Jeong, 1999). A recent review proposes that the cholinergic function modulates cognitive processes by having direct effects on basic stimulus processing, ranging from healthy to dysfunctional cognition in neuropathological conditions, including dementia and mood disorders (Furey, 2011).

The available protocols to study learning and memory in zebrafish are mostly based on longer training periods and/or have been based on reward or avoidance (Blank et al., 2009; Sison & Gerlai, 2010). An alternative approach to study less emotional cognitive responses in rodents has been the use of mazes, including T-maze, radial-maze and Y-Maze (Cognato et al., 2010; Gerlai, 1998; Shama, Rakoczy, & Brown-Borg, 2010). A two-trial memory protocol in a Y-Maze, based on a free-choice exploration, has been previously developed to study recognition processes in rats (Dellu, Fauchey, Le Moal, & Simon, 1997; Dellu, Mayo, Cherkaoui, Le Moal, & Simon, 1992) and has proven to be a useful tool for evaluating several behavioral and pharmacological conditions in rats (Conrad, Lupien, Thanasoulis, & McEwen, 1997; Dellu et al., 1997; Vallée et al., 1997). This task provides many advantages. First, the Y-Maze does not involve conditioned learning and thus enables specific testing of memory. Second, factors that may particularly influence performance, such as motivational or emotional states, are minimized. Third, the Y-Maze task is based on the natural tendency to explore novelty and this motivational component can be assessed first by the use of a short TTI when the mnemonic demand is minimal. Once preferential exploration of novelty is established, the measure of memory can be evaluated with longer TTIs. Fourth, since retention does not last longer than a few hours, performance can be assessed several times in the same animal (i.e., 1 week later). Fifth, locomotor activity, recorded as the number of arm visits or distance traveled, can be evaluated. Finally, measurement of behavior is quick, precise, and entirely automated, permitting a detailed analysis of performance.

The aim of this study was to establish a new two trial Y-Maze task for zebrafish and characterize the contribution of the glutamatergic and cholinergic systems in memory acquisition and consolidation for this task.

2. Materials and methods

2.1. Animals and housing

Adult (<8 months old) male wild type zebrafish with Tuebingen background (Capiotti et al., 2011; Maximino, Lima, Olivera, Picanço-Diniz, & Herculano, 2011) were obtained from a local supplier (Redfish Agroloja, RS, Brazil). Animals were maintained in 20 L housing tanks, divided into six compartments and filled with tap water previously treated with Tetra's AquaSafe® (Blacksburg, VA) to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to the fish. The tanks were also continuously aerated (7.20 mgO_2/L) and had mechanical and biological filtration systems purchased by Tetra® in order to avoid accumulation of organic toxins. Also, water quality parameters were monitored daily and kept in the following ranges: pH (6.5 and 7.5), conductivity (400–600 μS), ammonium concentration (<0.004 ppm) and temperature (25–28 °C). The fish were maintained in a 14–10 h light/dark photoperiod at a density of up to five animals per liter. Animals were acclimated for at least 2 weeks before the experiments. During the acclimation period, animals occupied only three compartments of the tank, and were moved to another compartment of the same tank daily, to avoid manipulation stress and the influence of a new environment on memory consolidation. They were fed three times a day with TetraMin Tropical Flake Fish®. Protocol was approved by the institutional Animal Ethics Committee under the number 11/00245 – CEUA-PUCRS.

2.2. Y-Maze apparatus

Animals were tested in a Y-Maze glass aquarium with three arms (25 cm long, 8 cm wide and 15 cm high). Visual cues made of white paper cut in different geometric forms were placed on the external maze walls, which were made of transparent glass, making them visible from inside the maze. The remaining area of the external maze walls was covered with black plastic self-adhesive film. The external floor of the apparatus was white to create a contrast between fish and maze and to facilitate the video analysis. Three liters of the same water used in the home aquarium was used in the apparatus. In order to evaluate the spatial learning memory without place preference, appropriate visual cues for each arm were established in such a way that the fish did not show signs of avoidance or preference. Primarily, geometric forms such as squares, triangles and crosses were used in each arm of the maze. Since the fish spent less time in the arm with crosses, these were substituted by circles in the final apparatus.

2.3. Y-Maze task

The arms of the Y-Maze were randomly designated: the start arm, in which fish started to explore (always open), the novel arm, which was blocked during the first trial, but open during the second trial, and the other arm (always open). The center of the maze (neutral zone) was not computed in the analysis. The Y-Maze task consisted of two trials separated by a TTI to assess response to novelty (fish placed in the apparatus when the novel arm was unblocked by a pulley and string system) and spatial recognition memory (1 h, 3 h and 6 h TTI) (Dellu et al., 1997). Different groups of fish were tested in each TTI. Thus, animals were exposed to novel arm only once. During the first trial (training, 5 min), fish were allowed to explore only two arms (start and other arm), with the third arm (novel arm) closed. For the second trial (after different TTIs), the fish were placed back in the same starting arm, with free access to all three arms for 5 min. Fish were placed in different arms as starting points and the maze was rotated in every experiment in order to randomize the maze cues. Training and test sections were recorded using Logitech Quikcam PRO 9000 and further analyzed using the ANY-Maze recording software (Stoelting Co., Wood Dale, IL, USA). The time spent in each arm was determined, along with measures of locomotion (such as total distance, mean speed, turn angle and number of line crossings). Fig. 1 shows the Y-Maze glass tank.

2.4. Cognitive deficit induced by MK-801 or scopolamine

To demonstrate that learning and memory on the zebrafish Y-Maze task involved evolutionary conserved mechanisms, and the task's potential use in pharmacological studies of memory mechanisms, we investigated the role of glutamatergic and cholinergic systems on memory acquisition and consolidation. MK-801 (Dizocilpine hydrogen maleate, $C_{16}H_{16}N.C_4H_4O$, CAS number 77086-22-

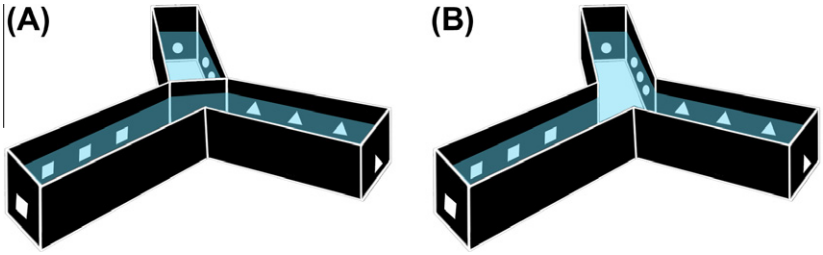

Fig. 1. Schematic representation of the Y-Maze glass tank with (A) the novel arm blocked with a sliding partition, as in a training session, and with (B) the novel arm open, as in the test sessions. The cues were distributed between both sides and the back of each arm. Three liters of water were necessary to cover the cues in order for the fish to see them and differentiate the arms.

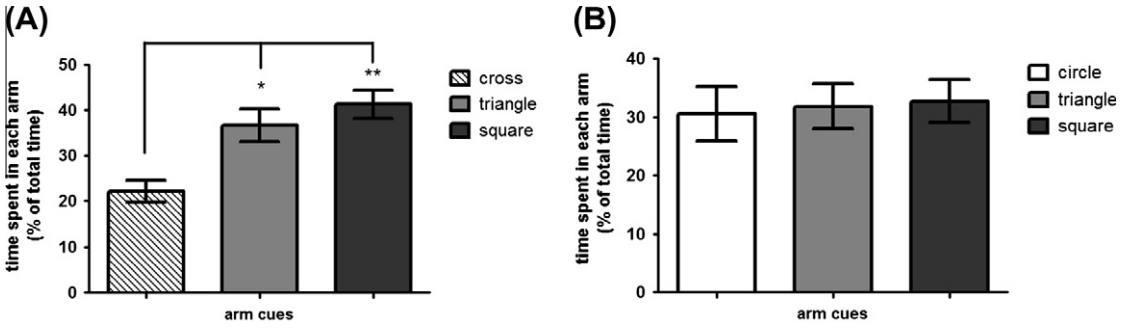

Fig. 2. Establishment of visual cues for the Y-Maze tank. Fish preferences between triangles, squares and crosses are presented in panel A ($n = 20$) and the preferences between triangles, squares and circles are presented in panel B ($n = 19$). The time spent in each arm was expressed as a percentage of the total time spent in the Y-Maze tank. Data is shown as mean ± SEM and was analyzed by One Way ANOVA followed by Tukey's multiple comparison test with $p < 0.01$ and $p < 0.001$ represented by ** and *** respectively.

77), a non-competitive glutamate NMDA receptor antagonist, or scopolamine ((−)-scopolamine hydrobromide trihydrate, $C_{17}H_{21}NO_4 \cdot HBr \cdot 3H_2O$, CAS number 6533-68-2), a muscarinic antagonist, were used to verify their influence on the Y-Maze memory task (both reagents were purchased from Sigma–Aldrich Corp., St. Louis, MO, USA). Before, or immediately after the training session, treated animals were placed in a compartment of a home tank with 5, 10 or 20 μM MK-801 for 15 min or 50, 100 or 200 μM scopolamine for 1 h and the control group was kept in the original compartment of the home tank. The doses used in the dose response curves were chosen based on the literature (Blank et al., 2009; Richetti et al., 2010). Animals were kept separated according to their group, until 1 h after their test session.

2.5. Statistical analysis

Data is shown as mean ± SEM. The time spent in each arm during the test session was compared with One-Way ANOVA, followed by Tukey's multiple comparison test. Comparisons between groups (controls versus MK-801 or scopolamine-treated animals) were performed using One-Way ANOVA, followed by Tukey's multiple comparison test. In all comparisons, $p < 0.05$ was considered to indicate statistical significance. GraphPad Prism 5 software was used for statistical analysis.

3. Results

3.1. Establishment of arm cues

Since the aim of this study was to evaluate the learning and memory, without using place preference, the first goal was to establish appropriate visual cues for each arm, in a way that the fish did not show signs of avoidance or preference. Primarily, geometric forms such as squares, triangles and crosses were used in each arm of the maze. As shown in Fig. 2A, the zebrafish spent less time in the arm with crosses (22.09% ± 2.47 of the total time spent in the maze), spending more time in the arms with squares (41.25% ± 3,12) or triangles cues (36.60% ± 3.51) ($P < 0.01$ for crosses versus triangles and $P < 0.001$ for crosses versus squares). After this result, crosses were substituted by circles, and the preference between circles, squares or triangles was evaluated. As shown in Fig. 2B, the zebrafish spent statistically comparable times in each arm (30.5% ± 4.7, 31.9% ± 3.7, and 32.8% ± 3.9 for circles, triangles and squares respectively), demonstrating a similar preference for these cues ($P = 0.93$). Therefore, the final Y-Maze apparatus consisted of squares, triangles and circles for arm cues.

3.2. Y-Maze response to novelty and spatial memory

The Y-Maze task takes advantage of the natural tendency to explore novelty using shorter TTIs. First, response to novelty was evaluated with no TTI. After 5 min of exploration with only two arms in the Y-Maze, the novel arm was unblocked slowly (over a period of 1 min) while the fish were still in the maze. After the novel arm was completely open, the test session started and lasted for 5 min. As shown in Fig. 3A, the zebrafish spent 69.2% ± 4.4 of total time in the novel arm and only 14.1% ± 3.8 and 14.8% ± 2.9 in the start and other arms respectively ($P < 0.0001$). Once preferential exploration of novelty was established, the measure of memory was evaluated with 1, 3, and 6 h of TTI. One hour after the training session, the fish spent 49.5% ± 3.8 of the total time in the novel arm, and 20.9% ± 3.3 and 22.0% ± 2.9 in the start and other arms respectively (Fig. 3B; $P < 0.0001$). Three hours after the training session, the fish spent 34.5% ± 2.1 of the total time in the novel arm and 26.7% ± 2.2 and 28.9% ± 2.2 in the start and other start arms respectively (Fig. 3C; $P < 0.05$). Six hours after training

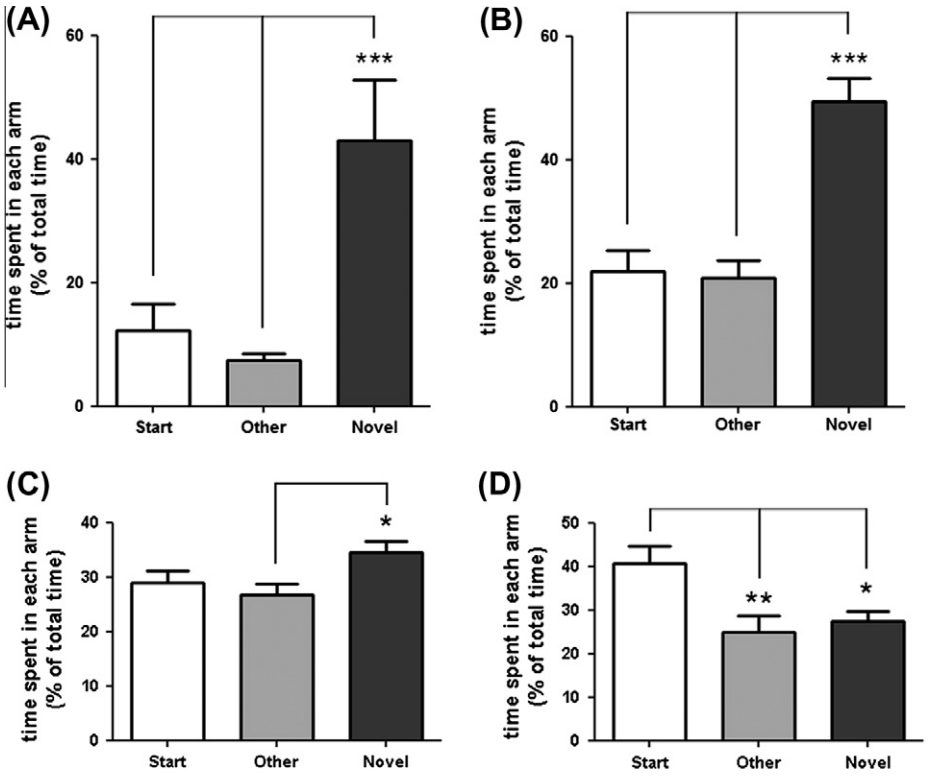

Fig. 3. The Y-Maze response by the fish to novelty is represented in panel A ($n = 12$) and spatial memory is represented in panel B (1 h TTI; $n = 12$), C (3 h TTI; $n = 12$), and D (6 h TTI; $n = 12$). The time spent in each arm was expressed as a percentage of the total time spent in the Y-Maze tank (300 s). Data is shown as mean ± SEM and was analyzed by One Way ANOVA followed by Tukey's multiple comparison test with $p < 0.05$, $p < 0.01$, and $p < 0.001$ represented by *, **, and *** respectively.

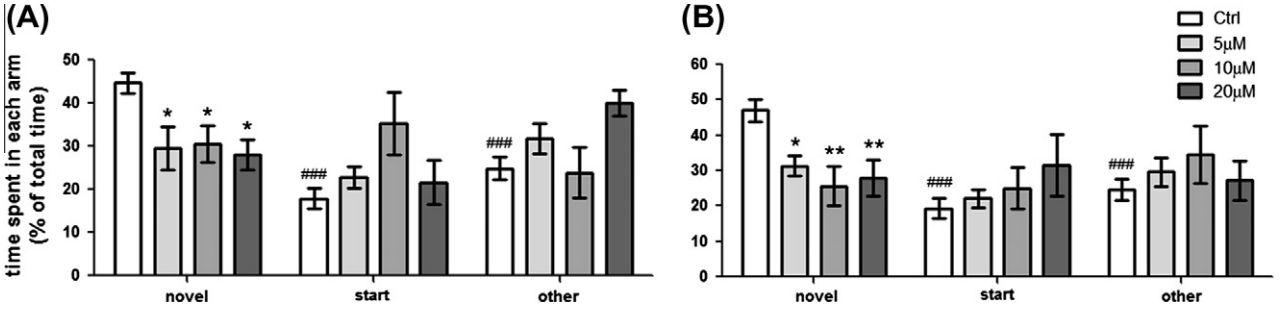

Fig. 4. Fish submitted to pre-training (panel A; $n = 15$) or post-training MK-801 (panel B; $n = 15$). Data is shown as mean ± SEM and the difference of the time spent in the novel arm between the control and the treated groups was analyzed by 1 way ANOVA followed by Tukey's multiple comparison test with $p < 0.05$ and $p < 0.01$ of significance represented by * and **, respectively. The difference between the time spent in each arm for the control group was analyzed by Tukey's multiple comparison test with $p < 0.0001$ and represented by ###.

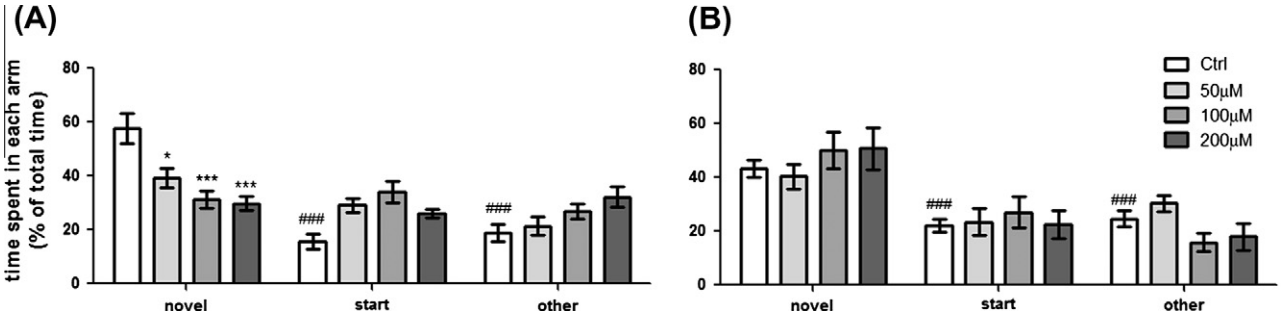

Fig. 5. Fish submitted to pre-training (panel A; $n = 15$) or post-training scopolamine treatment (panel B; $n = 15$) in the Y-Maze task. Data is shown as mean ± SEM and the difference of the time spent in the novel arm between the control and treated groups was analyzed by 1 way ANOVA followed by Tukey's multiple comparison test with $p < 0.05$ and $p < 0.001$ of significance represented by * and ***, respectively. The difference between the time spent in each arm by the control group was analyzed by 1 way ANOVA followed by Tukey's multiple comparison test with $p < 0.0001$ and represented by ###.

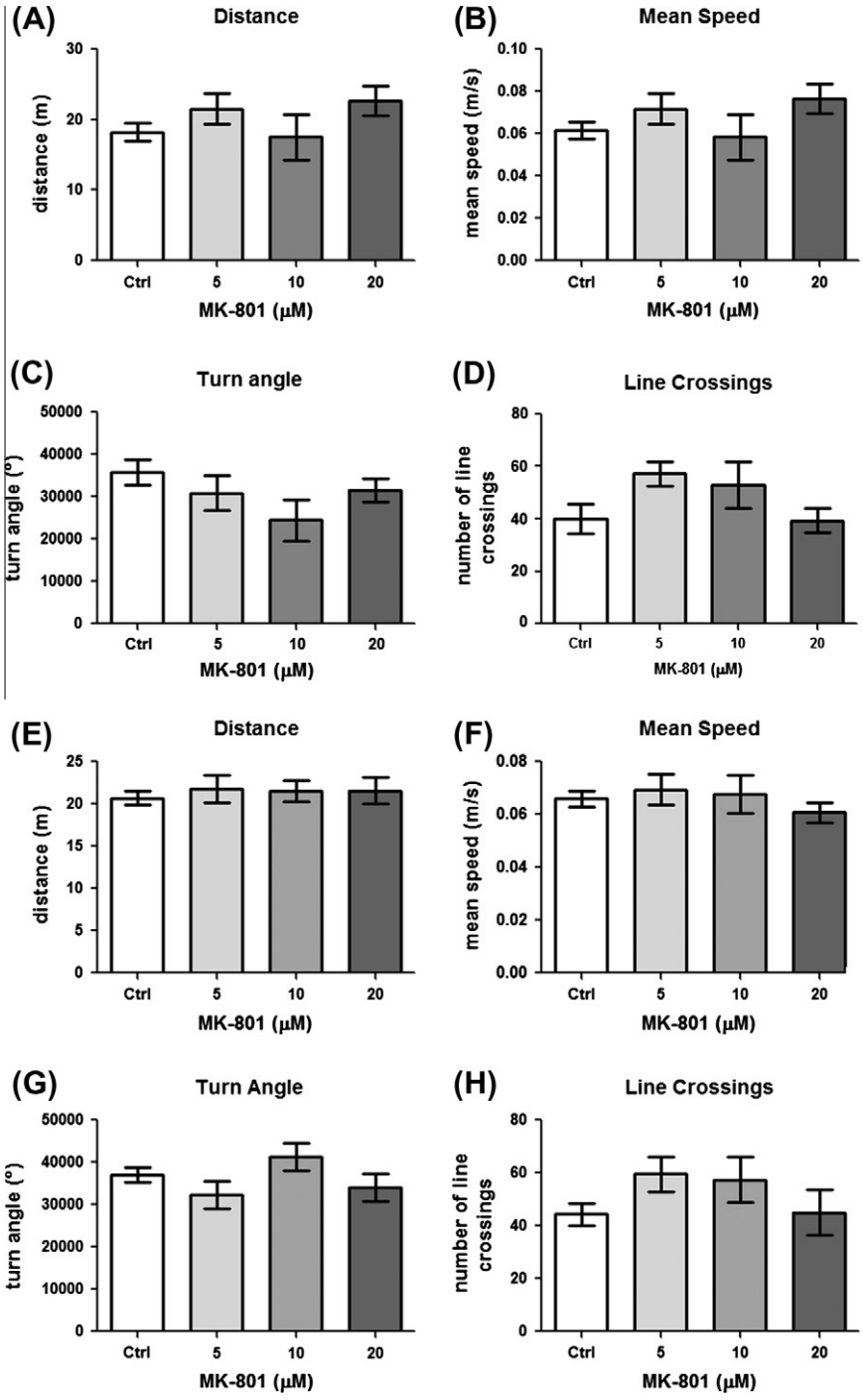

Fig. 6. Locomotion parameters of pre- (A–D) and post- (E–H) training of MK-801 treated fish. Data for the total distance traveled (A and E), the mean speed (B and F), the absolute turn angle (C and G), and the number of line crossings (D and H) are shown as mean ± SEM and the difference between the control and treated groups were analyzed by 1 way ANOVA followed by Tukey's multiple comparison test with $p < 0.05$ of significance.

session, the fish spent 27.3% ± 2.3 of the total time in the novel arm and 24.9% ± 3.7 and 40.6% ± 4.2 in the start and other arms respectively (Fig. 3D; $P < 0.05$ novel versus start arm; $P < 0.01$ start versus other arm).

3.3. Memory Impairment Induced by MK-801 and scopolamine

As the zebrafish were able to recognize the novel arm after 1 h of TTI, we evaluated whether the glutamatergic and cholinergic systems were involved in the acquisition and consolidation of recognition memory. Concerning acquisition, the MK-801-treated zebrafish spent less time in the novel arm in all doses tested (5 μM: 29.5% ± 5.0; 10 μM: 28.9% ± 4.4; 20 μM: 28.7% ± 3.2) when compared to the control group (44.7% ± 2.3) (Fig 4A; $P < 0.01$). Fish submitted to MK-801 treatments after training also spent less time in the novel arm (5 μM: 31.4% ± 3.0; 10 μM: 26.8% ± 5.1; 20 μM: 27.8% ± 3.7) when compared to the control group (47.0% ± 3.0) (Fig 4B; $P < 0.05$).

Animals treated with all doses of scopolamine before training spent less time in the novel arm (50 μM: 37.4% ± 3.5; 100 μM: 32.3% ± 3.2; 200 μM: 31.6% ± 3.0) when compared to the control group (54.1% ± 4.5) (Fig 5A; $P < 0.001$). In contrast, fish submitted

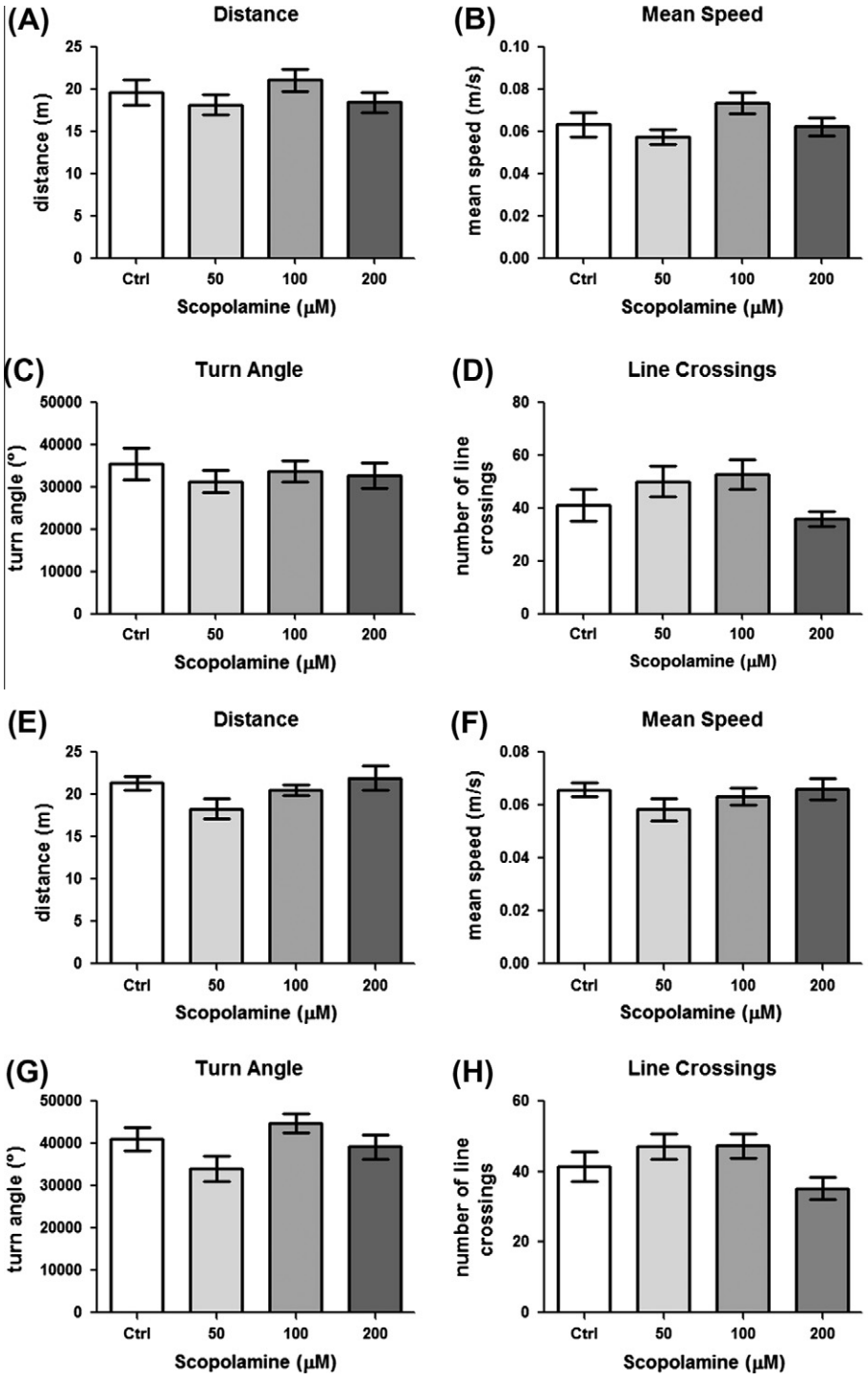

Fig. 7. Locomotion parameters of pre- (A–D) and post- (E–H) training of scopolamine treated fish. Data for the total distance traveled (A and E), the mean speed (B and F), the absolute turn angle (C and G), and the number of line crossings (D and H) are shown as mean ± SEM and the difference between the control and treated groups was analyzed by 1 way ANOVA followed by Tukey's multiple comparison test with $p < 0.05$ of significance.

to all doses of scopolamine after training spent as much time as the control group in the novel arm (Fig 5B; 50 μM: 38.3% ± 3.7; 100 μM: 50.0% ± 6.8; 200 μM: 50.7% ± 7.8; and control group: 44.4 ± 3.2), showing a significant preference for novelty ($P < 0.05$).

3.4. Locomotion of MK-801 and scopolamine treatments during pre-training

Since any alteration in locomotor activity could influence the results of the memory Y-Maze task, we evaluated exploratory behavior after treatment with MK-801 and scopolamine-Treated zebrafish. As observed in Fig. 6 and 7, the total distance traveled, the mean speed, the turn angle, and the number of line crossings were not statistically different between the control group and pre- and post-training MK-801 and scopolamine-treated animals.

4. Discussion

In this study, we aimed to characterize the zebrafish Y-Maze memory task, a simple and rapid task to study cellular and molec-

ular time-dependent processes involved in acquisition and consolidation of memory. As expected for this task, the animals showed a preference for the unexplored arm. Moreover, the Y-Maze task as described was useful to test the effects of pharmacological agents on memory acquisition and consolidation. We showed that MK-801 and scopolamine, dissolved in the tank water, were able to produce memory deficits (Blank et al., 2009; Richetti et al., 2010). The ability of the zebrafish to rapidly and efficiently absorb small molecules in water represents a major advantage for rapid and non-invasive behavioral screenings in zebrafish when compared to other model organisms, especially mammals (Blank et al., 2009).

Glutamate NMDA receptors have been implicated in the mechanisms underlying acquisition, consolidation, as well as recall (Sweatt, 2010). In our study, MK-801 efficiently blocked memory acquisition when given immediately before or after training. However, there is discrepant data in literature (Castellano, Cestari, & Ciamei, 2001; Dix, Gilmour, Potts, Smith, & Tricklebank, 2010). Briefly, there may be several reasons for the controversies, including the type of pharmacological tools employed, or the timing of the manipulations, the type of learning task utilized, and the species studied. Regarding the type of memory task, MK-801 disrupted or retarded the memory acquisition of mice or rats in several spatial tasks, such as the spatial Morris task, T-maze alternation tasks, and object recognition tasks (van der Staay, Rutten, Erb, & Blokland, 2011). In zebrafish, cognitive impairment induced by MK801 has already been observed in one-trial inhibitory avoidance tasks (Blank et al., 2009; Seibt et al., 2010) and associative learning plus maze tasks (Sison & Gerlai, 2011). Our results are in accordance with the literature for both pre- and post-training MK-801 treatment in a Y-Maze task. Additionally, MK-801 can increase locomotor activity, leading to impairment for place preference (Swain, Sigstad, & Scalzo, 2004). We found no effect of this low MK-801 on these behavioral parameters that could account for the memory deficit observed, including swimming activity and orientation. This is in accordance with the available literature, whereby an MK-801 deleterious effect on swimming activity was only observed on much longer treatments and higher dose regimens (Swain et al., 2004).

The cholinergic system is involved in many physiological processes, including synaptic plasticity and learning and memory (Power, Vazdarjanova, & McGaugh, 2003; Weinberger, 2006). Cholinergic agonists can facilitate memory, whereas cholinergic antagonists can impair memory (Mattson, 2004). Studies of the effects on brain plasticity of cholinergic agents, particularly those engaging muscarinic receptors, have provided robust and clarifying information about learning and memory processes (Hasselmo, 2006). In addition, the cholinergic hypothesis of geriatric memory dysfunction and the evidence of the involvement of this system in the etiology of AD have brought attention to cholinergic interventions as a treatment for this disease. Scopolamine, an acetylcholine muscarinic receptor antagonist, has largely been used as an animal model of cognitive impairment and memory loss since 1974, mimicking a type of dementia observed in Alzheimer's disease (Deiana, Harrington, Wischik, & Riedel, 2009; Drachman & Leavitt, 1974; Kim et al., 2008). The cognitive deficit induced by scopolamine, which easily penetrates the blood–brain barrier, impairs the acquisition and consolidation of memory in healthy rats, primates, and humans (Spinelli, Ballard, Feldon, Higgins, & Pryce, 2006). In fact, scopolamine has been already studied in T-maze alternation task in mice, resulting in an impaired performance of animals treated previously with scopolamine (Spowart-Manning & van der Staay, 2004).

In zebrafish, pre-training scopolamine treatment has induced memory deficits on one-trial avoidance tasks (Richetti et al., 2010) and passive avoidance tests (Kim, Lee, Kim, Jung, & Lee, 2010). Our data is in accordance with literature, since we observed cognitive deficits in the Y-Maze task induced by scopolamine. A key behavioral finding is that acetylcholine is more critical for memory encoding than consolidation (Hasselmo, 1999), with cholinergic stimulation being counterproductive if occurring after encoding (Bunce, Sabolek, & Chrobak, 2004; Gais & Born, 2004). Our results are in accordance with this information, since after training, scopolamine-treated fish did not show cognitive impairment, spending their time in the novel arm as much as the control group. Scopolamine data in literature has shown a substantial discrepancy in relation to scopolamine effects in locomotion. Some studies, in fact, challenge the viability of scopolamine use as a cognitive impairer, questioning if the alterations in behavior are related to peripheral locomotor effects, instead of memory disruption (for a review, see Klinkenberg & Blokland, 2010). To address this problem, we performed a general analysis on zebrafish locomotor behavior and observed no changes in none of the parameters analyzed. In light of this evidence, we believe that the scopolamine induced memory deficits observed were solely due to the drug effect on the cholinergic system and not due to altered locomotor behavior.

All the advantages of the Y-Maze memory task and the characterization of neurotransmitter systems related to memory processes in zebrafish indicate that this small teleost can be a good animal model for the study of learning and memory. In conclusion, the Y-Maze memory task for zebrafish is a new, rapid, and reward/avoidance free task for the study of memory in this teleost. In addition, our results highlight the implication of glutamatergic and cholinergic systems in memory as assessed by the Y-Maze task in zebrafish.

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