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Novel object recognition and object location tasks in zebrafish: Influence of habituation and NMDA receptor antagonism

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ABSTRACT

This study aims to establish a protocol for evaluating the object recognition memory and object location tasks in zebrafish. We evaluated novel the object recognition memory and analyzed the exploration time of the objects during training and testing. Zebrafish explored more the new object in comparison to the familiar object (61% of exploration time during test session). We also tested the object location task and measured the exploration time of each object in the familiar and novel object location. There was a preference to explore the object in the novel location (63% of exploration time during test session). The effect of the non-competitive NMDA receptor antagonist MK-801 was investigated on the object recognition and object location memory. Control (water only) and treated animals (5 μM MK-801) presented a significant preference in exploring the familiar object in comparison to the new object (66 and 68% of exploration time, respectively, during test session); however, 10 μM MK-801-treated animals did not show differences in the exploration time of the objects. In the object location task, the animals treated with the 5 or 10 μM MK-801 did not show a preference for the familiar or novel location whereas the control group had a higher preference in exploring the object in the familiar location (64% of exploration time during test session). Considering the different responses of the control group between original task and in the regimen treatment, we evaluated the impact of habituation on cortisol levels of animals in three different protocols: (1) habituated at the experiment apparatus for 3 days (C1 condition), (2) habituated at the experiment apparatus for 3 days plus treatment tank exposure at fourth day (C2 condition), (3) habituated at the treatment tank and experiment apparatus for 3 days and exposed to treatment tank again at fourth day (C3 condition). The results showed higher levels of cortisol in animals submitted to C2 and C3 conditions compared to animals submitted to C1. When introduced to an acute stressor during C1 condition, we observed an increase in the cortisol levels and an absence of preference for the objects in comparison to control group, which had a preference for novel object and novel location. Fluoxetine treatment induced a decrease in cortisol levels and an absence of preference for the objects in C2 and C3 conditions in comparison to control group, which had a preference for familiar object. However, fluoxetine treatment induced a preference to the novel location in C2 and C3 conditions in comparison to control group, which had a preference for familiar location. These results indicate that treatment tank exposure induced a different performance in object recognition and object location memory due to stress responses. Therefore, these tasks are prone to evaluate memory in physiological and pathological conditions, but its use is limited due to sensitivity to stress caused by manipulation.

1. Introduction

Zebrafish can be used to model complex human behavioral traits, such as reward responsiveness, learning and memory, aggression, anxiety, and sleep (Norton and Bally-Cuif, 2010). Several tasks used in the zebrafish explicitly target memorial processes, for example maze learning, which is related to the context of spatial discrimination learning and certainly relies on memory (Braida, Ponzoni, Martucci, Sparatore, 2014; Cognato et al., 2012; Grossman et al., 2010). Different tasks of avoidance learning have been established in zebrafish (Blank et al., 2009). In these tests, avoidance learning is inferred by the amount of time spent outside the compartment previously associated with an aversive stimulus. This passive avoidance learning is frequently used to characterize associative learning and short- and long-term

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memory in zebrafish and has been used to describe the effects of antipsychotics (Seibt et al., 2011), scopolamine (Kim, Lee, Kim, Jung, & Lee, 2010; Richetti et al., 2011), MK-801 (Ng et al., 2012; Seibt et al., 2011) and adenosine agonists and antagonists (Bortolotto, Melo, Cognato Gde, Vianna, & Bonan, 2015). Previous studies demonstrated that the telencephalon (with subdivisions homologous to the hippocampus and mammalian amygdala) is the area responsible for learning and memory in teleosteal fish (Portavella, Vargas, Torres, & Salas, 2002).

Among the tests used to evaluate amnesia models, there is the task of novel object recognition (NOR) (Ennaceur, 2010). The NOR is very useful for studying both short-term and long-term memory. By simply manipulating the retention interval, which is the amount of time between training and test sessions, it is possible to evaluate either type of memory (Taglialatela, Hogan, Zhang, & Dineley, 2009). The greatest advantage conferred by the test is the fact that it does not require external motivation, reward or punishment, but habituation is necessary, and can be completed in a relatively short period of time (Silvers, Harrod, Mactutus, & Booze, 2007). NOR was previously studied in zebrafish, where the ability to discriminate different objects and the object recognition memory was evaluated. Previous studies tested the effects of nicotine and phenylbutyrate (a histone acetylation inhibitor) in NOR memory in zebrafish, demonstrating that these drugs significantly modify the innate object preference (Braida, Ponzoni, Martucci, & Sala, 2014; Faillace, Pisera-Fuster, Medrano, Bejarano, & Bernbeu, 2017; May et al., 2016). In addition, conditions, such as total sleep deprivation, are able to impair the learning in an active avoidance conditioning paradigm (Pinheiro-da-Silva, Silva, Nogueira, & Luchiari, 2017).

Changes may be made in the object recognition test to assess the animal ability to recognize the specific position of objects within an arena. The object location memory is an important aspect of spatial memory, allowing us to remember the position of objects in our environment. Different cognitive processes are involved in the recall object and the position it finds (Moscovitch et al., 1995).

Glutamate is the major excitatory neurotransmitter of the central nervous system and is involved in many basic neuronal functions and in processes of the central nervous system, especially in learning, memory and synaptic plasticity (Tarabeux et al., 2011), acting pre- and postsynaptically by the activation of glutamate receptors. NMDA receptors are required for synaptic plasticity, learning and memory formation (Tsai, 2016). The non-competitive NMDA receptor antagonist MK-801 is used almost exclusively as an experimental drug to study behavioral processes mediated by the glutamatergic pathway. Memory deficits were demonstrated in zebrafish treated with MK-801 in the Y-maze task (Cognato et al., 2012). Avoidance tasks have been used to measure cognitive deficits associated with MK-801 administration in zebrafish (Blank et al., 2009; Seibt et al., 2011).

This study aims to establish a protocol for evaluating the memory of object recognition and object location in zebrafish. We also evaluated the influence of NMDA receptor antagonism, testing the effect of MK-801 on the object recognition or object localization memory in zebrafish and the impact of changes in habituation protocols on cortisol levels of animals.

2. Materials and methods

2.1. Animals

Adults (6–7 months) wild-type zebrafish (*Danio rerio*) were used. Animals were obtained from our breeding colony and kept in automated re-circulating systems (Zebtec, Tecniplast, Italy) with reverse osmosis filtered water equilibrated to reach the species recommended temperature (28 °C ± 2 °C), pH (7.0 and 7.5), conductivity and ammonia, nitrite, nitrate and chloride levels. Animals were subjected to a light/dark cycle of 14/10 h, respectively. Animals were fed with paramecium between 6 and 14 days post fertilization (dpf) of age and received commercial flakes (TetraMin Tropical Flake Fish®) three times a day supplemented with brine shrimp (Westerfield, 2000) after 14 dpf. Fish were transferred from one tank to another using a fish net. All protocols were approved by the Institutional Animal Care Committee from Pontifícia Universidade Católica do Rio Grande do Sul (CEUA-PUCRS, protocol number 16/7574).

2.2. Innate color preference

The objects used were simple 3D (cube) geometric shapes made of non-toxic plastic with ≈2 cm in size in blue and yellow colors that did not show high preference in previous studies (Faillace et al., 2017).

The experimental apparatus consisted of a 10 L - glass tank (35 cm × 35 cm × 10 cm, length, wide and height, respectively) filled with 6 cm of water. The sides of the apparatus were covered externally by black plastic (to reduce external interference). The animal was exposed to two objects with different colors (blue vs. yellow) for 20 min. The preference was evaluated over periods of 0–5 min, 0–10 min, 0–15 min, and 0–20 min to determine the time to be used for the object recognition task. We evaluated the exploration time of each object, which was defined as the time each animal remained in the area defined as 8 × 8 cm centered on the object.

2.3. Novel object recognition task

The experimental apparatus and objects used were previously mentioned in the Section 2.2 of the experiment for which the animals showed no innate preference (yellow and blue cubes). Before training, each animal was habituated to experimental apparatus in the absence of objects for 5 min twice a day (5-h interval between habituation sessions) over three consecutive days. On the fourth day, in the training phase, animals were exposed to two identical cubes (with the same color) for 15 min. After the training, the animals were submitted to a retention interval of 1 h. In the test, a new object (with different color) replaced one of the copies of the familiar object and the exploration time of each object was evaluated for 15 min. To avoid thigmotaxis influence, the distances between the objects and the walls were kept the same. Moreover, respecting the aforementioned distance to the walls, object locations in the tank were random to avoid the influence of external factors and the position of the familiar and new object were counterbalanced. We evaluated the exploration time of each object (%). The exploration area was defined as 8 × 8 cm area centered on the object and preference percentages were calculated as: [time of exploration of novel object/time of exploration of familiar object + time of exploration of novel object × 100].

2.3.1. Principal Component Analysis (PCA)

The Principal Component Analysis (PCA) was used to classify different fish regarding novel object recognition performance in a multivariate ordination (Chen et al., 2016). In brief, PCA is a technique that makes clusters of samples according to similarity, based on several variables simultaneously. Essentially, samples grouped on the plot are quite similar to each other. Point clusters in different regions of the graph indicate that the samples are different in the analyzed variables. Each variable has a vector (line). The values of the variable increase in the direction of the vector: if the vector points far to the right, samples to the right have larger values of the vector variable, while the samples to the left have smaller values of the same variable represented by the vector. This allows to observe if the variables (vectors = rows) are correlated (both pointing to the same direction), independent (90°angle between them) or inversely correlated (each pointing to a different side of the graph). The following parameters were considered for training and testing characteristics: % of time exploring the new object, total time exploring both objects in training, total time exploring both objects in test, time in zone A in training, time in zone B in training,

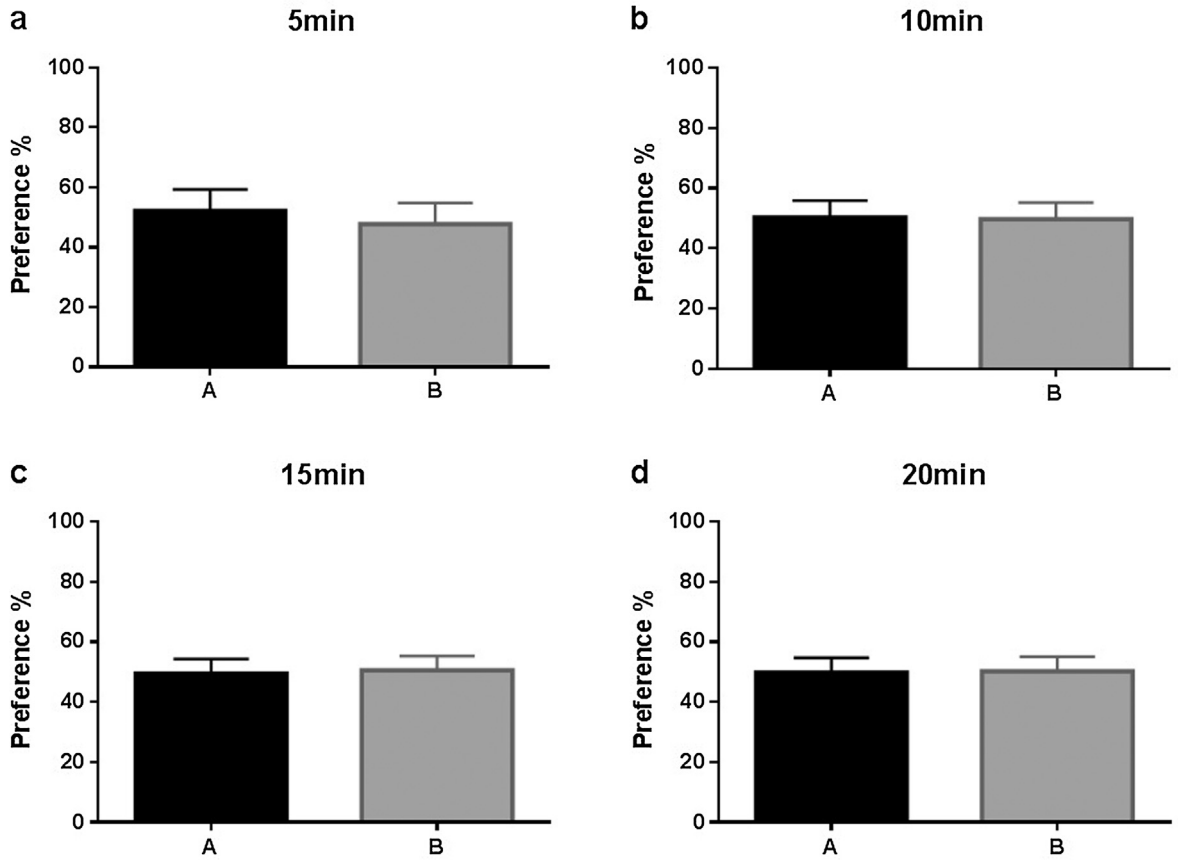

Fig. 1. Innate color preference. The exploration time of each object (%) was analyzed and the preference was evaluated in periods of 0–5 min (a), 0–10 min (b), 0–15 min (c) and 0–20 min (d). A and B represent the two objects tested (blue or yellow cubes). The data are expressed as the mean ± S.E.M (n = 18), and were analyzed by Student' s *t*-test.

latency to zone B in training, mobile time in training, mobile time in test, and latency to zone B in test. The locomotor parameters were analyzed using Noldus EthoVision XT7 software. Fish were considered immobile when less than 5% of their body area changed. The data were analyzed by a Principal Component Analysis (PCA).

2.4. Innate cue preference

For the object location task (Section 2.5), spatial cues were used and the animal is aware of the location of the object within the apparatus. To evaluate the innate preference for spatial cues, visual cues made of white paper cut (2 × 2 cm) in different geometric forms were placed on the external maze walls. Combinations of different shapes (square, circle, triangle, and diamond) were used in different sectors. The experimental apparatus (a 10 L-tank glass; 35 cm × 35 cm × 10 cm) was externally covered in black and the spatial cues attached to the outside of the apparatus having the same color and size, as performed in previous studies (Braida, Ponzoni, Martucci, & Sala, 2014; Cognato et al., 2012), where the preference for forms was not observed. The tank was divided virtually in two sectors, with different geometric shapes as spatial indications. The preference for the sectors was evaluated in the periods of 0–5 min, 0–10 min, 0–15 min and 0–20 min to determine the time to be used for the object location task. The exploration time of each sector (%) was evaluated.

2.5. Object location task

The animals were tested in the same experimental apparatus used for the analysis of the cue innate preference. The objects were previously selected in the Section 2.2, in which the animals showed no innate preference. The sides of the apparatus were covered externally by black plastic containing the spatial cues selected according Section 2.4. Before training, each zebrafish was habituated to experimental apparatus in the absence of objects for 5 min twice a day (5-h interval between habituation sessions) over three consecutive days. On the fourth day, in the training phase, animals were exposed to two identical cubes for 15 min in the same sector (initial location). After the training, the animals were submitted to a retention interval of 1 h. In the test, one object remained in the initial location and another was moved to a novel location and evaluated for 15 min. To avoid thigmotaxis influence, the distances between the objects and the walls were kept the same. Moreover, respecting the aforementioned distance to the walls, object locations in the tank were random to avoid the influence of external factors and the position of the familiar and new object were counterbalanced. We evaluated the exploration time of each object (%).

2.6. MK-801 or water treatment

All groups were previously submitted to habituation protocol, which consists in the exposure to the treatment tank (600 mL Becker) for 15 min and, subsequently, to experimental apparatus in the absence of objects for 5 min twice daily (5-h interval between habituation sessions) over three consecutive days. In the fourth day, the animals were exposed to 5 or 10 μM MK-801 (Cognato et al., 2012) or only water for control group. Treatments were administered by immersing the fish in a treatment tank filled with 400 mL of solution (MK-801) for 15 min prior to the object recognition or location task.

In the treatment tank, water parameters were kept the same as those from the housing tanks. Moreover, to avoid chemical signaling from one fish to another, water was completely changed and the tank was

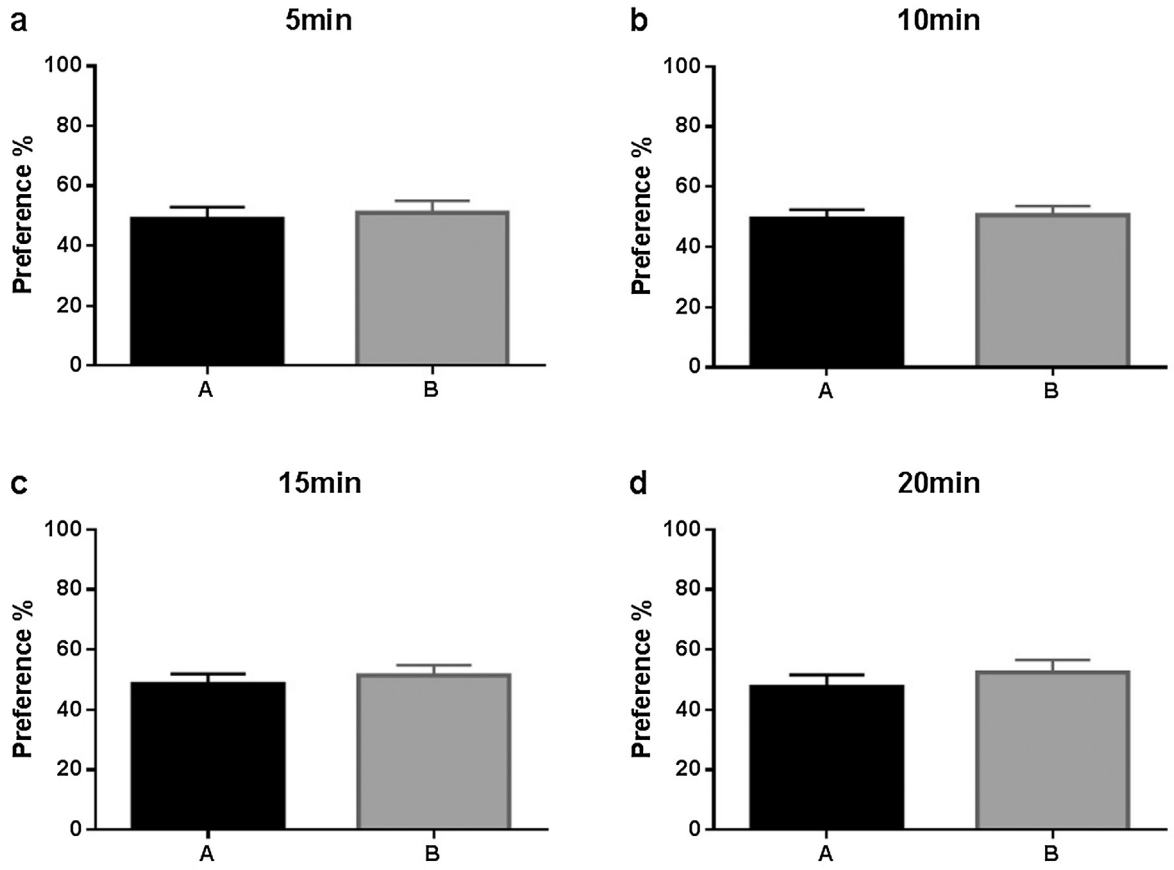

Fig. 2. Innate cue preference. The exploration time of each object (%) was analyzed and the preference was evaluated in periods of 0–5 min (a), 0–10 min (b), 0–15 min (c) and 0–20 min (d). To evaluate the innate preference for spatial cues, visual cues made of white paper cut (2 × 2 cm) in different geometric forms (circle or diamond) were placed on the external maze walls. A and B represent the two sectors tested (circle or diamond cues). The data are expressed as the mean ± S.E.M (n = 15), and were analyzed by Student' s *t*-test.

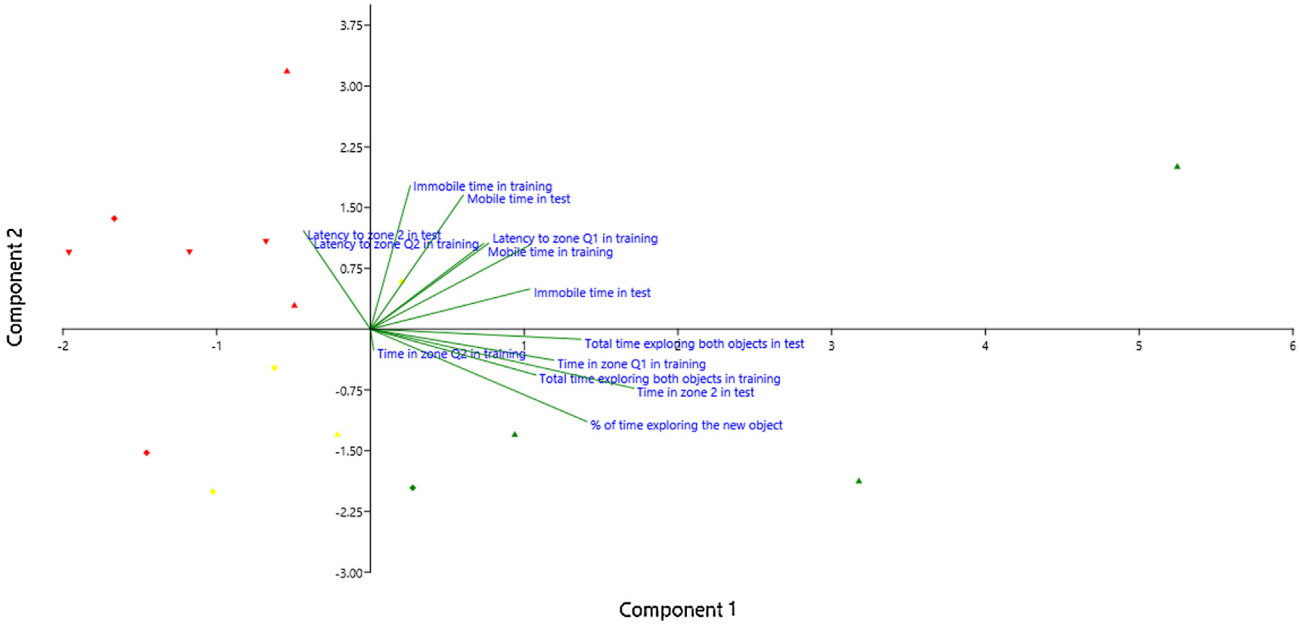

Fig. 3. Principal Component Analysis (PCA).The Principal Component Analysis (PCA) was used to classify different fish regarding novel object recognition performance in a multivariate ordination. Symbol and color coded: Green = % exploration of the new object above 70%; Yellow = % of exploration of the new object above 40–69%; Red = % exploration of the new object below 39%; Triangle = high time of exploration of objects during training; Diamond = intermediate time of exploration of objects during training; Inverted triangle = low time of exploration of objects during training. The data were analyzed by a Principal Component Analysis (PCA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

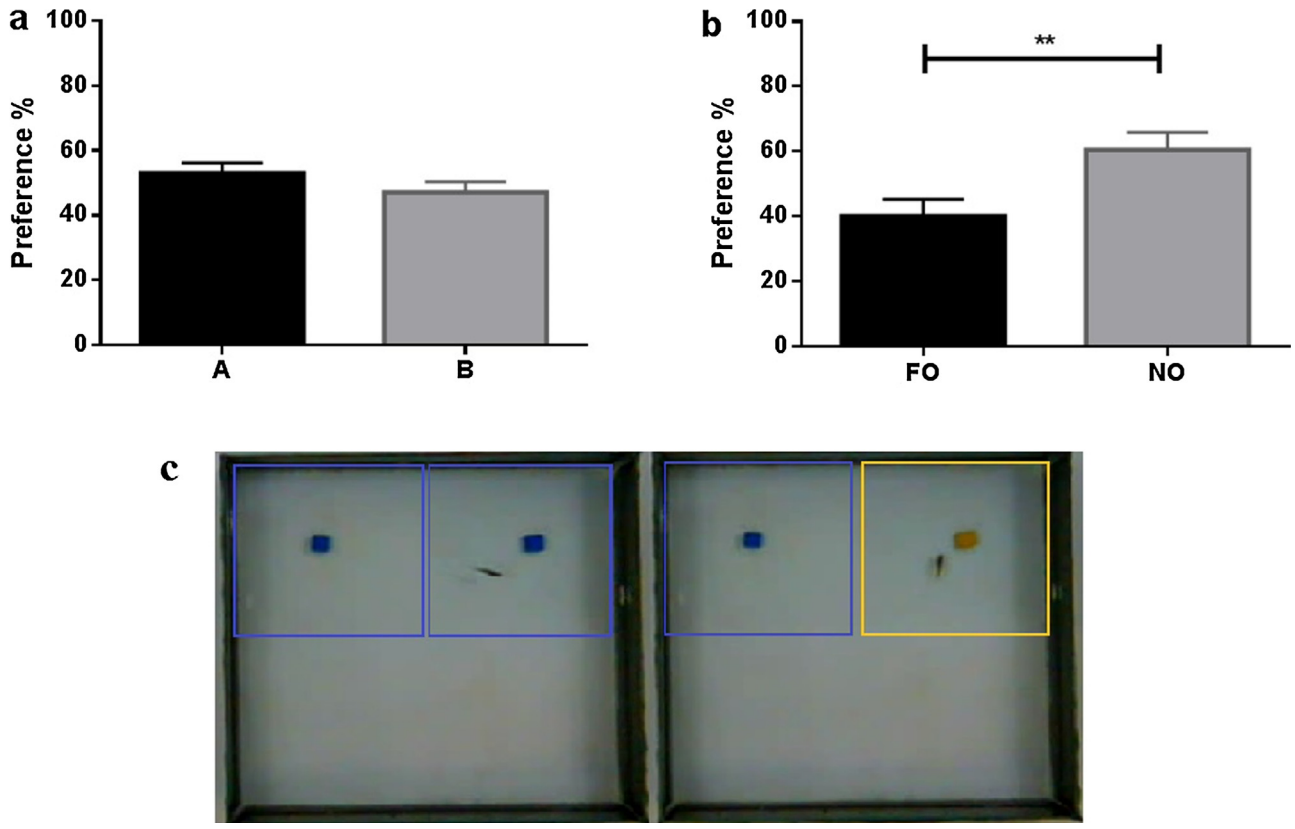

Fig. 4. Object recognition task. The exploration time of each object (%) was analyzed during training between two identical objects A and B (a) and between the new object (NO) and the familiar object (FO) and in the test session (b). The image (c) shows the task during training (left) and test (right), with exploration areas indicated. The data are expressed as the mean ± S.E.M (n = 22), and were analyzed by Student's *t*-test, ** represents significant difference at $p \leq 0.01$.

washed for each fish treated.

2.7. Cortisol levels

Each fish was weighed, minced, and placed into a disposable stomacher bag with 2 mL of phosphate buffered saline (PBS, pH 7.4) for 6 min. The contents were then transferred to a 10-mL screw top disposable test tube to which 5 mL of laboratory grade ethyl ether was added. The tube was vortexed for 1 min, which was immediately frozen in liquid nitrogen. The unfrozen portion (ethyl ether containing cortisol) was decanted and transferred to a new tube, where it was completely evaporated under a gentle stream of nitrogen for 2 h, yielding a lipid extract containing the cortisol, which was stored at −20 °C. Body extracts were re-suspended in 200 µL of phosphate buffered saline (PBS) and whole-body cortisol levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (EIAgen™CORTISOL test, BioChem ImmunoSystems). Whole-body cortisol was extracted using the method described by Sink, Lochmann, and Fecteau (2008).

2.8. Influence of habituation protocols

The control animals were submitted to three different habituation protocols named Condition 1, 2 or 3 (C1, C2, and C3, respectively) to evaluate the effect of placing the fish in the treatment tank just prior to experimentation as well as the repeated habituation of the fish to the treatment tank on cortisol levels of animals in situations similar to those tested previously. In the Condition 1 (C1), the animals were habituated individually, exploring experimental apparatus without objects for 5 min twice a day (5 h-interval between sessions) for 3 consecutive days and, on the fourth day, fish were removed from home tank aquaria, cryoeuthanized and samples were collected. In Condition 2 (C2), the animals were habituated individually, exploring the experimental apparatus without objects for 5 min twice daily (5 h-interval between sessions) for 3 consecutive days; in the fourth day, they were exposed to the treatment tank with water for 15 min and, immediately after, samples were collected. Therefore, this condition introduced the presence of the treatment tank on day 4. In Condition 3 (C3), the animals were habituated individually; first to the treatment tank for 15 min and immediately after to the experiment apparatus without objects for 5 min twice daily (5 h-interval between sessions) for 3 days consecutive. In the fourth day, they were exposed to the treatment tank with water for 15 min and immediately after samples were collected. Therefore, this condition introduced the habituation to the treatment tank on days 1–3 and like in C2 placed the fish in the treatment tank on day 4.

2.8.1. C1 plus stress

To evaluate if adding a stressor in C1 condition changes the preference of objects and cortisol levels, the control group were habituated individually, exploring experimental apparatus without objects for 5 min twice a day (5 h-interval between sessions) for 3 consecutive days and, on the fourth day, fish were removed from home tank aquaria. One group of animals was cryoeuthanized and samples were collected to evaluate cortisol levels and another group of animals was submitted to novel object recognition or object location tasks. The stressed group was habituated individually, exploring experimental apparatus without objects for 5 min twice a day (5 h-interval between sessions) for 3 consecutive days and, on the fourth day, fish were then submitted to a stress stimulus, consisting of chasing fish with a net for two minutes. After, fish were removed from home tank aquaria. One group of animals was cryoeuthanized and samples were collected to evaluate cortisol levels and the other group of animals was submitted to novel object recognition or object location tasks.

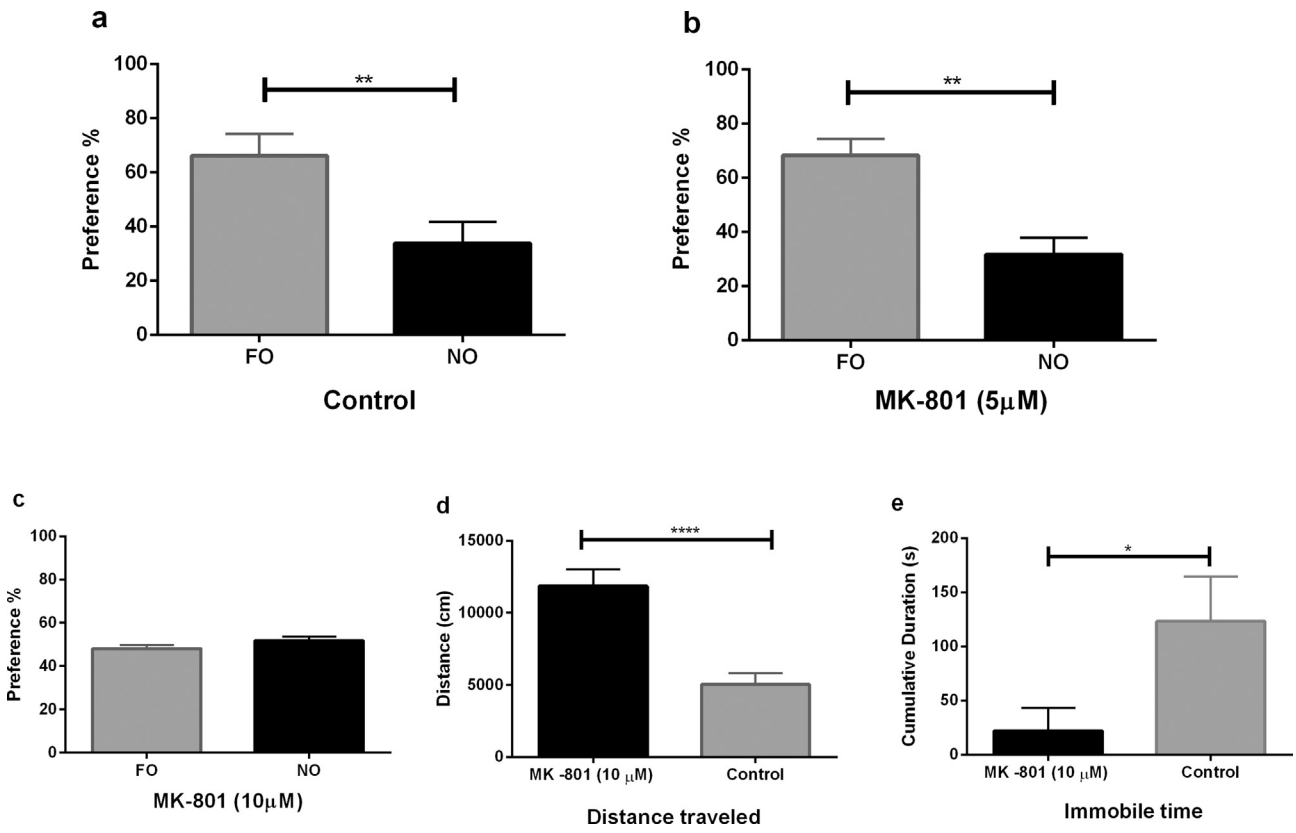

Fig. 5. Effects of MK-801 on object recognition memory. The exploration time of each object (%) was analyzed in the test session between new object (NO) and familiar object (FO). The animals were exposed to water for controls group (a), 5 (b) or 10 μM (c) MK-801. The group treated with 10 μM MK-801 showed an increase in distance traveled (d) and a decrease in the immobile time (e). The data are expressed as the mean ± S.E.M (n = 12 per group), and were analyzed by Student's *t*-test, ** represents significant difference at $p \leq 0.01$ and **** $p \leq 0.0001$.

2.8.2. C2 plus acute fluoxetine exposure

To evaluate whether the use of fluoxetine in C2 condition may result in changes in the preference of objects and in cortisol levels, the animals were habituated individually, exploring the experimental apparatus without objects for 5 min twice daily (5 h-interval between sessions) for 3 consecutive days; in the fourth day, they were exposed to the treatment tank with water or fluoxetine 50 μg/L for 15 min. This dose of fluoxetine was chosen based on previous zebrafish studies where a clear decrease in stress response was observed (Abreu, Koakoski, Ferreira, Oliveira, & Rosa, 2014; Giacomini et al., 2016). After, fish were removed from home tank aquaria. One group of animals was cryoeuthanized and samples were collected to evaluate cortisol levels and another group of animals was submitted to novel object recognition or object location tasks.

2.8.3. C3 plus acute fluoxetine exposure

To evaluate whether the use of fluoxetine in Condition 3 may result in changes in the preference of objects and in cortisol levels, the animals were habituated individually, first to the treatment tank for 15 min and immediately after to the experiment apparatus without objects for 5 min twice daily (5 h-interval between sessions) for 3 days consecutive. In the fourth day, they were exposed to the treatment tank with water or 50 μg/L fluoxetine for 15 min. After, fish were removed from home tank aquaria. One group of animals was cryoeuthanized and samples were collected to evaluate cortisol levels and another group of animals was submitted to novel object recognition or object location tasks.

2.9. Statistical analysis

All trials were video recorded and the videos were analyzed using the Noldus EthoVision XT7 software. The normality of the data was analyzed by D'Agostino's K-squared test and over 95% of them were normal, indicating the use of parametric statistics. The behavioral analysis were evaluated by Student' s *t*-test and whole body cortisol levels were analyzed by one-way ANOVA followed by Tukey post-hoc test, using the Graphpad software, where $p < 0.05$ indicates significant difference.

3. Results

3.1. Innate color preference

The exploration time of each object (%) was analyzed and the preference was evaluated in different time periods (Fig. 1). The results showed that animals had no preference between objects (blue cube and yellow cube) during periods of 0–5 min ($p = 0.6630$; Fig. 1a), 0–10 min ($p = 0.9337$; Fig. 1b), 0–15 min ($p = 0.8812$; Fig. 1c) and 0–20 min ($p = 0.9534$; Fig. 1d). As there was no preference between the objects, they were used for the subsequent experiments. For the object recognition task, the evaluation time of 15 min was chosen in order to ensure a time enough to explore the both objects.

3.2. Innate cue preference

The exploration time of each sector (%), containing different spatial cues was analyzed and the preference was evaluated in different time periods (Fig. 2). The results did not show preference between the spatial cues circle and diamond during the 0–5 min ($p = 0.7164$; Fig. 2a), 0–10 min ($p = 0.7691$; Fig. 2b), 0–15 min ($p = 0.5504$; Fig. 2c) and 0–20 min ($p = 0.3950$; Fig. 2d). As there was no preference among these spatial cues, they were used for the object localization task. For the object location task, the evaluation time of 15 min was determined

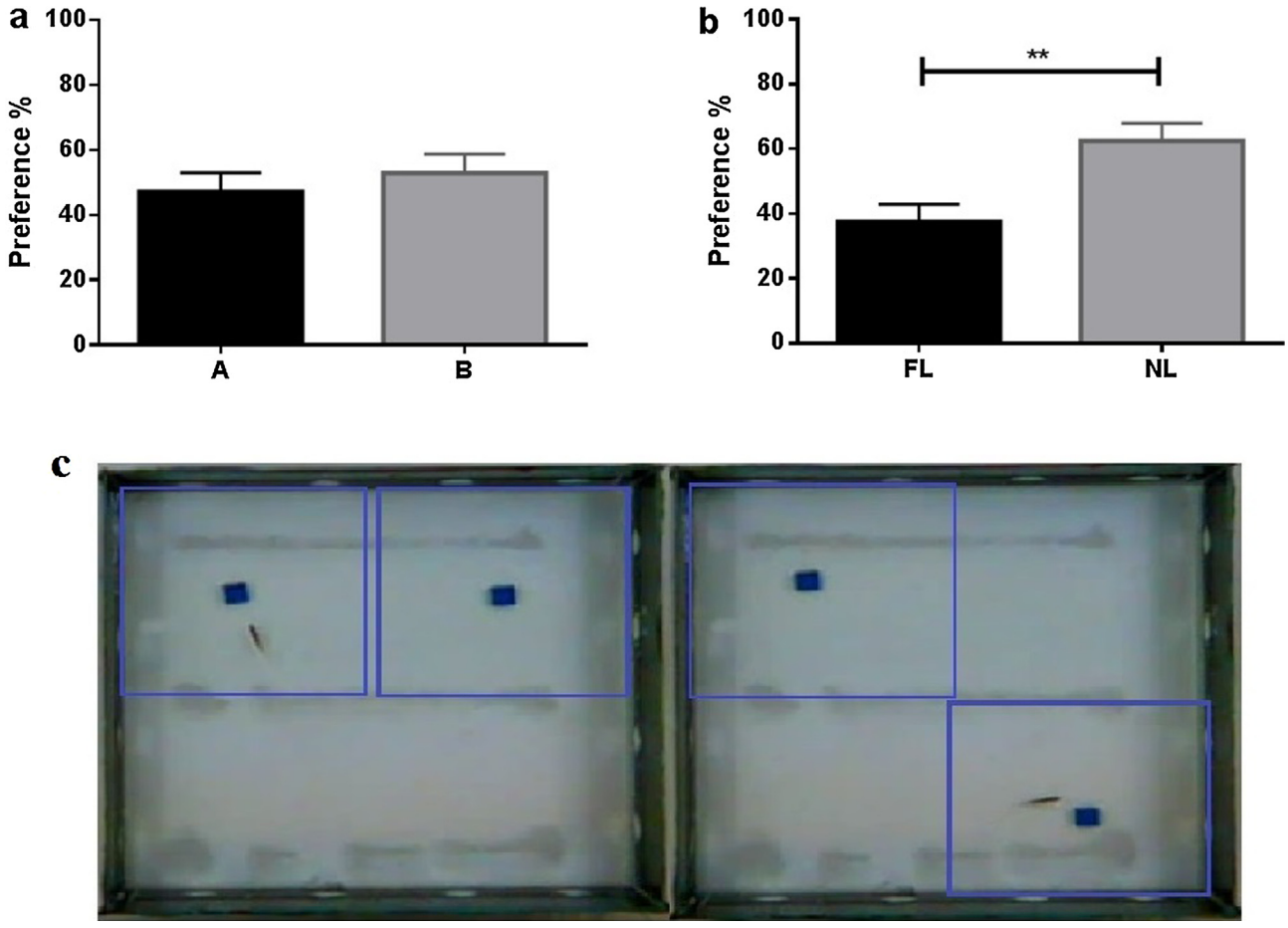

Fig. 6. Object location task. The exploration time of each object (%) was analyzed during training between A and B objects in the initial location (a) and between the object in the novel location (NL) and in the familiar location (FL) in the test session (b). The image (c) shows the task during training (left) and test (right). The data are expressed as the mean ± S.E.M (n = 21), and were analyzed by Student' s *t*-test, ** represents significant difference at $p \leq 0.01$.

chosen in order to ensure a time enough to explore the both objects.

3.3. Object recognition task

A PCA analysis was performed to evaluate the factors in the training session that would be determinant for the performance of the animal during the test session, creating a correlation. Many inferences can be made from PCA (Fig. 3). If we consider the vector "% of time exploring the new object", this is the most important vector, because it determines whether the animals learned or not the task. In addition, it may be related with the other vectors that have positive values in X-axis and negative Y-axis (i.e. located in the same quadrant). Thus, it is possible to observe a correlation of the following parameters: time in zone 2 in the test, total time exploring both objects in training, total time exploring each zone (Q1 and Q2) in the training and the total time exploring both objects in the test. Based on these results, a filter was determined for the object recognition task, where only the animals that explored both objects during the training in a total time equal to or exceeding 400 s were considered.

Fig. 4 shows the exploration time of each object (%) in the object recognition task. In the training, no preference was observed between objects A and B ($p = 0.3340$; Fig. 4a). In the test session, we observed a preference of the animal to explore the new object in comparison to the familiar object ($p < 0.01$; Fig. 4b).

3.3.1. Effects of MK-801 on object recognition memory

Fig. 5 shows that control animals (Fig. 5a) had a preference in exploring the familiar object rather than the new object ($p < 0.01$). Similarly, the animals treated with 5 µM MK-801 group (Fig. 5b) also presented a significant preference in exploring the familiar object in comparison to the new object ($p < 0.01$). When the 10 µM MK-801-treated group (Fig. 5c) was evaluated, there was no difference in the exploration of the objects ($p = 0.1639$); however, 10 µM MK-801-treated group showed increase in distance traveled ($p < 0.0001$; Fig. 5d) and a decrease in the immobile time ($p < 0.05$; Fig. 5e), which was not observed 5 µM MK-801- treated animals (data not shown).

3.4. Object location task

We evaluated the exploration time of each object (%) in the object localization task. In the training session, no preference was observed between objects A and B ($p = 0.5096$; Fig. 6a). In the test session, we observed a preference of the animal to explore the object in the new location in comparison to the familiar location during the test ($p < 0.01$; Fig. 6b).

3.4.1. Effects of MK-801 on object location memory

Fig. 7 shows that the control group (Fig. 7a) had a preference in exploring the object in the familiar location rather than in the new location ($p < 0.01$). However, the animals treated with 5 or 10 µM MK-801 (Fig. 7b and 7c, respectively) did not show a significant preference ($p = 0.2397$ and $p = 0.6599$, respectively). However, there is an increase in distance traveled ($p < 0.001$) for 10 µM MK-801-treated group (Fig. 7d), which was not observed for 5 µM MK-801-treated animals (data not shown). The immobile time was not altered for 5 µM (data not shown) and 10 µM MK-801-treated animals (Fig. 7e).

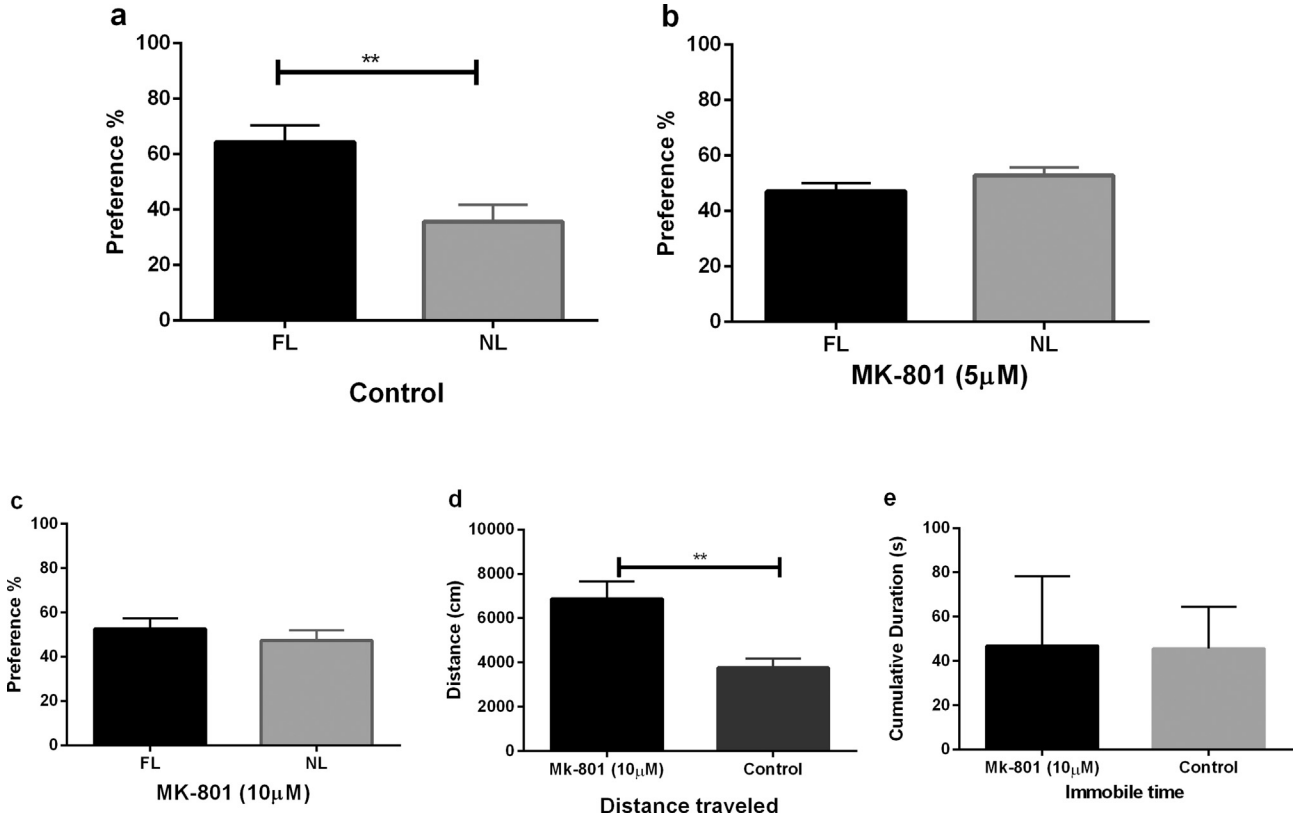

Fig. 7. Effects of MK-801 on object location task. The exploration time of each object (%) was analyzed in the test session between object in the new location (NL) and in the familiar location (FL). The animals were exposed to either water for the control group (a) or to 5 (b) or (c) 10 μM MK-801. The group treated with 10 μM MK-801 showed an increase in distance traveled (d) and no changes in immobile time (e). The data are expressed as the mean ± S.E.M (n = 12 per group), and were analyzed by Student's *t*-test, ** represents significant difference at $p \leq 0.01$.

3.5. Influence of habituation protocols

3.5.1. Cortisol levels

Considering that the control group had a preference for the novel object or location and, after exposure to treatment tank, showed opposite results, in which developed preference for the familiar object or location, we evaluated the impact of habituation on cortisol levels of animals. The results showed higher levels of cortisol in animals submitted to C2 and C3 conditions compared to animals submitted to C1 conditions ($p < 0.001$; Fig. 8a).

We evaluated the impact of adding a stress stimulus on cortisol levels of animals submitted to C1 condition. The results showed higher levels of cortisol in animals submitted to C1 plus stress compared to animals submitted only to C1 condition ($p < 0.05$; Fig. 8b). The effect of acute fluoxetine exposure also was investigated on cortisol levels of animals submitted to C2 condition, demonstrating lower levels of cortisol in animals treated with fluoxetine in C2 condition ($p < 0.05$; Fig. 8b). Similarly, the results also showed lower levels of cortisol in animals exposed to fluoxetine in C3 conditions ($p < 0.001$; Fig. 8b).

3.5.2. Influence on the novel object recognition memory

3.5.2.1. C1 plus stress. Fig. 9 shows the exploration time of each object (%) in the object recognition task. The control and stressed groups, in the training, did not have a preference between objects A and B (data not shown). In the test session, we observed a preference of the control group to explore the new object in comparison to the familiar object ($p < 0.01$; Fig. 9a). In the test, the stressed group did not show a significant preference ($p = 0.6599$; Fig. 9a).

3.5.2.2. C2 plus acute fluoxetine exposure. In the object recognition task, the control and fluoxetine groups did not have a preference between objects A and B in the training (data not shown). In the test session, the control group showed a preference for the familiar object ($p < 0.05$; Fig. 9b) whereas no preference was observed between the objects in the fluoxetine group ($p = 0.2026$; Fig. 9b).

3.5.2.3. C3 plus acute fluoxetine exposure. In the object recognition task, the control group and fluoxetine groups did not have a preference between objects A and B in the training (data not shown). In the test session, control group had a preference in exploring the familiar object ($p < 0.01$; Fig. 9c). In the test, there was no preference between the objects in fluoxetine group ($p = 0.0881$; Fig. 9c).

3.5.3. Influence on the object location memory

3.5.3.1. C1 plus stress. We evaluated the exploration time of each object (%) in the object localization task. The control and stressed groups, in the training, did not have a preference between objects A and B (data not shown). In the test session, we observed a preference of the control group to explore the object in the new location in comparison to the familiar location ($p < 0.05$; Fig. 10a). However, in the test session, the stressed group did not show a significant preference between locations ($p = 0.6197$; Fig. 10a).

3.5.3.2. C2 plus acute fluoxetine exposure. The control and fluoxetine groups did not have a preference between objects A and B in the training (data not shown). In the test session, the control group had a preference in exploring the object in the familiar location ($p < 0.05$; Fig. 10b). However, the fluoxetine group demonstrated a preference to explore the object in the new location in comparison to the familiar location during the test session ($p < 0.01$; Fig. 10b).

3.5.3.3. C3 plus acute fluoxetine exposure. The control group and

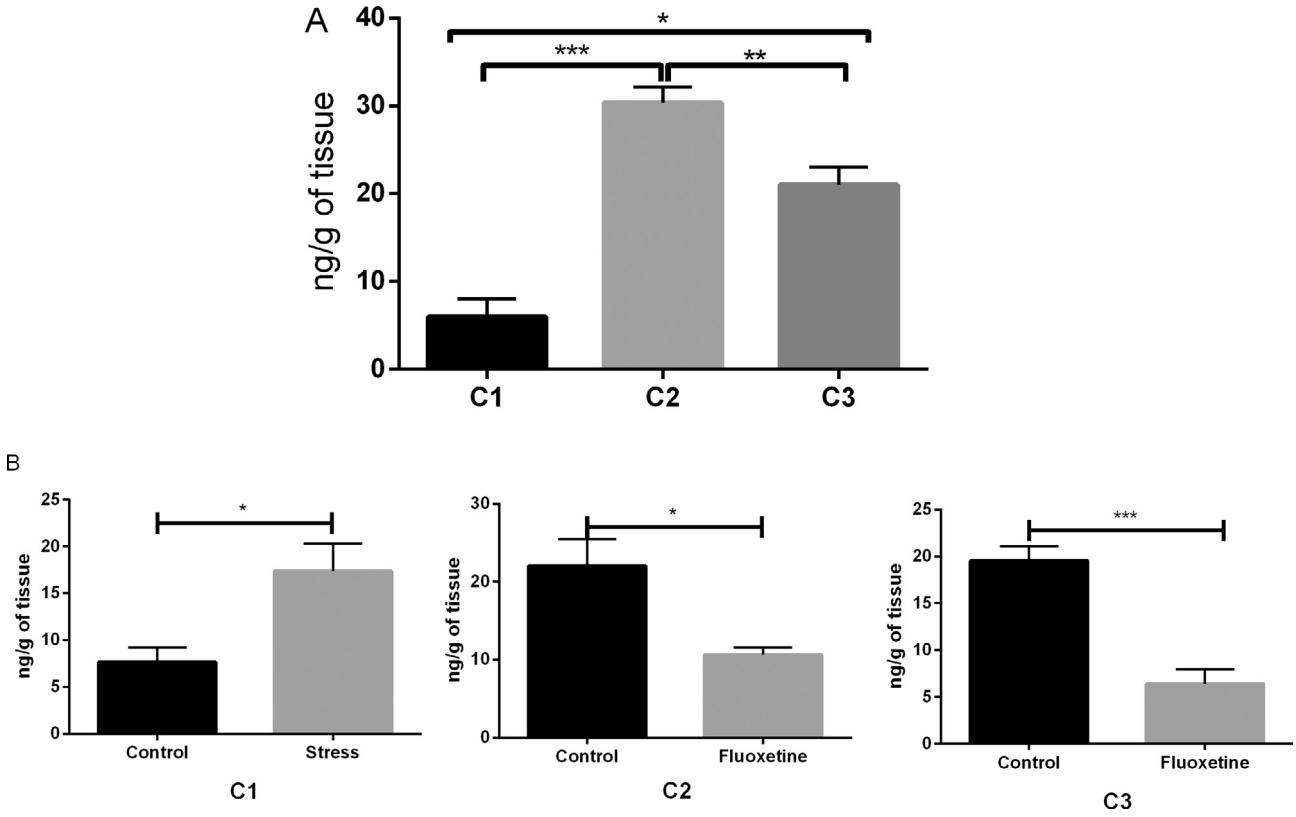

Fig. 8. Influence of habituation protocols on whole-body cortisol levels. The animals were submitted to three different habituation protocols named Condition (C1, C2, and C3). (A) Cortisol levels in C1, C2 and C3 condition in the control group. The data are expressed as the mean ± S.E.M (N = 3 per group) and were analyzed by One-way ANOVA, followed by a post-hoc Tukeýs test. (B) Influence of acute stress in C1 condition and exposure to fluoxetine on C2 and C3 conditions on cortisol levels. The data are expressed as the mean ± S.E.M (N = 5 per group) and were analyzed by Student' s *t*-test. * represents significant difference at $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

fluoxetine groups did not have a preference between objects A and B in the training (data not shown). In the test session, the control group had a preference in exploring the object in the familiar location ($p < 0.05$; Fig. 10c). The fluoxetine group had a preference to explore the object in the new location in comparison to the familiar location during the test session ($p < 0.01$; Fig. 10c).

4. Discussion

In this study, we evaluated the memory of object recognition and object location in zebrafish as well as the influence of habituation and NMDA antagonism in these tasks. We observed that MK-801 treatment impaired the object location memory and that occurred a change in preference pattern in object recognition and object location memory due to stress responses.

The novel object recognition task is very useful for studying short-term memory and long-term memory, manipulating the retention interval, which is the amount of time between training and testing sessions (Taglialatela et al., 2009). Firstly, we evaluated the innate preference of object colors, since the object choice is one of the most important and underappreciated aspects of conducting an object recognition or object location memory protocol (Ennaceur, 2010). Since this task relies on innate preference for novelty, all objects used for this protocol should meet the following criteria: (1) are adequately explored during a test session, (2) if used for novel object recognition, the two objects have equal innate preference and can be discriminated. Oliveira, Silveira, Chacon, and Luchiari (2015) observed that the zebrafish has the ability to discriminate between objects based on color and shape but not on size. In addition, Faillace et al. (2017) demonstrated that zebrafish was better at discriminating color changes than object shape or size and the color directly influences the preference between a new or familiar object, reinforcing the importance of evaluating innate color preference in this type of task. Peeters, et al.. (2016) also showed that the location of the color appears to be of critical importance. Presentation of the color on the walls induces an approach response whereas presentation on the bottom induces an aversion. Therefore, it is important to evaluate the preference directly for the objects to be used, since zebrafish seems to have a different perception of being in an environment of a certain color and having an object of a certain color in the same environment. This can be an explanation for controversial results in the literature considering the method of presentation of the color in the task. Since there was no preference between the objects, they were considered suitable for the tasks.

Spatial learning is the process that allows animals (humans and others) to acquire spatial cues and dynamic relationships among these cues. Therefore, spatial learning leads to the establishment of a spatial map, a neural representation of the external environment (Karnik and Gerlai, 2012). Previous studies confirmed that zebrafish has the ability to perform well in associative learning tasks (Sison and Gerlai, 2010). Braida, Ponzoni, Martucci, and Sala (2014) showed that the obtained discrimination indices are comparable to those previously found in mice submitted to the same task. For the object location task, spatial cues were used and the animal is aware of the location of the object within the apparatus. To evaluate the innate preference for spatial cues, combinations of different shapes (square, circle, triangle, and diamond) in different sectors were evaluated. The apparatus was externally covered in black and the spatial cues were attached to the outside of the apparatus having the same color and size, as performed in previous memory studies (Braida, Ponzoni, Martucci, & Sala, 2014; Cognato et al., 2012), where the preference for forms was not observed. Our

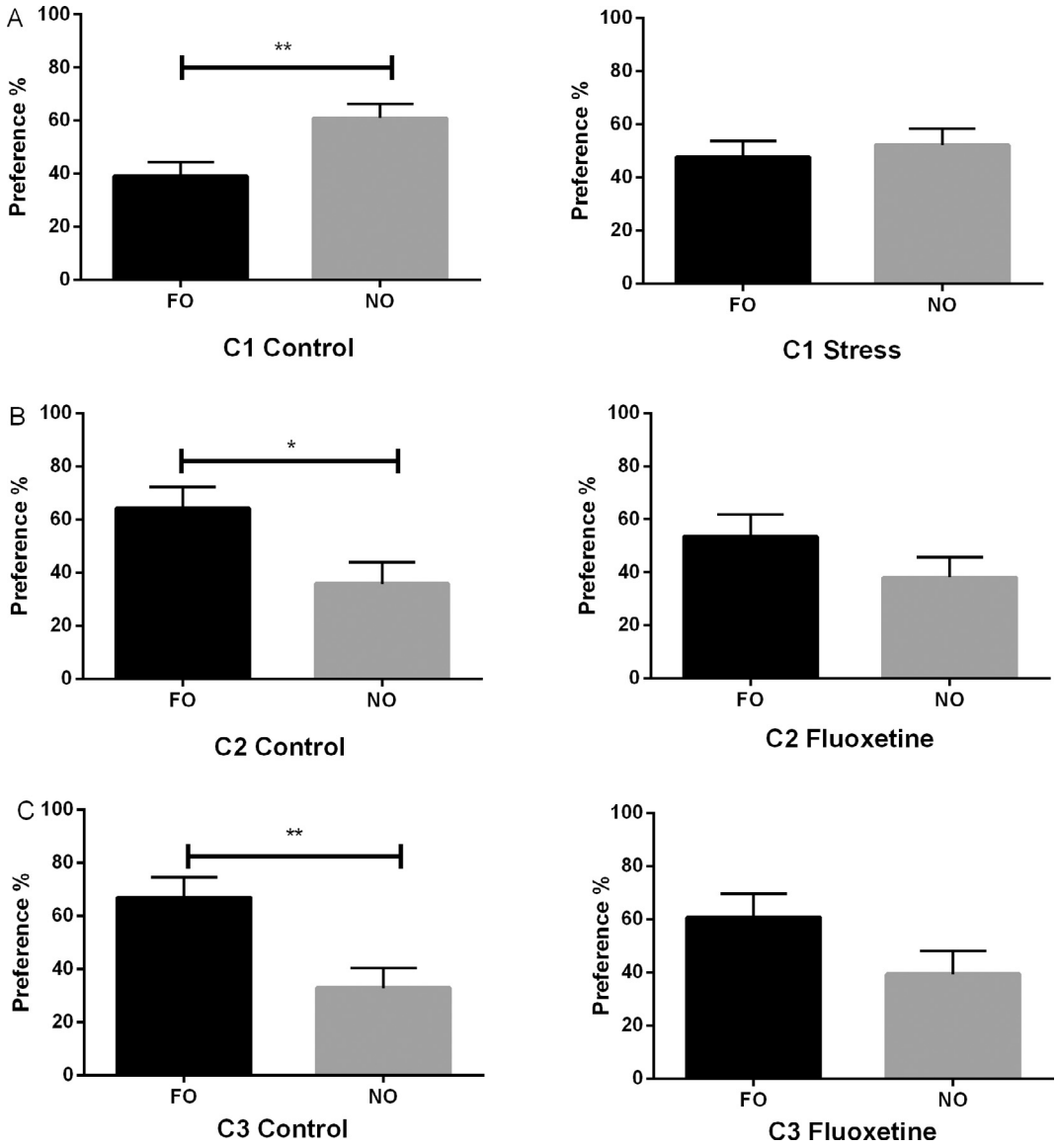

Fig. 9. Influence of habituation protocols on the novel object recognition memory. The exploration time of each object (%) was analyzed. We demonstrated the preference between the new object (NO) and the familiar object (FO) in the test session. (A) The effect of C1 condition plus stress; (B) The effect of fluoxetine in the C2 condition; (C) The effect of fluoxetine in the C3 condition. The data are expressed as the mean ± S.E.M (n = 12), and were analyzed by Student's *t*-test, * represents significant difference at $p \leq 0.05$; ** $p \leq 0.01$.

results did not show preference between the spatial cues circle and diamond and they were used for the object localization task.

The novel object recognition memory was previously studied in zebrafish, where the ability to discriminate different objects and the memory of object recognition was evaluated. May et al. (2016) showed that fish spent more time exploring the familiar object during the test, suggesting a neophobic behavior. In contrast, Braida, Ponzoni, Martucci, and Sala (2014) observed that zebrafish showed preference for new shapes when presented in a virtual object recognition test. Lucon-Xiccato and Dadda (2014) also observed that animals have a tendency to explore the new object in a modified version of the NOR test. Pinheiro-da-Silva et al. (2017) demonstrated that the control animals explored more the new object than the familiar object whereas there was no preference for the objects in sleep-deprived animals. The contrasting results may be explained by factors, such as the complexity level of the task and the objects as well and the type of habituation that would influence the performance during tasks. May et al. (2016) used objects (Lego® figures) with high complexity combining several colors and shapes and a short habituation period. Such factors may have contributed to a neophobic behavior due to the several variants presented as novelty at one trial. Studies demonstrating a neophilic behavior used simpler 2D or 3D forms, longer or repeated habituation periods and/or environmental enrichment to reduce stress (Lucon-Xiccato and Dadda, 2014). Our findings demonstrated a preference for the novel object in the object recognition memory task, which are in agreement with the previous studies using simpler forms and repeated habituation periods. However, when we tested the effect of MK-801, we observed contrasting effects with preference for the familiar object for the control and 5 μM MK-801-treated groups. There was no preference for the 10 μM MK-801-treated group possibly due to a hyperlocomotor effect caused by this drug. Previous studies have already demonstrated that 5 μM MK-801 did not alter locomotor activity whereas higher MK-801 concentrations induced an increase of locomotor parameters (Seibt et al., 2010, 2011).

For the object location task, we observed a preference of the animal to explore the new object in comparison to the familiar object during the test session. Hamilton et al. (2016) demonstrated the same exploration preference when zebrafish were exposed to familiar object in

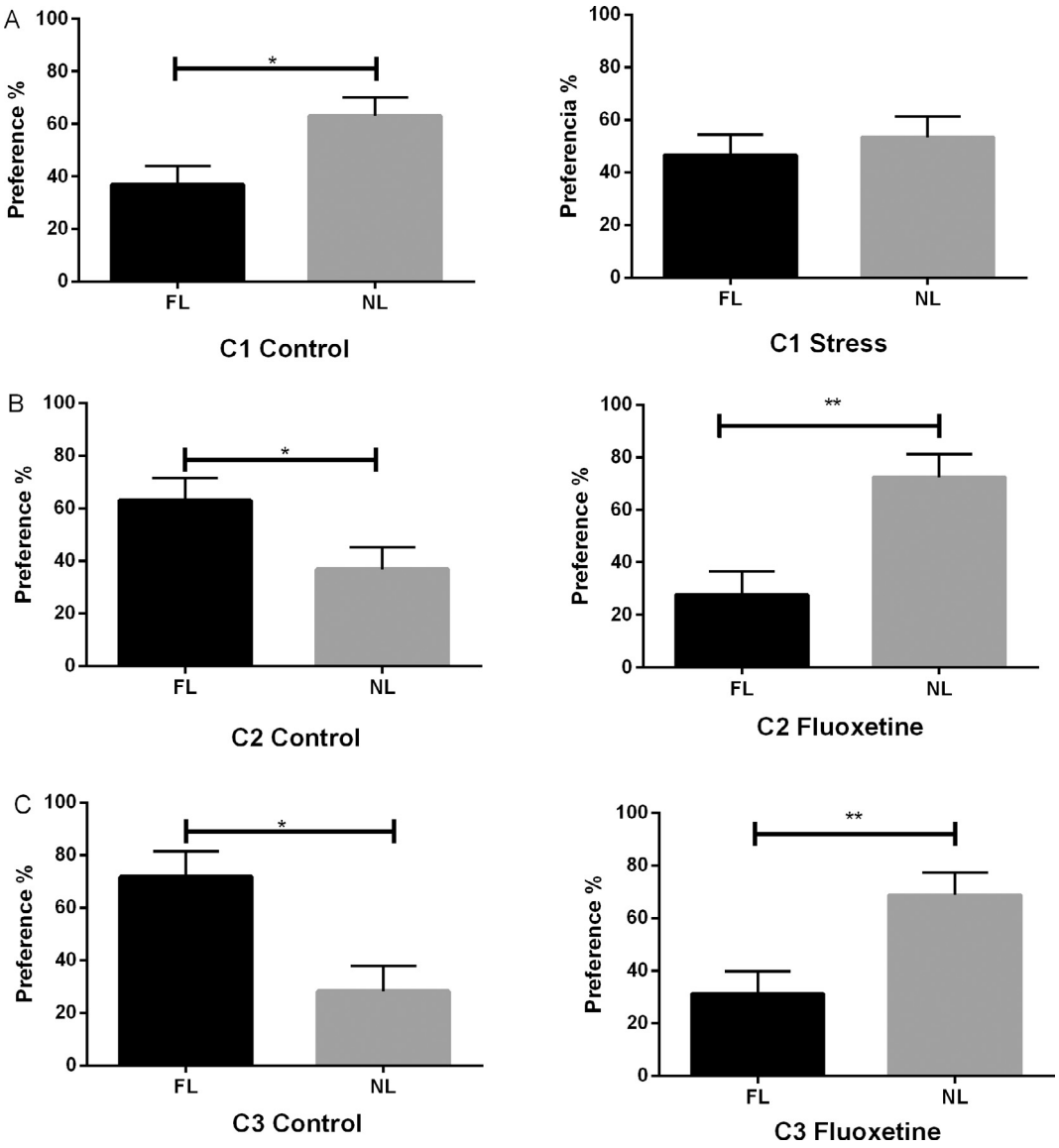

Fig. 10. Influence of habituation protocols on the object location memory. The exploration time of each object (%) was analyzed. We demonstrated the preference between the object in the novel location (NL) and in the familiar location (FL) in the test session (A) The effect of C1 condition plus stress; (B) The effect of fluoxetine in the C2 condition; (C) The effect of fluoxetine in the C3 condition. The data are expressed as the mean ± S.E.M (n = 12), and were analyzed by Student's *t*-test, * represents significant difference at $p \leq 0.05$; ** $p \leq 0.01$.

a familiar context, but at novel location within that context, they spend more time in the novel quadrant. This indicates that zebrafish are capable to display episodic-like memory as they discriminate the type, location and the context in the object was presented (Hamilton et al., 2016). However, in our study, the animals treated with the 5 or 10 μM MK-80 did not show a significant difference in the exploration of the objects, but an increase in distance traveled ($p < 0.001$) was observed in the animals treated with 10 μM MK-801. Therefore, it is possible to suggest that 5 μM MK-801 impaired the memory formation in an object location task, which is in agreement with previous studies demonstrating the cognitive deficit induced by MK-801 treatment in aversive (Seibt et al., 2011) and spatial memory (Cognato et al., 2012). The different effects observed by 5 μM MK-801 in novel object recognition and object location memory indicate that NMDA antagonism may induce distinct effects in these tasks, according to the dose tested.

Our findings also demonstrated a change of preference pattern for the objects, when a new element (treatment tank) was introduced in the protocol, since the animals spent more time exploring the familiar object or location after treatment tank exposure. Our results demonstrated a higher cortisol levels to C2 and C3 conditions, in which there was treatment tank exposure than C1 condition (no exposure to treatment tank). However, when we introduced an acute stressor during C1 condition, we observed an increase in the cortisol levels. In addition, in relation to novel object recognition and object location memory, the inclusion of an acute stressor induced an absence of preference for the objects in comparison to control group, which had a preference for novel object and novel location. Previous studies demonstrated that adrenal stress hormones, epinephrine and cortisol released by emotional arousal play an important role in enabling the significance of an experience to regulate the strength of memory of an experience (Meir Drexler and Wolf, 2017). The influence of stress on these memory tasks was confirmed, since animals submitted to fluoxetine treatment had a decrease in cortisol levels when exposed to C2 and C3 conditions. In relation to novel object recognition memory, fluoxetine treatment induced an absence of preference for the objects in C2 and C3 conditions in comparison to control group, which had a preference for familiar object. However, in the object location memory, fluoxetine treatment induced a preference for the novel location in C2 and C3 conditions in

comparison to control group, which had a preference for familiar location. Such findings reinforce the role of stress during habituation affecting the performance of the animals during object recognition and location memory tasks. Previous studies demonstrated that the use of mirrors during memory task reduces the stress promoted by social isolation in guppies (Milleto Petrazzini, Agrillo, Piffer, Dadda, & Bisazza, 2012). Regardless of stress reduction, animals showed preference to the familiar object, indicating that even with habituation there may be other factors (experimental protocols, or species differences) that lead to differences in object preferences (familiar versus novel). Leighton, Nadolski, Morrill, Hamilton, & Allison, 2018 evaluated novel object recognition and additional tests were done to evaluate in anxious behavior. Therefore, mechanisms able to minimize the stress during habituation or the use of additional tests to evaluate anxiety may be considered in the experimental design for these memory tasks. Our findings indicate that these tasks are highly sensitive to experimental manipulation, which suggest that the memory tested is labile in zebrafish. In addition, fluoxetine treatment modulates the novel object recognition and object location memory, in a different manner, which open promising perspectives to evaluate the neurochemical pathways involved in these memory tasks in zebrafish. It is important to highlight that the use of these tasks may be restricted due to the sensitivity to stress caused experimental manipulation, which may impair the development of pharmacological studies.

In summary, the novel object recognition and object location tasks are promising to evaluate memory in physiological and pathological conditions in zebrafish that may modulate the memory processing.

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