Aquatic Toxicology 182 (2017) 172–183

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Manganese(II) chloride alters behavioral and neurochemical parameters in larvae and adult zebrafish

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ARTICLE INFO

Article history:
Received 8 September 2016
Received in revised form 24 October 2016
Accepted 16 November 2016
Available online 17 November 2016

Keywords:
Cell death
Dopaminergic system
Locomotor behavior
Manganese(II) chloride
Zebrafish

ABSTRACT

Manganese (Mn) is an essential metal for organisms, but high levels can cause serious neurological damage. The aim of this study was to evaluate the effects of $MnCl_2$ exposure on cognition and exploratory behavior in adult and larval zebrafish and correlate these findings with brain accumulation of Mn, overall brain tyrosine hydroxylase (TH) levels, dopamine (DA) levels, 3,4-dihydroxyphenylacetic acid (DOPAC) levels and cell death markers in the nervous system. Adults exposed to $MnCl_2$ for 4 days (0.5, 1.0 and 1.5 mM) and larvae exposed for 5 days (0.1, 0.25 and 0.5 mM) displayed decreased exploratory behaviors, such as distance traveled and absolute body turn angle, in addition to reduced movement time and an increased number of immobile episodes in larvae. Adults exposed to $MnCl_2$ for 4 days showed impaired aversive long-term memory in the inhibitory avoidance task. The overall brain TH levels were elevated in adults and larvae evaluated at 5 and 7 days post-fertilization (dpf). Interestingly, the protein level of this enzyme was decreased in larval animals at 10 dpf. Furthermore, DOPAC levels were increased in adult animals exposed to $MnCl_2$. Protein analysis showed increased apoptotic markers in both the larvae and adult nervous system. The results demonstrated that prolonged exposure to $MnCl_2$ leads to locomotor deficits that may be associated with damage caused by this metal in the CNS, particularly in the dopaminergic system.

1. Introduction

The water pollution, especially metal contamination, including that by manganese (Mn), has become a serious problem throughout the world. Mn is an essential element occurring in the aquatic ecosystem naturally with elevated levels as a result of agricultural and industrial emissions (Harangi et al., 2016; Hu et al., 2013; Wang et al., 2015). In addition, it is commonly associated to the production of steel and batteries and is an important ingredient in products such as ceramics, paints, fertilizers, and pesticides (Harford et al., 2015; Najamuddin et al., 2016). This metal, when present in high concentrations in water, accumulates in plants and other organisms in the aquatic food chain (Harangi et al., 2016). Typically living organisms can regulate the internal concentration of manganese; however, when there is a high concentration of this metal in the external environment, the internal control is not effective, leading to intoxication (Marks et al., 2016).

The effects of Mn in aquatic environments demonstrate different sensitivities of tropical fish species to this metal, which may be attributed to pH and water hardness (Harford et al., 2015). The mechanism(s) of Mn toxicity in aquatic species are not well understood, but there is evidence about the inhibition induced by this metal on various calcium-mediated physiological processes in mammals (Gavin et al., 1999) and invertebrates (Baker et al., 1973). Studies have also demonstrated that Mn functions as a cofactor of various enzymes required for neuronal and glial cells, as well as of enzymes involved in neurotransmitter synthesis and metabolism (for review see Erikson and Aschner, 2003; Roth et al., 2013), such

Abbreviations: DOPAC, 3,4-dihydroxyphenylacetic acid; DA, dopamine; Mn, manganese; TH, tyrosine hydroxylase.

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http://dx.doi.org/10.1016/j.aquatox.2016.11.013
0166-445X/

as glutamine synthetase, arginase and pyruvate decarboxylase (for review see Wedler and Denman, 1984). Mn toxicity mechanisms are associated with neurochemical changes, such as alteration of iron homeostasis, excitotoxicity, mitochondrial dysfunction, oxidative stress, protein aggregation and changes in homeostasis of other divalent metals that share the same transporters (for review see Aschner et al., 2002; Erikson et al., 2005; Normandin et al., 2004; Seth and Chandra, 1984). These alterations have been associated with damage to the dopaminergic neurons (for review see Aschner et al., 2005; Morello et al., 2008).

Dopaminergic signaling is known to regulate a variety of physiological and behavioral processes, such as motor activity, cognition, memory, mood, and the reward system (for review see Bjorklund and Dunnett, 2007; Goldman-Rakic, 1998; Jones and Miller, 2008). The synthesis of dopamine (DA) is modulated by the activity of tyrosine hydroxylase (TH). TH converts the L-amino acid tyrosine to L-DOPA, which is decarboxylated to form dopamine (DA) (for review see Missale et al., 1998; Yamamoto et al., 2010). DA is released by exocytosis in the synaptic cleft (for review see Sesack et al., 2003) and acts through its receptors (D1, D2, D3, D4 and D5) (Ciliax et al., 1999; Han et al., 2007). Dopaminergic neurons have been identified in developing zebrafish embryos (Holzschuh et al., 2001) and adults (Rink and Wullimann, 2001). Moreover, two nonallelic genes encoding TH (*th1* and *th2*) have been identified in zebrafish as a result of teleost genome duplication (Candy and Collet, 2005).

Acute exposure to neurotoxins, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) or 6-OHDA (6-hydroxydopamine), by intramuscular injection has been shown to diminish DA and noradrenaline levels in the brains of zebrafish; additionally, zebrafish exhibited a decrease in swimming velocity and total distance traveled (Anichtchik et al., 2004; Bretaud et al., 2004). Furthermore, chronic exposure to paraquat decreased the distance traveled and absolute turn angle, and DA levels increased while 3,4-dihydroxyphenylacetic acid (DOPAC) levels decreased in the zebrafish brain (Bortolotto et al., 2014). On the other hand, paraquat and rotenone diluted in the water of the zebrafish tank did not alter locomotion or the number of DA neurons in zebrafish (Bretaud et al., 2004). Bakthavatsalam et al. (2014) showed Mn decreased TH immunoreactivity in almost all catecholaminergic nuclei except the area postrema of the hindbrain, which induced a reduction in movements in zebrafish larvae.

Because prolonged exposure to Mn is a potential hazard to the CNS, the aim of this study was to evaluate the toxicological effects of this metal on zebrafish larvae and adults. Thus, the influence of manganese(II) chloride ($MnCl_2$) on locomotion, overall brain TH, DA, DOPAC and cell death markers was assessed in zebrafish larvae and adults. We also analyzed the survival and morphological changes in the zebrafish during the larval stage as well as memory in adult animals.

2. Materials and methods

2.1. Animals

Larval (0–10 days post-fertilization) and adult stage (6–8 months, 0.2–0.4 g) wild-type *Danio rerio* from the Tübingen background were used. Animals were obtained from our breeding colony, which was maintained in recirculating systems (Zebtec, Tecniplast, Italy) with reverse osmosis-filtered water equilibrated to reach the species standard temperature (28 °C ± 2° C), pH (7.0 and 7.5), and ammonia, nitrite, nitrate and chloride levels. Animals were subjected to a light/dark cycle of 14/10 h. Animals were fed with paramecium between 6 and 14 days post-fertilization (dpf), and after 14 dpf with commercial flakes (TetraMin Tropical Flake Fish®) three times a day supplemented with brine shrimp (Westerfield, 2000).

For breeding, females and male (1:2) were placed in breeding tanks overnight separated by a transparent barrier that was removed after the lights went on the following morning. The fertilized eggs retained in the fitted tank bottom were used for the experiments. For the experiments with larvae, the embryos were collected, sanitized and immediately subjected to the treatment. For the experiments in adult animals, after collection, the embryos were maintained for up to 7 dpf at a density of one larva per 7 mL in cell culture dishes in a biochemical oxygen demand (BOD) incubator. They were immediately transferred to a tank with a density of one larva per 60 mL. When the animals reached 30 dpf, they were maintained at a density of one animal per 200 mL until adulthood.

Water used in the experiments was obtained from a reverse osmosis apparatus (18 MOhm/cm) and was reconstituted with marine salt (Crystal Sea™, Marinemix, Baltimore, USA) at 0.4 ppt. The total organic carbon concentration was 0.33 mg/L. The total alkalinity (as CO_3^{2-}) was 0.030 mEq/L. During fish maintenance, water parameters were monitored daily and maintained in the following ranges: pH: 6.5 to 7.5, conductivity: 400 to 600 μS, ammonium concentration: <0.004 ppm, and temperature: 25 to 28 °C. All protocols were approved by the Animal Care Committee from Pontifícia Universidade Católica do Rio Grande do Sul (13/00354-CEUA- PUCRS).

2.2. Treatments

2.2.1. Larval treatment

Embryos were placed in six-well plates (10 embryos per well), and subjected to $MnCl_2$ (Sigma-Aldrich, St. Louis, MO) treatment at nominal concentrations of 0 (control group), 0.1, 0.25, 0.5, 1.0 and 1.5 mM (Cheng et al., 2003; Tamm et al., 2008) for five days (1 h post fertilization (hpf) to 5 dpf). After this treatment, the animals were placed in six-well plates with water. The medium was changed daily (Fig. 1a), including when the animals were only in the water. Animals were monitored daily for survival as determined by the lack of heart beat.

2.2.2. Adult treatment

Adult animals, aged between 6 and 7 months, were exposed to nominal concentrations of 0 (control group), 0.1, 0.25, 0.5, 1.0 and 1.5 mM (Cheng et al., 2003; Tamm et al., 2008) $MnCl_2$ in 2 L aquaria (10 animals per tank) for 96 h. The exposure water was changed daily (Fig. 1b).

2.3. Morphological defects

Potential $MnCl_2$ teratogenicity was estimated by monitoring morphological defects in 5, 7, and 10 dpf larvae under a stereomicroscopy. Body length (μm), ocular distance (μm), and surface area of the eyes (μm²) were evaluated using NIS-Elements D software for Windows 3.2 (Nikon Instruments Inc., Melville, USA). Body length was estimated using the method described by Capiotti et al. (2011) with modifications; the distance from the larval mouth to the pigmented tip of the tail was measured. The ocular distance was evaluated by the distance between the inner edge of the two eyes (similar to the inner intercantal distance in humans), and the size of the eyes was determined by measuring the surface area of the eyes (Lutte et al., 2015).

2.4. $MnCl_2$ brain accumulation

$MnCl_2$ accumulation in the brain was assessed using inductively coupled plasma mass spectrometry (ICP-MS) according to

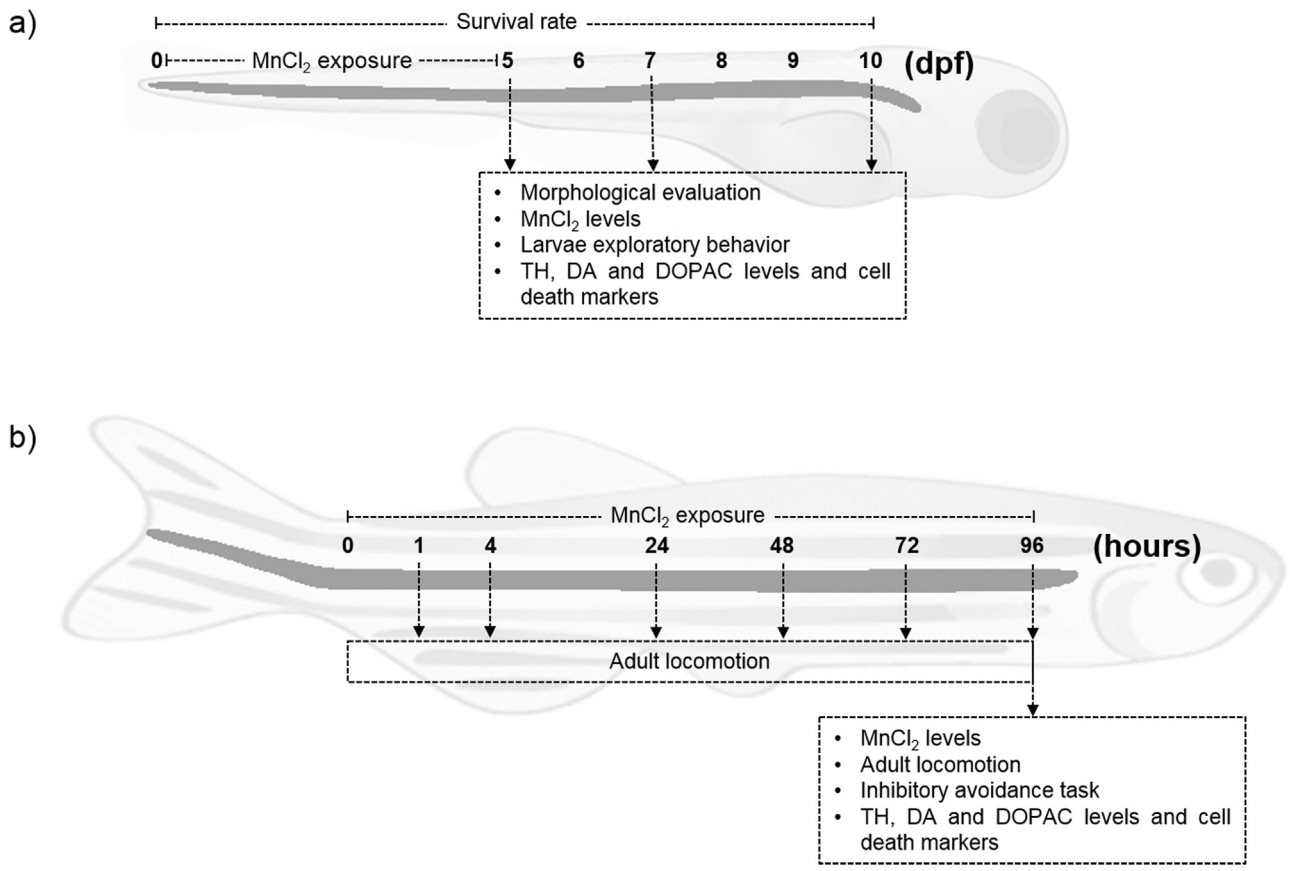

Fig. 1. Timeline of the experimental procedure. (a) Zebrafish larvae were exposed to water (control group) or $MnCl_2$ at concentrations of 0.1, 0.25, 0.5, 1.0 and 1.5 mM. The exposure occurred from 1 hpf to 5 dpf and the analysis lasted 10 dpf, including survival rate, teratogenicity, $MnCl_2$ accumulation, exploratory behavior, TH, DA and DOPAC levels and cell-death markers. (b) Zebrafish adults were exposed to water (control group) or $MnCl_2$ at a concentration of 0.1, 0.25, 0.5, 1.0, 1.5 mM. The exposure took place over 96 h, when $MnCl_2$ levels, exploratory/locomotory behavior, inhibitory avoidance memory, TH, DA, and DOPAC levels and cell-death markers.

the method described by Ashoka et al. (2009) with modifications. In adult animals, analysis was performed after 96 h of exposure to $MnCl_2$. In larvae, independent measurements were made at 5, 7, and 10 dpf. A pool of three brains of adult animals and 15 for larvae was washed with saline solution (0.9%) and digested with 0.5 mL of HNO_3 and 0.1 mL of HCl in a glass tube. Samples were then incubated for two hours at 85 °C and diluted to 5 mL with a 1% solution of HNO_3. Subsequently, the samples were placed in the autosampler for analysis. The $MnCl_2$ calibration curve was linear over the range of 10–1000 ppb (μg/L), and the results were expressed as μg per brain.

2.5. Behavioral analyses

2.5.1. Exploratory behavior of larvae

The exploratory behavior of the larvae was based on Colwill and Creton (2011) and evaluated at 5, 7, and 10 dpf. The experiments were performed in a temperature-controlled room (27 ± 2 °C) between 13:00 and 17:00. Each larva was individually placed in a 24-well cell culture plate containing 2 mL of water per well, and the total distance traveled, absolute body turn angle, movement time, and immobile episodes of each animal were evaluated. The immobile episode was characterized by total loss of movement for two seconds. After a 60-s habituation, the sessions were filmed and recorded for five minutes for later analysis using ANY-Maze software (Stoelting Co., Wood Dale, IL, USA), which is able to track the swimming activity of the animals at a rate of 15 positions per second. The video-tracking data were then used to determine the relevant measures through detecting animals by looking at the contrast between them and the background of the apparatus.

2.5.2. Adult exploratory behavior

Adult exploration was evaluated at six different times after the start of exposure (1, 4, 24, 48, 72, and 96 h). The experiments were performed in a temperature-controlled room (27 ± 1 °C) between 9:00 and 13:00. Animals were placed individually in experimental tanks (30 cm long × 15 cm high × 10 cm wide), and after 60 s of habituation, their locomotor behavior was recorded for five minutes. The videos were analyzed using the ANY-Maze software (Stoelting Co. Wood Dale, IL, USA), with the experimental tank divided into equal parts by three digital vertical lines and one horizontal line. The behavioral parameters analyzed were: distance traveled, line crossings, and time spent in the upper zone. The time spent in upper zone indicated an anxiolytic-like behavior index because normal exploratory behavior of the zebrafish, when introduced into a new environment, is to spend more time at the tank bottom and to gradually move to the upper zone after a few minutes (Levin et al., 2007). Additionally, the absolute turn angle of the animal body during exploration was also measured. This measure reports the sum of the absolute angle between each movement vector of the animal. To calculate this parameter a vector of movement from one position of the animal's center point to the next is created. For each vector the angle between it and the previous vector is calculated with anti-clockwise movement being negative and clockwise movement being positive (*i.e.* the angle is from −180° to 180°). The absolute value of this angle is summed for all the positions of the animal throughout the test or time period. To mea-

sure this parameter, the fish was placed in a round aquarium with an approximately 20 cm diameter, habituated for 60 s, and video recorded from the top of the aquarium for five minutes (Anichtchik et al., 2004). The behavioral analysis was performed in a computer using ANY-maze® software (Stoelting CO, USA) to track the swimming activity of the animals at a rate of 15 positions per second. The video-tracking data were then used to determine the relevant measures through detecting animals by looking at the contrast between them and the background of the apparatus.

2.5.3. Inhibitory avoidance task

To assess whether $MnCl_2$ could impair long-term memory in adult animals, the inhibitory avoidance test was carried out after 96 h of exposure (Blank et al., 2009). There were two sessions, training and test, with a 24 h interval between them. In each session, animals were placed individually in an experimental aquarium (18 cm long × 9 cm wide × 7 cm high) divided by a guillotine door (9 cm high × 7 cm wide) into two compartments of equal size, one black and one white. During the training session, the animal was placed in the white compartment, with the door closed for one minute for habituation and environment recognition. After this period, the division was lifted so that the fish could cross over to the dark side of the tank. Once the animal passed into the dark side, the guillotine door was again closed, and an electric shock pulse of 3 ± 0.2 V was applied for five seconds. The animal was removed from the apparatus and returned to the aquarium housing with only water. Twenty-four hours later, the animals were subjected to the test session, which consisted of the same training protocol, but without the shock. The latency to enter the dark compartment during the two sessions was determined, and the expected increase in the test session is used as an index of memory retention.

2.6. Western blot analysis of overall brain TH levels and cell death markers

Each sample was composed of five brains of adult animals or 30 larval brains. After dissection, brains were placed in a solution containing protease inhibitors (Complete Mini, Roche Applied Science) and stored at −80° C for subsequent analysis (Nornes et al., 2003). The protein extract was prepared in RIPA buffer (Sigma-Aldrich). From the total protein, 25 μg was separated on a SDS-polyacrylamide 12% gel and electrophoretically transferred to a nitrocellulose membrane. Then, the membranes were blocked with 5% albumin (Sigma-Aldrich) in TBS containing 0.05% Tween-20 and incubated overnight with the following primary antibodies: anti-β-actin (ab34731, Abcam, 1:2000), anti-tyrosine hydroxylase (MAB318, Millipore, 1:1000), anti-p53 (55342, Anaspec, 1:1000), anti-Bax-α (55469, Anaspec, 1:750) and anti-caspase-8 (55375, Anaspec, 1:750). Goat anti-mouse (L-21040, Molecular Probes, 1:2000) and goat anti-rabbit (ab97069, Abcam, 1:2000) conjugated secondary antibodies were used to detect the primary antibodies using 1 h as incubation time, and the resulting signal was visualized with a Western Lighting chemiluminescence kit (NEL 104001EA, Perkin Elmer). The antibodies used had their efficiency confirmed by previous studies in zebrafish or manufacturer information and prestained molecular weight protein markers (BenchMark, Invitrogen) were used to determine the molecular weight of the bands and to confirm target-specific antibodies. Since the membranes were incubated with different antibodies with almost the same molecular weight we dehydridised the membrane (stripped-off the antibodies) incubating the membrane at 60 °C for 30 min (10 mL SDS 10%, 3.2 mL 1 M Tris-HCl pH6.8; 0.35 mL 2-mercaptoethanol in 50 mL of ddH2O) after each membrane exposure and image capture of the bands. After 30 min of incubation membranes were washed 5 times for 15 min with TBS containing 0.05% Tween-20, blocked and incubated with another primary antibody (Nery et al., 2014). The densitometric quantification of each gel was conducted using Carestream software (Carestream Health). The total protein levels were normalized using the β-actin protein levels in each sample.

2.7. Determination of DA and DOPAC levels

Levels of DA and its metabolite DOPAC were determined using the method described by González et al. (2011) with some modifications. The experiments were conducted using four samples containing a pool of five brains for adults and 25 for larvae. Samples were homogenized on ice with an Ultra-Turrax (T10 basic IKA®) in 500 μL of formic acid (0.1 M) and were centrifuged at 20,000*g* for 20 min at 4 °C. The supernatant was filtered (0.22 μM filter) and injected into the UHPLC (ultra-high performance liquid chromatography) 1290/MS 6460 TQQQ (Agilent, all HPLC components and the MassHunter software were obtained from Agilent Technologies®). Chromatographic separations were performed using a Zorbax Eclipse Plus C18 2.1 × 50 mm 1.8 μm column. The flow rate of the methanol (eluent): 0.05% formic acid with 1 mM heptafluorobutyric acid (HFBA) (eluent B) mobile phase was 0.2 mL/min with a column temperature of 30 °C. The gradient was a 95%–0% B eluent gradient, with 95% eluent B for 0.5 min and a subsequent decrease to 0% in 3.5 min. A volume of 5 μL of the samples was injected in the UHPLC system. The transitions monitored were DA (154 > 137 and 154 > 91) and DOPAC (169 > 123 and 169 > 77). The results were expressed as ng of analyte per mg protein in the brain homogenate. Protein was measured by the Coomassie blue method (Bradford, 1976), and bovine serum albumin was used as a standard.

2.8. Statistical analysis

Larval survival during the 10 experimental days was analyzed by Kaplan-Meier analysis. Differences in locomotor parameters, considering time (larvae at 5, 7 and 10 dpf; adults at 1, 4, 24, 48, 72 and 96 h of treatment) and concentrations (0.1, 0.25 and 0.5 mM for larvae; 0.5, 1.0 and 1.5 for adults) of $MnCl_2$ exposure as factors, were evaluated by two-way analysis of variance (ANOVA) followed by *post-hoc* comparisons using Bonferroni corrections and are expressed as the mean ± S.E.M. Inhibitory avoidance memory data are presented as the mean ± S.E.M. Training and test latencies for each group were compared by the Wilcoxon matched pairs test. Latencies of multiple groups were compared using Kruskal–Wallis and Mann–Whitney *U* tests. $MnCl_2$ quantification, western blot protein levels and DA and DOPAC levels were evaluated by one-way ANOVA followed by a *post-hoc* Tukey's test in larvae and adult zebrafish. Data were expressed as the mean ± S.D. For all comparisons, the significance level was set at $p < 0.05$.

3. Results

3.1. Manganese(II) chloride exposure in zebrafish larvae

3.1.1. Survival

The potential embryotoxicity of $MnCl_2$ was tested, and the results demonstrated that animals exposed to concentrations of 0.1, 0.25, and 0.5 mM showed survival percentages of 84%, 82% and 72%, respectively, percentages which were not significantly different from the control group (88%). Animals exposed to concentrations of 1.0 and 1.5 mM, however, died over the 10 days of observation, with a survival percentage of 0%; thus, these concentrations were excluded from the study (Fig. 2a). Fig. 2b shows the larvae treated with all concentrations of $MnCl_2$ at 5 dpf and the control group.

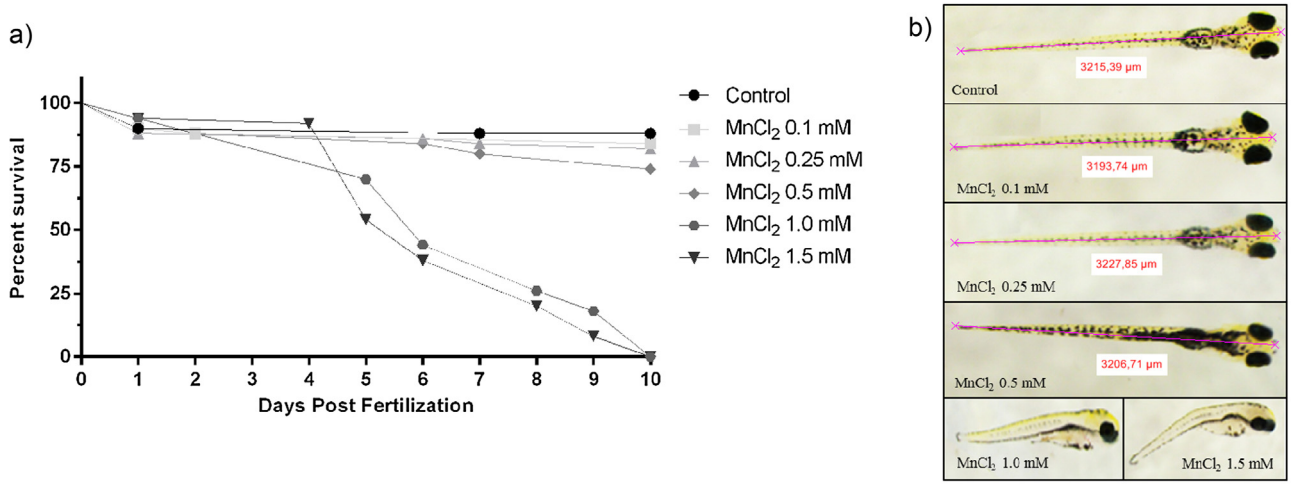

Fig. 2. (a) Kaplan-Meier survival for $MnCl_2$-treated zebrafish larvae. $MnCl_2$ significantly impaired survival at 1.0 and 1.5 mM when compared to control group (Log-rank (Mantel-Cox) test, $p < 0.05$). There was no significant difference between animals exposed to concentrations of 0.1, 0.25 and 0.5 mM $MnCl_2$ and control group (Log-rank (Mantel-Cox) test, $p = 0.3446$). Data are expressed as mean from 50 animals analyzed individually for each group. (b) Pictures represent morphological alterations induced by $MnCl_2$ at 5 dpf, with scale bar showing the body length of the larvae exposed to concentrations of 0.1, 0.25 and 0.5 mM.

Table 1
Effects of $MnCl_2$ exposure on morphological parameters in zebrafish larvae.

Group	*n*	Body Length (µm)	Surface Area (µm²)	Ocular Distance (µm)
5 dpf				
Control	30	3317.83 ± 54.96	52356.81 ± 2189.14	105.17 ± 3.46
$MnCl_2$ 0.1 mM	30	3387.57 ± 62.24	57440.07 ± 2026.27	112.59 ± 4.35
$MnCl_2$ 0.25 mM	30	3269.68 ± 56.82	57482.29 ± 1984.17	111.40 ± 6.48
$MnCl_2$ 0.5 mM	30	3179.53 ± 51.66	55975.67 ± 2102.90	109.46 ± 6.03
7 dpf				
Control	30	3396.87 ± 39.60	55916.05 ± 2118.76	110.45 ± 3.74
$MnCl_2$ 0.1 mM	30	3399.71 ± 47.28	57987.92 ± 1749.53	111.07 ± 5.01
$MnCl_2$ 0.25 mM	30	3301.18 ±35.73	58936.70 ± 2912.99	110.27 ± 5.59
$MnCl_2$ 0.5 mM	30	3198.18 ± 40.76	56421.27 ± 2324.66	109.05 ± 3.17
10 dpf				
Control	30	3514.30 ± 60.56	54751.10 ± 2783.78	109.92 ± 1.87
$MnCl_2$ 0.1 mM	30	3503.36 ± 65.29	58099.20 ± 1944.80	111.39 ± 2.59
$MnCl_2$ 0.25 mM	30	3469.68 ± 56.41	58309.51 ± 2925.42	110.61 ± 5.19
$MnCl_2$ 0.5 mM	30	3499.53 ± 73.00	58390.30 ± 2808.34	111.12 ± 4.53

Data are expressed as means ± S.D.

3.1.2. Morphological evaluation

The teratogenic effects of $MnCl_2$ on the morphology of larvae exposed to $MnCl_2$ were evaluated at 5, 7 and 10 dpf. There were no differences in body length, ocular distance and surface area of the eyes between the control and the $MnCl_2$-exposed groups at concentrations of 0.1, 0.25 and 0.5 mM (Table 1).

3.1.3. Manganese brain levels after $MnCl_2$ exposure

Mn quantification was performed at 5, 7, and 10 dpf to assess the deposition of this metal in the larva and its subsequent accumulation after the exposure (Fig. 3). At 5 dpf, larvae treated with $MnCl_2$ concentrations of 0.1, 0.25, and 0.5 mM had significantly higher levels of Mn than the control group ($F_{(3,12)} = 506.2$; $p < 0.05$). At 7 dpf, the animals treated with 0.25 and 0.5 mM of the metal had higher Mn levels compared to the control group ($F_{(3,12)} = 61.00$; $p < 0.05$). At 10 dpf, only the 0.5 mM group had higher concentrations than the control group ($F_{(3,12)} = 34.63$; $p < 0.05$).

3.1.4. Exploratory behavior

The exploratory behavior of the larvae was examined at 5, 7, and 10 dpf to determine whether $MnCl_2$ exposure could alter larvae locomotion and orientation. Animals exposed to $MnCl_2$ at concentrations of 0.1, 0.25, and 0.5 mM displayed reduced travel distances (Fig. 4a) compared to the control group at 5, 7 and 10 dpf ($F_{(3,332)} = 30.93$; $p < 0.05$). The parameter of absolute turn angle (Fig. 4b) was decreased at 5 dpf in the 0.25 and 0.5 mM $MnCl_2$ groups and at all concentrations at 7 and 10 dpf ($F_{(3,332)} = 20.39$; $p < 0.05$). Moreover, it was found that animals exposed to $MnCl_2$ concentrations of 0.25 and 0.5 mM had reduced movement times (Fig. 4c) compared with the control group at all the analyzed times ($F_{(3,332)} = 54.00$; $p < 0.05$). The number of immobile episodes (Fig. 4d) was increased at 5 and 7 dpf with concentrations of 0.25 and 0.5 mM compared to the controls ($F_{(3,332)} = 40.45$; $p < 0.05$). At 10 dpf, only the 0.5 mM group showed increases compared to the control group ($F_{(3,332)} = 40.45$; $p < 0.05$).

3.1.5. Dopaminergic system evaluation

Changes in the dopaminergic system may be associated with changes in the locomotor pattern and the overall brain TH, DA and DOPAC levels; thus, these parameters were measured. Overall brain TH protein levels (Fig. 5a and b; Supplementary Fig. S1) were increased with 0.5 mM of $MnCl_2$ at 5 dpf ($F_{(3,8)} = 8.102$; $p < 0.05$) and at all concentrations at 7 dpf ($F_{(3,8)} = 17.36$; $p < 0.05$) compared with the control group. On the other hand, at 10 dpf, all concentrations tested caused a decrease in the relative protein levels of TH compared with the control group ($F_{(3,8)} = 77.54$; $p < 0.05$). No significant

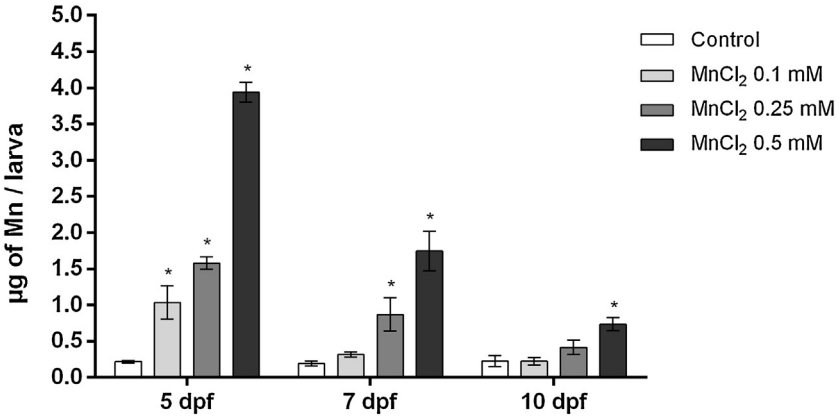

Fig. 3. Mn levels on larvae zebrafish brain measured by ICP-MS in 5, 7 and 10 dpf individuals. Data are expressed as mean ± S.D of four independent experiments (pool of 15 larvae per *n*). Results were analyzed by a one-way ANOVA followed by Tukey's *post-hoc* test. $^{*}p < 0.05$.

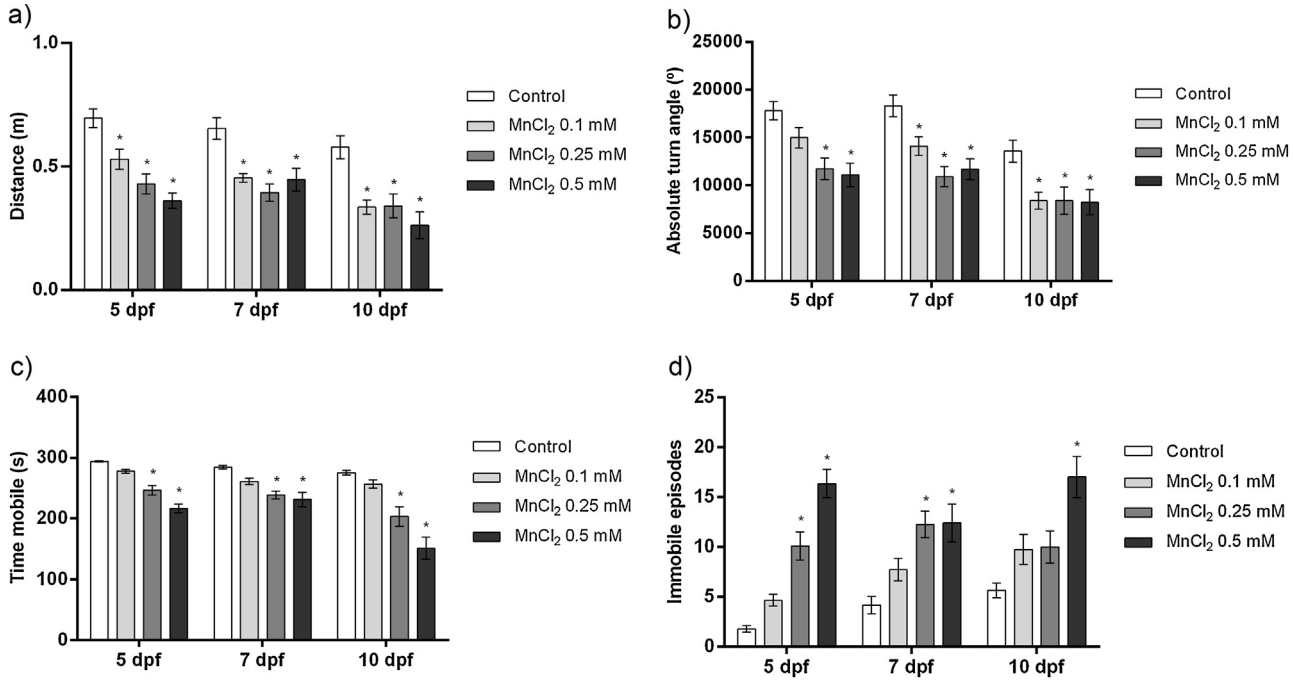

Fig. 4. Exploratory behavior of control and $MnCl_2$-treated zebrafish larvae. Distance traveled (a), absolute turn angle (b), time mobile (c) and immobile episodes (d) were evaluated at 5, 7 and 10 dpf. Data are expressed as the mean ± S.E.M. from 30 animals analyzed individually for each group and were analyzed by two-way ANOVA, considering as factors time and concentrations of $MnCl_2$ exposure, followed by Bonferroni *post-hoc* test. $^{*}p < 0.05$.

changes were observed in DA measurements (Fig. 5c) performed at 5 ($F_{(3,12)} = 1.775$; $p = 0.2053$), 7 ($F_{(3,12)} = 0.4757$; $p = 0.7050$) and 10 dpf ($F_{(3,12)} = 0.1195$; $p = 0.9469$). Similarly, DOPAC levels (Fig. 5d) also showed no differences compared to the control group at 5 ($F_{(3,12)} = 1.332$; $p = 0.3129$), 7 ($F_{(3,12)} = 2.520$; $p = 0.1074$) and 10 dpf ($F_{(3,12)} = 0.1762$; $p = 0.9105$).

3.1.6. Cell death markers

Apoptotic markers were analyzed at 5, 7 and 10 dpf to determine changes in the protein levels of these markers in larvae exposed to $MnCl_2$ (Fig. 6a; Supplementary Fig. S1). Analysis of p53 showed an increase in protein levels with 0.1 mM $MnCl_2$ at 5 dpf ($F_{(3,8)} = 6.079$; $p < 0.05$), with all concentrations at 7 dpf ($F_{(3,8)} = 39.57$; $p < 0.05$) and only for the 0.5 mM $MnCl_2$ group at 10 dpf ($F_{(3,8)} = 4.943$; $p < 0.05$) compared with the control group (Fig. 6b). The protein levels of caspase-8 (Fig. 6c) were increased with $MnCl_2$ concentrations of 0.25 and 0.5 mM at 5 dpf compared to the control group ($F_{(3,8)} = 21.14$; $p < 0.05$), and the same pattern was observed at 10 dpf ($F_{(3,8)} = 175.3$; $p < 0.05$). At 7 dpf, an increase in protein levels was observed at all concentrations tested compared to the control group ($F_{(3,8)} = 26.69$; $p < 0.05$). The Bax-α marker of cell death was also analyzed (Fig. 6d). There was an increase in protein levels of this marker in all concentrations tested at 5 ($F_{(3,8)} = 83.51$; $p < 0.05$) and 7 dpf ($F_{(3,8)} = 75.29$; $p < 0.05$) compared to the control group. At 10 dpf, an increase in Bax-α was observed only at concentrations of 0.25 and 0.5 mM compared to the control group ($F_{(3,8)} = 25.51$; $p < 0.05$).

3.2. Manganese(II) chloride in zebrafish adults

3.2.1. Manganese levels

Mn quantification was performed after 96 h of exposure to assess the accumulation of this metal in the brain of adult animals. No significant difference was found with concentrations of 0.1 and 0.25 mM compared to the control group. However, $MnCl_2$ concentrations of 0.5, 1.0, and 1.5 mM resulted in Mn levels in the brain that

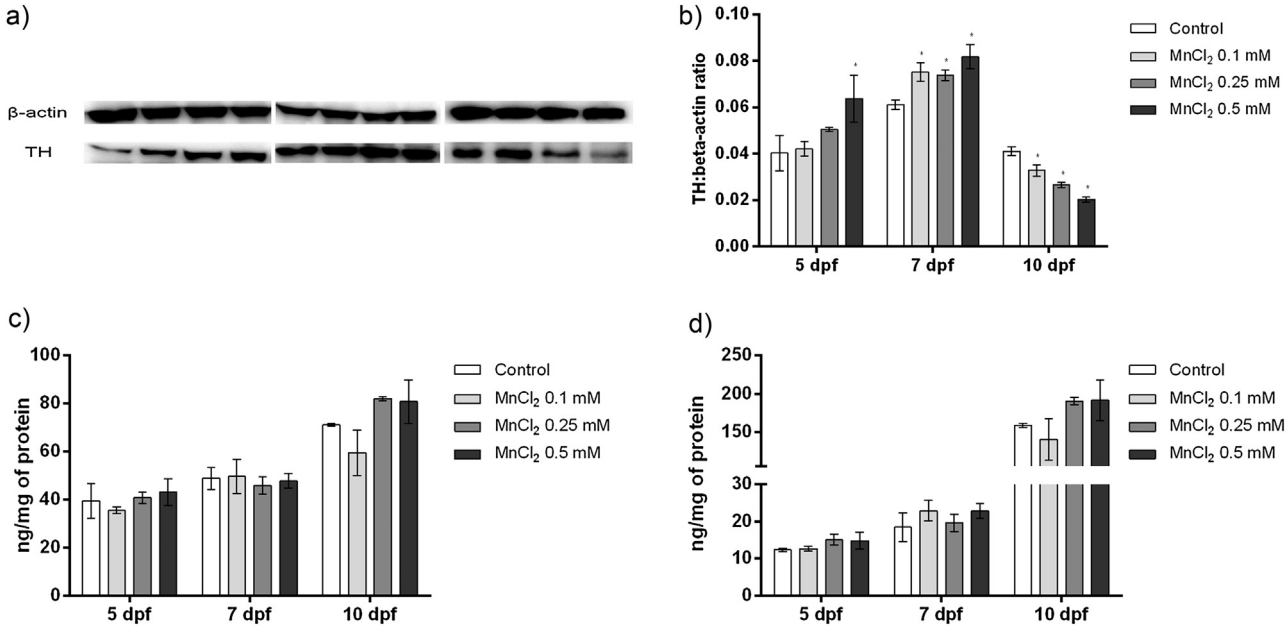

Fig. 5. Representative Western blots showing immunoreactivity of indicated proteins normalized to β-actin (a). The influence of $MnCl_2$ on TH immunocontent (b) in the brain of zebrafish larvae, DA (c) and DOPAC (d) levels in zebrafish larvae measured at 5, 7 and 10 dpf by HPLC. Data are expressed as mean ± S.D of three (TH) and four (DA and DOPAC) independent experiments (pool of 30 larval brains and 25 larvae per *n*, respectively). Results were analyzed by a one-way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$.

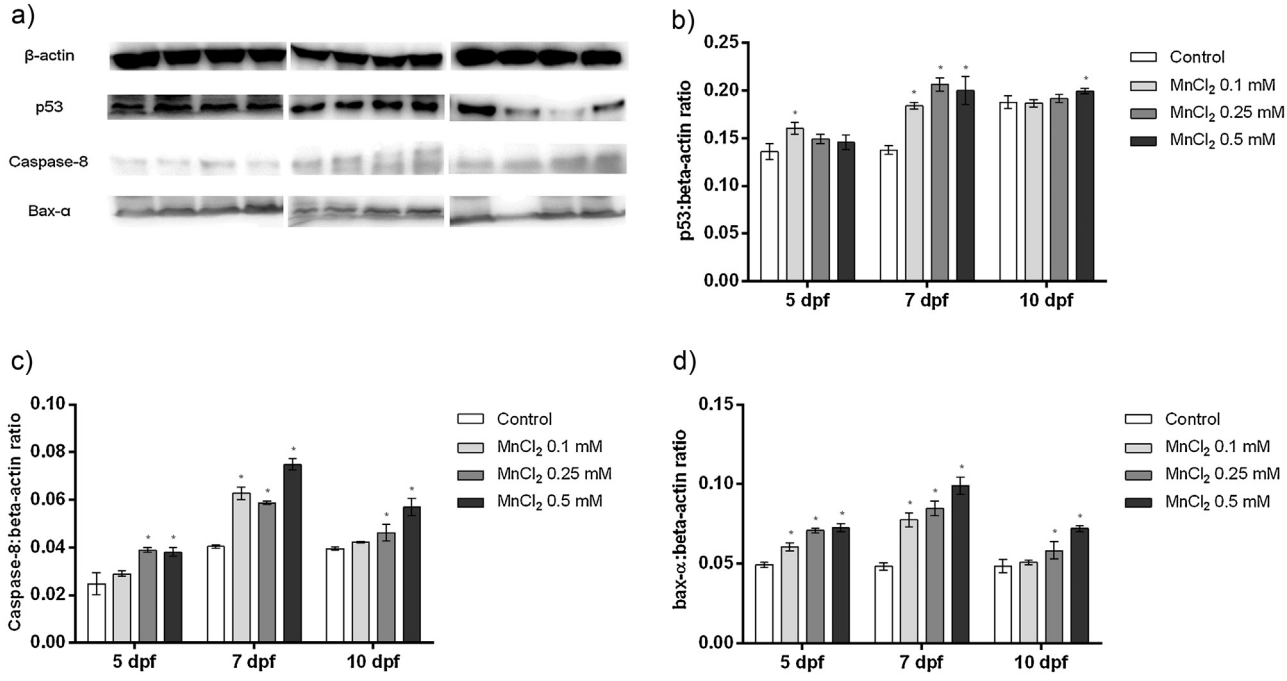

Fig. 6. Representative Western blots showing immunoreactivity of indicated proteins normalized to β-actin (a). The influence of $MnCl_2$ on apoptotic targets, such as p53 (b), caspase-8 (c) and bax-α (d) protein levels in larval zebrafish brains was determined at 5, 7 and 10 dpf by Western Blot. Data are expressed as mean ± S.D of three independent experiments (pool of 30 larval brains per *n*). Results were analyzed by a one-way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$.

were significantly higher than in the control group ($F_{(5,18)} = 54.01$; $p < 0.05$; Fig. 7).

3.2.2. Locomotor behavior

The behavior pattern of adult animals was analyzed at six different time periods (1, 4, 24, 48, 72, and 96 h) to determine if exposure to $MnCl_2$ could cause persistent changes. It was found that exposure to 0.5, 1.0 and 1.5 mM $MnCl_2$ resulted in decreases in the distance traveled ($F_{(3,570)} = 700.6$; $p < 0.05$; Fig. 8a), the number of line crossings ($F_{(3,570)} = 373.2$; $p < 0.05$; Fig. 8b) and the absolute turn angle ($F_{(3,516)} = 450.8$; $p < 0.05$; Fig. 8c) at all times analyzed. Furthermore, treatment with 1.5 mM $MnCl_2$ at 72 h reduced the time spent in the upper zone of the tank ($F_{(3,570)} = 5.701$; $p < 0.05$; Fig. 8d), with animals showing anxiogenic effects. However, at 96 h, the 1.0 mM concentration increased the time spent in the upper zone of the tank by the animals ($F_{(3,570)} = 5.701$; $p < 0.05$; Fig. 8d), demonstrating an anxiolytic behavior.

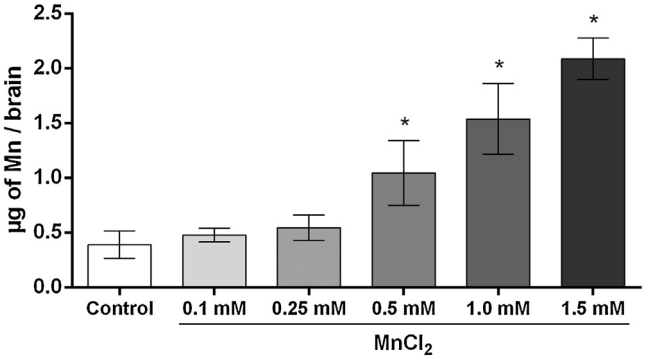

Fig. 7. Mn levels on adult zebrafish brains after 96 h of exposure measured by ICP-MS. Data are expressed as mean ± S.D of four independent experiments (pool of three adult brains per *n*). Results were analyzed by a one-way ANOVA followed by Tukey's *post-hoc* test. *$p < 0.05$.

3.2.3. Inhibitory avoidance task

Because long-term Mn exposure can cause memory impairments in humans, the effects of $MnCl_2$ on long-term memory were analyzed. It was found that $MnCl_2$ treatment with all concentrations tested (0.5, 1.0, and 1.5 mM) was able to impair the adult zebrafish long-term memory compared to the control group ($p < 0.05$; Fig. 9).

3.2.4. Dopaminergic system evaluation

An increase in the relative protein levels of TH ($F_{(3,20)} = 154.6$; $p < 0.05$; Fig. 10a and b; Supplementary Fig. S2) after exposure to 0.5, 1.0, and 1.5 mM $MnCl_2$ was observed at 96 h compared to the control group levels. Moreover, the metal had no significant effect on DA levels in the zebrafish brain compared to the control group ($F_{(3,12)} = 1.020$; $p = 0.4181$; Fig. 10c). However, DOPAC levels were increased with all concentrations tested after 96 h of exposure compared to the control group ($F_{(3,12)} = 11.67$; $p < 0.05$; Fig. 10d).

3.2.5. Cell death markers

Apoptotic markers in the adult brain were measured to determine whether $MnCl_2$ could cause alterations in the relative protein levels of cell death markers (Fig. 11a; Supplementary Fig. S2). $MnCl_2$ at concentrations of 0.5, 1.0, and 1.5 mM increased p53 protein levels ($F_{(3,20)} = 501.5$; $p < 0.05$; Fig. 11b) and caspase-8 ($F_{(3,20)} = 45.82$; $p < 0.05$; Fig. 11c) at 96 h compared to the control group. The analysis of Bax-α showed that animals exposed to 0.5 mM $MnCl_2$ for 96 h had reduced protein levels in zebrafish brains ($F_{(3,20)} = 24.75$; $p < 0.05$; Fig. 11d). However, the animals exposed to 1.0 and 1.5 mM $MnCl_2$ for 96 h showed an increase in protein levels of this marker ($F_{(3,20)} = 24.75$; $p < 0.05$; Fig. 11d) compared to the control group.

4. Discussion

Mn exists in various chemical forms, including oxidation states (Mn^{2+}, Mn^{3+}, Mn^{4+}, Mn^{6+}, Mn^{7+}), salts (sulfate and gluconate) and chelates (aspartate, fumarate, succinate). Mn pollution is an environmental concern due to water contamination (Krachler and Rossipal, 2000). Environmental risk assessments are conducted regularly throughout the world in order to monitor the levels of concentration of metal in the water. The WHO (2011) recommends that the maximum concentration of manganese present in water should not exceed 0.1 mg/L; however, studies have shown higher values in several countries around the world, such as Brazil, where 0.2 mg/L concentrations were found (Harangi et al., 2016; Harford et al., 2015; Puig et al., 2016; Siddiqi et al., 2016). It has been shown that excessive absorption of this metal leads to changes in metabolic functions, depending on factors such as age, weight and length, besides feeding habits of animals, a phenomenon observed in fish (Harangi et al., 2016). Our study demonstrated that $MnCl_2$ exposure induces behavioral and neurochemical changes in different developmental stages of zebrafish, which may be due to cell death induced by this metal.

The exposure to different $MnCl_2$ concentrations (from 200 µM to 10 mM) starting at 3.5 dpf during 24–48 h can cause systemic damage in zebrafish larvae, and it has a LC_{50} of 2.0 mM (Bakthavatsalam et al., 2014). Hernández et al. (2015) demonstrated that sensitivity in zebrafish embryos strongly depends on the period when Mn is applied. The study showed that longer $MnCl_2$ exposure starting earlier in the zebrafish development induced more toxic effects. The LC_{50} of $MnCl_2$ of the different exposure periods ranged from about 6 to 100 mM. The highest toxicity (LC_{50}) was verified to be between 6 and 30 mM, for period above 48 hpf or from 24 to 72 hpf for dechorionated embryos (Hernández et al., 2015). Bakthavatsalam et al. (2014) demonstrated that larvae

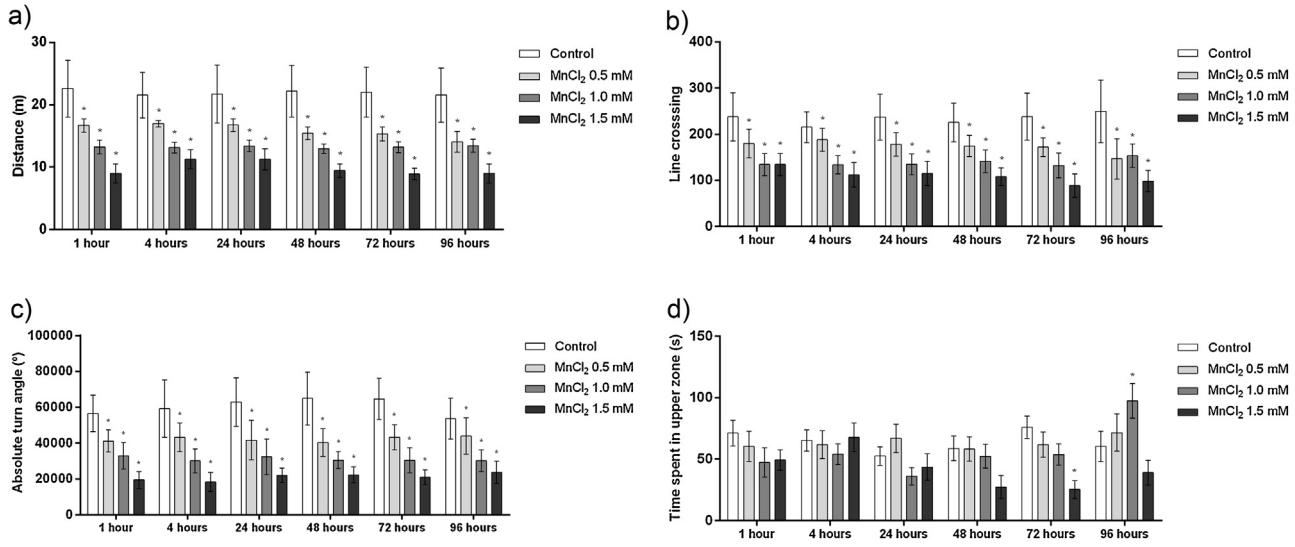

Fig. 8. Exploratory behavior of control and $MnCl_2$-treated adult zebrafish. Distance traveled (a), line crossing (b), absolute turn angle (c) and time spent in the tank upper zone (d) were evaluated at 1, 4, 24, 48, 72 and 96 h after treatment. Data are expressed as the mean ± S.E.M. from 25 animals analyzed individually for each group and were analyzed by two-way ANOVA, considering as factors time and concentrations of $MnCl_2$ exposure, followed by Bonferroni *post-hoc* test. *$p < 0.05$.

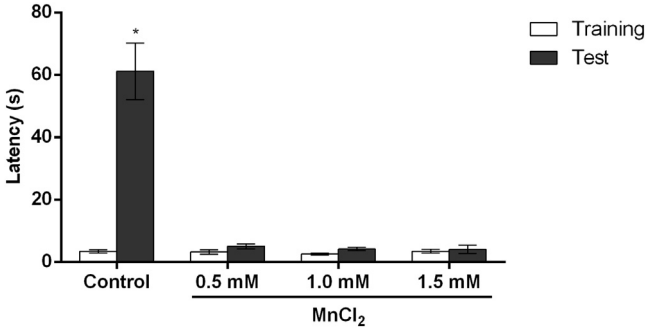

Fig. 9. Inhibitory avoidance task performance on training and long-term memory test sessions of control and $MnCl_2$-treated adult zebrafish after 96 h of exposure. Data are presented as mean ± S.E.M from 20 animals analyzed individually for each group; $^*p < 0.05$ indicates the differences between training and test sessions for each group compared using Wilcoxon matched pair test. No differences were found between training performance among all groups as evaluated by Kruskal–Wallis test.

exposed to 0.8 and 1.0 mM of $MnCl_2$ from 3.5 dpf for 24 and 48 h showed morphological changes, such as underdevelopment of the swim bladder and postural defects that may cause tail bending. They discovered that postural defects were caused by interference with mechanotransduction at the neuromasts because $MnCl_2$ was not able to affect neuronal innervation of muscles (Bakthavatsalam et al., 2014). Our findings showed that lower concentrations (0.1, 0.25, and 0.5 mM) of $MnCl_2$ did not alter morphological measurements, such as body length, ocular distance, and surface area of the eyes. However, the exposure to high concentrations of Mn (1.0 and 1.5 mM) in early stages of development (1 hpf–5 dpf) may induce long-term effects able to increase the mortality at 10 dpf.

Our study demonstrated that exposure to $MnCl_2$ in the early stages (1 hpf–5 dpf) of development was able to alter the swimming behavior of zebrafish larvae at 5, 7 and 10 dpf, reducing the distance traveled and the absolute turn angle of the animals, which indicates that $MnCl_2$ is able to change the swimming pattern of the larvae. Moreover, a decrease in the movement time and an increase in the number of immobile episodes were observed. Bakthavatsalam et al. (2014) found that $MnCl_2$ exposure (from 200 μM to 10 mM) starting at 3.5 dpf during 24–48 h reduced locomotor behavior, causing the animals to swim in circles. This phenomenon could be due to prolonged disruption of the motor neurons, which can lead to tail curving in the $MnCl_2$-treated larvae (Bakthavatsalam et al., 2014). Bakthavatsalam et al. (2014) also observed that $MnCl_2$-treated larvae exhibited fewer startle movements after 24 and 48 h of exposure. Therefore, it is possible to suggest that locomotor changes displayed by the larvae may be associated with damage caused by $MnCl_2$.

Because damage of the dopaminergic system is associated with locomotor disorders (Bowton et al., 2010) and prolonged Mn exposure (for review see Burton and Guilarte, 2009; Sanchez-Betancourt et al., 2012; Settivari et al., 2009), changes in this neurotransmitter system were examined in zebrafish larvae after $MnCl_2$ exposure. Our study showed an increase in overall brain TH protein levels

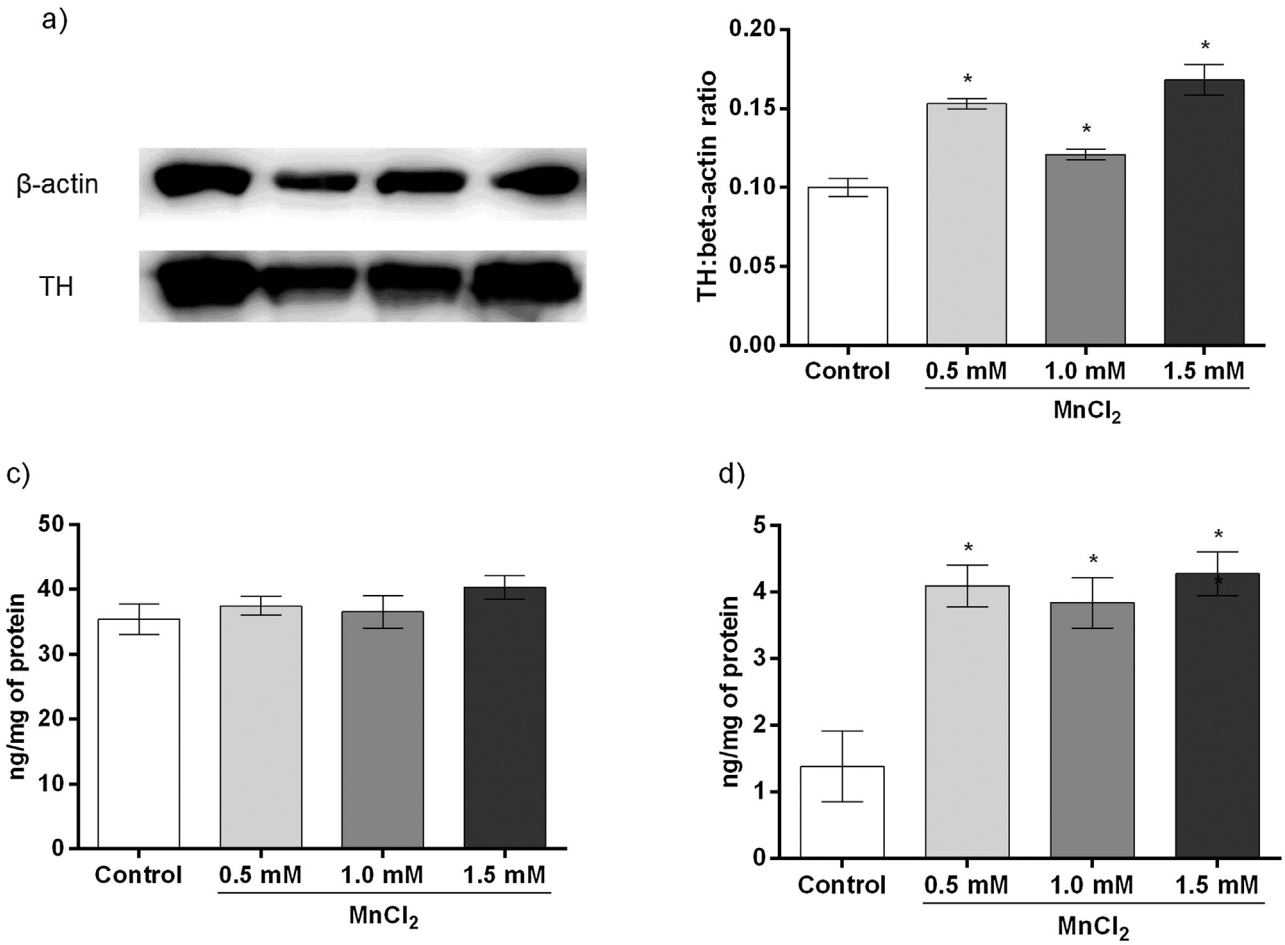

Fig. 10. Representative Western blots showing immunoreactivity of indicated proteins normalized to β-actin (a). The influence of $MnCl_2$ on TH immunocontent (b), DA (c) and DOPAC (d) levels in adult zebrafish brain after 96 h of exposure measured by HPLC. Data are expressed as mean ± S.D of six (TH) and four (DA and DOPAC) independent experiments (pool of five adult brain per *n*). Results were analyzed by a one-way ANOVA followed by Tukey's post-hoc test. $^*p < 0.05$.

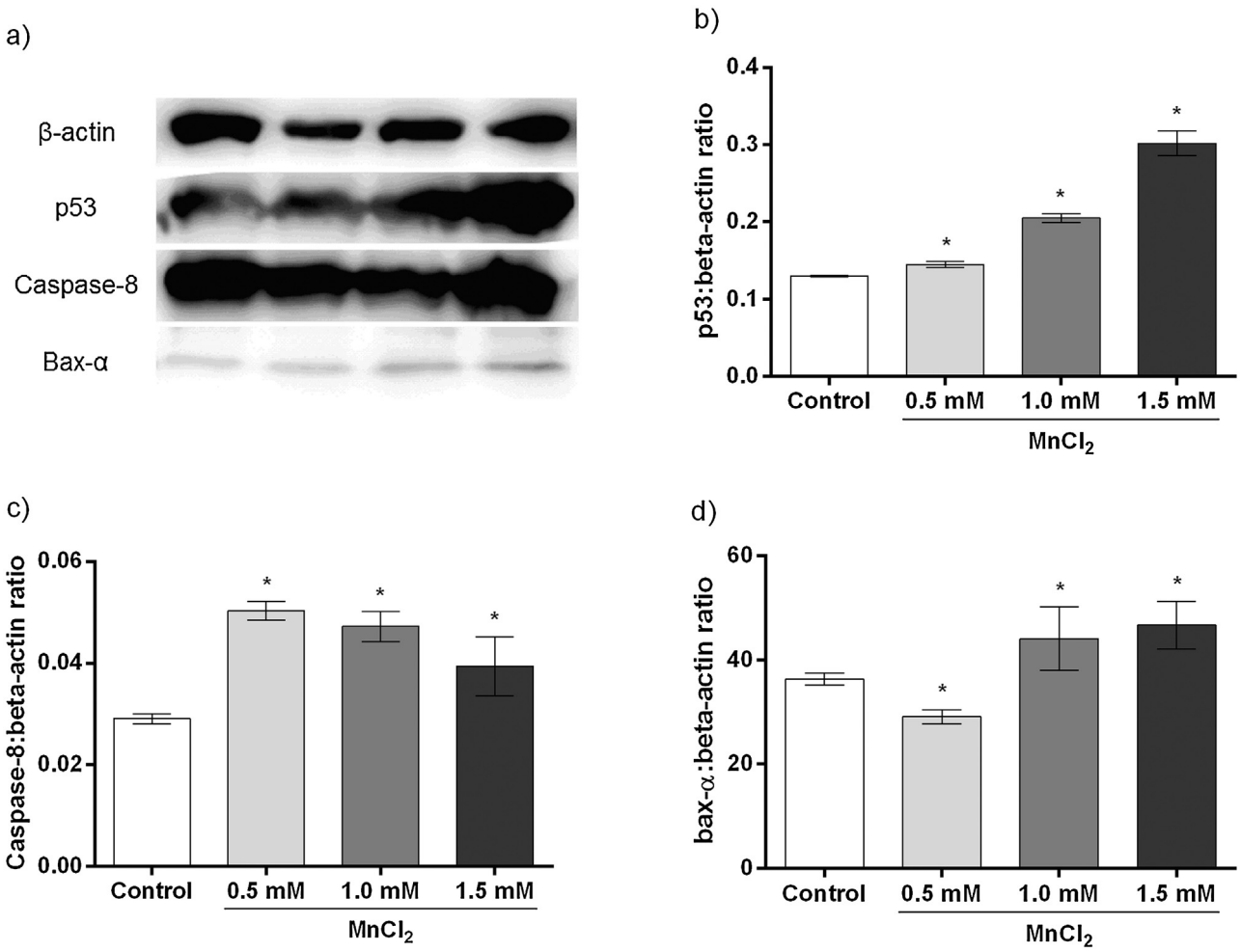

Fig. 11. Representative Western blots showing immunoreactivity of indicated proteins normalized to β-actin (a). The influence of $MnCl_2$ on apoptotic target protein levels, such as p53 (b), caspase-8 (c) and bax-α (d) in adult zebrafish brain was determined after 96 h of exposure by Western Blot. Data are expressed as mean ± S.D of six independent experiments (pool of five adult brains per *n*). Results were analyzed by a one-way ANOVA followed by Tukey's post-hoc test. $^*p < 0.05$.

at 5 and 7 dpf, followed by a reduction at 10 dpf. However, we did not observe a difference in DA and DOPAC levels after $MnCl_2$ exposure compared to the controls. Interestingly, when we compared all groups on different days, we observed a significant increase in DA and DOPAC levels at 10 dpf compared to 5 and 7 dpf. This difference occurs because zebrafish larvae present increased swimming after 7 dpf, when the animal begins to feed and hunt prey, thus accelerating the locomotor activity (Girdhar et al., 2015).

Despite the behavioral and neurochemical changes in the early stages of development, prolonged exposure of adults to Mn may also cause alterations in the CNS. Our findings demonstrated that $MnCl_2$ exposure reduced the distance traveled, the line crossing and the absolute turn angle at all times analyzed. We also observed changes in the anxiety, with anxiogenic-like and anxiolytic-like behaviors after 72 and 96 h of Mn exposure, respectively. In addition, we verified a significant impairment in long-term memory in the inhibitory avoidance task for $MnCl_2$-treated adult zebrafish, suggesting that this metal induces significant neurotoxic effects.

To investigate the possible mechanism related to the locomotor and cognitive deficits induced by $MnCl_2$ exposure in zebrafish, we evaluated their impact on dopaminergic system. Our results showed that adult animals exposed to $MnCl_2$ presented an increase in overall brain TH and DOPAC levels, with no changes in the DA levels in zebrafish brains. The increased TH protein levels could promote an increase in tyrosine to L-DOPA conversion and its subsequent decarboxylation to DA. Then, DA would be metabolized to DOPAC, increasing the levels of this metabolite (Molinoff and Axelrod, 1971).

In vitro studies demonstrated that Mn activates various intracellular pro-apoptotic signaling pathways in neuronal cultures (Hirata, 2002; Hirata et al., 1998; Ma et al., 2015). Our results showed that $MnCl_2$ exposure increased the protein levels of apoptosis markers, such as p53, caspase-8 and Bax-α. This work presents the first evidence that $MnCl_2$ is able to alter apoptotic markers in larvae and adult zebrafish, demonstrating that this metal can increase cellular death. Our results showed that there is a concentration-dependent increase of bax-α in zebrafish larvae and adults after exposure to $MnCl_2$. Although there was not a concentration-dependent effect on caspase in adult zebrafish and p53 at 5dpf, a significant increase of these apoptotic markers were induced by Mn exposure. Therefore, such effects may be associated with activation of the apoptotic cascade, leading to cell death.

In summary, our findings indicate that $MnCl_2$ exposure produced a decrease in exploratory behavior in zebrafish larvae, such as reducing the distance travelled, absolute turn angle and time mobile, as well as it caused an increase in immobile episodes. This metal also altered the locomotor and cognitive parameters in adult zebrafish, leading to a decrease in distance travelled, line crossing and absolute turn angle as well as an impaired long-term memory. These deficits may result from alterations in the dopaminergic system and a possible increase in cell death.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Proc. 446025/2014-3). S.A. was the recipient of a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). M.T.W, L.R.N, M.R.M.R.V and C.D.B were recipients of a fellowship from CNPq.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.11.013.

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