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ORGAN TOXICITY AND MECHANISMS

Long-term and low-dose malathion exposure causes cognitive impairment in adult mice: evidence of hippocampal mitochondrial dysfunction, astrogliosis and apoptotic events

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Abstract The organophosphorus (OP) pesticide malathion is a neurotoxic compound whose acute toxicity is primarily caused by the inhibition of acetylcholinesterase (AChE), leading to cholinergic syndrome-related symptoms. Some lines of evidence indicate that long-term exposure to low levels of OP may produce neuropsychiatric and/or neurobehavioral signs that do not necessarily involve the AChE inhibition. This study evaluated the effects of a repeated (15-day period) and low-dose malathion exposure on spatial memory and discrimination (object location task), as well as on biochemical parameters in the hippocampus of mice [AChE and mitochondrial chain complexes activities; levels of proapoptotic proteins (Bax and Bak) and cholinergic neuronal and astroglial markers (ChAT and GFAP, respectively)]. Malathion treatments (30 and 100 mg/kg, s.c.) did not affect the body weight of animals and caused no evident signs of cholinergic toxicity throughout the treatment, although the highest dose (100 mg/kg) was associated with inhibition of AChE activity. Malathion-exposed animals showed a significant

impairment on spatial memory and discrimination, which was correlated with a decrease in the mitochondrial complex I activity in the hippocampus. Moreover, malathion increased the levels of proapoptotic proteins and induced astroglial activation. The results show that long-term malathion exposure, at a dose that does not affect hippocampal AChE activity (30 mg/kg), caused impaired spatial memory and discrimination in mice that was related to hippocampal mitochondrial dysfunctional, astrogliosis and apoptosis. When extrapolated to humans, such results shed light on noncholinergic mechanisms likely related to the neurobehavioral and cognitive deficits observed in individuals chronically exposed to this pesticide.

Keywords Organophosphorus pesticides · Malathion · Chronic exposure · Mice · Cognitive impairment

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Introduction

Organophosphorus (OP) pesticides have broad household, agricultural and military applications worldwide; they are among the most ubiquitous synthetic chemicals in our environment and the most often associated with toxicity to humans (Buckley et al. 2004; Rohlman et al. 2007). The primary mechanism of OP-induced toxicity is related to acetylcholinesterase (AChE) inhibition, which results from phosphorylation of the serine residue within the active site of the enzyme, thus blocking the degradation and inactivation of the neurotransmitter acetylcholine. The resulting accumulation of acetylcholine in the synaptic cleft causes overstimulation of cholinergic pathways (Bajgar 2004; Bartling et al. 2007; Kwong 2002), thus leading to a variety of signs and symptoms such as muscle fasciculations, respiratory muscle paralysis, seizures, diarrhea, urination, meiosis, salivation

and lacrimation, which are observed primarily in acute OP poisonings (Rusyniak and Nanagas 2004; Sungurtekin et al. 2006). In addition to the classical cholinergic symptoms (mentioned above, resulting mainly from acute OP poisoning), neurobehavioral effects have also been reported as consequence of exposures to OP pesticides; however, in this scenario, prolonged exposures to relatively low pesticide levels are also relevant (Colosio et al. 2009; Dassanayake et al. 2008; Rosenstock et al. 1991; Steenland et al. 1994). Notably, the molecular mechanisms by which OP pesticides induce neurobehavioral effects are not understood.

Malathion (O,O-dimethyl-S-1,2-bis ethoxy carbonyl ethyl phosphorodithioate) is an OP compound extensively used in agriculture and veterinary practices, as well as in attempts at suicide (Maroni et al. 2000). Whereas the insecticidal actions of malathion and its acute toxicity have been attributed to inhibition of cholinesterases (ChEs), mainly AChE (Taylor et al. 1995), there is a lack of data on the potential mechanisms modulating malathion toxicity after long-term and "low-level" exposures. Based on the facts that malathion is one of the most commonly used insecticides in the world (Donaldson et al. 2002) and that there is evidence of the association between low levels of malathion exposure and deficits in neurobehavioral performance (Rothlein et al. 2006), knowledge concerning the mechanisms modulating such association is well warranted.

As previously mentioned, there is significant evidence to suggest that chronic exposure to OP can be associated with neurobehavioral abnormalities including anxiety, depression, psychotic symptoms, deficits in short-term memory, learning, attention and information processing (Amr et al. 1997; Salvi et al. 2003; Singh and Sharma 2000; Stephens et al. 1995). It is noteworthy that these neurobehavioral abnormalities can occur without previous cholinergic symptoms, and they do not appear to be dependent on AChE inhibition (Brown and Brix 1998; Ray and Richards 2001; Singh and Sharma 2000). In fact, OP pesticides might alter the function of a number of proteins others than ChEs (noncholinergic targets) (Terry 2012), which may contribute to the more delayed and persistent OP effects observed following chronic exposure (Costa 2006; Lotti and Moretto 2005). With particular emphasis on malathion, evidence suggests that neurobehavioral alterations observed after long-term exposures may be associated, at least in part, to oxidative damage in the brain mitochondria (Abdel-Rahman et al. 2004; Delgado et al. 2006). However, there are no data on the molecular events downstream to mitochondrial dyshomeostasis, which likely culminate in the neurotoxicity and behavioral impairments observed after malathion exposure.

As already mentioned, although there is convincing evidence that long-term exposures to OP pesticides (including malathion) cause neurobehavioral impairments (Colosio et al. 2009; Daniell et al. 1992; Farahat et al. 2003;

Jamal et al. 2002; Kamel and Hoppin 2004; Rohlman et al. 2007; Roldan-Tapia et al. 2005), data on molecular mechanisms involved in this event are scarce. The purpose of this study was to investigate the effects of repeated subclinical doses of malathion (over a 15-day period) in adult mice. We sought for potential neurobehavioral changes (related to spatial memory and discrimination), as well as for biochemical and cellular changes in the mouse hippocampus, in an attempt to elucidate mechanisms mediating the potential deleterious effects of long-term malathion exposure on cognitive performance. We hypothesize that malathion exposure (at dosages that do not change hippocampal AChE activity) can lead to hippocampal neurotoxicity in mice via noncholinergic events, resulting in memory deficits similar to those observed in humans chronically exposed to OP pesticides (Colosio et al. 2009; Rothlein et al. 2006).

Materials and methods

Chemicals

Commercial-grade malathion (O,O-dimethyl-S-1,2-bis ethoxy carbonyl ethyl phosphorodithioate, 95 % purity, CAS 121–75–5) was purchased from Dipil Chemical Ind. (Brazil). Antibody anti-choline acetyltransferase (ChAT) was purchased from Abcam and the antibody anti-glial fibrillary acidic protein (GFAP) from Sigma-Aldrich. Antibodies anti-Bax, anti-Bak, anti-synaptophysin and anti-β-actin were purchased from Santa Cruz Biotechnology. All other chemicals were of the highest grade available commercially.

Animals

Male adult Swiss albino mice (2 months old) were kept on a 12-h light/dark cycle (light on at 07.00 a.m.) at temperature of 22 ± 1 °C. They were housed in plastic cages with free access to food and water. All experiments were conducted in accordance with the guiding principles in the use of animals in toxicology, adopted by the Society of Toxicology (1989) and were approved by the ethics committee for animal use at the Universidade Federal de Santa Catarina (PP00765). In behavioral tests, the experimental number was 10. After the behavioral tests, six animals per group were used for biochemical analyses (mitochondrial complex activities, AChE activity) and four animals per group were used for Western blotting and histological studies.

Pesticide exposure schedule

Mice were randomly divided into three experimental groups. The animals were injected daily with a subcutaneous (s.c.) malathion injection (5 mL/kg), at the doses of

30 or 100 mg/kg body weight. Physiological saline (NaCl 0.9 %) was used as vehicle for malathion (s.c., 5 mL/kg), as well as injected s.c. to control group. Mice were weighed and individually monitored (in their home cages) for visible cholinergic symptoms (e.g., diarrhea, excessive salivation or lacrimation, respiratory difficulties, or muscle fasciculations) throughout the study. Sixteen days after beginning the treatment (24 h after the last injection), locomotor activity and exploratory behavior (open-field test), as well as spatial memory and discrimination (object location task) were performed as following described. After the behavioral analyses, the animals were killed by decapitation, their brains were immediately removed from the skull and the hippocampus was dissected for the biochemical analyses, as following described.

Behavioral analysis

Open-field test

Twenty-four hours after the last malathion administration, the open-field test was used to investigate locomotor activity and exploratory behavior ($n = 8$–10 animals). Mice were individually placed in a blue plastic circular open field ($d = 44$; $h = 22$ cm) with sectors drawn on the floor. Number of crossings (number of squares crossed by the animal with the four paws) was used to evaluate locomotor activity whereas number of rearings (number of times the mice stood on its hind legs or vertical exploratory activity) to assess the exploratory behavior. All the parameters were registered in a 5-min period. The apparatus was cleaned with a solution of ethanol 10 % between tests in order to remove animal odors.

Object location task

Object location task was conducted for evaluation of spatial memory and discrimination ability ($n = 8$–10 animals). The experimental apparatus used in this study was a blue plastic circular open field ($d = 44$; $h = 22$ cm). The objects were: two identical triangular rubber stoppers brown ($l = 9$; $w = 6.5$; $h = 12$ cm). The protocol was based on the previous studies described by Assini et al. (2009), with minor modifications (Santos et al. 2012). Mice were placed at the center of the apparatus, for 5 min, with two identical objects and visual clues, composed by a black and white vertical striped panel attached to the wall. The objects were placed 7 cm away from the walls of the open field. Exploration of the objects was timed by a stopwatch when mice sniffed, whisked, or looked at the objects from no more than 1 cm away. After this training phase, the mice were removed from the apparatus for 90 min. Thereafter, one object was moved to a new location. The time spent exploring the objects in new (novel) and old (familiar) locations was recorded during 5 min. Sessions were recorded and later analyzed. All locations for the objects were counterbalanced among the groups. After each session, the experimental apparatus was cleaned with ethanol 10 % thus minimizing the interference of potential unfamiliar smells. In order to analyze the cognitive performance, a location index was calculated as previously described by Murai et al. (2007): (Tnovel)/(Tnovel + Tfamiliar), where Tnovel is the time spent exploring the displaced object and Tfamiliar is the time spent exploring the non-displaced object (Murai et al. 2007).

Tissue preparation for biochemical analysis

Twenty-four hours after the behavioral analyses, animals ($n = 6$) were killed by decapitation and the hippocampus was removed and homogenized (1:10 w/v) in phosphate buffer (pH 7.4), containing 0.3 M sucrose, 5 mM MOPS, 1 mM EGTA and 0.1 % bovine serum albumin HEPES buffer (20 mM, pH 7.0). The tissue homogenates were centrifuged at $1,000 \times g$, at 4 °C for 10 min, and an aliquot of the low-speed supernatant (S1) was used for the determination of AChE activity. For the analysis of mitochondrial respiratory chain complexes activity, disrupted mitochondria were obtained by freezing/thawing the S1 three times. NADH dehydrogenase (complex I), succinate dehydrogenase (complex II) and cytochrome c oxidase (complex IV) activities were measured.

Biochemical analyses

Acetylcholinesterase (AChE) activity

Hippocampal AChE activity was measured as previously described (Ellman et al. 1961), using acetylthiocholine iodide as a substrate. The rate of hydrolysis of acetylthiocholine iodide was measured at 412 nm through the release of the thiol compound (thiocholine), which produces the color-forming compound TNB after reaction with DTNB.

Respiratory chain complex activities

Complex I activity was measured by the rate of NADH-dependent ferricyanide reduction as described in Cassina and Radi (1996). The activity of succinate-2,6-dichloroindophenol (DCIP)-oxidoreductase (complex II) was determined according to the method of Fischer et al. (1985) and cytochrome c oxidase (complex IV) activity according to Rustin et al. (1994). The methods described were slightly modified, as detailed in a previous report (Latini et al. 2005). The activities of the respiratory chain complexes were calculated as $nmol \times min^{-1} \times mg\ protein^{-1}$.

Western blotting

After the previously described treatments, the animals ($n = 4$) were killed by decapitation and the hippocampi were quickly removed and homogenized in ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 1 % Triton X-100, 100 mM NaCl, 5 mM EDTA pH 8.0, 40 mM β-glycerophosphate, 50 mM NaF, 200 μM orthovanadate, 5 % glycerol and protease inhibitors) and sonicated for 5 min. The samples were centrifuged at $3{,}000 \times g$ at 4 °C for 45 min, and the supernatant was separated for protein content measurement. Fifty milligrams of protein extract was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) using 15 % gels, and the proteins were transferred to nitrocellulose membranes using a tank transfer system at 100 V and 400 mA for 90 min. The membranes were blocked (1 h) with 5 % skim milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.5). Membranes were incubated overnight at 4 °C with the following antibodies: monoclonal antibody anti-Bax (1/300; 23 kDa; Santa Cruz Biotechnology), polyclonal antibody anti-Bak (1/300; 28 kDa; Santa Cruz Biotechnology), monoclonal antibody anti-synaptophysin (1/300; 38-48 kDa; Santa Cruz Biotechnology), polyclonal antibody anti-ChAT (1/2000; 70 kDa; Abcam) and anti-β-actin (1:3,000; 43 kDa, Santa Cruz Biotechnology), diluted in TBS–Tween–BSA buffer (20 mM Tris base, 140 mM·NaCl, 0.05 % Tween-20). After washing, the membranes were incubated for 60 min at room temperature with protein A/G-horseradish peroxidase conjugate (1:1,000) in TBS–Tween buffer. Then, membranes were washed and developed with Immun-Star HRP Chemiluminescent reagents (Luminol Reagent sc-2048, Santa Cruz, CA, USA), and chemiluminescence was viewed with the ECL system. β-actin was used as loading control. Band intensity was quantified by using the Scion Image software. Densitometric values were normalized with respect to β-actin bands.

Immunofluorescence staining

To visualize the glial fibrillary acidic protein (GFAP) in different hippocampal regions, a new group of animals was treated with malathion (30 and 100 mg/kg) or vehicle (NaCl 0.9 %) for a 15-day period ($n = 4$). At the end of the treatment, the animals were deeply anesthetized with ketamine/xylazine (75 and 10 mg/kg, respectively, *i.p.*) and were perfused through the left cardiac ventricle with 0.9 % saline solution, followed by 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. After perfusion, the brains were removed, post-fixed in the same fixative solution for 24 h at room temperature and cryoprotected by immersion in 30 % sucrose solution in PBS at 4 °C. The brains were then frozen by immersion in cooled isopentane and stored in a freezer (−80 °C) for later analyses. Serial coronal Sects. (40 μm) of hippocampus were obtained with a cryostat (Leica) at −20 °C. The free-floating sections were first blocked using 5 % horse serum (HS) diluted in PBS containing 2 % Triton X-100 (PBS-Tx) for 2 h at room temperature. Next, the sections were incubated for overnight at 4 °C with anti-GFAP from mouse in 1 % HS diluted in PBS-Tx 0.5 %. After three washes in PBS, tissue sections were incubated with anti-mouse Alexa 488 in 1 % HS diluted in PBS-Tx 0.5 % for 2 h at room temperature. Thereafter, the sections were washed three times in PBS, mounted on slides with Fluor Save and covered with coverslips. After that, images from mouse hippocampus were obtained with a confocal Olympus FV-1000 microscope and examined with Image G software (adapted from (Rodrigues et al. (2010)).

Protein determination

Sample protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Statistical analysis

Comparisons between groups were made using analysis of variance (with repeated measures when indicated) followed by Duncan's post hoc test. Pearson's correlations were performed for associations between object location task and complex I activity. The object location task was analyzed by one-sample *t* tests to determine whether the location index was different from chance performance (50 %). The accepted level of significance for the tests was $p \leq 0.05$. All tests were performed using the Statistica® software package (StatSoft Inc., Tulsa, OK, USA).

Results

Repeated exposure to low-dose malathion does not induce cholinergic symptoms

Prolonged and acute exposure to high doses of OP compounds elicits well-defined signs of cholinergic toxicity primarily due to inhibition of AChE activity and overactivation of acetylcholine receptors in the central and peripheral nervous systems (Bajgar 2004; Bartling et al. 2007). The present study focused on the effects of repeated exposure to low doses of malathion, which do not elicit evident signs of overt cholinergic toxicity. To ensure that mice received subclinical exposure to malathion, signs of OP poisoning were qualitatively observed over a 15-day period. ANOVA with repeated measures showed no significant effects
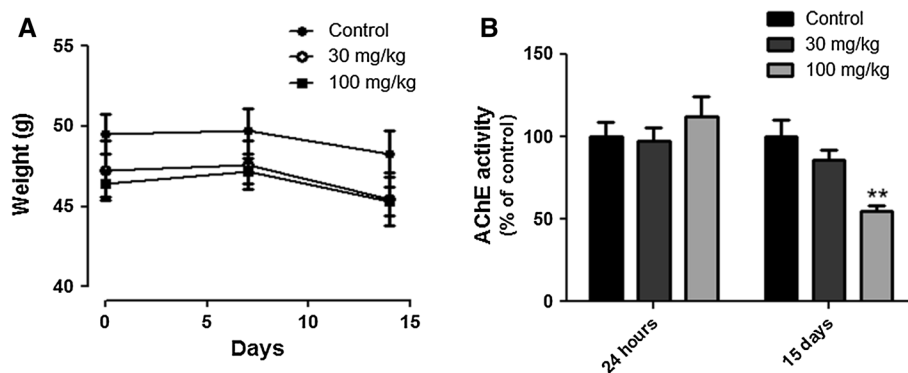
Fig. 1 Effect of repeated malathion exposure on body weight (**a**) and AChE activity in the hippocampus of mice (**b**). Two doses of malathion (30 and 100 mg/kg), dissolved in saline solution, were administered subcutaneously (s.c.) once a day for 15 consecutive days. Control mice received injections of saline solution. AChE activity measured as % of control ($n = 6$ animals per group) at two different time points during the treatment period. Each value represents the mean $\pm$ SEM. **$p < 0.01$ when compared with the control treated with saline for the analysis of variance (ANOVA) followed by Duncan's multiple-range test

of treatments toward body weight of animals, suggesting the absence of overt systemic toxicity. Furthermore, no evident signs of cholinergic toxicity, such as diarrhea, excessive lacrimation and respiratory difficulties, were observed throughout the treatment (data not shown). Figure 1b shows the effects of malathion exposure (30 and 100 mg/kg) on hippocampal AChE activity. Significant effects were observed after the 15-day treatment period [$F(2.15) = 11.97$; $p < 0.001$], but only for the 100 mg/kg dose. In fact, post hoc analyses showed no significant effects of malathion (at 30 mg/kg) on hippocampal AChE activity after 15 days of treatment, indicating that this dosage (30 mg/kg) does not affect hippocampal AChE activity. Hippocampal AChE activity was also measured in a parallel group of animals exposed acutely (24 h) to the same malathion doses (30 and 100 mg/kg) (Fig. 1b), and no significant effects of treatments were observed [$F(2.15) = 0.6059$; $p = 0.5628$]. These results indicate that acute (24 h) and long-term (15 days) exposures to 30 mg/kg of malathion do not inhibit hippocampal AChE activity and do not cause overt signs of cholinergic toxicity.

Repeated exposure to malathion impairs spatial memory in the object location task

The effects of repeated exposures to malathion on mouse spatial memory performance are illustrated in Fig. 2. Firstly, we determined whether malathion exposure had significant effects on exploratory activity or motor function (effects that influence the performance in the memory-related tests). There were no significant treatment-related effects on horizontal (number of crossed squares [$F(2.27) = 0.5752$; $p = 0.5696$] and vertical (rearing behavior [$F(2.27) = 0.2999$; $p = 0.7433$] activities after a 15-day treatment period (Fig. 2a, b).

In order to evaluate the effects of repeated exposure to malathion on mouse spatial memory performance, the object location task was performed. In the training session, all the groups showed the similar interests for both objects, spending around 50 % of the time exploring each object (Fig. 2c). On the other hand, in the test session, malathion caused a significant decline in the spatial memory following the treatment for a 15-day period with both doses, 30 and 100 mg/kg, as indicated by a decrease in the time spent in the displaced object when compared to controls, which showed a time index significantly higher than chance performance ($p < 0.01$) (Fig. 2d). The changes observed in the object location task were not related to motor impairment because motor activity and performance were not affected by malathion exposure (Fig. 2a, b). The occurrence of spatial memory impairment in animals exposed to 30 mg/kg malathion, with no significant hippocampal AChE inhibition, suggests the possibility of non-cholinergic-related events in the modulation of cognitive performance in these animals.

Repeated exposure to malathion impaired the mitochondrial complex I activity

Because significant spatial memory impairments were observed in malathion-exposed animals with no changes in hippocampal AChE activity, we sought for noncholinergic molecular events potentially involved in long-term malathion (30 mg/kg)-induced memory deficits. The effect of repeated subclinical malathion exposure on mitochondrial respiratory chain complexes in the hippocampus is shown in Fig. 3. One-way ANOVA showed significant effects of treatments on hippocampal NADH dehydrogenase activity (complex I) [$F(2.15) = 32.29$; $p < 0.001$]. We found that animals treated for a 15-day period with 30 and 100 mg/kg
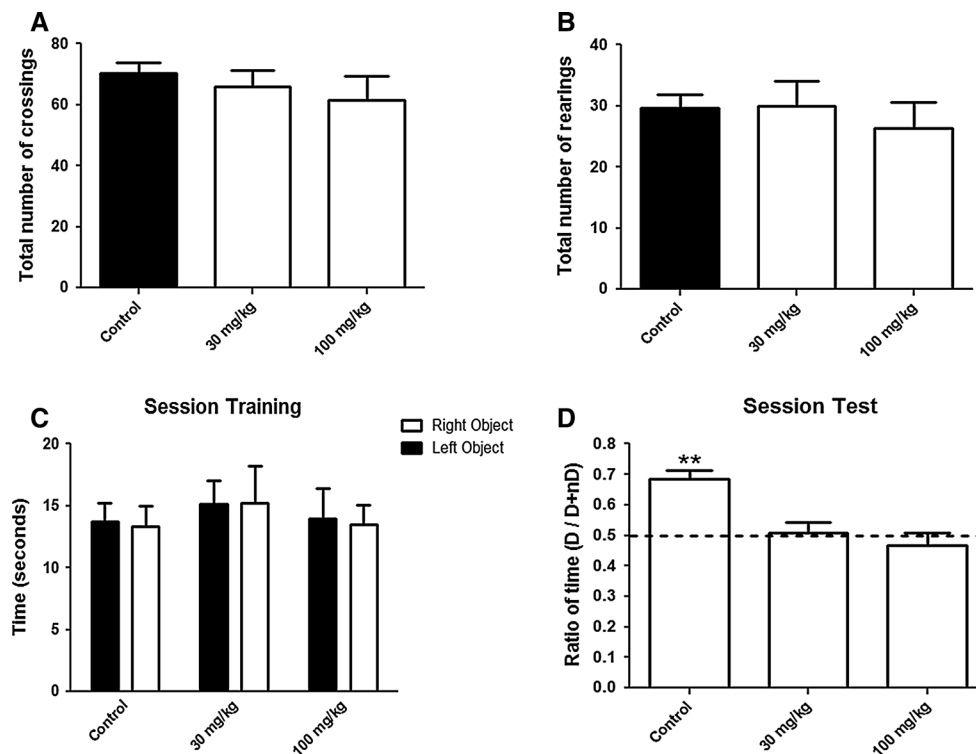
Fig. 2 Effect of repeated malathion exposure on object location memory. Two doses of malathion (30 and 100 mg/kg), dissolved in saline solution, were administered subcutaneously (s.c.) once a day for 15 consecutive days. Control mice group received injections of saline solution. Locomotor (**a**) and exploratory (**b**) activities, as well as the time spent in the objects exploration in the training test (**c**) and the discrimination ratio in the test session (**d**) were evaluated 24 h after the last malathion administration. The results are expressed as the total number of crossings (**a**), total number of rearings (**b**), the time spent in both objects in the training session (**c**) and (**d**) the ratio between time spent in the displaced and the total time spent in both objects (displaced + nondisplaced). Data are presented as mean ± SEM ($n = 10$ animals per group). **$**p < 0.01$ versus chance level (50 % of displaced object investigation in the session test)

of malathion presented significant inhibition of the NADH dehydrogenase activity in the hippocampus compared with vehicle-treated control group ($p < 0.001$) (Fig. 3a). No significant malathion effects were observed toward hippocampal complex II and complex IV activities (Fig. 3b, c). Interestingly, the hippocampal activity of NADH dehydrogenase was positively correlated with the object location task (Pearson's coefficient = 0.6902; $p = 0.0015$) (Fig. 4), suggesting a potential link between damaged hippocampal respiratory chain function and impaired cognitive performance.

Repeated exposure to malathion causes glial activation and stimulates proapoptotic proteins in the hippocampus, but does not affect synaptophysin and choline acetyl transferase levels

The presented results pointed to energetic mitochondrial dyshomeostasis (without significant AChE inhibition) in the hippocampus as a likely cause of malathion (30 mg/kg)-induced spatial memory impairment. In order to further investigate mechanisms mediating this impairment, we

focused on events downstream to mitochondrial dysfunction and investigated the potential occurrence of glial activation, apoptosis, synaptic dyshomeostasis and a marker of cholinergic neurons. Figure 5 shows that the treatments with malathion (30 and 100 mg/kg) did not affect the levels of synaptophysin, a protein marker of synapses, in the hippocampus [$F(2.9) = 1.626$, $p = 0.2495$] (Fig. 5), suggesting that hippocampal synaptic homeostasis seems to be not involved in the behavioral effects induced by malathion. A significant gliosis (detected by increased GFAP levels) was observed in the hippocampus of malathion-exposed animals (Fig. 6). One-way ANOVA demonstrated significant effect of malathion treatments on the levels of GFAP in the hippocampal CA1 [$F(2.9) = 7.94$, $p < 0.05$], CA3 [$F(2.9) = 12\ 45$, $p < 0.01$] and dentate gyrus (DG) [$F(2.9) = 16.44$, $p < 0.01$] subregions. Post hoc analyses showed that malathion significantly increased the levels of GFAP in CA1 [30 and 100 mg/kg, $p < 0.05$], CA3 [30 mg/kg, $p < 0.01$ and 100 mg/kg, $p = 0.055$] and DG [30 and 100 mg/kg, $p < 0.01$] subregions of the hippocampus when compared with the control group (Fig. 6a, b). Likewise, the levels of proapoptotic proteins Bax [$F(2.9) = 12.69$,

Fig. 3 Effect of repeated malathion exposure on **a** NADH dehydrogenase activity, **b** respiratory chain complex II and **c** respiratory chain complex III in the hippocampus. Two doses of malathion (30 and 100 mg/kg), dissolved in saline solution, were administered subcutaneously (s.c.) once a day for 15 consecutive days. Control mice group received injections of saline solution. Enzyme activities are expressed as % of control ($n = 6$ animals per group). Each value represents the mean $\pm$ SEM. ***$p < 0.001$ when compared with the control treated with saline for the analysis of variance (ANOVA) followed by Duncan's multiple-range test
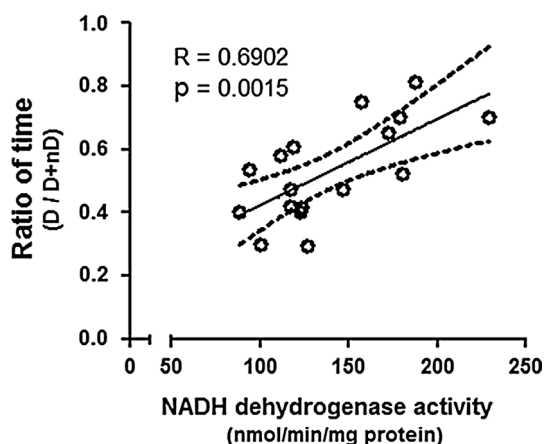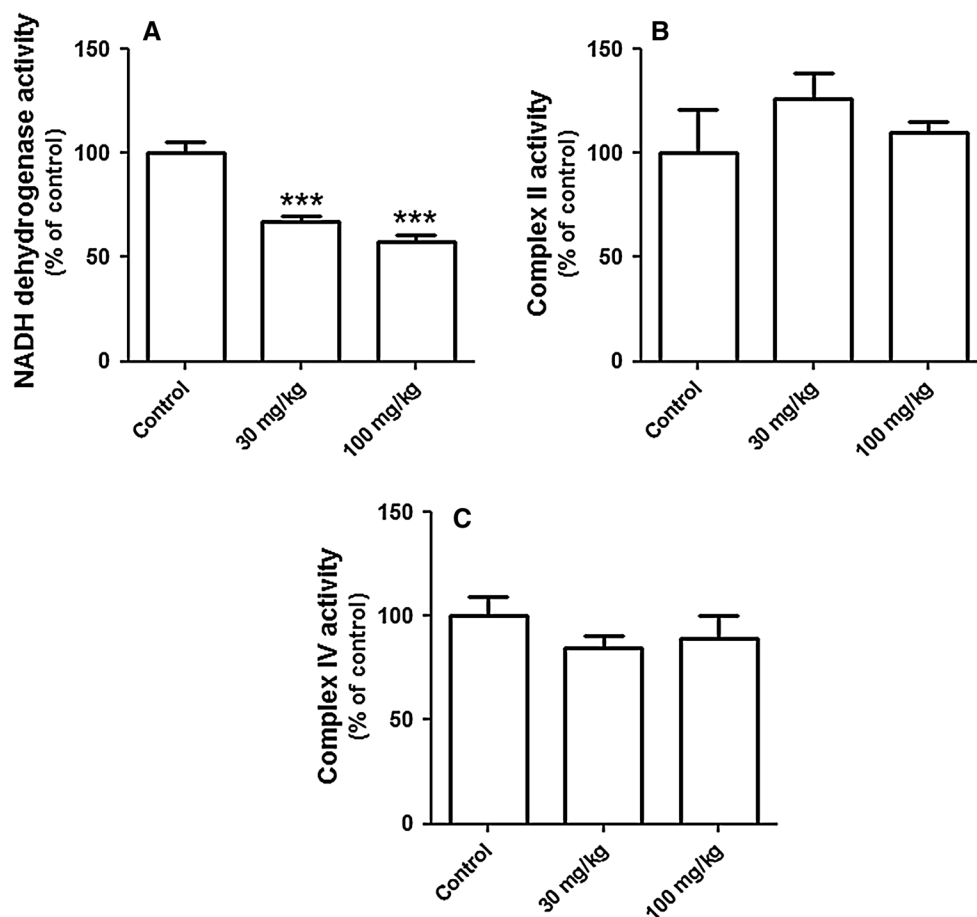
Fig. 4 Malathion-induced decrease in hippocampal NADH dehydrogenase activity is significantly correlated with object location task. NADH dehydrogenase activity was plotted versus the discrimination ratio in the object location task. The relationship between changes in the enzymatic activity and the decline in the spatial memory was significant ($r = 0.6902$, $p < 0.01$)

$p < 0.05$] and Bak [$F(2.9) = 12.69$, $p < 0.05$], which are proteins involved in apoptotic pathways, were significantly increased in the hippocampus of malathion-exposed animals (Fig. 7). Post hoc analyses showed that malathion caused a significant increase in the levels of Bax [30 mg/kg, $p < 0.05$ and 100 mg/kg, $p < 0.01$ (Fig. 7a)] and Bak [30 mg/kg, $p < 0.05$ and 100 mg/kg, $p < 0.01$ (Fig. 7b)] compared with controls. These results suggest that the damage of mitochondrial energy metabolism induced by malathion, even at a dose that does not affect the AChE activity (30 mg/kg), could be activating the apoptotic pathway involving the mitochondria. On the other hand, the hippocampal levels of ChAT, a marker of cholinergic neurons, were not altered after exposure of 30 or 100 mg/kg of malathion [$F(2.9) = 0.4422$, $p = 0.6559$] (Fig. 8). These results suggest that the cognitive deficit induced after malathion exposure is not necessarily related to the loss of hippocampal cholinergic neurons (commonly associated with mechanisms of learning and memory).

Discussion

The present results are the first to show that the exposure to repeated doses of the OP malathion (over a 15-day period) caused impaired spatial memory and hippocampal biochemical changes (decreased mitochondrial complex
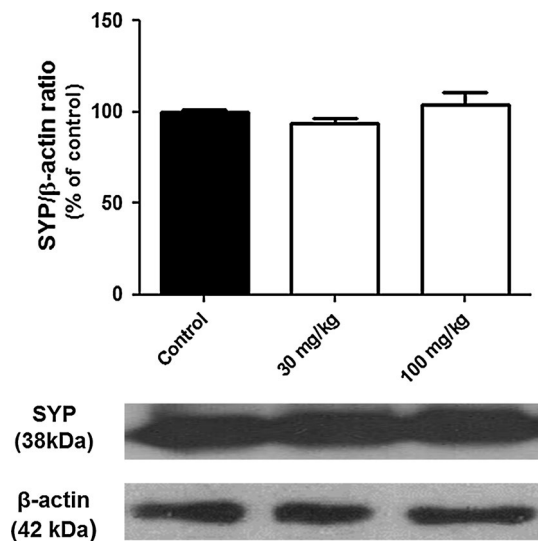
Fig. 5 Repeated malathion treatment does not modify the expression of synaptic protein synaptophysin. Two doses of malathion (30 and 100 mg/kg), dissolved in saline solution, were administered subcutaneously (s.c.) once a day for 15 consecutive days. Control mice group received injections of saline solution. Densitometric levels of synaptophysin protein were normalized by β-actin, and the image shows the levels of these proteins in the control and malathion (30 and 100 mg/kg) groups. Data are expressed as % of control ($n = 4$) and represented as mean ± SEM for the analysis of variance (ANOVA) followed by Duncan's multiple-range test

I activity, astroglial activation and increased levels of proapoptotic proteins Bax and Bak) in mice with neither decreased hippocampal AChE activity nor overt signs of cholinergic toxicity. These results suggest a possible dissociation between the malathion-induced neurotoxicity from its well-known cholinergic effects, shedding light on additional (noncholinergic) mechanisms mediating neurological injury induced by this OP pesticide. Anyway, the present results do not allow for stating whether the observed results are persistent and additional analyses at longer time points following the exposures are necessary to study the potential reversibility of the mentioned effects.

OP pesticides-induced inhibition of AChE activity has been recognized as a primary mechanism underlying the neurotoxicity elicited by these compounds. Acute cholinergic toxicity is mediated by overstimulation of nicotinic and muscarinic receptors, resultant from AChE inhibition and increased acetylcholine levels in cholinergic synapses (Costa 2006). Despite the long history of research on the neurotoxicity of OP, the current knowledge on mechanisms other than AChE inhibition is scarce. Nevertheless, some lines of evidence suggest that the neurotoxicity associated with chronic (or repeated) exposures to OP can occur without antecedent cholinergic symptoms, which likely are not dependent on AChE inhibition (Ray and Richards 2001;

Singh and Sharma 2000). Our results are in line with such observations, corroborating them.

The 30 mg/kg dose of malathion used in this study did not cause significant AChE inhibition in the adult mouse hippocampus, when given systemically over a 15-day period. In other words, we did not see considerable buildup of AChE inhibition following the last malathion injection. This data suggests, in addition to no significant effect of malathion on the levels of ChAT, that the central cholinergic pathways probably are not involved in the neurotoxicity induced by repeated low dose of malathion. On the other hand, the data found in the literature normally show an additive effect of AChE inhibition with repeated OP exposures, even in the absence of overt clinical signs of cholinergic toxicity (Ellison et al. 2011; Speed et al. 2012; Terry et al. 2003, 2007). In fact, the AChE inhibition observed in our long-term exposure protocol (significant only at a dose of 100 mg/kg) likely represents the consequence of cumulative phenomena, related to (1) the relative low rate of malathion metabolism/excretion (Chambers and Levi 1992) and (2) the relative long replacement time for AChE (half-life is between 3 and 12 days) (Krejci 2006). Furthermore, it has been shown for a long time that the chronic exposure of animals to OP usually results in the development of tolerance to their cholinergic effects, and an essential component of this compensation is the down-regulation of ACh receptors (Costa et al. 1982). According to this view, the repeated exposure to AChE inhibitors may cause adaptive changes in the nervous system that permit the animal to maintain, or regain, normal function despite chronically suppressed AChE activity (Russell and Overstreet 1987). Taking into account that the AChE turnover half-life is between 3 and 12 days (Krejci 2006), it is possible that small doses of OP compounds may result in a significant inhibition of AChE activity after a chronic exposure protocol. Actually, this kind of intoxication is the most common found in the literature nowadays. However, in our study, malathion exposure to 30 mg/kg during 15 days, which did not significantly change whole hippocampal AChE activity, caused significant behavioral and hippocampal biochemical changes. These results clearly disassociate malathion-induced neurotoxicity from its well-known effects toward AChE. Anyway, it is important to note that the method used in this study for the detection of AChE (Ellman et al. 1961) measures the total AChE activity. In other words, both soluble (located at the synapses) and insoluble (located at the membranes) enzymes can be detected. This feature makes this method effective to measure the degree of AChE inhibition by OP compounds, despite it does not provide information about the subcellular enzyme localization.

Cognitive deficits have been reported as common and persistent behavioral outcomes in individuals exposed to OP (Chen 2012). Epidemiologic evidence suggests that

Fig. 6 Micrographs showing immunofluorescence for glial fibrillary acidic protein (GFAP) in the hippocampal regions CA1, CA3 and dentate gyrus (DG) of adult mice. Two doses of malathion (30 and 100 mg/kg), dissolved in saline solution, were administered subcutaneously (s.c.) once a day for 15 consecutive days. Control mice group received injections of saline solution. **a** Immunofluorescence images for GFAP and **b** immunofluorescence quantification. All images were obtained in confocal microscope Olympus FV-1000. *Scale bar* 25 mm. Data are expressed as mean ± SEM. ***$p < 0.001$ compared with control group treated with saline for the analysis of variance (ANOVA) followed by Duncan's multiple-range test
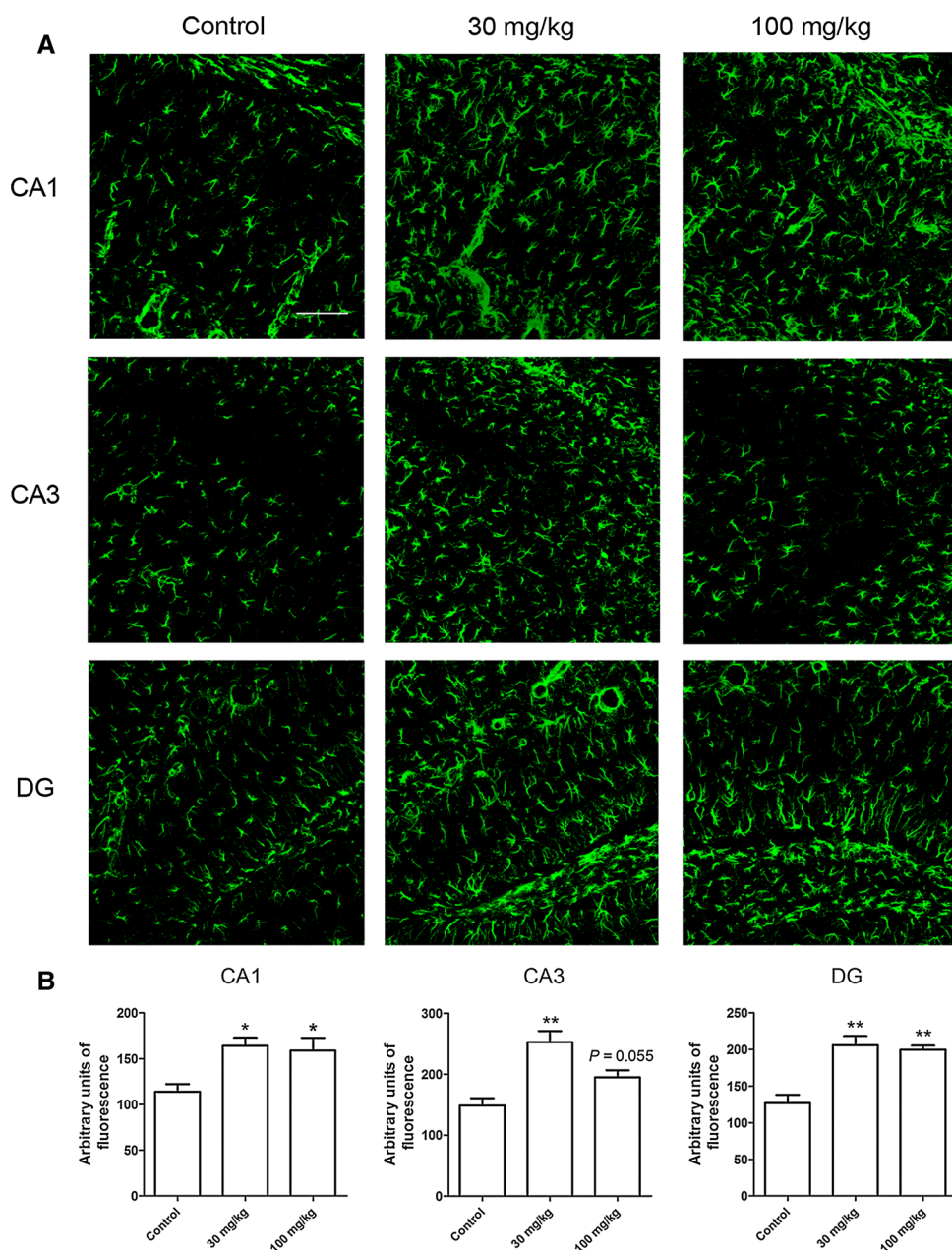
long-term "subclinical" exposures to OP pesticides can lead to long-lasting neurological impairment in adults (Jamal et al. 2002) and in children (Jurewicz and Hanke 2008; Rauh et al. 2006). However, the low chronic exposure to different OP compounds is very contradictory. It has been pointed out that the pattern of symptoms varies with different OP and cannot be explained by inhibition of AChE alone, suggesting the involvement of additional targets (Lockridge et al. 2005; Moser 1995; Pope 1999). For example, Terry et al. (2003) showed that a daily treatment with chlorpyrifos (CPF) for 2 weeks resulted in a decrement in the water maze performance, which was evident from 1 to 5 days after CPF discontinuation. However,

2 weeks after discontinuation, the performance was similar to the controls. Further, it has been shown that intermittent exposure to CPF for 30 days, resulted in a decrement in the water maze performance, which was evident for up 2 weeks after CPF discontinuation (Terry et al. 2007). Prendergast et al. (1997) has demonstrated that spatial learning is impaired for up to 21 days after withdrawal from a 14-day treatment regimen with low doses of diisopropylfluorophosphate (DFP) and that this effect was associated with the AChE activity recovery. As already mentioned, the present experimental protocol caused significant impairments in spatial memory in the object location task (which is highly dependent upon the hippocampal system (Assini
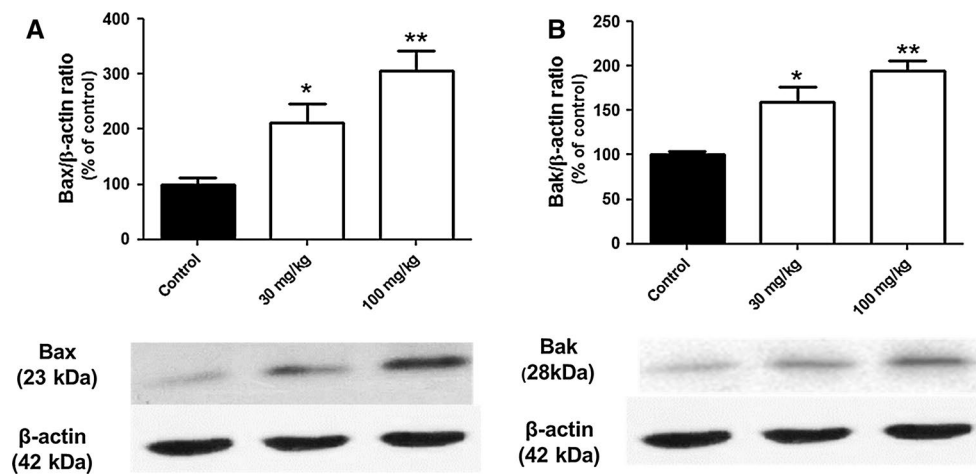
Fig. 7 Repeated malathion treatment increases the expression of proteins associated with apoptosis (Bax and Bak). Two doses of malathion (30 and 100 mg/kg), dissolved in saline solution, were administered subcutaneously (s.c.) once a day for 15 consecutive days. Control mice group received injections of saline solution. Densitometric levels of Bax (**a**) and Bak (**b**) proteins were normalized by β-actin, and the images show the levels of these proteins in the control and malathion (30 and 100 mg/kg) groups. Data are expressed as % of control ($n = 4$) and represented as mean ± SEM. *$p < 0.05$ and **$p < 0.01$ compared with control group treated with saline for the analysis of variance (ANOVA) followed by Duncan's multiple-range test
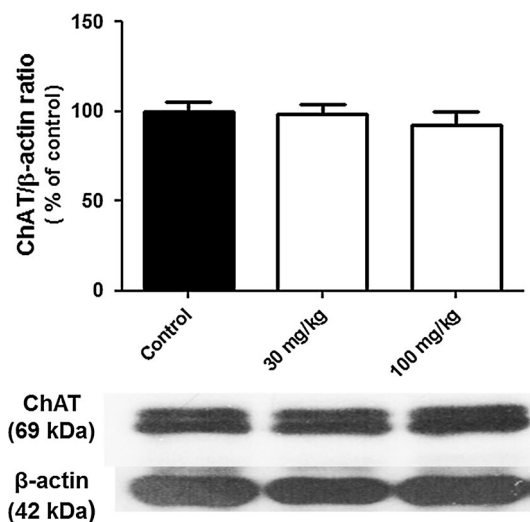

Fig. 8 Repeated malathion treatment does not affect the expression of choline acetyltransferase (ChAT), a marker enzyme of cholinergic neurons. Two doses of malathion (30 and 100 mg/kg), dissolved in saline solution, were administered subcutaneously (s.c.) once a day for 15 consecutive days. Control mice group received injections of saline solution. Densitometric levels of ChAT protein were normalized by β-actin, and the image shows the levels of these proteins in the control and malathion (30 and 100 mg/kg) groups. Data are expressed as % of control ($n = 4$) and represented as mean ± SEM for the analysis of variance (ANOVA) followed by Duncan's multiple-range test

et al. 2009; Broadbent et al. 2010), even at a dose that did not cause significant inhibition of hippocampal AChE activity and evident signs of cholinergic toxicity. These results are in agreement with Valvassori et al. (2007), who

have shown that OP exposure can impair aversive-memory retention in rats without affecting hippocampal AChE activity. However, the mechanisms of this noncholinergic toxicity induced by malathion, likely responsible for the cognitive deficits, have not yet been reported. In this context, we observed a significant decrease in the activity of hippocampal mitochondrial complex I in mice exposed to the 30 mg/kg dose (Fig. 3), which also displayed impaired spatial memory (Fig. 2d). Mitochondrial complex I, which is the first protein component of the mitochondrial respiratory chain (playing a crucial role in ATP production and mitochondrial function in general), has been identified as a potential target for neurotoxins that contribute to the development of neurodegenerative processes (for a review, see Schapira (2010)). Although it is not possible to propose a cause–effect relationship between decreased hippocampal complex I activity and memory impairment in malathion-exposed mice, our data (added to the current literature) suggest that this protein might represent an important noncholinergic target in malathion-induced neurotoxicity. This idea is reinforced by the significant correlation between hippocampal complex I activity and spatial memory in the object recognition task (Fig. 4).

Most studies of moderate pesticide exposure have found increased prevalence of neurological symptoms and changes in neurobehavioral performance, which reflects cognitive and psychomotor dysfunction (Kamel and Hoppin 2004). An extensive literature suggests that pesticide exposure may increase the risk of Parkinson's disease (PD), where the decrease in mitochondrial complex I activity may be closely related with this disorder (Binukumar et al.

2010; Manthripragada et al. 2010). Occupational pesticide exposure was also associated with mild cognitive dysfunction (Bosma et al. 2000), amyotrophic lateral sclerosis (ALS) (Nelson 1995–1996) and dementia as Alzheimer's disease (AD) (McDowell et al. 1994). In our experimental model, the malathion-induced memory deficits were not followed by changes in the hippocampal levels of synaptophysin (Fig. 5), the major synaptic vesicle protein (a specific pre-synaptic marker), suggesting that the number of synapses were not affected by malathion treatment. On the other hand, it is possible that the significant decrease in mitochondrial function (decrease in complex I activity) might have affected the quality of hippocampal synapses and contributed to cognitive decline. In this regard, Reddy and Beal (2008) demonstrated that defective mitochondria found in the synaptic terminals may be a critical factor that contribute to synaptic damage and cognitive decline observed in people with AD.

There is a growing body of evidence suggesting a link between OP exposure and inflammation (Banks and Lein 2012). Low-level repeated exposure to OP has been shown to induce inflammatory responses in cultured astrocytes (Mense et al. 2006) and to upregulate inflammatory cytokines in vivo (Singh and Jiang 2003). Acute soman (a nerve agent OP) intoxication upregulates the glial fibrillary acidic protein (GFAP) expression and activates microglia in many regions of the brain, including the hippocampus (Angoa-Perez et al. 2010; Baille-Le Crom et al. 1995; Collombet et al. 2005; Zimmer et al. 1997). Similarly, chronic administration of the OP CPF increases GFAP expression in the hippocampus of rats, without inhibiting serum cholinesterase (Lim et al. 2011). Interestingly, the overproduction of proinflammatory cytokines and excessive inflammation in the central nervous system is characteristic of many neurodegenerative diseases (Blasko et al. 2004; Eikelenboom et al. 1998; Whitton 2007) and, in this scenario, has been proposed that mitochondria may exert a crucial role (Di Filippo et al. 2010). With particular emphasis on malathion, the administration of low levels of this OP for 14 or 90 days increased macrophage function and mast cell degranulation in mice (Rodgers and Xiong 1997a, b); nonetheless, studies linking malathion exposure and neuroinflammation have not been reported in the literature. Our results showed an increase in the GFAP expression (Fig. 6), a protein expressed in the astrocytes and a sensitive and early biomarker of neurotoxicity, in the hippocampal CA1, CA3 and dentate gyrus (DG) subregions, after a 15-day treatment period with malathion (30 and 100 mg/kg). These results indicate that repeated exposure to malathion can trigger astroglial response in different regions of the hippocampus even in the absence of AChE inhibition. The present result also suggests a dissociation between cholinergic from noncholinergic events in malathion-induced

neurotoxicity, pointing to increased astroglial response as a significant cellular event.

Recently, Abdel-Rahman et al. (2004) showed that subacute dermal exposure to malathion (44 mg/kg, for 30 days) induced significant neurobehavioral deficits and significant reduction in the density of surviving neurons in the DG, CA1 and CA3 subfields of the hippocampus, midbrain, brainstem and cerebellum in rats (Abdel-Rahman et al. 2004). Another study using adult mice injected with low-dose of malathion (40 mg/kg, *i.p.*, for 14 days) showed a significant reduction on the dendritic morphology of neurons from hippocampus and prefrontal cortex, brain regions implicated in cognition and behavior (Campana et al. 2008). In this study, we evaluated whether a 15-day treatment period with malathion could change the levels of two major proteins involved in the mitochondrial apoptotic pathway, Bax and Bak (Dewson and Kluck 2009). These proteins are members of proapoptotic Bcl-2 family and act as the essential gateway to the intrinsic cell death pathway operating at the mitochondria, by perturbing the permeability of the mitochondrial outer membrane and facilitating the release of cytochrome c (Hetz et al. 2005; Sheridan et al. 2008). Our results showed a significant increase in the levels of both proteins in the hippocampus after the treatment with 30 and 100 mg/kg of malathion (Fig. 7). Some studies in cell cultures show that OP compounds induce apoptosis through mitochondria-dependent pathway. For example, Saleh et al. (2003) showed that the OP paraoxon caused a direct effect on mitochondria by disrupting its transmembrane potential and releasing the cytochrome c, in cultured murine lymphocytes cells. Masoud et al. (2003) suggested that the cytotoxicity of malathion in cultured mouse fibroblasts is mediated through caspase-dependent apoptosis. Furthermore, Brocardo et al. (2007) reported that malathion-induced apoptosis in the DG region of the hippocampus of rats in doses that did not affect the AChE activity. Based on these findings, it is suggested that the mechanism of cytotoxicity observed upon low exposures to malathion may be related, at least in part, to the induction of apoptosis. In this regard, it is important to mention that specific apoptotic pathways are resultant from previous mitochondrial dysfunction, which corroborate with the observed decrease in the hippocampal complex I activity in malathion-exposed mice (Fig. 3a).

Because cholinergic neurons play a key role in the mechanisms of memory and in the pathophysiology of OP-induced neurotoxicity (Bajgar 2004; Nordberg 2001), we evaluated the hippocampal levels of ChAT in malathion-exposed animals. This enzyme, which catalyzes a pivotal reaction in acetylcholine biosynthesis, represents a specific marker for the functional state of cholinergic neurons. Nevertheless, its hippocampal levels were not changed in malathion-exposed mice, suggesting that the viability of

the hippocampal cholinergic neurons was not affected following treatment with this OP in our experimental protocol. Our results indicate that the proapoptotic events (Bax and Bak upregulation) were likely detected at early periods when significant cholinergic neuronal death was not detected. In fact, we cannot rule out the idea that cholinergic neuronal death could occur in more advanced stages of malathion intoxication, but additional studies are necessary to answer this question.

In summary, our results are the first to show that the repeated low-dose malathion exposure (over a 15-day period) caused impaired spatial memory and hippocampal biochemical changes (decreased mitochondrial complex I activity, astroglial activation and increased levels of proapoptotic proteins Bax and Bak) in mice with neither decreased hippocampal AChE activity nor overt signs of cholinergic toxicity. These findings suggest that the malathion-induced impairments in spatial memory and discrimination are probably the result of the mitochondrial dysfunction, astrogliosis and activation of proapoptotic proteins in the hippocampus. As already mentioned, these results strongly suggest a dissociation between the malathion-induced neurotoxicity from its well-known cholinergic effects, shedding light on additional (noncholinergic) mechanisms mediating neurological injury induced by this OP pesticide. When extrapolated to humans, such experimental results likely related to the neurobehavioral and cognitive deficits observed in individuals chronically exposed to malathion.

Conflict of interest The authors declare that there are no conflict of interest.

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