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Toxicology

Neurochemical dysfunction in motor cortex and hippocampus impairs the behavioral performance of rats chronically exposed to inorganic mercury

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ABSTRACT

Chronic exposure to mercury chloride ($HgCl_2$) has been shown to promote oxidative stress and cell death in the central nervous system of adult rats displaying motor and cognitive impairments. However, there are no investigations about neurochemical function after this type of exposure in rodents that may be associated with those behavioral changes already reported. Thus, the aim of this study was to analyze glutamatergic and GABAergic dysfunctions in the motor cortex and hippocampus of adult rats, in a model of chronic exposure to $HgCl_2$ in. Twenty rats were exposed to a daily dose of 0.375 mg/kg for 45 days. After this period, they were submitted to motor and cognitive functions tests and euthanized to collect the motor cortex and hippocampus for measurement of mercury (Hg) levels in the parenchyma and neurochemical assays for analysis of glutamatergic and GABAergic functions. It was observed that chronic exposure to $HgCl_2$ promoted increase in total Hg levels in these two brain areas, with changes in glutamatergic transport, but without changes in GABAergic transport. Functionally this model of exposure caused the decrease of the spontaneous motor locomotion and in the process of learning and memory. In this way, our results provide evidences that glutamatergic neurochemical dysfunction can be pointed out as a strong causal factor of motor and cognitive deficits observed in rats exposed to this $HgCl_2$.

1. Introduction

The mercurial elements present a complex biogeochemical cycle, once they exist in different chemical forms and oxidized states in aquatic and terrestrial ecosystems [1,2]. Mercury (Hg) is a metal that can be found in two major categories in environment: inorganic forms (elementary or metallic form, Hg° and mercury chloride, $HgCl_2$) and organic species (mainly represented by methylmercury, MeHg) [2], and depending on the chemical nature, it has different susceptibility to bioaccumulate in the body after exposure.

The natural and anthropogenic emissions of Hg represent a high risk to human health, becoming a global concern [3]. Besides, Hg is among the metals of high density that are quite toxic in low concentrations [4–7]. This metal is known to be used in gold-mining activity [8], contaminating the environment and directly exposing humans by inhalation or feeding and increase the risk of inducing disasters as those occurred in Japan [9] and Iraq [10,11].

Inorganic Hg has been used in the manufacture of cosmetics and personal care products, exposing humans to its toxic effect [12–15] and others studies that show its role in renal [16–18], hepatic [19], cardiovascular impairments [20,21] and high neurotoxic effects [22–24].

In previous studies [25,26], we observed that a long-term exposure to $HgCl_2$, besides increases Hg levels in neural parenchyma, also compromises motor and cognitive abilities and cellular death. However, there are no data on literature about the modulation of neurochemical homeostasis after the exposure to this toxicant. In this way, the present study aimed to analyze whether the chronic exposure to $HgCl_2$ is able to promote neurochemical modulation in motor cortex and hippocampus of adult rats associated to functional impairments.

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2. Materials and methods

2.1. Ethics statement

The animal protocols used in this work were approved by the Ethics Committee on Experimental Animals of Federal University of Pará, under the protocol BIO139-13. They are in accordance with NIH Guide for the Care and Use of Laboratory Animals and national law for laboratory experimentation [27].

2.2. Animals and experimental groups

Male *Wistar* rats (n = 20; 90 days old) were obtained from the Federal University of Pará (UFPA) and kept in collective cages (four animals per cage). Animals were maintained in a climate-controlled room on a 12 h light/dark cycle (lights on 7:00 a.m.), with food and water *ad libitum*. Distilled water or $HgCl_2$ (dose of 0.375 mg/kg/day) were orally administered, 10 animals/group, over a period of 45 days, with weekly weighting for dose adjustment, according to a procedure previously established by our group in Teixeira et al. [28].

The experimental design is summarized in Fig. 1.

2.3. Behavioral assays

Twenty-four hours after the last exposure to $HgCl_2$, animals were led to the assay room and acclimated for at least 1 h before the beginning of the behavioral test with attenuation of noise levels and low illumination (12 lux).

2.3.1. Open field test

Spontaneous locomotor activity was evaluated through the open field test. Briefly, animals were individually positioned at the center of a dark wooden arena (100 × 100 × 40 cm virtually divided into 25 squares of 20 × 20 cm by Anymaze software Stoelting Co., UK) and allowed to free ambulation for 5 min. Total, central and peripheral distance travelled were evaluated [29].

2.3.2. Morris water maze

Hg intoxication disrupts cognitive functions [28]. Thus, we employed the Morris Water Maze test to analyze learning, spatial and long-term memory impairments [30]. Firstly, animals were submitted to the training session, which consists of four consecutive exposures (each section cut-off 120 s) to a circular tank (150 cm × 60 cm) filled with water dark-colored by a nontoxic soluble dye (45 cm), with 5 min of interval. Briefly, the equipment was virtually divided into four quadrants (Q1-Q4), where in the Q4 (target quadrant was randomly chosen) it was positioned an acrylic platform (10 cm^2), hidden under the water at a height of 1 cm below water level. During the training section, the animals were able to stay on the secure platform for 20 s. In addition, the animal start position in each session was modified, according to the cardinal orientation (North, South, West, East). Animals that were not able to find the platform by themselves were gently conducted to the

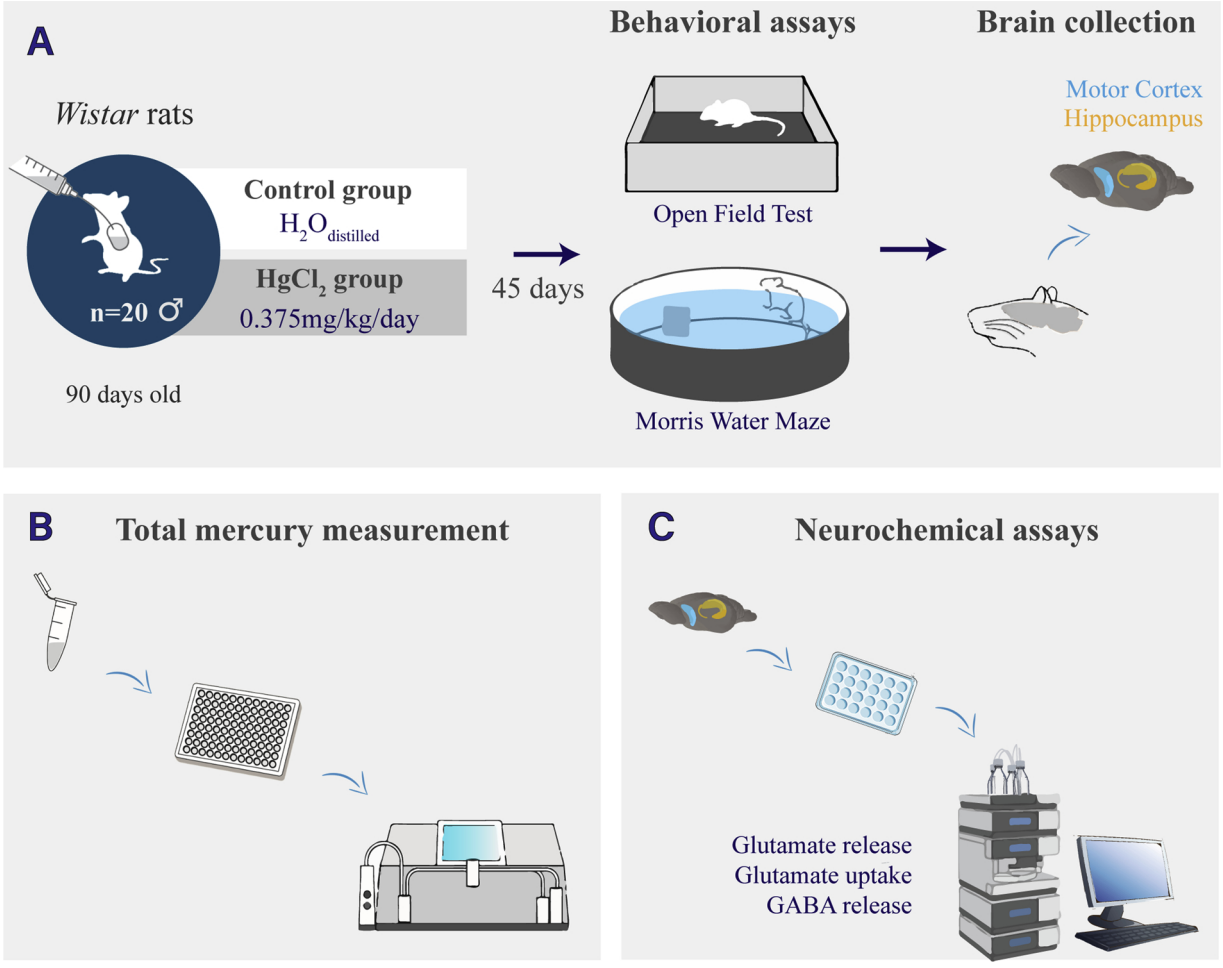

Fig. 1. Sample description and experimental stages: (A) The sample and model of exposure to $HgCl_2$; (B) the behavioral evaluation through (1) Open Field test and (2) Morris Water Maze; (C) euthanasia and brain collection for analyzes in motor cortex and hippocampus; (D) biochemical analyses based on (1) mercury levels and (2) determination of glutamate and GABA uptake and release.

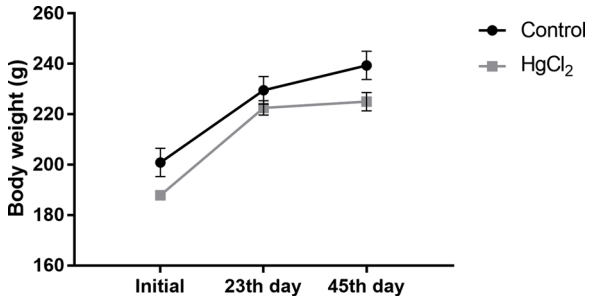

Fig. 2. Effects of chronic exposure to $HgCl_2$ on body weight of adult *Wistar* rats (g). Results are expressed as mean ± standard error. No significant differences ($p > 0.05$) between groups in any time (One-way ANOVA analysis for repeated measures).

platform. The escape latency time (ELT) to find the underwater platform was adopted as an index of learning. On the test trial (24 h after training section), the platform was removed and the animals were placed on the Q1, in which the latency spent in Q4, as well as the latency to arrive in Q4 (ALT) were considered as parameter of long-term /spatial memory [31,32].

2.4. Fresh samples collection

Following behavioral assays, the animals were euthanized by cervical dislocation and their brains immediately removed. The hippocampus and motor cortex were dissected into two hemispheres in order to quantify e total Hg (THg) (submitted to dry ice afterward) and neurochemical assays, both described below.

2.5. THg levels measurement

For THg measurement, each sample of motor cortex were weighted (0.5 g maximum of wet weight) in a sample digestion bottle, and 1 mL of distilled water, 2 mL of nitric acid-perchloric acid with HNO_3-$HClO_4$ (equal proportions) and 5 mL of sulfuric acid (H_2SO_4) were sequentially added, followed by heat treatment on a hot plate (200–230 °C) for 30 min. THg content in the samples was estimated by wet digestion, reduction and cold vapor atomic absorption spectrometry (CVAAS) (Semi-automated Mercury Analyzer, model Hg-201, Sanso Seisakusho Co. Ltd., Tokyo, Japan); this method was as previously described by Suzuki et al. [33]. The detailed methodology of this analysis was described in previously published work. In this work, we used this analysis as counterproof of reproducibility of this intoxication model previously used by our group [25,26,28,34].

2.6. Neurochemical assays

Hippocampus and brain motor cortex were quickly dissected and transferred to culture plates containing HANK´s sodium solution (128 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 12 mM glucose and 20 mM HEPES) pH 7.4. The release assays were performed by tissues incubation in Hank´s solution for 20 min at 37 °C in CO^2 oven. After the incubation period, the medium was collected and stored at -20 °C for further glutamate and GABA quantification utilizing high performance liquid chromatography (HPLC). The protocol used for glutamate uptake assay was performed as described previously by Moraes et al. [35].

2.6.1. Determination of glutamate and GABA uptake and release levels by HPLC

HPLC system (Shimadzu - model LC-10 AD, Tokyo, Japan was coupled to a fluorescence detector (RF-10AXL) and LC-20AT pump. Sample separation was made using an analytical column (C18 – Shimpack VP-ODS 4.6 * 250LC, internal diameter 4.6 mm) which was maintained at 29 °C with a thermostatic system (CTO-20a). The mobile phase A (50 mM sodium acetate, methanol 5% and 2-propanol, pH 5.67) and mobile phase B containing (70% (v/v) methanol) were used to sample elution through C18 column. Neurotransmitter detection was made utilizing fluorescence detector set at 340 nm (excitation wavelength) and 460 nm (emission wavelength). The concentrations of glutamate and GABA were quantified considering a standard curve and the values were normalized per sample protein content. The protein content was measured using Bradford's method.

2.7. Statistical analysis

All values are expressed as means ± S.E.M. For normality analyses, we performed the Shapiro-Wilk test. The body weight was analyzed by one-way ANOVA test for repeated measures. Statistical comparisons between groups were performed using the Student's t-test. Values of $p < 0.05$ were considered statistically significant. The GraphPad Prism 7.0 (San Diego, CA, USA) software was used to perform statistical analyses.

3. Results

3.1. Effects of $HgCl_2$ chronic exposure on body weight of rats

During the period of exposure, the animals of control group and the animals exposed to $HgCl_2$ had gained weight ($p < 0.0001$), but no difference after the daily exposure to 0.375 mg/kg/day to $HgCl_2$ for 45 days was observed ($p > 0.05$) (Fig. 2).

3.2. A long term exposure to 0.375 mg/kg/day of $HgCl_2$ is capable of promoting Hg deposits on motor cortex and hippocampus of adult rats

$HgCl_2$ is able to cross the blood-brain barrier and increase Hg levels in CNS areas, as motor cortex and hippocampus of adult animals, being found about 30–40% more metal on exposed group (Motor cortex: $HgCl_2 = 0.029 \pm 0.004$; Hippocampus: $HgCl_2 = 0.043 \pm 0.003$) than in the control (Motor cortex: Control = 0.015 ± 0.001; Hippocampus: Control = 0.012 ± 0.0002; $p < 0.0001$; Fig. 3).

3.3. $HgCl_2$ exposure causes glutamate levels changes, but not on GABA levels on motor cortex and hippocampus of rats

Our results have shown that $HgCl_2$ exposure induces significant increase in glutamate uptake in the motor cortex of rats when compared with control (Control = 0.033 ± 005μM and $HgCl_2 = 0.053 \pm 007$μM; $p = 0.001$; Fig. 4A). We also have described similar results in the hippocampal tissue of rats exposed to $HgCl_2$ (Control = 0.04 ± 003μM and HgCl2 = 0.06 ± 009μM; $p = 0.04$; Fig. 4A). As observed in the Fig. 4B, $HgCl_2$ exposure did not promote significant changes on glutamate release in motor cortex (Control = 0.017 ± 005μM and

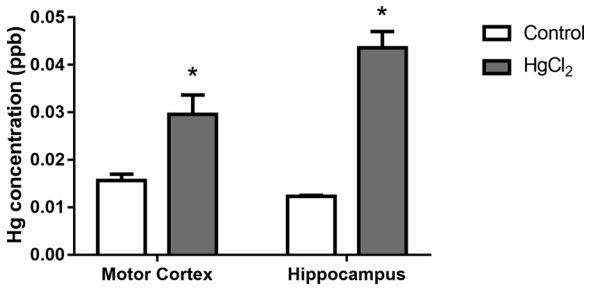

Fig. 3. The total Hg (THg) levels found in the motor cortex and hippocampus of adults *Wistar* rats after 45 days exposure to 0.375 mg/kg/day $HgCl_2$. The results are expressed as mean ± standard error of the mean (SEM). *$p < 0.05$ compared to the control group (Student's t-test).

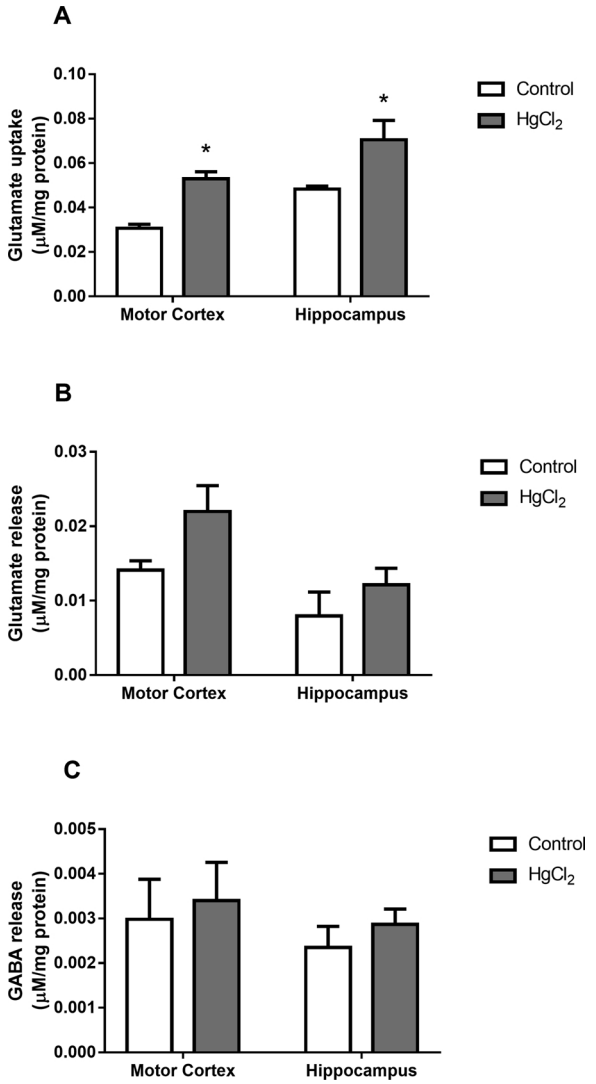

Fig. 4. Effects of chronic exposure to $HgCl_2$ on biochemical parameters analyzed in the motor cortex and hippocampus of adult *Wistar* rats. The results are expressed as mean ± standard error of; (A) glutamate uptake in the motor cortex and hippocampus; (B) glutamate release in the motor cortex and hippocampus; (C) GABA uptake in the motor cortex and hippocampus. *p < 0.05 compared to the control group (Student's t-test).

$HgCl_2$ = 0.018 ± 007µM; p = 0.5) or in hippocampal tissue (Control = 0.008 ± 006µM and $HgCl_2$ = 0.014 ± 003µM; p = 0.09).

We also evaluated GABA release in both cerebral regions of rats exposed to $HgCl_2$. Our data demonstrated that $HgCl_2$ exposure did not induce significant changes on GABA release in motor cortex of rat (Control = 0.002 ± 0.0004 µM and $HgCl_2$ = 0.003 ± 0.002 µM; p = 0.7; Fig. 4C). Similar results were observed in the hippocampal tissue of animals exposed to $HgCl_2$ when compared with control (control = 0.002 ± 0.001µM and $HgCl_2$ = 0.005 ± 0.003µM; p = 0.4; Fig. 4C).

3.4. $HgCl_2$ exposure displayed motor performance alteration as well as cognitive impairment in rats

In the motor evaluation (Fig. 5), our data revealed that chronic $HgCl_2$ exposure reduced spontaneous locomotor activity. The total distance executed by exposed animals was lower than in the control group ($HgCl_2$ = 11,90 ± 1,10; control = 16,08 ± 1,48; p = 0,04; Fig. 5A and B). To confirm the motor impairment, it was measured the peripheral distance performed and the exposed animals they got lower values in travelled distance compared to the control group. ($HgCl_2$ = 10,98 ± 0,96; control = 14,30 ± 0,98; p = 0,04; Fig. 5C). No difference was observed in the central distance performed between the two groups ($HgCl_2$ = 0,75 ± 0,14; control = 1,26 ± 0,40; p = 0,17; Fig. 5D), reinforcing that the motor impairment was not related to an anxiety-like behavior, once the central distance performed did not show differences in comparison to the control group.

In addition to behavioral status, learning and memory were assessed by Morris Water Maze (Fig. 6). Our data demonstrated that Hg levels disrupts learning across the learning phase of the test, increasing the time to find the submerse platform (P < 0.01; Fig. 6A and B). Besides, subjects exposed to $HgCl_2$ increased the latency to reach the target quadrant, as well reduced the time spent on the Q4, which reflects the mnemonic impairment (P < 0.01; Fig. 6C and D).

4. Discussion

This study shows that long-term exposure to inorganic Hg promotes Hg deposits in the cerebral parenchyma and glutamatergic dysfunction in hippocampus and motor cortex. In this way, this neurochemical dysfunction is for the first time associated toa possible causal agent for cognitive and motor dysfunctions triggered by exposure to $HgCl_2$.

Hg is a metal that presents itself in different forms in the environment and throughout life, humans can be exposed by these different species [2]. The toxic, behavioral properties and clinical manifestations of mercurial compounds are directly related to their chemical specie - elemental, organic or inorganic form [2,36,37]. The toxicity of $HgCl_2$, the inorganic form of Hg, is modulated by its pharmacokinetic properties of low liposolubility, thus, low body absorption and low passage through the blood brain barrier, in which contribute to a lower toxic effect on tissues [36,37]. However, the amount of metal that is absorbed by the action of specific cellular pathways, such as ion channels and membrane transporter proteins, causes homeostatic, biochemical and structural changes in the cell [25,26,28,34].

In this study, the model of chronic exposure to $HgCl_2$ was based on the study of Szász et al. [22], in which a model was developed associating the average daily consumption of water of rats during the mercurial exposure with the tolerance of the treated animals to different intoxication parameters. The exposure to 0.8 mg $HgCl_2$/kg body weight per day (3.99 mmol/kg per day) chosen by these authors did not cause symptoms of toxicity in pregnant rats and their offspring. For this study, we used less than half that dose, which is a relatively low concentration exposure that did not cause animal death but has been able to promote Hg deposits in organs such as the brain and salivary glands [25,26,28].

The result of this investigation shows that chronic exposure to inorganic Hg promotes the deposition of total Hg in the motor cortex and hippocampus of adult rats, corroborating our previous work [25,26,28] that reported deposits of Hg in the cerebral parenchyma. In fact, the Hg concentrations deposit were lower than that reached by CELDS of MeHg, mainly on the cortex areas, i.e. prefrontal (up to 15 ppb) and motor cortex (up to 5 ppb) [38]. Thus, we hypothesize that although its low liposolubility, inorganic Hg is able to deposit on the brain tissues, however, under lower capacity of brain deposit. Besides, such data elicited gaps related to the probable mechanisms that inorganic Hg performs to reach cerebral parenchyma to promote mercury deposits.

Possibilities in this casuistry indicate that inorganic Hg promotes interference in enzymatic activity and affects ionic movement in Na^+/K^+ ATPase in cerebral cortex layers [39], which could in fact explain their accumulation in these brain regions investigated. In addition, another hypothesis is the use of amino acid transporters, particularly the cysteine transporter, the same as MeHg used to cross the blood-brain barrier [37].

Hippocampus and motor cortex consist of brain areas that play a pivotal role on mnemonic process and motor function [40,41]. Our previous studies evaluated motor and memory parameters, proving

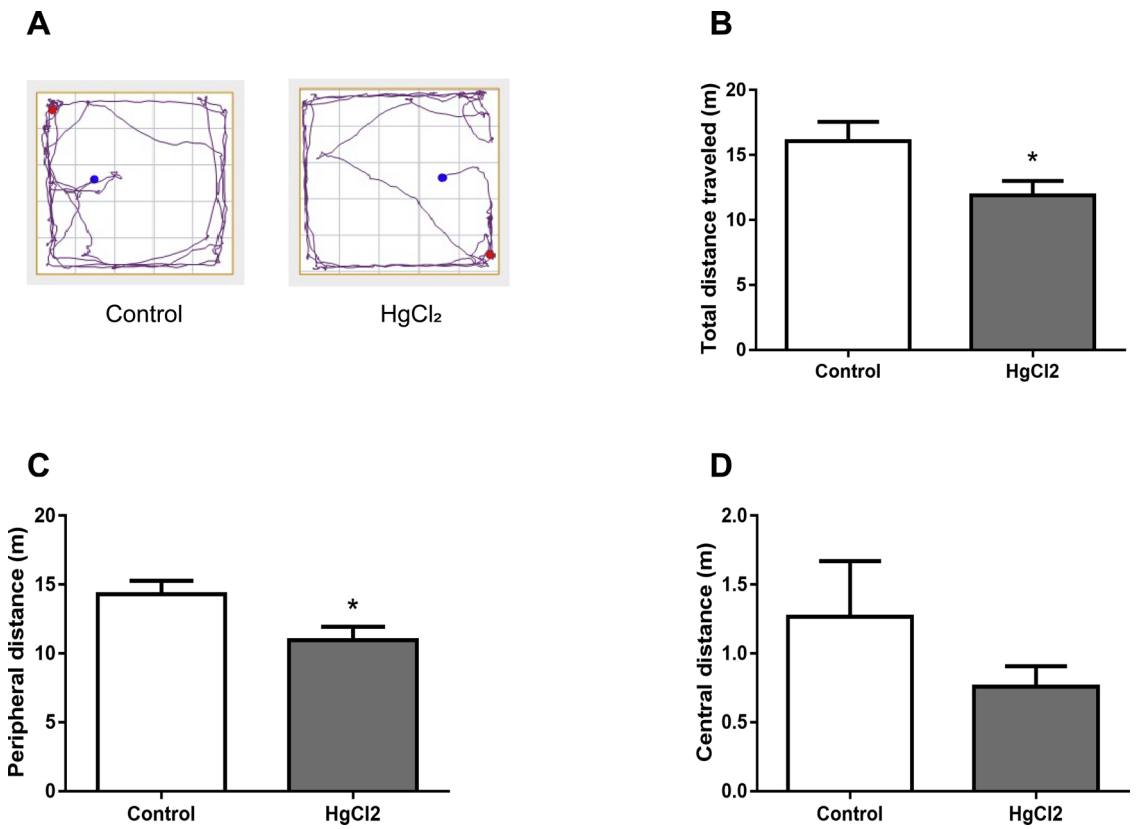

Fig. 5. Effects of chronic exposure to HgCl2 on locomotor activity of adult Wistar rats. (A) Representation of the horizontal locomotor activity analysis in the Open Field test. The results are expressed as mean ± standard error of (B) Total distance traveled (m); (C) Peripheral distance (m) and (D) Central distance performed (m). *$p < 0.05$ compared to the control group (Student's t-test).

alterations in fine motor coordination and balance [22], spontaneous locomotor activity [24] and short and long memories [21,24]. However, the present study aimed to evaluate other parameters, such as freezing time, anxiety profile and spatial memory, which ratify the results already demonstrated previously, in order to prove the harmful effects of inorganic Hg even in low doses.

The motor cortex is an important brain area involved in motor control [42,43], sensory-motor integration [44] and spontaneous motor locomotion [28,45]. The behavioral test used in this study (open field) is essentially to measure the behaviors by an animal submitted in an open space, which represents a new environment and from which there is no possibility of escape. The evaluation of rodents in an arena or open field is a procedure widely used for the purpose of observing the locomotor activity of small animals [46]. Our results showed that inorganic Hg promotes motor deficit in relation to horizontal exploration. Furthermore, the motor impairment was not related to an anxiety-like behavior, once the central distance performed in group exposed to metal did not show differences in comparison to the control. The minor total and peripheral distance travelled by exposed group reinforce this motor function impairment.

In addition to motor function, memory disruption caused by $HgCl_2$ intoxication has been reported [47]. In fact, our results demonstrate that 0.04 ppb of total Hg deposit in hippocampus was sufficient. The hippocampus is a region associated with anxiety-like behaviors, learning processes and memory and due to its connections with other limbic regions is involved in emotional behaviors [48,49]. Because of this, several behavioral tests can be used to evaluate hippocampal function [50]. In previous studies we verified that inorganic Hg promoted emotive mnemonic alterations [25], object recognition [51] and memory and learning [28]. In this study, we used the Morris aquatic labyrinth which is one of the most sensitive tests for evaluation of learning and spatial memory of long and short duration [50] and that in this study demonstrates the presence of cognitive alteration in the animals exposed to Hg.

In addition to the behavioral analyses, we evaluated the transport of glutamate in the motor cortex and hippocampus of exposed rats to reinforce our hypothesis that the inorganic species of Hg alter cerebral physiology. As observed in our results, mercurial exposure induced a significant increase in glutamate transport in the motor cortex and hippocampus of the exposed animals. The change in glutamate transport represents one of the main phenomena related to Hg toxicity in the CNS [52]. Previous studies using in vitro models have shown that intoxication with organic and inorganic forms of Hg promote blockade of glutamate transport in astrocytes [52–54]. The authors attributed this blockage to the significant increase of glutamate levels in the synaptic cleft and the consequent glutamatergic excitotoxicity in neurons. The results observed in our in vivo experiments suggest that the mechanism of toxicity of the inorganic form of Hg in the CNS differs from that observed in cultured astrocytes. Elevation of glutamate-uptake nerve tissue ability observed in our results was also described by Farina et al. [55] in experiments with rats exposed to the organic form of mercury. Like these authors, we consider that elevation of uptake may represent the physiological response of nerve tissue to an increase in glutamate levels in the synaptic cleft. In fact, Ming-Chi Tsai et al. [56] demonstrated that changes in extracellular glutamate levels increase the expression of excitatory amino acid transporters (EAATs) in the CNS. Confirming our hypothesis, the literature reports that Hg intoxication induces an increase in the vesicular release of glutamate, which would lead to an increase in the expression of EAATs and, consequently, an increase in the uptake observed in our study. However, further studies are needed to confirm this hypothesis.

Regarding the GABAergic system, our results demonstrate that exposure to $HgCl_2$ did not alter GABAergic neurochemistry. These results are in accordance with that previously described by Fitsanakis and

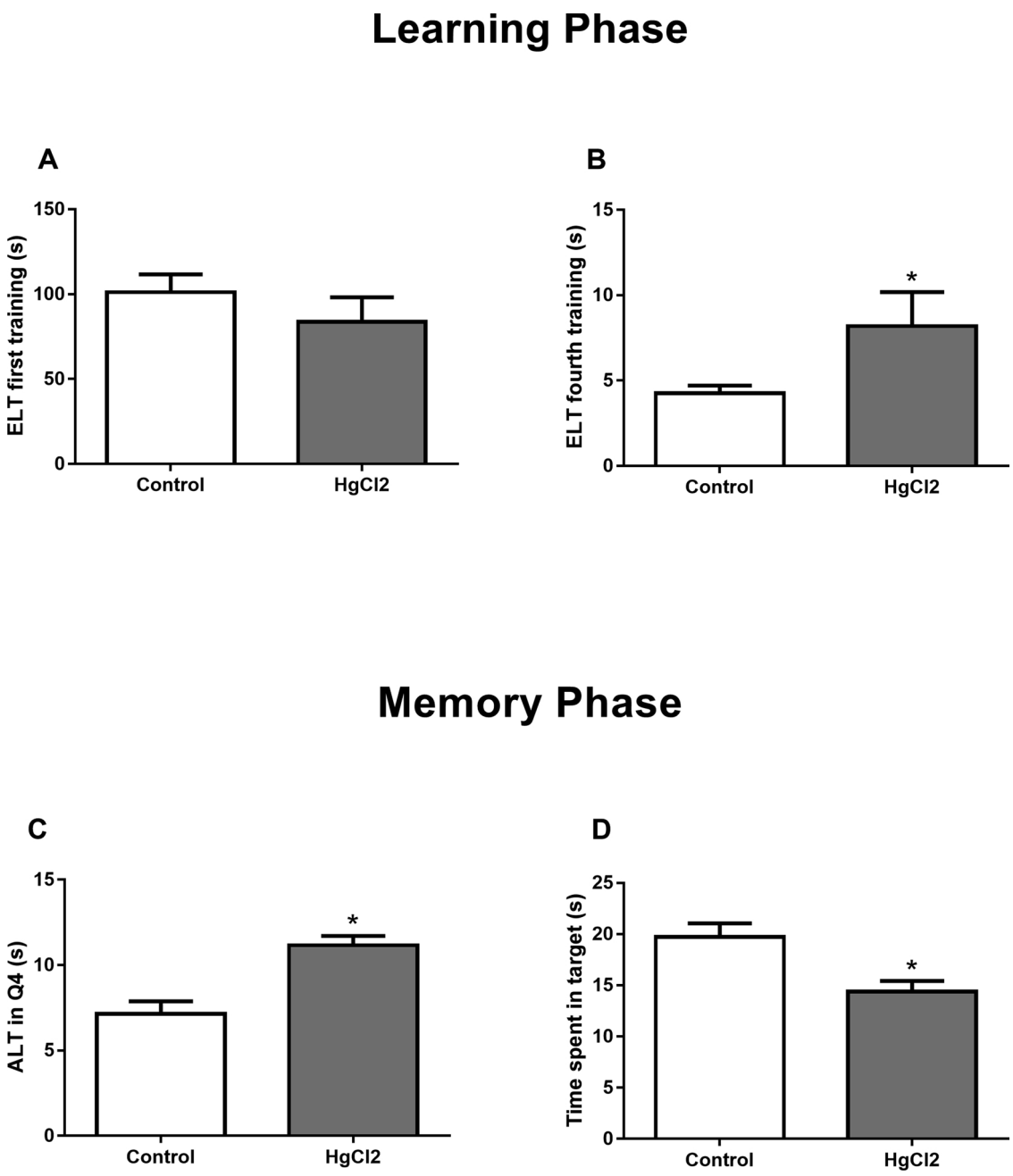

Fig. 6. Effects of chronic exposure to $HgCl_2$ on learning and memory of adult *Wistar* rats. The results are expressed as mean ± standard error of (A) escape latency time (ELT) first training; (B) ELT fourth training (C) latency to arrive (ALT) in target quadrant (Q4) and (D) time spent in target (s). *$p < 0.05$ compared to the control group (Student's t-test).

Aschner [57]. In this sense, our work contributes to the elucidation of the neurochemical toxicity mechanism of the inorganic form of Hg in brain areas important for motor control and memory acquisition, where we show that the exposure to metal changes a specific neurotransmission system.

From our results, we confirmed that chronic exposure to low-dose of inorganic Hg promotes neurochemical dysfunctions with repercussions on motor and cognitive functions. Motor cortex neurons form a well-characterized glutamatergic / GABAergic neural circuit where motor experience may alter its morphology and the efficacy of existing synaptic transmission [58–60]. Activation of AMPA-like glutamate receptors induces rapid excitatory neurotransmission that facilitates motor memory and performance enhancement related to motor tasks, whereas activation of the NMDA receptor is implicated in the maintenance of spatial memory as well as in associative learning [60,61].

NMDA, AMPA and kainate receptors are critical for the rapid regulation of synaptic plasticity, including long-term potentiation and long-term depression, both of which are important for learning and memory [62–65]. In the hippocampus, NMDA receptor hypofunction is thought to be a factor that triggers impairment of learning and spatial memory [65,66].

Finally, our previous studies [26,67] show that alterations in the oxidative balance, Hg deposits (fact also observed in this study) and neuronal and astrocytic death are related to the damages that the Hg in the motor cortex and hippocampus. In fact, the decrease in the number of neuronal cells can consequently alter the neurochemical balance of some neurotransmitters. Based on this, our study indicates that this

glutamatergic dysfunction may be associated with the development of motor and cognitive deficits observed in adult rats after chronic exposure to inorganic Hg.

Conflict of interest

The authors declare no conflict of interests.

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