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ORIGINAL PAPER

The Administration of Cadmium for 2, 3 and 4 Months Causes a Loss of Recognition Memory, Promotes Neuronal Hypotrophy and Apoptosis in the Hippocampus of Rats

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Abstract

Cadmium (Cd) is a toxic metal and classified as a carcinogen whose exposure could affect the function of the central nervous system. There are studies that suggest that Cd promotes neurodegeneration in different regions of the brain, particularly in the hippocampus. It is proposed that its mechanism of toxicity maybe by an oxidative stress pathway, which modifies neuronal morphology and causes the death of neurons and consequently affecting cognitive tasks. However, this mechanism is not yet clear. The aim of the present work was to study the effect of Cd administration on recognition memory for 2, 3 and 4 months, neuronal morphology and immunoreactivity for caspase-3 and 9 in rat hippocampi. The results show that the administration of Cd decreased recognition memory. Likewise, it caused the dendritic morphology of the CA1, CA3 and dentate gyrus regions of the hippocampus to decrease with respect to the time of administration of this heavy metal. In addition, we observed a reduction in the density of dendritic spines as well as an increase in the immunoreactivity of caspase-3 and 9 in the same hippocampal regions of the animals treated with Cd. These results suggest that Cd affects the structure and function of the neurons of the hippocampus, which contribute to the deterioration of recognition memory. Our results suggest that the exposure to Cd represents a critical health problem, which if not addressed quickly, could cause much more serious problems in the quality of life of the human population, as well as in the environment in which they develop.

Keywords The novel object recognition · CA1–CA3–DG hippocampus · Dendritic spines · Caspases · Cell death · Golgi–Cox

Introduction

In recent years, economic growth and globalization have brought great benefits to humankind, however, this has also brought new risks to human health. Reports indicate that 20% of the total incidence of disease can be attributed to environmental factors. Particularly those related to the consumption or exposure to contaminated water, food and air [1]. At the same time, heavy metal contamination has had an impact on world health [2]. Though safety standards have been implemented to reduce the risk of heavy metal poisoning, it has been shown that exist a continuous exposure, even at low concentrations, these can have an impact on human health [3]. An example is the cadmium (Cd); a non-essential transition metal with carcinogenic activity, considered one of the top five most hazardous environmental contaminants by the Agency for

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Toxic Substances and Disease Registry [4]. Cd is released naturally into the atmosphere through volcanic activity. However, anthropogenic activity is responsible for major emissions, through mining, metallurgy, paint manufacturing, batteries, and fertilizers, which are important foci of exposure for the world's population [5–7]. Cadmium oxide (CdO) and cadmium chloride ($CdCl_2$) are mainly compounds in dust and smoke generated by both industry and tobacco [8].

In a common daily adult diet, up to 30 µg of Cd per day can be ingested by food and water. The acidic environment of the digestive tract favors the transport of Cd in the apical membrane of the enterocytes [9]. In addition, when accompanied by an iron deficiency, Cd absorption and accumulation is promoted. Likewise, inhaling high amounts of CdO (especially in active and passive smokers) can lead to emphysema, anosmia and chronic rhinitis, acute pneumonitis with pulmonary edema and at high enough concentrations lung cancer may develop [5].

After a Cd exposure, the metal is distributed and progressively bio-accumulated due to the fact that it is a non-biodegradable substance with a very long biological half-life. The persistence of Cd in the body has a range of half-lives from 6 to 38 years for humans; the liver and kidneys being the organs with the greatest accumulation (4–19 years). However, other tissues can be affected and can display toxic effects, such as the lungs, gut, pancreas, ovaries, testes and central nervous system (CNS). The CNS is particularly affected by Cd accumulation, with symptoms including headaches, vertigo, olfactory dysfunction, peripheral neuropathy, impaired balance and cognitive impairment [7, 10].

Exposure to Cd promotes indirectly way the generation of reactive oxygen species (ROS). Also, enzymes such as superoxide dismutase, catalase, and glutathione peroxidase reduce its activity or concentration favoring an oxidative environmental. Consequently, this triggers oxidative stress, which could contribute to the structural and functional damage of neurons in the cerebral cortex and hippocampus [11, 12]; brain regions involved in the functioning of spatial and recognition memory, respectively. At the morphological level, in vitro studies indicate that cortical neurons exposed to Cd for 24 h, not only loss neuronal integrity [13, 14], but also dendritic retraction and apoptosis happen. Also, it reported that exposure to this heavy metal inhibits proliferation and neurogenesis [15, 16]. Therefore, it could be thought that Cd is a possible neurodegenerative factor that causes neuronal death through apoptosis, particularly in cortical and hippocampal neurons, causing dementia events similar to Alzheimer's disease (AD) [17, 18]. However, the mechanism of action has not been defined and it is unknown how morphological deterioration occurs in hippocampal neurons in response to chronic intoxication with Cd, as well as its consequences on recognition memory.

In this sense, the aim of the present work was to study the effect of Cd exposure at different times on recognition memory, neuronal morphology and the apoptosis in rat hippocampi.

Methodology

Experimental Animals

For this work, 1-month-old male Wistar rats with an approximate weight of 80–100 g were used. The animals were obtained from the bioterium "Claude Bernard" from the Autonomous University of Puebla. The animals were housed in a temperature and humidity-controlled environment on a 12 h–12 h light–dark cycle with free access to food and water. All procedures described in this study were approved by the ethics committee and Guide for the Care and Use of Laboratory Animals in Mexico (NOM-062-ZOO-1999). Every effort was made to minimize the number of animals used and to ensure minimal animal pain and/or discomfort.

Administration of Cd

The experimental animals were divided into two groups (n = 45/group): (1) control (drinking water) and (2) treatment with Cd. 32.5 ppm of Cd was added to the water in the form of $CdCl_2$ for the treatment group, while the control group received only purified water to, provided daily "ad libitum". It should be noted that the gastrointestinal pathway represents low absorption (< 10%). This the reason for selecting a lowest-observed-adverse-effect level dosage (LOAEL), since this is the dose known that increases the Cd levels in circulating over time, according to the report of Treviño et al. [19]. Each group was subdivided into three (n = 15), with the purpose of evaluating the effect of Cd-exposure over 2, 3- and 4-month periods. After each exposure time, a cognitive test was performed on the animals.

The Novel Object Recognition Test (NORT)

Once the period of exposure to Cd was completed, the animals were evaluated by the novel objects recognition test (NORT) to assess the recognition memory. This test is based on the tendency of animals to explore a novel object for a long time with respect to a familiar object [20, 21].

The NORT was performed in an opaque plastic box measuring 80 cm width × 80 cm length × 80 cm height. In this task four plastic objects were used that varied in color and size (width = 9.0–12.0 cm, height = 6.0–9.0 cm). The objects were fixed to the floor of the box using velcro strips located ~ 30 cm away, located diagonally opposite one another. Also, a video camera fixed on the wall directly above the box was

used to record the test session for offline analysis. The lighting was done with a white light bulb located above the box.

The first stage of the task was habituation, the rats were previously acclimated in the behavior room for two consecutive days before beginning the habituation phase for 45 min. On the day of the test, the animals were placed 45 min in the behavior room and subsequently, the animals explored the empty box for 5 min. Once time finished the task, rats were returned to their home cage. In this phase of the NORT, the animal´s trajectory of displacement in the box was analyzed. In addition, the average of the distance traveled and the velocity recorded by the animals from each experimental group in the box (for 5 min test) was plotted.

In the familiarization phase (24 h later of habituation phase) rats were familiarized with two identical objects, previously acclimatized in the behavior room for 45 min and then placed in the box with two identical objects (two square-based pyramids) centrally at 30 cm. The time spent to explore each object was quantified by a period of 5 min. The exploration of objects was later qualified with a video recording of each trial by an experimenter. The exploration time of each object was quantified when the rat's nose was 1 cm from the object and the vibrissae were moving. The object exploration time was not recorded when the rat used the object to back up with the rat's nose facing the ceiling.

To assess short-term recognition memory (STRM), 2 h after exposure to the two objects, one of the familiar objects was replaced with a new one (cylinder), and the animal was left in the box for 5 min. Exploration time for each object was recorded similarly as described in the familiarization phase. The preference for the novel object has been manifested as the time spend for a rat exploring the new object (compared to the familiar object). The long-term memory recognition (LTMR) was evaluated 24 h later of the assessment of STRM, previously the animals were acclimated in the behavior room for 45 min and then placed in the box to be exposed to two objects. The object who was presented as a novel in the previous phase was replaced with a new object (cube) to evaluate the LTMR, as previously described in the familiarization phase.

We obtained the discrimination index (DI) = (TN − TF)/(TN + TF), identifying TF and TN as the time of exploring the familiar and novel objects, for each of the animals in the experimental groups. To exclude odor cues, the box and the objects were cleaned with 80% ethanol after each session.

Morphological Assessment by Golgi–Cox Stain Method

At the end of the novel object's recognition test, ten animals from each group were anesthetized with sodium pentobarbital (75 mg/kg, ip) and perfused intracardially with an isotonic saline solution. The brains were removed and stained using the modified Golgi–Cox method, as previously described [22]. Coronal sections of 200-µm thickness were obtained from the hippocampus using a vibratome (Campden Instrument, MA752, Leicester, UK). These sections were placed on pre-gelatinized slides. The samples were subsequently treated with ammonium hydroxide, followed by a Kodak Film Fixer and finally rinsed with distilled water and mounted on a resinous medium [23].

Microscopic Observation and Sholl Analysis

The hippocampus was evaluated in 3 sub-regions corresponding to pyramidal cells from CA1 and CA3, as well as granule cells of the dentate gyrus (DG) [24], from each animal. Neurons were drawn from both the left and the right hemispheres using a lucid camera at a magnification of ×40 (DMLS, Leica Microscope) by a trained observer who was blind to the experimental conditions [25]. Sequential two-dimensional reconstructions of the entire dendritic tree were generated for each neuron and the dendritic tracings were quantified by Sholl analysis [26, 27] as follows: a transparent grid with equidistant (10 µm) concentric rings was centered over the dendritic tree tracings and the number of ring intersections was used to estimate the total dendritic length (TDL) and dendritic arborization [21, 25]. The total number of dendritic branches (branching indicated by Y bifurcated), another estimate of dendritic arborization, was counted at each order away from the cell body or dendritic shaft. To calculate the spine density, a length of dendrite (at least > 10-µm long) was traced (at ×1000) and the exact length of the dendritic segment was calculated and the number of spines along the length counted (to yield spines/10-µm). Dendritic length and spine density of the neurons (n = 10 per each animal; total n = 600 per group) were analyzed for the basilar dendrites.

Immunohistochemistry Examination

After the novel objects recognition test, the rats of experimental groups (n = 5/group) were anesthetized with pentobarbital sodium (75 mg/kg; ip) and perfused with paraformaldehyde (4%). The brains were removed and post-fixed and embedded in paraffin. 5-µm thick coronal sections were taken from each brain at the level of the anterior temporal area, approximately 3.8 to 6.8 mm from bregma. And the coronal sections were placed on slides. The paraffin was subsequently removed, and the slides were rehydrated. The non-specific binding sites were blocked by incubating in 2% IgG-free bovine serum albumin (Sigma) at room temperature. Afterward, samples were incubated with 0.2% Triton X-100. Sections were then incubated overnight at 4–8 °C with anti-caspase-3 and 9 (1:100, Santa Cruz Biotechnology), which were identified using anti-mouse FITC-labelled secondary

antibodies (1:100, Jackson Immuno Research Laboratories Inc.) shown in green. Slides were counterstained with VectaShield with DAPI (Vector Labs., CA, USA) for nuclei staining (blue channel).

Photomicrographs were taken using a fluorescence microscope (Leica), similar to those reported by Diaz et al. [27]. Three consecutive slices of each brain tissue were used to observe the CA1–CA3–DG regions of the hippocampus neurons at ×40.

The number of caspase-3 and 9 immunoreactive cells was counted in the CA1–CA3–DG subfields of the hippocampus. Four fields per slide were analyzed and graphically expressed as an average per group ± SE. The images [×40] were projected with the Leica Microsystems program. All counting measures were made by a specialist in morphology unaware of the specifics of the study.

Statistical Analysis

First, it was realized a normality test Shapiro–Wilk to verify that different data comes from a normally distributed population. For tests of recognition memory, dendritic length, and spine density, paired groups data were reported as the mean ± standard error (SEM), and comparisons were made with an unpaired Student's *t* test, considering $p < 0.05$ as significant. Meanwhile, the number of cells immunoreactive to caspase-3 and caspase-9 was analyzed mean Mann–Whitney a nonparametric test due to the nature of the variable, with $p < 0.05$ as significant. Finally, a Two-Way ANOVA followed by a Bonferroni post-test was used to analyze the branch order length, with $p < 0.05$ as significant. The factor corresponding to the means dendrite length of a specific brain region was analyzed in relation to variables dendritic arbor morphology, number of dendritic intersections and cadmium exposition.

Results

Effect of Cd Exposure on Recognition Memory

To demonstrate that the Cd administration modifies the recognition memory, the NORT was evaluated. In the first stage of the test, the animals freely explored the box for 5 min. The trajectory that the animals went through is shown in a representative trajectory in Fig. 1a. The values of the

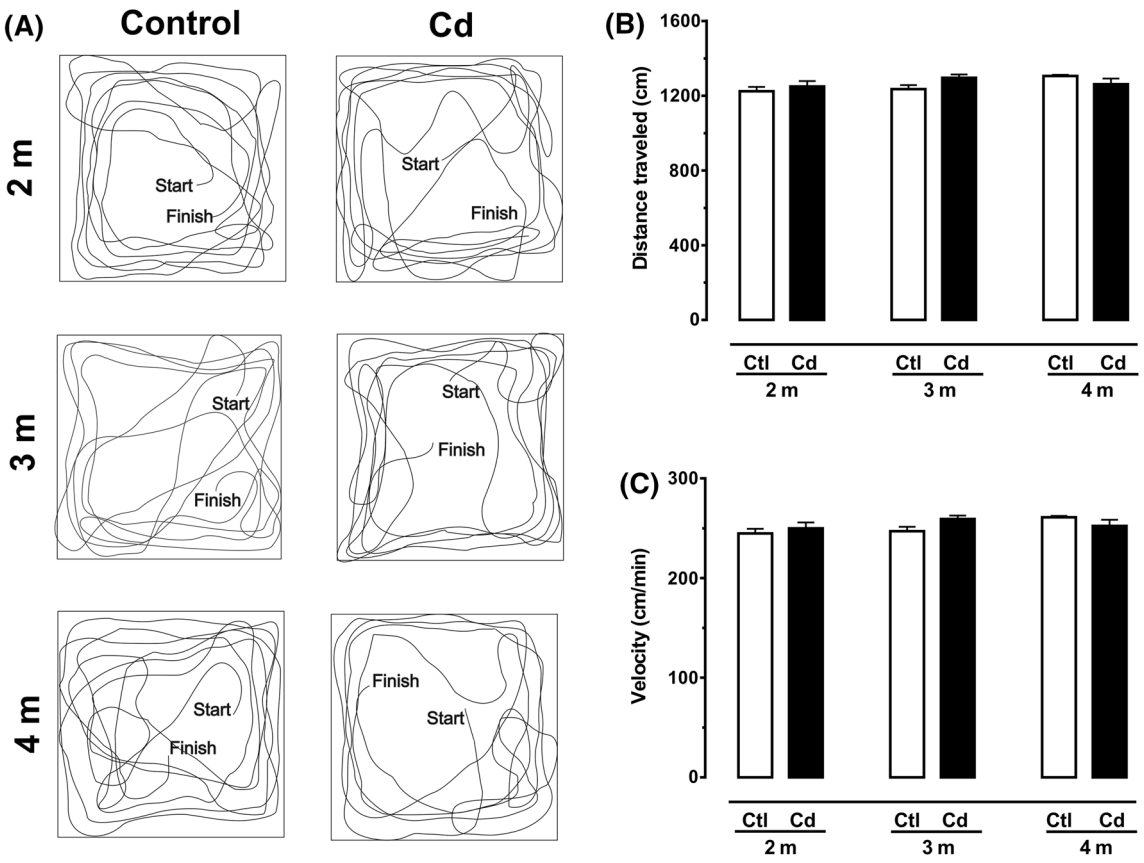

Fig. 1 Chronic exposure to Cd on locomotor activity. Representative travel drawings of the locomotor activity (**a**). Open field measurements quantified distance traveled (**b**) and velocity (**c**) after 2, 3 and 4 months of treatment. The values show the mean ± SEM; *P < 0.05

distances traveled by the rats treated with water and Cd at the different administration times were the following: 1224 ± 7 and 1249 ± 29 cm (2 months, P = 0.3330); 1235 ± 22 and 1296 ± 17 cm (3 months; P = 0.0594), as well as 1305 ± 7 and 1261 ± 30 cm (4 months; P = 0.2470), respectively (Fig. 1b). Another parameter analyzed was the velocity recorded by the animals. Results of the control group were 244.8 ± 4.5; 247 ± 4 and 261 ± 4 cm/min, after 2, 3 and 4 months of treatment, respectively, while the velocity of the group treated with Cd was 250 ± 6; 260 ± 3 and 252 ± 6 cm/min, at the same evaluated times (Fig. 1c). When comparing between groups, these did not show significant differences (P = 0.3330; P = 0.0594 and P = 0.2470). The statistical analysis of these two parameters reveals that the administration of Cd does not modify the locomotor activity in animals.

During the familiarization phase, the animals were placed in the box with two identical objects (object A and B). The purpose of this test was to analyze the exploration time that the animals made to each object. The data obtained from the average time of exploration in the animals of the control group indicate that at 2, 3 and 4 months it was 12.3 ± 0.5, 12.6 ± 0.5, 14.5 ± 0.4 s. for object "A" and 13.2 ± 0.7, 11.15 ± 1.1, 13.4 ± 0.5 s. for object "B". On the other hand, the group administered with Cd recorded an average of 13.8 ± 1, 13.3 ± 1.1, 12.2 ± 0.5 s. for object "A", while for object "B" it was 13.5 ± 1, 13.6 ± 0.7, 13.0 ± 0.8 s. for each of the evaluated times (Fig. 2a). The statistical analysis revealed that the animals of each experimental group at 2-months (P = 0.4782), 3-months (P = 0.6746) showed no preference for object "A", but 4-months cadmium group lost interest in this one (P = 0.0200). For the object "B" the animals of each experimental group at 2-months (P = 0.0630), 3-months (P = 0.1356) and 4-months (P = 0.7458) showed no preference.

Subsequently, the discrimination index was analyzed to evaluate the STMR and LTMR when the animals were exposed to a novel object. The results indicate that in both groups (treated with and without Cd) they do not show significant differences after 2 months of administrations (P = 0.3215). However, after 3 (P < 0.0001) and 4 months (P < 0.0001), the animals exposed to Cd showed a significant decrease in the discrimination index compared to the control group (Fig. 2b, c).

Effect of Cd on Hippocampal Dendritic Morphology

The technique of impregnation with the Golgi–Cox solution was used in this work to identify the dendritic structures of pyramidal neurons of CA1 and CA3, as well as the granular cells of the DG of the hippocampus. Figure 3 shows, in addition, the Sholl analysis was applied to the samples. Golgi–Cox staining allowed us to determine the dendritic branching (dendritic order), TDL and to count dendritic spine density in the CA1, CA3 and DG regions of the hippocampus.

The dendritic order analysis indicates that in the CA1 region of animals exposed to Cd for 2 months, only the dendrites of fourth order showed a significative difference [F = 3.433 (Dfw 10, Dfb 1); P < 0.01]. In the animals exposed for 3 months, a significant reduction in the second [F = 6.098 (Dfw 18, Dfb 1), P < 0.01], third [F = 24.117 (Dfw 18, Dfb 1), P < 0.01] and fourth [F = 19.952 (Dfw 18, Dfb 1), P < 0.01] dendritic order exists. Meanwhile, the group exposed for 4 months showed a reduction in the third [F = 24.117 (Dfw 15, Dfb 1), P < 0.001] and fourth order [F = 22.54 (Dfw 15, Dfb 1), P < 0.001] in comparison to the control group (Fig. 4a, c). In the CA3 region of the hippocampus, treatment with Cd for 2 months did not affect the dendritic order of pyramidal neurons. However, the neurons of the CA3 region of the animals exposed to Cd during 3 months [F = 12.943 (Dfw 15, Dfb 1), P < 0.001; F = 21.473 (Dfw 15, Dfb 1), P < 0.001] and 4 months [F = 9.499 (Dfw 15, Dfb 1), P < 0.001; F = 3.491 (Dfw 15, Dfb 1), P < 0.01] showed a significant reduction in the fourth

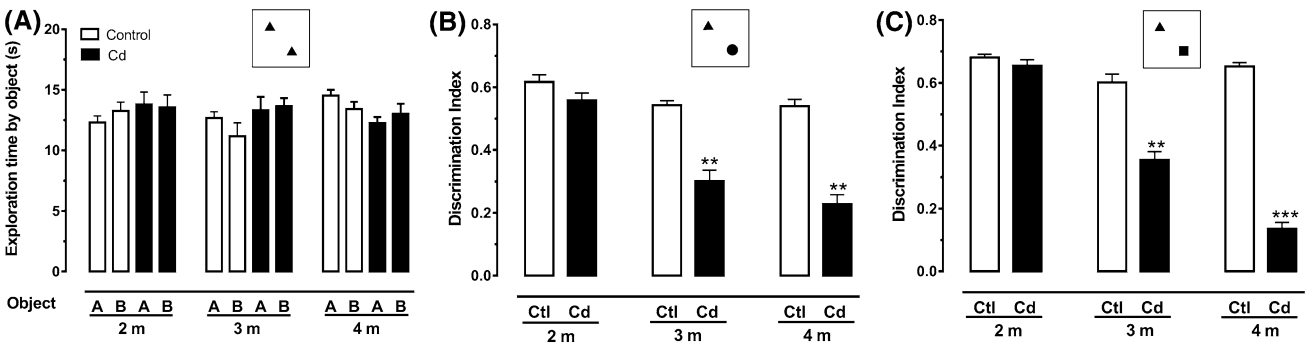

Fig. 2 Chronic exposure to Cd progressively deteriorates recognition memory. The animals of the Cd group and the control group developed the novel objects recognition test (NORT), quantified the exploration time with identical objects (**a**), the discrimination index during the short-term recognition memory (STRM) (**b**) and long-term recognition memory (LTRM) (**c**) after 2, 3 and 4 months of treatment. The values show the mean SE; **P < 0.01 and ***P < 0.001

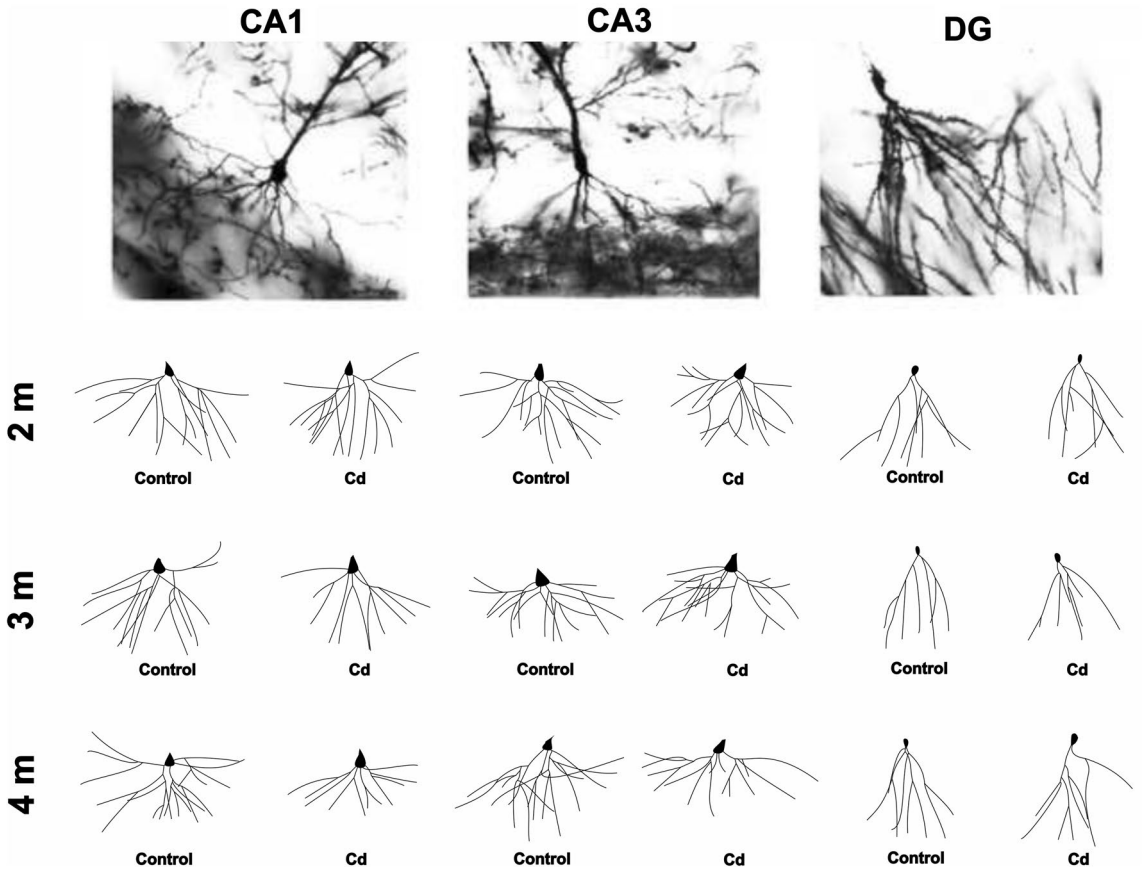

Fig. 3 Effect of Cd exposure on morphological changes in the hippocampus after 4 months of exposure. Photomicrograph showing representative Golgi–Cox-impregnated neurons and schematic drawings of dendritic tree of the pyramidal neurons of the CA1 and CA3 of the dorsal hippocampus and the granule cells of the dentate gyrus (DG) of rats treated with vehicle and Cd after 2, 3 and 4 months of treatment

and fifth dendritic order, in comparison with their control group (Fig. 4d–f). The analysis by two-way ANOVA from the dendritic order of the DG granular cells, show no significant differences between groups after 2, 3 and 4 months (Fig. 4g–i).

The effect of Cd exposure on TDL of the CA1 hippocampal region showed a significant reduction after 2 ($P < 0.0001$), 3 ($P < 0.0001$) and 4 months ($P = 0.0001$) in relation to control groups, respectively (Fig. 5a). The CA3 and DG regions revealed a statistically significant decrease only after 4 months of exposure to Cd (Fig. 5b, c), to CA3 $P = 0.6346$, $P = 0.2285$ and $P < 0.0001$, meanwhile to DG $P = 0.0594$, $P = 0.0506$ and $P < 0.0001$.

Finally, the dendritic spines density in the CA1 region was lower in the groups exposed to Cd for 3 ($P < 0.0001$) and 4 months ($P \leq 0.0001$), compared to their control groups, respectively, while after 2 months ($P = 0.3263$) of treatment, there was no change between the control and Cd group (Fig. 5d). In the CA3 region, the dendritic spine density did not show statistical changes between the animals with 2 and 3 months of Cd exposure and the vehicle (control group), $P = 0.5459$, $P = 0.1613$. But, in the 4 months of treatment, the density of dendritic spines significantly decreased, $P < 0.0001$ (Fig. 5e). Whereas, in the DG of animals with exposure to Cd, the density of dendritic spines was significantly lower after 3 and 4 months, $P < 0.0001$ for both cases (Fig. 5f).

Effect of Cd on the Immunoreactivity of Caspase-3 and Caspase-9 in the Hippocampus

To determine the effect of Cd on the immunoreactivity of caspase-3 and caspase-9 in the CA1–CA3–DG hippocampal areas, sections were evaluated after NORT application. Figures 6 and 7 shows the photomicrographs of caspase-3 and caspase-9 immunoreactivity in hippocampal areas, respectively. The qualitative analysis showed a low immunoreactivity of caspase-3 and caspase-9 (both in green) in control groups, with an irregular pattern, localized in the three hippocampal regions (CA1–CA3–DG), after 2, 3 and 4 months. In contrast, a higher caspase-3 and caspase-9 immunoreactivity were

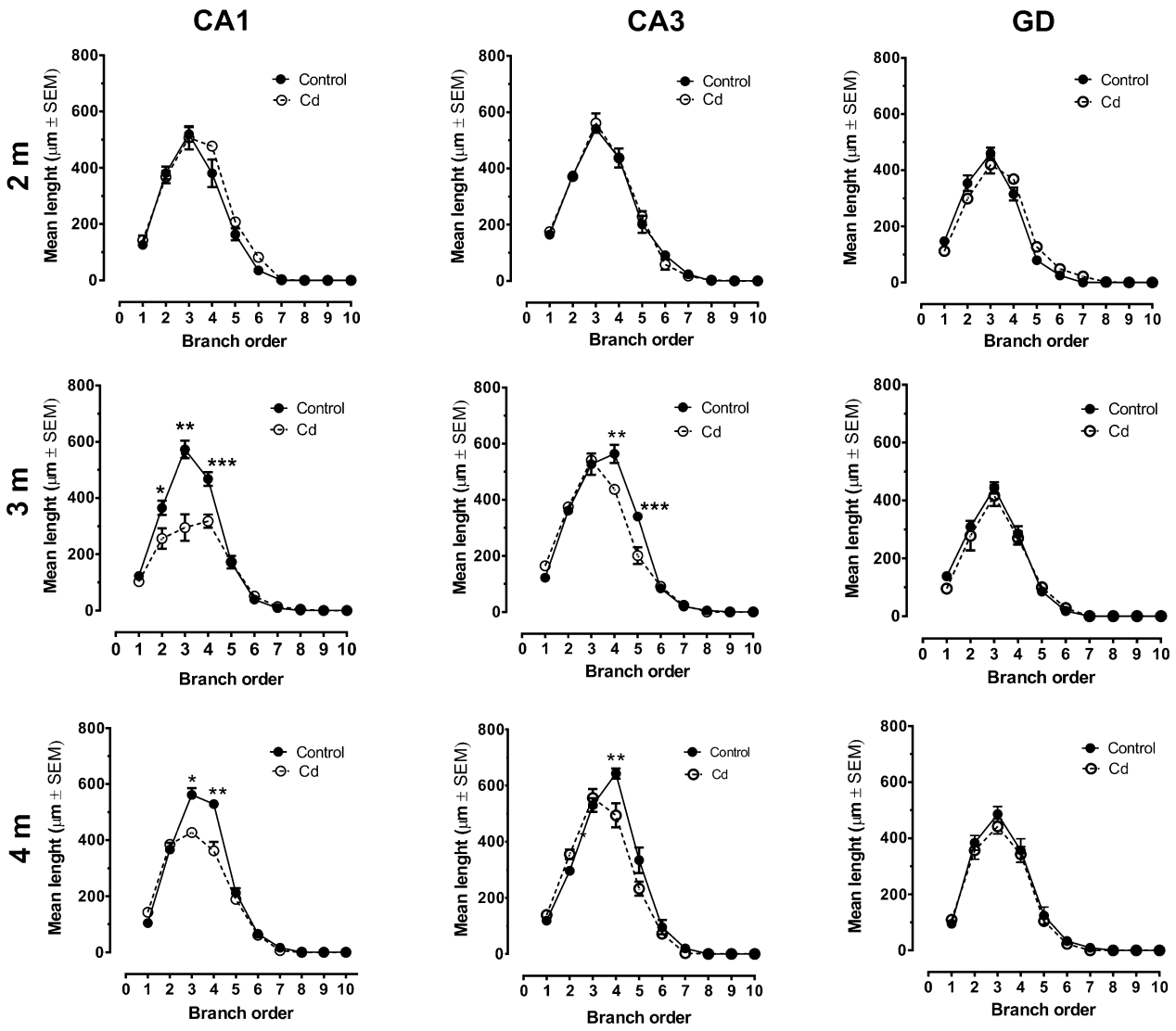

Fig. 4 Effect of Cd exposure on the length of dendritic branches in rats. The pyramidal neurons of the CA1 and CA3 of the dorsal hippocampus and granule cells of the DG of the dorsal hippocampus in Cd-exposed rats at 2, 3 and 4 months showed a decrease in dendritic length at distinct levels of the branching order compared to the vehicle-treated animals. *P < 0.05; ** P < 0.01; ***P < 0.001

observed for Cd-exposure groups in the brain regions analyzed, and the intensity increased proportionally depending on exposure time.

Statistical analysis indicates that in CA1 region of the hippocampus in animals Cd-exposed for 3 and 4-months, immunoreactivity were significative different (P = 0.0097 for both groups). Similar results were observed on CA3 region, thus 3 and 4-months Cd-exposed groups showed differences (P = 0.0129 for both groups). Meanwhile, the DG region showed differences in all time of Cd exposition (P = 0.0290, P = 0.0290 and P = 0.0109, respectively) (Fig. 6b). As for caspase-9, the CA1 region showed differences since the second until the fourth month of exposition (P = 0.0129, P = 0.0109 and P = 0.0109, respectively). The Cd-exposition had the same effect in CA3 region (P = 0.0232, P = 0.0109 and P = 0.0109, respectively) and the DG region (P = 0.0259, P = 0.0097 and P = 0.0109, respectively) (Fig. 7b).

Discussion

In recent years, cellular and animal models have demonstrated the toxic potential induced by chronic Cd exposure. Cd accumulation in different tissues (liver, kidney and recently, in the brain) detonates a series of molecular events that cause cell death. In the present work, we demonstrate that chronic Cd exposure causes severe impairment in the dendritic morphology of hippocampal neurons, as well as in the density of dendritic spines. Simultaneously, the exposure

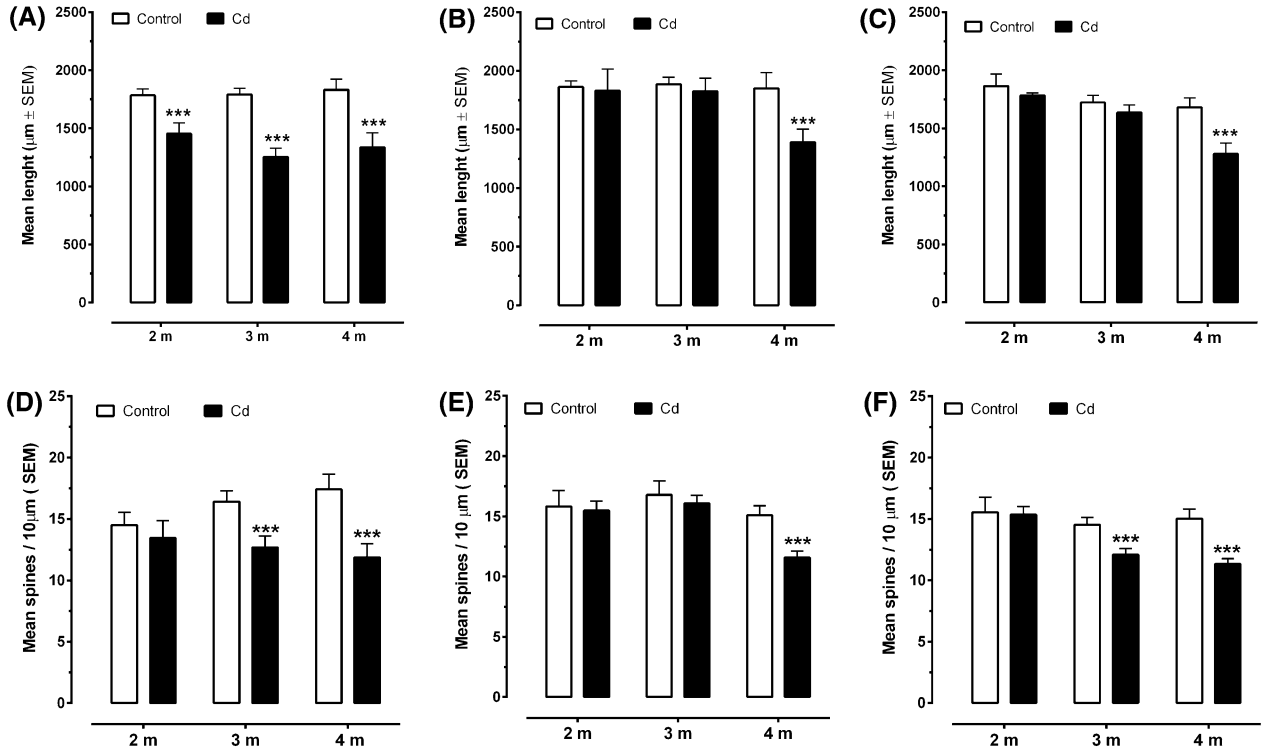

Fig. 5 Analysis of the effect of Cd exposure on total dendritic length and dendritic spine density in rats. The total dendritic length and dendritic spine density of the pyramidal neurons of CA1 (**a, d**) and CA3 (**b, e**) and granule cells of the DG (**c, f**) of the hippocampus in Cd-exposed rats at 2, 3 and 4 months showed a decrease in dendritic length and dendritic spine compared to the control group. *P < 0.05; **P < 0.01

to Cd generated a higher caspase-3 and caspase-9 immunoreactivity, which suggests a neuronal death in this cerebral region, and consequently, there occurs a deterioration of cognitive processes, such as in recognition memory.

In this paper, we provide direct evidence that relates to the progressive deterioration of the recognition memory, evaluated by the NOR test, with chronic Cd exposure [27, 28]. Previous studies indicate that Cd triggers a dysfunction of recognition memory [29], probably associated with the ability of this heavy metal to cross the blood–brain barrier (BBB) and its progressive accumulation [30, 31]. Reports indicate that Cd can accumulate in higher concentrations in the choroid plexus, at a greater amount than even cerebrospinal fluid [32]. This could damage the structure of the choroid plexus and therefore affect the integrity of the BBB, leading to a progressive accumulation at the intracerebral level, and could be responsible for dysfunction and neuronal death [10].

Our results suggest that a 2-month exposure of Cd is not enough to activate toxicity mechanisms, thus, it does not affect recognition memory. However, in the proceeding months, the effects of the Cd increased progressively to toxic levels, producing cognitive dysfunction in the exposed animals. It can be deduced from the above that the hippocampal neurons are damaged. Notably, animals exposed to Cd did not exhibit locomotor problems. Therefore, the main complication in these animals was their poor cognitive performance in the development of specific tasks, suggesting that Cd exposure only affects short and long-term memory [33–35]. This corroborates the theory that the neurotoxicity caused by Cd promotes neuronal changes that affect cognitive functions [10].

Moreover, the results strongly indicate that Cd is associated with the deterioration of hippocampal memory and synapse dysfunction and modification of neuronal plasticity [36, 37]. Consequently, after a chronic exposure to Cd, we observed the deterioration of synaptic plasticity, which impairs the hippocampal innervation and thus, recognition memory [38]. In this sense, our results on neuronal morphology show that the CA1 and CA3 hippocampal regions of the animals exposed to Cd, exhibit a dendritic order reduction of TDL and the dendritic spines density, which corroborates our findings that the neurotoxicity of the Cd is critical for neuronal plasticity.

The mechanism by which Cd induces morphological changes is not yet fully understood, however, the evidence indicates that Cd affects the integrity of white matter [39, 40], because it modifies both the axons and dendritic

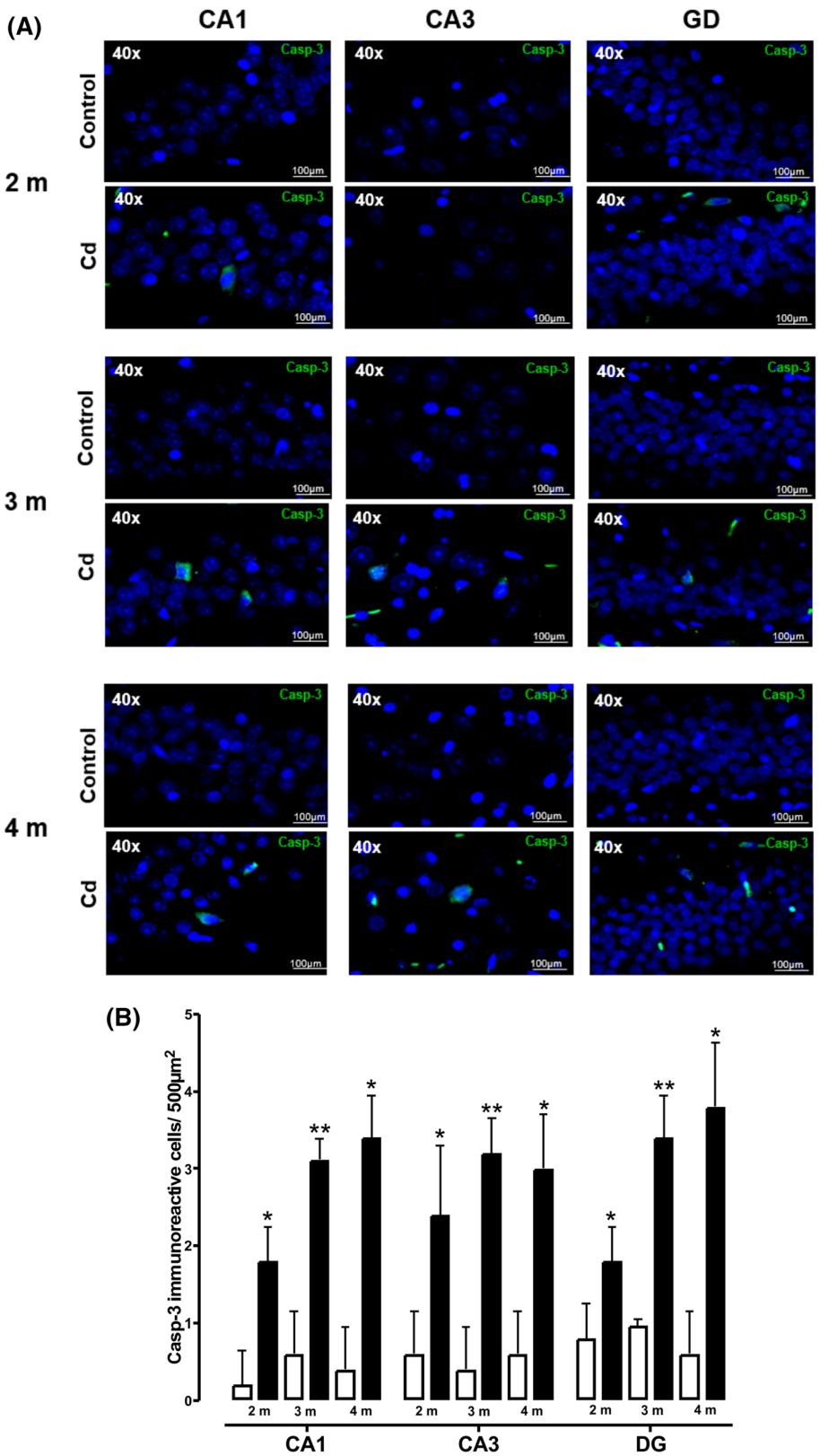

Fig. 6 Chronic exposure to Cd induces an increase of caspase-3 (casp-3) immunoreactivity in rat hippocampi. **a** The photomicrographs show the immunoreactivity of casp-3 (green) in the CA1–CA3–DG hippocampal areas of rats after 2, 3 and 4 months of Cd-exposure (n = 5) and Control group (n = 5) (magnification × 40). **b** shows that the number casp-3-immunoreactive cells in CA1, CA3 and DG of the group with Cd increases with respect to the control group in each of the study times. Data correspond to the mean ± SE; *P < 0.05 and **P < 0.01. (Color figure online)

morphology, as well as promoting the formation of ROS in neurons of parietal cortex, striatum and the hippocampus [41–43]. At the same time, Cd exposure reduces the concentration and activity of antioxidant enzymes, such as catalase, glutathione peroxidase, and superoxide dismutase, among others [11, 42, 44]. Altogether, this leads to a state of

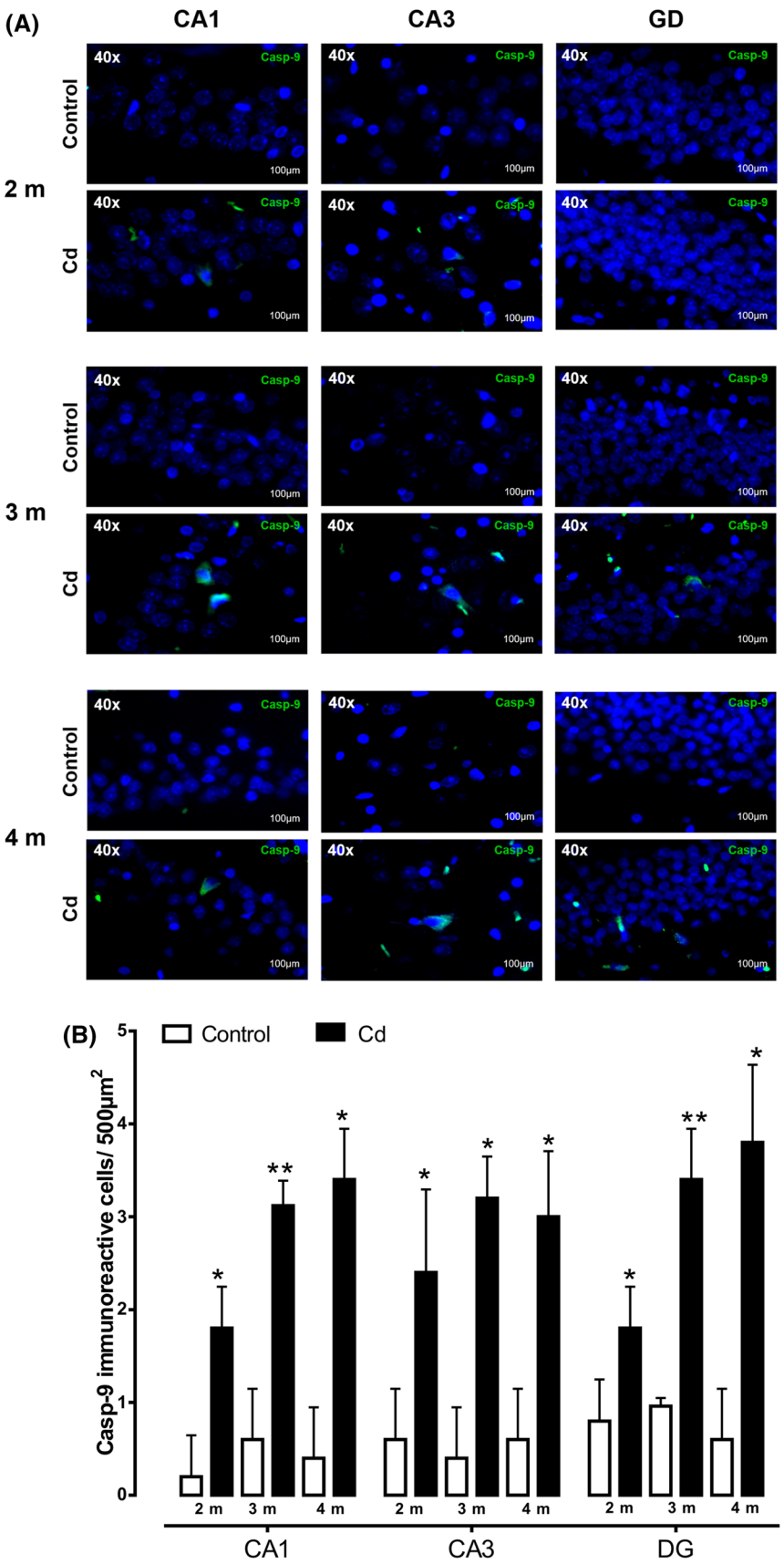

Fig. 7 The Cd increase of caspase-9 (casp-9) immunoreactivity in rat hippocampi. The immunoreactivity of casp-9 (green) in the hippocampus (CA1–CA3–DG) of rats after 2, 3 and 4 months of Cd-treatment and water (Control group) (n = 5/group) are shown (magnification ×40) in **a**. The number casp-3-immunoreactive cells in CA1, CA3 and DG of the group with Cd which, is shown in **b**, increases with respect to the control group in each of the study times. Data correspond to the mean ± SE; *P < 0.05 and **P < 0.01. (Color figure online)

oxidative stress, which elevates lipid peroxidation, causing damage to neurons of different brain regions [10, 45].

On the other hand, it is suggested that Cd affects the excitability of neurons since it blocks calcium channels at the level of the membrane in the presynaptic terminals that diminishes the synthesis and release of neurotransmitters, such as acetylcholine and glutamate, causing a dysfunction of learning and memory [46, 47].

Additionally, it has been observed that exposure to Cd could affect neurogenesis, but reduces both axogenesis and spinogenesis, leading to neuronal death mediated by apoptosis [10, 16]. Cd also promotes the inhibition of the synthesis of neurotrophins, such as the brain-derived growth factor (BDNF), which is an important factor for dendritic remodeling [40, 48].

Cd-induced neuronal apoptosis occurs in a time-dependent manner. In this regard, it is known that Cd activates a network of signaling in which JNK, Erk1/2 and mTOR participate [10, 41]. Recently, it has been reported that in exposure to Cd, the MAPK pathway combined with mTOR activity, induces apoptosis through the ROS production by via cytosolic Ca^{2+} increase, affecting the mitochondrial membrane potential, resulting in the cleavage of caspase 9 and 3 and poly (ADP-ribose) polymerase (PARP) causing programmed neuronal death [49]. Our results show an increase in the number of immunoreactivity cells to caspase-3 and caspase-9 (time-dependent manner) in the three hippocampal regions studied, corroborating that Cd could be a key factor in the etiology of neurodegenerative diseases, such as dementia or AD, initially associated with a loss of recognition memory by synaptic dysfunction [17].

In conclusion, it is suggested that the neurotoxic effects of the exposure to Cd for 2, 3 and 4 months cause neuronal-death and progressive hypotrophy, which is associated with a decrease in density of dendritic spines in the CA1, CA3 and DG regions of the hippocampus. Synaptic loss reduces neuronal activity and, consequently, causes a cognitive deficit in recognition tasks. Daily exposure to Cd in LOAEL dosage represents a critical situation that threatens the quality of life and must be considered a serious pathophysiological risk factor for neurodegenerative diseases.

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Author Contributions AD and ST designed the study and wrote the protocol. GP, UPR, RV-R, and AM-R performed the experiments. AD, ST, GF, EB, JG managed the literature searches and analysis, AHS undertook the statistical analysis. AD and ST wrote the first draft of the manuscript. All contributing authors have approved the final manuscript. JLMP participated in the managed the literature searches and analysis and undertook the statistical analysis.

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Compliance with Ethical Standards

Conflict of interest Authors have no conflicts of interest to declare.

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