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ORIGINAL PAPER

Trans-resveratrol Inhibits Tau Phosphorylation in the Brains of Control and Cadmium Chloride-Treated Rats by Activating PP2A and PI3K/Akt Induced-Inhibition of GSK3β

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Abstract

This study investigated if resveratrol (RES) can protect against cadmium chloride ($CdCl_2$)-induced memory loss and Tau protein hyperphosphorylation in rats and explored its effect on AMPK/PI3K/Akt signaling pathway. Rats (n = 10/group) were divided into seven groups as: control; control + DMSO; control + LY294002, a selective PI3K inhibitor (0.25 µg/100 g, i.p); control + RES (300 mg/kg, orally); $CdCl_2$ (5 mg/kg, orally); $CdCl_2$ + RES and $CdCl_2$ + RES + LY294002. All treatments were carried out for 30 consecutive days on a daily basis. RES improved both short and long-term memory as analyzed by novel object recognition task and significantly increased brain levels of glutathione in both control and $CdCl_2$-treated rats. It also inhibited ROS levels of malondialdehyde in the brains of $CdCl_2$-treated rats. In both groups, RES decreased the phosphorylation rate of Tau at Ser^{199} and Ser^{296}. Concomitantly, it significantly increased protein levels of p-GSK3β (Ser9) and p-PP2A and decreased p-GSK3β (Tyr^{216}). Also, RES activated PI3K/Akt signaling pathway in both control and $CdCl_2$ treated rats by increasing levels of p-PI3K (Tyr^{607}) and p-Akt (Ser^{473}). This was concomitant with significant increase in the levels of AMPK and p-AMPK, known upstream regulators of PI3K/Akt signaling pathway. Interestingly, all the above listed beneficial effects of RES, except their effect on AMPK/p-AMPK, were completely abolished in $CdCl_2$ + RES + LY294002-treated rats. In conclusion, in addition to its antioxidant potential, RES inhibits Tau phosphorylation in rat's brain by activating PP2A protein and AMPK/PI3K/Akt-induced inhibition of GSK3β.

Keywords Resveratrol · Cadmium · Brain · Tau · Memory, PI3K/Akt

Introduction

Alzheimer's disease (AD) is one of the most common progressive neurodegenerative diseases and is characterized by severe cognitive deficits and memory loss [1]. The hallmark of the disease is the over-aggregation of intracellular neurofibrillary tangles (TNF) and aggregates of paired helical filaments (PHFs), mainly due to Tauopathies defined as hyperphosphorylation of Tau protein and/or loss of Tau normal function [2–5], which eventually leads to microtubule instability, neurodegeneration and neural apoptosis [5].

Tau protein phosphorylation of is a tightly regulated mechanism that requires a delicate balance between certain phosphatases and kinases. In the brain, protein phosphatase 2A (PP2A) is the major Tau protein phosphatase [6] whereas glycogen synthase kinase-3β (GSK3β) is the major Tau protein kinase [7]. Over-activation of GSK3β and/or decreased expression and activity of PP2A have been reported during the development and progression of AD [7, 8]. In this regard, the enhanced activity of GSK3β was shown in cultured neural cells exposed to β-amyliod (Aβ) [9, 10]. In these studies, GSK3β induced Tau protein hyper-phosphorylation at Ser^{396}, Ser^{199}, Thr^{205} and Thr^{231} sites [7–10].

However, the activity of GSK3β itself can be also regulated by phosphorylation at specific sites. Indeed phosphorylation of GSK-3β at Ser^{9} or dephosphorylation at Tyr^{216} site can inhibit the activity of GSK-3β and so Tau protein phosphorylation [8, 11, 12]. In this regard, phosphoinositide

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3-kinase (PI3K) dependent activation of protein kinase B (Akt) can induce a direct phosphorylation of GSK3β activity at Ser^9 leading to inhibition of its activity [8, 11, 13]. In support, pharmacological inhibition of PI3K/Akt signaling induced apoptosis and activation of GSK3β [8, 14, 15]. Very interestingly, reactive oxygen species (ROS) can directly inhibit PI3K/Akt activity in numerous tissues including neurons, thus inducing a direct activation of GSK3β [8, 14–17].

On the other hand, both genetic (70%) and environmental factors (30%) participate in AD pathogenesis [1, 18]. Cadmium (Cd) is one of the major animals and humans environmental neurotoxic pollutants [19]. This has been attributed to its high bioavailability in our daily consumables including fertilizers, pigments, smoking and plastics products, high exposure rats through the gastrointestinal, pulmonary and dermal routes, very slow elimination rate, its ability to increase blood–brain barrier (BBB) permeability, and its pro-apoptotic properties [18–22]. Currently, Cd has been listed as one of the major risk factors for the development of AD [23–25], by depleting levels of intracellular reduced glutathione (GSH) and enhancing the production of ROS [26–29]. In addition, Cd ions can hyper-phosphorylate Tau protein and increase the aggregation of Tau filaments via activation of GSK-3β and inhibition of PP2A [25, 30, 31]. Also, Cd-induced neurodegeneration is mediated by a sustained activation of the mammalian target of rapamycin (mTOR) signaling pathway [26, 31–33], which was also shown to modulate Tau protein phosphorylation [34, 51].

On the other hand, *trans*-resveratrol (3,5,4-trihydroxy-*trans*-stilbene) (RES) is a well-reported neuroprotective agent for both animals and humans due to its potent antioxidant and ROS scavenging potentials [35–37]. In human, moderate RES intake for 3 years was associated with the inverse relationship of the incidence of AD [38, 39]. Using animal's models of the AD and cultured neuronal cells, it has been shown that RES inhibited neural apoptosis via inhibition of ROS generation and caspase activation, improved learning, and memory formation enhanced the clearance of β-amyloid (Aβ) peptides and inhibition of neuronal apoptosis [40–43]. However, the mechanism by which RES acts is remaining unclear.

In in vitro cultured cortical neurons, RES effectively prevented Cd-induced apoptosis by inhibiting ROS generation, down-regulating mTOR pathway and activating of PP2A [44, 45]. This highlights an inhibitory role of RES on the activity of PP2A. Further support comes from the study of Schweiger et al. [46] who elegantly showed that RES administration to cultured cortical neurons or in vivo in wild type mice reduces dephosphorylation of Tau by interfering with the with microtubule-associated ubiquitin ligase (MID1)-PP2A complex, thus prevents the degradation of PP2A. In spite of these findings, it is still unknown if RES may act in a similar mechanisms under stress conditions such as those induced by cadmium chloride ($CdCl_2$). On the other hand, the inhibitory effect of RES on the PI3K/Akt/GSK3β axis has been also shown in vitro in oxygen–glucose-deprived hippocampal cultured cells model [47]. In this study, RES induced an activation of Akt and phosphorylation/inactivation of GSK3β, an effect that was prevented by the use of LY294002, a PI3K inhibitor [47].

In spite of these supportive findings, the regulatory effect of RES on the brain activity and expression of PP2A and PI3K/Akt/GSK3β in rats in relation to Tau hyperphosphorylation and memory deficits induced by chronic administration $CdCl_2$ remains unsecured. Based on this evidence, this in vivo study was designed with the hypothesis that RES ameliorates Tau hyperphosphorylation in the brain of rats by inhibiting of PI3K/Akt/GSK3β signaling pathways and activating that of P22A.

Materials and Methods

Drugs and Chemicals

Dimethyl sulfoxide (DMSO) and hydroxypropyl cyclodextrin (HDCD) were purchased from Sigma Aldrich, UK (Cat No. 276855, Cat No. H107, respectively). LY294002 was purchased from Cell Signaling Technology, USA (Cat No. 9901, USA). LY294002 was first freshly prepared in 100% DMSO to a final concentration of 100 µg/ml and then diluted with PBS (1:10) to give a final concentration of 10 µg/ml (0.50 µg/50 µl), used for the experiment. RES was purchased from Sigma Aldrich, UK (Cat No. R5010) and was always freshly prepared in a saline solution (0.9% NaCl) of 20% HDCD.

Animals

All of the experiments involving animals in present study were performed in the animal center of King Khalid University (KKU), Abha, Suadi Arabia and approved by the animal ethical committee of the College of Science that follows the guidelines established by the US National Institutes of Health animal (NIH publication No. 85-23, revised 1996). Disease-free healthy adult male white albino Sprague Dawley rat (220–230 g) were used in the experimental procedure and were housed in the same room with controlled temperature (22 °C), humidity (60%), and a 12 h light/dark cycle. During the adaptation period for 1 week and during the whole period of the experimental procedure, all rats were fed a standard AIN-93G diet (Harlan Teklad, Madison, WI) and had free access to drinking water, ad libitum.

Experimental Protocol

Rats were divided into six groups (n = 10/group) of (1) a control + HDCD group that received of an oral dose of 1 ml of a saline solution prepared in of 20% HDCD; (2) a control + DMSO that received an i.p. injection of 50 µl of 100% DMSO diluted with PBP (1:10); (3) a control + LY294002 (a PI3K inhibitor) which were administered an i.p. injection of 50 µl of LY294002 (0.25 µg/100 body weight); (4) a control + RES-treated group: control rats which received an oral dose of RES (300 mg/kg body weight), freshly prepared in a saline solution of 20% HDCD; (5) a $CdCl_2$-intoxicated group which were administered an oral dose of 1 ml of $CdCl_2$ (5 mg/kg) freshly prepared in normal saline; (6) a $CdCl_2$ + RES-treated group which received concomitant doses of $CdCl_2$ and RES as prepared and given in groups 2 and 3, respectively; (7) a $CdCl_2$ + RES + LY294002-treated group which were treated with LY294002 as performed in groups 5, 2 h before being treated with the combined dose of $CdCl_2$ + RES as performed in group 4. All treatments were carried out for 45 days on daily basis.

Dose Selection

The dose of RES and rout of administration was based on the study of Karuppagounder et al. [41] who showed that administration of such dose of RES for 30 days is able to reduce plaque formation in the medial cortex, striatum, and hypothalamus of a transgenic mice model of AD. The dose and route of administration of $CdCl_2$ were selected based on other studies which showed induced neurotoxicity, Tau hyperphosphorylation and AD-like symptoms when such dose was chronically administered to rats for 30 days [48]. The dose of LY294002 was selected based on the in vivo study of Xiaobin et al. [49], who reported that such dose of LY294002 is able to selectively and specifically block PI3K activity with no reported toxicity in normal tissues. It also recommended by the manufacturer who indicates that LY294002 specifically abolished PI3K activity (IC50 = 0.43 µg/ml; 1.40 µM) but no other lipid and protein kinases such as PKC, MAP kinase or c-Src. However, this dose was also tested in our preliminary studies which confirmed not to cause any apparent toxicity but successfully abolished the activation of PI3K and Akt (data are included in the "Results" section).

Assessment of Recognition Memory

To measure the effect of $CdCl_2$ and RES on short and long-term memory, we used novel object recognition task (NORT) test, which measures the spontaneous tendency of rodents to explore novel objects, according to the gridlines established by Bevins and Besheer [50]. The task was performed in a black woody apparatus that is impermeable to light (dimensions, 40 × 50 × 50 cm). In every test, two objects of the same color, size, and texture but having different shapes were placed on opposite sides of the testing room. The objects chosen in this task were two cuboidal, a cylindrical and a triangle wooden blocks which were heavy enough to prevent the rat from moving them. The test was started 1 day post the last treatment (day 31), where the rats were first exposed to a habituation session (three times) to explore the apparatus (10 min/each) without inserting any objects. One day later, rats were acclimated to the testing room for 1 h before the beginning of the training sessions. Training was carried out by allowing each rat to explore two identical objects (two identical cuboidal blocks, object A1 and A2) and then returned for its cage. Testing of short-term recognition memory was done 3 h post the training session, but this time the rats were allowed to explore two dissimilar objects; a familiar object from the training session (a cuboidal block) and a new novel object (a cylindrical block) (objects A1 and B). The rat was returned to its cage for another 21 h. Then long-term recognition memory was tested (24 h post the training session) as done in the short memory but this time the novel object was placed with a triangle block (object C). All rats were allowed to explore the room for a maximum time of 5 min and the room was cleaned with alcohol between sessions. A higher ratio was considered an evidence of enhanced cognitive performance. Effective exploration was defined only as directing or touching the nose with the object at a distance of no more than 2 cm. In all tests, turning around, rearing up onto, and sitting on the object were not considered an exploration behavior and were ignored. Discrimination ratio was determined by dividing the time spent to explore the familiar or novel object over the total time needed to explore both familiar and novel.

Brain Collection and Lysate Preparation for Western Blotting

Preparation of brain homogenates for western blotting, biochemical analysis and in vivo measurements total free radicals generation were done in accordance with procedures published by Zhang et al. [51]. Directly post the NORT test, each rat was anesthetized by an i.p. the dose of sodium pentobarbital (70 mg/kg) (Cat. No. P3761, Sigma Aldrich, UK) and killed by cervical dislocation. The brain was rapidly removed, placed on ice-cold dishes and washed with cold phosphate buffer saline (PBS, pH 7.4). Each brain was bisected in the mid-sagittal plane into two identical halves, each of which was snap frozen in liquid nitrogen. Brain powder was either homogenized in an immunoprecipitation buffer A or lysis buffer B (See supplementary methods).

The supernatants of both lysates were stored at − 80 °C and used later for western blotting or biochemical analysis. In both cases, proteins levels were determined by a PierceTM bicinchoninic acid protein (BCA) assay kit (Cat. No. 23225, NJ, USA).

Biochemical Analysis in the Brain Homogenates

Malondialdehyde (MDA) levels (nmol/mg protein) were measured using a colorimetric assay kit a (Cat. No. ab118970, Abcam, UK). Levels of reduced GSH and glutathione disulfide (GSSG) (μM) were measured using a colorimetric assay kit (Cat. No. 786-075, GBioscience, MO, USA). Total levels of ROS and nitrogen reactive species (NRS) were measured using used OxiSelect™ in vitro ROS/RNS Assay Kit (Cat. STA-347, Cell Biolabs, Inc. San Diego, CA) and expressed as % of the control. Levels of acetylcholine esterase (AChE) (mU/mg protein) were measured using a colorimetric assay kit (Cat. No. ab138871, Abcam, UK). Levels of Choline Acetyltransferase (ChAT) (ng/mg protein) were measured using chemiluminescent immunoassay Kit (Cat. No. SCB929Ra Cloud-Clone Corp., USA). Levels of Ach (ng/mg protein) were measured using a colorimetric assay Kit (Cat. No. STA-603, Cell Biolabs, Inc, USA). Details procedures are shown in supplementary methods.

Western Blotting Analysis

All protein samples were prepared in the loading buffer and boiled for 5 min. Equal amounts of protein (60 μg protein/well) were subjected to SDS polyacrylamide gel electrophoresis (10 and 12%) for 2 h at 100 V and then transferred to nitrocellulose membranes (Whatman, Dassel, Germany) for another 2 h at 70 V. The blots were then blocked with 5% non-skimmed milk, washed three times with 1 × 0.1 TBST buffer and then incubated with primary antibodies against total Tau-5, p-Tau (Ser^{396}), p-Tau (Ser^{199}), GSK3β, p-GSK3β (Ser^{9}), p-GSK3β (Tyr^{216}), PI3K, p-PI3K (^{tyr607}), PP2A, p-PP2A(Tyr^{307}), Akt, p-Akt (Ser^{473}), cleaved caspase-3, Bcl2, Bax, and β-actin. Characteristics of all antibodies and their suppliers are listed in Supplementary Table 1. The blots were then incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h. All developed bands were detected with an a Pierce-enhanced chemiluminescence (ECL) kit (Cat. No. 32106, Thermofisher, USA, Piscataway, NJ). Photographs were scanned and analyzed using the C-DiGit Blot Scanner (model: LI-COR, USA) with the supplied Image Studio DiGits software. Individual band densities were adjusted between different blots for inter-gel variability using an internal standard. Protein expressions were presented as relative expressions to that of the reference protein, β-actin. Data were performed in duplicate of 6 rats/group. Example data of detected protein are shown in the result section using the same the rat's brain/group.

Statistical Analysis

All data were analyzed using GraphPad Prism statistical software package (version 6) using one-way ANOVA followed by Tukey's test post-hoc. Different times obtained from the novel object recognition times were compared between groups by two-way ANOVA followed by Bonferroni post-hoc test. Data are presented as mean ± SD of a number of rats included in each experimental part. $p < 0.05$ is considered significantly meaningful.

Results

RES Increases GSH Levels in the Brains of Both Control and $CdCl_2$-Treated Rats and Inhibited ROS Generation and Lipid Peroxidation in $CdCl_2$-Treated Rats

Levels of GSH, GSSG, MDA and intracellular ROS in the brain homogenates of rats are shown in Fig. 1A–D. Administration of DMSO or LY294002 didn't affect the brain level of any of these biochemical parameters during the experimental period, as compared to control rats. While administration of RES to control rats didn't affect levels of ROS, MDA or GSSG, it significantly increased levels of GSH, indicating increase synthesis (Fig. 1A). On the other hand and as compared to the control group, administration of $CdCl_2$ to rats significantly lowered levels of GSH and increased those of MDA, GSSG, and ROS. Rats co-treated with RES + $CdCl_2$ showed significant increase in GSH levels with parallel significant decrease in levels of GSSG, MDA, and ROS, as compared to $CdCl_2$-treated rats. However, as compared to $CdCl_2$ + RES-treated rats, rats co-treated with $CdCl_2$ + RES + LY294002, showed significant increase in the levels of GSSG, MDA and ROS and significant decrease levels of GSH, levels, which all were not significantly different from their corresponding values measured in $CdCl_2$-treated rats (Fig. 1A–D).

RES Increases Levels of Ach and Activity of ChAT and Decreases the Activity of AChE in the Brain of Both Control and $CdCl_2$-Treated Rats

While control rats co-treated with DMSO showed normal levels of Ach and activities of ChAT and AchE in their brains homogenates as compared to control rats, levels of Ach and activity of ChAT were significantly decreased whereas activity of AchE was significantly increased in the brain of rats administered either LY294002 or $CdCl_2$,

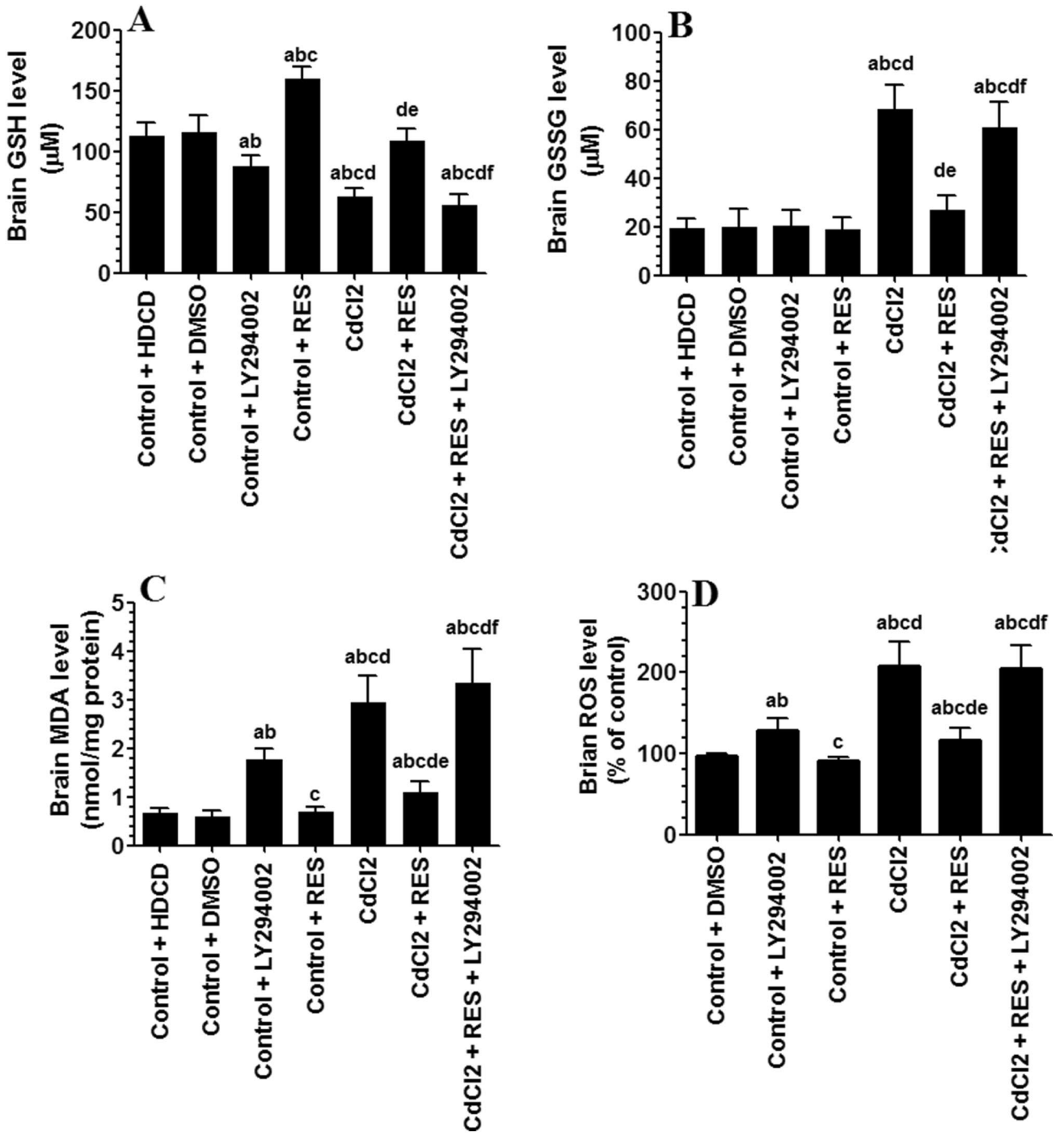


Fig. 1 Levels of reduced glutathione (GSH, **A**), glutathione disulfide (GSSG, **B**), malondialdehyde (MDA, **C**), and reactive oxygen species (ROS, **D**) in the brain homogenates of all experimental groups. Data are presented as mean ± SD of n = 10 rats/group. [a]Versus control = HDCD, [b]versus control + DMSO, [c]versus control + LY294002, [d]versus control + RES control, [e]versus $CdCl_2$, and [f]versus $CdCl_2$ + RES. *HDCD* hydroxypropyl cyclodextrin, *DMSO* dimethyl sulfoxide (DMSO), *RES* resveratrol

individually (Fig. 2A–C). On the contrary, control or $CdCl_2$-treated rats which were co-treated with RES showed significant decrease in brain activity of AchE and increase in levels of Ach and ChAT as compared to their control groups administered normal saline or $CdCl_2$, respectively (Fig. 2A–C). However, significant decrease in the levels of Ach and activity of ChAT with a concomitant increase in the activity of AchE were seen in the group of rats administered $CdCl_2$ + RES + LY294002, as compared to RES + $CdCl_2$-treated rats. Interestingly, the decrease in the brain level or

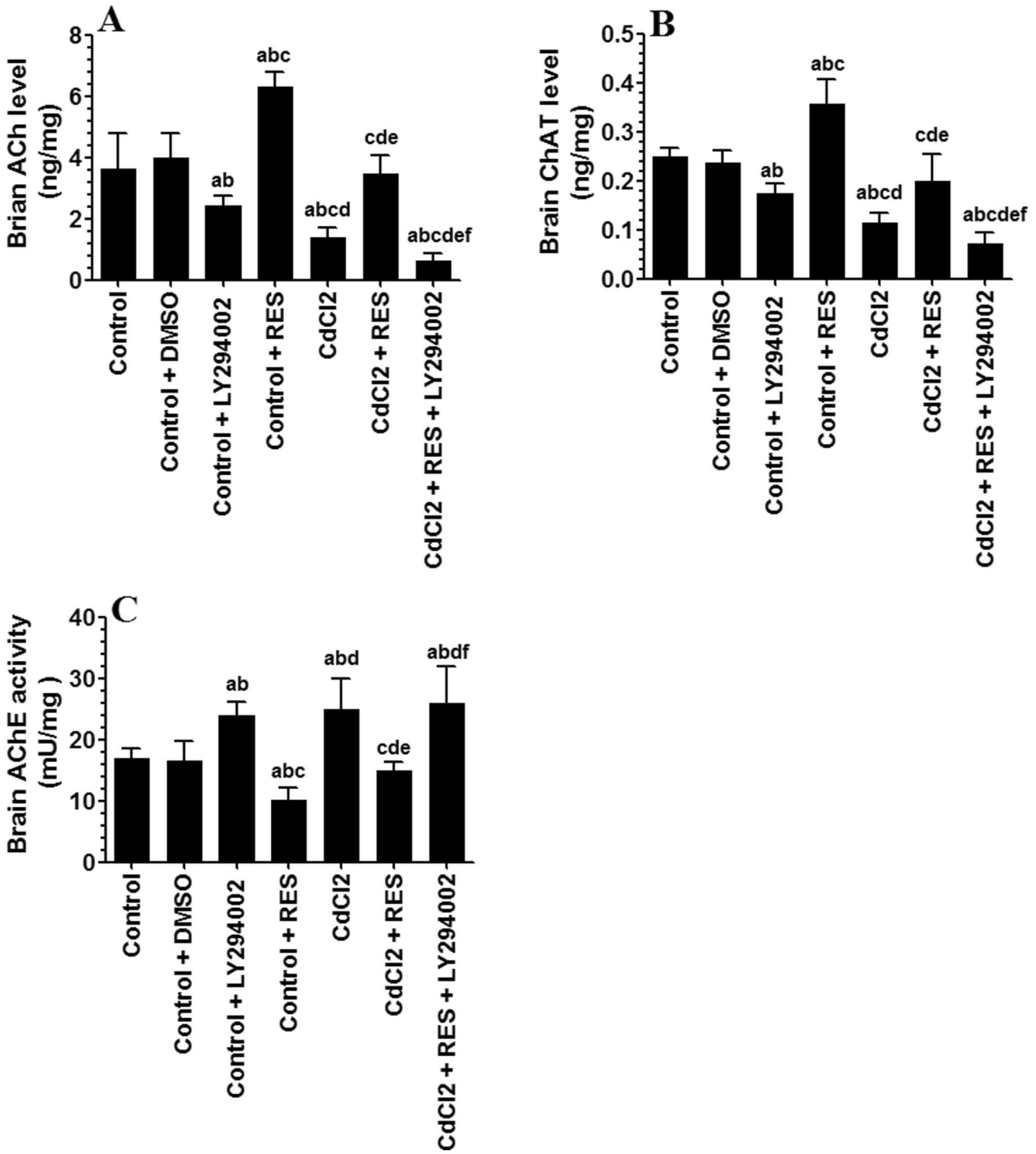


Fig. 2 Brian levels of acetylcholine (Ach, **A**) and activities of acetyltransferase (ChAT, **B**) and acetylcholinterase (AChE, **C**) in all experimental groups. Data are presented as mean ± SD of n = 10 rats/group. [a]Versus control = HDCD, [b]versus control + DMSO, [c]versus control + LY294002, [d]versus control + RES control. [e]versus $CdCl_2$, and [f]versus $CdCl_2$ + RES. *HDCD* hydroxypropyl cyclodextrin, *DMSO* dimethyl sulfoxide (DMSO), *RES* resveratrol

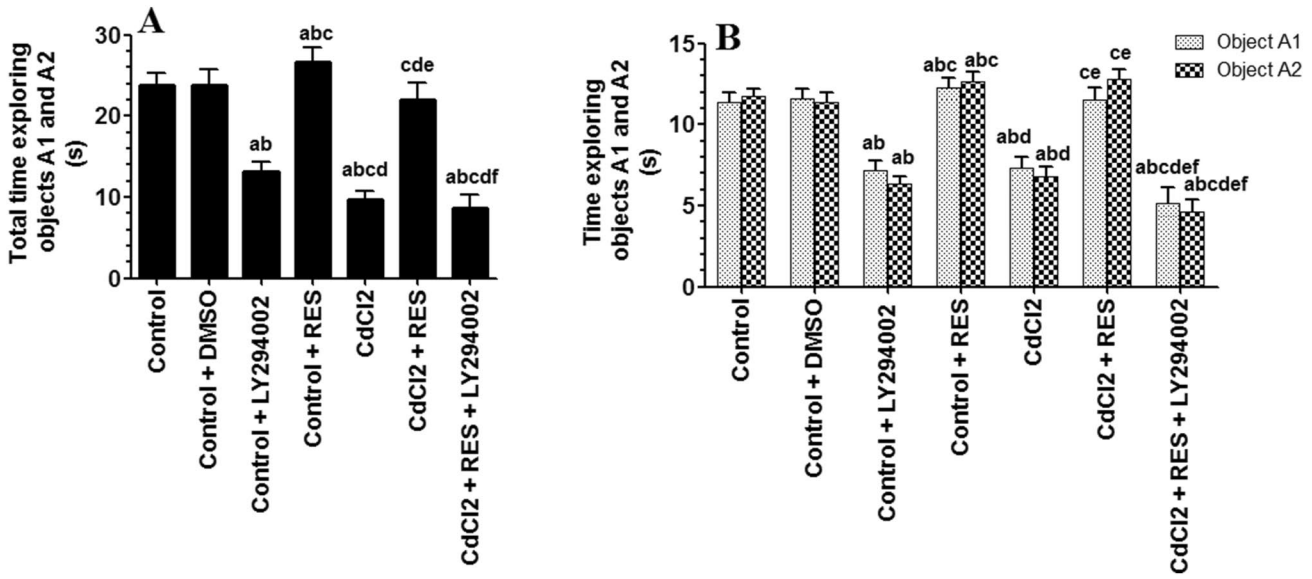


Fig. 3 Object recognition memory test results during the training session of all groups of rats. Training was carried out by allowing each rat to explore two identical objects (two identical cuboidal wooden blocks, object A1 and A2, respectively), and the time needed to explore each object was recorded. The analysis in figure **A** was done by one-way ANOVA followed by Tukey's t posthoc test. The analysis in figure **B** was done using two-way ANOVA followed by Bonferroni posthoc test. Data are presented as mean ± SD of n = 10 rats/group. [a]Versus control = HDCD, [b]versus control + DMSO, [c]versus control + LY294002, [d]versus control + RES control. [e]versus $CdCl_2$, and [f]versus $CdCl_2$ + RES. *HDCD* hydroxypropyl cyclodextrin, *DMSO* dimethyl sulfoxide (DMSO), *RES* resveratrol

activities of these cholinergic markers were more profound than their decrease measured in the brain of rats treated with either LY294002 or $CdCl_2$.

RES Enhances Short and Long-Term Recognition Memory in Both Control and $CdCl_2$ Treated Rats

We evaluated the effects of all treatments on short and long-term recognition memory by exposing all rats to a NORT (Figs. 3, 4, 5). During the training session (Fig. 3), rats administered either LY294002 or $CdCl_2$ showed decreased total and individual time to explore two identical objects (A1 and A2), whereas the RES-treated control rats showed an increase in total time needed for this task (Fig. 3A). On the other hand, RES + $CdCl_2$-treated rats showed significant increase in total and individual times needed to explore the same identical object as compared to $CdCl_2$-treated rats (Fig. 3A, B). However, rats co-treated with $CdCl_2$ + RES + LY294002 required less total and individual times to explore the same identical object as compared to CdCl2 + RES-treated rats, time periods which were not significantly different as compared to $CdCl_2$ + RES-treated rats (Fig. 3A).

Also, we tested the short-term memory 3 h post the training session (Fig. 4). In this part of the experiment, we have replaced one of the familiar objects (A2) with a new novel object (B). Normally, healthy rats spend a longer time to explore the new novel object. Data show that total time needed to explore objects A1 and B as well as the individual times needed to explore object A1 or B were significantly decreased in rats treated with LY294002 or $CdCl_2$ (Fig. 4A–C). Those rats were not able to discriminate between the familiar and novel objects, as indicated by the similar exploration times for both objects (Fig. 4B), which lead to a significant decrease in their calculated discrimination ratio, as compared to control rats (Fig. 4C). RES-treated rats had longer total exploration time to explore objects A1 and B and significantly longer individual time needed to explore object B, as compared to control rats (Fig. 4A–C). In addition, these rats had a higher calculated discrimination ratio. However, the group of rats administered $CdCl_2$ + RES + LY294002 showed similar results like those obtained in $CdCl_2$-treated rats but the decrease in their total time to explore both objects and object B as well as the in discrimination ratio were the most profound.

We also evaluated the effect of all treatments on long-term recognition memory in all groups of rats 24-h post the training session by replacing object B with a new novel object (C) (Fig. 5A–C). Similar results to those obtained in the short memory task were obtained in this task for all groups. However, administration of DMSO to control rats didn't affect time periods or discrimination periods during the training and short-or long memory testing and all measured time intervals were not significantly different to those measured in the control rats (Figs. 3, 4, 5).

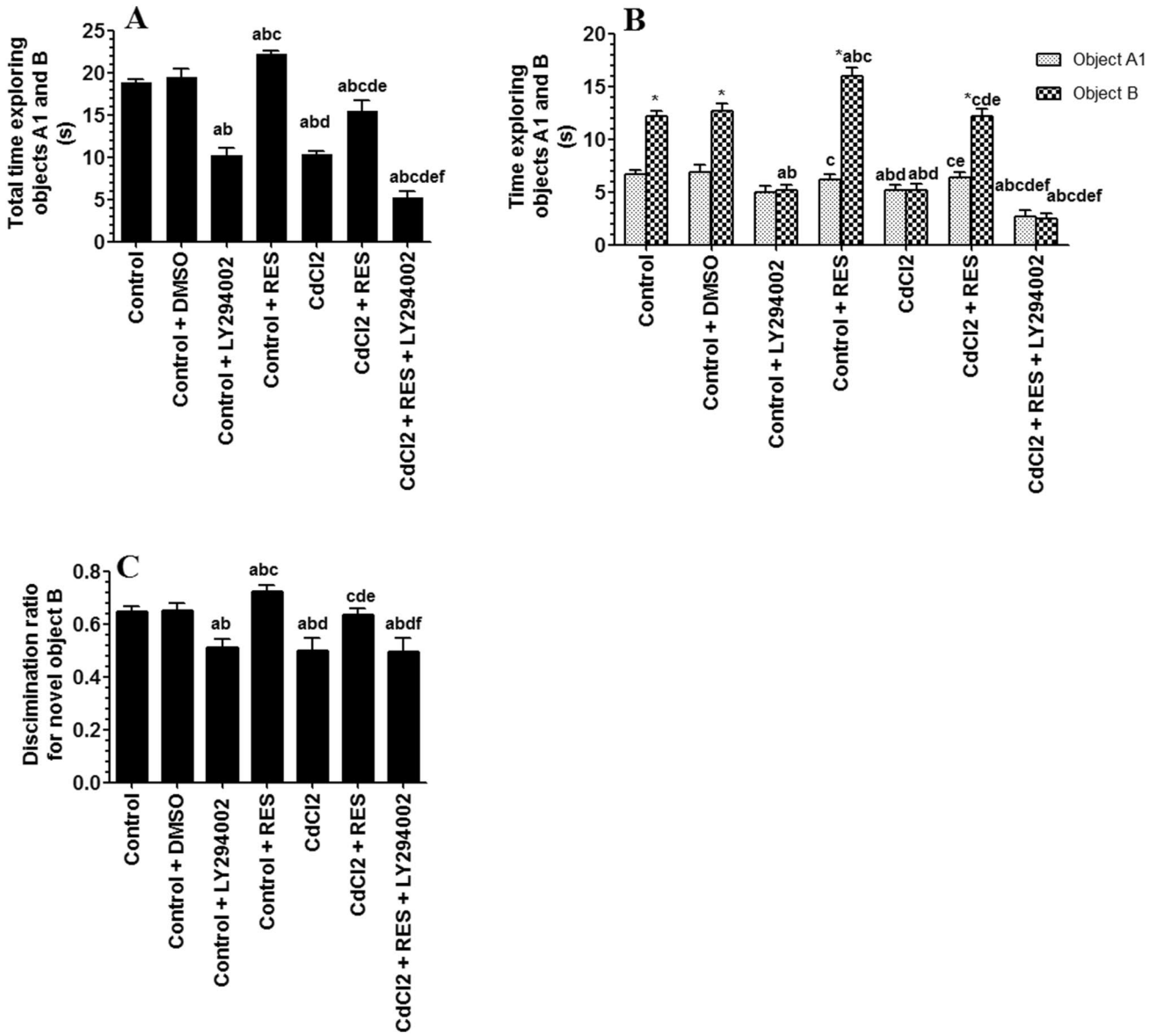


Fig. 4 Short-term object recognition memory test results in all groups of rats. The test was carried out 3 h post the training session to explore two dissimilar objects (a cuboidal and a cylindrical wooden blocks) (A1 and B, respectively), and time needed to explore each object was recorded. The analysis in figures **A** and **C** was done by one-way ANOVA followed by Tukey's t posthoc test. The analysis in figure **B** was done using two-way ANOVA followed by Bonferroni posthoc test. Data are presented as mean ± SD of n = 10 rats/group. [a]Versus control = HDCD, [b]versus control + DMSO, [c]versus control + LY294002, [d]versus control + RES control. [e]versus $CdCl_2$, and [f]versus $CdCl_2$ + RES. *HDCD* hydroxypropyl cyclodextrin, *DMSO* dimethyl sulfoxide (DMSO), *RES* resveratrol

RES Down-Regulates Tau Phosphorylation and Inhibits GS3Kβ Activity in the Brains of Both Control and $CdCl_2$-Treated Rats

As shown in Figs. 6 and 7, levels of total Tau and GS3K remained constant in all experimental rats. A significant increase in protein levels of p-Tau (Ser^{199} andSer^{396}) (Fig. 6A, B) as well as p-GS3Kβ (Tyr^{216}) (Fig. 7B) with a concomitant decrease in protein levels of p-GS3Kβ (Ser^{9}) (Fig. 7A) were seen in the brains of rats administered either $CdCl_2$ or LY294002, as compared to control rats. The protein levels of these proteins remained unaltered in control rats administered DMSO. RES administration to either the control or $CdCl_2$-treated rats significantly decreased the phosphorylation of Tau at its Ser^{199} and Ser^{216} (Fig. 6A, B) and that of p-GS3Kβ (Tyr^{216}) (Fig. 7B) and significantly increased protein levels of p-GS3Kβ (Ser^{9}) (Fig. 7A), as compared to control or $CdCl_2$-treated rats, respectively. Co-administration of

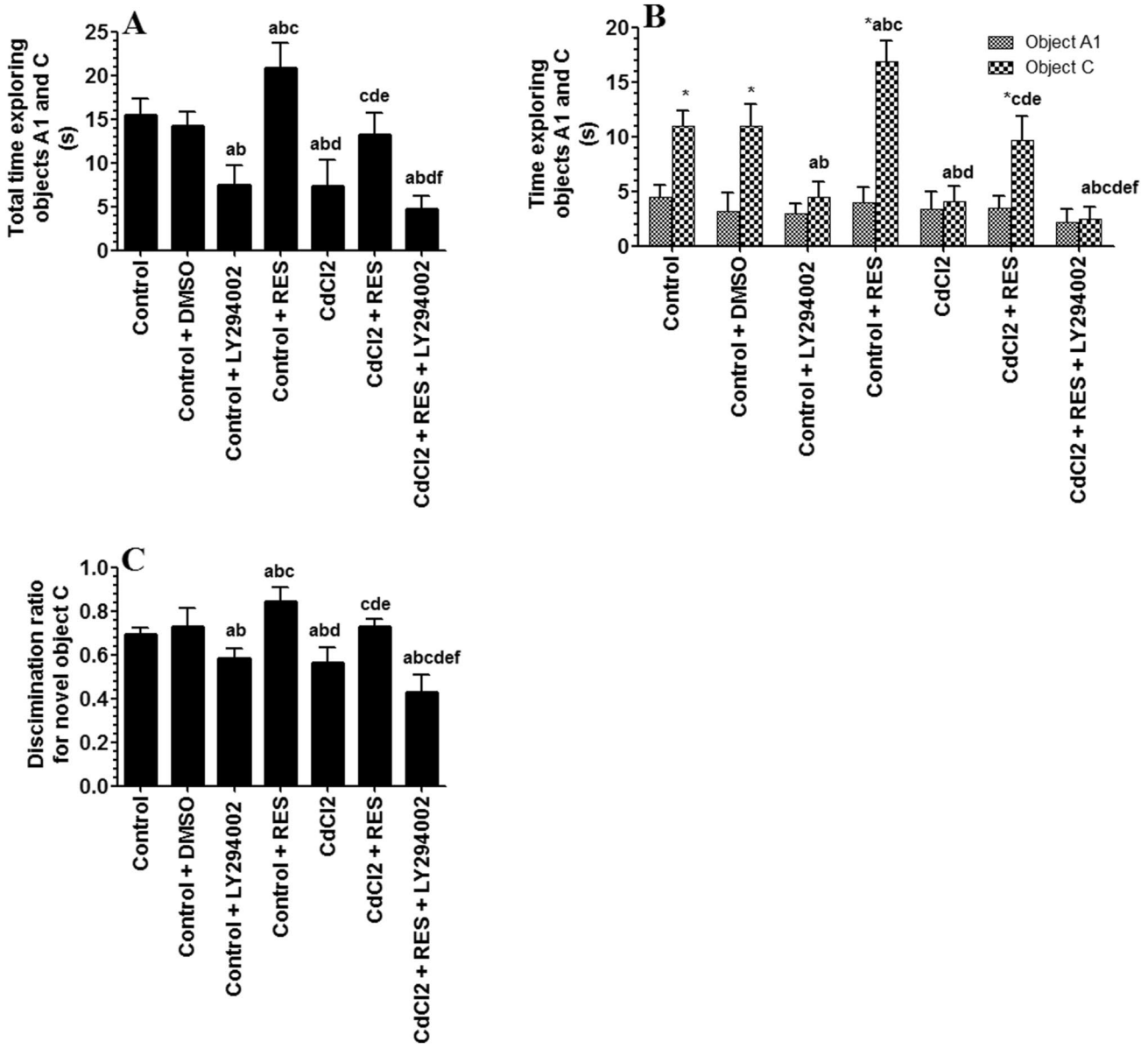


Fig. 5 Long-term object recognition memory test results in all groups of rats. The test was carried out 24 h post the training session to explore two dissimilar objects (a cuboidal and a triangle wooden blocks) (A1 and C, respectively), and time needed to explore each object was recorded. The analysis in figures **A** and **C** was done by one-way ANOVA followed by Tukey's t posthoc test. The analysis in figure **B** was done using two-way ANOVA followed by Bonferroni posthoc test. Data are presented as mean ± SD of n = 10 rats/group. [a]Versus control = HDCD, [b]versus control + DMSO, [c]versus control + LY294002, [d]versus control + RES control, [e]versus $CdCl_2$, and [f]versus $CdCl_2$ + RES. *HDCD* hydroxypropyl cyclodextrin, *DMSO* dimethyl sulfoxide (DMSO), *RES* resveratrol

LY294002 with RES + $CdCl_2$ abolished the effect of RES o these protein and led to significant increase in the levels of p-Tau (Ser^{199} and Ser^{396}) (Fig. 6A, B) and p-GS3Kβ (Tyr^{216}) (Fig. 7B) and significant decrease in protein levels of p-GS3Kβ (Ser^{9}) (Fig. 7A), as compared to $CdCl_2$ + RES-treated rats.

RES Enhances the Levels and Activity of AMPK and Activates PI3K/Akt Signaling in the Brains of Both Control and $CdCl_2$-Treated Rats

Total protein levels of PI3K-p85α and Akt were not varied and were abundantly available between all experimental groups (Fig. 8A, B). DMSO didn't affect levels of total or phosphorylated forms of PI3K and Akt nor AMPK as compared to control rats (Fig. 8A–C). Rats administered $CdCl_2$

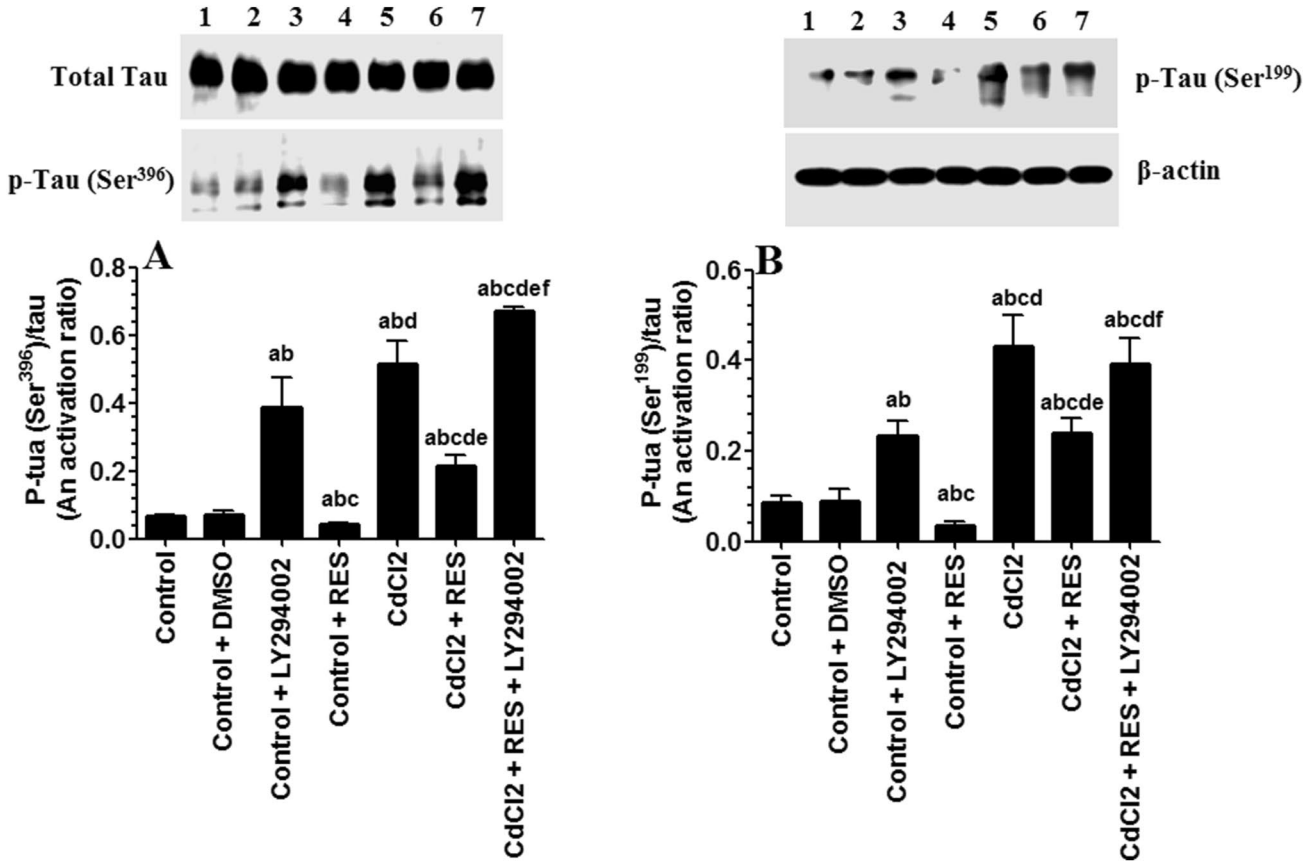


Fig. 6 Protein levels of total Tau (**A**), phopho-tau (Ser^{394} and Ser^{199}, **A**, **B**) in the brains of all experimental groups. Data are presented as mean ± SD of n = 6 rats/group. [a]Versus control = HDCD, [b]versus control + DMSO, [c]versus control + LY294002, [d]versus control + RES control, [e]versus $CdCl_2$, and [f]versus $CdCl_2$ + RES. Lane 1: control (rat #1); lane 2: control + DMOS (rat#1); lane 3: control + LY294002 (rat#1); lane 4: control + RES (rat#1); lane 5: $CdCl_2$ (rat#1); lane 6: $CdCl_2$ + RES (rat#1); lane 7: CdCl2 + RES + LY294002 (rat#1). *HDCD* hydroxypropyl cyclodextrin, *DMSO* dimethyl sulfoxide (DMSO)

or LY294002 had decreased levels of p-PI3K (Tyr^{697}) and Akt (Ser^{473}) leading to decrease in their activation ratios as compared to control rats (Fig. 8A, B). Also, $CdCl_2$-treated rats showed decreased levels of both AMPKα1 and p-AMPKα1/2 (Thr^{172}) in their brains as compared to all other groups. On the opposite, control or $CdCl_2$-treated rats and co-administered RES showed significant increase in the proteins levels of p-PI3K (Tyr^{697}) and Akt (Ser^{473}) and p-AMPKα1/2 as well as in total AMPKα1 with higher activation ratios of these proteins as compared to control rats or $CdCl_2$-treated rats, respectively (Fig. 8A–C). As compared to CdC_2-treated + RES rats, the concomitant administration of LY294002 with RES in $CdCl_2$-treated rats significantly lowered protein levels of both p-PI3K (Tyr^{697}) and Akt (Ser^{473}) and their activation ratios to a similar level reported in $CdCl_2$-treated rats (Fig. 8A, B), However, administration of LY294002 with RES to $CdCl_2$-treated rats didn't affect levels of AMPK nor p-AMPK, confirming that AMPK is an upstream inducer of PI3K/Akt signaling pathway, not vice versa.

RES Decreases Levels of Bax and Cleaved Caspase-3 and Increases Levels of Bcl2 in the Brains of Both Control and $CdCl_2$-Treated Rats

Stable levels of Bax, Bcl-2 and cleaved caspase-3 were observed in the brain of control rats administered DMSO as compared to their basal levels observed in the control groups (Fig. 9A–C). There were significant increase in the levels of Bax and cleaved caspase-3 with a parallel decrease in levels of Bcl-2 in the brain of rats administered either LY294002 or $CdCl_2$, as compared to control rats (Fig. 9A–C). When compared to control rats administered normal saline or $CdCl_2$-treated rats, respectively, control or $CdCl_2$-treated rats and co-administered RES had significantly higher levels of Bcl-2 and significantly lowered levels of both Bax and cleaved caspase-3 (Fig. 9A–C). Of

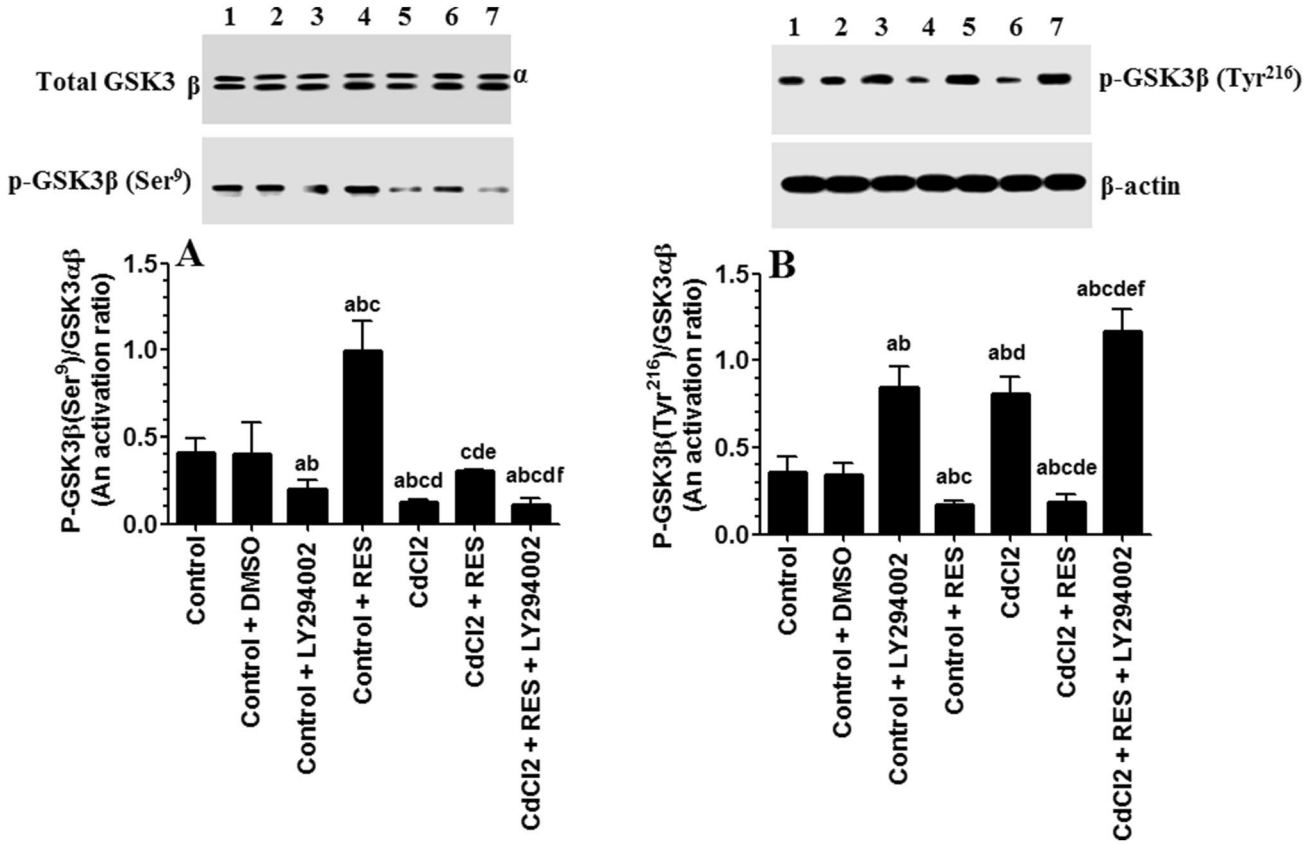


Fig. 7 Protein levels of total glycogen synthase kinase-3β (GSK3β, **A**), phospho-GSK3β (Ser^{9} and Tyr^{216}, **A**, **B**) in the brains of all experimental groups. Data are presented as mean ± SD of n = 6 rats/group. [a]Versus control = HDCD, [b]versus control + DMSO, [c]versus control + LY294002, [d]versus control + RES control. [e]versus $CdCl_2$, and [f]versus $CdCl_2$ + RES. Lane 1: control (rat #1); lane 2: control + DMOS (rat#1); lane 3: control + LY294002 (rat#1); lane 4: control + RES (rat#1); lane 5: $CdCl_2$ (rat#1); lane 6: $CdCl_2$ + RES (rat#1); lane 7: CdCl2 + RES + LY294002 (rat#1). *HDCD* hydroxypropyl cyclodextrin, *DMSO* dimethyl sulfoxide (DMSO)

interest, the levels of Bax and cleaved caspase-3 and levels of Bcl-2 were not significantly varied between groups of rats administered $CdCl_2$ + RES and that administered $CdCl_2$ + RES + LY294002 (Fig. 9A–C).

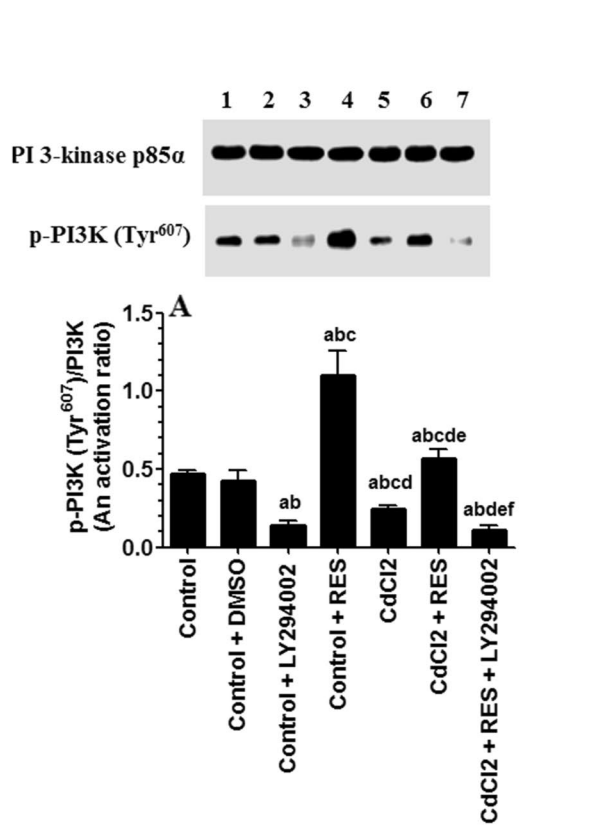
RES Enhances PP2A Activity in the Brain of Both Control and $CdCl_2$-Treated Rats

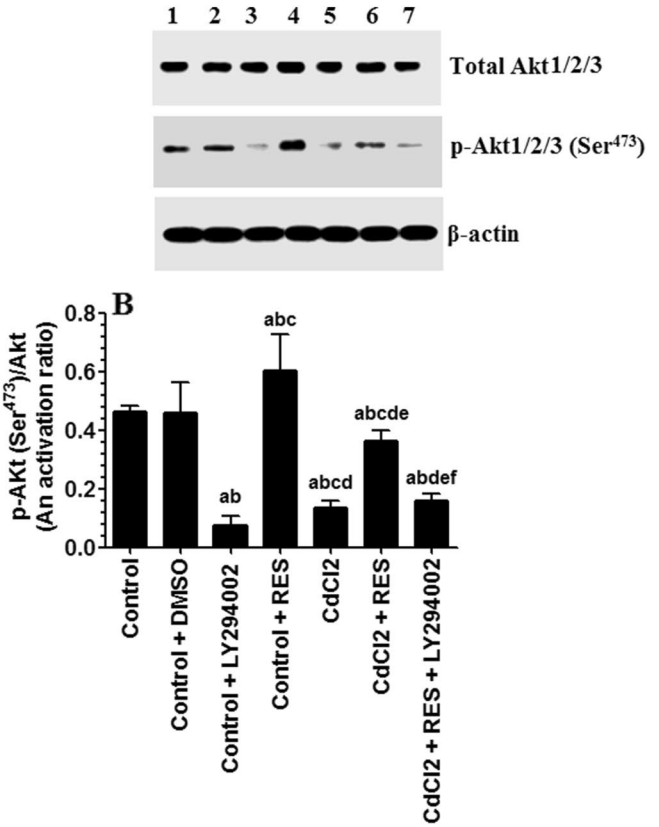
Protein levels of PP2A and p-PP2A (Tyr^{307}) remained stable in the groups of rats administered DMOS or LY294002, as compared to their protein levels detected in the control rats (Fig. 9A). RES administration to either the control or $CdCl_2$-treated rats significantly increased protein levels of p-PP2A (Tyr^{307}) but didn't affect total protein levels of PP2A, leading to increasing its activation ratio as compared to control rats or $CdCl_2$-treated rats, respectively (Fig. 9A). Levels of p-PP2A (Tyr^{307}) were significantly decreased and returned to their levels detected in CdCl2-treated rats when LY294002 was co-administered with $CdCl_2$ and RES (Fig. 9A).

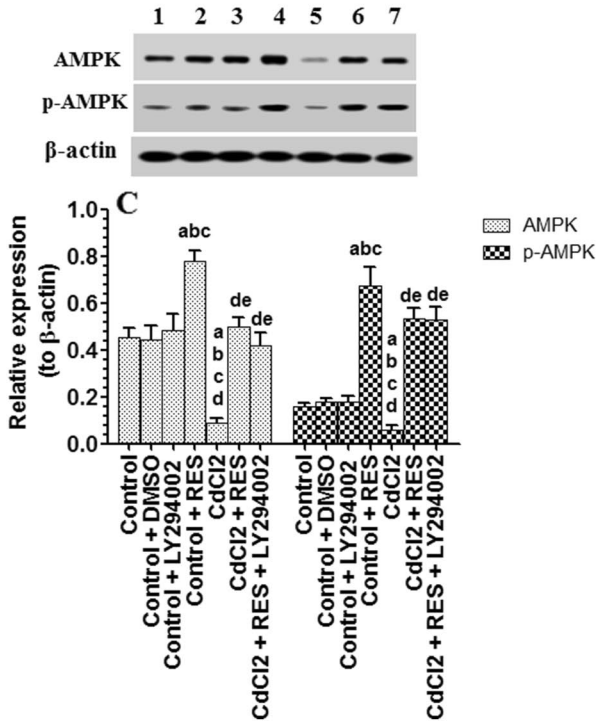
Discussion

The findings of this in vivo study support our hypothesis that RES protects against $CdCl_2$-induced Tau hyperphosphorylation and memory deficits and show that the mechanism of protection is mediated by concurrent activation of PP2A and inhibition of GSK3β mediated by activation of AMPK/PI3K/Akt signaling pathway. Interestingly, the protective effect of RES on tau hyperphosphorylation was completely abolished by co-administration LY294002, a selective PI3K inhibitor, suggesting that activation of AMPK/PI3K/Akt signaling pathway, rather than PP2A, is indispensable mechanism of action of RES in this animal model.

Cd ions can easily cross the Blood brain barrier (BBB) and induce mitochondrial-mediated cell apptosis via depletion of cellular antioxidant systems and generation of ROS [26–32, 52]. This has been also confirmed in this study as evident by the higher brain levels of ROS and MDA, lower levels of GSH, higher protein levels of cleaved caspase-3 and Bax and the lower protein levels of Bcl-2. In accordance,







low ratio of Bcl-2/Bax was seen in the cortex and hippocampus of rats administered $CdCl_2$ for 15 days [53].

High levels of ROS production can inhibit PI3K/Akt signaling pathway [54]. Akt is an important survival molecule that can inhibit apoptosis by inhibiting Bax translocation to

◂**Fig. 8** Protein levels of total phosphoinositide 3-kinase (PI3K, **A**), phospho-PI3K (Tyr697, **A**), total kinase B (Akt, **B**), phosphor-Akt (Ser^{473}, **B**), total 5′-AMP-activated protein kinase (AMPK, **C**) and phospho-AMPK (Thr^{172}, **C**) in the brains of all experimental groups. Data are presented as mean ± SD of n = 6 rats/group. [a]Versus control = HDCD, [b]versus control + DMSO, [c]versus control + LY294002, [d]versus control + RES control, [e]versus $CdCl_2$, and [f]versus $CdCl_2$ + RES. Lane 1: control (rat #1); lane 2: control + DMOS (rat#1); lane 3: control + LY294002 (rat#1); lane 4: control + RES (rat#1); lane 5: $CdCl_2$ (rat#1); lane 6: $CdCl_2$ + RES (rat#1); lane 7: CdCl2 + RES + LY294002 (rat#1)

mitochondria and the activity of procaspase-9 and stimulating GSH biosynthesis [55–57]. Given the inhibited activity of PI3K/Akt signaling pathway in the brains of control rats treated with LY294002 (a PI3K/Akt inhibitor) or in $CdCl_2$-treated rats, we could speculate that the decrease in levels of GSH and Bcl-2, and the increase in Bax levels is mediated by the effect $CdCl_2$ on this signaling pathway. Hence, $CdCl_2$ may act as a prooxidant in the brain by indirectly by inhibition of PI3K/Akt activity.

On the other hand, the oxidative stress response, the increase in ROS and the alterations in these biochemical and apoptotic markers in the brain of rats treated with $CdCl_2$ were prevented by RES co administration. This is expected with the well-known free-radical scavenging properties of RES [35–37]. In addition, a similar mirror image of these changes were also observed in the brain of control rats treated with RES. Again, all these changes was coincided with an activation of the PI3K/Akt signaling pathway in both groups which were completely abolished in the brain of $CdCl_2$ + RES + LY294002-treated rats. Hence, in addition to its free radical scavenging properties, these finding suggest that the anti-oxidant and anti-apoptotic effects of RES are also mediated by activation of PI3K/Akt signaling pathway. Similar to our findings but in vitro, RES activated PI3K/Akt signaling in organotypic hippocampal cultures exposed to oxygen-glucose deprivation (OGD), an effect that was prevented by LY294002 [46]. Similar results were also reported in cardiomyocytes [58].

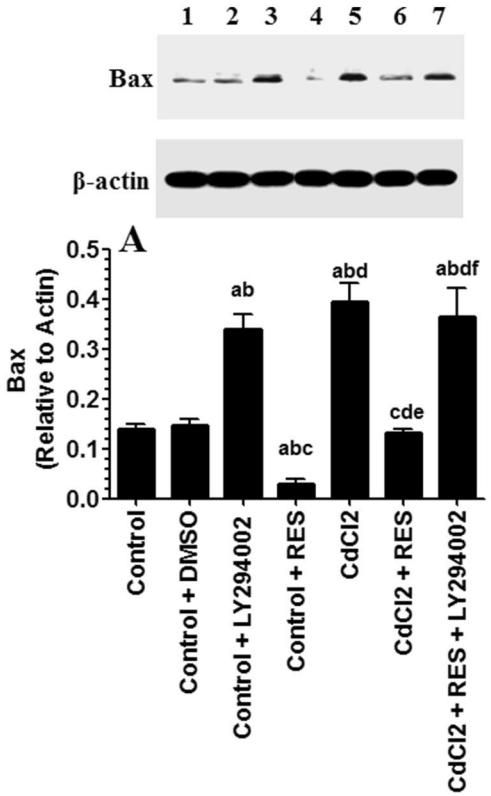
However, impairment of cognitive function, loss of memory, and decreased intelligence are classical signs observed in most AD patients [1, 59] as well as in human or animals exposed to Cd [60–62]. This is attributed to the loss and apoptosis of cholinergic and non-cholinergic neurons and the subsequent loss of Ach [63, 64]. Indeed, AD patients experienced decreased levels of Ach their brains [59, 65]. In the same line, control + LY294002 or $CdCl_2$-treated rats showed significant decrease in levels of Ach and activity of ChAT with a parallel increase in AchE activity in their brain homogenates. These effects were also reported by other authors and were explained by the active cell death of cholinergic neurons [48, 62–66].

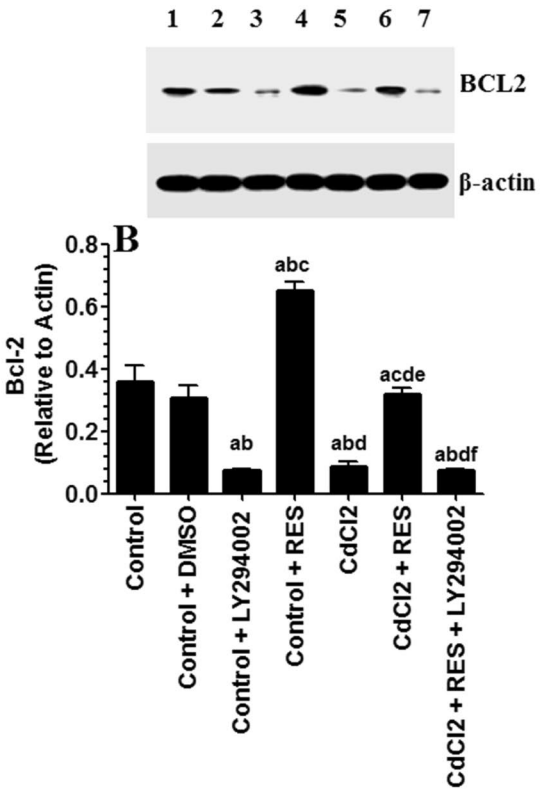
Concomitant with these cholinergic alterations, both short and long-term memory, were significantly impaired in these groups of rats. Of interest, RES enhanced and improved both types of memory in both control and $CdCl_2$-treated rats, respectively. Similar to our findings, RES improved the recognition index performance in AβPP/PS1 mice, a murine model of the familial AD and in a rat model of Aβ-induced AD [42, 43]. This could be explained by the concurrent increase in brain levels of Ach and activity of ChAT and inhibited brain activity of AChE in both groups with RES therapy. Given the stable levels of ROS and absence of apoptosis in the brains of control rats, these interesting findings may list RES as an AChE inhibitor, independent of its anti-oxidant potential. Even this not listed yet in literature, but it can be supported by other studies. Indeed, similar flavonoids has been reported to exert an AChE inhibitory activity, in vivo, such naringenin and quercetin [67, 68]. However, this requires a further research.

On the other hand, $CdCl_2$ was shown to induce Tau protein hyperphosphorylation in vivo and in vitro [25, 69]. $CdCl_2$-treated rats showed significant increase in Tau protein phosphorylation at Ser^{199} and Ser^{396}. Tau phosphorylation at these sites is associated with NFT formation and with loss of memory and cognitive deficits in the neurodegenerative disorders [70]. In animal models of induced-AD and $CdCl_2$-induced brain injury, increased activity of GS3Kβ, or decreased activity of PP2A can directly hyperphosphorylated Tau protein [45–74]. p-Akt can directly inhibit GS3Kβ activity by increasing phosphorylation at its Ser^9 or decreased phosphorylation at Tyr^{216} [75]. However, Akt should be phosphorylated p-by PI3K (Tyr^{607}) at its Ser^{473} for the maximum activity to be achieved [76].

Associated with the Tau hyperphosphorylation, the brains of $CdCl_2$-treated rats showed significant decrease in protein levels of p-PI3K (Tyr^{607}) and p-Akt (Ser^{473}), and significant increase in the activity of GS3Kβ as evident by the significant decrease in protein levels of p-GS3Kβ (Ser^9) and increased p-GS3Kβ (Tyr^{216}). In addition, they showed significant decrease in protein levels of PP2A (Tyr^{307}), a major phosphatase of Tau in the brain of $CdCl_2$-treated rats. Supporting our in vivo evidence, $CdCl_2$ treatment inhibited PP2A in cultured SH-SY5Y cells [31].

Interestingly, RES co-therapy significantly increased levels of p-PP2A (Tyr^{307}) and its activation ratio in the brain of control and $CdCl_2$-treated rats. These findings illustrate a regulatory role of RES on PP2A activity under healthy and stress conditions. However, this is not a unique finding. The effect of RES on PP2A activity has been previously shown in vitro and in other animal models. Even the possible mechanisms behind this is not investigated here, it has been previously explained by Schweiger et al. [46]. Using control cultured neural cells and wild type mice, those authors have shown that RES enhances the activity of PP2A by the reducing MID1 protein expression and interferes with the MID1-α4-PP2A degradation complex. On the other hand, what is


1 2 3 4 5 6 7
Bax
β-actin
A
Bax (Relative to Actin)
0.5
0.4
0.3
0.2
0.1
0.0
ab
abd
abdf
abc
cde
Control
Control + DMSO
Control + LY294002
Control + RES
CdCl2
CdCl2 + RES
CdCl2 + RES + LY294002


1 2 3 4 5 6 7
BCL2
β-actin
B
Bcl-2 (Relative to Actin)
0.8
0.6
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0.2
0.0
abc
acde
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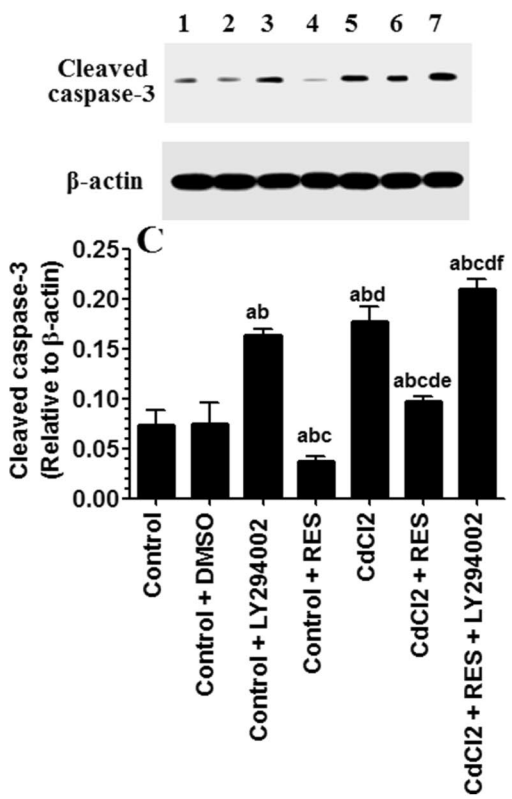
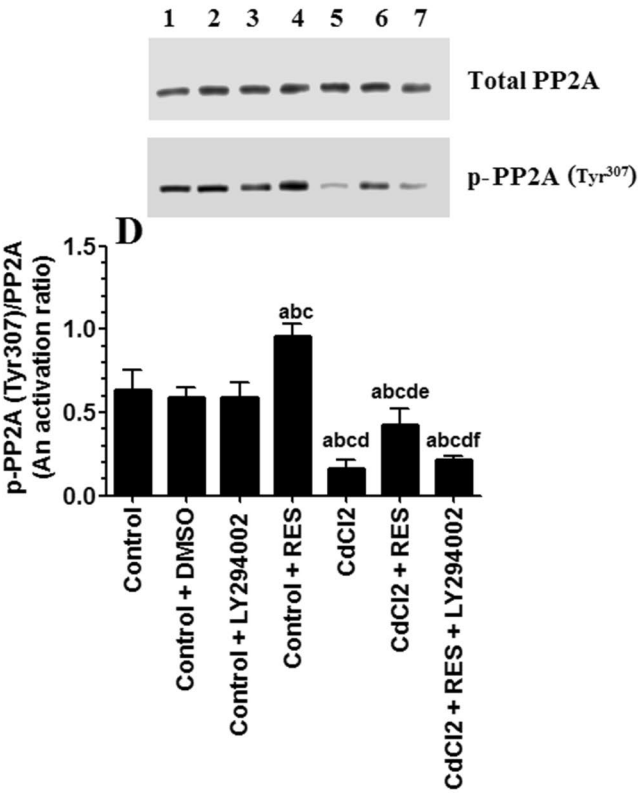
Control
Control + DMSO
Control + LY294002
Control + RES
CdCl2
CdCl2 + RES
CdCl2 + RES + LY294002


1 2 3 4 5 6 7
Cleaved caspase-3
β-actin
C
Cleaved caspase-3 (Relative to β-actin)
0.25
0.20
0.15
0.10
0.05
0.00
ab
abd
abcdf
abc
abcde
Control
Control + DMSO
Control + LY294002
Control + RES
CdCl2
CdCl2 + RES
CdCl2 + RES + LY294002


1 2 3 4 5 6 7
Total PP2A
p-PP2A (Tyr307)
D
p-PP2A (Tyr307)/PP2A (An activation ratio)
1.5
1.0
0.5
0.0
abc
abcde
abcd
abcdf
Control
Control + DMSO
Control + LY294002
Control + RES
CdCl2
CdCl2 + RES
CdCl2 + RES + LY294002

◂**Fig. 9** Protein levels of Bax (**A**), Bcl-2-t (**B**), cleaved caspase-3 (**C**), total protein phosphatase 2A (PP2A, **D**), phosphp-PP2A (Tyr^{307}, **D**) in the brains of all experimental groups. Data are presented as mean ± SD of n = 6 rats/group. [a]Versus control = HDCD, [b]versus control + DMSO, [c]versus control + LY294002, [d]versus control + RES control, [e]versus $CdCl_2$, and [f]versus $CdCl_2$ + RES. Lane 1: control (rat #1); lane 2: control + DMOS (rat#1); lane 3: control + LY294002 (rat#1); lane 4: control + RES (rat#1); lane 5: $CdCl_2$ (rat#1); lane 6: $CdCl_2$ + RES (rat#1); lane 7: CdCl2 + RES + LY294002 (rat#1). *HDCD* hydroxypropyl cyclodextrin, *DMSO* dimethyl sulfoxide (DMSO)

unique in this study is the increased activity of PI3K/Akt and concomitant inhibition of GS3Kβ activity in the brain of control and $CdCl_2$-treated rats which received a co-therapy of RES. These findings are of much interest as they show that RES may act as a survival molecule by direct stimulation of this pathway even in absence of stress. Hence, it was of our interest to go further to investigate the mechanism by which RES activate PI3K/Akt signaling pathway.

RES is a potent AMPK/SIRT1 agonist [77, 78]. Interestingly AMPK can stimulate PI3K/Akt signaling pathway [79]. In addition, AMPK can modulate oxidative stress, mitochondrial damage and GS3Kβ [79–81]. In CB2R deficient mice, RES restored the behavioral impairment, reduced Tau phosphorylation, and reduced oxidative stress by inducing AMPK induced inhibition of GS3Kβ [82]. In this study, levels of AMPK were significantly reduced by $CdCl_2$. On the other hand, RES significantly increased the phosphorylation of AMPK and so its activity in both control and $CdCl_2$-treated rats. Hence, we could conclude that RES effects seen in this study in both the control are mediated at least by activation of AMPK-induced activation of PI3K/Akt signaling pathway.

RES levels in the tissues depends on its solubility and bioavailability in the blood [81]. However, one limitation in this study is that we were not able to measure $CdCl_2$ or RES levels in the serum or in the brain of all experimental groups. Knowing the levels of Cd ions in the serum of or brains of these rats exposed to this dose of $CdCl_2$ may provide us with bioavailability and pharmacokinetics of these drugs in the blood of these animals. This will allow us also to compare the severity of neurodegenerative effects produced by our dose used in human. This could be of much interest given that Cd has long half time (about 30 years) in tissues.

Overall, this study is a unique study that shows RES is able to protect against $CdCl_2$-induced-neurodegeneration and memory loss by boosting neurons antioxidant potential, preventing apoptosis and inhibition of Tau phosphorylation. This is mediated by activation of PP2A and AMPK-induced activation of PI3K/Akt signaling pathway which inhibits the activity of GS3K.

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Author Contributions Both AS and MA contributed equally to this work. AS and MA designed the experimental procedure and supervised the treatment and sample collections. MA performed the behavioral analysis and measured some of the biochemical parameters of this study. AS performed all the western blotting measurements. AS and MA collected the data, analyzed them and graphed the data. AS wrote the draft of the manuscript and MA finalized the final version.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interests.

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