Neurotoxicity Research (2019) 36:334–346
https://doi.org/10.1007/s12640-019-00045-7

ORIGINAL ARTICLE

Effects of Al Exposure on Mitochondrial Dynamics in Rat Hippocampus

Jisheng Nie[1] · Shengjie Lv[1] · Xueying Fu[1] · Qiao Niu[1]

Received: 16 October 2018 / Revised: 8 April 2019 / Accepted: 10 April 2019 / Published online: 4 May 2019

Abstract

Aluminum (Al) exposure impairs learning and memory function in humans and in animal models. Several studies have shown that the neurotoxicity of Al is associated with damage to mitochondrial morphology and mitochondrial dysfunction, but the molecular mechanism is unclear. The present study was performed to elucidate the possible molecular mechanism related to the Al-induced abnormal mitochondrial dynamics that lead to learning and memory disorders. SD rats were exposed to Al-maltolate complex ($Al(mal)_3$) (blank, 0, 0.41, 0.81, or 1.62 mg/kg) for 30, 60, or 90 days, and neurobehavior, mitochondrial morphology, mitochondrial function, the levels of fission proteins such as dynamin-related protein 1 (Drp1) and fission protein 1 (Fis1), and the levels of fusion proteins such as optic atrophy 1 (Opa1), mitofusin 1 (Mfn1), and mitofusin 2 (Mfn2) were explored. The results indicated that exposure to $Al(mal)_3$ increased the concentration of Al in the brain in a time- and dose-dependent manner and impaired spatial learning and memory. $Al(mal)_3$ damaged mitochondrial morphology and impaired mitochondrial function in the hippocampus. Dose-dependent elevations in the levels of mitochondrial fission (Drp1 and Fis1) and fusion (Opa1, Mfn1, and Mfn2) proteins were observed. In addition, the upregulation of calcineurin (CaN) and the reduced phosphorylation of Drp1 (s637) may have disturbed the balance of mitochondrial fission and fusion in the hippocampus. These results showed that Al-induced learning and memory impairment may be related to mitochondrial fission and fusion disorders.

Keywords Al · Neurotoxicity · Mitochondria · Fusion · Fission

Abbreviations

Al	Aluminum
COX IV	Cytochrome oxidase IV
Drp1	Dynamin-related protein 1
Fis1	Fission protein 1
Opa1	Optic atrophy 1
Mfn1	Mitofusin1
Mfn2	Mitofusin2
Maltol	3-Hydroxy-2-methyl-4-pyrone
$Al(mal)_3$	Aluminum-maltolate complex
MWM	Morris water maze
GFAAS	Graphite furnace atomic absorption spectrometry
TEM	Transmission electron microscopy
ETC	Electron transport chain
CaN	Calcineurin
pDrp1 (s637)	Drp1 ser 637 phosphorylation

Shengjie Lv co-first author

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12640-019-00045-7) contains supplementary material, which is available to authorized users.

✉ Jisheng Nie
niejisheng@sxmu.edu.cn

✉ Qiao Niu
niuqiao55@163.com

[1] Department of Occupational Health, School of Public Health, Shanxi Medical University, Xinjiannan Road 56, Taiyuan 030001, Shanxi, China

Introduction

Al is the third most abundant element after oxygen and silicon and constitutes approximately 8% of the earth's crust. Some of the major sources of human Al consumption are food, drinking water, beverages, and Al-containing drugs (Kandimalla et al. 2016). Because of its low molecular weight, Al^{3+} permeates the blood-brain barrier and accumulates in different areas of the hippocampus and cerebral cortex, and many epidemiological and clinical studies have related it to

neurodegenerative disorders including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and AD-type dementia in Parkinson's disease patients (Bondy 2014; Hu et al. 2007; Yokel 2002). Various animal studies have also shown that Al exposure causes neuropathological, neurobehavioral, and neurochemical changes that result in impaired learning ability (Kaur et al. 2006; Silva et al. 2013; Zhang et al. 2014). The molecular mechanisms involved in these effects have been studied; nevertheless, the exact mechanism by which Al exerts its toxic effects in the brain still needs to be delineated.

Mitochondria are key cytoplasmic organelles responsible for generating cellular energy, regulating intracellular calcium levels, altering the reduction-oxidation potential of cells, and regulating cell death. Impaired mitochondrial dynamics have increasingly been implicated in neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's diseases (Deng et al. 2008; Manczak et al. 2016; Mortiboys et al. 2008; Wang et al. 2009a, b). Several studies have indicated that Al has the potential to induce deleterious effects in terms of mitochondrial morphology and function. Al-induced cognitive dysfunction is associated with mitochondrial damage in terms of altering the activity of mitochondrial electron transport chain complexes as well as ATP synthesis in various brain regions of rats (Kumar et al. 2008; Prakash and Kumar 2012). In vivo studies have provided evidence that the neurotoxicity of Al affects ATP synthesis involved in the impairment of electron transfer along the respiratory chain, including the alteration of cytochrome oxidase (COX) subunits, i.e., COX I, COX II, COX III, COX IV, and ATP synthase (ATPase) (Dua and Gill 2004; Iglesias-Gonzalez et al. 2017; Kumar et al. 2008; Sood et al. 2015). Similarly, in vitro experiments have shown that exposure to Al causes decreases in ATP synthesis and damage to mitochondrial morphology in neuronal cells (Niu et al. 2005; Wang et al. 2017). The molecular mechanism by which Al causes damage to mitochondrial morphology and mitochondrial dysfunction remains unclear.

Mitochondria are dynamic organelles that continuously fuse with one another to form larger tubular networks and divide into smaller structures, a process regulated by the balance of fission/fusion machinery that mainly involves several large GTPases. Fission requires fission 1 protein (Fis1) and dynamin-like protein (Drp1). Conversely, fusion involves mitofusins 1 and 2 (Mfn1, Mfn2) and optic atrophy 1 (Opa1) (Chen and Chan 2009). The delicate balance between mitochondrial fission and fusion is crucial to the maintenance of a healthy population of mitochondria and proper mitochondrial distribution, morphology and function, the disruption of which causes human diseases, including neurological diseases like AD, Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD) (Liu et al. 2013; Manczak et al. 2011; Shirendeb et al. 2011; Wang et al. 2015). However, no study has focused on the correlation between the neurotoxicity of Al in relation to learning and memory impairment and the imbalance of mitochondrial fission and fusion in in vivo models. In the present study, we hypothesized that Al leads to neurotoxicity by interfering with the balance of mitochondrial fission and fusion proteins.

The dynamin-related GTPase Drp1 is an evolutionarily conserved protein that mediates mitochondrial division, and its functional impairment results in the aggregation of large, interconnected mitochondria within cells. Drp1 is associated with several mitochondrial functions, including fragmentation, phosphorylation, SUMOylation, ubiquitination, and cell death, thus linking Drp1 to neurodegenerative diseases (Ishihara et al. 2009; Wakabayashi et al. 2009). Although the functions of Drp1 in cells have been extensively studied, the mechanisms underlying its regulation remain less clear. The cAMP-dependent protein kinase-dependent phosphorylation of Drp1 within the GED domain at Ser637 (pDrp1 (s637)) inhibits Drp1 GTPase activity and results in clear alterations in Drp1 function and mitochondrial morphology that are likely involved in the dynamic regulation of mitochondrial division in cells (Chang and Blackstone 2007). On the other hand, other findings have demonstrated the involvement of calcineurin (CaN), which promotes the dephosphorylation of Drp1 at Ser637, leading to mitochondrial fragmentation, in the regulation of mitochondrial dynamics (Park et al. 2016). Primarily, the present study aimed to illuminate the role of mitochondrial fission/fusion proteins in Al-induced abnormal mitochondrial dynamics.

Materials and Methods

Al-Maltol ($Al(mal)_3$) Solution

Al-maltolate complex ($Al(mal)_3$) was prepared according to the procedure described in previous publications (Langui et al. 1990; Liang et al. 2012). $AlCl_3 \cdot 6H_2O$ (Tianjin Fenchuan Chemical Reagent Technologies, Co., Ltd., Tianjin, China) and maltolate (Sigma, MO, USA) were dissolved in distilled water and phosphate-buffered saline (PBS), respectively. $Al(mal)_3$ was freshly prepared for each experiment by mixing equal volumes of these solutions, adjusting the pH to 7.4 with NaOH, and filtering with 0.22 μmol/L syringe filters.

Animals and Treatment

One hundred eighty male Sprague-Dawley rats (8 weeks old) were used in this study and were purchased from the Laboratory Animal Center of China Food and Drug Certification Research Institute (certificate: SCXK (Jing) 2014–0013). All animals were kept under standard temperature conditions (22 ± 2 °C) with a 12:12 h light-dark cycle, were housed three per cage, and were given rat chow and

water ad libitum. The studies were performed in accordance with the Regulations of Institute Animal Care and Use Committee of Shanxi Medical University. The rats were weighed before being treated with $Al(mal)_3$.

One hundred eighty male SD rats were weighed and numbered, and the numbered rats were sorted according to their weights. Next, the rats were divided into 15 groups according to a random number table, and finally, each group of rats was randomly assigned to an $Al(mal)_3$ exposure group (blank, 0, 0.41, 0.81, or 1.62 mg/kg bw $Al(mal)_3$ for 30 or 60 or 90 days; $n = 12$/per dose). The blank control group received no treatment, the 0 mg/kg bw group received saline in a volume of 1 mL/kg bw via intraperitoneal injection, and the $Al(mal)_3$ treatment groups received $Al(mal)_3$ solutions of different concentrations (0.41, 0.81, or 1.62 mg/mL), which were prepared fresh. The volume of the intraperitoneal injection was determined by body weight (1 mL/kg bw). The rats were weighed to determine the volume of vehicle or $Al(mal)_3$. The rats were treated every other day for 30, 60, or 90 days.

Morris Water Maze

The Morris water maze apparatus was purchased from the Institute of Chinese Medical Science (Beijing, China) and included software that could be used to record latency, the time spent in the target space, the number of platform crossings, and other indexes. The rats from each group were tested on the MWM after exposure to $Al(mal)_3$. The Morris water maze apparatus was a stainless steel pool 130 cm in diameter and 50 cm in height with a nonreflective black interior surface. The pool was filled with water to a depth of 30 cm, and the temperature was maintained at 21 ± 2 °C. The platform was 10 cm in diameter and was submerged 1 cm below the water surface. The apparatus was placed in a room with ample surrounding visual cues.

Place Navigation Test

In the place navigation test, all the rats were tested for six successive days. On the first day, the platform was removed from the tank, and each rat was released into the water for 2 min from one of the four starting locations (N, E, NW, and SE) with its heads pointing toward the wall of the tank. After the rats acclimated to the water maze, the test was started. Beginning on the second day on, the platform was placed in the NE quadrant. Each rat was released into the water from a random starting point. If any rat failed to find the submerged platform within 2 min, the experimenter placed it gently on the platform for 10 s. Each rat underwent four trials each day.

Spatial Probe Test

After the place navigation test, the spatial probe trial was performed on the sixth day. Each rat was allowed to swim for 120 s from the SW starting point in the absence of the hidden platform. A series of test indexes, including the time spent in the target quadrant, the number of platform crossings, and the swimming path, which were recorded by an automatic tracking system (Morris Maze Experimental Assistant System; Institute of Material Medical, Chinese Academy of Medical Science, Beijing, China), were used to measure spatial memory in the probe trial.

Graphite Furnace Atomic Absorption Spectrometry

The tissues collected for graphite furnace atomic absorption spectrometry (GFAAS) were snap frozen in liquid nitrogen and stored at − 80 °C for analysis. Preceding analysis, the tissues were lyophilized for 48 h, weighed, digested in concentrated HNO3, and slowly evaporated on a warming plate for 72 h. The samples were resuspended in 20 μg/L of 1.0% HNO3 for analysis. The concentration of Al in the tissues was quantified using a Thermo iCE3500 graphite furnace (Thermo Fisher, Massachusetts, USA) with a wavelength of 309.3 nm, a slit of 0.5 nm, and an injection volume of 10 μL.

Transmission Electron Microscopy

The hippocampal tissues collected from the animals for transmission electron microscopy (TEM) (approximately 1 mm^3) were finely minced on dental wax and immediately fixed in 2.0% phosphate-buffered glutaraldehyde (pH 7.4) for 1–4 h at 4 °C. Subsequently, the samples were postfixed in 1.0% phosphate-buffered (pH 7.4) osmium tetroxide for 1 h at 4 °C. The tissues were washed in buffer and dehydrated in a series of water/acetone solutions up to 100% acetone. The hippocampal samples were infiltrated in sequentially increasing concentrations of LX112-Araldite to 100%, embedded in BEEM capsules, and placed in a 60 °C oven for 4 days. Semithin sections (0.5 μm) were stained with a 1:1 mixture of 1.0% methylene blue and 1.0% azure B and observed with a light microscope, and subsequently, selected regions were thin-sectioned and collected on 300-mesh copper grids. The tissue sections were stained with uranyl acetate followed by lead citrate and viewed with a JEM-100CX electron microscope (JEOL, Tokyo, Japan) at 80 kV.

Determination of Na^+-K^+ ATPase and Ca^{2+}-Mg^{2+}ATPase Activity

Na+-K+ ATPase and Ca2+-Mg2+ATPase activity was measured using a commercial kit (Nanjing Jiancheng Technology

Co., Ltd., Nanjing, China) according to the manufacturer's protocols.

Western Blot Analysis

The rats were sacrificed at the indicated times, and the brain tissue was extracted and immediately frozen at − 80 °C until use for western blot analysis. The hippocampal tissue was homogenized, sonicated on ice using a protein extraction reagent (Protein Extraction Reagent: CWBiotech, Beijing, China) containing protease and protein phosphatase inhibitors (CWBiotech, Beijing, China) and centrifuged (10,000×*g* for 20 min), and the supernatants were collected. The protein concentration was determined with the BCA Protein Assay Kit (CWBiotech, Beijing, China). The tissue lysates (30 μg of protein) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride membranes. Antibodies that recognize COX IV, Opa1, Mfn1, Mfn2, Fis1, Drp1, pDrp1 (s637), and CaN (Abcam, USA) were used to detect the relevant proteins by a chemiluminescence system with an eECL kit (CWBiotech, Beijing, China). Densitometric quantification of the immunoblots was performed by Universal Hood II (Bio-Rad, California, USA).

Statistical Analysis

Data analyses were performed with Statistical Product and Service Solutions 13.0 (SPSS 13.0), and the data were analyzed using analysis of variance (ANOVA). The data obtained during the hidden platform training days were analyzed by repeated measures ANOVA, and the remaining data were analyzed by one-way ANOVA; statistical significance was determined by the least significant difference (LSD) test. The interaction between the dose and time of each index was analyzed by factorial design ANOVA. The data were expressed as the mean ± SEM. A value of $P < 0.05$ was considered statistically significant for all analyses.

Results

Subchronic Al Exposure Increased the Al Concentration in the Hippocampus of Rats

The hippocampal tissue of the SD rats treated with $Al(mal)_3$ for 30, 60, or 90 days in the present study was weighed, and no change in the mass of the rat hippocampus among the groups was found (Table S1). We performed GFAAS to detect the Al concentration in the rat hippocampus. As shown in Fig. 1, subchronic Al exposure resulted in time- and dose-dependent increases in the concentration of Al in the hippocampus; as the administered dose and exposure time increased, the Al concentration in the hippocampus increased. Additionally, there was no significant interaction between dose and exposure time ($F = 1.097$, $P > 0.05$).

Subchronic Al Exposure Impaired Learning and Memory Ability in Rats

To investigate whether subchronic Al exposure impaired cognitive function, hippocampus-dependent learning and memory were assessed using the Morris Water Maze. As shown in Fig. 2a, rats from all groups showed similar

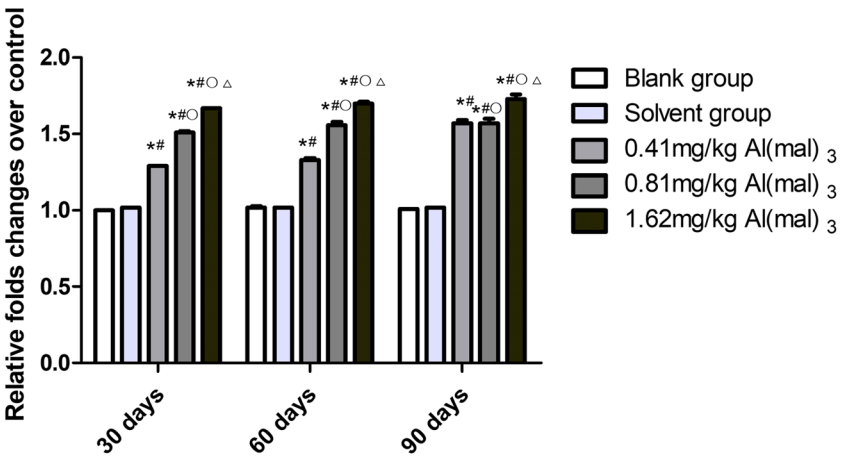

Fig. 1 Subchronic Al exposure resulted in an increased concentration of Al in the hippocampus rats. Male SD rats were administered different doses of $Al(mal)_3$ (0, 0.41, 0.81, or 1.62 mg/kg) intraperitoneally or left untreated for 30, 60, or 90 days. The Al concentration in the hippocampus of the rats in the different groups was tested by graphite furnace atomic absorption spectrophotometry (GFAAS). Factorial analysis of related factors (dose and time) showed a statistically significant interaction ($F = 1.097$, $P > 0.05$). The results are expressed as the mean ± SD, $n = 6$; * means versus blank control, $P < 0.05$; # means versus solvent, $P < 0.05$; ○ means versus 0.41 mg/kg $Al(mal)_3$, $P < 0.05$; △ means versus 0.81 mg/kg $Al(mal)_3$, $P < 0.05$

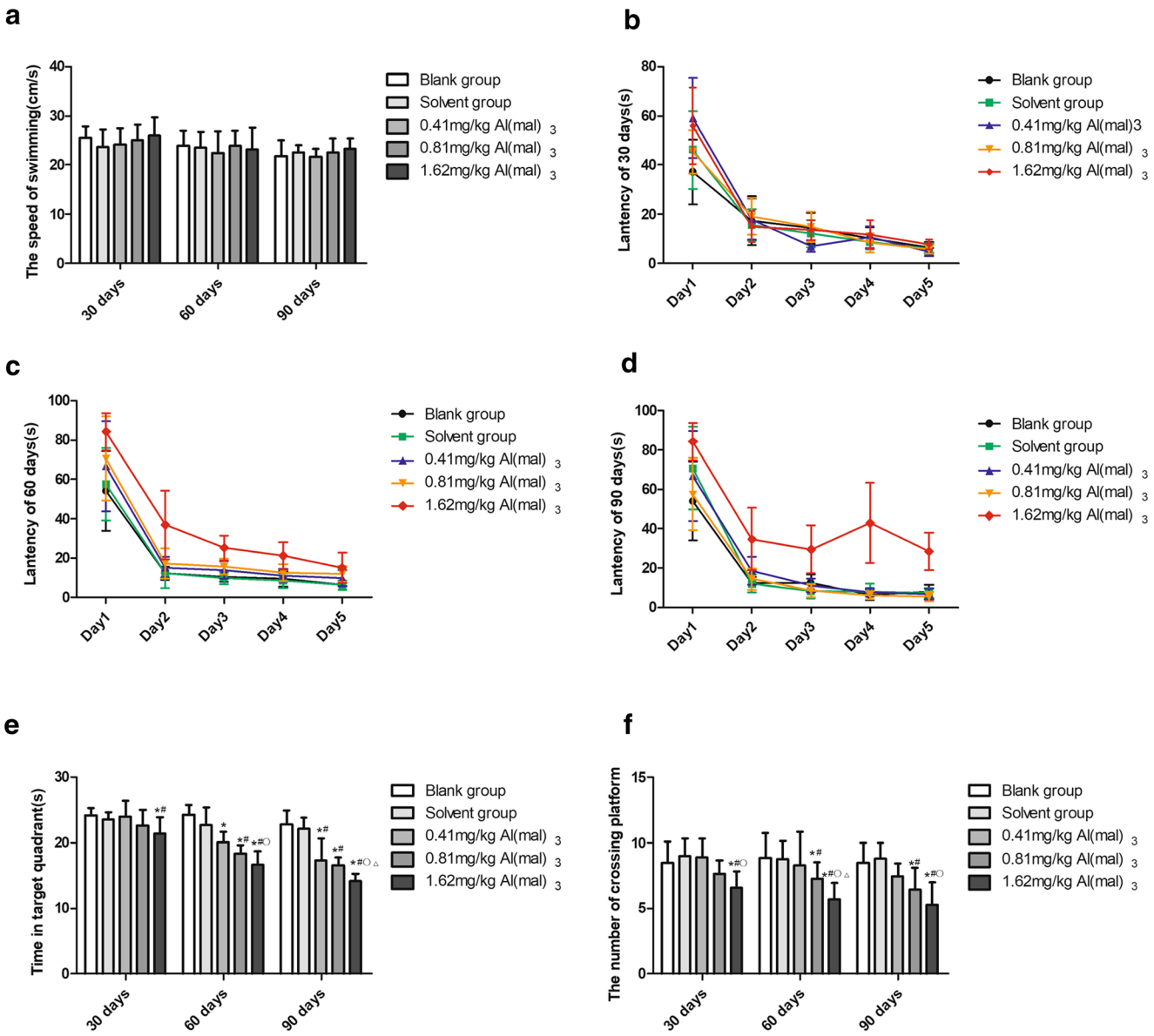

Fig. 2 Subchronic Al exposure resulted in the impairment of learning and memory ability in rats. The Morris water maze test was performed to assess the spatial learning ability and memory of the rats. **a** The average swimming speed at 30, 60, and 90 days before the place navigation test and showed no statistically significant interaction ($F_1 = 0.349$, $P = 0.946$). **b**, **c**, and **d** The escape latencies at 30, 60, and 90 days. Repeated-measures ANOVA of related factors (training day and dose) showed no statistically significant interaction ($F_1 = 1.648$, $P = 0.125$; $F_2 = 0.986$, $P = 0.453$; $F_3 = 0.763$, $P = 0.664$). **e** and **f** The effects of $Al(mal)_3$ exposure on the behavior of the rats in the probe trial: **e** the time spent in the target quadrant and **f** the number of platform crossings in the target area by the rats. Factorial analysis showed that there was a statistically significant interaction between the administered dose and the exposure time on the time spent in the target quadrant ($F = 4.853$, $P < 0.001$) but not the number of platform crossings ($F = 0.409$, $P = 0.913$). The results are expressed as the mean ± SD, $n = 8$; * means versus blank control, $P < 0.05$; # means versus solvent, $P < 0.05$; ○ means versus 0.41 mg/kg $Al(mal)_3$, $P < 0.05$; △ means versus 0.81 mg/kg $Al(mal)_3$, $P < 0.05$

swimming speeds at 30, 60, and 90 days. As shown in Fig. 2b–d, the escape latency of the 1.62 mg/kg Al-treated group decreased after exposure to Al for 60 and 90 days when compared with that of the blank and solvent groups. There was no statistically significant interaction of Al dose and training days on escape latency at 30, 60, and 90 days ($F_1 = 1.648$, $P = 0.125$; $F_2 = 0.986$, $P = 0.453$; $F_3 = 0.763$, $P = 0.664$). In the probe trial, as shown in Fig. 2e and f, Al exposure decreased the time spent in the target quadrant and the number of platform crossings in a time-dependent and dose-dependent manner, particularly in groups exposed to 0.81 and 1.62 mg/kg Al for 60 and 90 days. There was a statistically significant interaction between Al exposure time and dose on the time spent in the target quadrant ($F = 4.853$, $P < 0.001$) but not in the number of platform crossings ($F = 0.409$, $P = 0.913$). The data from the probe trial suggest that Al causes deleterious effects on spatial memory.

Subchronic Al Exposure Led to Mitochondrial Morphology Alterations

We assessed the effect of exposure on mitochondrial ultrastructure in hippocampal cells at 90 days by transmission electron microscopy. As shown in Fig. 3, marked pathological alterations were present in the 0.81 kg/kg and 1.62 mg/kg groups, which exhibited mitochondria that were rounder and swollen with broken cristae. These alterations became more severe in the mitochondria, which exhibited an extreme loss of internal cristae structure and vacuolation. Exposure to elevated concentrations of $Al(mal)_3$ (1.62 mg/kg) (Fig. 3e) also led to more prominent fission at 90 days. Altogether, these results reveal that subchronic Al exposure results in altered mitochondrial morphology.

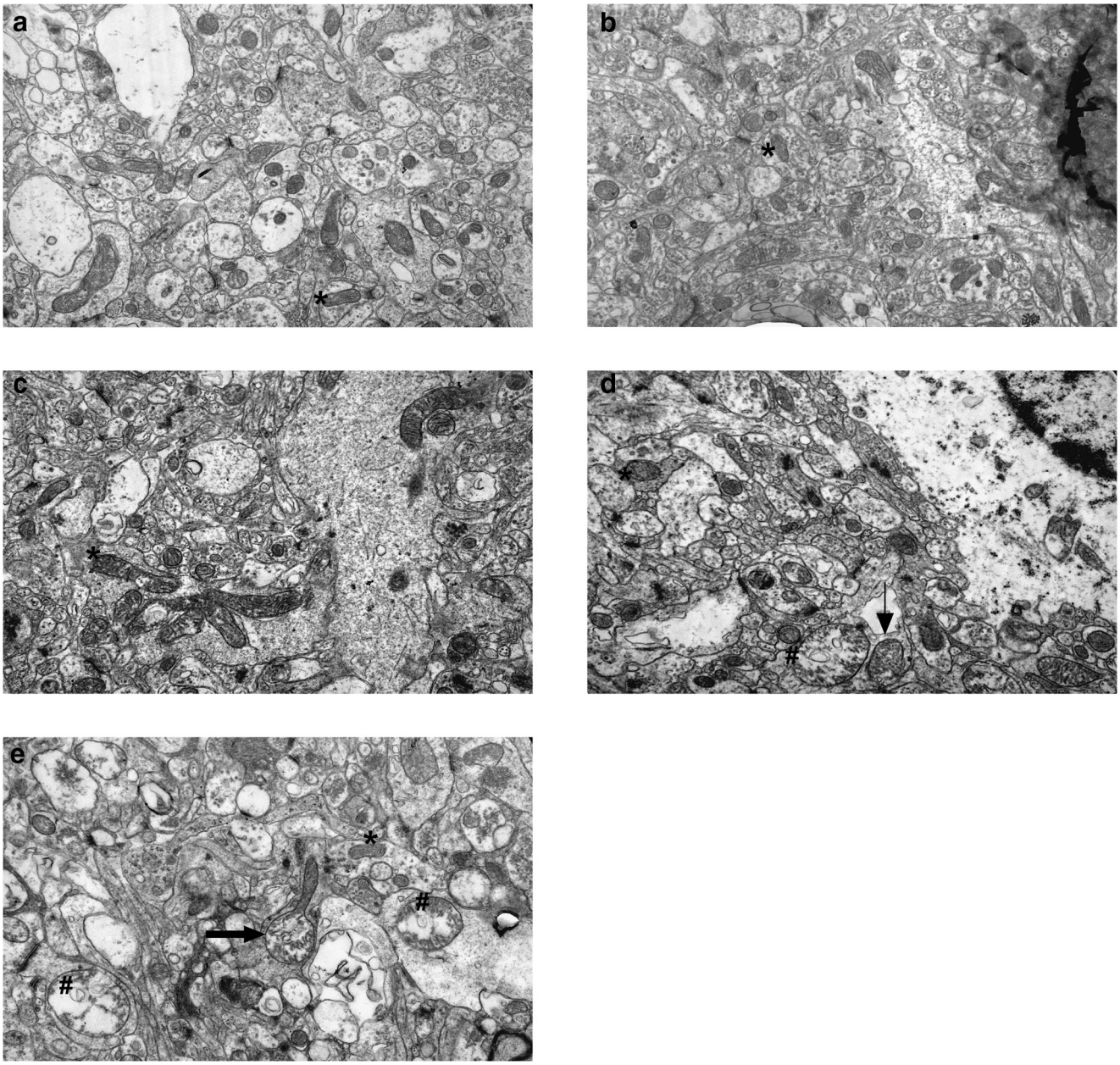

Fig. 3 Subchronic Al exposure led to mitochondrial morphology alterations. The detrimental effects of $Al(mal)_3$ exposure on the ultrastructure of the mitochondria in rats from the **a** blank group, **b** solvent group, **c** 0.41 mg/kg group, **d** 0.81 mg/kg group, and **e** 1.62 mg/kg group after 90 days of treatment, × 20,000 magnification. Normal mitochondria (*) are shown in **a**–**e**, and mitochondrial swelling, crista disorder and vacuolization (#) are shown in **d** and **e**. The thin arrows indicate the sites of mitochondrial fragment in **d**, and the thick arrow indicates the fission of broken mitochondria in **e**. ($n = 3$)

Subchronic Al Exposure Resulted in Mitochondrial Dysfunction in the Hippocampus

Cellular mitochondria generate abundant ATP through the electron transport chain (ETC) to maintain endergonic processes. These reactions are coupled to the creation of a proton gradient across the mitochondrial inner membrane by three proton pumps (complex I, III, IV), and the protein level of these proton pumps and ATP synthase activity reflect the function of mitochondria. We examined Na^+-K^+ ATPase and Ca^{2+}-Mg^{2+} ATPase activity in the hippocampus using a colorimetric technique. As shown in Fig. 4a and b, Na^+-K^+ ATPase and Ca^{2+}-Mg^{2+}ATPase activity decreased with increasing exposure dose and time; Na^+-K^+ ATPase activity was significantly decreased in the groups exposed to 0.81 mg/kg and 1.62 mg/kg Al compared with the blank group in all investigations. The activity of Ca^{2+}-Mg^{2+}ATPase in the group that received 1.62 mg/kg Al markedly decreased compared with that in the blank group at 60 and 90 days; interaction effects of exposure time and dose on Na^+-K^+ ATPase activity and on Ca^{2+}-Mg^{2+} ATPase activity were observed, and both interactions were found to be statistically significant ($F = 2.675$, $P = 0.012$; $F = 3.665$, $P < 0.001$, respectively). Western blot analysis was used to detect the protein level of COX IV in the hippocampus of the rats. As shown in Fig. 4c, Al treatment, especially the 0.81 and 1.62 mg/kg doses, downregulated the COX IV protein, and the COX IV protein level was inversely associated with the dose of Al. This phenomenon was particularly apparent at 60 and 90 days, and there was an interaction effect of exposure time and dose on the protein expression of COX IV ($F = 4.297$, $P < 0.001$).

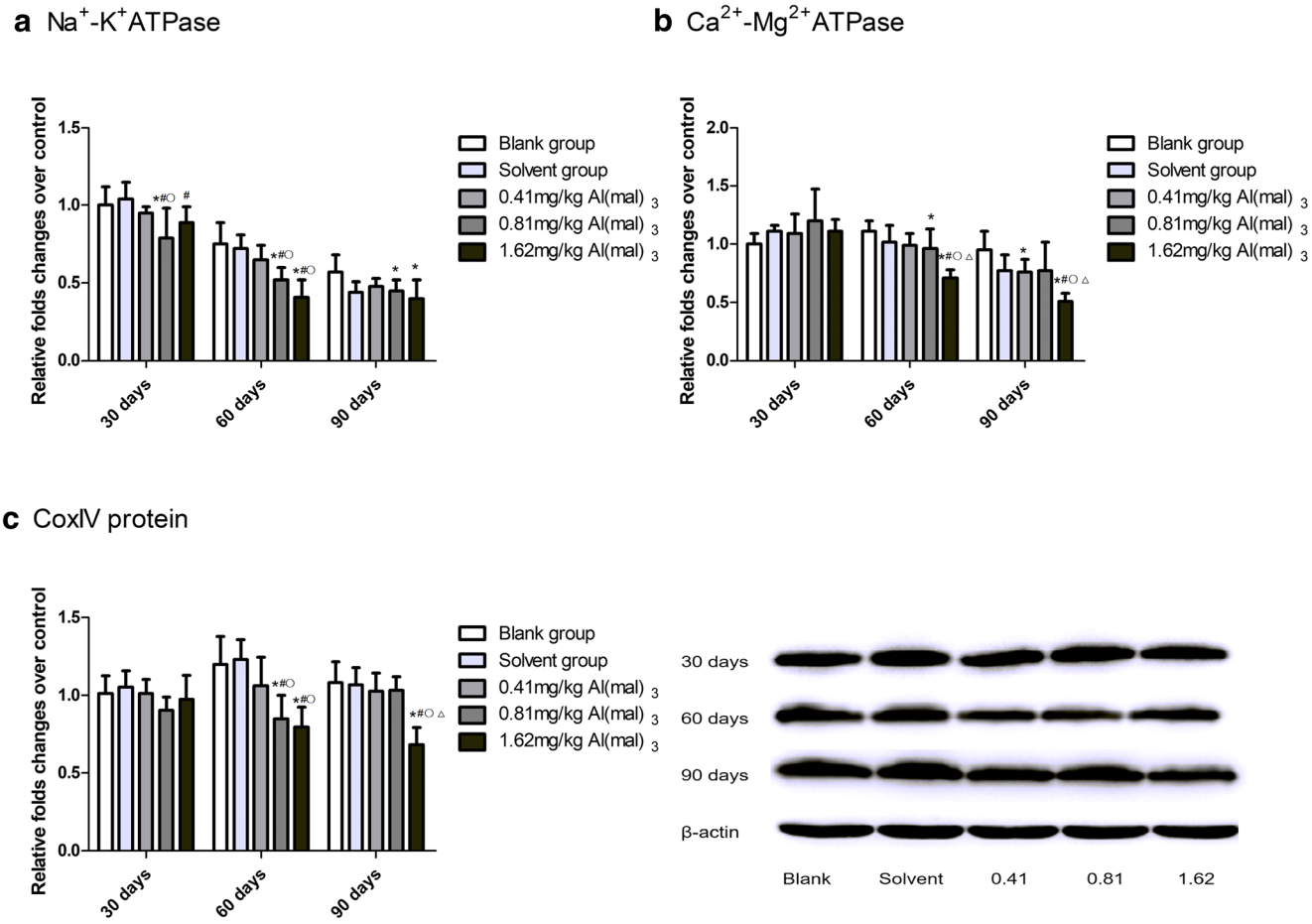

Fig. 4 Subchronic Al exposure resulted in mitochondrial dysfunction in the hippocampus. **a** and **b** Na^+-K^+ ATPase and Ca^{2+}-Mg^{2+}ATPase activity was measured using a commercial kit according to the manufacturer's protocol. The interactions between dose and exposure time on Na^+-K^+ ATPase activity and on Ca^{2+}-Mg^{2+}ATPase activity were both statistically significant ($F = 2.675$, $P = 0.012$; $F = 3.665$, $P < 0.001$, respectively). **c** The expression level of the COX IV protein was determined by western blot analysis at 30 days, 60 days, and 90 days. The interaction between dose and exposure time on the expression level of the COX IV protein was statistically significant ($F = 4.297$, $P < 0.001$). The results are expressed as the mean ± SD, $n = 6$; * means versus blank control, $P < 0.05$; # means versus solvent, $P < 0.05$; ○ means versus 0.41 mg/kg $Al(mal)_3$, $P < 0.05$; △ means versus 0.81 mg/kg $Al(mal)_3$, $P < 0.05$

Subchronic Al Exposure Increased Drp1 and Fis1 Protein Levels

The levels of mitochondrial fission proteins, including Drp1 and Fis1, in the hippocampus were detected by western blot analysis. As shown in Fig. 5a and b, Al strongly upregulated the protein expression of Drp1 in a dose- and time-dependent manner. In particular, the significantly increasing levels of Drp1 were identified in the 1.62 mg/kg groups when compared with the blank group at all investigated times. When compared with the blank control group, the groups that received all doses showed a dose-dependent improvement in Fis1 levels at 90 days. We observed a statistically significant interaction between treatment dose and the duration of Al exposure on the protein expression of Fis1 ($F = 7.188$, $P < 0.001$) but not of Drp1 ($F = 1.646$, $P = 0.126$).

Subchronic Al Exposure Upregulated the Protein Levels of Opa1, Mfn1, and Mfn2

In addition, we also investigated the protein levels of Opa1, Mfn1, and Mfn2, which regulate mitochondrial fusion and found dose- and time-associated increases in Mfn1 and Mfn2 protein levels, in the 1.62 mg/kg dose groups. The protein levels of Opa1 and Mfn1 exhibited significant improvement when compared to that in the blank control group at 30, 60, and 90 days (Fig. 6a and b). The protein levels of Mfn2 followed a similar pattern to that of Opa1 and Mfn1 when $Al(mal)_3$ was administered for 60 or 90 days (Fig. 5c). There were significant statistical interactions between dose and exposure time on the levels of the Opa1 and Mfn2 proteins ($F = 2.358$, $P = 0.026$; $F = 2.274$, $P = 0.031$) but not on that of Mfn1 ($F = 0.596$, $P = 0.778$).

Subchronic Al Exposure Decreased Drp1 Phosphorylation at Ser637 and Elevated CaN Protein Levels

To explore the regulatory mechanism of the Drp1 protein, we measured the protein phosphorylation level of Drp1 at Ser637 and the CaN protein level in the hippocampus, as shown in Fig. 7a and b. Al, compared to no treatment in the blank groups, contributed to decreased pDrp1 (s637) levels, and the pDrp1 (s637) level was negatively correlated with dose, especially the 0.81 mg/kg and 1.62 mg/kg doses. Al conversely led to a dose-dependent increase in the CaN protein level, which was positively correlated with the treatment dose. The 1.62 mg/kg $Al(mal)_3$ dose induced a marked upregulation of

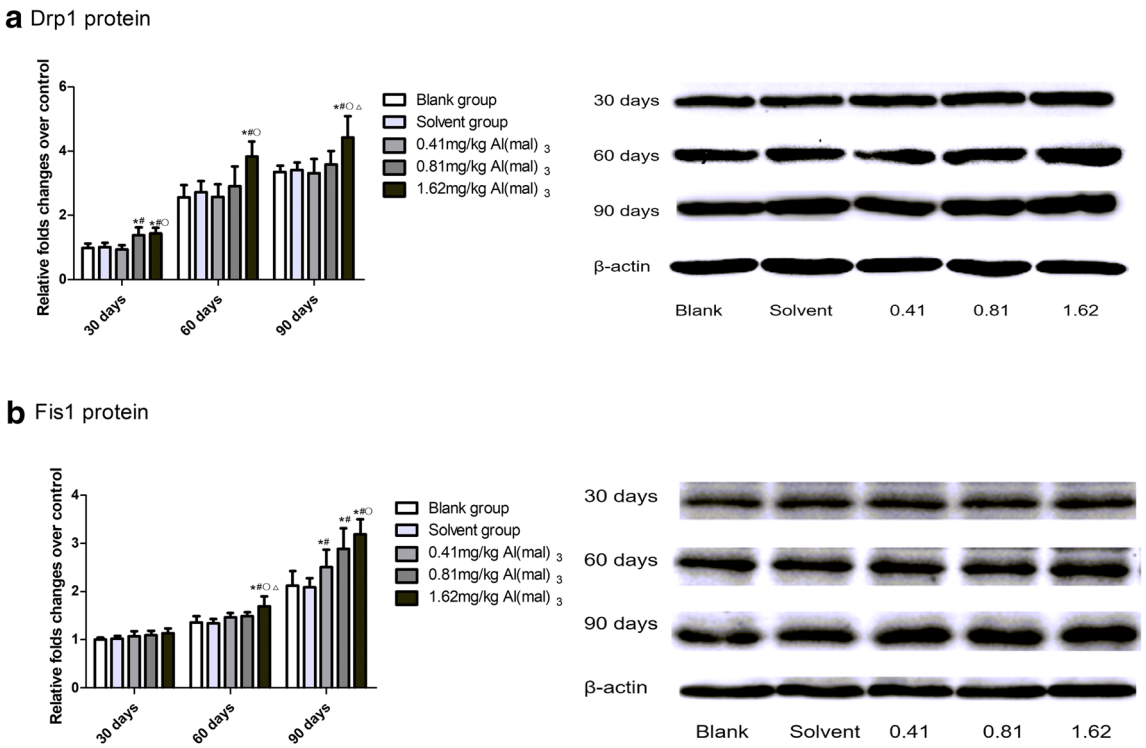

Fig. 5 Subchronic Al exposure led to increased protein levels of Drp1 and Fis1. **a** and **b** The expression levels of the Drp1 and Fis1 proteins were determined by western blot. The interaction between dose and time on the protein level of Fis1 ($F = 7.118$, $P < 0.001$) but not on the protein level of Drp1 ($F = 1.646$, $P = 0.126$) was statistically significant. The results are expressed as the mean ± SD, $n = 6$; * means versus blank control, $P < 0.05$; # means versus solvent, $P < 0.05$; ○ means versus 0.41 mg/kg $Al(mal)_3$, $P < 0.05$; △ means versus 0.81 mg/kg $Al(mal)_3$, $P < 0.05$

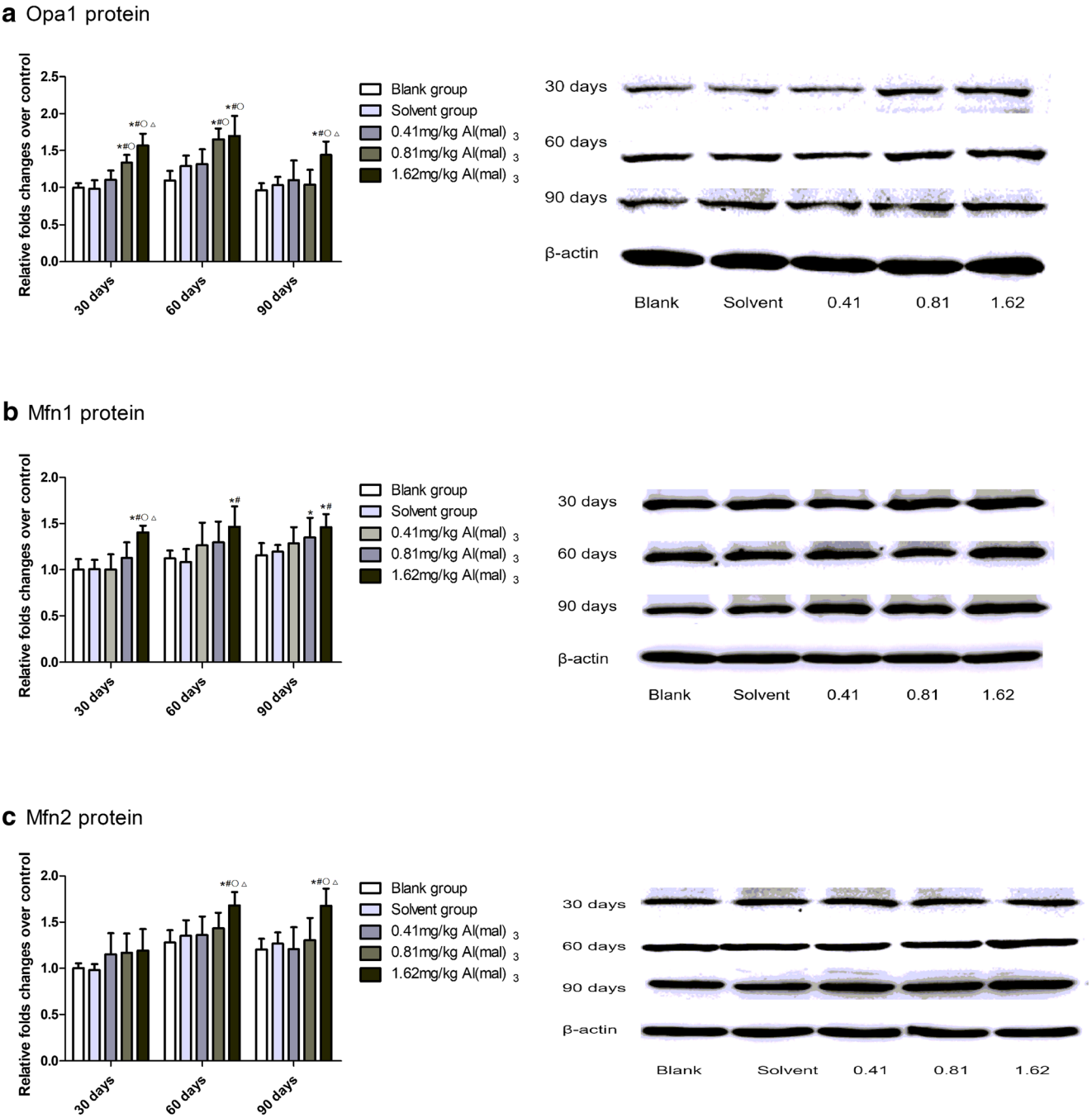

Fig. 6 Subchronic Al exposure upregulated the protein levels of Opa1, Mfn1, and Mfn2. **a**, **b**, and **c** The expression levels of the Opa1, Mfn1, and Mfn2 proteins in the blank control, solvent, 0.41 mg/kg Al-treated, 0.81 mg/kg Al-treated, and 1.62 mg/kg Al-treated groups at 30 days, 60 days, and 90 days were determined by western blot. The interactions between dose and exposure time on the protein levels of Opa1 and Mfn2 protein were statistically significant ($F = 2.358$, $P = 0.026$; $F = 2.274$, $P = 0.031$, respectively), but there was no interaction between these variables on the protein level of Mfn2 ($F = 0.596$, $P = 0.778$). The results are expressed as the mean ± SD, $n = 6$; * means versus blank control, $P < 0.05$; # means versus solvent, $P < 0.05$; ○ means versus 0.41 mg/kg $Al(mal)_3$, $P < 0.05$; △ means versus 0.81 mg/kg $Al(mal)_3$, $P < 0.05$

CaN protein levels when compared with no treatment in the blank control groups at all testing times, and the interactions between dose and exposure time on the protein levels of pDrp1 (s637) and CaN were statistically significant ($F = 2.488$, $P = 0.019$; $F = 2.451$, $P = 0.021$).

Discussion

In the present study, the subchronic exposure of male SD rats to $Al(mal)_3$ increased the Al concentration in the hippocampus in a time- and dose-dependent manner. Along with spatial

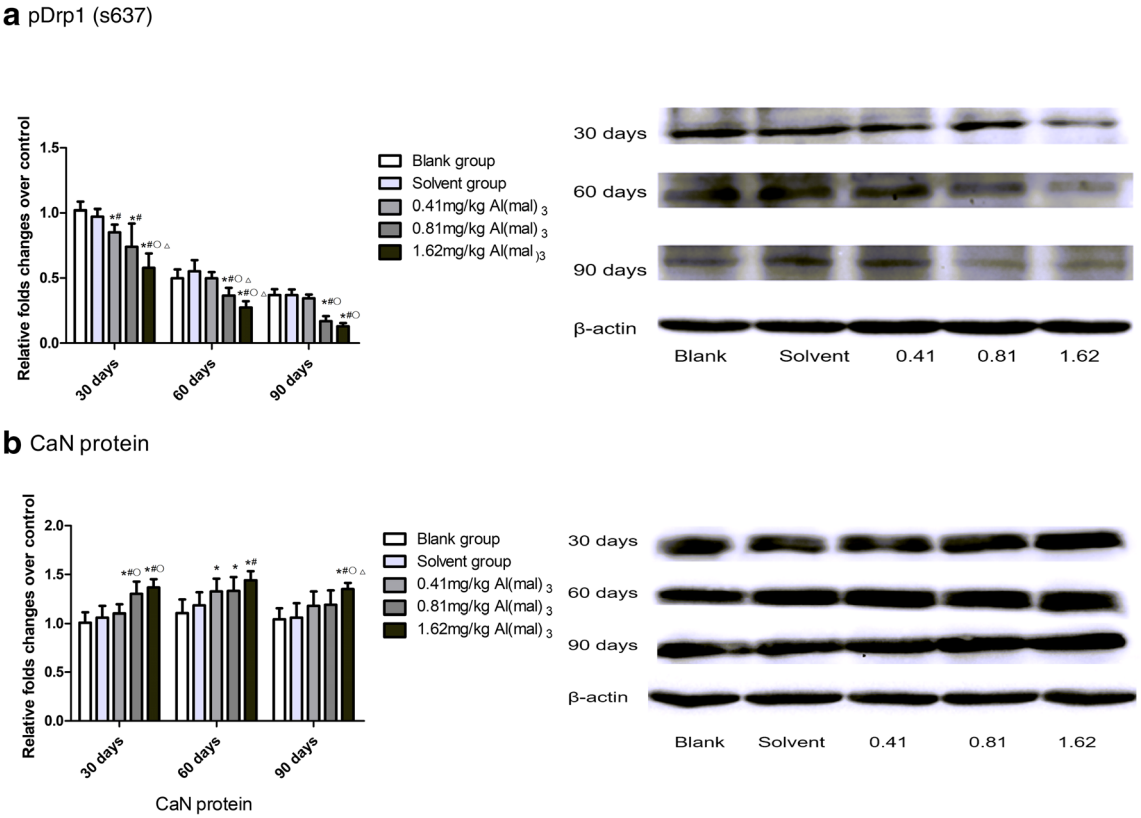

Fig. 7 Al decreased the protein phosphorylation level of Drp1 and elevated the expression of the CaN protein. **a** and **b** The protein phosphorylation level of Drp1 at Ser637 and the CaN protein expression levels in the blank control, solvent, 0.41 mg/kg Al-treated, 0.81 mg/kg Al-treated, and 1.62 mg/kg Al-treated groups at 30 days, 60 days, and 90 days were determined by western blot. The interactions between dose and exposure time on the protein levels of pDrp1 (s637) and CaN were statistically significant ($F = 2.488$, $P = 0.019$; $F = 2.451$, $P = 0.021$). The results are expressed as the mean ± SD, $n = 6$; * means versus blank control, $P < 0.05$; # means versus solvent, $P < 0.05$; ○ means versus 0.41 mg/kg $Al(mal)_3$, $P < 0.05$; △ means versus 0.81 mg/kg $Al(mal)_3$, $P < 0.05$

learning and memory deficits, we observed breached membranes, swollen mitochondria, cristae disorder, and mitochondrial fission in the animals exposed to $Al(mal)_3$ for 90 days. Na^+-K^+ ATPase and Ca^{2+}-Mg^{2+}ATPase activity and the protein expression of COX IV were decreased in the Al-administered groups. Additionally, $Al(mal)_3$ upregulated the levels of mitochondrial fission (Drp1 and Fis1) and fusion (Opa1, Mfn1, and Mfn2) proteins. We also detected the level of CaN and the protein phosphorylation level of Drp1 at Ser637 and found that $Al(mal)_3$ upregulated CaN and downregulated the protein phosphorylation level of Drp1 at Ser637 in the hippocampus. In this study, we elucidated the connection between fusion (Drp1 and Fiss1) and fission (OPA1, Mfn1, and Mfn2) proteins in the mitochondria and the neurotoxicity of Al.

The effect of exposure to Al on learning and memory has been studied extensively in animal models. Al-maltolate complex has been used to study the neurotoxicity of Al in animals, and there is evidence to suggest that maltolate may facilitate the entry of Al into the brain (Christen 2000), thereby increasing the potential for neurotoxicity (Griffioen et al. 2004). In the present study, the Al content in the brains of rats treated with the highest dose of Al (1.62 mg/kg) was similar after 30, 60, and 90 days of treatment, but most of the detrimental effects of this dose of Al were found after 90 days of exposure. This raises the possibility that there is a threshold for the accumulation of Al in the brain, suggesting that high levels of Al in the brain cause the subsequent accumulation of Al in the brain to slow down, which is related to the ability to transport eliminate Al from the brain. The relevant regulatory mechanism needs to be further studied (Hichem et al. 2014). The detrimental effects of Al not only depend on Al content in the rat brain but also on exposure duration, which raises the possibility that time is critical for the toxic effects of Al (Liu et al. 2018, 2017). Our research group intraperitoneally injected $Al(mal)_3$ at doses of 0.41, 0.82, or 1.23 mg/kg into rats for 8 weeks and found dose-dependent suppressive effects on long-term potentiation (LTP) with 0.82, or 1.23 mg/kg $Al(mal)_3$ (Song et al. 2014). Consistent with previous studies, male SD rats received intraperitoneal injections $Al(mal)_3$ (0, 0.41, 0.81, or 1.62 mg/kg bw) or were left untreated for 30, 60, or 90 days, and the $Al(mal)_3$-exposed rats exhibited learning and memory deficits.

Mitochondrial dysfunction, such as abnormal ATP generation, is a significant aging-related concern in the nervous system and has been associated with major neurodegenerative disorders, including Parkinson's, Alzheimer's, and Huntington's diseases (Chen and Chan 2009; Sheng and Cai 2012). In vitro and in vivo studies have indicated that mitochondrial swelling, the loss of mitochondria cristae, and reduced numbers of mitochondria as well as increased levels of reactive oxygen species (ROS) and an increased rate of cell death are observed upon treatment with Al^{3+} (Kumar et al. 2008; Niu et al. 2005); the analysis of cultured hippocampal neurons from Wistar rats has revealed that Na^{+}-K^{+}-ATPase activity and Ca^{2+}-Mg^{2+}-ATPase activity are the most sensitive indicators when mitochondrial function and structure are impaired (Li et al. 2016), and an in vivo study showed mitochondrial dysfunction along with decreasing COX IV protein levels in the hippocampus of rats chronically administered Al (Cardoso et al. 2004). In the present study, alterations in mitochondrial morphology and function, including decreases in Na^{+}-K^{+} ATPase and Ca^{2+}-Mg^{2+}ATPase activity and the expression of COX IV, were apparent in the hippocampus of the Al-exposed rats.

Mitochondrial damage is compensated for by fusion and eliminated by fission. Mitochondrial fusion and fission are proposed to reconcile two competing processes. In the present study, we found that $Al(mal)_3$ upregulated the level of mitochondrial fusion proteins (Opa1, Mfn1, and Mfn2) while also altering mitochondrial morphology; the mitochondrial morphology disruptions that were observed included breached membranes, swollen mitochondria, and cristae disorder. Swollen mitochondria may lead to mitochondrial membrane dysfunction and rupture. Fusion allows mitochondria to compensate for defects by sharing components, and this compensation helps maintain energy output in the face of stress as long as the stress is below a critical threshold. This differs from the Al-induced decreased levels of the fusion proteins Opa1, Mfn1, and Mfn2 observed in an AD model in a study by Manczak (Manczak et al. 2011). This raises the possibility that defective mitochondria may not be able to synthesize healthy mitochondria by increasing the levels of mitochondrial fusion proteins and may ultimately prematurely die and contribute to neurodegenerative disorders, including Parkinson's disease and Alzheimer's disease. Meanwhile, the upregulation of fission proteins (Drp1 and Fis1) was also found in an AD model and in our present study (Chen and Chan 2009), which indicates that irreversibly damaged mitochondria are segregated by fission, which can result in abnormal alterations in mitochondrial morphology, such as fragmented and scattered mitochondria. The findings agree with the TEM images.

In our study, the dose- and time-dependent upregulation of Drp1 after Al exposure indicates that Drp1 may play an important role in Al-induced abnormal dynamics. Various existing pieces of evidence show that calcineurin (CaN) promotes the dephosphorylation of Drp1 at Ser637 and increases Drp1 protein levels, eventually contributing to mitochondrial abnormal morphology (Lee et al. 2016; Park et al. 2013, 2015). Consistent with the changes in mitochondrial morphology, Al led to the downregulation of Drp1 phosphorylation at Ser637 and the upregulation of Drp1 and CaN protein levels. CaN is a Ca^{2+}-dependent protein phosphatase, and Al delays the closure of voltage-dependent calcium channels and blocks Ca^{2+}/Mg^{2+}-ATPase, which increases intracellular calcium levels in the hippocampus of rats (Exley 1999). Consistent with the results of these studies, decreased Ca^{2+}/Mg^{2+}-ATPase activity was also observed in the present study. In summary, we speculate that Al leads to a decrease in the Ca^{2+}/Mg^{2+}-ATPase activity, which results in abnormal calcium homeostasis, and then upregulates CaN, which may be responsible for the increased levels of Drp1 phosphorylation at Ser637 and the upregulation of Drp1 after Al exposure.

Conclusion

Al exposure impaired spatial learning and memory and induced abnormal mitochondrial dynamics, and this abnormal neurobehavior was accompanied by the induction of fusion- and fission-related mitochondrial dysfunction in the rat hippocampus. This study provides the basis for understanding mitochondrial abnormality-related neuropathological dysfunction in response to Al exposure and the frangibility of the central nervous system.

Acknowledgments We sincerely thank our colleagues for their help and work on the research.

Funding information This research was financially supported by the National Nature Science Foundation of China (No. 81430078 and 81872599).

Compliance with Ethical Standards The use of rats in this study was approved by the Institutional Animal Care and Use Committee at Shanxi Medical University (Taiyuan, China), and the protocol was approved by the Experimental Animal Ethics Committee of the Shanxi Medical University.

Conflict of Interest The authors declare that they have no conflict of interest.

References

Bondy SC (2014) Prolonged exposure to low levels of aluminum leads to changes associated with brain aging and neurodegeneration. Toxicology 315:1–7. https://doi.org/10.1016/j.tox.2013.10.008

Cardoso SM, Proença MT, Santos S, Santana I, Oliveira CR (2004) Cytochrome c oxidase is decreased in Alzheimer's disease platelets.

Neurobiol Aging 25:105–110. https://doi.org/10.1016/s0197-4580(03)00033-2
Chang CR, Blackstone C (2007) Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. J Biol Chem 282:21583–21587. https://doi.org/10.1074/jbc.C700083200
Chen H, Chan DC (2009) Mitochondrial dynamics-fusion, fission, movement, and mitophagy-in neurodegenerative diseases. Hum Mol Genet 18:R169–R176. https://doi.org/10.1093/hmg/ddp326
Christen Y (2000) Oxidative stress and Alzheimer disease. Am J Clin Nutr 71:621S–629S
Deng H, Dodson MW, Huang H, Guo M (2008) The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in Drosophila. Proc Natl Acad Sci U S A 105:14503–14508. https://doi.org/10.1073/pnas.0803998105
Dua R, Gill KD (2004) Effect of aluminium phosphide exposure on kinetic properties of cytochrome oxidase and mitochondrial energy metabolism in rat brain. Biochim Biophys Acta 1674:4–11. https://doi.org/10.1016/j.bbagen.2004.05.003
Exley C (1999) A molecular mechanism of aluminium-induced Alzheimer's disease. J Inorg Biochem 76:133–140
Griffioen KS, Ghribi O, Fox N, Savory J, DeWitt DA (2004) Aluminum maltolate-induced toxicity in NT2 cells occurs through apoptosis and includes cytochrome c release. Neurotoxicology 25:859–867. https://doi.org/10.1016/j.neuro.2003.12.004
Hichem N, May ME, Ladhari N, Mrabet A, Gharbi R (2014) Aluminum chloride impacts dentate gyrus structure in male adult albino Wistar rats. Tissue & cell 46:409–414. https://doi.org/10.1016/j.tice.2014.05.006
Hu WP, Li XM, Chen JG, Li ZW (2007) Potentiation of the nicotinic acetylcholine receptor by aluminum in mammalian neurons. Neuroscience 149:1–6. https://doi.org/10.1016/j.neuroscience.2007.07.018
Iglesias-Gonzalez J, Sanchez-Iglesias S, Beiras-Iglesias A, Mendez-Alvarez E, Soto-Otero R (2017) Effects of aluminium on rat brain mitochondria bioenergetics: an in vitro and in vivo study. Mol Neurobiol 54:563–570. https://doi.org/10.1007/s12035-015-9650-z
Ishihara N, Nomura M, Jofuku AKH, Suzuki SMK, Otera H, Nakanishi Y et al (2009) Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nat Cell Biol 11:958–966. https://doi.org/10.1038/ncb1907
Kandimalla R, Vallamkondu J, Corgiat EB, Gill KD (2016) Understanding aspects of aluminum exposure in Alzheimer's disease development. Brain Pathol 26:139–154. https://doi.org/10.1111/bpa.12333
Kaur A, Joshi K, Minz RW, Gill KD (2006) Neurofilament phosphorylation and disruption: a possible mechanism of chronic aluminium toxicity in Wistar rats. Toxicology 219:1–10. https://doi.org/10.1016/j.tox.2005.09.015
Kumar V, Bal A, Gill KD (2008) Impairment of mitochondrial energy metabolism in different regions of rat brain following chronic exposure to aluminium. Brain Res 1232:94–103. https://doi.org/10.1016/j.brainres.2008.07.028
Langui D, Probst A, Anderton B, Brion JP, Ulrich J (1990) Aluminium-induced tangles in cultured rat neurones. Acta Neuropathol 80:649–655
Lee DG, Park J, Lee H, Lee SR, Lee DS (2016) Iron overload-induced calcium signals modulate mitochondrial fragmentation in HT-22 hippocampal neuron cells. Toxicology 365:17–24. https://doi.org/10.1016/j.tox.2016.07.022
Li Y, Wang M, Wang S (2016) Effect of inhibiting mitochondrial fission on energy metabolism in rat hippocampal neurons during ischemia/reperfusion injury. Neurol Res 38:1027–1034. https://doi.org/10.1080/01616412.2016.1215050
Liang RF, Li WQ, Wang XH, Zhang HF, Wang H et al (2012) Aluminium-maltolate-induced impairment of learning, memory and hippocampal long-term potentiation in rats. Ind Health 50:428–436
Liu MY, Lou HQ, Chen WW, Piñeros MA, Xu JM, Fan W, Kochian LV, Zheng SJ, Yang JL (2018) Two citrate transporters coordinately regulate citrate secretion from rice bean root tip under aluminum stress. Plant Cell Environ 41:809–822. https://doi.org/10.1111/pce.13150
Liu WT, Yamashita T, Tian FF, Morimoto N, Ikeda Y, Deguchi K, Abe K (2013) Mitochondrial fusion and fission proteins expression dynamically change in a murine model of amyotrophic lateral sclerosis. Curr Neurovasc Res 10:222–230
Liu X, Lin Y, Liu D, Wang C, Zhao Z, Cui X, Liu Y, Yang Y (2017) MAPK-mediated auxin signal transduction pathways regulate the malic acid secretion under aluminum stress in wheat (Triticum aestivum L.). Sci Rep 7:1620. https://doi.org/10.1038/s41598-017-01803-3
Manczak M, Calkins MJ, Reddy PH (2011) Impaired mitochondrial dynamics and abnormal interaction of amyloid beta with mitochondrial protein Drp1 in neurons from patients with Alzheimer's disease: implications for neuronal damage. Hum Mol Genet 20:2495–2509. https://doi.org/10.1093/hmg/ddr139
Manczak M, Kandimalla R, Fry D, Sesaki H, Reddy PH (2016) Protective effects of reduced dynamin-related protein 1 against amyloid beta-induced mitochondrial dysfunction and synaptic damage in Alzheimer's disease. Hum Mol Genet 25:5148–5166
Mortiboys H, Thomas KJ, Koopman WJ, Klaffke S, Abou-Sleiman P et al (2008) Mitochondrial function and morphology are impaired in parkin-mutant fibroblasts. Ann Neurol 64:555–565. https://doi.org/10.1002/ana.21492
Niu PY, Niu Q, Zhang QL, Wang LP, He SE et al (2005) Aluminum impairs rat neural cell mitochondria in vitro. Int J Immunopathol Pharmacol 18:683–689
Park J, Choi H, Kim B, Chae U, Lee DG, Lee SR, Lee S, Lee HS, Lee DS (2016) Peroxiredoxin 5 (Prx5) decreases LPS-induced microglial activation through regulation of Ca 2+ /calcineurin-Drp1-dependent mitochondrial fission. Free Radic Biol Med 99:392–404. https://doi.org/10.1016/j.freeradbiomed.2016.08.030
Park J, Choi H, Min JS, Park SJ, Kim JH, Park HJ, Kim B, Chae JI, Yim M, Lee DS (2013) Mitochondrial dynamics modulate the expression of pro-inflammatory mediators in microglial cells. J Neurochem 127:221–232. https://doi.org/10.1111/jnc.12361
Park J, Lee DG, Park J, Kim B, Park SJ et al (2015) Iron overload triggers mitochondrial fragmentation via calcineurin-sensitive signals in HT-22 hippocampal neuron cells. Toxicology 337:39–46. https://doi.org/10.1016/j.tox.2015.08.009
Prakash A, Kumar A (2012) Mitoprotective effect of Centella asiatica against aluminum-induced neurotoxicity in rats: possible relevance to its anti-oxidant and anti-apoptosis mechanism. Neurol Sci 34:1403–1409. https://doi.org/10.1007/s10072-012-1252-1
Sheng ZH, Cai Q (2012) Mitochondrial transport in neurons: impact on synaptic homeostasis and neurodegeneration. Nat Rev Neurosci 13:77–93. https://doi.org/10.1038/nrn3156
Shirendeb UP, Calkins MJ, Manczak M, Anekonda V, Dufour B, McBride JL, Mao P, Reddy PH (2011) Mutant huntingtin's interaction with mitochondrial protein Drp1 impairs mitochondrial biogenesis and causes defective axonal transport and synaptic degeneration in Huntington's disease. Hum Mol Genet 21:406–420. https://doi.org/10.1093/hmg/ddr475
Silva AF, Aguiar MS, Carvalho OS, Santana LN, Franco EC et al (2013) Hippocampal neuronal loss, decreased GFAP immunoreactivity and cognitive impairment following experimental intoxication of rats with aluminum citrate. Brain Res 1491:23–33. https://doi.org/10.1016/j.brainres.2012.10.063
Song J, Liu Y, Zhang HF, Zhang QL, Niu Q (2014) Effects of exposure to aluminum on long-term potentiation and AMPA receptor subunits in

rats in vivo*. Biomed Environ Sci 27:77–84. https://doi.org/10.3967/bes2014.006

Sood PK, Verma S, Nahar U, Nehru B (2015) Neuroprotective role of lazaroids against aluminium chloride poisoning. Neurochem Res 40:1699–1708. https://doi.org/10.1007/s11064-015-1653-7

Wakabayashi J, Zhang ZY, Wakabayashi N, Tamura Y, Fukaya M, Kensler TW, Iijima M, Sesaki H (2009) The dynamin-related GTPase Drp1 is required for embryonic and brain development in mice. J Cell Biol 186:805–816. https://doi.org/10.1083/jcb.200903065

Wang H, Lim PJ, Karbowski M, Monteiro MJ (2009a) Effects of overexpression of huntingtin proteins on mitochondrial integrity. Hum Mol Genet 18:737–752. https://doi.org/10.1093/hmg/ddn404

Wang W, Wang X, Fujioka H, Hoppel C, Whone AL, Caldwell MA, Cullen PJ, Liu J, Zhu X (2015) Parkinson's disease–associated mutant VPS35 causes mitochondrial dysfunction by recycling DLP1 complexes. Nat Med 22:54–63. https://doi.org/10.1038/nm.3983

Wang X, Fan X, Yuan SJ, WX J, Liu B et al (2017) Chlorogenic acid protects against aluminium-induced cytotoxicity through chelation and antioxidant actions in primary hippocampal neuronal cells. Food Funct 8:2924–2934. https://doi.org/10.1039/c7fo00659d

Wang X, Su B, Lee HG, Li X, Perry G, Smith MA, Zhu X (2009b) Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. J Neurosci Off J Soc Neurosci 29:9090–9103. https://doi.org/10.1523/JNEUROSCI.1357-09.2009

Yokel RA (2002) Brain uptake, retention, and efflux of aluminum and manganese. Environ Health Perspect 110:699–704

Zhang L, Jin C, Lu X, Yang J, Wu S, Liu Q, Chen R, Bai C, Zhang D, Zheng L, du Y, Cai Y (2014) Aluminium chloride impairs long-term memory and downregulates cAMP-PKA-CREB signalling in rats. Toxicology 323:95–108. https://doi.org/10.1016/j.tox.2014.06.011