Journal of Trace Elements in Medicine and Biology 52 (2019) 199–208

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

A multifunctional bis-(−)-nor-meptazinol-oxalamide hybrid with metal-chelating property ameliorates Cu(II)-induced spatial learning and memory deficits via preventing neuroinflammation and oxido-nitrosative stress in mice

Xiaofang Tan[a,1], Yan Zhou[a,1], Ping Gong[a], Huifeng Guan[a], Baichuan Wu[a], Lina Hou[a], Xuemei Feng[a], Wei Zheng[b,*], Juan Li[a,*]

[a] Department of Pharmacology and Chemical Biology, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai 200025, PR China
[b] NPFPC Key Laboratory of Contraceptives and Devices, Shanghai Institute of Planned Parenthood Research, 2140 Xietu Road, Shanghai 200032, PR China

ARTICLE INFO

Keywords:
Bis-(−)-nor-meptazinol-oxalamide
Hybrid
Copper
Cognitive deficit
Neuronal injury
Neuroinflammation
Oxido-Nitrosative stress

ABSTRACT

Excess copper exposure is a risk factor of neurodegeneration related to Alzheimer's disease (AD). Evidence indicates that, besides promoting amyloid β aggregation, activation of neuroinflammation and oxido-nitrosative stress (two key pathophysiological processes of AD) may also play important roles in Cu(II)-induced neuronal injury. Therefore, the copper-chelating strategy has gained attention in search for new anti-AD drugs. We previously reported a novel multifunctional compound N^1,N^2-bis(3-(*S*)-meptazinol-propyl) oxalamide (ZLA), a bis-(−)-nor-meptazinol-oxalamide hybrid with properties of dual binding site acetylcholinesterase (AChE) inhibition and Cu(II)/Zn(II) chelation. The present study was aimed to explore its effect on cognitive deficits caused by intrahippocampal injection of Cu(II) in mice. Results showed that ZLA (2, 5 mg/kg; i.p.) treatment significantly ameliorated the Cu(II)-induced impairment of hippocampus-dependent learning and memory, whereas rivastigmine, an AChE inhibitor showing a similar potency of enzyme inhibition to ZLA, had no obvious effect. Immunohistochemical and Western blot analyses revealed that ZLA attenuated the decrease in hippocampal expression of microtubule-associated protein 2 (MAP2, a dendritic marker) in Cu(II)-challenged mice. Further analysis showed that ZLA suppressed the Cu(II)-evoked microglial activation. Moreover, it inhibited the Cu(II)-evoked production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-1β and expression of inducible nitric oxide synthase in the hippocampus. The Cu(II)-induced oxidative and nitrosative stress in the hippocampus was also attenuated after ZLA treatment. Collectively, these results suggest that ZLA ameliorates the Cu(II)-caused cognitive deficits. Inhibition of neuroinflammation and oxido-nitrosative stress, and thus ameliorating neuronal injury, may be the potential mechanism for the anti-amnesic effect of ZLA.

1. Introduction

Alzheimer's disease (AD), characterized by a progressive cognitive decline, is one of the most devastating neurodegenerative disorders among the elderly people. The pathogenesis of AD involves multiple factors including formation of neurotoxic amyloid β (Aβ) aggregates, hyperphosphorylation of tau protein, chronic neuroinflammation, oxidative and nitrosative stress, and synaptic loss [1–3]. Currently, FDA-approved drugs for AD therapy, including acetylcholinesterase inhibitors (AChEIs) and the N-methyl-D-aspartic acid (NMDA) receptor antagonist memantine, cannot overtly modify the pathophysiological processes although benefit in improving cognitive and behavioral

Abbreviations: AChE, acetylcholinesterase; AChEIs, acetylcholinesterase inhibitors; AD, Alzheimer's disease; Aβ, amyloid β peptide; ANOVA, analysis of variance; DTNB, 5,5′-dithiobis-(2-nitrobenzoic) acid; GSH, glutathione; IHC, immunohistochemistry; IL-6, interleukin-6; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; MAP2, microtubule-associated protein 2; MCI, mild cognitive impairment; MDA, malondialdehyde; MWM, Morris water maze; TNF-α, tumor necrosis factor-α; ZLA, N^1,N^2-bis(3-(*S*)-meptazinol-propyl) oxalamide

* Corresponding authors.
E-mail addresses: zhengwei726@126.com (W. Zheng), lijuanpharm@163.com (J. Li).
[1] Contributed equally to this work.

https://doi.org/10.1016/j.jtemb.2018.12.014
Received 24 October 2018; Received in revised form 18 December 2018; Accepted 29 December 2018

symptoms.

Evidence suggests that copper exposure is a risk factor of mild cognitive impairment (MCI) and AD [4]. Chronic Cu(II) exposure causes elevated free copper content in the hippocampus (a region that is crucial for memory encoding and is profoundly affected by AD [5]), with concomitant loss of hippocampal synaptic proteins and spatial memory impairment [6]. Clinical data shows that copper concentration is elevated in serum and cerebrospinal fluid of patients with AD [7,8]. Moreover, James et al. [9] identified elevated levels of exchangeable (as opposed to tightly bound) Cu(II) in postmortem cortical tissues from AD patients and in cerebral tissues of transgenic AD mice. The normal extracellular copper concentration in brain is 0.2–1.7 μM [10]. The free copper concentration in the synaptic cleft can reach to 15 μM upon activation of synaptic NMDA receptors in the hippocampal neurons of healthy brain; in the hippocampus of Alzheimer's brain it overtly rises due to reduced metal reuptake [11]. In AD brain the copper concentration can reach to 390 μM in senile plaques [12]. Besides promoting Aβ aggregation and tau hyperphosphorylation [10,13–15], Cu (II) at subneurotoxic doses can promote microglial secretion of pro-inflammatory mediators such as TNF-α and nitric oxide and thus cause microglia-mediated neuronal death [16]. Therefore, Cu(II) may directly trigger neuroinflammation, which plays a major role in the etiopathogenesis of AD [2,3]. In addition, evidence shows that oxidative stress is the earliest event in AD and is a feature of MCI [17,18]. As a redox-active metal ion, Cu(II) can promote free radical formation *via* Fenton reaction in AD brain [19]. It is reported that the elevation of Cu(II) levels in postmortem brain tissues from AD patients are correlated with tissue oxidative damage [9]. The *in vitro* studies reveal that Cu(II) activates oxidative and nitrosative stress pathways in microglia, neurons and astrocytes [16,20]. Therefore, Cu(II) may act as an important trigger in AD pathology. In recent years copper-chelating strategy has gained increasing attention for its potential role in AD treatment *via* inhibiting the deleterious effect of Cu(II) [21–23]. The Cu(II)/Zn(II) chelators such as PBT2 have been studied in clinical trials, with encouraging results in some AD patients [24,25].

We previously designed and synthesized two derivatives of bis-(−)-nor-meptazinol (bis-Mep), a potent dual binding site AChEI [26–28], in an effort to identify novel drug candidates for AD by introducing metal-chelating pharmacophores into the middle chain of bis-Mep. Among them, N^1,N^2-bis(3-(*S*)-meptazinol-propyl) oxalamide (ZLA, Fig.1), a bis-Mep-oxalamide hybrid, showed a potent dual binding AChE inhibition activity, a moderate Cu(II)/Zn(II)-chelating property and a favorable blood brain barrier penetrating property [29]. In the present study, we examined whether ZLA treatment would ameliorate cognitive dysfunction caused by intrahippocampal injection of Cu(II) in mice. Furthermore, we assessed the alteration of microglial functional status, cytokine levels, and oxido-nitrosative stress parameters to probe the possible underlying mechanisms.

2. Materials and methods

2.1. Animals

Adult male Kunming mice (25–30 g) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Science (Shanghai, China). They were maintained under standard conditions with a 12 h: 12 h light-dark cycle, a temperature and humidity controlled environment and *ad libitum* access to food and water. All experimental protocols met the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Shanghai Jiao Tong University School of Medicine.

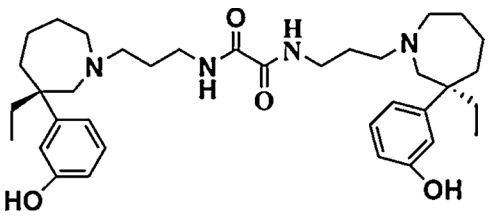

Fig. 1. Chemical structure of ZLA.

2.2. Reagents

ZLA was synthesized in our laboratory. Copper(II) chloride, acetylthiocholine, 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) and rivastigmine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were analytical grade and obtained from commercial sources. Copper(II) chloride was dissolved in ultrapure water to get a Cu(II) stock solution (100 mM) and was diluted with normal saline to get the working solutions. ZLA and rivastigmine were dissolved in dimethyl sulfoxide (DMSO) and normal saline respectively to get a stock solution (10 mg/ml). Fresh working solutions were prepared daily with normal saline. The final concentration of DMSO in ZLA working solutions were ≤ 10%.

2.3. Intrahippocampal injection of Cu(II)

Mice were anaesthetized with intraperitoneal (i.p.) injection of pentobarbital sodium (50 mg/kg) and fixed in a stereotaxic instrument (ASI Instruments, Inc., USA). A bilateral injection of Cu(II) (0.1, 0.25, or 0.5 nmol, 2.5 μL/side) into the hippocampus (-2.0 mm posterior to bregma, ± 1.8 mm lateral to midline, -2.0 mm ventral to skull surface) was performed using a 10 μL Hamilton at a rate of 1 μL/min. The needle was left in place for an additional 5 min at the end of injection. The control mice received injection of normal saline.

2.4. Experimental design

As demonstrated in Table 1, in the first behavioral experiment animals were divided into 4 groups and subjected to intrahippocampal injection of saline or Cu(II) on day 1. Cognitive function was assessed by performing Morris water maze (MWM) test 4 days later. In the second behavioral experiment, animals were divided into 6 groups and subjected to various treatments on days 1-4. MWM or open field test was performed on day 5. Different animal groups were used for MWM or open field test and biochemical evaluation to avoid the mutual influence of various behavioral tests and possible influence of behavioral experiments on biochemical measurements. A schematic diagram showing the experimental timeline and study plan was shown in Fig. 2.

2.5. Immunohistochemistry (IHC)

Mice were given a lethal dose of pentobarbital sodium 4 days after hippocampal saline or Cu(II) injection. After transcardial perfusion with fixative containing 4% paraformaldehyde, brain tissues were removed, fixed in 10% formalin for 24 h, and then were paraffin-embedded and sectioned (4 μm). After epitope retrieval treatment (at 98 °C for 15 min in 0.01 M citrate buffer solution, pH 6.0), sections were incubated with rat anti-CD45 (1:50; BD Biosciences Pharmingen, San Jose, CA, USA) or rabbit anti-microtubule-associated protein 2 (MAP2; 1:50; Cell Signaling Technology, Danvers, MA, USA) antibody overnight at 4 °C. They were then incubated with goat anti-rat or rabbit horseradish peroxidase (HRP)–conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature. After being stained with diaminobenzidine the sections were counterstained with hematoxylin before mounted onto pre-coated glass slides. The images were captured under a Leica DFC 320 digital camera (Leica, Wetzlar, Germany).

Table 1
Behavioral experimental design.

	Day1	Days 2-4
Experiment 1 (n = 10)		
Control group	IH injection of saline	—
Group II	IH injection of Cu(II) (0.1 nmol/side)	—
Group III	IH injection of Cu(II) (0.25 nmol/side)	—
Group IV	IH injection of Cu(II) (0.5 nmol/side)	—
Experiment 2 (n = 10)		
Control group	Vehicle (10% DMSO, i.p.) treatment 30 min before IH injection of saline	Daily vehicle treatment
Group II	Vehicle (i.p.) treatment 30 min before IH injection of Cu(II) (0.5 nmol/side)	Daily vehicle treatment
Group III	ZLA (1 mg/kg, i.p.) treatment 30 min before IH injection of Cu(II) (0.5 nmol/side)	Daily ZLA treatment
Group IV	ZLA (2 mg/kg, i.p.) treatment 30 min before IH injection of Cu(II) (0.5 nmol/side)	Daily ZLA treatment
Group V	ZLA (5 mg/kg, i.p.) treatment 30 min before IH injection of Cu(II) (0.5 nmol/side)	Daily ZLA treatment
Group VI	Riva (2 mg/kg, i.p.) treatment 30 min before IH injection of Cu(II) (0.5 nmol/side)	Daily Riva treatment

Note: IH, intrahippocampal; Riva, rivastigmine.

2.6. Western blot

The hippocampal tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing a protease inhibitor cocktail (Sigma-Aldrich). Equal amount (30 μg) of proteins was loaded on 10% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST; 50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and then were incubated with a rabbit anti-inducible nitric oxide synthase (iNOS; 1:1000; Cell Signaling Technology), MAP2 (1:1000) or β-actin (1:1000; Cell Signaling Technology) antibody overnight at 4 °C. After being washed with TBST, membranes were incubated with a HRP-conjugated secondary antibody (1:5000; Abcam, Cambridge, MA, USA) for 1 h. The bands were visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA) and analyzed by ImageJ software.

2.7. MWM test

The hippocampus-dependent spatial learning and memory function was evaluated as previously described [27]. Briefly, mice were acclimatized to the experimental environment 2 days before the start of evaluation procedures. A black circular pool (140 cm diameter) was placed in the middle of an experimental room containing a few cues, including wall posters, blue window curtain, and a computer rack. The pool was filled with water (22 ± 1.0 °C) to a depth of 30 cm. The tank was divided into 4 equal quadrants, and a black platform (9 cm diameter), which was made invisible to mice and remained in one location for the entire test, was submerged 1 cm below the water surface at the center of one quadrant. A video camera was placed above the center of the pool to capture images of the swimming trace of mice. During the acquisition testing phase, each mouse was given 4 trials per day for 4 consecutive days, with 30 s interval between trials. The starting position was randomized over days but remained the same order for all the mice. Each trial lasted either until the mice had found the platform or for a maximum of 90 s. The mice that were unsuccessful within 90 s were guided to the platform. Animals were allowed to rest on the platform for 30 s. On the 5th day, the platform was removed from the pool and a probe trial was performed to examine retention of spatial memory. Each mouse was allowed to search for 90 s. All the traces were recorded and analyzed with automated tracking software (Morris Water Maze Video Analysis System, DigBeh-MM, Jiliang Software Technology Co. Ltd., Shanghai, China).

2.8. Open field test

The open field test was conducted to explore the locomotor activity and exploratory behavior as described previously [30]. Briefly, each mouse was individually placed in the center area of an open field chamber (40 cm × 40 cm × 40 cm) and was allowed to move freely for 5 min. The floor and the wall of the chamber were cleaned with 70% ethanol solution before commencing a new test to eliminate the odor traces. The movement trace of each mouse was recorded and analyzed with a video camera connected to a tracking system (Jiliang Software Technology Co., Ltd.) to obtain the ratios of distance and time in the center area.

2.9. Biochemical studies

2.9.1. Preparation of hippocampal homogenates

Mice were performed with euthanasia 4 days after hippocampal saline or Cu(II) injection and the hippocampus was immediately isolated and homogenized (10% w/v) in 0.1 M phosphate buffer (pH 7.4) for further analysis.

2.9.2. AChE activity assay

AChE activity was determined by a modified Ellman's method. Briefly, the hippocampal homogenates were centrifuged at 4000 g for

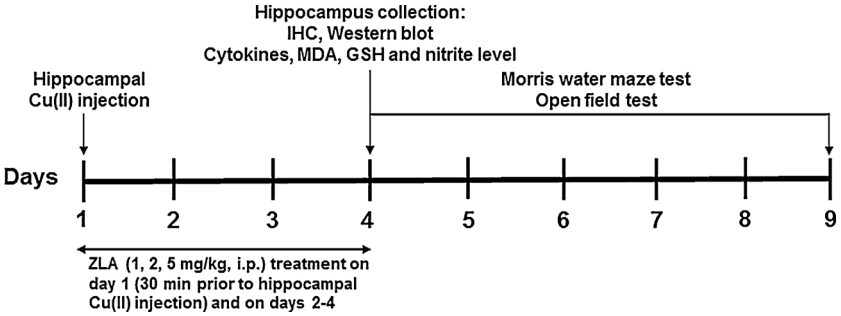

Fig. 2. A schematic diagram showing the experimental timeline and study plan.

10 min at 4 °C. The supernatant was mixed with 0.05 M phosphate buffer solution (pH 7.2) containing 0.25 mM DTNB. Then 1 mM acetylthiocholine was added and the change in absorbance at 412 nm was recorded for a period of 5 min at intervals of 1 min using a Varioskan Flash multimode reader (Thermo, Waltham, MA, USA). Protein content in the supernatant was measured by BCA method. AChE activity was expressed as μmol/min/mg of protein.

2.9.3. Cytokine detection

Quantitative determination of hippocampal TNF-α, IL-6, and IL-1β levels were determined using ELISA kits (Pierce) according to the manufacturer's protocol. Cytokine levels were expressed as pg/mg of protein.

2.9.4. Oxidative stress parameter estimation

Lipid peroxidation and reduced glutathione (GSH) were detected to assess the oxidative stress status in the hippocampus. The content of malondialdehyde (MDA) was determined as previously described [31]. Absorbance for the thiobarbituric acid reactive substances (TBARS) level estimation was measured at 532 nm. GSH level was determined by the method of Beutler et al. [32]. The estimation of MDA and GSH were expressed as μM/g of wet tissue.

2.9.5. Nitrosative stress parameter estimation

Hippocampal nitrite estimation, an indirect measurement of nitric oxide content in the biological samples, was performed as described by Green et al. [33]. Griess reagent (Sigma-Aldrich) and supernatant of homogenates were mixed in a ratio of 1:1 and incubated for 10 min at room temperature. Then the absorbance was measured at 560 nm in a microplate reader. Nitrite content was determined from a standard nitrite curve generated by using sodium nitrite as a standard substance. Results were expressed as μM/g of wet tissue.

2.10. Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post-test comparison. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. ZLA improves the learning and memory deficits caused by intrahippocampal injection of Cu(II)

We first examined the effect of various concentrations of Cu(II) on spatial learning and memory ability (evaluated by MWM test) of mice. Two-way ANOVA analysis in Fig. 3A revealed significant effects for Cu (II) treatment [$F(3, 144) = 13.62$, $P < 0.0001$] and days of acquisition training [$F(3, 144) = 12.10$, $P < 0.0001$] on escape latency (*i.e.* time spent in finding the platform). The representative swimming traces for different groups on day 4 were shown in Fig. 3B. In the probe trial on day 5, the percentage of distance travelled and the percentage of time spent in the target quadrant were detected to evaluate the spatial memory retention. One-way ANOVA analysis showed that these indexes were significantly different among the experimental groups [$F(3, 39) = 4.84$, $P = 0.0063$; Fig. 3C and $F(3, 39) = 4.76$, $P = 0.0068$; Fig. 3D]. The swimming speed on day 5 did not differ among all groups [$F(3, 39) = 0.13$, $P = 0.94$; Fig. 3E], indicating that the locomotor ability of mice was not affected. In contrast to mice receiving intrahippocampal injection of Cu(II) at doses of 0.1 and 0.25 nmol per side, mice receiving injection of Cu(II) at 0.5 nmol per side showed longer escape latency on days 3 and 4 ($P < 0.05$ *vs*. control, Fig. 3A). Moreover, they showed smaller percentage of distance travelled ($P < 0.05$ *vs*. control; Fig. 3C) and smaller percentage of time spent ($P < 0.05$ *vs*. control; Fig. 3D) in the target quadrant on day 5. Therefore, we adopted this dose of Cu(II) in the following experiments. The results of open field test showed that the ratios of the distance [$P = 0.89$; Student *t* test] and time [$P = 0.97$; Student *t* test] in the center area in mice receiving intrahippocampal injection of saline were not different from those in naïve mice (Supplemental Fig. S1). Thus, the operation of intrahippocampal injection might not affect the locomotor activity of mice.

We next examined the effect of ZLA on Cu(II)-induced cognitive deficits. Two-way ANOVA analysis in Fig. 4A demonstrated that escape latency was significantly different among the experimental groups [$F(5, 216) = 14.10$, $P < 0.0001$] and days of acquisition training [$F(5, 216) = 11.75$, $P < 0.0001$]. In mice receiving hippocampal Cu(II) injection and 4-day systemic ZLA (at 2 or 5 mg/kg) treatment, a significantly reduced latency was observed on day 4 ($P < 0.05$ *vs*. Cu(II)-treated group; Fig. 4A). The representative swimming traces for different groups on day 4 were shown in Fig. 4B. One-way ANOVA analysis showed that in the probe trial the percentage of distance travelled [$F(5, 59) = 4.74$, $P = 0.0012$; Fig. 4C] and the percentage of time spent [$F(5, 59) = 5.10$, $P = 0.0007$; Fig. 4D] in the target quadrant were significantly different among the experimental groups. Mice receiving hippocampal Cu(II) injection and 4-day systemic ZLA (at 2 or 5 mg/kg) treatment exhibited significantly greater percentage of distance travelled ($P < 0.05$; Fig. 4C) and percentage of time spent ($P < 0.05$; Fig. 4D) in the target quadrant compared with mice receiving Cu(II) alone. However, rivastigmine, an AChE inhibitor showing a similar potency of enzyme inhibition to ZLA [29], had no effect on Cu(II)-induced elevation of escape latency ($P > 0.05$) and decrease in percentage of distance travelled ($P > 0.05$) and percentage of time spent ($P > 0.05$) in the target quadrant (Fig. 4A-D). ZLA, rivastigmine and vehicle of ZLA (10% DMSO) alone had no effect on escape latency [$F(5, 120) = 1.36$, $P = 0.68$; Supplemental Fig. S2A] and percentage of distance travelled [$F(5, 35) = 0.83$, $P = 0.54$; Supplemental Fig. S2B] and percentage of time spent [$F(5, 35) = 0.28$, $P = 0.92$; Supplemental Fig. S2C] in the target quadrant. The swimming speed on day 5 did not differ among all groups [$F(5, 59) = 0.23$, $P = 0.94$; Fig. 4E]. Moreover, one-way ANOVA analysis of the results of open field test showed that the ratios of the distance [$F(5, 35) = 0.07$, $P = 0.99$] and time [$F(5, 35) = 0.06$, $P = 0.99$] in the center were not different among the experimental groups (Supplemental Fig. S3). These data indicate that the observed improvement in learning and memory was not due to the difference in locomotor ability.

3.2. Hippocampal AChE activity

One-way ANOVA analysis showed that hippocampal AChE activity was not significantly different among the experimental groups [$F(6, 55) = 0.21$, $P = 0.97$]. Intrahippocampal injection of Cu(II) did not evoke an alteration in AChE activity as compared with control group. AChE activity in the hippocampus of mice receiving hippocampal Cu(II) injection plus systemic ZLA or rivastigmine treatment was not different from that of Cu(II)-treated group (Fig. 5).

3.3. ZLA attenuates Cu(II)-induced dendritic damage in the hippocampus

The dendritic marker MAP2 is essential for the function of the somatodendritic compartment of neurons [34]. IHC analysis revealed a dense MAP2-immunoreactivity in the CA1 region of the hippocampus in control mice receiving intrahippocampal injection of saline. By contrast, MAP2 staining showed diffuse loss in the CA1 region of the hippocampus in mice receiving intrahippocampal injection of Cu(II) (Fig. 6A, B). In mice subjected to hippocampal Cu(II) injection plus 4-day systemic ZLA (at 2 or 5 mg/kg) treatment, MAP2-immunoreactivity was denser than in mice receiving Cu(II) alone (Fig. 6C, D). Consistently, one-way ANOVA analysis of Western blot results revealed that hippocampal protein levels of MAP2 differ significantly among the experimental groups [$F(2, 11) = 15.27$, $P = 0.0013$]. Hippocampal Cu

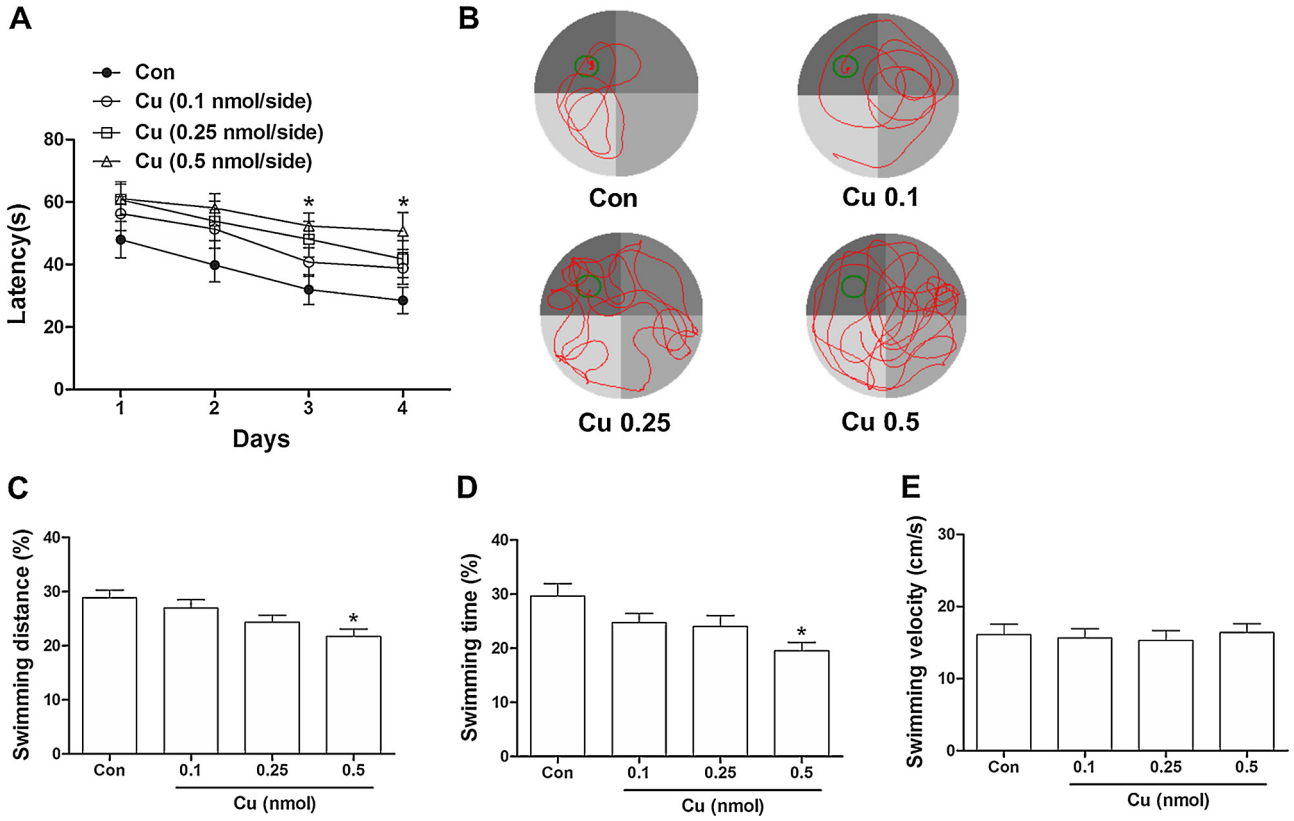

Fig. 3. Intrahippocampal injection of Cu(II) induces learning and memory deficits in mice. (A) Escape latency during the acquisition testing phase (days 1–4). (B) Representative swimming traces on day 4 for different groups. Percentage of swimming distance travelled (C) and percentage of time spent (D) in the target quadrant during the probe trial (day 5). (E) Swimming velocity on day 5. Data are expressed as mean ± SEM (n = 10). *$P < 0.05$ *vs.* control.

(II) injection caused a significantly decrease (by 42.25%) in MAP2 level compared with saline injection ($P < 0.05$). This effect was attenuated by ZLA treatment ($P < 0.05$ *vs.* Cu(II)-treated group) (Fig. 6E, F)

3.4. ZLA inhibits Cu(II)-induced microglial activation in the hippocampus

We next examined the effect of ZLA on Cu(II)-induced microglial activation with an antibody to CD45, a marker of microglial activation [35]. In contrast to saline treatment, a robust microglial activation (indicated by increased CD45-immunoreactivity) in the CA1 region of mouse hippocampus was observed 4 days after intrahippocampal injection of Cu(II) (Fig. 7A,B,E). ZLA (at 2 or 5 mg/kg; i.p.) repressed the elevation of CD45-immunoreactivity in the hippocampus of Cu(II)-treated mice, with a staining intensity similar to that observed in control mice (Fig. 7C-E).

3.5. ZLA inhibits Cu(II)-induced pro-inflammatory cytokine production and iNOS expression in the hippocampus

One-way ANOVA analysis showed that 4 days after intrahippacampal injection of Cu(II), the hippocampal levels of TNF-α, IL-6 and IL-1β were significantly different among the experimental groups [TNF-α: $F(5, 47) = 9.56$, $P < 0.0001$; IL-6: $F(5, 47) = 9.07$, $P < 0.0001$; IL-1β: $F(5, 47) = 8.53$, $P < 0.0001$]. Cu(II) induced an increase in hippocampal levels of TNF-α, IL-6 and IL-1β ($P < 0.01$ *vs.* control group). Four-day systemic ZLA (at 2 or 5 mg/kg) treatment repressed the increase of cytokine levels in Cu(II)-treated mice ($P < 0.05$ at 2 mg/kg and $P < 0.01$ at 5 mg/kg *vs.* Cu(II)-treated group) (Fig. 8A–C). Moreover, one-way ANOVA analysis of Western blot results revealed that hippocampal levels of iNOS were significantly different among the experimental groups [$F(3, 15) = 10.34$, $P = 0.0012$]. The levels of iNOS in the hippocampus of Cu(II)-treated mice were overtly upregulated compared with those of control group (by 63.39%; $P < 0.05$). This effect was inhibited by 4-day systemic treatment with ZLA at doses of 2 and 5 mg/kg ($P < 0.05$ *vs.* Cu(II)-treated group) (Fig. 8D, E).

3.6. ZLA ameliorates Cu(II)-induced oxido-nitrosative stress in the hippocampus

We next evaluated the oxido-nitrosative status in mouse hippocampus. One-way ANOVA analysis revealed a significant difference in hippocampal levels of MDA [$F(5, 47) = 6.83$, $P < 0.0001$], GSH [$F(5, 47) = 6.65$, $P < 0.0001$] and nitrite [$F(5, 47) = 5.44$, $P = 0.0006$] among the experimental groups. MDA and nitrite levels were significantly increased ($P < 0.01$ *vs.* control group) while GSH level was significantly reduced ($P < 0.01$ *vs.* control group) in the hippocampus of mice subjected to hippocampal Cu(II) injection. These effects were inhibited by 4-day systemic ZLA (at 2 or 5 mg/kg) treatment ($P < 0.05$ at 2 mg/kg and $P < 0.01$ at 5 mg/kg *vs.* Cu(II)-treated group) (Fig. 9).

3.7. The safety of ZLA

No obvious adverse reactions, such as salivation and trembling, were observed in mice receiving i.p. injection of ZLA at doses of 1, 2, or 5 mg/kg, even at the dose of 100 mg/kg (data not shown). However, rivastigmine (the reported LD50 in male mice is approximately 6 mg/kg [36]) caused obvious trembling, fasciculation and decreased locomotor activity at 5 mg/kg and death of all tested mice at 100 mg/kg.

4. Discussion

AD involves a variety of neurobiological etiologies, among which neuroinflammation and oxido-nitrosative stress play essential roles

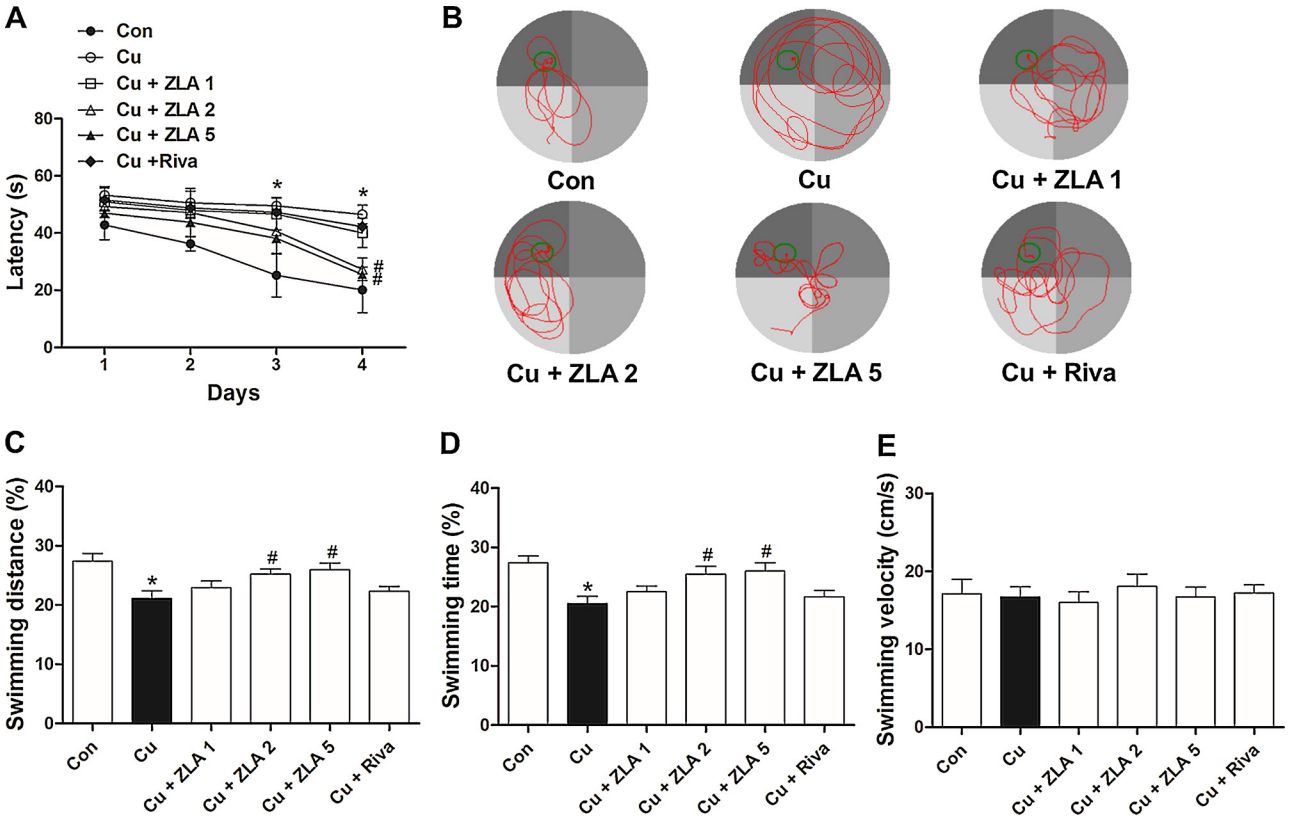

Fig. 4. ZLA ameliorates learning and memory deficits caused by intrahippocampal injection of Cu(II) in mice. (A) Escape latency during the acquisition testing phase (days 1–4). (B) Representative swimming traces on day 4 for different groups. Percentage of swimming distance travelled (C) and percentage of time spent (D) in the target quadrant during the probe trial (day 5). (E) Swimming velocity on day 5. ZLA (1, 2, or 5 mg/kg, i.p.) or rivastigmine (2 mg/kg, i.p.) was administered 30 min before intrahippocampal injection of saline or Cu(II) (0.5 nmol/side) and was daily administered for the following 3 days. Data are expressed as mean ± SEM (n = 10). $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* Cu(II)-treated group.

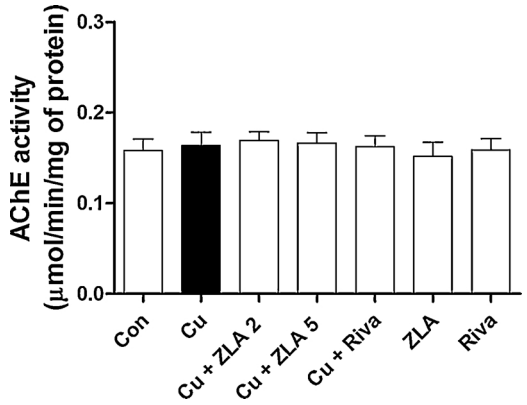

Fig. 5. Effect of Cu(II) exposure and ZLA or rivastigmine treatment on AChE activity in mouse hippocampus. ZLA (2, 5 mg/kg, i.p.) or rivastigmine (2 mg/kg, i.p.) was administered 30 min before intrahippocampal injection of saline or Cu(II) and was daily administered for the following 3 days. Data are expressed as mean ± SEM (n = 8).

[2,3]. This multifactorial nature suggests that compounds capable of hitting several molecule targets involved in AD pathogenesis may yield disease-modifying effects. In recent years, a variety of multi-targeted agents have been developed and evaluated for their potential in AD therapy [37,38]. Accumulating evidence suggests that copper imbalance also contributes to AD pathogenesis *via* promoting Aβ aggregation and tau hyperphosphorylation [10,13–15,39]. Moreover, *in vitro* and *in vivo* studies indicate that Cu(II) might elicit neurotoxicity *via* promoting neuroinflammation and oxido-nitrosative stress pathway [16,20,40]. Therefore, development of biocompatible metal-chelating agents may offer a potential therapeutic solution to target mislocalized Cu(II) [11]. In this study, we demonstrate that ZLA, a multifunctional compound with properties of dual binding site AChE inhibition and Cu (II) chelation [29], exerts protective effects against cognitive impairment caused by intrahippocampal injection of Cu(II). The underlying mechanism may involve attenuation of neuroinflammation and oxido-nitrosative stress.

In AD brain the copper concentration can reach to 390 μM in senile plaques [12]. Therefore, we injected Cu(II) to the mouse hippocampus to mimic this micro-environmental milieu in AD. We observed a dose-dependently deleterious effect of Cu(II) on performance of MWM task. Injection of Cu(II) at 0.5 nmol per side evoked a significant impairment of cognitive function. Therefore, this dose of Cu(II) was adopted in the following experiments. ZLA, when systemically administered at doses of 1, 2 and 5 mg/kg, reversed the Cu(II)-caused prolonged escape latency (in the acquisition test) and decreased percentage of distance travelled and percentage of time spent in the target quadrant (in the probe trial) in a dose-dependent manner. These results, similar to the observations showing that clioquinol, an agent with metal-chelating property, significantly improved the memory dysfunction induced by Cu(II)–cholesterol [41], suggest that ZLA ameliorated the Cu(II)-caused cognitive impairment. However, different from the results of Mehra et al. [41] demonstrating that treatment with Cu(II)–cholesterol for 8 weeks resulted in a rise in brain AChE activity, we did not observe an increase in hippocampal AChE activity after intrahippocampal injection of Cu(II). This may be due to the shorter duration of Cu(II) treatment in our study. Our results, similar to those of Simonato et al. [42] demonstrating that Cu(II) exposure did not significantly change brain AChE activity, indicate that behavioral changes after Cu(II) challenge might

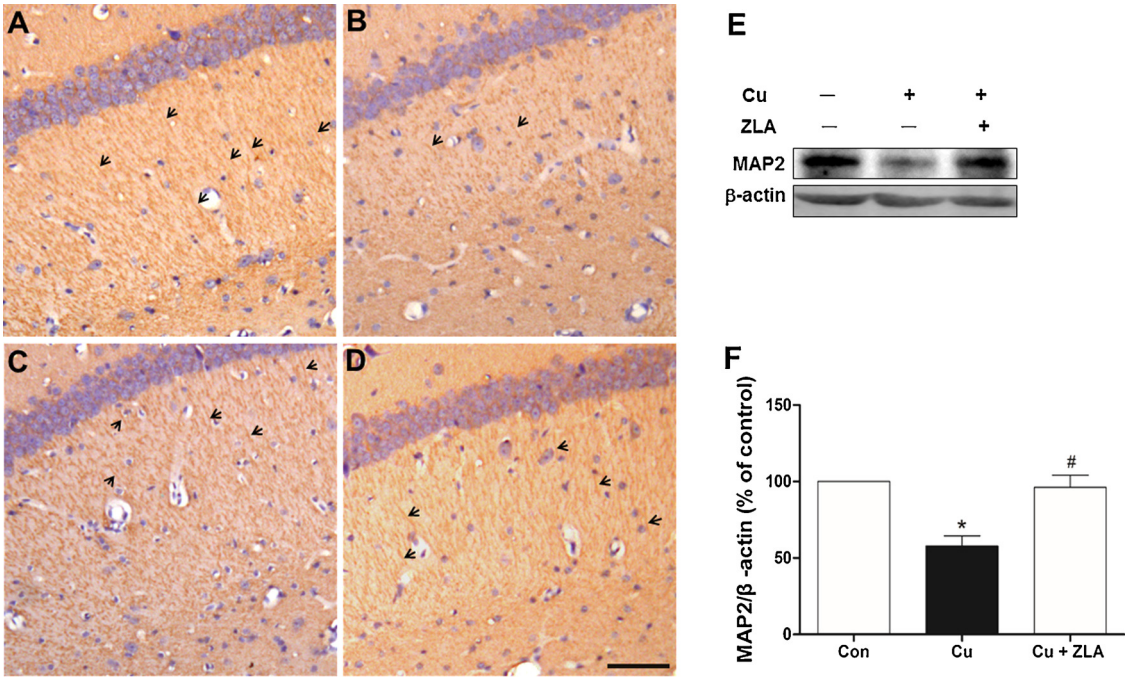

Fig. 6. ZLA attenuates Cu(II)-induced dendritic loss in the hippocampus. (A) A representative photomicrograph showing dense MAP2-immunoreactivity (arrow) in the CA1 region of the hippocampus of a mouse subjected to intrahippocampal injection of saline. (B) Four days after intrahippocampal injection of Cu(II), an obvious loss of MAP2 staining was observed. (C, D) ZLA (2 or 5 mg/kg, i.p.) attenuated Cu(II)-induced dendritic loss. ZLA was administered 30 min before hippocampal Cu(II) injection and was daily administered for the following 3 days. Scale bar = 200 μm. (E, F) Western blot analysis showed that ZLA (5 mg/kg, i.p.) rescued Cu(II)-caused decrease in hippocampal MAP2 expression. Data are expressed as mean ± SEM (n = 4). $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* Cu(II)-treated group.

not be attributed to a modulatory effect on hippocampal enzyme activity. Moreover, different from the results of Mehra et al. [41] showing that an AChEI donepezil improved the performance of MWM task in mice treated with Cu(II)–cholesterol, we found that rivastigmine did not attenuate Cu(II)-caused dementia. These findings indicate that alleviation of spatial memory deficits by ZLA may not involve AChE inhibition activity. Rather, it could be associated with free copper chelation.

The dendritic marker MAP2, the most abundant cytoskeletal protein in the brain, is involved in microtubule assembly and is particularly vulnerable to brain injury, with a rapid (about 3 h) loss of staining [43,44]. IHC analysis revealed that MAP2 immunoreactivity exhibited diffuse loss 4 days after hippocampal Cu(II) injection, an effect that was rescued by ZLA (at 2 or 5 mg/kg) treatment. Similarly, the results of Western blot analysis showed that Cu(II)-induced decrease in MAP2 protein level was attenuated by ZLA treatment. So we suppose that Cu (II)-caused dementia was associated with the dendritic damage and that prevention of Cu(II)-induced neuronal dendritic damage in the hippocampus may explain, at least in part, the anti-amnesic effect of ZLA.

Microglia-mediated neuroinflammation plays important roles in neuronal injury in early stages of AD [45,46]. It has been suggested that Cu(II) can indirectly (*via* promoting Aβ aggregation) and directly

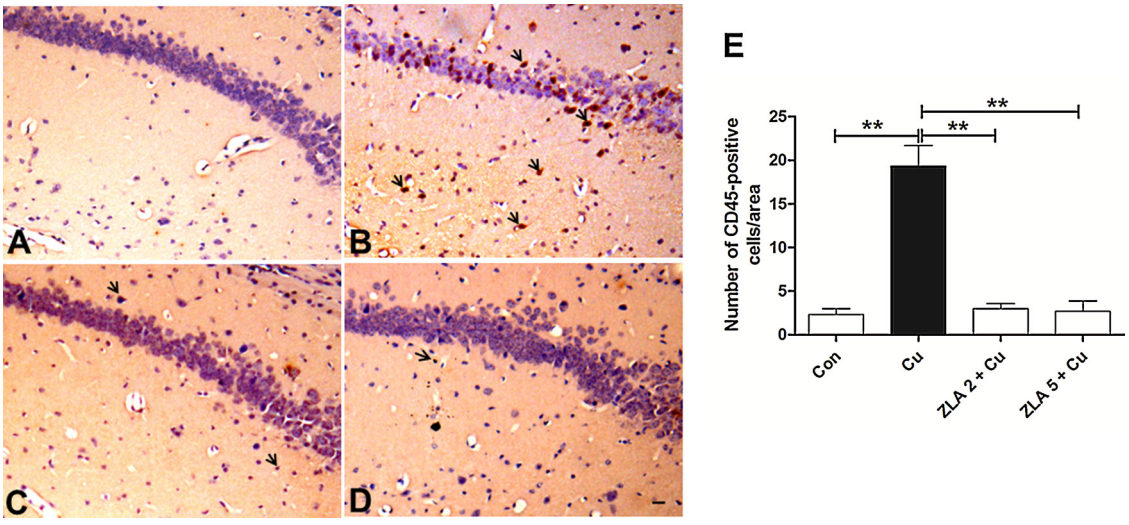

Fig. 7. ZLA attenuates Cu(II)-induced microglial activation in the hippocampus. Representative photomicrographs of CD45-immunoreactivity (arrows) in the CA1 region of mouse hippocampus 4 days after intrahippocampal injection of saline (A) or Cu(II) (B). (C, D) ZLA (2, 5 mg/kg, i.p.) decreased CD45-immunoreactivity in the hippocampus of Cu(II)-treated mice. ZLA was administered 30 min before hippocampal Cu(II) injection and was daily administered for the following 3 days. Scale bar = 100 μm. (E) Quantitative analysis of CD45-positive cells. Data are expressed as mean ± SEM (n = 4). ** $P < 0.01$.

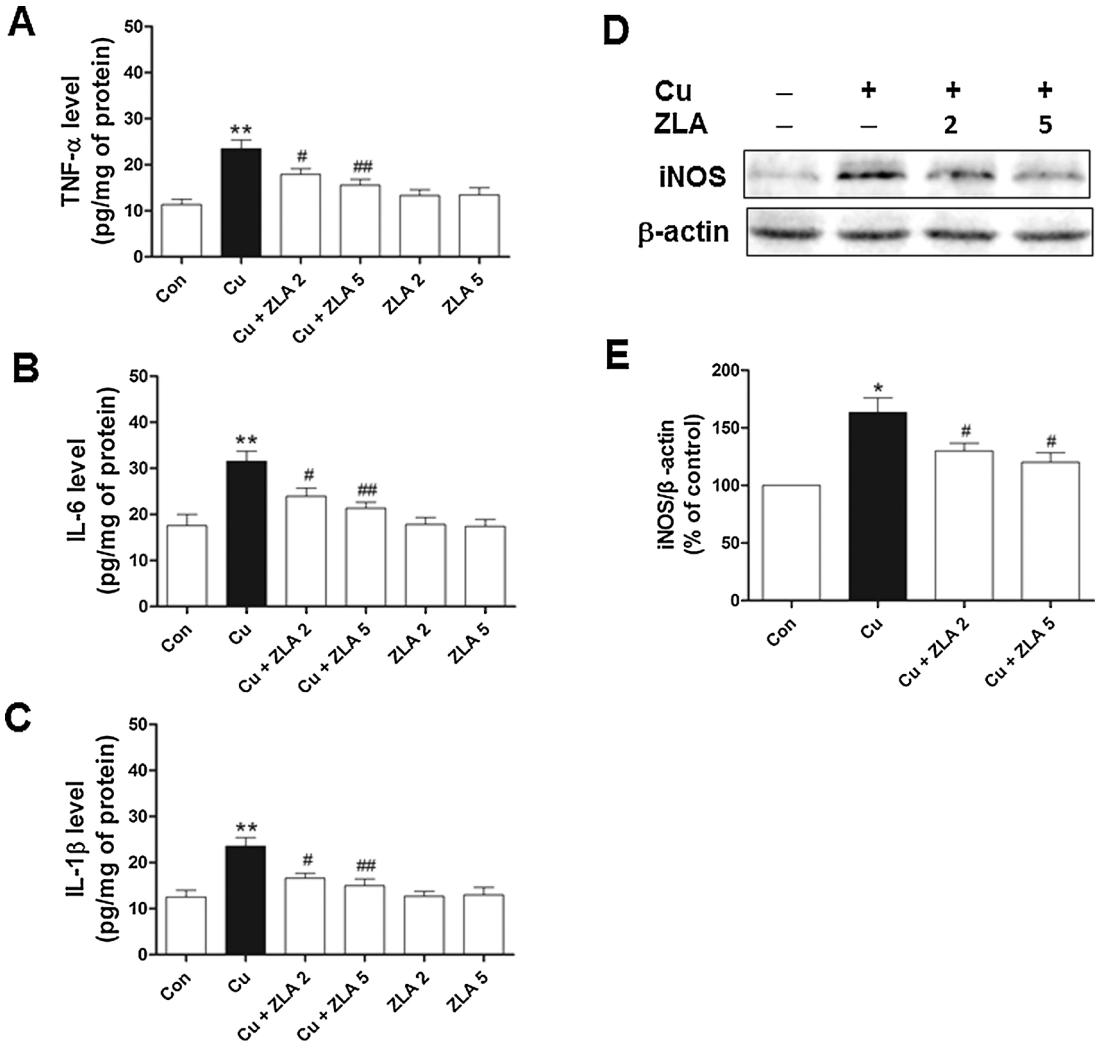

Fig. 8. ZLA represses Cu(II)-induced production of pro-inflammatory cytokines and expression of iNOS in mouse hippocampus. Effect of ZLA treatment on hippocampal content of TNF-α (A), IL-6 (B), and IL-1β (C) in Cu(II)-challenged mice. ZLA (2, 5 mg/kg, i.p.) was administered 30 min before intrahippocampal injection of saline or Cu(II) and was daily administered for the following 3 days. Data are expressed as mean ± SEM (n = 8). (D, E) Western blot analysis of iNOS expression in the hippocampus 4 days after Cu(II) challenge, with or without ZLA (2 or 5 mg/kg, i.p.) treatment. Data are expressed as mean ± SEM (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* Cu(II)-treated group.

induce microglia-mediated neuroinflammatory processes in AD [16,47,48]. In line with our findings in cultured microglial cells [16], we observed in mice that Cu(II) evoked overt microglial activation in the hippocampus 4 days after intrahippocampal injection. Moreover, the hippocampal content of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β was enhanced. These effects were significantly inhibited by systemic ZLA (at 2 or 5 mg/kg) treatment. The pro-inflammatory cytokines (such as TNF-α) can induce expression of iNOS, which catalyzes formation of nitric oxide from guanidine nitrogen of L-arginine [49]. We found a significantly increased hippocampal iNOS expression in Cu(II)-challenged mice, an effect that was repressed by ZLA treatment. These results indicate that microglia-mediated neuroinflammatory responses may contribute to Cu(II)-induced neuronal dendritic damage and cognitive deficits and that ZLA ameliorates Cu(II) effects *via*, at least in part, inhibition of microglia-mediated neuroinflammation.

Oxidative and nitrosative stress, which leads to oxidation and nitration of proteins and lipids as well as adduct formation in DNA, also plays essential roles in neurodegeneration related to AD [2]. Neuroinflammation and oxido-nitrosative stress are closely interacted and reinforce each other [2,50]. Evidence shows that the pro-inflammatory cytokines (such as TNF-α) increase reactive oxygen species (ROS) and nitric oxide (*via* triggering iNOS expression) production, leading to aggravation of oxido-nitrosative stress [2,49]. Evidence shows that Cu (II) can promote free radical formation in AD brain [19]. We observed enhanced levels of MDA (a marker of lipid peroxidation) and nitrite (reflection of nitric oxide production) and decreased level of GSH in the hippocampus of Cu(II)-challenged mice, indicating a higher oxidative and nitrosative status and a lower antioxidant status. The observations that ZLA significantly attenuated the increase in MDA and nitrite levels and the decrease in GSH level in hippocampus of Cu(II)-challenged mice indicate that inhibition of Cu(II)-induced oxido-nitrosative stress may also contribute to the memory-improving effect of ZLA.

Our previous *in vitro* study revealed that ZLA at a concentration of 100 μM had no obvious effect on the viability of human neuron-like SH-SY5Y cells [29]. In the present study, we found no obvious adverse reactions (such as salivation and trembling) in mice receiving i.p. injection of ZLA, even at a dose of 100 mg/kg. By contrast, rivastigmine at this dose caused animal death. Moreover, the results of MWM test and open field test demonstrated that ZLA treatment did not affect the swimming speed and the locomotor activities. These observations suggest a good safety of ZLA. It should be noted that as a multifunctional agent with chelating property, it cannot be ruled out that ZLA could interact with other metabolically active metals in the brain

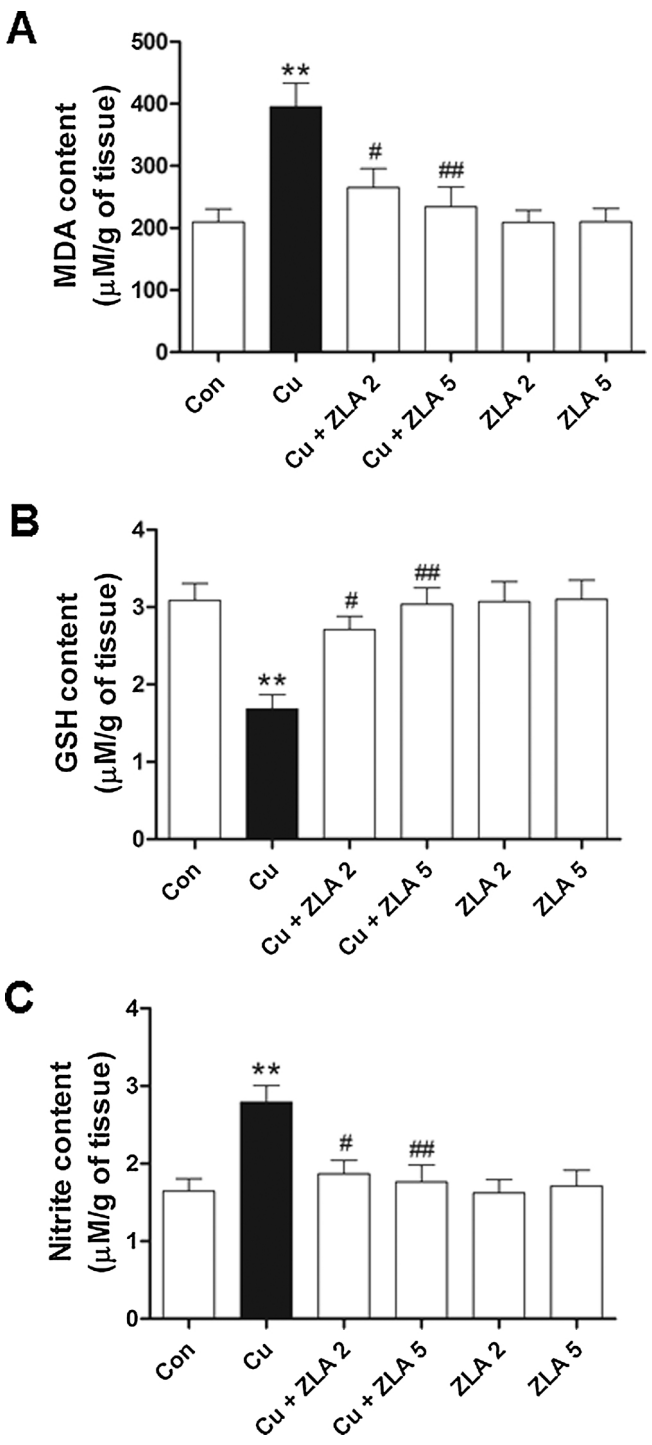

Fig. 9. ZLA represses Cu(II)-induced oxido-nitrosative stress in mouse hippocampus. Effect of ZLA treatment on hippocampal content of MDA (A), GSH (B), and nitrite (C) in Cu(II)-challenged mice. ZLA (2, 5 mg/kg, i.p.) was administered 30 min before intrahippocampal injection of saline or Cu(II) and was daily administered for the following 3 days. Data are expressed as mean ± SEM (n = 8). $^{**}P < 0.01$ *vs.* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* Cu(II)-treated group.

such as iron, zinc, and the most importantly calcium. Accumulating evidence shows that transition metals such as copper, zinc, and iron play critical roles in pathogenesis of AD and that multi-functional agents with chelating property could be effective against AD [9,23]. Therefore, the interactions of ZLA with iron and zinc might be beneficial for AD intervention. ZLA could also interact with calcium, an important intracellular second messenger. As we did not observe obvious toxic effect of ZLA even at a dose of 100 mg/kg, this possible interaction could hardly yield overtly deleterious effect.

Elevated brain free Cu(II) levels, which could trigger oxidative damage, has been indicated in brain tissues of AD patients and transgenic APP/PS1 mice [9,19]. As intrahippocampal injection of Cu(II) simultaneously triggered neuroinflammation and oxidative stress, two key processes that can act in concert to play a major role in the etiopathogenesis of AD [2,50], we chose this animal model to test if ZLA can act as a possible anti-AD agent. We acknowledge that this animal model has limitations and cannot mimic all the pathophysiological processes in AD brain. Notably, in addition to the nootropic effect of ZLA we observed in the animal model subjected to intrahippocampal injection of Cu(II), we also found that ZLA (1, 2, 5 mg/kg, i.p.) ameliorates the dementia induced by i.p. injected scopolamine (a muscarinic receptor antagonist that can pass through the blood-brain barrier) in a dose–dependent manner (unpublished data). These data provide behavioral evidence for the multi-targeted property of ZLA as a potential anti-AD agent. We will further examine its efficacy in other AD animal models (such as APP/PS1 transgenic mice) in the future research.

In summary, this study demonstrates that ZLA effectively improves the Cu(II)-induced learning and memory dysfunction. The potential mechanisms may involve attenuation of neuroinflammation and oxido-nitrosative stress. Future studies are needed to further examine if ZLA has potential disease-modifying effect in transgenic animal models of AD.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

This research was supported by National Natural Science Foundation of China (grant numbers 81373417, 81503044, 30973538 and 21202098) and Shanghai Municipal Science and Technology Commission (grant number 14431901800).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2018.12.014.

References

[1] S. Kocahan, Z. Doğan, Mechanisms of Alzheimer's disease pathogenesis and prevention: the brain, neural pathology, n-methyl-d-aspartate receptors, tau protein and other risk factors, Clin. Psychopharmacol. Neurosci. 15 (2017) 1–8.
[2] M.A. Daulatzai, Fundamental role of pan-inflammation and oxidative-nitrosative pathways in neuropathogenesis of Alzheimer's disease in focal cerebral ischemic rats, Am. J. Neurodegener. Dis. 5 (2016) 102–130.
[3] S. Piirainen, A. Youssef, C. Song, A.V. Kalueff, G.E. Landreth, T. Malm, L. Tian, Psychosocial stress on neuroinflammation and cognitive dysfunctions in Alzheimer's disease: the emerging role for microglia? Neurosci. Biobehav. Rev. 77 (2017) 148–164.
[4] G.J. Brewer, Divalent copper as a major triggering agent in Alzheimer's disease, J. Alzheimers Dis. 46 (2015) 593–604.
[5] W. Jaroudi, J. Garami, S. Garrido, M. Hornberger, S. Keri, A.A. Moustafa, Factors underlying cognitive decline in old age and Alzheimer's disease: the role of the hippocampus, Rev. Neurosci. 28 (2017) 705–714.
[6] Q. Ma, M. Ying, X. Sui, H. Zhang, H. Huang, L. Yang, X. Huang, Z. Zhuang, J. Liu, X. Yang, Chronic copper exposure causes spatial memory impairment, selective loss of hippocampal synaptic proteins, and activation of PKR/eIF2α pathway in mice, J. Alzheimers Dis. 43 (2015) 1413–1427.
[7] H. Basun, L.G. Forssell, L. Wetterberg, B. Winblad, Metals and trace elements in plasma and cerebrospinal fluid in normal aging and Alzheimer's disease, J. Neural Transm. Park. Dis. Dement. Sect. 3 (1991) 231–258.
[8] F.N.C. Vaz, B.L. Fermino, M.V.L. Haskel, J. Wouk, G.B.L. de Freitas, R. Fabbri, E. Montagna, J.B.T. Rocha, J.S. Bonini, The relationship between copper, iron, and selenium levels and Alzheimer disease, Biol. Trace Elem. Res. 181 (2018) 185–191.

[9] S.A. James, I. Volitakis, P.A. Adlard, J.A. Duce, C.L. Masters, R.A. Cherny, A.I. Bush, Elevated labile Cu is associated with oxidative pathology in Alzheimer disease, Free Radic. Biol. Med. 52 (2012) 298–302.
[10] S. Keskitalo, M. Farkas, M. Hanenberg, A. Szodorai, L. Kulic, A. Semmler, M. Weller, R.M. Nitsch, M. Linnebank, Reciprocal modulation of Aβ42 aggregation by copper and homocysteine, Front. Aging Neurosci. 6 (2014) 237.
[11] J.A. Duce, A.I. Bush, Biological metals and Alzheimer's disease: implications for therapeutics and diagnostics, Prog. Neurobiol. 92 (2010) 1–18.
[12] M.A. Lovell, J.D. Robertson, W.J. Teesdale, J.L. Campbell, W.R. Markesbery, Copper, iron and zinc in Alzheimer's disease senile plaques, J. Neurol. Sci. 158 (1998) 47–52.
[13] H. Tamano, A. Takeda, Is interaction of amyloid β-peptides with metals involved in cognitive activity? Metallomics. 7 (2015) 1205–1212.
[14] S. Martic, M.K. Rains, H.B. Kraatz, Probing copper/tau protein interactions electrochemically, Anal. Biochem. 442 (2013) 130–137.
[15] M.G. Savelieff, S. Lee, Y. Liu, M.H. Lim, Untangling amyloid-β, tau, and metals in Alzheimer's disease, ACS Chem. Biol. 8 (2013) 856–865.
[16] Z. Hu, F. Yu, P. Gong, Y. Qiu, W. Zhou, Y. Cui, J. Li, H. Chen, Subneurotoxic copper (II)-induced NF-κB-dependent microglial activation is associated with mitochondrial ROS, Toxicol. Appl. Pharmacol. 276 (2014) 95–103.
[17] A. Nunomura, G. Perry, G. Aliev, K. Hirai, A. Takeda, E.K. Balraj, P.K. Jones, H. Ghanbari, T. Wataya, S. Shimohama, S. Chiba, C.S. Atwood, R.B. Petersen, M.A. Smith, Oxidative damage is the earliest event in Alzheimer disease, J. Neuropathol. Exp. Neurol. 60 (2001) 759–767.
[18] J.N. Keller, F.A. Schmitt, S.W. Scheff, Q. Ding, Q. Chen, D.A. Butterfield, W.R. Markesbery, Evidence of increased oxidative damage in subjects with mild cognitive impairment, Neurology. 64 (2005) 1152–1156.
[19] K. Jomova, D. Vondrakova, M. Lawson, M. Valko, Metals, oxidative stress and neurodegenerative disorders, Mol. Cell. Biochem. 345 (2010) 91–104.
[20] P.V. Reddy, K.V. Rao, M.D. Norenberg, The mitochondrial permeability transition, and oxidative and nitrosative stress in the mechanism of copper toxicity in cultured neurons and astrocytes, Lab. Invest. 88 (2008) 816–830.
[21] M. Nguyen, C. Bijani, N. Martins, B. Meunier, A. Robert, Transfer of copper from an amyloid to a natural copper-carrier peptide with a specific mediating ligand, Chemistry. 21 (2015) 17085–17090.
[22] H. Segal-Gavish, O. Danino, Y. Barhum, T. Ben-Zur, E. Shai, D. Varon, D. Offen, B. Fischer, A multifunctional biocompatible drug candidate is highly effective in delaying pathological signs of Alzheimer's disease in 5XFAD mice, J. Alzheimers Dis. 58 (2017) 389–400.
[23] A. Sharma, V. Pachauri, S.J.S. Flora, Advances in multi-functional ligands and the need for metal-related pharmacology for the management of Alzheimer disease, Front. Pharmacol. 9 (2018) 1247.
[24] L. Lannfelt, K. Blennow, H. Zetterberg, S. Batsman, D. Ames, J. Harrison, C.L. Masters, S. Targum, A.I. Bush, R. Murdoch, J. Wilson, C.W. Ritchie, PBT2-201-EURO study group. Safety, efficacy, and biomarker findings of PBT2 in targeting Abeta as a modifying therapy for Alzheimer's disease: a phase IIa, double-blind, randomised, placebo-controlled trial, Lancet Neurol. 7 (2008) 779–786.
[25] N.G. Faux, C.W. Ritchie, A. Gunn, A. Rembach, A. Tsatsanis, J. Bedo, J. Harrison, L. Lannfelt, K. Blennow, H. Zetterberg, M. Ingelsson, C.L. Masters, R.E. Tanzi, J.L. Cummings, C.M. Herd, A.I. Bush, PBT2 rapidly improves cognition in Alzheimer's Disease: additional phase II analyses, J. Alzheimers Dis. 20 (2010) 509–516.
[26] Q. Xie, H. Wang, Z. Xia, M. Lu, W. Zhang, X. Wang, W. Fu, Y. Tang, W. Sheng, W. Li, W. Zhou, X. Zhu, Z. Qiu, H. Chen, Bis-(-)-nor-meptazinols as novel nanomolar cholinesterase inhibitors with high inhibitory potency on amyloid-beta aggregation, J. Med. Chem. 51 (2008) 2027–2036.
[27] T. Liu, Z. Xia, W.W. Zhang, J.R. Xu, X.X. Ge, J. Li, Y. Cui, Z.B. Qiu, J. Xu, Q. Xie, H. Wang, H.Z. Chen, Bis(9)-(-)-nor-meptazinol as a novel dual-binding AChEI potently ameliorates scopolamine-induced cognitive deficits in mice, Pharmacol. Biochem. Behav. 104 (2013) 138–143.
[28] Y. Shi, W. Huang, Y. Wang, R. Zhang, L. Hou, J. Xu, Z. Qiu, Q. Xie, H. Chen, Y. Zhang, H. Wang, Bis(9)-(-)-Meptazinol, a novel dual-binding AChE inhibitor, rescues cognitive deficits and pathological changes in APP/PS1 transgenic mice, Transl. Neurodegener. 7 (2018) 21.
[29] W. Zheng, J. Li, Z. Qiu, Z. Xia, W. Li, L. Yu, H. Chen, J. Chen, Y. Chen, Z. Hu, W. Zhou, B. Shao, Y. Cui, Q. Xie, H. Chen, Novel bis-(-)-nor-meptazinol derivatives act as dual binding site AChE inhibitors with metal-complexing property, Toxicol. Appl. Pharmacol. 264 (2012) 65–72.
[30] T. Wei, M. Yi, W. Gu, L. Hou, Q. Lu, Z. Yu, H. Chen, The potassium channel KCa3.1 represents a valid pharmacological target for astrogliosis-induced neuronal impairment in a mouse model of Alzheimer's disease, Front. Pharmacol. 7 (2017) 528.
[31] A. Jangra, A.K. Datusalia, S. Khandwe, S.S. Sharma, Amelioration of diabetes-induced neurobehavioral and neurochemical changes by melatonin and nicotinamide: implication of oxidative stress-PARP pathway, Pharmacol. Biochem. Behav. 114-115 (2013) 43–51.
[32] E. Beutler, O. Duron, B.M. Kelly, Improved method for the determination of blood glutathione, J. Lab. Clin. Med. 61 (1963) 882–888.
[33] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids, Anal. Biochem. 126 (1982) 131–138.
[34] G.P. Ballough, L.J. Martin, F.J. Cann, J.S. Graham, C.D. Smith, C.E. Kling, J.S. Forster, S. Phann, M.G. Filbert, Microtubule-associated protein 2 (MAP-2): a sensitive marker of seizure-related brain damage, J. Neurosci. Methods 61 (1995) 23–32.
[35] D.L. Herber, M. Mercer, L.M. Roth, K. Symmonds, J. Maloney, N. Wilson, M.J. Freeman, D. Morgan, M.N. Gordon, Microglial activation is required for Aβ clearance after intracranial injection of lipopolysaccharide in APP transgenic mice, J. Neuroimmune Pharmacol. 2 (2007) 222–231.
[36] C. Mustazza, A. Borioni, M.R. Del Giudice, F. Gatta, R. Ferretti, A. Meneguz, M.T. Volpe, P. Lorenzini, Synthesis and cholinesterase activity of phenylcarbamates related to rivastigmine, a therapeutic agent for Alzheimer's disease, Eur. J. Med. Chem. 37 (2002) 91–109.
[37] C. Lecoutey, D. Hedou, T. Freret, P. Giannoni, F. Gaven, M. Since, V. Bouet, C. Ballandonne, S. Corvaisier, A. Malzert Fréon, S. Mignani, T. Cresteil, M. Boulouard, S. Claeysen, C. Rochais, P. Dallemagne, Design of donecopride, a dual serotonin subtype 4 receptor agonist/acetylcholinesterase inhibitor with potential interest for Alzheimer's disease treatment, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) E3825–30.
[38] L. Wang, G. Esteban, M. Ojima, O.M. Bautista-Aguilera, T. Inokuchi, I. Moraleda, I. Iriepa, A. Samadi, M.B. Youdim, A. Romero, E. Soriano, R. Herrero, A.P. Fernández Fernández, Ricardo-Martínez-Murillo, J. Marco-Contelles, M. Unzeta, Donepezil + propargylamine + 8-hydroxyquinoline hybrids as new multifunctional metal-chelators, ChE and MAO inhibitors for the potential treatment of Alzheimer's disease, Eur. J. Med. Chem. 80 (2014) 543–561.
[39] Y.H. Hung, A.I. Bush, R.A. Cherny, Copper in the brain and Alzheimer's disease, J. Biol. Inorg. Chem. 15 (2010) 61–76.
[40] M. Kitazawa, H.W. Hsu, R. Medeiros, Copper exposure perturbs brain inflammatory responses and impairs clearance of amyloid-beta, Toxicol. Sci. 152 (2016) 194–204.
[41] R. Mehra, R.K. Sodhi, N. Aggarwal, Memory restorative ability of clioquinol in copper-cholesterol-induced experimental dementia in mice, Pharm. Biol. 53 (2015) 1250–1259.
[42] J.D. Simonato, M. Mela, H.B. Doria, I.C. Guiloski, M.A.F. Randi, P.S.M. Carvalho, P.C. Meletti, H.C. Silva de Assis, A. Bianchini, C.B.R. Martinez, Biomarkers of waterborne copper exposure in the neotropical fish Prochilodus lineatus, Aquat. Toxicol. 170 (2016) 31–41.
[43] S.C.1 Hellewell, E.B. Yan, D.S. Alwis, N. Bye, M.C. Morganti-Kossmann, Erythropoietin improves motor and cognitive deficit, axonal pathology, and neuroinflammation in a combined model of diffuse traumatic brain injury and hypoxia, in association with upregulation of the erythropoietin receptor, J. Neuroinflammation 10 (2013) 156.
[44] R.M.1 Posmantur, A. Kampfl, W.C. Taft, M. Bhattacharjee, C.E. Dixon, J. Bao, R.L. Hayes, Diminished microtubule-associated protein 2 (MAP2) immunoreactivity following cortical impact brain injury, J. Neurotrauma 13 (1996) 125–137.
[45] C.H. Latta, H.M. Brothers, D.M. Wilcock, Neuroinflammation in Alzheimer's disease; A source of heterogeneity and target for personalized therapy, Neuroscience. 302 (2015) 103–111.
[46] Z. Cai, M.D. Hussain, L.J. Yan, Microglia, neuroinflammation, and beta-amyloid protein in Alzheimer's disease, Int. J. Neurosci. 124 (2014) 307–321.
[47] X.Y. Choo, L. Alukaidey, A.R. White, A. Grubman, Neuroinflammation and copper in Alzheimer's disease, Int. J. Alzheimers Dis. 2013 (2013) 145345.
[48] F. Yu, P. Gong, Z. Hu, Y. Qiu, Y. Cui, X. Gao, H. Chen, J. Li, Cu(II) enhances the effect of Alzheimer's amyloid-β peptide on microglial activation, J. Neuroinflammation 12 (2015) 122.
[49] J.L. Madrigal, O. Hurtado, M.A. Moro, I. Lizasoain, P. Lorenzo, A. Castrillo, L. Boscá, J.C. Leza, The increase in TNF-alpha levels is implicated in NF-κB activation and inducible nitric oxide synthase expression in brain cortex after immobilization stress, Neuropsychopharmacology 26 (2002) 155–163.
[50] J. Leszek, G.E. Barreto, K. Gąsiorowski, E. Koutsouraki, M. Ávila-Rodrigues, G. Aliev, Inflammatory mechanisms and oxidative stress as key factors responsible for progression of neurodegeneration: role of brain innate immune system, CNS Neurol. Disord. Drug Targets 15 (2016) 329–336.