Original Article

L-3-n-butylphthalide may attenuate Aβ$_{1-42}$-induced neuronal apoptosis and synaptotoxicity in primary cultured cortical neurons possibly via modulating both PI3K/AKT and ERK signaling pathways

Yunxia Zhao[1], Yong Zhang[1], Xiuying Chen[1], Wei Dong[2], Yifeng Du[1]

Departments of [1]Neurology, [2]Thoracic Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, P.R. China

Abstract: Background: Alzheimer's disease (AD), which is characterized by progressively cognitive decline and neuronal loss, is one of the most common neurodegenerative diseases. The β-amyloid (Aβ)-mediated mitochondrial dysfunction and eventually neuronal apoptosis contribute to the pathogenesis of AD. L-3-n-butylphthalide (L-NBP) has been proven to have significant protective effects in many stoke and AD models. Our study is to investigate the protective effects and mechanisms of L-NBP against Aβ induced cell injury in cortical neurons. Material and method: The primary cortical neurons treated with Aβ$_{1-42}$ were used as the AD cell model. Then the cells were treated with or without L-NBP. MTT assay were used to assess the cell viability. Apoptosis of cortical neurons was analyzed by Hoechst 33342 staining. The flow cytometry was used to assess the mitochondrial membrane potential (Δψm). The apoptotic and signaling associated protein expression levels were measured using Western blotting. Results: Pretreatment with L-NBP effectively inhibited Aβ$_{1-42}$-induced cytotoxicity, apoptosis, mitochondrial dysfunction, and synaptotoxicity in primary cultured cortical neurons in a dose-dependent way. The protective effects of L-NBP were accompanied by increased phosphorylation of AKT and decreased phosphorylation of the extracellular protein kinase (ERK). The neuroprotective effects could be abolished by LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K)-dependent protein kinase B (AKT). Furthermore, ERK pathway inhibitor U0126 enhanced the beneficial effects of L-NBP in inhibiting Aβ-induced apoptosis. Conclusions: L-NBP may exert protective effects on Aβ induced cell injury in cortical neurons through regulating both PI3K/AKT and ERK signaling pathways.

Keywords: Alzheimer's disease (AD), β-amyloid (Aβ), apoptosis, PI3K/AKT pathway, ERK pathway

Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease featured by extracellular β-amyloid (Aβ) peptide deposition and intracellular neurofibrillar tangles, which is followed by loss of cortex and hippocampus neurons and cognitive dysfunction [1]. The mechanisms underlying AD pathogenesis largely remain elusive. However, increasing evidence suggests that Aβ deposition can initiate a cascade and lead progressively to inflammation, tau hyperphosphorylation, synaptic dysfunction, neuronal apoptosis, and finally dementia [2]. The hippocampi of AD patients and cultured neurons under Aβ exposure show characteristics of apoptosis [3]. However, the mechanism underlying Aβ induced neural apoptosis remains to be elucidated.

Aβ might initiate a succession of detrimental changes that ultimately lead to neuronal apoptosis through varying mechanisms, including increased reactive oxygen species production and cytochrome C release, mitochondrial dysfunction, and caspase-3 activation [4]. Aβ may induce neuronal apoptosis via inactivation of phosphoinositide 3-kinase (PI3K)-dependent protein kinase B (AKT), activation of p38 mitogen activated protein kinase (MAPK) or extracellular regulated protein kinase (ERK), nuclear factor-kappa B, and Wnt pathways [5-9]. In pAPPswe-transfected SH-SY5Y cells, neuroglobin attenuated Aβ induced mitochondrial dys-

function and neuron apoptosis by inhibiting the activation of caspase-3 and caspase-9 [10]. The beneficial effect of neuroglobin was accompanied by an increased expression of p-AKT and an inhibitor of PI3K (LY294002) could block the protective effect [10]. Previous study has shown that gastrodin protected against neurotoxicity induced by $Aβ_{1-42}$ in primary neural progenitor cells and improved hippocampal neurogenesis of C57BL/6 mice injected with $Aβ_{1-42}$ through reverse phosphorylation of MEK-1/2, ERK, and c-Jun terminal kinase (JNK). Furthermore, the MEK inhibitor U0126 and JNK inhibitor SP600125 showed a synergistic effect with gastrodin [11].

L-3-n-butylphthalide (L-NBP) was originally extracted from the seeds of *Apium graveolens* Linn (Chinese celery). Studies have shown that L-NBP protects against ischemic stroke through multiple mechanisms, such as inhibiting platelet aggregation, improving microcirculation in arterioles [12, 13], decreasing oxidative damage and neuronal apoptosis [14], improving mitochondrial function and inhibiting inflammatory response [15]. Furthermore, L-NBP can improve Aβ-induced learning and memory impairments, oxidative damage, and mitochondrial dysfunction [16, 17]. Lei *et al.* found that L-NBP attenuated Aβ-induced toxicity through modulating mitochondrial apoptosis and MAPK signaling pathway in SH-SY5Y cells [18]. However, it is not yet known whether L-NBP can prevent $Aβ_{1-42}$ induced apoptosis and synaptotoxicity through modulating the PI3K/AKT and/or ERK pathway.

The present study was designed to analyze the protective effects of L-NBP against neuronal apoptosis and synaptotoxicity induced by Aβ *in vitro*. In addition, the potential mechanisms including mitochondrial function and possible signaling pathway underlying the neuroprotective effects of L-NBP were further analyzed and discussed.

Materials and methods

Animals

Pregnant Sprague-Dawley rats on gestation day 18 were provided by the Laboratory Animal Research Center of Shandong University, China. All experimental procedures involving animals were carried out in accordance with the Guidance Suggestion for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering.

Primary cortical neuron isolation and culture

Primary cortical neurons were cultured as previously described [19]. Briefly, the cerebral cortices were separated from embryonic Sprague-Dawley rat fetuses (E18D), cut into pieces, and then digested with 0.25% trypsin for 20 min in 37°C. After adding DMEN medium (with 10% fetal bovine serum) for 10 min, tissues were dissociated mechanically with a fire-polished pipette. Then after centrifugation and re-suspension, about $1×10^5$ cells in 1 ml were plated on 6-well plates coated with poly-D-lysine. The medium was replaced by Neurobasal medium (Gibco, CA, USA) supplemented with 2% B-27 supplement (Gibco) and 0.5 mM L-glutamine 4 h later. For maintenance, the medium was changed by half every 4 days. Cells were cultured in a humidified incubator containing 5% CO_2 at 37°C for 8 days before experiments.

Cell treatment

$Aβ_{1-42}$ (Sigma-Aldrich Inc, St. Louis, MO, USA) was dissolved in distilled water and aged for 6 days at 37°C. L-NBP (purity >98%), which was synthesized by the Department of Synthetic Medicinal Chemistry, Institute of Materia Medica (Shijiazhuang, China), was dissolved in dimethyl sulfoxide (DMSO) as 200 mM stock solution. The LY294002 and U0126 were from Cell Signaling Technology, Inc. (Beverly, MA, USA) and were dissolved by DMSO. Cells were divided into control group (without treatment), Aβ group (10 μM $Aβ_{1-42}$ for 24 h), Aβ+L-NBP group (first treated with 0.1, 1.0, or 10 μM L-NBP for 4 h and then treated with 10 μM $Aβ_{1-42}$ for 24 h), Aβ+L-NBP+LY294002 group (first treated with 60 uM LY294002 for 1 h, then with 10 μM L-NBP for 4h and finally with 10 μM $Aβ_{1-42}$ for 24 h), and Aβ+L-NBP+U0126 group (first treated with 10 uM U0126 for 1 h, then with 10 μM L-NBP for 4h and finally with 10 μM $Aβ_{1-42}$ for 24 h).

MTT assay

Primary cortical neurons ($1×10^4$ cells/well) were plated on 96-well plates and treated as above described. After treatment, DMEM and 5 mg/ml of freshly prepared MTT (Sigma-Aldrich Inc, St. Louis, MO, USA) were added to culture plates at a final concentration of 10%, and incu-

bated for 4 h. DMSO (200 µl) was added to the plates and vibrated for 10 minutes at 37°C to solubilize MTT formazan crystals. Then the treatment wells were quantified spectrophotometrically at 570 nm using a LAS-4000 microplate reader (FUJIFILM, Japan). The cell viability was presented as the ratio of the experimental group to the control group.

Immunofluorescence assay

For immunofluorescence analysis, cortical neurons were plated on coverslips coated with poly-L-lysine. Then the cells were fixed with freshly prepared 4% paraformaldehyde in PBS for 30 min, permeabilized with Triton X-100 (0.1%) for 15 min, and rinsed with PBS for 3 times. After blocking with 5% goat serum for 30 min, cells were incubated with the primary antibody of rabbit anti-synaptophysin antibody (1:100, Abcam, UK) overnight at 4°C. After washing with PBS, secondary antibodies of Alexa Fluor 568 goat anti-rabbit IgG (1:500, Abcam, UK) were added and incubated for 1 h under darkness. Then, cells were incubated in 10 µg/ml DAPI for 10 min. After rinsed with PBS for 3 times, the cortical neurons were analyzed on an inverted fluorescence microscopy (Zeiss Jenalumar, Jena, Germany). Cell incubated without primary antibodies was as a negative control. The fluorescence intensity of synaptophysin was quantified using Image J 1.48u software (National Institutes of Health).

Hoechst 33342 staining

The cortical neurons were seeded on coverslips. After treatment described above, the cells were fixed with freshly prepared 4% paraformaldehyde for 30 min. Then, Hoechst 33342 (1 µM) was added to the cells and incubated for 30 min in the dark. After washed with PBS for 3 times, the cortical neurons were analyzed on an inverted fluorescence microscopy (UV excitation and emission at 360 and 450 nm, respectively). The cells with condensed nuclear chromatin and fragmentation were confirmed as apoptotic cells. The apoptotic ratio was calculated as the apoptotic cells to the total cells counted.

Flow cytometry to assess mitochondrial membrane potential (Δψm)

After treatments, 5 µM of JC-1 was added into the neurons and incubated for 15 min at 37°C under darkness. Then a flow cytometer was applied to analyze the ratio of red (590 nm)/green (529 nm) fluorescence and determine the percentage of cells with Δψm collapse.

Western blotting

Cells were harvested and incubated with lysis buffer containing phosphatase and protease inhibitors on the ice. The lysed cells were centrifuged (12,000× g) for 10 min at 4°C. The supernatants were collected and the total protein was qualified by a BCA protein assay kit (Beyotime, Nanjing, China). Then, proteins were electrophoresed on SDS-PAGE and then transferred to PVDF membrane. Membranes were blocked with blocking buffer (Thermo Scientific, Waltham, USA) for 1 h at room temperature and then incubated with primary antibodies against pro-caspase-3, cleaved caspase-3 (c-caspase-3), synaptophysin, phospho-Tau (Ser 199/202), phospho-Akt (Ser 473), phospho-Erk1/2 (Thr202/thy204), Akt, Erk1/2, total Tau and β-actin (Abcam, Cambridge, UK) overnight at 4°C, respectively. After rinsed with PBS, membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature. Finally, the antibody-bound proteins were detected by an ECL Western blot detection kit (Santa Cruz, CA, USA). The densities of protein bands were measured by Image J software.

Statistical analysis

All the results are expressed as mean ± SD and statistical analysis was performed using SPSS statistical software (version 17.0). The differences were analyzed by one-way variance analysis combined with Bonferroni-Dunn's test or Student's *t* test. A *P* value less than 0.05 was considered as statistically significant.

Results

L-NBP can attenuate the cytotoxicity, apoptosis, mitochondrial dysfunction, and synaptotoxicity of primary cultured neurons under Aβ exposure

Cell viability was measured by the MTT assay. Pilot experiment showed that the survival rate was approximately 50% under exposure to 10 µM Aβ$_{1-42}$ for 24 h (data not shown). Thus, the concentration of Aβ$_{1-42}$ used in this study was determined at 10 µM. We found that 10 µM of Aβ$_{1-42}$ markedly decreased cell viability (**Figure 1A**), suggesting that Aβ$_{1-42}$ induced cytotoxicity
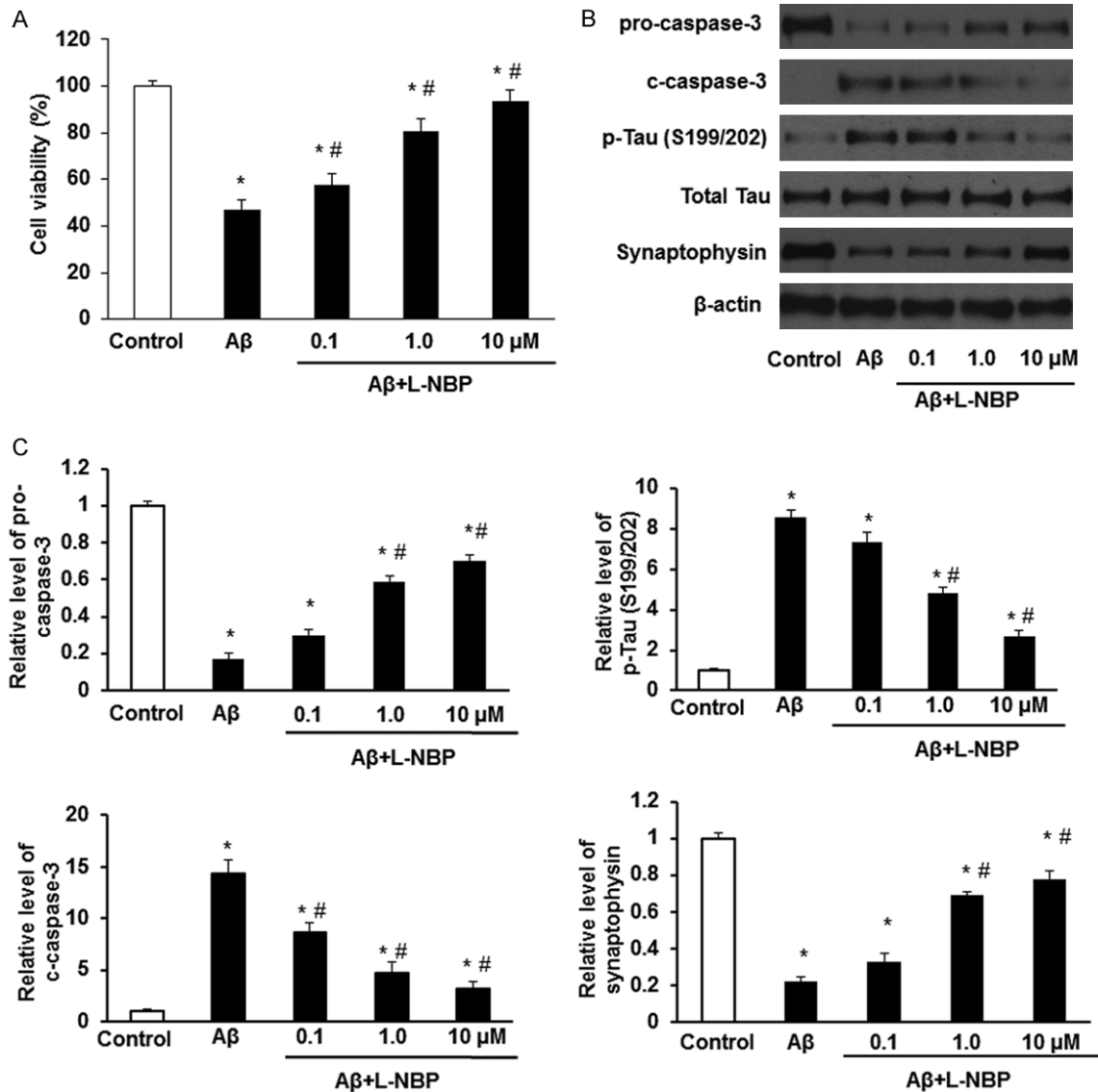
Figure 1. L-NBP attenuated cytotoxicity and apoptosis induced by Aβ. A. Viability of cortical neurons. B. Western blots analysis showed the expression of pro-caspase-3, c-caspase-3, p-Tau and synaptophysin. β-actin was shown as an internal control. C. The intensities of pro-caspase-3, c-caspase-3, p-Tau and synaptophysin bands were quantified by densitometry. The data were shown as mean ± SD from the three independent experiments. Control, cells without treatment. Aβ$_{1-42}$, cells treated with 0, 0.1, 1.0, or 10 μM L-NBP and Aβ$_{1-42}$, respectively. *p<0.01 for Aβ vs control, #p<0.05 for Aβ+L-NBP vs Aβ.

in cortical neurons. Next, the expression of pro-caspase-3, c-caspase-3, p-Tau and synaptophysin was detected by Western Blot. Results showed that 10 μM of Aβ$_{1-42}$ markedly increased the expression of c-caspase-3 and p-Tau, and decreased the expression of pro-caspase-3, synaptophysin in cortical neurons (**Figure 1B** and **1C**). Immunofluorescence assay also detected the Aβ$_{1-42}$ induced decreased synaptophysin expression (**Figure 2A**). The Hoechst 33342 staining showed Aβ$_{1-42}$ increased the ratio of apoptotic cells significantly (**Figure 2B**).

This indicates the synaptotoxicity and pro-apoptosis ability of Aβ$_{1-42}$. Flow cytometry analysis showed that cells with Δψm collapse were increased (**Figure 2C**), demonstrating that Aβ$_{1-42}$ induced mitochondrial dysfunction in cortical neurons. However, when the neurons were pretreated with 0.1, 1, and 10 μM L-NBP, the cytotoxicity, apoptosis, mitochondrial dysfunction, and synaptotoxicity induced by Aβ$_{1-42}$ in cultured neurons were all attenuated by L-NBP in a dose-dependent manner (**Figures 1** and **2**). There were significant differences between Aβ
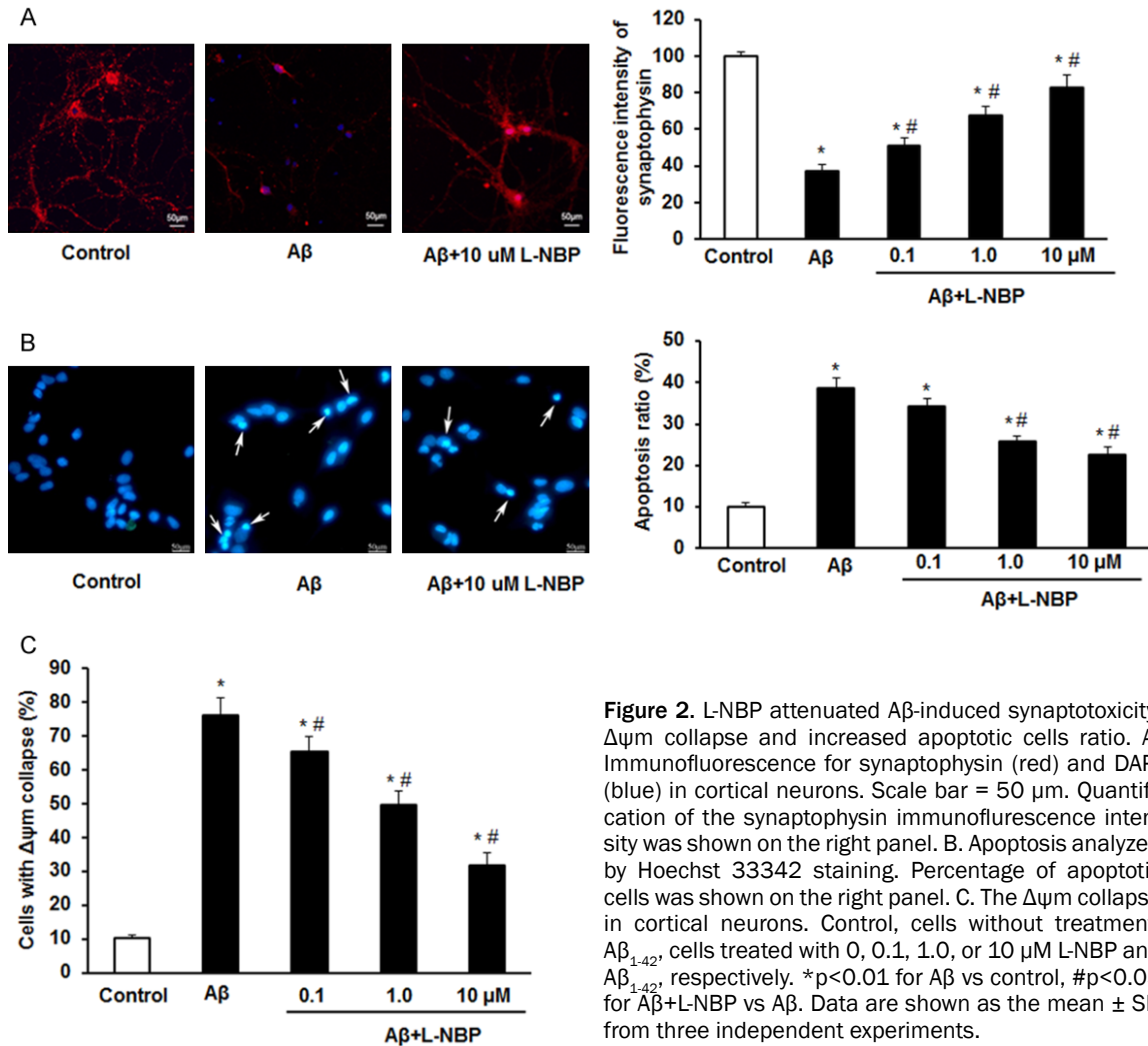
Figure 2. L-NBP attenuated Aβ-induced synaptotoxicity, Δψm collapse and increased apoptotic cells ratio. A. Immunofluorescence for synaptophysin (red) and DAPI (blue) in cortical neurons. Scale bar = 50 μm. Quantification of the synaptophysin immunoflurescence intensity was shown on the right panel. B. Apoptosis analyzed by Hoechst 33342 staining. Percentage of apoptotic cells was shown on the right panel. C. The Δψm collapse in cortical neurons. Control, cells without treatment. Aβ$_{1-42}$, cells treated with 0, 0.1, 1.0, or 10 μM L-NBP and Aβ$_{1-42}$, respectively. *$p<0.01$ for Aβ vs control, #$p<0.05$ for Aβ+L-NBP vs Aβ. Data are shown as the mean ± SD from three independent experiments.

group and Aβ+L-NBP group ($P<0.05$). Significant differences were also found between control group and Aβ group, and, between control group and Aβ+L-NBP group ($P<0.05$). The basal levels of cortical cell viability, pro-caspase-3, c-caspase-3, p-Tau, Δψm collapse and synaptophysin were affected by L-NBP insignificantly ($p>0.05$; data are not shown here). These results demonstrate that L-NBP can protect cortical neurons against injuries induced by Aβ$_{1-42}$.

The protective effects of L-NBP are accompanied by increased phosphorylation of AKT and decreased phosphorylation of ERK

To further explore the neuroprotective mechanism of L-NBP, the phosphorylation of AKT and ERK was detected by Western blot. We found that Aβ$_{1-42}$ inhibited the level of AKT phosphorylation whereas increased the level of ERK phosphorylation. However, L-NBP treatment rever-

sed the effect of Aβ$_{1-42}$ on AKT and ERK phosphorylation in a dose-dependent way (**Figure 3A-C**). There were significant differences among control group, Aβ group and Aβ+L-NBP group ($P<0.05$). Additionally, the basal levels of AKT or ERK were insignificantly affected by L-NBP ($p>0.05$; data are not shown here). These results indicate that L-NBP pretreatment could reverse the decreased phosphorylation of AKT and increased phosphorylation of ERK induced by Aβ$_{1-42}$.

LY294002 can block the protective effects of L-NBP

To further investigate the change of PI3K/AKT signaling pathways underlying the neuroprotective of L-NBP. We found that LY294002, an inhibitor of the PI3K [20], blocked the beneficial role of L-NBP in reducing the cytotoxicity, apoptosis, synaptotoxicity, and mitochondrial dys-
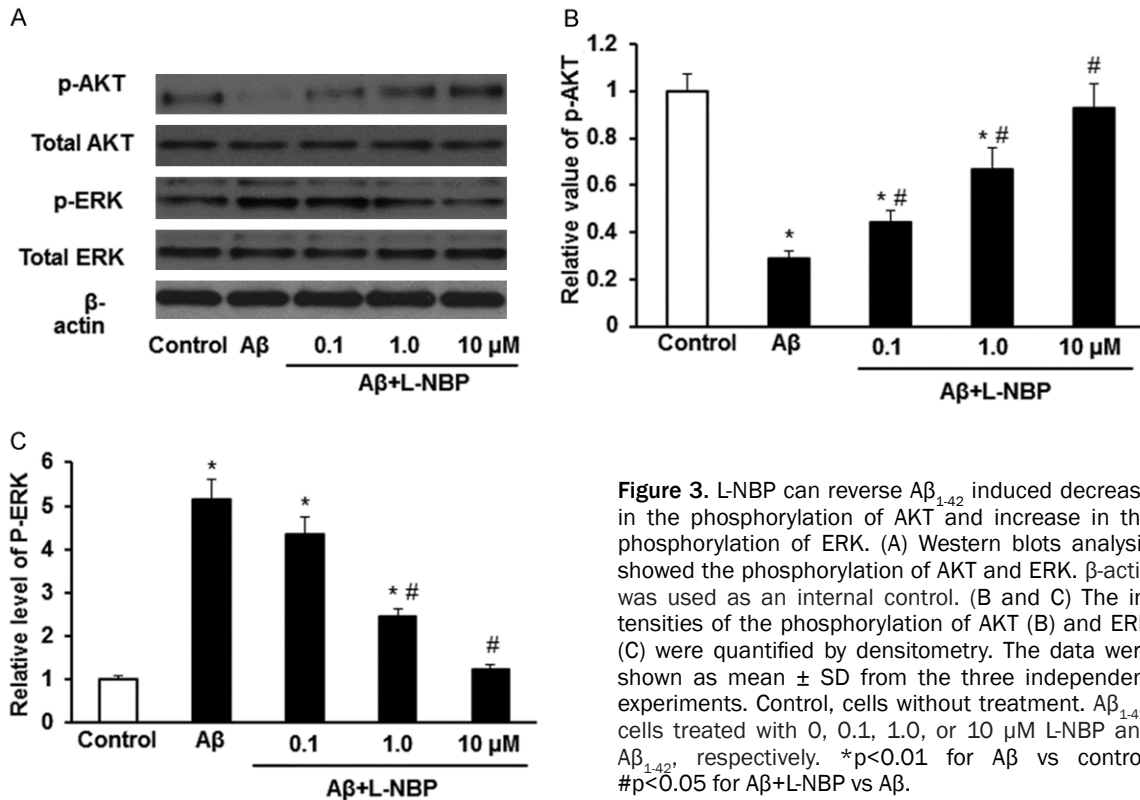
Figure 3. L-NBP can reverse $Aβ_{1-42}$ induced decrease in the phosphorylation of AKT and increase in the phosphorylation of ERK. (A) Western blots analysis showed the phosphorylation of AKT and ERK. β-actin was used as an internal control. (B and C) The intensities of the phosphorylation of AKT (B) and ERK (C) were quantified by densitometry. The data were shown as mean ± SD from the three independent experiments. Control, cells without treatment. $Aβ_{1-42}$, cells treated with 0, 0.1, 1.0, or 10 μM L-NBP and $Aβ_{1-42}$, respectively. *p<0.01 for Aβ vs control, #p<0.05 for Aβ+L-NBP vs Aβ.

function induced by Aβ (**Figure 4A-F**). Pretreatment with L-NBP could significantly attenuate 10 μM $Aβ_{1-42}$ induced increased expression of c-caspase-3 and p-Tau, and decreased the expression of synaptophysin and phosphorylation of AKT in cortical neurons. However, Aβ+L-NBP+LY294002 group showed increased expression of c-caspase-3 and p-Tau and decreased phosphorylation of AKT than Aβ+L-NBP group. And the Aβ+L-NBP+LY294002 group showed increased Δψm collapse in contrast with Aβ+L-NBP group. There were significant differences among control group, Aβ group, Aβ+L-NBP group and Aβ+L-NBP+LY294002 group (P<0.05). Additionally, Aβ-induced decreased phosphorylation of AKT in primary cultured neurons could be reversed by LY294002. These results show that L-NBP may protect against Aβ induced cytotoxicity, apoptosis, synaptotoxicity, and mitochondrial dysfunction by increasing the phosphorylation of AKT.

U0126 showed a significant synergistic effect with L-NBP against $Aβ_{1-42}$-induced apoptosis

To determine the change of ERK signaling pathway underlying the neuroprotective of L-NBP, Western blot was performed. The ERK pathway inhibitor U0126 showed a significant synergis-

tic effect with L-NBP against $Aβ_{1-42}$-induced increase expression of p-ERK and c-caspase-3 (**Figure 5A-C**). Pretreat with L-NBP could significantly decrease the increased expression of p-ERK and c-caspase-3 induced by Aβ, and the Aβ+L-NBP+U0126 group showed lower expression of p-ERK and c-caspase-3 than Aβ+L-NBP group. There were significant differences among control group, Aβ group, Aβ+L-NBP group and Aβ+L-NBP+U0126 group (P<0.05). These results show that L-NBP may protect against Aβ induced apoptosis by decreasing the phosphorylation of ERK in cortical neurons.

Discussion

In this study, we used primary cultured cortical neurons under $Aβ_{1-42}$ exposure as an AD cell model *in vitro* and the protective effects of L-NBP were assessed from different aspects. First, our results found that the cytotoxicity, apoptosis, mitochondrial dysfunction, and synaptotoxicity induced by $Aβ_{1-42}$ in cultured neurons were all attenuated by L-NBP in a dose-dependent manner. Besides, the protective effects of L-NBP were concomitant with increased phosphorylation of AKT and decreased phosphorylation of ERK. Furthermore, we found that the inhibitor of the PI3K, LY294002, abol-
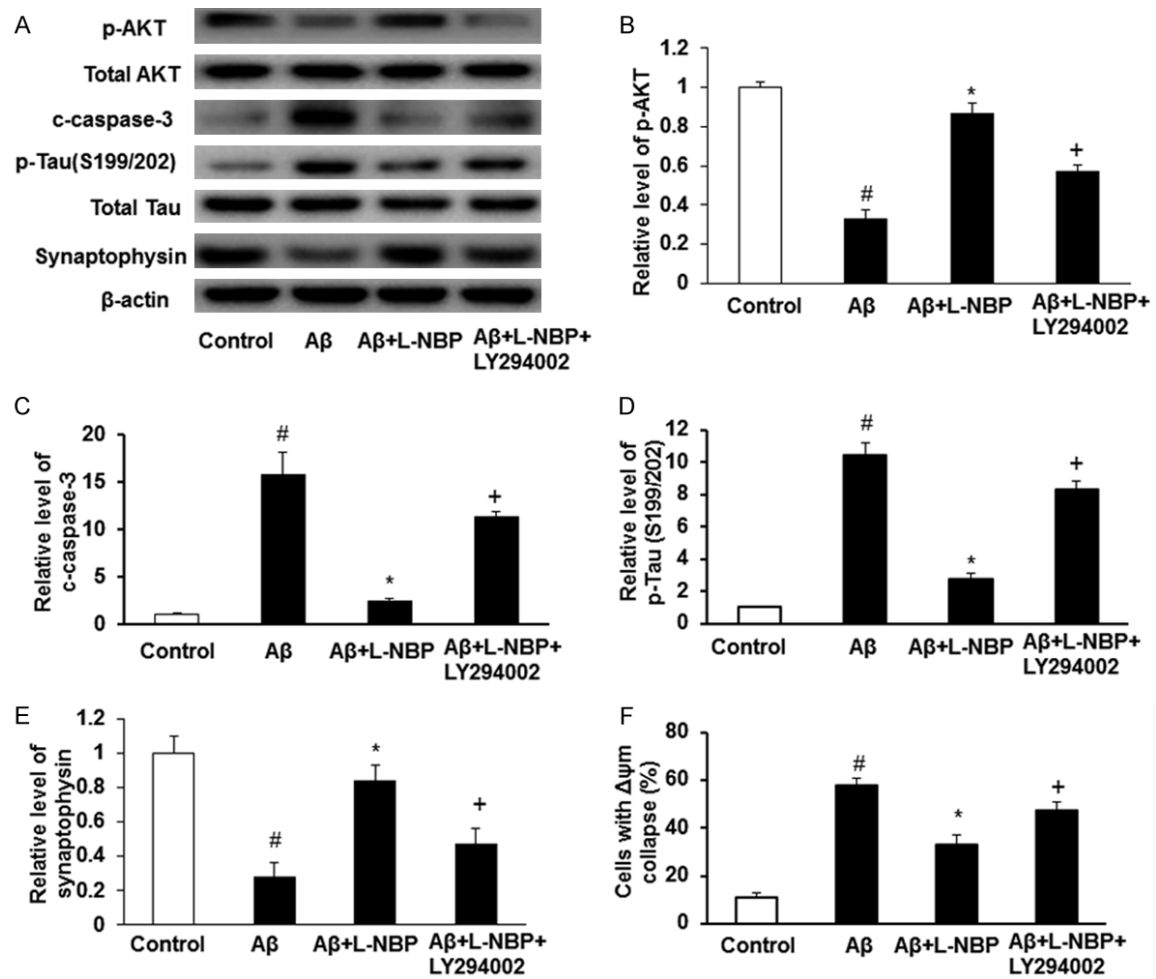
Figure 4. LY294002 can block the beneficial effects of L-NBP. A. Western blots analysis showed the expression of c-caspase-3, p-Tau, p-AKT, and synaptophysin. B-E. The intensities of the indicated protein bands were quantified by densitometry. F. The Δψm collapse in cortical neurons. The data were shown as means ± SD from the three independent experiments. Control, cells without treatment. Cells were treated with Aβ only, Aβ with 10 uM L-NBP, and Aβ with 10 uM L-NBP and LY294002, respectively. #$p<0.05$ for Aβ vs control, *$p<0.05$ for Aβ+L-NBP vs Aβ, +$p<0.05$ for Aβ+L-NBP+LY294002 vs Aβ+L-NBP.

ished the beneficial role of L-NBP in reducing the cytotoxicity, apoptosis, synaptotoxicity, and mitochondrial dysfunction induced by Aβ. And, the ERK inhibitor, U0126, which can inhibit the ERK pathway, enhanced the beneficial effects of L-NBP in reducing Aβ-induced apoptosis. As a result, we conclude that L-NBP may protect against the neuronal apoptosis, mitochondrial dysfunction and synaptotoxicity induced by Aβ through modulating PI3K/AKT and ERK signaling pathways.

Accumulated studies demonstrate that both extracellular and intracellular $Aβ_{1-42}$ can activate caspases, which lead to synaptic and neuronal degeneration [21]. The activation of caspases by $Aβ_{1-42}$ is mediated by both extrinsic

and intrinsic pathways [22]. Extracellular $Aβ_{1-42}$ can bind to cell receptors [23], while intracellular $Aβ_{1-42}$ can trigger mitochondrial stress by binding to a mitochondrial alcohol dehydrogenase [24]. Previous studies [5-9] have shown that PI3K/AKT, MAPK, nuclear factor-kappa B and Wnt signaling pathways are involved in $Aβ_{1-42}$ induced neuronal apoptosis. The PI3K/AKT/GSK-3β signaling pathway is critical in the pathogeneses of AD because it can boost tau hyperphosphorylation [25], which is the main component of neurofibrillary tangles. Specifically, decreased activation of PI3/AKT causes increased GSK-3β activity and lead to tau hyperphosphorylation [26, 27]. Our current study demonstrated that the decreased expression of p-AKT, increased expression of p-Tau and
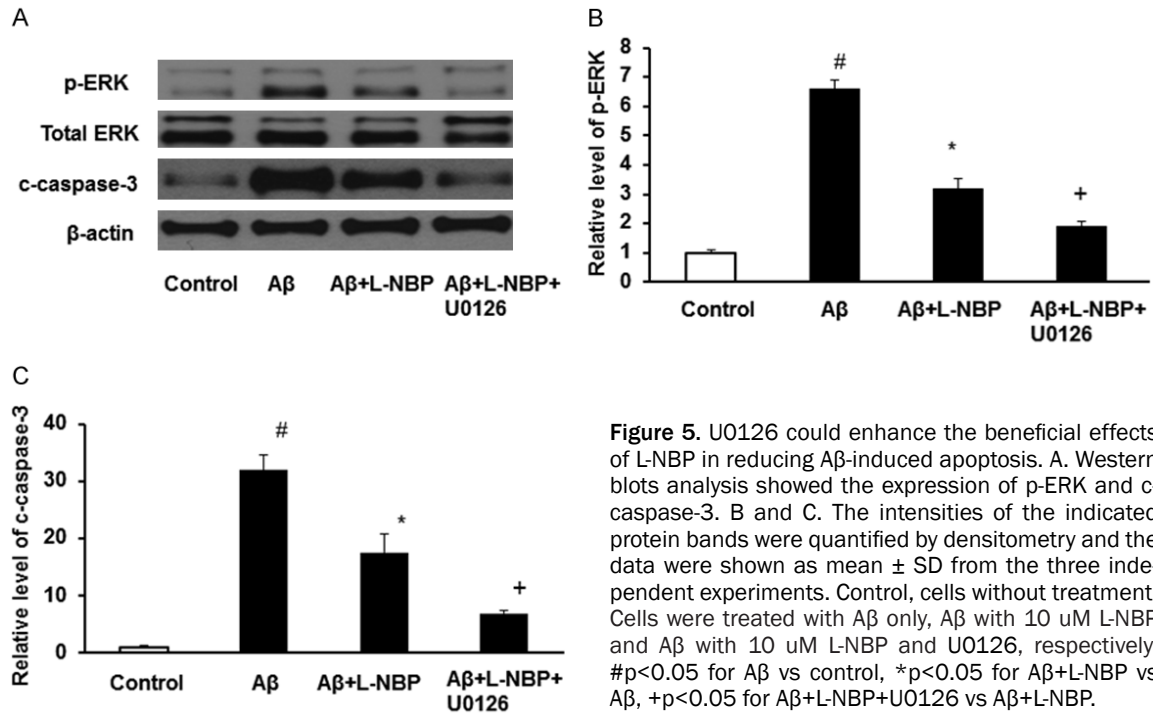
Figure 5. U0126 could enhance the beneficial effects of L-NBP in reducing Aβ-induced apoptosis. A. Western blots analysis showed the expression of p-ERK and c-caspase-3. B and C. The intensities of the indicated protein bands were quantified by densitometry and the data were shown as mean ± SD from the three independent experiments. Control, cells without treatment. Cells were treated with Aβ only, Aβ with 10 uM L-NBP and Aβ with 10 uM L-NBP and U0126, respectively. #$p<0.05$ for Aβ vs control, *$p<0.05$ for Aβ+L-NBP vs Aβ, +$p<0.05$ for Aβ+L-NBP+U0126 vs Aβ+L-NBP.

c-caspase-3 were associated with $Aβ_{1-42}$ induced neurotoxicity in cortical neurons. The L-NBP pre-incubation can significantly increase the p-AKT level and decrease c-caspase-3 and p-Tau expression. And, these beneficial effects were blocked by LY294002. Our results are partly consistent with previous researches [28, 29]. For instance, curcumin can improve synaptic plasticity and cognitive function in rats via the PI3K/AKT signaling pathway [28]. Furthermore, melatonin protects against Aβ-induced neurotoxicity through decreasing memory deterioration, synaptic disorder, tau hyperphosphorylation, and neurodegeneration through PI3K/AKT/GSK3β signaling in the $Aβ_{1-42}$-treated mouse model of AD [29]. These results suggest that PI3K/AKT/GSK-3β pathway may be used as an effective therapeutic target in treating AD.

MAPK pathways, the critical mediators that propagate extracellular signals from the membrane to the nucleus, include ERK, JNK/stress-activated protein kinase, and p38. It is generally accepted that ERK activity is typically involved in cell survival, proliferation, differentiation, and memory formation in nervous system [30]. Accumulating evidence [31, 32] have shown that the phosphorylation of ERK is actively involved in AD pathology. Nevertheless, views about the function of ERK under $Aβ_{1-42}$ expo-

sure are not conclusive. Some studies demonstrate that decreased ERK activity induced by $Aβ_{1-42}$ can protect against cytotoxicity and apoptosis [33, 34]. However, others studies indicate that Aβ-induced ERK activation leads to neural apoptosis [35, 36]. The current study showed that $Aβ_{1-42}$-induced increased expression of both p-ERK and c-caspase-3 in cortical neurons and that these increases were attenuated by L-NBP significantly. Meanwhile, the ERK pathway inhibitor U0126 enhanced beneficial role of L-NBP in inhibiting $Aβ_{1-42}$-induced apoptosis. These results suggest that L-NBP exhibit protective effects through inhibition of ERK phosphorylation in $Aβ_{1-42}$-induced neuronal apoptosis. Our results are partly consistent with previous investigations, which reported that in isolated hippocampal cell culture, increased phosphorylated ERK by Aβ exposure was in parallel with that in cell death and apoptosis and insulin could inhibit ERK phosphorylation induced by $Aβ_{1-42}$ [37].

As the source of energy, mitochondria play a crucial role in regulating cell apoptosis and redox balance, and the "mitochondrial cascade hypothesis" has been proposed to be involved in the pathogenesis of AD [38]. Increasing evidence has reported that $Aβ_{1-42}$ exposure impairs mitochondrial permeability and that mitochon-

drial permeability transition pore openings lead to Δψm collapse and pro-apoptotic factors release from mitochondrial to cytosol [39, 40]. Accumulating evidence suggests that mitochondrial perturbation plays a critical role in synaptic failure and degeneration in AD [41, 42]. The present study further showed that L-NBP significantly attenuated $Aβ_{1-42}$-induced Δψm collapse and synaptotoxicity, and that LY294002 blocked this effect of L-NBP. The present results are consistent to the study of Zhang Yu *et al.* [43], which has found that L-NBP can reconstruct synaptic and spine function in aged APP/PS1 AD transgenic mice by suppressing $Aβ_{1-42}$ plaques deposition and neuroinflammatory response and that Wnt/β-catenin signaling pathway may be involved [43].

In conclusion, our findings indicate that L-NBP has protective effects on $Aβ_{1-42}$ induced cell injury possibly by regulating both PI3K/AKT and ERK signaling pathways. The PI3K/AKT and ERK pathways may become potential therapeutic targets in AD treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Yifeng Du, Department of Neurology, Shandong Provincial Hospital Affiliated to Shandong University, No. 324 Jingwu Road, Jinan 250021, P.R. China. Tel: +86-0531-68776451; Fax: +86-0531-68776451; E-mail: duyifengzr@163.com; Wei Dong, Department of Thoracic Surgery, Shandong Provincial Hospital Affiliated to Shandong University, No. 324 Jingwu Road, Jinan 250021, P.R. China. Tel: +86-0531-68776387; Fax: +86-0531-68777100; E-mail: dongwei318@163.com

References

[1] Cordonnier C, van der Flier WM. Brain microbleeds and Alzheimer's disease: innocent observation or key player? Brain 2011; 134: 335-44.

[2] De Strooper B, Karran E. The cellular phase of Alzheimer's disease. Cell 2016; 164: 603-15.

[3] Majd S, Zarifkar A, Rastegar K and Takhshid MA. Different fibrillar Abeta 1-42 concentrations induce adult hippocampal neurons to re-enter various phases of the cell cycle. Brain Res 2008; 1218: 224-229.

[4] Rohn TT. The role of caspases in Alzheimer's disease; potential novel therapeutic opportunities. Apoptosis 2010; 15: 1403-1409.

[5] Li J, Ding X, Zhang R, Jiang W, Sun X, Xia Z, Wang X, Wu E, Zhang Y and Hu Y. Harpagoside ameliorates the amyloid-beta-induced cognitive impairment in rats via up-regulating BDNF expression and MAPK/PI3K pathways. Neuroscience 2015; 303: 103-114.

[6] Morishima Y, Gotoh Y, Zieg J, Barrett T, Takano H, Flavell R, Davis RJ, Shirasaki Y and Greenberg ME. Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand. J Neurosci 2001; 21: 7551-7560.

[7] Modi PK, Komaravelli N, Singh N and Sharma P. Interplay between MEK-ERK signaling, cyclin D1, and cyclin-dependent kinase 5 regulates cell cycle reentry and apoptosis of neurons. Mol Biol Cell 2012; 23: 3722-3730.

[8] Srinivasan M, Bayon B, Chopra N and Lahiri DK. Novel nuclear factor-kappaB targeting peptide suppresses beta-amyloid induced inflammatory and apoptotic responses in neuronal cells. PLoS One 2016; 11: e0160314.

[9] Zheng Y, Wang J, Li D, Guo M, Zhen M and Chang Q. Wnt/ss-catenin signaling pathway against Abeta toxicity in PC12 cells. Neurosignals 2016; 24: 40-47.

[10] Ashraghi MR, Pagano G, Polychronis S, Niccolini F and Politis M. Parkinson's disease, diabetes and cognitive impairment. Recent Pat Endocr Metab Immune Drug Discov 2016; 10: 11-21.

[11] Li M and Qian S. Gastrodin protects neural progenitor cells against amyloid beta (1-42)-induced neurotoxicity and improves hippocampal neurogenesis in amyloid beta (1-42)-injected mice. J Mol Neurosci 2016; 60: 21-32.

[12] Peng Y, Zeng X, Feng Y and Wang X. Antiplatelet and antithrombotic activity of L-3-n-butylphthalide in rats. J Cardiovasc Pharmacol 2004; 43: 876-881.

[13] Ye J, Zhai L, Zhang S, Zhang Y, Chen L, Hu L and Ding Z. DL-3-n-butylphthalide inhibits platelet activation via inhibition of cPLA2-mediated TXA2 synthesis and phosphodiesterase. Platelets 2015; 26: 736-744.

[14] Chang Q and Wang XL. Effects of chiral 3-n-butylphthalide on apoptosis induced by transient focal cerebral ischemia in rats. Acta Pharmacol Sin 2003; 24: 796-804.

[15] Xu HL and Feng YP. Inhibitory effects of chiral 3-n-butylphthalide on inflammation following focal ischemic brain injury in rats. Acta Pharmacol Sin 2000; 21: 433-438.

[16] Peng Y, Sun J, Hon S, Nylander AN, Xia W, Feng Y, Wang X and Lemere CA. L-3-n-butylphthalide improves cognitive impairment and reduces amyloid-beta in a transgenic model of Alzheimer's disease. J Neurosci 2010; 30: 8180-8189.

[17] Peng Y, Hu Y, Xu S, Li P, Li J, Lu L, Yang H, Feng N, Wang L and Wang X. L-3-n-butylphthalide reduces tau phosphorylation and improves cognitive deficits in Abeta PP/PS1-Alzheimer's transgenic mice. J Alzheimers Dis 2012; 29: 379-391.

[18] Lei H, Zhao CY, Liu DM, Zhang Y, Li L, Wang XL and Peng Y. l-3-n-Butylphthalide attenuates beta-amyloid-induced toxicity in neuroblastoma SH-SY5Y cells through regulating mitochondrion-mediated apoptosis and MAPK signaling. J Asian Nat Prod Res 2014; 16: 854-864.

[19] Troy CM, Rabacchi SA, Friedman WJ, Frappier TF, Brown K and Shelanski ML. Caspase-2 mediates neuronal cell death induced by beta-amyloid. J Neurosci 2000; 20: 1386-1392.

[20] Lesne S, Gabriel C, Nelson DA, White E, Mackenzie ET, Vivien D and Buisson A. Akt-dependent expression of NAIP-1 protects neurons against amyloid-{beta} toxicity. J Biol Chem 2005; 280: 24941-24947.

[21] Gotz J, Eckert A, Matamales M, Ittner LM and Liu X. Modes of Abeta toxicity in Alzheimer's disease. Cell Mol Life Sci 2011; 68: 3359-3375.

[22] Dickson DW. Apoptotic mechanisms in Alzheimer neurofibrillary degeneration: cause or effect? J Clin Invest 2004; 114: 23-27.

[23] Salminen A, Ojala J, Suuronen T, Kaarniranta K and Kauppinen A. Amyloid-beta oligomers set fire to inflammasomes and induce Alzheimer's pathology. J Cell Mol Med 2008; 12: 2255-2262.

[24] Lustbader JW, Cirilli M, Lin C, Xu HW, Takuma K, Wang N, Caspersen C, Chen X, Pollak S, Chaney M, Trinchese F, Liu S, Gunn-Moore F, Lue LF, Walker DG, Kuppusamy P, Zewier ZL, Arancio O, Stern D, Yan SS and Wu H. ABAD directly links Abeta to mitochondrial toxicity in Alzheimer's disease. Science 2004; 304: 448-452.

[25] Kitagishi Y, Nakanishi A, Ogura Y and Matsuda S. Dietary regulation of PI3K/AKT/GSK-3beta pathway in Alzheimer's disease. Alzheimers Res Ther 2014; 6: 35.

[26] Manning BD and Cantley LC. AKT/PKB signaling: navigating downstream. Cell 2007; 129: 1261-1274.

[27] Lee KY, Koh SH, Noh MY, Kim SH and Lee YJ. Phosphatidylinositol-3-kinase activation blocks amyloid beta-induced neurotoxicity. Toxicology 2008; 243: 43-50.

[28] Wang R, Li YH, Xu Y, Li YB, Wu HL, Guo H, Zhang JZ, Zhang JJ, Pan XY and Li XJ. Curcumin produces neuroprotective effects via activating brain-derived neurotrophic factor/TrkB-dependent MAPK and PI-3K cascades in rodent cortical neurons. Prog Neuropsychopharmacol Biol Psychiatry 2010; 34: 147-153.

[29] Ali T and Kim MO. Melatonin ameliorates amyloid beta-induced memory deficits, tau hyperphosphorylation and neurodegeneration via PI3/Akt/GSk3beta pathway in the mouse hippocampus. J Pineal Res 2015; 59: 47-59.

[30] Roskoski R Jr. ERK1/2 MAP kinases: structure, function, and regulation. Pharmacol Res 2012; 66: 105-143.

[31] Kirouac L, Rajic AJ, Cribbs DH and Padmanabhan J. Activation of Ras-ERK signaling and GSK-3 by amyloid precursor protein and amyloid beta facilitates neurodegeneration in Alzheimer's disease. eNeuro 2017; 4.

[32] Deng LJ, Cheng C, Wu J, Wang CH, Zhou HB and Huang J. Oxabicycloheptene sulfonate protects against beta-amyloid-induced toxicity by activation of PI3K/Akt and ERK signaling pathways via GPER1 in C6 cells. Neurochem Res 2017; 42: 2246-2256.

[33] Fan CD, Li Y, Fu XT, Wu QJ, Hou YJ, Yang MF, Sun JY, Fu XY, Zheng ZC and Sun BL. Reversal of beta-amyloid-induced neurotoxicity in PC12 cells by curcumin, the important role of ROS-mediated signaling and ERK pathway. Cell Mol Neurobiol 2017; 37: 211-222.

[34] Savage MJ, Lin YG, Ciallella JR, Flood DG and Scott RW. Activation of c-Jun N-terminal kinase and p38 in an Alzheimer's disease model is associated with amyloid deposition. J Neurosci 2002; 22: 3376-3385.

[35] Frasca G, Carbonaro V, Merlo S, Copani A and Sortino MA. Integrins mediate beta-amyloid-induced cell-cycle activation and neuronal death. J Neurosci Res 2008; 86: 350-355.

[36] Yang H, Wang S, Yu L, Zhu X and Xu Y. Esculentoside A suppresses Abeta(1-42)-induced neuroinflammation by down-regulating MAPKs pathways in vivo. Neurol Res 2015; 37: 859-866.

[37] Ghasemi R, Moosavi M, Zarifkar A, Rastegar K and Maghsoudi N. The interplay of Akt and ERK in Abeta toxicity and insulin-mediated protection in primary hippocampal cell culture. J Mol Neurosci 2015; 57: 325-334.

[38] Swerdlow RH, Burns JM and Khan SM. The Alzheimer's disease mitochondrial cascade hypothesis: progress and perspectives. Biochim Biophys Acta 2014; 1842: 1219-1231.

[39] Santos RX, Correia SC, Wang X, Perry G, Smith MA, Moreira PI and Zhu X. Alzheimer's disease: diverse aspects of mitochondrial malfunctioning. Int J Clin Exp Pathol 2010; 3: 570-581.

[40] Lu Y, Wang R, Dong Y, Tucker D, Zhao N, Ahmed ME, Zhu L, Liu TC, Cohen RM and Zhang Q. Low-level laser therapy for beta amyloid toxicity in rat hippocampus. Neurobiol Aging 2017; 49: 165-182.

[41] Cai Q and Tammineni P. Mitochondrial aspects of synaptic dysfunction in Alzheimer's disease. J Alzheimers Dis 2017; 57: 1087-1103.

[42] Du H, Guo L, Fang F, Chen D, Sosunov AA, McKhann GM, Yan Y, Wang C, Zhang H, Molkentin JD, Gunn-Moore FJ, Vonsattel JP, Arancio O, Chen JX and Yan SD. Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer's disease. Nat Med 2008; 14: 1097-1105.

[43] Zhang Y, Huang LJ, Shi S, Xu SF, Wang XL and Peng Y. L-3-n-butylphthalide rescues hippocampal synaptic failure and attenuates neuropathology in aged APP/PS1 mouse model of Alzheimer's disease. CNS Neurosci Ther 2016; 22: 979-987.