Effects of diazoxide on $A\beta_{1-42}$-induced expression of the NR2B subunit in cultured cholinergic neurons

JIN ZHU[1*], QINGXI FU[2*], CHUNFENG XIA[1] and GUOZHAO MA[1]

[1]Department of Neurology, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan, Shangdong 250014; [2]Department of Neurology, Linyi People's Hospital, Linyi, Shandong 276003, P.R. China

Received January 18, 2015; Accepted September 10, 2015

DOI: 10.3892/mmr.2015.4457

Abstract. The accumulation of amyloid-β protein (Aβ) is significant in the pathogenesis of Alzheimer's disease. Several previous studies indicate that the NR2B-containing N-methyl-D-aspartate receptors are critically involved in the Aβ mediated disruption of neuronal function. Diazoxide (DZ), a highly selective drug capable of opening mitochondrial ATP-sensitive potassium channels, has neuroprotective effects against neuronal cell death. However, the mechanism by which DZ protects cholinergic neurons against Aβ-induced cytotoxicity remains to be elucidated. The present study was designed to investigate the effects of DZ pretreatment against $A\beta_{1-42}$-induced expression of NR2B in order to gain novel insights into the neuroprotective mechanisms. Following exposure to $A\beta_{1-42}$ for 24 h, the expression of the NR2B subunit remained unchanged compared with the control group. However, a significant increase in the expression of the NR2B subunit was observed following treatment with $A\beta_{1-42}$ for 72 h ($P<0.05$); and the upregulation of the expression of the NR2B subunit was reversed by pretreatment with DZ ($P<0.05$). These results suggested that DZ may counteract $A\beta_{1-42}$-mediated cytotoxicity by alleviating the expression of NR2B.

Introduction

Alzheimer's disease (AD), the most common neurodegenerative disorder, is characterized by the accumulation of the amyloid-β (Aβ) peptide, a hyper-phosphorylation of tau protein and loss of neurons (1). Aβ has a complex role in the molecular events, which lead to the progressive loss of function and eventually to neurodegeneration in this devastating disease (2). Previous studies have confirmed that the activity of N-methyl-D-aspartic acid receptors (NMDARs) may be involved in the cytotoxic mechanism of Aβ (3). NMDARs are widely involved in neuronal excitatory synaptic transmission, synaptic plasticity and long range enhancement. Previous studies indicate that activation of NMDARs by Aβ accumulation may occur at the early stages of the disease and that Aβ evokes an immediate Ca^{2+} rise via the activation of NR2B-containing NMDARs in cultured cortical neurons (4,5). Ronicke *et al* (6) demonstrated that early neuronal dysfunction induced by Aβ is mediated by an activation of NR2B subunits in primary neuronal cultures and hippocampal slices from rat and mouse. The NR2B subunit is important for the pharmacological features of the NMDAR, which is a potential target for the treatment of NMDAR-associated diseases (7).

Mitochondrial ATP sensitive potassium (mitoKATP) channels are involved in the regulation of mitochondrial volume, ionic homeostasis, pH gradient and membrane potential (8). Pharmacological activation of mitoKATP channels using drugs, including diazoxide (DZ), has been demonstrated to protect neurons against ischemic damage and death (9), and our previous study reported that DZ protects cultured primary cholinergic neurons against $A\beta_{1-42}$-induced cytotoxicity (1,10). Although it has been previously reported that DZ reduces neuronal dysfunction caused by Aβ, the underlying mechanisms remain to be elucidated. Since NR2B subunits have been implicated in increasing neuronal vulnerability, the present study investigated the effect of activation of mitoKATP with DZ on the expression of NR2B in cultured primary rat basal forebrain cholinergic neurons following exposure to $A\beta_{1-42}$.

Materials and methods

Ethics statement. The present study was performed in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Shandong Provincial Qianfoshan Hospital. Female wistar rats (age, 24 h; weight, 300-350 g) were housed under controlled temperature (21˚C) and light conditions (12 h light/dark cycle) with free access to water and standard diet.

Correspondence to: Professor Guozhao Ma, Department of Neurology, Shandong Provincial Qianfoshan Hospital, Shandong University, 16766 Jingshi Road, Jinan, Shandong 250014, P.R. China
E-mail: maguozhao@aliyun.com

*Contributed equally

Key words: diazoxide, $A\beta_{1-42}$, N-methyl-D-aspartate receptor, NR2B subunit

Reagents. $A\beta_{1-42}$, DZ and polyl-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO), USA; fetal bovine serum, B27 supplement and Neurabasal were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA); Dulbecco's modified Eagle's medium (DMEM) was obtained from HyClone Laboratories, Inc. (Logan, UT, USA); dimethyl sulfoxide (DMSO) was purchased from Shengbo Biotech Co., Ltd. (Shanghai, China). The following antibodies were used: Rabbit anti-NMDAR 2B (cat no. ab65783; 1:100; Abcam, Cambridge, UK), goat anti-choactase (D-16; cat no. sc-19056; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-beta actin antibody (cat no. ab8226; 1:1,000; Abcam). All other chemicals and reagents, unless otherwise specified, were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). $A\beta_{1-42}$ (1 mg) was initially dissolved in DMSO at a concentration of 0.5 mol/l, subsequently diluted with 500 μl phosphate buffered saline (PBS; 0.2 μM; pH 7.4), incubated at 37˚C for 36 h and stored at 4˚C. Stock solutions were diluted in serum-free medium prior to use in the experiments. The final concentration of DMSO in cell cultures was <0.1%.

Cell cultures. Embryos were removed from pregnant Wistar rats at 17-19 days of gestation. The basal forebrain was dissected and digested with 0.125% trypsinogen for 20 min at 37˚C. Proliferation growth medium, comprising a 1:9 mixture of fetal bovine serum and DMEM, and 2% B27 supplement was added, and the cells were subsequently dissociated mechanically using a fire-polished pipette. The dispersed cells were seeded into a culture dish or 12-well plate (Falcon, Franklin Lakes, NJ, USA) coated with poly-D-lysine (5 μg/ml), and were maintained at 37˚C in 95% air and 5% CO_2. The density of the cells was $6.0x10^5$/ml in 12-well plates or $2.0x10^6$/ml in culture dishes. The cells were treated with cytosine 1-beta-D-arabinofuranoside (cytarabine; 5 μM) when cultured for 3-5 days in order to inhibit the proliferation of gliocytes (11).

Identification of cholinergic neurons by immunocytochemistry. For the identification of cholinergic neurons, the cells were seeded onto glass coverslips precoated with poly-L-lysine, incubated for 7 days in growth medium and were subsequently fixed with 4% paraformaldehyde in PBS for 30 min at 4˚C. Choactase was used as a marker protein for identifying cholinergic neurons. Goat anti-Choactase (1:100) was applied overnight at 4˚C, biotinylated secondary immunoglobulin G (Biostar, Shanghai, China) was subsequently applied for 1 h, followed by streptavidin-Cy3 (Biostar) for 30 min. Antibody binding was visualized using diaminobenzidine substrate and the slides were subsequently restained with hematoxylin prior to observation under a light microscope (IM50; Leica Microsystems, Tokyo, Japan) with 10 random 20x20 fields sampled for microscopic observation. The number of positive cells in each field and total cell numbers were determined. Primary rat fetal neurons were propagated *in vitro*. Prior work in our laboratory revealed that >90% of these cells were cholinergic neurons (11).

Cell viability measurements. Mitochondrial dehydrogenase cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to determine cell survival in quantitative colorimetric assays. Cultured cells were incubated with MTT (0.5 mg/ml) for 2 h at 37˚C and the blue-colored formazan product, produced by dehydrogenase enzyme activity in active mitochondria, was dissolved in DMSO and quantified spectrophotometrically at 570 nm (752N; Shanghai Precision & Scientific Instrument Co., Ltd., Shanghai, China).

Experimental treatment. The cells were randomly divided into four groups: (i) Control; (ii) $A\beta_{1-42}$; (iii) DZ; (iv) DZ + $A\beta_{1-42}$. The cells in the $A\beta_{1-42}$ group were cultured for 7 days and treated with $A\beta_{1-42}$ for 24 or 72 h, the cells in the DZ + $A\beta_{1-42}$ group were pretreated with DZ for 1 h prior to treatment with $A\beta_{1-42}$ for 24 or 72 h, and the DZ group was pretreated with DZ for 1 h and subsequently treated with equal volumes of PBS rather than $A\beta_{1-42}$ for 24 or 72 h.

Western blot analysis. The cells were collected and washed with PBS three times and the total cellular proteins were extracted with radioimmunoprecipitation buffer for 20 min on ice. The cell lysates were centrifuged at 1,200 x g for 10 min at 4˚C and the protein concentration in the extracts was determined using a bicinchoninic acid Protein assay kit. A total of 60 mg samples were loaded per lane for SDS-PAGE on 8% polyacylamide gels, followed by transfer onto polyvinylidene membranes. The membranes were blocked with 5% dried non-fat milk for 60 min at 25˚C and were subsequently probed with primary antibody (1:100) overnight at 4˚C. The blots were washed three times in Tris-buffered saline, containing 0.05% Tween-20 and were subsequently incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, followed by washing three times. Signal detection was performed using enhanced chemiluminescence. β-actin was used as loading control, with image analysis software BandScan 5.0 software (Glyko Inc., Novato, CA, USA) being used for integral optical density value analysis.

Statistical analysis. Statistical analyses were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance followed by a two-tailed Student's t-test, or multiple comparison tests were performed as appropriate. Data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DZ protects against $A\beta_{1-42}$-induced neurotoxicity. $A\beta_{1-42}$ was demonstrated to induce cytotoxicity in cholinergic neurons, as assessed using an MTT assay. The MTT levels were significantly decreased by $A\beta_{1-42}$ in a concentration-dependent manner, indicating a reduction in the number of viable cells (Fig. 1A). Treatment with 2 μM $A\beta_{1-42}$ for 72 h was selected for subsequent experiments, since it reduced cell viability to ~48% of the control. The results obtained with the MTT reduction assay revealed that $A\beta_{1-42}$ (2 μM) induced cytotoxicity and significantly reduced cell viability ($P<0.05$, 24 h; $P<0.001$, 72 h), as compared with the control cells. Pretreatment of cholinergic neurons with DZ resulted in a reduction of

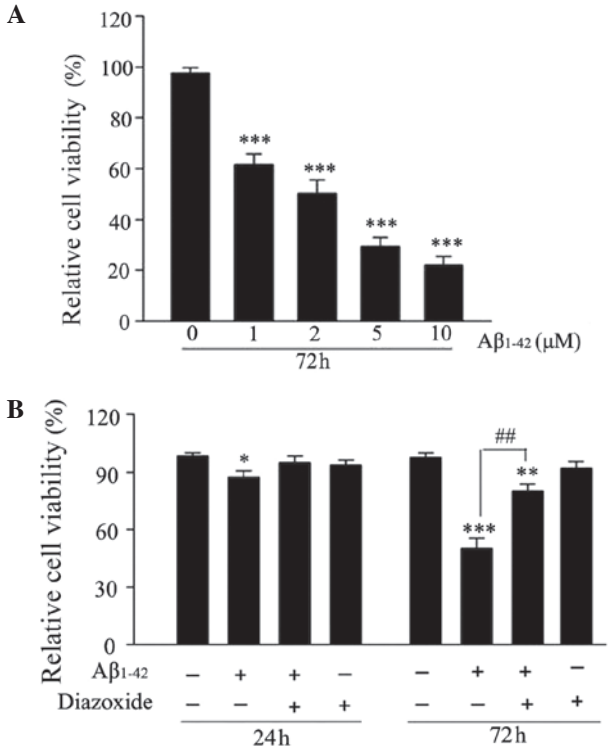

Figure 1. (A) Effect of $A\beta_{1-42}$ on cell viability. The cells were treated with different concentrations of $A\beta_{1-42}$ for 72 h and (B) the effect of 500 μM diazoxide pretreatment on $A\beta_{1-42}$ (2 μM) induced cell death was determined. The cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assay (*P<0.05 and **P<0.01, vs. control; ***P<0.001, vs. control; $^{\#\#}$P<0.01).

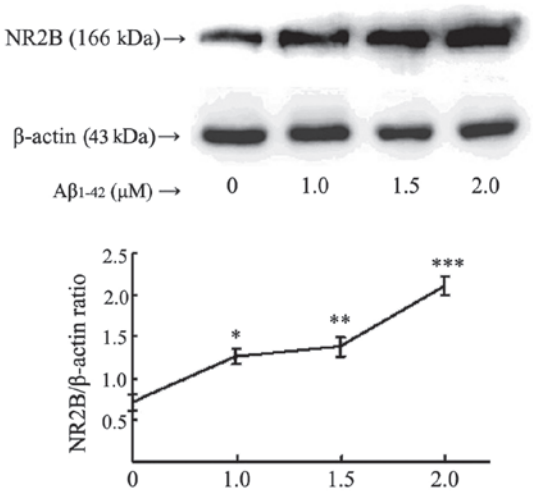

Figure 2. Protein expression of NR2B following treatment with $A\beta_{1-42}$. The neurons were incubated for 72 h with increasing concentrations of $A\beta_{1-42}$ (0, 1, 1.5 and 2 μM). The proteins were collected and subjected to western blot analysis for NR-2B, and the expression level of NR-2B increased in a concentration-dependent manner (*P<0.05, **P<0.01 and ***P<0.001, $A\beta_{1-42}$, vs. control).

$A\beta_{1-42}$-induced cytotoxicity and significantly increased cell viability (Fig. 1B).

Expression of the NR-2B subunit is induced by different concentrations of $A\beta_{1-42}$. The neurons were incubated for 72 h with different concentrations of $A\beta_{1-42}$ (0, 1, 1.5 and 2 μM).

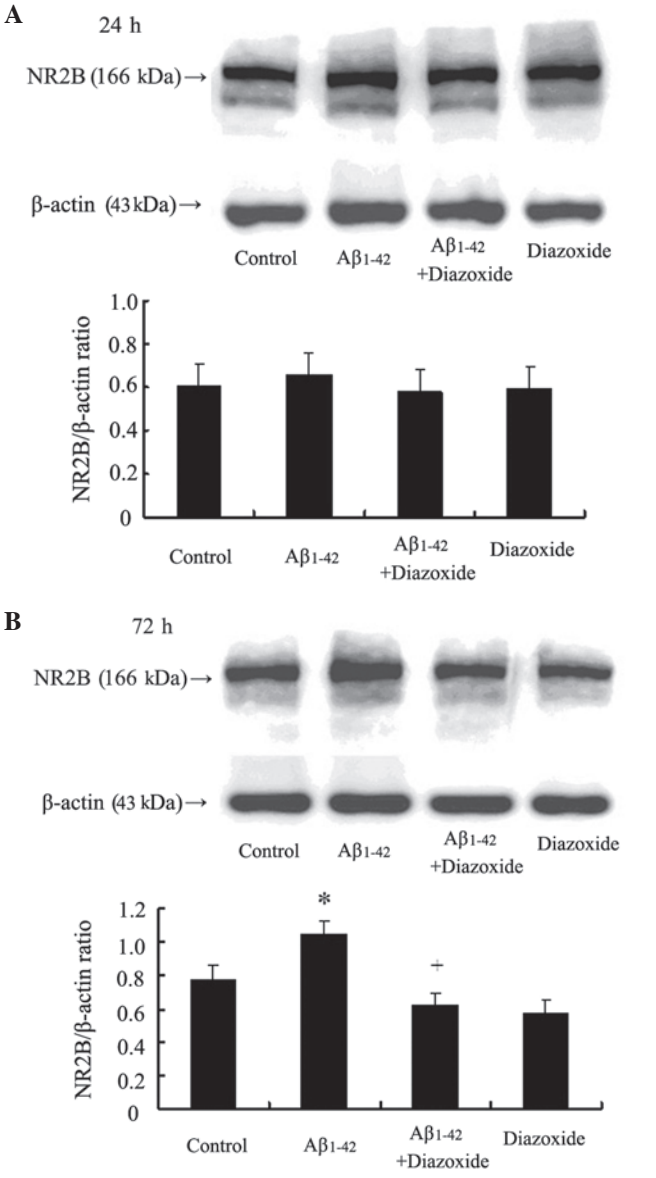

Figure 3. Expression of the NR2B subunit was determined by immunoblotting following exposure to $A\beta_{1-42}$ or $A\beta_{1-42}$ + diazoxide for 24 or 72 h. The neurons were randomly divided into control, $A\beta_{1-42}$, $A\beta_{1-42}$ + diazoxide or diazoxide groups. (A) No significant difference was observed at 24 h. (B) A significant difference was observed at 72 h (*P<0.05, $A\beta_{1-42}$, vs. control; $^{+}$P<0.05, $A\beta_{1-42}$ + diazoxide, vs. $A\beta_{1-42}$).

Western blot analysis for NR2B revealed that the expression of the NR2B subunit was increased in a concentration-dependent manner (Fig. 2).

DZ alleviates the expression of NR2B induced by $A\beta_{1-42}$. Exposure to $A\beta_{1-42}$ (2 μM) for 24 h compared with the control group revealed no significant difference in the expression of the NR2B subunit in the $A\beta_{1-42}$ group and $A\beta_{1-42}$ + DZ group (P>0.05; Fig. 3A). Compared with control group, the expression of the NR2B subunit in the $A\beta_{1-42}$ group was significantly upregulated for 72 h (P<0.05), and compared with the $A\beta_{1-42}$ group, the expression of the NR2B subunit in the $A\beta_{1-42}$ + DZ group was significantly decreased (P<0.05). The NR2B expression in the control and the DZ groups was not significantly difference (P>0.05; Fig. 3B).

Discussion

Mitochondrial dysfunction, hyperphosphorylation of Tau protein, apoptosis in neurons, and degradation and disappearance of synapses are all associated with the generation and aggregation of Aβ (12). It can be observed by the toxicology model that Aβ oligomers can lead to extensive damage to the dendritic spines of neurons, reduction of synaptic transmission, weakening of synaptic connections and excitotoxicity of hippocampal neurons (13). Currently, the mechanism of Aβ on synaptic plasticity injury is inconclusive. Previous research indicates that it is closely associated with the system activation of glutamatergic. Aβ can reduce astrocytic glutamate reuptake and influence glutamate transporter-1 and glutamate-aspartate transporter activity function, leading to an abnormally high concentration of glutamate in the synaptic cleft and thereby cause the sustained arousal of the NMDA receptor (14). Mattson *et al* (15) also identified a direct association between Aβ and the NMDA receptors. Aβ directly enhanced NMDA receptor-mediated excitotoxicity. In the hippocampus, when the NMDA receptor was activated, a substantial increase of intracellular Ca^{2+} and the phenomenon of NMDA receptor activation was also observed (15). Therefore, NMDA receptor-mediated glutamate excitotoxicity is vital in Aβ-induced synaptic plasticity injury.

The NMDA receptor is a typical isomer consisting of NR1 and NR2 subunits (NR2A-NR2D). NR1 is a basic subunit of the NMDA receptor, which is necessary for the channel function; NR2 is a regulation subunit, which assists to form the diversified structure of NMDA receptor. NR2B is important for the structure and function of NMDA receptors (16). The *in vitro* experiments revealed that the NR2B subunit regulated NMDA receptor-mediated excitatory postsynaptic potential and influenced the synaptic plasticity in the central nervous system (17). Additionally, NR2B-contained NMDA receptor ion channels revealed a high Ca^{2+} permeability (18). The experiments in the present study revealed that the protein expression of the NR2B subunit was not significantly changed following treatment with $A\beta_{1-42}$ for 24 h in cultured neurons. After 72 h exposure of $A\beta_{1-42}$, the protein expression levels of the NR2B subunit were significantly increased compared with the control. This indicated that Aβ changed the structure and function of the NMDA receptor complex, particularly the NMDAR1/NMDAR2B type NMDA receptor complex, further activated the NMDA receptor, causing extracellular Ca^{2+} influx augmentation and intracellular calcium overload, and was involved in NMDA-mediated cell excitotoxic damage and destruction process of synaptic plasticity (19).

KATP channels may be crucial in coupling metabolic energy to the membrane potential of cells and potassium channel dysfunction has been indicated in AD. It has been reported that activation of mitoKATP increases cell viability against rotenone-induced cell death (20) and in cultured cerebellar granule neurons subjected to H_2O_2, DZ can contribute to the stability of mitochondrial membrane potentials (21). A previous study revealed that DZ preconditioning may improve the learning ability and memory of rat models of AD (22), Teshima *et al* (21) reported that DZ preconditioning may inhibit the release of excitatory glutamate of cerebellar granule cells by activating mitoKATP channels (21). The present study demonstrated that DZ pretreatment diminished the expression of NR2B induced by $A\beta_{1-42}$ in cholinergic neurons, which indicated that DZ may suppress the $A\beta_{1-42}$-induced NMDA-mediated cell excitotoxic damage and destruction process of synaptic plasticity (18), in part, by alleviating the expression of NR2B. However, the exact mechanism of molecule signaling remains to be elucidated.

ATP-sensitive potassium channels may be a trigger and/or effector for a variety of oxidative stress preconditioning in the brain. KATP channel openers as brain protective agents has become a research hotspot (23). The present study demonstrated that KATP opener DZ preconditioning antagonized the upregulation of the expression of the NR2B subunit induced by $A\beta_{1-42}$, indicating that DZ may affect $A\beta_{1-42}$-induced cytotoxicity via the NMDA receptor pathway, and may provide a potential insight into the theoretical basis for the prevention of AD.

Acknowledgements

The present study was supported by the Young Scholars General Program of the National Natural Science Foundation of the People's Republic of China (no. 30600202) and the General Program of the National Natural Science Foundation of the People's Republic of China (no. 30870874).

References

1. Fu Q, Gao N, Yu J, Ma G, Du Y, Wang F, Su Q and Che F: Diazoxide pretreatment prevents Aβ1-42 induced oxidative stress in cholinergic neurons via alleviating NOX2 expression. Neurochem Res 39: 1313-1321, 2014.
2. Mota SI, Ferreira IL and Rego AC: Dysfunctional synapse in Alzheimer's disease-A focus on NMDA receptors. Neuropharmacology 76: 16-26, 2014.
3. Roselli F, Tirard M, Lu J, Hutzler P, Lamberti P, Livrea P, Morabito M and Almeida OF: Soluble beta-amyloid1-40 induces NMDA-dependent degradation of postsynaptic density-95 at glutamatergic synapses. J Neurosci 25: 11061-11070, 2005.
4. Parameshwaran K, Dhanasekaran M and Suppiramaniam V: Amyloid beta peptides and glutamatergic synaptic dysregulation. Exp Neurol 210: 7-13, 2008.
5. Ferreira IL, Bajouco LM, Mota SI, Auberson YP, Oliveira CR and Rego AC: Amyloid beta peptide 1-42 disturbs intracellular calcium homeostasis through activation of GluN2B-containing N-methyl-d-aspartate receptors in cortical cultures. Cell Calcium 51: 95-106, 2012.
6. Ronicke R, Mikhaylova M, Rönicke S, Meinhardt J, Schröder UH, Fändrich M, Reiser G, Kreutz MR and Reymann KG: Early neuronal dysfunction by amyloid β oligomers depends on activation of NR2B-containing NMDA receptors. Neurobiol Aging 32: 2219-2228, 2011.
7. Loftis JM and Janousky A: The N-methyl-D-aspartate receptor subunit NR2B: Localization, function properties, regulation and clinical implication. Pharmacol Ther 97: 55-85, 2003.
8. Virgili N, Mancera P, Wappenhans B, Sorrosal G, Biber K, Pugliese M and Espinosa-Parrilla JF: K(ATP) Channel opener diazoxide prevents neurodegeneration: A new mechanism of action via antioxidative pathway activation. PLoS One 8: e75189, 2013.
9. Liu D, Lu C, Wan R, Auyeung WW and Mattson MP: Activation of mitochondrial ATP-dependent potassium channels protects neurons against ischemia-induced death by a mechanism involving suppression of Bax translocation and cytochrome c release. J Cereb Blood Flow Metab 22: 431-443, 2002.
10. Zeng X, Wang T, Jiang L, Ma G, Tan S, Li J, Gao J, Liu K and Zhang Y: Diazoxide and cyclosporin A protect primary cholinergic neurons against beta-amyloid (1-42)-induced cytotoxicity. Neurol Res 35: 529-536, 2013.

11. Ma G, Fu Q, Zhang Y, Gao J, Jiang J, Bi A, Liu K, Du Y, Chen C, Cui Y and Lu L: Effects of Abeta1-42 on the subunits of KATP expression in cultured primary rat basal forebrain neurons. Neurochem Res 33: 1419-1424, 2008.
12. Hardy J and Selkoe DJ: The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. Science 297: 353-356, 2002.
13. Lacor PN, Buniel MC, Furlow PW, Clemente AS, Velasco PT, Wood M, Viola KL and Klein WL: Abeta oligomer-induced aberrations in synapse composition, shape and density provide a molecular basis for loss of connectivity in Alzheimer's disease. J Neurosci 27: 796-807, 2007.
14. Matos M, Augusto E, Oliveira CR and Agostinho P: Amyloid-beta peptide decreases glutamate uptake in cultured astrocytes: Involvement of oxidative stress and mitogen-activated protein kinase cascades. Neuroscience 156: 898-910, 2008.
15. Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I and Rydel RE: beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J Neurosci 12: 376-389, 1992.
16. Thomas CG, Miller AJ and Westbrook GL: Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. J Neurophysiol 95: 1727-1734, 2006.
17. Köhr G, Jensen V, Koester HJ, Mihaljevic AL, Utvik JK, Kvello A, Ottersen OP, Seeburg PH, Sprengel R and Hvalby Ø: Intracellular domains of NMDA receptor subtypes are determinants for long-term potentiation induction. J Neurosci 23: 10791-10799, 2003.
18. Cais O, Sedlacek M, Horak M, Dittert I and Vyklicky L Jr: Temperature dependence of NR1/NR2B NMDA receptor channels. Neuroscience 151: 428-438, 2008.
19. Zempel H, Thies E, Mandelkow E and Mandelkow EM: Abeta oligomers cause localized Ca(2+) elevation, missorting of endogenous Tau into dendrites, Tau phosphorylation and destruction of microtubules and spines. J Neurosci 30: 11938-11950, 2010.
20. Tai KK, McCrossan ZA and Abbott GW: Activation of mitochondrial ATP-sensitive potassium channels increases cell viability against rotenone-induced cell death. J Neurochem 84: 1193-1200, 2003.
21. Teshima Y, Akao M, Li RA, Chong TH, Baumgartner WA, Johnston MV and Marbán E: MitoKATP channel activation protects cerebellar granule neurons from apoptosis induced by oxidative stress. Stroke 34: 1796-1802, 2003.
22. Liu D, Pitta M, Lee JH, Ray B, Lahiri DK, Furukawa K, Mughal M, Jiang H, Villarreal J, Cutler RG, *et al*: The KATP channel activator diazoxide ameliorates amyloid-β and Tau pathologies and improves memory in the 3xTgAD mouse model of Alzheimer's Disease. J Alzheimers Dis 22: 443-457, 2010.
23. Wickenden AD: K+ channels as therapeutic drug targets. Pharmacol Ther 94: 157-182, 2002.