

# Neuregulin-1 (Nrg1) signaling has a preventive role and is altered in the frontal cortex under the pathological conditions of Alzheimer's disease

QIONG JIANG\*, SHUANGXI CHEN\*, CHENGLIANG HU, PEIZHI HUANG, HUIFAN SHEN and WEIJIANG ZHAO

Center for Neuroscience, Shantou University Medical College, Shantou, Guangdong 515041, P.R. China

Received March 17, 2016; Accepted July 14, 2016

DOI: 10.3892/mmr.2016.5542

**Abstract.** Alzheimer's disease (AD), one of the neurodegenerative disorders that may develop in the elderly, is characterized by the deposition of  $\beta$ -amyloid protein ( $A\beta$ ) and extensive neuronal cell death in the brain. Neuregulin-1 (Nrg1)-mediated intercellular and intracellular communication via binding to ErbB receptors regulates a diverse set of biological processes involved in the development of the nervous system. In the present study, a linear correlation was identified between Nrg1 and phosphorylated ErbB (pNeu and pErbB4) receptors in a human cortical tissue microarray. In addition, increased expression levels of Nrg1, but reduced pErbB receptor levels, were detected in the frontal lobe of a patient with AD. Western blotting and immunofluorescence staining were subsequently performed to uncover the potential preventive role of Nrg1 in cortical neurons affected by the neurodegenerative processes of AD. It was observed that the expression of Nrg1 increased as the culture time of the cortical neurons progressed. In addition,  $H_2O_2$  and  $A\beta_{1-42}$ , two inducers of oxidative stress and neuronal damage, led to a dose-dependent decrease in Nrg1 expression. Recombinant Nrg1 $\beta$ , however, was revealed to exert a pivotal role in preventing oxidative stress and neuronal damage from occurring in the mouse cortical neurons. Taken together, these results suggest that changes in Nrg1 signaling may influence the pathological development of AD, and exogenous Nrg1 may serve as a potential candidate for the prevention and treatment of AD.

## Introduction

Alzheimer's disease (AD) is the most common age-associated disorder, accounting for ~60-80% of all cases of dementia (1). Previous studies have shown that atrophy of the hippocampus and amygdala may occur in AD, even at the preclinical stages (2-4). AD is typically characterized by a progressive loss of memory, impairment of higher cognitive functions and major degeneration in the brain cortex. This degeneration includes the production and deposition of  $\beta$ -amyloid ( $A\beta$ ) peptide, intracellular neurofibrillary tangles (5,6) and extensive neuronal cell death (7) in specific cortical and subcortical zones. AD shares a number of common pathological features with other neurodegenerative diseases, including activated apoptotic biochemical cascades, up-regulated oxidative stress levels, abnormal protein processing, and so forth (8). Age-associated oxidative insults have been associated with neurodegenerative diseases, including AD and Parkinson's disease (9,10).  $A\beta$  peptide fragments are capable of inducing neuronal cell death directly or indirectly (11-13). In addition, transgenic mice with mutant amyloid precursor protein are considered a valuable animal model to test preventative and therapeutic interventions for AD due to the occurrence of biochemical, behavioral and histopathological changes that are similar to those observed in patients with AD (14). Although current therapeutic candidates for the treatment of AD that are similar to cholinesterase inhibitors such as meserine (15) and memantine (16) may modestly improve memory and cognitive function in this transgenic mouse model, these drugs do not show disease-modifying effects in patients. To date, the available therapies for AD only serve the purpose of ameliorating disease symptoms, and there are no effective therapeutic approaches that address the underlying pathological processes of AD (17).

Neuregulin-1 (Nrg1), a protein encoded by the *NRG1* gene, has been identified as an active epithelial growth factor (EGF) family member (18). At least 31 isoforms and six types of Nrg1, including Nrg1 $\alpha$  and Nrg1 $\beta$ , types I to VI, have been identified, due to alternative splicing (19). These types, or isoforms, perform a broad spectrum of functions. Nrg1 has been implicated in glioma malignancy (20), gastrointestinal systems (21) and prolactin secretion (22-24). Specific direct binding of Nrg1 to ErbB receptors, including ErbB3 and ErbB4 (25,26), activates a diverse set of biological processes,

---

*Correspondence to:* Professor Weijiang Zhao, Center for Neuroscience, Shantou University Medical College, 22 Xinling Road, Shantou, Guangdong 515041, P.R. China  
E-mail: neuromancn@aliyun.com

\*Contributed equally

**Key words:** Alzheimer's disease, neuregulin-1, frontal lobe, ErbB receptors, cortical neurons

including myelination, neurite outgrowth, cell proliferation, differentiation and protection against apoptosis (27,28). Nrg1, as well as its receptor ErbB tyrosine kinase, is expressed in the developing nervous system and the adult brain, where they exert a key role in regulating the development and regeneration of the nervous system (29-31). Nrg1 has been reported to prevent brain injury following stroke (32), and to exert a protective role for dopaminergic neurons in a mouse model of Parkinson's disease (33). A burgeoning body of evidence suggests that Nrg1 is associated with traumatic brain injury (34) and AD (35).

Given the alteration of Nrg1 signaling in patients with AD and the protective role of Nrg1 in the lesioned nervous system (29,30), it was hypothesized that Nrg1 may exert a preventive role in the maintenance of cell survival-associated signaling under the pathological conditions present in AD. In the present study, it has been shown that the levels of Nrg1 are altered in response to hydrogen peroxide ( $H_2O_2$ )- or  $A\beta_{1-42}$ -induced oxidative stress and neuronal damage in an attempt to protect the cortical neurons from abnormal changes in cell signaling. Notably, exogenous Nrg1 was revealed to have a pivotal role in preventing neurons from oxidative damage and in triggering changes in Nrg1-ErbB signaling in response to the harmful situation. These results demonstrated that Nrg1 signaling is perturbed under the pathological conditions of AD, and this alteration may be partially reversed by the exogenous application of Nrg1. Taken together, these data indicate a neuroprotective role of Nrg1 against pathological damage during the development of AD.

## Materials and methods

**Tissue microarray.** Human brain tissue microarray containing 4- $\mu$ m-thick cortical tissues was purchased from Shaanxi Chaoying Biotechnology Co., Ltd. (BN 126; Xi'an, Shanxi, China). In addition, human brain frontal lobe sections from a normal individual (cat. no. ab4304; Abcam, Cambridge, MA, USA) and from a patient with AD (cat. no. ab4582; Abcam) at a thickness of 5  $\mu$ m were used.

**Animals.** Female and male C57BL/6 mice (n=20; age, 3 months) were purchased from Guangdong Medical Laboratory Animal Center (Foshan, Guangdong, China) and maintained in the animal center of Shantou University Medical College (SUMC). All the animals were housed in the SUMC animal center at 25°C in a reversed 12/12 h dark-light cycle, and food and water were provided *ad libitum*. All experiments conducted on animals were reviewed and approved by the Animal Ethics Committee of SUMC and the authorities of the Guangdong Province. All efforts were made to minimize the suffering of animals and to reduce the number of animals used in these experiments.

**Preparation of recombinant Nrg1 $\beta$  (rNrg1 $\beta$ ) and oligomeric  $A\beta_{1-42}$ .** The *Escherichia coli*-derived rNrg1 $\beta$  (CYT-407; ProSpec-Tany TechnoGene Ltd., Ness Ziona, Israel) was dissolved in phosphate-buffered saline (PBS, pH 7.4) and used for *in vitro* cell culture experiments.

Synthetic  $A\beta_{1-42}$  powder (cat. no.1932-2-15; ChinaPeptides Co., Ltd., Shanghai, China) was dissolved in 0.1% dimethyl

sulfoxide and diluted 1:100 in Dulbecco's modified Eagle's medium (DMEM)/F-12 culture medium (HyClone™; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The diluted solution was subsequently incubated at 4°C for 24 h prior to centrifugation at 14,000 g for 10 min. The supernatant was used as oligomeric  $A\beta_{1-42}$  for *in vitro* cell culture experiments.

**Primary culture of the mouse cortical neurons.** Mouse frontal cortical tissues were obtained from postnatal C57BL/6 mice on day 0 (P0) and crudely homogenized by chopping following the removal of the vessels and meninges. The tissues were kept on ice in DMEM/F-12 culture medium (HyClone™; Thermo Fisher Scientific, Inc.) without serum, and subsequently digested with 0.125% trypsin (Solarbio Biotech Corp., Beijing, China) at 37°C in a humidified 5%  $CO_2$  atmosphere for 30 min. The finely separated cortical neurons were seeded in a volume of 200  $\mu$ l at a density of  $2 \times 10^5$  cells per well in 48-well cell culture plates pre-coated with 100  $\mu$ g/ml poly-D-lysine (C0312; Beyotime Institute of Biotechnology, Shanghai, China). Cells were cultured in DMEM/F-12 culture medium supplemented with 10% fetal bovine serum (FBS; Sijiqing Biotech Corp., Hangzhou, China) and 1% penicillin/streptomycin (Solarbio Biotech Corp.) for 6 h to enable cell adhesion to the plates. The medium was subsequently aspirated and replaced with Neurobasal-A (cat. no. 21103-049; Gibco; Thermo Fisher Scientific, Inc.) culture medium supplemented with 2% B-27 (cat. no. 17504-001, Gibco, Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin.

To investigate changes in Nrg1 signaling *in vitro* at the protein level, the primary cortical neurons were treated using two different paradigms: i) The cortical neurons were maintained at 37°C in a humidified 5%  $CO_2$  atmosphere for 24 h, and subsequently the culture medium was replaced with Neurobasal-A medium containing  $H_2O_2$  at various concentrations: 0, 1, 2.5, 5, 10 and 20  $\mu$ M for 24 h; ii) the cells were cultured for 6 h, 1, 3, 6 and 10 days. Cells cultured for 6 h were used as a control (0 days). At the indicated time points, whole-cell lysates were collected.

To study the neuroprotective role of rNrg1 $\beta$  in regulating Nrg1 signaling at the protein level, cortical neurons were utilized with two different cell models: i) Cortical neurons were treated with 0, 5 or 10 nM rNrg1 $\beta$  for 2 h, followed by an exposure to 2.5  $\mu$ M  $H_2O_2$  for 24 h; ii) after a 24-h incubation period, cells were treated with 0, 5 or 10 nM rNrg1 $\beta$  for 2 h prior to incubation with a sublethal dose of 10  $\mu$ M oligomeric  $A\beta_{1-42}$  for 24 h. Finally, whole-cell lysates were collected for western blotting.

**Immunofluorescence staining.** Paraffin-embedded human brain cortical tissue microarray and human frontal cortical sections from a normal individual and a patient with AD were deparaffinized, rehydrated via a graded array of ethanol to PBS, and antigen retrieval was then performed using 10 mM citrate buffer (pH 6.0) for 40 min. Non-specific protein binding sites were blocked by incubation with 10% normal donkey serum diluted in PBS at room temperature (RT, 25°C) for 1 h. The sections were incubated at 4°C overnight with mouse monoclonal anti-Nrg1 antibody (1:200, cat. no. MS-272-P1, Thermo Fisher Scientific, Inc.) without or with either rabbit polyclonal anti-phosphorylated (p)ErbB4 antibody (1:200, cat.

no. sc-33040, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or rabbit polyclonal anti-pNeu antibody (1:200, cat. no. sc-12352-R, Santa Cruz Biotechnology, Inc.). Mouse monoclonal anti- $\beta$ -III tubulin (1:1,000, cat. no. sc-80016, Santa Cruz Biotechnology, Inc.) was applied to indicate the mouse cortical neurons. Following washing with PBS three times (5 min each wash), the samples were incubated with a donkey anti-mouse secondary antibody conjugated to Dylight™ 488 (1:1,000) and a donkey anti-rabbit secondary antibody conjugated to Dylight™ 594 (1:1,000) at RT in the dark for 90 min. The samples were finally mounted using ProLong® Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; P36935, Gibco; Thermo Fisher Scientific, Inc.). Double-immunofluorescence images were acquired using an Olympus laser confocal system (FV-1000; Olympus, Tokyo, Japan). DAPI was excited at 405 nm. Dylight™ 488 and Dylight™ 594 were excited at 488 and 594 nm, respectively, in a multi-track configuration.

The protein levels of Nrg1, pNeu and pErbB4 in the human brain cortical tissue microarray were evaluated using integrated fluorescence intensity (IFI). The IFI at each tissue point was obtained using the MultiImage Light Cabinet CY3 filter for Nrg1, and the CY5 filter of the FluorChem HD2 gel-imaging system for pErbB4 or pNeu (Alpha Innotech, San Leandro, CA, USA). The IFI was analyzed using Image Tool II software 3.0 (University of Texas Health Science Center, San Antonio, TX, USA).

**Immunohistochemical staining.** Deparaffinized human frontal cortical sections from a normal individual and a patient with AD were rehydrated via a graded array of ethanol to PBS. Subsequently, hit-induced antigen retrieval was performed in citrate buffer (10 mM, pH 6.0) at 95°C for 40 min, followed by cooling down to RT for at least 60 min. Sections were then incubated in a 3% H<sub>2</sub>O<sub>2</sub> solution for endogenous peroxidase clearance at RT for 10 min. Sections were subsequently washed in PBS for 5 min three times. Following blocking in 10% PBS-buffered normal goat serum for 30 min, sections were incubated with primary antibodies, including mouse monoclonal anti-Nrg1 antibody (1:200, cat. no. MS-272-P1, Thermo Fisher Scientific, Inc.), rabbit polyclonal anti-pErbB4 antibody (1:200, cat. no. sc-33040, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-pNeu antibody (1:200, cat. no. sc-12352-R, Santa Cruz Biotechnology, Inc.) and rabbit polyclonal anti-A $\beta$ <sub>1-42</sub> antibody (1:1,000, cat. no. ab39377, Abcam) overnight at 4°C, followed by incubation with an Enhanced Polymer DAB Detection kit (cat. no. PV-900; ZSGB-Bio, Beijing, China) and an AEC kit (cat. no. ZLI-9036; ZSGB-Bio). Stained sections were mounted on slides, dehydrated and sealed with coverslips using a commercial water-soluble mounting kit (cat. no. AR1018; Boster Biological Technology, Wuhan, China). Counterstaining was performed with Mayer's hematoxylin in certain of the tissue sections.

Congo red reagent (cat. no. DG0025, Beijing Leagene Biotech. Co., Ltd., Beijing, China) was used to confirm the formation of amyloid plaques in frontal lobe sections from a patient with AD, according to the manufacturer's protocol.

**Western blotting analysis.** Tissue lysates of the frontal lobe from either normal adults (cat. no. ab29969, Abcam)

or the patient with AD (cat. no. ab29971, Abcam) and the whole-cell lysates from cultured mouse cortical neurons were mixed with 20% sample loading buffer [0.125 M Tris/HCl (pH 6.8), 20% glycerol, 10% sodium dodecylsulfate, 0.1% bromophenol blue and 5%  $\beta$ -mercaptoethanol]. All samples were heated for 15 min at 95°C. Protein samples were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently electroblotted onto polyvinylidene difluoride membranes (Millipore Corp., Billerica, USA). Incubation in 5% non-fat milk or bovine serum albumin diluted in Tris/HCl saline buffer supplemented with 0.1% Tween-20 (TBST, pH 7.4) for 1 h was used to block non-specific protein-binding sites. Membranes were incubated with antibodies specific for mouse monoclonal anti-Nrg1 antibody (1:1,000, cat. no. MS-272-P1, Thermo Fisher Scientific, Inc.), rabbit polyclonal anti-pErbB4 antibody (1:500, cat. no. sc-33040, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-ErbB4 antibody (1:1,000, cat. no. sc-283, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-pNeu antibody (1:500, cat. no. sc-12352-R, Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-Neu antibody (1:1,000, cat. no. sc-33684, Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-phosphorylated extracellular signal-regulated kinase 1/2 (anti-pErk1/2) antibody (1:1,000, cat. no. sc-7383, Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-Erk1/2 antibody (1:1,000, cat. no. sc-135900, Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-pAkt1 antibody (1:1,000, cat. no. sc-81433, Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-Akt1 antibody (1:1,000, cat. no. sc-55523, Santa Cruz Biotechnology, Inc.) and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GADPH) antibody (1:1,000, cat. no. sc-365062, Santa Cruz Biotechnology, Inc.) overnight at 4°C. After washing the membrane with TBST three times at RT (5 min each wash), membranes were further incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:1,000, cat. no. BA1051, Boster Biological Technology, Wuhan, China) or anti-rabbit secondary antibody (1:1,000, cat. no. BA1055, Boster Biological Technology) for 1 h. Subsequently, membranes were washed in TBST three times (5 min each wash) at RT. The immunoreactive bands were visualized using an enhanced chemiluminescence kit (Bio-Rad Laboratories, Richmond, CA, USA) and an imaging system (Alpha Innotech, San Leandro, CA, USA). The signal intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** Statistical analyses were performed with SPSS 17.0 software (Chicago, IL, USA). Data were expressed as the mean  $\pm$  standard error of the mean and analyzed with one-way analysis of variance (ANOVA) with Tukey's post-hoc test for independent samples.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Co-immunostaining correlation analysis of Nrg1 with the phosphorylation levels of ErbB4 and Neu receptors in a human cortical tissue microarray.** To determine a functional correlative relationship between the level of Nrg1 and the phos-



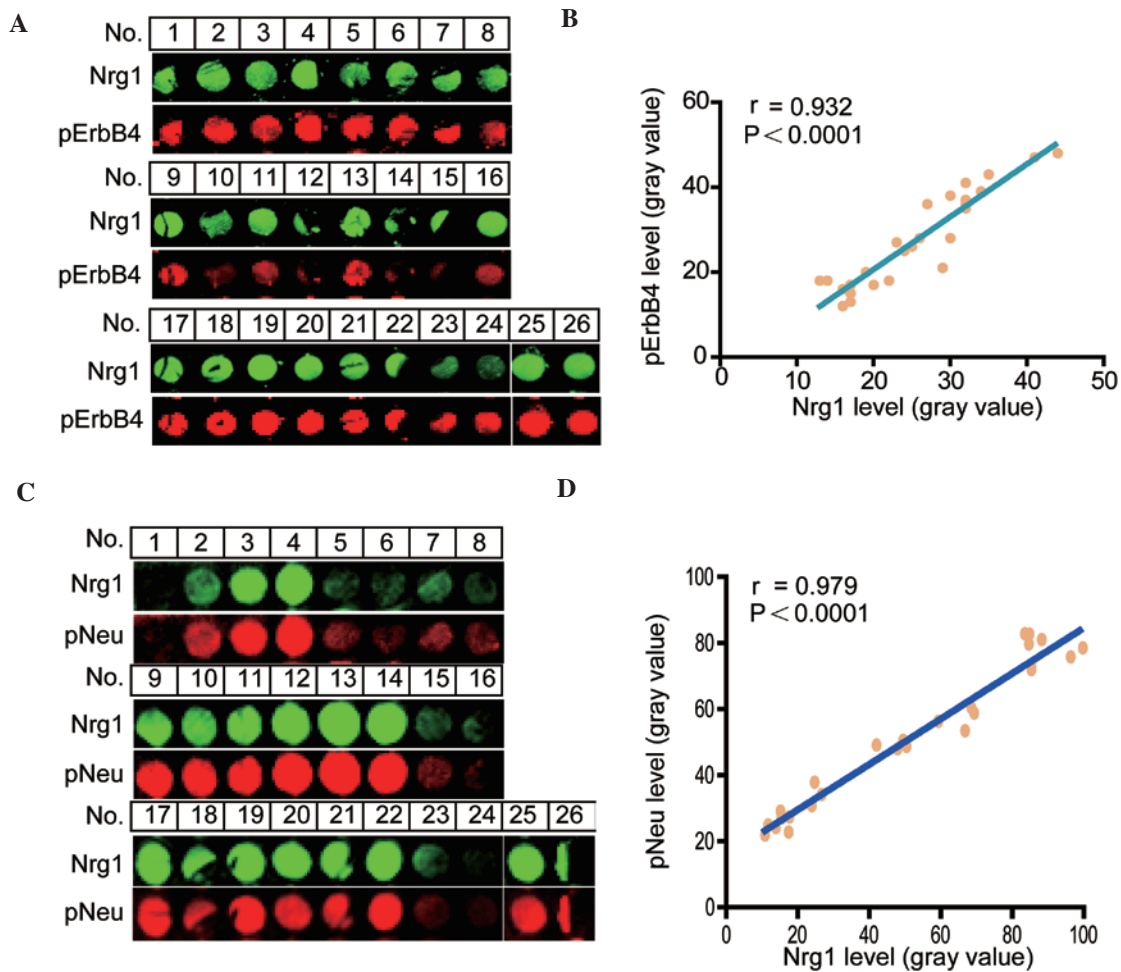


Figure 1. Linear correlation analyses for Nrg1 with either pErbB4 (A and B) or pNeu (C and D) based on integrated fluorescence intensity of the human frontal cortex tissue array. Double immunofluorescence staining of Nrg1 with either pErbB4 (A) or pNeu (C) at each tissue point is shown. Linear correlation graph depicting the association between Nrg1 and pErbB4 (B) or pNeu (D). Each corresponding Pearson correlation coefficient ( $r$ ) is shown. Nrg1, neuregulin 1; pErbB4, phosphorylated ErbB4; pNeu, phosphorylated Neu.

phorylation levels of either the ErbB4 or the Neu receptors, the co-localization of Nrg1 with either pErbB4 or pNeu receptors was examined. The signal intensity for Nrg1 with pErbB4 receptors was revealed by double immunofluorescence (Fig. 1A), and a positive correlation between Nrg1 and pErbB4 ( $r=0.932$ ,  $P<0.0001$ ) (Fig. 1B) was identified. Similarly, the signal intensity for Nrg1 with Neu receptors was also revealed by double immunofluorescence (Fig. 1C) and an apparent positive correlation between Nrg1 and pNeu ( $r=0.979$ ,  $P<0.0001$ ) (Fig. 1D) was revealed in the human cortical region.

**Detection of Nrg1 signaling in the frontal lobe of a human AD brain.** Congo red staining was used to confirm the formation of the amyloid plaques in the frontal lobe of the brain of a human patient with AD. The results demonstrated that there were numerous amyloid plaques distributed in the frontal lobe of an AD brain, whereas no amyloid plaque was detected in the normal control (Fig. 2A). In addition, immunohistochemical staining also revealed the formation of  $A\beta_{1-42}$  positive plaques in the frontal cortical zone, whereas few  $A\beta_{1-42}$  positive plaques were found in the normal individual (Fig. 2A).

To study the changes in Nrg1 signaling in the frontal cortex of an AD brain, western blotting was used to determine the

protein levels of Nrg1 and the phosphorylation levels of ErbB4 and Neu. The protein level of Nrg1 was increased in the frontal cortical gray matter from an AD brain (Fig. 2B). In contrast, the levels of pErbB4 and pNeu showed a tendency towards a decrease when compared with a normal control. Notably, the phosphorylation level of Erk1/2, which is involved in downstream Nrg1-ErbB signaling, was increased when compared with that in the normal control (Fig. 2B).

To further investigate changes in Nrg1 signaling under the conditions of AD, Nrg1, pErbB4 and pNeu in the frontal cortical gray matter from a patient with AD and a normal control were immunohistochemically stained. A tendency for there to be an increased level of Nrg1 was observed in the gray matter of an AD brain compared with a normal control (Fig. 2C). By contrast, the staining intensities for pErbB4 and pNeu were clearly reduced (Fig. 2C).

To evaluate the expression and potential co-localization of Nrg1 with pErbB4 or pNeu, co-immunostaining of Nrg1 with these receptors was performed using the frontal cortical tissue from an AD brain. Co-localization of Nrg1 (green) with pErbB4 (red) or with pNeu (red) was observed. In the human AD brain, a tendency towards an up-regulation of the levels of Nrg1 was observed, whereas the levels of pErbB4 and

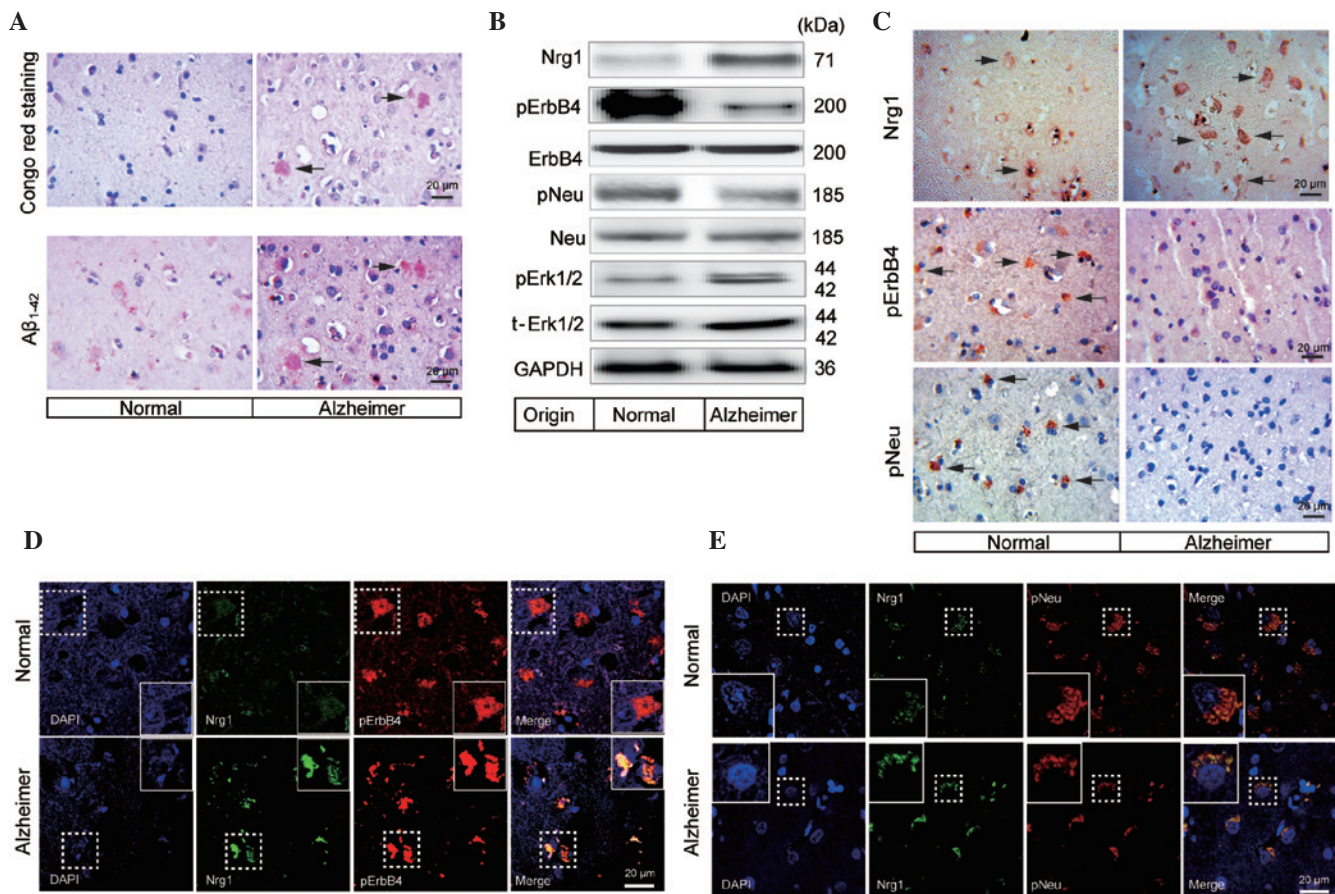


Figure 2. Changes in the Nrg1 signaling pathway molecules in the frontal lobe of a human AD brain. (A) Congo red staining and abnormal aggregation of Aβ<sub>1-42</sub>, indicating the formation of the amyloid plaques (indicated by the arrowheads) in the frontal cortical gray matter of a human AD brain. The scale bar represents 20 μm. (B) Western blotting analysis of Nrg1, phosphorylation levels of ErbB4, Neu and Erk1/2 in the frontal lobe of a human AD patient brain. (C) Immunohistochemical detection of Nrg1, pErbB4 and pNeu (indicated by the arrowheads) in the frontal cortical gray matter from either the normal individual or the human AD patient. Double immunofluorescence staining images are shown for co-localization of Nrg1 with either (D) pErbB4 or (E) pNeu in the frontal cortical gray matter from either the normal individual or the human AD patient. The scale bar represents 20 μm. AD, Alzheimer's disease; Aβ, β-amyloid; Nrg1, neuregulin 1; pErbB4, phosphorylated ErbB4; pNeu, phosphorylated Neu; Erk, extracellular-signal regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

pNeu were not up-regulated compared with those of normal brain tissue (Fig. 2D and E). In addition, pErbB4 and pNeu were detected in a smaller number of the neuronal cells in the AD-affected cerebral cortex, which may explain, in part, the reduced levels of the two molecules observed in the western blots.

**Western blotting analysis of Nrg1 isoform expression and the ErbB receptor phosphorylation level in primary cortical neurons during the progression of cell senescence.** The primary cortical neurons were routinely cultured for 1, 3, 6 and 10 days without any treatment. The expression of Nrg1 isoforms, including the 123-, 95-, 75-, 71-, 60-, 54- and 36-kDa variants, showed a time-dependent increase that reached statistical significance at 3, 6 and 10 days when compared with the 0-day control (Fig. 3A). A marked increase in the protein levels of the pNeu and pErbB4 receptors was observed at 1 to 10 days when compared with the 0-day control (Fig. 3B).

**Investigation of the protective role of Nrg1β in primary cortical neuronal cultures oxidatively stressed by H<sub>2</sub>O<sub>2</sub>.** To determine the role of Nrg1 in preventing oxidative insults at the protein level, western blotting was employed to analyze

the protein level of Nrg1 and the phosphorylation levels of Neu and ErbB4 receptors in primary cortical neurons in response to a 24 h treatment with H<sub>2</sub>O<sub>2</sub> at concentrations ranging from 0 to 20 μM. Multiple isoforms of Nrg1, including the 123-, 71-, 60-, 54- and 36-kDa variants, were expressed in the primary cortical neurons. Compared with the vehicle control, all Nrg1 isoforms showed a dose-dependent decrease in response to the gradually increased concentrations of H<sub>2</sub>O<sub>2</sub> (Fig. 4A). In comparison with the vehicle control, the expression of Nrg1 was down-regulated, accompanied by a marked reduction in the receptor levels of pNeu and pErbB4 (Fig. 4B).

Furthermore, in order to explore the role of rNrg1β in alleviating oxidative stress and axonal damage, western blotting was conducted to evaluate whether the Nrg1-ErbB signaling pathway was involved in the preventive mechanism on exposure to H<sub>2</sub>O<sub>2</sub>. Based on the data from a previous study (Chen *et al*, unpublished) a concentration of 2.5 μM was adopted as the optimal concentration of H<sub>2</sub>O<sub>2</sub> for treatment of the cortical neurons following pretreatment with 0, 5 or 10 nM rNrg1β for 2 h. It was revealed that the levels of pErbB4 and pNeu were markedly decreased following treatment with H<sub>2</sub>O<sub>2</sub>, and this effect was reversed on addition of rNrg1β, to a maximal extent at 10 nM for pErbB4 and at 5 nM for pNeu

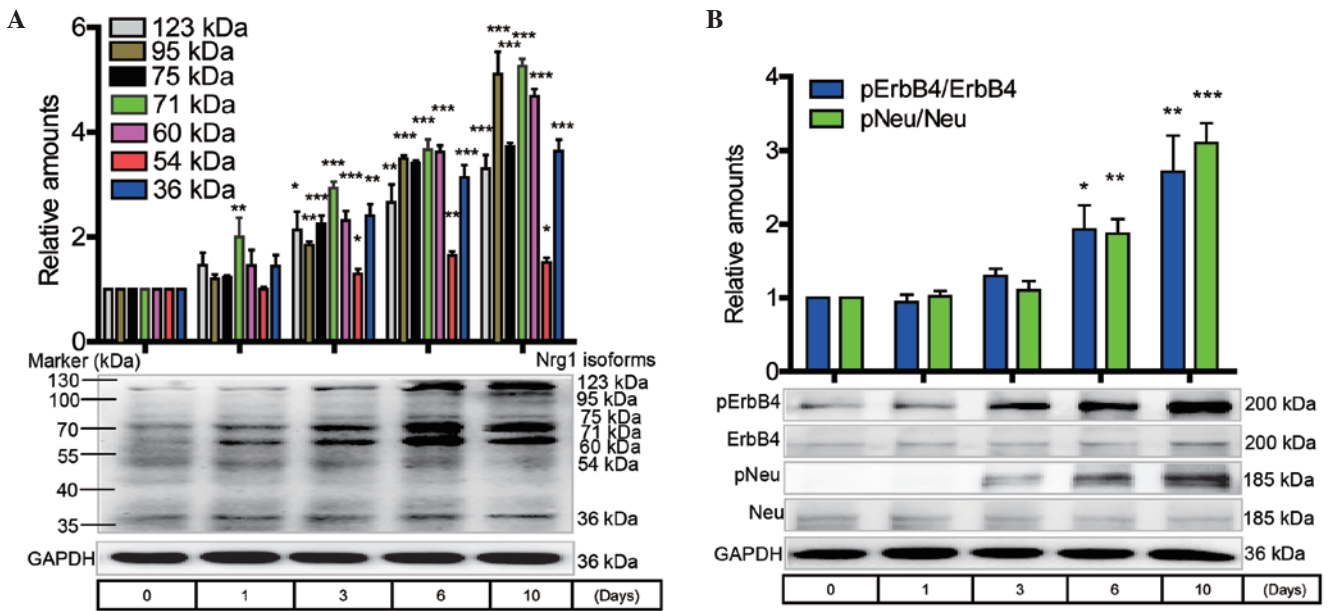


Figure 3. Western blot analysis of Nrg1, pNeu and pErbB4 in primary mouse cortical neurons in response to cell senescence. (A) Protein levels of Nrg1 isoforms and (B) the phosphorylation levels of Neu and ErbB4 in cortical neurons cultured for 0 to 10 days ( $n=5$ , one-way analysis of variance with Tukey's post-hoc test; data are expressed as the mean  $\pm$  standard error of the mean). \* $P<0.05$ , \*\* $P<0.01$  and \*\*\* $P<0.001$  compared with the 0-day control. Nrg1, neuregulin 1; pErbB4, phosphorylated ErbB4; pNeu, phosphorylated Neu; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(Fig. 4C). The levels of Akt1 and Erk1/2 activation exhibited a similar trend to that of pErbB4 (Fig. 4D and E).

Double immunofluorescence staining was subsequently used to further confirm these observations. It was revealed that, compared with non-stressed neurons,  $H_2O_2$ -treated neurons exhibited diminished levels of neurite outgrowth, with the detection of reduced levels of Nrg1 and of pNeu or pErbB4. In contrast, pretreatment with 5 and 10 nM rNrg1 $\beta$  prior to  $H_2O_2$  exposure was able to partially reverse these effects by increasing the levels of both pNeu and pErbB4, with more clearly recognizable effects observed at a concentration of 10 nM (Fig. 4F and G).

*The preventive role of rNrg1 $\beta$  in counteracting the effects of  $A\beta_{1-42}$  on mouse cortical neurons.* The protective role of rNrg1 $\beta$  in mouse cortical neurons treated with  $A\beta_{1-42}$  was subsequently investigated. Western blotting was utilized to evaluate the influence of rNrg1 $\beta$  pretreatment on the phosphorylation levels of Neu and ErbB4 and on the downstream signaling pathways in primary cortical neurons following a 24 h incubation with 10  $\mu$ M oligomeric  $A\beta_{1-42}$ . Administration of 10  $\mu$ M oligomeric  $A\beta_{1-42}$  significantly downregulated the protein levels of several Nrg1 isoforms, including the 60 kDa and 36 kDa Nrg1, whereas pretreatment with 5 nM rNrg1 $\beta$  counteracted the effects of  $A\beta_{1-42}$  by increasing the Nrg1 isoforms. By contrast, pretreatment with 10 nM rNrg1 $\beta$  showed no apparent effects on the function of  $A\beta_{1-42}$  (Fig. 5A). It was also demonstrated that the relative levels of pErbB4 and pNeu were markedly decreased following treatment with  $A\beta_{1-42}$ , and this effect on pErbB4 was compensated for by pretreatment with 10 nM rNrg1 $\beta$  and on pNeu by pretreatment with 5 nM rNrg1 $\beta$  (Fig. 5B). In addition, pAkt1 levels were increased, and pErk levels were decreased when treated with  $A\beta_{1-42}$  alone (Fig. 5C and D). However, rNrg1 $\beta$  pretreatment upregulated the levels of pAkt1 and pErk in  $A\beta_{1-42}$ -challenged cortical neurons (Fig. 5C and D).

We subsequently applied double immunofluorescence staining to further confirm these observations. It was observed that, compared with non-stressed neurons,  $A\beta_{1-42}$ -treated neurons demonstrated a diminished neurite outgrowth, with reduced levels of pNeu or pErbB4 detected. In contrast, pretreatment with 5 or 10 nM rNrg1 $\beta$  prior to  $A\beta_{1-42}$  exposure was able to partially reverse these effects by increasing the levels of both pNeu and pErbB4, with the most marked effects being observed with 10 nM rNrg1 $\beta$  for pErbB4 and 5 nM rNrg1 $\beta$  for pNeu (Fig. 5E and F).

## Discussion

It is widely acknowledged that the Nrg1-ErbB signaling pathway exerts a crucial role in multiple biological processes, including cell differentiation, organ development and tumorigenesis. Receptors for Nrg1 signaling undergo phosphorylation of their cytoplasmic tyrosine residues, which elicits downstream effects and biological responses (36). The binding of the ligand results in the dimerization and activation of ErbB receptors. Phosphorylation of the intracellular domains creates docking sites for adaptor proteins, including growth factor receptor-bound protein 2 (Grb2) and Shc for the activation of the Erk pathway, and p85 for the activation of the phosphoinositide 3-kinase pathway (37). A previous study revealed an association between Nrg1 and ErbB4 immunoreactivity and the formation of neuritic plaques in patients with AD in a transgenic animal model of AD (35). In the present study, a linear correlation was observed between Nrg1 and the phosphorylation of Neu and ErbB4 receptors in a normal human cortical tissue microarray. To elucidate the exact mechanism by which Nrg1 contributes to AD development, two cell models were applied. Based on our results using cortical neurons under the pathological conditions of AD, multiple isoforms of Nrg1 were altered, including the 123-, 95-, 75-,



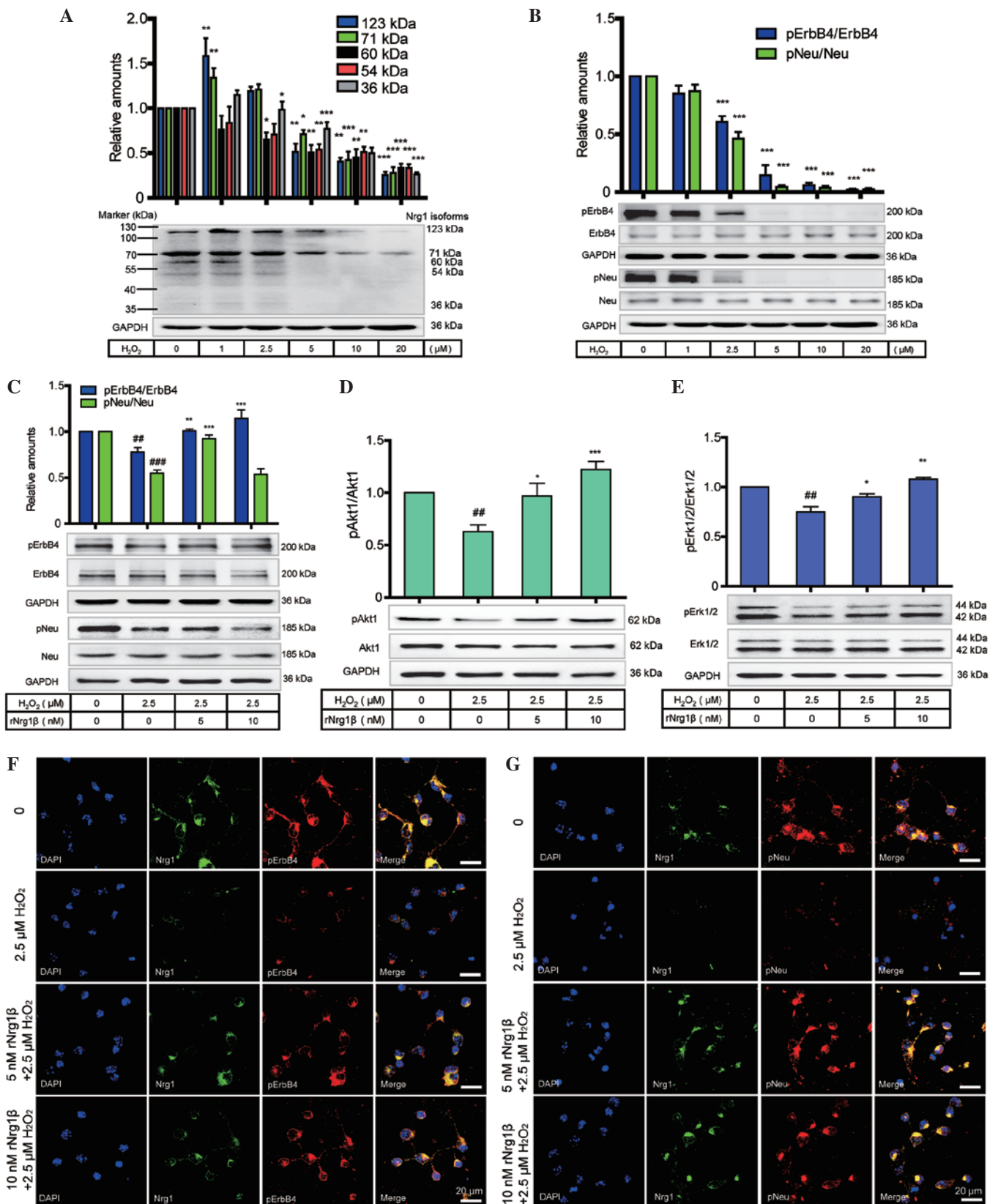


Figure 4. Protective role of rNrg1 $\beta$  in primary mouse cortical neurons in response to oxidative stress and neuraxon damage. (A) Protein levels of Nrg1 isoforms and (B) phosphorylation levels of Neu and ErbB4 receptors in primary cortical neurons after a 24 h treatment with 0-20  $\mu M$   $H_2O_2$  (n=6, one-way ANOVA with Tukey's *post-hoc* test; data are expressed as the mean  $\pm$  SEM). \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 compared with the control group. (C) The phosphorylation levels of Neu and ErbB4, and (D) the pAkt1 levels and (E) the pErk1/2 levels in primary cortical neurons pretreated with rNrg1 $\beta$  at a concentration of 5 or 10 nM for 2 h prior to a 24-h treatment with 2.5  $\mu M$  oligomeric  $H_2O_2$  (n=5, one-way ANOVA with Tukey's *post-hoc* test; data are expressed as the mean  $\pm$  SEM). \*\* $P$ <0.01 and \*\*\* $P$ <0.001 vs. the vehicle control and \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 vs. the  $H_2O_2$ -treated group. Double immunofluorescence staining of Nrg1 with either (F) pErbB4 or (G) pNeu in the cortical neurons pretreated with rNrg1 $\beta$  at a concentration of 5 or 10 nM for 2 h prior to a 24 h treatment with 2.5  $\mu M$  oligomeric  $H_2O_2$ . Scale bars=20  $\mu m$ . ANOVA, analysis of variance; SEM, standard error of the mean;  $H_2O_2$ , hydrogen peroxide; rNrg1 $\beta$ , recombinant neuregulin  $\beta$ ; pErbB4, phosphorylated ErbB4; pNeu, phosphorylated Neu; pErk1/2, phosphorylated extracellular-regulated signal kinase 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

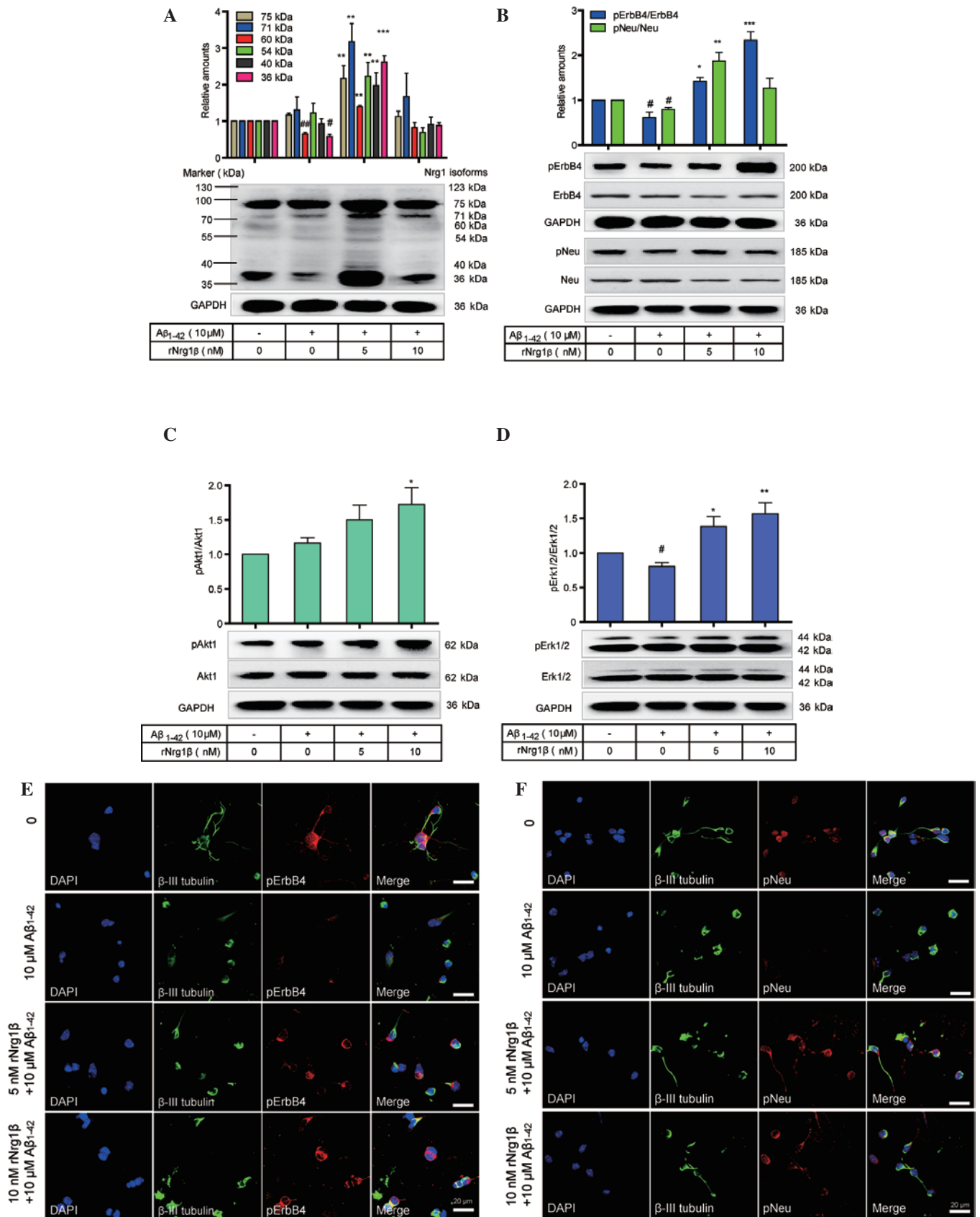


Figure 5. Effects of rNrg1β pretreatment on the Nrg1 signaling in primary mouse cortical neurons exposed to Aβ<sub>1-42</sub>. The (A) relative levels of the Nrg1 isoforms, (B) phosphorylation levels of Neu and ErbB4, (C) pAkt1 levels and (D) pErk1/2 levels in primary cortical neurons pretreated with rNrg1β at a concentration of 5 or 10 nM for 2 h prior to a 24-h treatment with 10 μM oligomeric Aβ<sub>1-42</sub> are shown (n=5, one-way analysis of variance with Tukey's post-hoc test; data are expressed as the mean ± standard error of the mean). Double immunofluorescence staining of β-III tubulin with either (E) pErbB4 or (F) pNeu in the cortical neurons pretreated with rNrg1β at a concentration of 5 or 10 nM for 2 h prior to a 24 h treatment with 10 μM oligomeric Aβ<sub>1-42</sub>. Scale bars=20 μm. rNrg1β, recombinant neuregulin 1β; pErbB4, phosphorylated ErbB4; pNeu, phosphorylated Neu; pErk1/2, phosphorylated extracellular-regulated signal kinase 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Aβ, amyloid-beta; DAPI, 4',6-diamidino-2-phenylindole. \*P<0.05 and \*\*P<0.01 vs. vehicle control and \*\*\*P<0.001 compared with the control group.



71-, 60-, 54-, 40- and 36-kDa proteins. These bands represent alternatively spliced products of the *NRG1* gene, post-translationally modified forms of the proteins, and/or a shedding of the ectodomains from the initial precursors. All isoforms of *Nrg1* contain an epidermal growth factor (EGF)-like signaling domain that is required for activation of the receptors (38). In addition, the interaction of *Nrg1* with its receptors was shown to be associated with the activation of intracellular signaling pathways that are associated with the development and regeneration of the nervous system (29,30). In the present study, it has been demonstrated that the changes in *Nrg1* isoform expression and receptor phosphorylation are highly influenced by the pathological conditions observed in AD. Thus, expression changes in *Nrg1* isoforms appear to be associated with the pathological development of AD, suggesting that *Nrg1* may be a critical molecule in the development of AD.

Oxidative stress plays an essential role in the onset and development of AD (39,40), and cellular oxidative stress levels are increased in vulnerable regions of the AD brain (41,42). The brain is particularly sensitive to oxidative stress due to special cellular features, including a large dependence on oxidative phosphorylation for energy production, low antioxidant concentrations, low levels of membrane lipids and high levels of iron, which are associated with free radical injury (43-45). Previous studies reported that *Nrg1* was up-regulated following nerve injury, and it served as an essential agent to protect the neurons from ischemic damage (34,46,47). In addition, *Nrg* proteins attenuated the release of free radicals and protected neuronal cells from  $H_2O_2$ -induced apoptosis (48,49). In the present study, the protein levels of multiple *Nrg1* isoforms and the phosphorylation of their receptors were observed to increase in a time-dependent manner. Changes in *Nrg1* signaling in cortical neurons exposed to oxidative stress were further investigated. It was observed that protein levels of *Nrg1*, and the phosphorylation of its receptors, were down-regulated in response to high concentrations of  $H_2O_2$ . Although the *Nrg1* protein level was up-regulated at low concentrations of  $H_2O_2$  compared with the control, no up-regulation of receptor phosphorylation was identified. This suggested that the interactions between *Nrg1* and the ErbB receptors were perturbed under conditions of oxidative stress. By contrast, exogenous r*Nrg1* $\beta$  was able to protect the cortical neurons from oxidative stress and neuraxonal damage via the up-regulation of the *Nrg1*-ErbB cell signaling pathway. Cui *et al* (50) proposed that endogenous *Nrg1* was increased in response to the production of A $\beta$  to protect the neurons against damage. However, the injured neurons were not capable of expressing sufficient *Nrg1* $\beta$ 1 to adapt to prolonged damage, which ultimately led to apoptosis. Increased oxidative stress occurs in response to increased A $\beta$  levels (51); therefore, in the present study, it was originally hypothesized that the up-regulation of endogenous *Nrg1* in primary cortical neurons exposed to  $H_2O_2$  may be an initial, local protective response against abnormal cell signaling. However, the cortical neurons were unable to express sufficient *Nrg1* over time, eventually resulting in the dysfunction of *Nrg1* signaling. These results suggest the existence of an intrinsic self-protective mechanism in which injured cortical neurons may adapt to, and automatically counteract, neuronal injury.

A $\beta$  is associated with the generation of reactive oxygen species, which cause cell damage, apoptosis, mitochondrial

dysfunction and the peroxidation of membrane lipids (52,53). In addition, the accumulation of A $\beta$  peptides has been identified as a key step in the multiple pathogenic changes associated with neurodegeneration and dementia (54,55). Previous studies demonstrated that the neurotoxicity induced by A $\beta_{1-42}$  may lead to apoptotic cell death (56), and that A $\beta$  is able to disrupt signaling pathways, including those involving Erk1/2 and Akt in the primary rat cortical neurons (57,58). In the present study, it was observed that exposure of primary cortical neurons to A $\beta_{1-42}$  caused an up-regulation in the level of *Nrg1* protein and in Akt1 phosphorylation, and a down-regulation of Neu/ErbB4 phosphorylation and pErk1/2 levels. *In vitro* studies have demonstrated that *Nrg1* $\beta$  treatment may protect neuronal cells (59-62). In the present study, A $\beta_{1-42}$  treatment increased the levels of *Nrg1* and Akt1 phosphorylation, and decreased the phosphorylated levels of ErbB4, Neu and Erk1/2. Moreover, the A $\beta_{1-42}$ -induced reduction in the levels of pErbB4, pNeu, pAkt1 and pErk1/2 was antagonized by r*Nrg1* $\beta$  pretreatment. Recombinant human *Nrg1* contains an EGF-like domain that is essential for the phosphorylation-dependent activation of Neu/ErbB4 receptors (63). *Nrg1* is able to signal to target cells via interactions with transmembrane tyrosine kinase receptors of the ErbB family. The interaction of *Nrg1* with ErbB receptors may result in the dimerization of receptors, tyrosine phosphorylation, and activation of intracellular signaling pathways (59,64). Activation of ErbB4 by *Nrg1* may induce a marked increase in ErbB4 phosphorylation (65) and lead to a sustained activation of Akt and Erk (66). In addition, the Akt and Erk1/2 signaling cascades have an essential role in regulating gene expression and in preventing apoptosis (67). A wide spectrum of *in vivo* and *in vitro* studies have demonstrated that phosphorylation of Erk facilitates cell survival (68), and that the dephosphorylation of Akt is involved in the development of AD (58,69,70). These results indicated that *Nrg1* signaling in mouse cortical neurons is altered in response to the accumulation of A $\beta$ , suggesting that *Nrg1* may function as a crucial candidate for the prevention and treatment of AD.

In view of these observations, it was our hypothesis that, although *Nrg1* is up-regulated in cortical neurons during the early stages of AD to protect against abnormal changes in cell signaling, the phosphorylation levels of its receptors are relatively less responsive due to some unknown interrupting factors. As a consequence, *Nrg1* signaling is not able to function properly when ErbB4 is not adequately activated. In addition, sufficient levels of *Nrg1* are not expressed when the damage is prolonged, thus failing to prevent the development and progression of AD. Notably, the present study revealed that pretreatment of neural cells with r*Nrg1* $\beta$  partially reversed the neurotoxicity of A $\beta_{1-42}$ . These findings have provided a foundational basis for *Nrg1* signaling as a potential therapeutic target for the prevention, and possibly the treatment, of AD.

## Acknowledgements

We would like to thank the National Natural Science Foundation of China (grant nos. 81171138 and 81471279 to W-J. Z.) for support. This work was also supported by a Talent Support Grant from Shantou University Medical College (grant no. 250122 0118 to W-J. Z.).

## References

- Alzheimer's Association: 2013 Alzheimer's disease facts and figures. *Alzheimers Dement* 9: 208-245, 2013.
- Golebiowski M, Barcikowska M and Pfeffer A: Magnetic resonance imaging-based hippocampal volumetry in patients with dementia of the Alzheimer type. *Dement Geriatr Cogn Disord* 10: 284-288, 1999.
- Heun R, Mazanek M, Atzor KR, Tintera J, Gawehn J, Burkart M, Gänssicke M, Falkai P and Stoeter P: Amygdala-hippocampal atrophy and memory performance in dementia of Alzheimer type. *Dement Geriatr Cogn Disord* 8: 329-336, 1997.
- Fox NC, Warrington EK, Freeborough PA, Hartikainen P, Kennedy AM, Stevens JM and Rossor MN: Presymptomatic hippocampal atrophy in Alzheimer's disease. A longitudinal MRI study. *Brain* 119: 2001-2007, 1996.
- Querfurth HW and LaFerla FM: Alzheimer's disease. *N Engl J Med* 362: 329-344, 2010.
- D'Aniello A, Fisher G, Migliaccio N, Cammis G, D'Aniello E and Spinelli P: Amino acids and transaminases activity in ventricular CSF and in brain of normal and Alzheimer patients. *Neurosci Lett* 388: 49-53, 2005.
- Selkoe DJ: Alzheimer's disease results from the cerebral accumulation and cytotoxicity of amyloid beta-protein. *J Alzheimers Dis* 3: 75-80, 2001.
- Culmsee C and Landshamer S: Molecular insights into mechanisms of the cell death program: Role in the progression of neurodegenerative disorders. *Curr Alzheimer Res* 3: 269-283, 2006.
- Honda K, Casadesus G, Petersen RB, Perry G and Smith MA: Oxidative stress and redox-active iron in Alzheimer's disease. *Ann N Y Acad Sci* 1012: 179-182, 2004.
- Jenner P: Oxidative stress in Parkinson's disease. *Ann Neurol* 53 (Suppl 3): S26-S36; discussion S36-S38, 2003.
- Su JH, Anderson AJ, Cummings BJ and Cotman CW: Immunohistochemical evidence for apoptosis in Alzheimer's disease. *Neuroreport* 5: 2529-2533, 1994.
- Yagami T, Ueda K, Asakura K, Sakaeda T, Nakazato H, Kuroda T, Hata S, Sakaguchi G, Itoh N, Nakano T, *et al*: Gas6 rescues cortical neurons from amyloid beta protein-induced apoptosis. *Neuropharmacology* 43: 1289-1296, 2002.
- Wang DM, Li SQ, Zhu XY, Wang Y, Wu WL and Zhang XJ: Protective effects of hesperidin against amyloid- $\beta$  (A $\beta$ ) induced neurotoxicity through the voltage dependent anion channel 1 (VDAC1)-mediated mitochondrial apoptotic pathway in PC12 cells. *Neurochem Res* 38: 1034-1044, 2013.
- Schenk D, Barbour R, Dunn W, Gordon G, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K, Khan K, *et al*: Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* 400: 173-177, 1999.
- Shao BY, Xia Z, Xie Q, Ge XX, Zhang WW, Sun J, Jiang P, Wang H, Le WD, Qiu ZB, *et al*: Meserine, a novel carbamate AChE inhibitor, ameliorates scopolamine-induced dementia and alleviates amyloidogenesis of APP/PS1 transgenic mice. *CNS Neurosci Ther* 20: 165-171, 2014.
- Liu MY, Wang S, Yao WF, Zhang ZJ, Zhong X, Sha L, He M, Zheng ZH and Wei MJ: Memantine improves spatial learning and memory impairments by regulating NGF signaling in APP/PS1 transgenic mice. *Neuroscience* 273: 141-151, 2014.
- Sadowski M and Wisniewski T: Disease modifying approaches for Alzheimer's pathology. *Curr Pharm Des* 13: 1943-1954, 2007.
- Ritch PS, Carroll SL and Sontheimer H: Neuregulin-1 enhances survival of human astrocytic glioma cells. *Glia* 51: 217-228, 2005.
- Mei L and Xiong WC: Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. *Nat Rev Neurosci* 9: 437-452, 2008.
- Zhao WJ and Schachner M: Neuregulin 1 enhances cell adhesion molecule II expression in human glioma cells and promotes their migration as a function of malignancy. *J Neuropathol Exp Neurol* 72: 244-255, 2013.
- Zhao WJ: The expression and localization of neuregulin-1 (Nrg1) in the gastrointestinal system of the rhesus monkey. *Folia Histochem Cytobiol* 51: 38-44, 2013.
- Zhao W and Ren SG: Neuregulin-1 (Nrg1) is mainly expressed in rat pituitary gonadotroph cells and possibly regulates prolactin (PRL) secretion in a juxtacrine manner. *J Neuroendocrinol* 23: 1252-1262, 2011.
- Zhao W, Shen Y and Ren S: Endogenous expression of Neuregulin-1 (Nrg1) as a potential modulator of prolactin (PRL) secretion in GH3 cells. *Cell Tissue Res* 344: 313-320, 2011.
- Zhao WJ, Jiang Q and Mei JP: Neurohypophyseal Neuregulin 1 is derived from the hypothalamus as a potential prolactin modulator. *Neuroendocrinology* 102: 288-299, 2015.
- Wilson TR, Lee DY, Berry L, Shames DS and Settleman J: Neuregulin-1-mediated autocrine signaling underlies sensitivity to HER2 kinase inhibitors in a subset of human cancers. *Cancer Cell* 20: 158-172, 2011.
- Zhao WJ and Ren SG: Endogenous neuregulin-1 expression in the anterior pituitary of female Wistar-Furth rats during the estrous cycle. *Nan Fang Yi Ke Da Xue Xue Bao* 31: 921-927, 2011 (In Chinese).
- Liu J and Kern JA: Neuregulin-1 activates the JAK-STAT pathway and regulates lung epithelial cell proliferation. *Am J Respir Cell Mol Biol* 27: 306-313, 2002.
- Puricelli L, Proietti CJ, Labriola L, Salatino M, Balañá ME, Aguirre Ghiso J, Lupu R, Pignataro OP, Charreau EH, Bal de Kier Joffé E and Elizalde PV: Heregulin inhibits proliferation via ERKs and phosphatidylinositol 3-kinase activation but regulates urokinase plasminogen activator independently of these pathways in metastatic mammary tumor cells. *Int J Cancer* 100: 642-653, 2002.
- Falls DL: Neuregulins: Functions, forms, and signaling strategies. *Exp Cell Res* 284: 14-30, 2003.
- Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S, Brynjolfsson J, Gunnarsdottir S, Ivarsson O, Chou TT, *et al*: Neuregulin 1 and susceptibility to schizophrenia. *Am J Hum Genet* 71: 877-892, 2002.
- Britsch S: The neuregulin-1/ErbB signaling system in development and disease. *Adv Anat Embryol Cell Biol* 190: 1-65, 2007.
- Xu Z, Crosland DR, Harris AE, Ford GD and Ford BD: Extended therapeutic window and functional recovery after intraarterial administration of neuregulin-1 after focal ischemic stroke. *J Cereb Blood Flow Metab* 26: 527-535, 2006.
- Carlsson T, Schindler FR, Höllerhage M, Depboylu C, Arias-Carrión O, Schnurrbusch S, Rösler TW, Wozny W, Schwall GP, Groebe K, *et al*: Systemic administration of neuregulin-1 $\beta$  protects dopaminergic neurons in a mouse model of Parkinson's disease. *J Neurochem* 117: 1066-1074, 2011.
- Tokita Y, Keino H, Matsui F, Aono S, Ishiguro H, Higashiyama S and Oohira A: Regulation of neuregulin expression in the injured rat brain and cultured astrocytes. *J Neurosci* 21: 1257-1264, 2001.
- Chaudhury AR, Gerecke KM, Wyss JM, Morgan DG, Gordon MN and Carroll SL: Neuregulin-1 and erbB4 immunoreactivity is associated with neuritic plaques in Alzheimer disease brain and in a transgenic model of Alzheimer disease. *J Neuropathol Exp Neurol* 62: 42-54, 2003.
- Yarden Y and Sliwkowski MX: Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2: 127-137, 2001.
- Mei L and Nave KA: Neuregulin-ERBB signaling in the nervous system and neuropsychiatric diseases. *Neuron* 83: 27-49, 2014.
- Kanzaki H, Mizobuchi S, Obata N, Itano Y, Kaku R, Tomotsuka N, Nakajima H, Ouchida M, Nakatsuka H, Maeshima K and Morita K: Expression changes of the neuregulin 1 isoforms in neuropathic pain model rats. *Neurosci Lett* 508: 78-83, 2012.
- Maiese K, Li F and Chong ZZ: Erythropoietin in the brain: Can the promise to protect be fulfilled? *Trends Pharmacol Sci* 25: 577-583, 2004.
- Mattson MP: Pathways towards and away from Alzheimer's disease. *Nature* 430: 631-639, 2004.
- Chong ZZ, Li F and Maiese K: Oxidative stress in the brain: Novel cellular targets that govern survival during neurodegenerative disease. *Prog Neurobiol* 75: 207-246, 2005.
- Mattson MP: Oxidative stress, perturbed calcium homeostasis, and immune dysfunction in Alzheimer's disease. *J Neurovirol* 8: 539-550, 2002.
- Facecchia K, Fochesato LA, Ray SD, Stohs SJ and Pandey S: Oxidative toxicity in neurodegenerative diseases: Role of mitochondrial dysfunction and therapeutic strategies. *J Toxicol* 2011: 683728, 2011.
- Viegas CM, Tonin AM, Zanatta A, Seminotti B, Busanello EN, Fernandes CG, Moura AP, Leipnitz G and Wajner M: Impairment of brain redox homeostasis caused by the major metabolites accumulating in hyperornithinemia-hyperammonemia-homocitrullinuria syndrome *in vivo*. *Metab Brain Dis* 27: 521-530, 2012.
- Herbert V, Shaw S, Jayatilake E and Stopler-Kasdan T: Most free-radical injury is iron-related: It is promoted by iron, hemin, holoferitin and vitamin C, and inhibited by desferoxamine and apoferritin. *Stem Cells* 12: 289-303, 1994.

46. Guo WP, Fu XG, Jiang SM and Wu JZ: Neuregulin-1 regulates the expression of Akt, Bcl-2, and Bad signaling after focal cerebral ischemia in rats. *Biochem Cell Biol* 88: 649-654, 2010.
47. Calvo M, Zhu N, Tsantoulas C, Ma Z, Grist J, Loeb JA and Bennett DL: Neuregulin-ErbB signaling promotes microglial proliferation and chemotaxis contributing to microgliosis and pain after peripheral nerve injury. *J Neurosci* 30: 5437-5450, 2010.
48. Erlich S, Goldshmit Y, Lupowitz Z and Pinkas-Kramarski R: ErbB-4 activation inhibits apoptosis in PC12 cells. *Neuroscience* 107: 353-362, 2001.
49. Dimayuga FO, Ding Q, Keller JN, Marchionni MA, Seroogy KB and Bruce-Keller AJ: The neuregulin GGF2 attenuates free radical release from activated microglial cells. *J Neuroimmunol* 136: 67-74, 2003.
50. Cui W, Tao J, Wang Z, Ren M, Zhang Y, Sun Y, Peng Y and Li R: Neuregulin1beta1 antagonizes apoptosis via ErbB4-dependent activation of PI3-kinase/Akt in APP/PS1 transgenic mice. *Neurochem Res* 38: 2237-2246, 2013.
51. Yu W, Bonnet M, Farso M, Ma K, Chabot JG, Martin E, Torriglia A, Guan Z, McLaurin J, Quirion R and Krantic S: The expression of apoptosis inducing factor (AIF) is associated with aging-related cell death in the cortex but not in the hippocampus in the TgCRND8 mouse model of Alzheimer's disease. *BMC Neurosci* 15: 73, 2014.
52. Cecchi C, Fiorillo C, Baglioni S, Pensalfini A, Bagnoli S, Nacmias B, Sorbi S, Nosi D, Relini A and Liguri G: Increased susceptibility to amyloid toxicity in familial Alzheimer's fibroblasts. *Neurobiol Aging* 28: 863-876, 2007.
53. Miranda S, Opazo C, Larrondo LF, Muñoz FJ, Ruiz F, Leighton F and Inestrosa NC: The role of oxidative stress in the toxicity induced by amyloid beta-peptide in Alzheimer's disease. *Prog Neurobiol* 62: 633-648, 2000.
54. Hardy JA and Higgins GA: Alzheimer's disease: The amyloid cascade hypothesis. *Science* 256: 184-185, 1992.
55. Karran E, Mercken M and De Strooper B: The amyloid cascade hypothesis for Alzheimer's disease: An appraisal for the development of therapeutics. *Nat Rev Drug Discov* 10: 698-712, 2011.
56. Thangnipon W, Puangmalai N, Chinchalongporn V, Jantrachotechatchawan C, Kitiyanant N, Soi-Ampornkul R, Tuchinda P and Nobsathian S: N-benzylcinnamide protects rat cultured cortical neurons from  $\beta$ -amyloid peptide-induced neurotoxicity. *Neurosci Lett* 556: 20-25, 2013.
57. Chen TJ, Wang DC and Chen SS: Amyloid-beta interrupts the PI3K-Akt-mTOR signaling pathway that could be involved in brain-derived neurotrophic factor-induced Arc expression in rat cortical neurons. *J Neurosci Res* 87: 2297-2307, 2009.
58. Tong L, Balazs R, Thornton PL and Cotman CW: Beta-amyloid peptide at sublethal concentrations downregulates brain-derived neurotrophic factor functions in cultured cortical neurons. *J Neurosci* 24: 6799-6809, 2004.
59. Parkinson DB, Langner K, Namini SS, Jessen KR and Mirsky R: beta-Neuregulin and autocrine mediated survival of Schwann cells requires activity of Ets family transcription factors. *Mol Cell Neurosci* 20: 154-167, 2002.
60. Xu Z, Jiang J, Ford G and Ford BD: Neuregulin-1 is neuroprotective and attenuates inflammatory responses induced by ischemic stroke. *Biochem Biophys Res Commun* 322: 440-446, 2004.
61. Xu Z, Ford GD, Crosland DR, Jiang J, Gates A, Allen R and Ford BD: Neuroprotection by neuregulin-1 following focal stroke is associated with the attenuation of ischemia-induced pro-inflammatory and stress gene expression. *Neurobiol Dis* 19: 461-470, 2005.
62. Liu Y, Yu Y, Schachner M and Zhao W: Neuregulin 1- $\beta$  regulates cell adhesion molecule L1 expression in the cortex and hippocampus of mice. *Biochem Biophys Res Commun* 441: 7-12, 2013.
63. An T, Zhang Y, Huang Y, Zhang R, Yin S, Guo X, Wang Y, Zou C, Wei B, Lv R, *et al*: Neuregulin-1 protects against doxorubicin-induced apoptosis in cardiomyocytes through an Akt-dependent pathway. *Physiol Res* 62: 379-385, 2013.
64. Burden S and Yarden Y: Neuregulins and their receptors: A versatile signaling module in organogenesis and oncogenesis. *Neuron* 18: 847-855, 1997.
65. Vaskovsky A, Lupowitz Z, Erlich S and Pinkas-Kramarski R: ErbB-4 activation promotes neurite outgrowth in PC12 cells. *J Neurochem* 74: 979-987, 2000.
66. Di Segni A, Farin K and Pinkas-Kramarski R: ErbB4 activation inhibits MPP<sup>+</sup>-induced cell death in PC12-ErbB4 cells: Involvement of PI3K and Erk signaling. *J Mol Neurosci* 29: 257-267, 2006.
67. McCubrey JA, Steelman LS, Abrams SL, Lee JT, Chang F, Bertrand FE, Navolanic PM, Terrian DM, Franklin RA, D'Assoro AB, *et al*: Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv Enzyme Regul* 46: 249-279, 2006.
68. Junttila MR, Li SP and Westermarck J: Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. *FASEB J* 22: 954-965, 2008.
69. Jo J, Whitcomb DJ, Olsen KM, Kerrigan TL, Lo SC, Bru-Mercier G, Dickinson B, Scullion S, Sheng M, Collingridge G and Cho K: A $\beta$ (1-42) inhibition of LTP is mediated by a signaling pathway involving caspase-3, Akt1 and GSK-3 $\beta$ . *Nat Neurosci* 14: 545-547, 2011.
70. Ma T, Du X, Pick JE, Sui G, Brownlee M and Klann E: Glucagon-like peptide-1 cleavage product GLP-1(9-36) amide rescues synaptic plasticity and memory deficits in Alzheimer's disease model mice. *J Neurosci* 32: 13701-13708, 2012.