Full Paper

Neuregulin-1 Exerts Protective Effects Against Neurotoxicities Induced by C-Terminal Fragments of APP via ErbB4 Receptor

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Abstract. Neuregulin-1 (NRG1) plays important roles in the development and plasticity of the brain, and it is also reported to have potent neuroprotective properties. We previously reported that NRG1 has neuroprotective actions against Swedish amyloid precursor protein–induced neurotoxicity. In addition to the amyloid beta peptide, other metabolites of amyloid precursor protein (APP) such as the C-terminal fragments of APP (APP-CTs) have been reported to possess cytotoxic effects in neuronal cells. In this study, we investigated whether NRG1 exerts neuroprotective effects against APP-CTs and attempted to determine its neuroprotective mechanisms. NRG1 attenuated the neurotoxicities induced by the expression of APP-CTs in neuronal cells. NRG1 also reduced the accumulation of reactive oxygen species and attenuated mitochondrial membrane potential loss induced by APP-CTs. In addition, NRG1 upregulated the expression of the anti-apoptotic protein Bcl-2. This effect was blocked by the inhibition of ErbB4, a key NRG1 receptor. Taken together, these results demonstrate the neuroprotective potential of NRG1 in Alzheimer's disease.

Keywords: Alzheimer's disease, neuregulin-1, ErbB4, C-terminal fragment of amyloid precursor protein

Introduction

Neuregulin-1 (NRG1), a family of polypeptides that plays an important role in neural development, is reported to be involved in nerve cell differentiation and synapse formation (1, 2), radial glia formation and neuronal migration (3, 4), oligodendrocyte development and axon myelination (5, 6), axon navigation (7), neurite extension and arborization (8), and the expression of neurotransmitter receptors (9, 10). NRG1 and its receptor ErbB tyrosine kinase are expressed not only in the developing nervous system, but also in the adult brain.

Among a class of receptor tyrosine kinases including ErbB2, ErbB3, and ErbB4, NRG1 binds directly to ErbB3 and ErbB4 (11), and binds to ErbB2 only after first binding ErbB3 or ErbB4 (12). ErbB4 is likely to be the major mediator of NRG1 function in the brain (13).

NRG1 is expressed at synaptic regions in many brain areas, and its processing can be regulated by neuronal activity. Recent biochemical studies showed that ErbB4 is highly enriched in the postsynaptic density (PSD) of excitatory synapses (14 – 16) and GABAergic presynaptic terminals in the cerebral cortex (17). In the hippocampus, NRG1 mRNA is highly expressed in area CA3, a region presynaptic to area CA1 which displays ErbB4 expression (17, 18). Indeed, NRG1 regulates both excitatory and inhibitory synaptic transmission in the adult brain. Moreover, *in vitro* studies indicate that NRG1 can be neuroprotective for cortical neurons (19), motor neu-

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rons (20), dopaminergic neurons (21), cochlear sensory neurons (22), and PC12 cells (23, 24); it also protects neurons following ischemia (25–27). Recently, we showed upregulation of ErbB4 immunoreactivity in neurons from Alzheimer's disease (AD) patients and amyloid precursor protein (APP) / PS1 mice, a model of AD (28, 29). In addition, NRG1 protects against neurotoxicities induced by Swedish APP expression or amyloid beta peptide₁₋₄₂ ($A\beta_{1-42}$) treatment (30). In addition, NRG1 exerts protective effects against $A\beta$ -induced impairment of long-term potentiation in hippocampal slices (31). These findings suggest that NRG1/ErbB4 signaling might be important in cognition, learning, and memory formation because it modulates synaptic plasticity and neuronal survival. It is, therefore, a critical component in neurodegenerative disease.

AD is a neurodegenerative disorder characterized by sporadic deterioration of cognitive function resulting from the progressive degeneration and loss of neurons in the brain (32). $A\beta$ is a principal constituent of senile plaques (33) in the brains of patients with AD (34, 35). It is derived from APP, a type I transmembrane protein that is proteolytically processed by three enzymatic activities. The middle of the $A\beta$ region is cleaved by α -secretase and generates an 83-residue carboxyl terminal fragment (APP-CT), C83, whereas β -secretase primarily cuts at another site to generate a 99-residue APP-CT, C99. These APP-CTs are retained in the membrane and become substrates for intramembrane cleavage mediated by γ -secretase, generating C59 and $A\beta$. APP-CTs were initially identified in the brains of AD patients (36, 37) and were shown to either induce apoptosis or enhance other apoptotic stimuli (36).

In AD brains, degenerating neurons show evidence of oxidative stress, excessive calcium-mediated proteolysis and aggregates of hyperphosphorylated tau protein that form aberrant structures such as neuropil threads or neurofibrillary tangles (38, 39). AD and other neurodegenerative disorders share the following pathological features: increased levels of oxidative stress, metabolic compromise, perturbed calcium regulation, ER (endoplasmic reticulum) stress, mitochondrial dysfunction, DNA damage, activation of apoptotic biochemical cascades, and abnormalities in protein processing (40).

In this study, we investigate the neuroprotective aspects of NRG1 and its mechanism of action in cells overexpressing APP-CTs. Using this model, we demonstrate that NRG1 has neuroprotective effects. In addition, these neuroprotective effects require ErbB4, a key NRG1 receptor.

Materials and Methods

Reagents and antibodies

The NRG1 used in this study is a recombinant polypeptide containing the entire EGF domain of the β -type NRG1 from Prospec-Tany TechnoGene. It was prepared in 1% bovine serum albumin (BSA). AG1478 and AG879 were from Calbiochem (Darmstadt, Germany) and were dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA). Antibodies ErbB4, sc-283, sc-8050, ErbB2, and sc-284 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Bcl-2 (#2870) and Bax (#2774) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rhodamine 123 (Rh123, 83702) was from Sigma Aldrich (St. Louis, MO, USA). Experimentally verified control siRNA (negative, 1027280; positive, 1022564), ErbB4 siRNA (SI02223893), and ErbB2 siRNA (SI02223571) were purchased from QIAGEN (Hilden, Germany).

Plasmid constructs

The cDNA constructs of APP-C59 and APP-C100 were generated by PCR from human APP695 cDNA and encompassed the last 59 or 100 amino-acid residues, respectively. They were subcloned into a pcDNA3-flag vector with the flag at the N terminus. All constructs were sequenced using an ABI310 Sequencer.

Cell culture and transfection

SH-SY5Y human neuroblastoma cells obtained from the American Type Cell Collection (ATCC; Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing a 10% FBS, penicillin–streptomycin–amphotericin B mixture (Invitrogen) at 37°C and 5% CO₂. SH-SY5Y cells were transiently transfected with 1 μ g of plasmid DNA (APP-CT59, APP-CT100) and 3 μ l Lipofectamine 2000 (Invitrogen) in 100 μ l of DMEM without serum according to the manufacturer's instructions. Transient transfection efficiencies using Lipofectamine 2000 were assessed with GFP vector in SH-SY5Y cells. For cotransfection, 33 nM ErbB4 siRNA plus 0.2 μ g of the cDNA constructs or 40 nM ErbB2 siRNA plus 0.2 μ g of the cDNA constructs were added to 3×10^4 cells in each well of a 96-well plate using Lipofectamine 2000 and Opti-MEM (Invitrogen) without antibiotics.

Lactate dehydrogenase (LDH) release assay

Degrees of cell death were assessed by the activity of LDH released into the culture medium using a Cytotox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

The results are expressed as a percentage of maximum LDH release obtained upon complete cell lysis.

Measurement of reactive oxygen species (ROS) generation

Intracellular ROS in SH-SY5Y cells were assayed using the dye 2',7'-dichlorofluorescein diacetate (DCFH-DA, Invitrogen). Cells were washed with HEPES-buffered saline and incubated in the dark for 1 h in HEPES-buffered saline containing 200 μ M DCFH-DA. Upon incubation, DCFH-DA is taken up by cells, where intracellular esterase cleaves the molecule to 2',7'-dichlorofluorescein, which is oxidized to dichlorofluorescein in the presence of H₂O₂. The total fluorescence was measured using a spectrofluorometer (Victor 2; PerkinElmer, Shelton, CT, USA) at an emission wavelength of 529 nm and an excitation wavelength of 504 nm (41).

Assessment of mitochondrial membrane potential ($\Delta\psi$ m)

The potentiometric probe Rh123 (Sigma) was used to measure the perturbations in the $\Delta\psi$ m. Rh123 is a fluorescence probe that selectively enters mitochondria with an intact membrane potential and is retained in the mitochondria. Its fluorescence intensity in the mitochondria quantitatively decreases in response to dissipation of the $\Delta\psi$ m. Cells were carefully washed with PBS and Rh123 (final concentration: 2.5 μ g/ml in 5 mM HEPES-buffered saline) was added to the cells. Cells were incubated at 37°C in the dark for 1 h. At the end of the exposure period, culture supernatants were discarded and cells were carefully rinsed with PBS before addition of 200 μ l per well of fresh HEPES-buffered saline. Fluorescence was directly measured in each well at an excitation wavelength of 485 $\pm$ 20 nm and an emission wavelength of 530 $\pm$ 25 nm with a spectrofluorometer (Victor 2, PerkinElmer) (42).

Superoxide dismutase (SOD) activity assay

Cells were washed with PBS and then collected by scraping. Collected cells were homogenized with lysis buffer (20 mM HEPES, pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose) and then centrifuged at 1500 $\times$ g for 5 min at 4°C. A 10- μ l aliquot of the supernatant was added to each well, followed by 190 μ l of the diluted radical detector. SOD activity was measured using a colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. The kit utilizes water-soluble tetrazolium salt, which produces a water-soluble formazan dye upon reaction with superoxide anion and this was detected at 450 nm using the Victor X3 Multilable plate reader (PerkinElmer). SOD activity was calculated on the basis

of the percent inhibition of superoxide anion.

Western blotting

Western blotting was performed as previously described (17). Briefly, cells were lysed in a modified RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.25% sodium-deoxycholate, 1 mM PMSF, 1 mM EDTA, 1 μ g/ml aprotinin, leupeptin, and pepstatin protease inhibitors). Proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane, which was blocked with TBS containing 5% nonfat dry milk and 0.05% Tween 20 for 1 h. The membrane was then incubated overnight at 4°C with primary antibodies and developed by horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis

Data are each presented as the mean $\pm$ S.E.M. of three or more independent experiments. Student's paired *t*-test was used for comparison of means from the same group of cells. Values of *P* < 0.05 were considered significant.

Results

NRG1 attenuates neuronal cell death induced by expression of APP-CTs in SH-SY5Y cells

In this study, we investigated the effects of NRG1 on cytotoxicities induced by APP-CTs, ROS production, SOD activity, $\Delta\psi$ m loss, and Bax/Bcl-2 expression in SH-SY5Y cells employing a transient transfection system. Under our experimental conditions, the efficiencies of transient transfections using Lipofectamine 2000 were assessed with a GFP vector in SH-SY5Y cells. The transfection efficiencies were 45% $\pm$ 5% (*n* = 8) in SH-SY5Y cells, as confirmed by the expression of GFP fluorescence (data not shown).

It was previously reported that APP-CTs play a role in inducing neuronal death in AD (43). To determine whether NRG1 affects the cytotoxicities observed in SH-SY5Y cells through the expression of APP-CTs, an LDH release assay was performed 36 h after the transfection of APP-CTs to determine whether NRG1 affected cytotoxicity induced by APP-CTs in SH-SY5Y cells. A 30-h treatment with NRG1 6 h after transfection reduced APP-CT100-induced LDH release in a concentration-dependent manner (concentration of NRG1: 0.5, 1, 5, and 10 nM) (Fig. 1A). Based on this result, we used the concentration of 5 nM for further experiments.

LDH release, as expressed by the percentage of maximal LDH release obtained upon complete cell lysis, increased to 14.33% $\pm$ 1.33% and 20.00% $\pm$ 1.16% (*n* = 6) 36 h after transfection of APP-CT59 and APP-CT100,

Fig. 1. NRG1 attenuates the neuronal cell death induced by expression of APP-CTs. A) NRG1 attenuates the cell death induced by APP-CT100 expression in a dose-dependent manner. After 36 h, the degrees of cell death were assessed by LDH activity in the medium. NRG1 inhibits LDH release in a dose-dependent manner. $n = 6$, $*P < 0.05$, $**P < 0.01$. B) NRG1 (5 nM) attenuates the cell death induced by expression of APP-CTs in SH-SY5Y cells. Cytotoxicity was assessed by LDH activity in the medium. $n = 6$, $*P < 0.05$ (vs. mock), $#P < 0.05$ (vs. NRG1 treated). C) Representative photographs of the cells. Scale bar, 100 μm .

respectively, versus non-treated control cells. Pretreatment with NRG1 at 5 nM for 30 h significantly reduced LDH release to $6.00\% \pm 2.00\%$ and $6.67\% \pm 1.33\%$ in APP-CT59- and APP-CT100-expressing SH-SY5Y cells, respectively (Fig. 1B). Representative images of the cells are shown in Fig. 1C. These results indicate that NRG1 exerts a significant protective effect over cell death induced by expression of APP-CTs.

NRG1 reduces ROS accumulation induced by expression of APP-CTs in SH-SY5Y cells

We examined whether NRG1 affects the ROS accumulation induced by the expression of APP-CTs. As shown in Fig. 2, A and B, treatment with NRG1 inhibited ROS accumulation induced by APP-CTs in SH-SY5Y cells. ROS levels increased to $273.33\% \pm 14.53\%$ and $296.67\% \pm 8.81\%$ in APP-CT59- and APP-CT100-expressing SH-SY5Y cells, respectively, versus nontreated control cells. NRG1 treatment reduced ROS levels to

$126.67\% \pm 8.81\%$ and $140.00\% \pm 15.28\%$ ($n = 10$) in APP-CT59- and APP-CT100-expressing SH-SY5Y cells, respectively (Fig. 2B). Taken together, these observations demonstrate that NRG1 may regulate the generation of ROS in AD.

NRG1 prevented the increase in SOD activity induced by APP-CTs in SH-SY5Y cells

SOD catalyzes the dismutation of the superoxide anion radical to hydrogen peroxide (44). We found that the expression of APP-CTs upregulated SOD activity and that NRG1 treatment prevented the upregulation of SOD activity caused by the expression of APP-CTs (Fig. 3).

It has been reported previously that treatment with $A\beta_{25-35}$ increased SOD activity whereas, olanzapine, an antipsychotic that showed protective effects against $A\beta_{25-35}$ -induced oxidative stress in PC 12 cells, reduced the increase in enzyme activity (44). It has been suggested that the increase in SOD activity indicates a

Fig. 2. NRG1 reduces ROS accumulation induced by expression of APP-CTs. A) After 24 h, ROS were detected by fluorescence microscopy using DCFH-DA dye in vehicle- or 5 nM NRG1-treated SH-SY5Y cells expressing APP-CTs. B) ROS production was also measured using DCFH-DA dye in vehicle- or 5 nM NRG1-treated SH-SY5Y cells expressing mock, APP-CT59, or APP-CT100. $n = 10$, * $P < 0.05$ (vs. mock), # $P < 0.05$ (vs. NRG1-treated). Fluorescence of cells in 96-well plates was read at 504 nm (excitation) and 529 nm (emission) using a fluorescence plate reader.

Fig. 3. NRG1 diminishes the upregulation of SOD activity induced by expression of APP-CTs. Cells were seeded at 8×10^5 in a six-well plate and transfected with APP-CTs. Then, cells were treated with 5 nM NRG1 for 24 h and then harvested. SOD activity was measured by utilizing water-soluble tetrazolium salt, which produces a water-soluble formazan dye upon reaction with superoxide anion, and the product that was detected at 450 nm using a Victor X3 Multilable plate reader (PerkinElmer). SOD activity was calculated on the basis of the percent inhibition of superoxide anion. * $P < 0.05$ (vs. mock), # $P < 0.05$ (vs. NRG1-treated).

compensatory action of the cells exposed to the neurotoxic stimuli to maintain cellular redox status. Our results show that NRG1 treatment prevented the upregulation of SOD activity caused by the expression of APP-CTs

expression.

NRG1 rescues $\Delta\psi_m$ loss induced by the expression of APP-CTs in SH-SY5Y cells

$\Delta\psi_m$ has been reported to play a key role in apoptosis induction by regulating the mitochondrial permeability transition pore opening (45). To determine whether NRG1 affects the regulation of $\Delta\psi_m$, we performed further experiments. The Rh123 fluorescent probe was used for evaluating the $\Delta\psi_m$ (46). As shown in Fig. 4, NRG1 reduced the number of the cells showing $\Delta\psi_m$ loss induced by the expression of APP-CTs (CT59, CT100). These results indicate that NRG1 protects against the mitochondrial dysfunction induced by the expression of APP-CTs.

NRG1 alters the protein levels of Bcl-2 induced by APP-CTs in SH-SY5Y cells

Programmed cell death or apoptosis, the critical process for neurodegeneration, involves multiple signaling pathways and crucial modulators of cell survival, such as the Bcl-2 family of proteins. To determine whether NRG1 regulates the Bcl-2 family of proteins, we investigated changes in the levels of Bax (a pro-apoptotic protein) and Bcl-2 (an anti-apoptotic protein). APP-CTs

Fig. 4. NRG1 attenuates membrane potential loss induced by expression of APP-CTs. SH-SY5Y cells were transfected with mock, APP-CT59, or APP-CT100 in the absence or presence of 5 nM NRG1 for 24 h. $n = 6$, * $P < 0.05$ (vs. mock), # $P < 0.05$ (vs. NRG1-treated) Mitochondrial membrane potentials ($\Delta\psi_m$) were measured by spectrofluorometer using Rhodamine 123.

resulted in a significant decrease in the protein levels of Bcl-2, whereas APP-CTs had no effect on the levels of Bax. NRG1 attenuated the reduction in Bcl-2 protein caused by APP-CTs (Fig. 5: A and B). These results suggest that NRG1 may regulate the altered Bcl-2/Bax expression observed in neuronal cell death induced by various neurotoxic stimuli.

ErbB4 is necessary for the effects of NRG1 on neuronal cell death

Of the three ErbB kinases, ErbB2 and ErbB4, but not ErbB3, are catalytically active (47). To determine which ErbB is involved in the regulation of cytotoxicity by NRG1, cells were treated either with AG1478 or AG879, specific inhibitors of ErbB4 or ErbB2, respectively. ErbB4 tyrosine phosphorylation in response to NRG1 was blocked in neurons pretreated with AG 1478, but not AG879 (17). ErbB4 or ErbB2 were detected in SH-SY5Y cells (Fig. 6C).

Treatment with AG1478 prevented NRG1 from reducing LDH release induced by APP-CTs (APP-CT59 or APP-CT100), but treatment with AG879 did not (Fig. 6A). Moreover, we confirmed that knock-down of ErbB4 by siRNA for ErbB4 also prevented the neuroprotective effects of NRG-1 against cytotoxicity induced by APP-CTs (Fig. 6B). These results suggest a role for ErbB4 in the regulation of neuronal cell death by NRG1. ErbB4-specific siRNA (33 nM) or ErbB2-specific siRNA (40 nM) downregulated ErbB4 and ErbB2 mRNA in SH-SY5Y cells by 85.00% and 90.00%, respectively. The knock-down effects of siRNAs for ErbB4 or ErbB2 are shown in Fig. 6C.

B

Fig. 5. The effect of NRG1 on the protein levels of Bcl-2 family members induced by expression of APP-CTs. A) Representative immunoblot for Bax and Bcl-2 is shown for SH-SY5Y cells treated with vehicle or combinations of 5 nM NRG1 and APP-CTs for 36 h. B) Quantification of data in panel A. $n = 6$, * $P < 0.05$. Bax or Bcl-2 was quantified and normalized to actin and as fold difference compared to that in vehicle or mock.

Discussion

Previously, we demonstrated that NRG1 treatment protected neuronal cells against Swedish APP expression or $A\beta_{1-42}$ treatment. In the present study, we investigated whether NRG1 has a protective effect against cytotoxicity induced by APP-CTs (APP-CT59 and APP-CT100) in SH-SY5Y cells. The expression of APP-CTs has been shown to induce apoptosis in NGF-differentiated PC 12 cells (41).

Here, we found that the expression of APP-CTs in-

Fig. 6. Inhibition of ErbB4 blocks NRG1 attenuation of neuronal cell death. **A)** SH-SY5Y cells were transfected with mock, APP-CT59, or APP-CT100. The cells were treated with 5 μ M AG1478 or 5 μ M AG879 followed by treatment with or without 5 nM NRG1 6 h after transfection of APP-CTs. The degree of cell death was assessed using LDH activity in the medium 36 h after transfection of APP-CTs. $n = 6$, $*P < 0.05$. **B)** Effects of treatment with ErbB4 or ErbB2 siRNA. SH-SY5Y cells were cotransfected with ErbB siRNA and a cDNA construct (mock, APP-CT59, or APP-CT100). The cells were cultured in the presence of 5 nM NRG1 for 30 h. The degree of cell death was assessed 36 h after cotransfection through the quantification of LDH activity in the medium. **C)** ErbB4 and ErbB2 protein levels after treatment with ErbB4 or ErbB2 siRNA. β -Actin was used as a loading control.

duces ROS accumulation, reduction of $\Delta\psi_m$, and down-regulation of Bcl-2 expression. In addition, expression of APP-CTs upregulates SOD activity, which seems to be a compensatory action for the increase in ROS induced by APP-CTs. NRG1 was shown to exert a significant protective effect over the cytotoxicity induced by the expression of APP-CTs. NRG1 reduces ROS and restores $\Delta\psi_m$ loss caused by the expression of APP-CTs. Moreover, NRG1 rescues the decrease in Bcl-2 protein levels by the expression of APP-CTs. We also confirmed that these neuroprotective effects required ErbB4, a key NRG1 receptor, by using AG1478, a specific inhibitor for ErbB4, and a knock-down strategy with siRNA for ErbB4.

AD is characterized by the progressive impairment of cognition and behavioral disturbance that strongly correlate with the degeneration and death of neurons in the cerebral cortex and limbic brain areas such as the hippocampus and the amygdala (38). Studies of postmortem brain tissue have provided evidence for increased levels of cellular oxidative stress in vulnerable regions of the AD brain in comparison with tissue from age-matched neurologically normal control patients (48, 49).

Furthermore, enhanced generation of ROS is mediated by $A\beta$ or other metabolites of APP, such as mitochondrial dysfunction by APP-CTs. Mitochondria are central to the maintenance of cell function and viability. Mitochondrial damage has been considered as the “point of no return” in the cell death cascade triggered in neurons after an apoptotic insult. The $\Delta\psi_m$ is an electrochemical potential that consists of a transmembrane electrical potential and a proton gradient. The $\Delta\psi_m$ reflects the functional status of mitochondria. The present study provides evidence that NRG1 modulates ROS levels and $\Delta\psi_m$ loss generated by APP-CTs, although further study is required to clarify the underlying mechanisms of how NRG1 prevents ROS generation or accumulation.

Multiple lines of evidence demonstrate that the regulation of the Bcl-2 protein family members is crucial for the maintenance of mitochondrial integrity and function, thereby deciding a cell’s fate after severe stress (50). Wide-spectrum gene expression studies show a depletion of the anti-apoptotic members of the Bcl-2 gene family in AD hippocampus and superior temporal lobe neocortex and a shift in expression toward the more pro-apoptotic Bcl-2 family species Bax, Bad, Bid, and Bik (51). It is noteworthy that in this study, NRG1 up-regulates Bcl-2 protein in cells transfected with APP-CTs. However, the detailed mechanism involved remains to be clarified.

ErbB4 is also a substrate for γ -secretase, similar to APP. A recent study showed that ErbB4 is first cleaved by TACE to release a soluble extracellular peptide that

contains the NRG1 binding site (ecto-ErbB4). The remaining membrane-anchored 80-kDa fragment (that is, ErbB4-CTF) is further cleaved in its transmembrane domain by presenilin-dependent γ -secretase to release the ErbB4 intracellular domain (ErbB4-ICD), which has been shown to translocate to the nucleus and regulate transcription (52, 53). More work is needed to determine whether ErbB4-ICD has a physiological function in neurons. It is possible that the balance between the generation of the APP intracellular domain (AICD) and ErbB4-ICD are associated with AD. Our results, however, suggest otherwise; NRG1/ErbB4 signaling may be involved in AD neuropathology. Further investigation of NRG1's role in AD could be useful in understanding the pathogenesis of AD.

Collectively, our results clearly show that NRG1 exerts neuroprotective actions in AD experimental systems mediated by APP-CTs and that ErbB4 is required for these effects of NRG1.

These results suggest that NRG1/ErbB4 signaling may regulate the activity of neurons, providing insight into potential mechanisms by which this trophic factor regulates neuronal stress and pathogenesis of AD.

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References

- Buonanno A, Fischbach GD. Neuregulin and ErbB receptor signaling pathways in the nervous system. *Curr Opin Neurobiol.* 2001;11:287–296.
- Corfas G, Roy K, Buxbaum JD. Neuregulin 1-erbB signaling and the molecular/cellular basis of schizophrenia. *Nat Neurosci.* 2004;7:575–580.
- Anton ES, Marchionni MA, Lee KF, Rakic P. Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. *Development.* 1997;124:3501–3510.
- Rio C, Rieff HI, Qi P, Khurana TS, Corfas G. Neuregulin and erbB receptors play a critical role in neuronal migration. *Neuron.* 1997;19:39–50.
- Calaora V, Rogister B, Bismuth K, Murray K, Brandt H, Leprince P, et al. Neuregulin signaling regulates neural precursor growth and the generation of oligodendrocytes in vitro. *J Neurosci.* 2001;21:4740–4751.
- Fernandez PA, Tang DG, Cheng L, Prochiantz A, Mudge AW, Raff MC. Evidence that axon-derived neuregulin promotes oligodendrocyte survival in the developing rat optic nerve. *Neuron.* 2000;28:81–90.
- Lopez-Bendito G, Cautinat A, Sanchez JA, Bielle F, Flames N, Garratt AN, et al. Tangential neuronal migration controls axon guidance: a role for neuregulin-1 in thalamocortical axon navigation. *Cell.* 2006;125:127–142.
- Gerecke KM, Wyss JM, Carroll SL. Neuregulin-1beta induces neurite extension and arborization in cultured hippocampal neurons. *Mol Cell Neurosci.* 2004;27:379–393.
- Liu Y, Ford B, Mann MA, Fischbach GD. Neuregulins increase alpha7 nicotinic acetylcholine receptors and enhance excitatory synaptic transmission in GABAergic interneurons of the hippocampus. *J Neurosci.* 2001;21:5660–5669.
- Okada M, Corfas G. Neuregulin1 downregulates postsynaptic GABAA receptors at the hippocampal inhibitory synapse. *Hippocampus.* 2004;14:337–344.
- Carraway KL 3rd, Weber JL, Unger MJ, Ledesma J, Yu N, Gassmann M, et al. Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. *Nature.* 1997;387:512–516.
- Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol.* 2001;2:127–137.
- Falls DL. Neuregulins: functions, forms, and signaling strategies. *Exp Cell Res.* 2003;284:14–30.
- Garcia RA, Vasudevan K, Buonanno A. The neuregulin receptor ErbB-4 interacts with PDZ-containing proteins at neuronal synapses. *Proc Natl Acad Sci U S A.* 2000;97:3596–3601.
- Huang YZ, Won S, Ali DW, Wang Q, Tanowitz M, Du QS, et al. Regulation of neuregulin signaling by PSD-95 interacting with ErbB4 at CNS synapses. *Neuron.* 2000;26:443–455.
- Li B, Woo RS, Mei L, Malinow R. The neuregulin-1 receptor erbB4 controls glutamatergic synapse maturation and plasticity. *Neuron.* 2007;54:583–597.
- Woo RS, Li XM, Tao Y, Carpenter-Hyland E, Huang YZ, Weber J, et al. Neuregulin-1 enhances depolarization-induced GABA release. *Neuron.* 2007;54:599–610.
- Law AJ, Shannon Weickert C, Hyde TM, Kleinman JE, Harrison PJ. Neuregulin-1 (NRG-1) mRNA and protein in the adult human brain. *Neuroscience.* 2004;127:125–136.
- Li BS, Ma W, Jaffe H, Zheng Y, Takahashi S, Zhang L, et al. Cyclin-dependent kinase-5 is involved in neuregulin-dependent activation of phosphatidylinositol 3-kinase and Akt activity mediating neuronal survival. *J Biol Chem.* 2003;278:35702–35709.
- Ricart K, J Pearson R Jr, Viera L, Cassina P, Kamaid A, Carroll SL, et al. Interactions between beta-neuregulin and neurotrophins in motor neuron apoptosis. *J Neurochem.* 2006;97:222–233.
- Zhang L, Fletcher-Turner A, Marchionni MA, Apparsundaram S, Lundgren KH, Yurek DM, et al. Neurotrophic and neuroprotective effects of the neuregulin glial growth factor-2 on dopaminergic neurons in rat primary midbrain cultures. *J Neurochem.* 2004;91:1358–1368.
- Stankovic K, Rio C, Xia A, Sugawara M, Adams JC, Liberman MC, et al. Survival of adult spiral ganglion neurons requires erbB receptor signaling in the inner ear. *J Neurosci.* 2004;24:8651–8661.
- Goldshmit Y, Erlich S, Pinkas-Kramarski R. Neuregulin rescues PC12-ErbB4 cells from cell death induced by H(2)O(2). Regulation of reactive oxygen species levels by phosphatidylinositol 3-kinase. *J Biol Chem.* 2001;276:46379–46385.
- Di Segni A, Shaharabani E, Stein R, Pinkas-Kramarski R. Neuregulins rescue PC12-ErbB-4 cells from cell death induced by beta-amyloid peptide: involvement of PI3K and PKC. *J Mol Neurosci.* 2005;26:57–69.
- Li Y, Xu Z, Ford GD, Crosland DR, Cairobe T, Li Z, et al. Neuroprotection by neuregulin-1 in a rat model of permanent focal cerebral ischemia. *Brain Res.* 2007;1184:277–283.
- Shyu WC, Lin SZ, Chiang MF, Yang HI, Thajeb P, Li H. Neu-

- regulin-1 reduces ischemia-induced brain damage in rats. *Neurobiol Aging*. 2004;25:935–944.
- 27 Crosland DR, Schoell MC, Ford GD, Pulliam JV, Gates A, Clement CM, et al. Neuroprotective effects of neuregulin-1 on B35 neuronal cells following ischemia. *Brain Res*. 2008;1210:39–47.
 - 28 Woo RS, Lee JH, Yu HN, Song DY, Baik TK. Expression of ErbB4 in the neurons of Alzheimer's disease brain and APP/PS1 mice, a model of Alzheimer's disease. *Anat Cell Biol*. 2011;44:116–127.
 - 29 Woo RS, Lee JH, Yu HN, Song DY, Baik TK. Expression of ErbB4 in the apoptotic neurons of Alzheimer's disease brain. *Anat Cell Biol*. 2010;43:332–339.
 - 30 Min SS, An J, Lee JH, Seol GH, Im JH, Kim HS, et al. Neuregulin-1 prevents amyloid beta-induced impairment of long-term potentiation in hippocampal slices via ErbB4. *Neurosci Lett*. 2011;505:6–9.
 - 31 Woo RS, Lee JH, Kim HS, Baik CH, Song DY, Suh YH, et al. Neuregulin-1 protects against neurotoxicities induced by Swedish amyloid precursor protein via the ErbB4 receptor. *Neuroscience*. 2012;202:413–423.
 - 32 McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology*. 1984;34:939–944.
 - 33 Glenner GG, Wong CW. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun*. 1984;120:885–890.
 - 34 Selkoe DJ. Alzheimer's disease: a central role for amyloid. *J Neuropathol Exp Neurol*. 1994;53:438–447.
 - 35 Checler F. Processing of the beta-amyloid precursor protein and its regulation in Alzheimer's disease. *J Neurochem*. 1995;65:1431–1444.
 - 36 Passer B, Pellegrini L, Russo C, Siegel RM, Lenardo MJ, Schettini G, et al. Generation of an apoptotic intracellular peptide by gamma-secretase cleavage of Alzheimer's amyloid beta protein precursor. *J Alzheimers Dis*. 2000;2:289–301.
 - 37 Kim HS, Kim EM, Lee JP, Park CH, Kim S, Seo JH, et al. C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3beta expression. *FASEB J*. 2003;17:1951–1953.
 - 38 Mattson MP. Pathways towards and away from Alzheimer's disease. *Nature*. 2004;430:631–639.
 - 39 Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev*. 2001;81:741–766.
 - 40 Culmsee C, Landshamer S. Molecular insights into mechanisms of the cell death program: role in the progression of neurodegenerative disorders. *Curr Alzheimer Res*. 2006;3:269–283.
 - 41 Joo Y, Kim HS, Woo RS, Park CH, Shin KY, Lee JP, et al. Mefenamic acid shows neuroprotective effects and improves cognitive impairment in in vitro and in vivo Alzheimer's disease models. *Mol Pharmacol*. 2006;69:76–84.
 - 42 Lautraite S, Bigot-Lasserre D, Bars R, Carmichael N. Optimisation of cell-based assays for medium throughput screening of oxidative stress. *Toxicol In Vitro*. 2003;17:207–220.
 - 43 Suh YH, Checler F. Amyloid precursor protein, presenilins, and alpha-synuclein: molecular pathogenesis and pharmacological applications in Alzheimer's disease. *Pharmacol Rev*. 2002;54:469–525.
 - 44 Wang H, Xu H, Dyck LE, Li XM. Olanzapine and quetiapine protect PC12 cells from beta-amyloid peptide(25-35)-induced oxidative stress and the ensuing apoptosis. *J Neurosci Res*. 2005;81:572–580.
 - 45 Petronilli V, Cola C, Bernardi P. Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore. II. The minimal requirements for pore induction underscore a key role for transmembrane electrical potential, matrix pH, and matrix Ca²⁺. *J Biol Chem*. 1993;268:1011–1016.
 - 46 Palmeira CM, Moreno AJ, Madeira VM, Wallace KB. Continuous monitoring of mitochondrial membrane potential in hepatocyte cell suspensions. *J Pharmacol Toxicol Methods*. 1996;35:35–43.
 - 47 Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. *Nat Rev Mol Cell Biol*. 2006;7:505–516.
 - 48 Chong ZZ, Li F, Maiese K. Oxidative stress in the brain: novel cellular targets that govern survival during neurodegenerative disease. *Prog Neurobiol*. 2005;75:207–246.
 - 49 Mattson MP. Oxidative stress, perturbed calcium homeostasis, and immune dysfunction in Alzheimer's disease. *J Neurovirol*. 2002;8:539–550.
 - 50 Polster BM, Fiskum G. Mitochondrial mechanisms of neural cell apoptosis. *J Neurochem*. 2004;90:1281–1289.
 - 51 Lukiw WJ, Bazan NG. Survival signalling in Alzheimer's disease. *Biochem Soc Trans*. 2006;34:1277–1282.
 - 52 Ni CY, Murphy MP, Golde TE, Carpenter G. gamma-Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science*. 2001;294:2179–2181.
 - 53 Lee HJ, Jung KM, Huang YZ, Bennett LB, Lee JS, Mei L, et al. Presenilin-dependent gamma-secretase-like intramembrane cleavage of ErbB4. *J Biol Chem*. 2002;277:6318–6323.