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Research Report

Neuroprotective effects of neuregulin-1 on B35 neuronal cells following ischemia

*DaJoie R. Croslan, Matthew C. Schoell, Gregory D. Ford, John V. Pulliam, Alicia Gates, Ceilessia M. Clement, Adalynn E. Harris, Byron D. Ford**

Department of Anatomy and Neurobiology, Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA 30310, USA

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ABSTRACT

We previously showed that neuregulin-1 (NRG-1) protected neurons from death *in vivo* following focal ischemia. The goal of this study was to develop an *in vitro* rat ischemia model to examine the cellular and molecular mechanisms involved in the neuroprotective effects of NRG-1 on ischemia-induced neuronal death. Rat B-35 neuroblastoma cells differentiated by serum withdrawal, developed enhanced neuronal characteristics including, neurite extension and upregulation of neuronal markers of differentiation. When B35 neurons were subjected to oxygen glucose deprivation (OGD)/reoxygenation or glutamate, widespread neuronal death was seen after both treatments. Treatment with NRG-1 immediately after OGD significantly increased neuronal survival. NRG-1 administration also resulted in a significant decrease in annexin V, an early marker of apoptosis. However, the neurotoxic actions of glutamate were unaffected by NRG-1. The neuroprotective effects of NRG-1 were prevented by an inhibitor of the phosphatidylinositol-3-kinase/Akt pathway. These results provide a new model to gain insight into the mechanisms employed by NRG-1 to protect neurons from ischemic brain injury.

1. Introduction

Ischemic stroke results in cellular energy depletion, excessive neuronal depolarization and massive glutamate release from neurons (del Zoppo et al., 2000; Dirnagl et al., 1999; Iadecola and Alexander, 2001; Lee et al., 2000). The excess glutamate released overstimulates *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, ultimately leading to glutamate-induced excitotoxicity and neuronal death. This excitotoxic neuronal injury also triggers inflammatory and oxidative stress responses that lead to a delayed apoptotic neuronal death. A number of recent reviews have described the delayed apoptotic and inflammatory mechanisms that occur in neuronal cells following ischemia (Chan, 2004; del Zoppo et al., 2000; Dirnagl et al., 1999; Iadecola and Alexander, 2001; Lee et al., 2000; Lo et al., 2005; Ouyang and Giffard, 2004).

The neuregulins are a family of multipotent growth factors that includes acetylcholine receptor inducing activities (ARIAs), glial growth factors (GGFs), heregulins and neu differentiation factors (NDFs) (Falls et al., 1993; Ho et al., 1995; Holmes et al., 1992; Marchionni et al., 1993; Wen et al., 1992). Neuregulin-1 (NRG-1) has been shown to promote the survival and function of neuronal and non-neuronal cell types (Bermingham-McDonogh et al., 1996; Buonanno and Fischbach, 2001; Erlich et al.,

* *Corresponding author*. Department of Anatomy and Neurobiology, Neuroscience Institute, Morehouse School of Medicine, 720 Westview Drive, SW; MRC 222, Atlanta, GA 30310, USA. Fax: +1 404 752 1041.
E-mail address: bford@msm.edu (B.D. Ford).

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2001; Goldshmit et al., 2001; Vaskovsky et al., 2000; Verdi et al., 1996). NRG-1 binds to erbB receptors and can signal through various pathways, including the phosphatidylinositol-3-kinase (PI3K)/Akt pathway (Buonanno and Fischbach, 2001; Burden and Yarden, 1997; Falls, 2003). Several recent reports from our laboratory and others demonstrated that NRG-1 is neuroprotective *in vivo* following ischemia (Guo et al., 2006; Shyu et al., 2004; Xu et al., 2006; Xu et al., 2005, 2004). The effects of NRG-1 were shown to be associated with an inhibition of mechanisms associated with inflammation and neuronal apoptosis (Shyu et al., 2004; Xu et al., 2005; Xu et al., 2004).

In this study, we use a rat B35 neuronal cell line (Schubert et al., 1974) to further elucidate the molecular mechanisms associated with the direct neuroprotective effects of NRG-1 in ischemia. Many neuronal cell lines require use of growth factors to differentiate into neurons. However, the use of growth factors for differentiation could complicate results seen when examining the effect of growth factors such as NRG-1. The advantage of B-35 neurons is the ability to differentiate simply upon serum withdrawal. They develop large growth cones and extend long neurites when grown in low serum in the absence of endogenous growth factors (Otey et al., 2003; Schubert et al., 1974). Here, we further characterize the differentiation of B35 neurons to determine their suitability to study ischemia *in vitro*. We also examine whether NRG-1 can directly prevent ischemia-induced neuronal death *in vitro* by activating the PI3K/Akt survival pathway.

2. Results

2.1. *Characterization of B35 neurons upon serum withdrawal*

Rat B35 neuroblastoma cells were derived from tumors of the neonatal rat central nervous system and display neuronal properties such as membrane excitability and expression of enzymes for neurotransmitter release, including acetylcholinesterase, and glutamic acid decarboxylase upon differentiation by serum withdrawal (Schubert et al., 1974; Vinores et al., 1984). We examined the expression specific neuronal markers in undifferentiated B35 cells and upon serum withdrawal. As previously reported, B35 cells grown in serum are round, phase-bright cells with few, short processes (Fig. 1A). Upon serum withdrawal, cells begin to elaborate long processes within 2–3 days of culture which continues for several days *in vitro* (Fig. 1B). Undifferentiated B-35 cells expressed neuron specific enolase (NSE) as previously described (Vinores et al., 1984), as well as neurofilament (NF200) and synaptophysin (SYN) when grown in the presence of serum (Fig. 2). These levels of these neuronal markers appeared virtually unchanged in differentiated cells after serum withdrawal. Consistent with previous studies (Schubert et al., 1974), undifferentiated B-35 cells express choline acetyltransferase (ChAT); however, unlike the neuronal markers, serum withdrawal resulted in an increase in the levels of this enzyme (Fig. 3). B-35 cells did not synthesize tyrosine hydroxylase (TH), a marker for catecholaminergic neurons (Fig. 3), or serotonin (not shown) in either differentiation state as previously shown (Schubert et al., 1974).

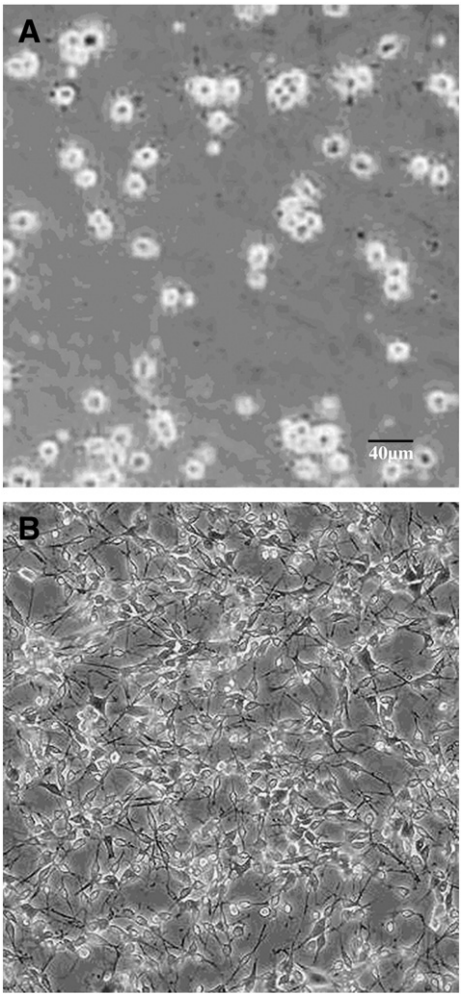

Fig. 1 – Differentiation of rat B-35 neuroblastoma cells. Rat B-35 neuroblastoma cells were grown in serum (A; undifferentiated) or serum-free medium (B; differentiated) for 3 days. Representative bright field images are shown. Upon serum withdrawal, cells begin to elaborate long processes within 2 days of culture which continues for several days *in vitro*. The scale bar is 40 μm.

2.2. *OGD and glutamate exposure decrease survival in differentiated B35 neurons*

Glutamate excitotoxicity and oxygen glucose deprivation (OGD) are well-characterized neuronal cellular injury methods that mimic events that occur following an ischemic insult *in vivo* (Bruno et al., 1994; Goldberg and Choi, 1993; Gwag et al., 1995; Noraberg et al., 2005; Tymianski et al., 1993). As an *in vitro* method of ischemia, B35 cells were subjected to OGD followed by reoxygenation for a total of 24 h. We observed a time-dependent decrease in cell survival with increased OGD duration (Fig. 4A and B). To examine whether these cells exhibited glutamate excitotoxicity, B35 neurons were exposed to various concentrations of glutamate for 1 h and allowed to recover for 23 h. Quantitative analysis showed a dose-dependent decrease in neuronal survival after glutamate exposure (Fig. 4C and D).

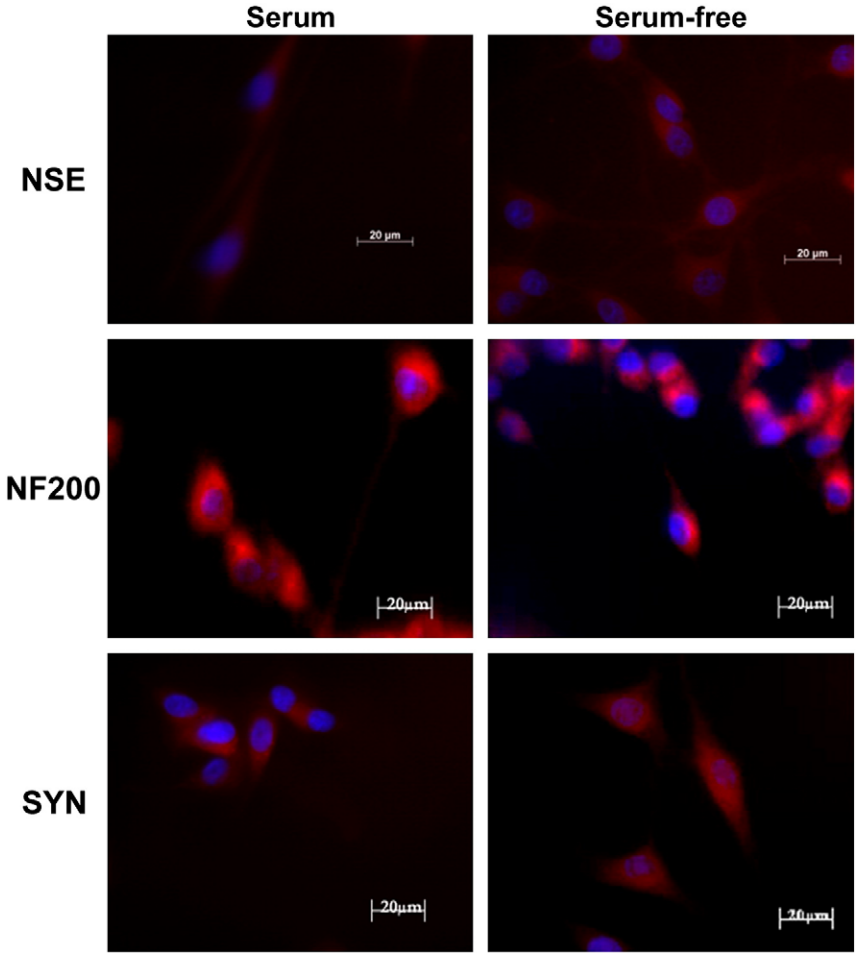

Fig. 2 – Rat B-35 neuroblastoma cells were grown in serum (left column) or serum-free medium (right column) for 3 days. Representative images are shown ($n=3$). B35 cell express neuron specific enolase (NSE) as well as neurofilament 200 (NF200) and synaptophysin (SYN) when grown in the absence or presence of serum. These levels of these neuronal markers appeared virtually unchanged in differentiated cells after serum withdrawal. The scale bar is 20 μm.

2.3. *NRG-1 increases neuronal survival after OGD, but not glutamate exposure*

The capability of B35 cells to respond to NRG-1 was examined by determining the expression of the erbB receptors. Our results showed expression mRNA for the erbB2 and erbB3, but not erbB4 (Fig. 5). No change in receptor expression was seen following differentiation. To determine the neuroprotective effects of NRG-1 on cell survival after ischemia/reoxygenation *in vitro*, cells were exposed to OGD and treated with NRG-1 immediately after ischemia and prior to reoxygenation. Administration of NRG-1 immediately after OGD resulted in a 2-fold increase in cell viability compared to untreated cells (Fig. 6A). To examine whether NRG-1 could protect neurons from glutamate excitotoxicity, B-35 cells were incubated with 50 μM glutamate. After a 60 min exposure, medium was removed and cells were re-incubated in conditioned medium for 24 h. NRG-1 was added at the indicated concentrations one h prior to glutamate exposure. Unlike OGD, when B35 cells were subjected glutamate treatment, no difference in survival was seen after NRG-1 treatment, even when up to 10-fold higher concentrations were used (Fig. 6B).

To examine whether anti-apoptotic mechanisms could be involved in neuroprotection by NRG-1, we analyzed the labeling of cells with annexin V, an early marker of apoptosis that binds to phosphatidyl serine transposed in the outer membrane during the apoptotic process (Stadelmann and Lassmann, 2000; van den Eijnde et al., 1997; Vincent and Maiese, 1999). Control samples do not show annexin V binding, however, a significant increase in annexin V labeling was detected in cells following OGD (Fig. 7). Annexin V levels were significantly attenuated by 40% in cells treated with NRG-1 after OGD.

2.4. *Neuroprotection by NRG-1 in OGD involves the PI3K/Akt pathway*

NRG-1 has been shown to stimulate the PI3K/Akt pathways, which has a well established role in cell survival (Buonanno and Fischbach, 2001; Burden and Yarden, 1997; Falls, 2003). Baseline pAkt levels are relatively low in control cultures, however, stimulation with NRG-1 results in a dramatic increase in pAkt levels while total Akt levels were unchanged following NRG-1 exposure (Fig. 8A). To determine whether

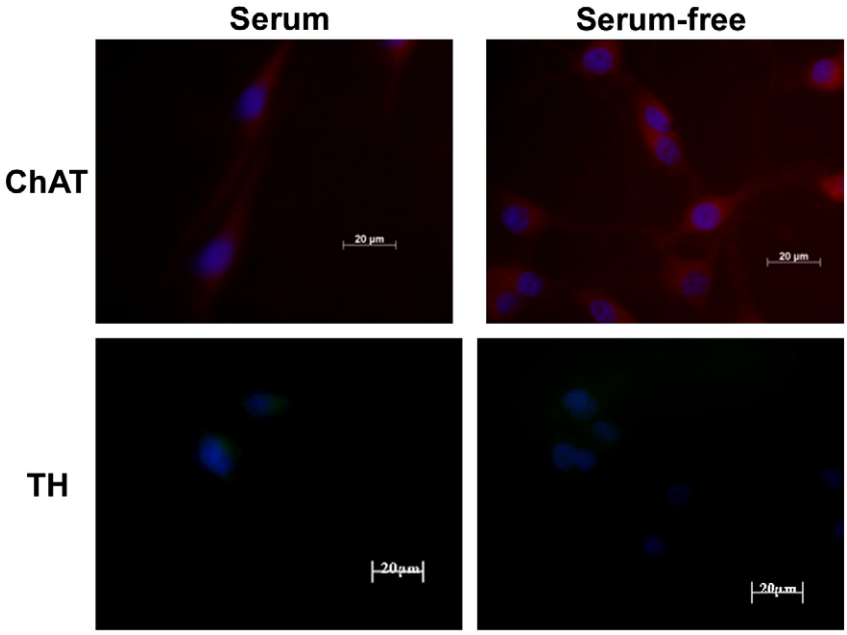

Fig. 3 – Rat B-35 neuroblastoma cells were grown in serum (left column) or serum-free medium (right column) for 3 days. Representative images are shown ($n=3$). Consistent with previous studies (Schubert et al., 1974), undifferentiated B-35 cells (left column) express choline acetyltransferase (ChAT). However, serum withdrawal (right column) resulted in an increase in the expression of ChAT. B-35 cells did not synthesize tyrosine hydroxylase (TH). The scale bar is 20 μm.

PI3K/Akt signaling pathways was responsible for NRG-1's neuroprotective capability, we applied wortmannin, an inhibitor of PI3K, to cultures prior to OGD. Wortmannin prevented NRG-1 from protecting neurons from OGD (Fig. 8B). Our data suggest that neuroprotective effect of NRG-1 in ischemia involves the activation of the PI3K/Akt pathway.

3. Discussion

Previous findings from our laboratory and others demonstrated that NRG-1 prevents neuronal death following ischemia *in vivo* (Guo et al., 2006; Shyu et al., 2004; Xu et al., 2006; Xu et al., 2005; 2004). However, the neuroprotection in the *in vivo* models could result either from direct effects on neurons or indirect effects on non-neuronal cells that facilitate ischemia-induced neuronal death. The results of this study clearly show the direct protective effects of NRG-1 on neurons following *in vitro* ischemia (OGD).

Cerebral ischemia results in an early glutamate-induced excitotoxicity (del Zoppo et al., 2000; Dirnagl et al., 1999; Iadecola and Alexander, 2001; Lee et al., 2000). Subsequently, delayed neuronal death occurs that is characterized by inflammation, oxidative stress and apoptosis. Glutamate excitotoxicity and OGD are two widely accepted methods for studying mechanisms associated with cerebral ischemia *in vitro* (Gwag et al., 1995; Noraberg et al., 2005). In this study, we examined the utility of B35 neurons as a model for ischemic neuronal injury. The advantage of B35 neurons is the ability to adopt neuronal characteristics upon serum withdrawal without the requirement of exogenously applied growth factors to induce neuronal differentiation (Schubert et al., 1974). The use of growth factors to stimulate differentiation as with many neuronal cells lines could confound the results seen when investigating the effects of growth factors like NRG-1 in neuronal cultures.

In this study, we showed that while NRG-1 protects neurons from OGD, it has no effect on glutamate neurotoxicity. This is consistent with our previous observations that NRG-1 protects neurons from delayed neuronal death and apoptosis *in vivo* (Xu et al., 2006; Xu et al., 2005, 2004). The PI3K/Akt signaling pathway is a well described survival and anti-apoptotic factor in neurons and other cells during ischemia (Hillion et al., 2006; Horn et al., 2005; Lawlor and Alessi, 2001; Malhotra et al., 2006; Zhao et al., 2005). Binding of NRG-1 erbB receptors has been shown to activate the PI3K/Akt pathway (Buonanno and Fischbach, 2001; Burden and Yarden, 1997; Falls, 2003). NRG-1 strongly stimulated the phophorylation of Akt in B35 cells. Blockade of PI3K prevented NRG-1 from protecting neurons from OGD. This is consistent with previous findings in PC-12 cells demonstrating that NRG-1 induced the activation of PI3K and inhibition of the PI3K/Akt pathway activity prevented the NRG-1 mediate survival effect. (Di Segni et al., 2006; Di Segni et al., 2005; Erlich et al., 2001; Goldshmit et al., 2001). The PI3K/Akt pathway has been shown to have direct effects on multiple apoptosis mechanisms (Downward, 2004; Mitsiades et al., 2004; Zhao et al., 2005). For example, Akt has been shown to phosphorylate and inactivate the pro-apoptotic Bcl-2 related protein, BAD as well as other downstream effectors, including NF-κB, Forkhead and GSK-3 and MDM-2, which mediate the effects of Akt on protection from pro-apoptotic stimuli.

In conclusion, we showed that B35 cells are suitable in both glutamate excitotoxicity and OGD initiated *in vitro* ischemia models. Additionally, we demonstrated that NRG-1 can directly protect neurons from ischemic injury. These findings may aid

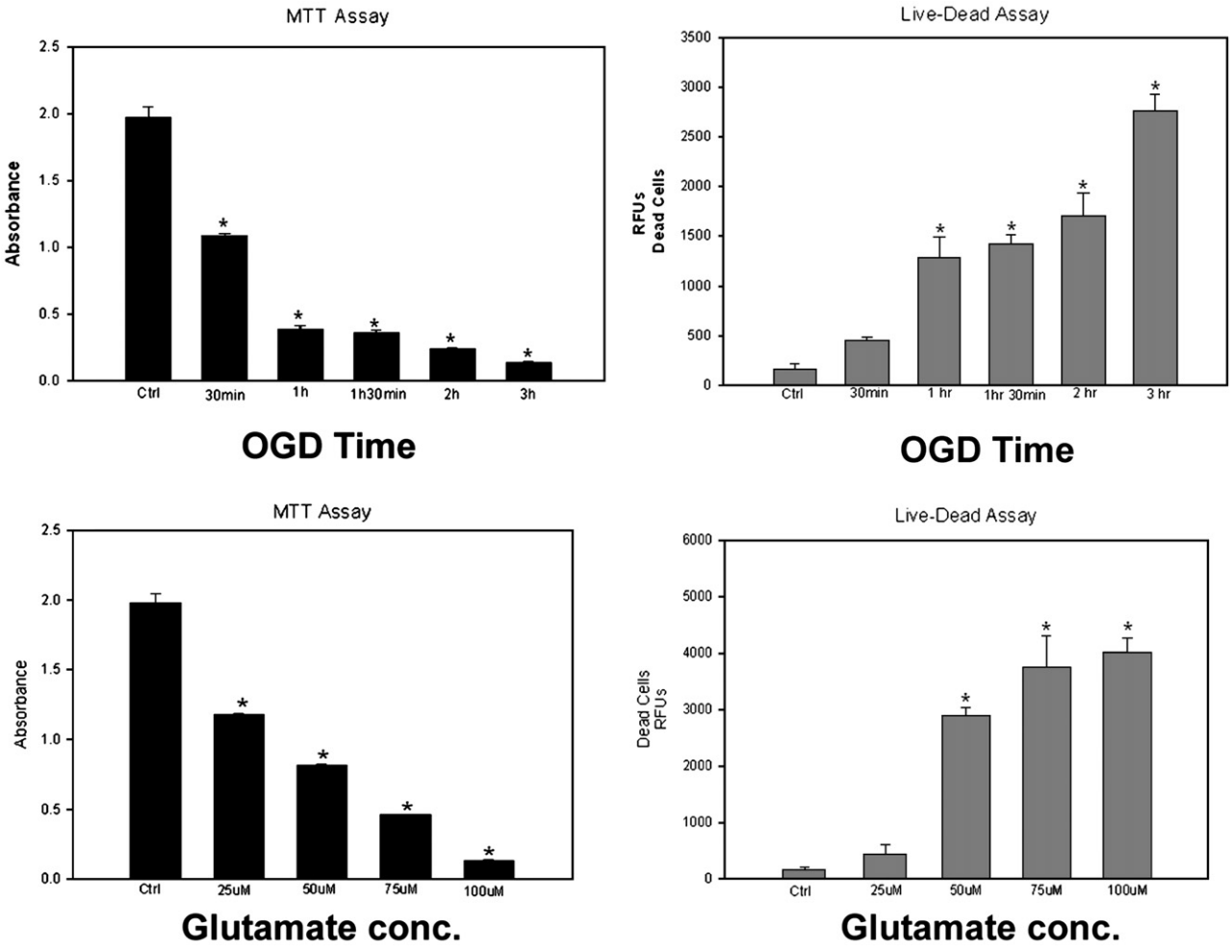

Fig. 4 – Effect of OGD and glutamate exposure time on B35 cell survival. B-35 cells were subjected to OGD at the indicated time followed by reoxygenation for a total of 24 h. Results revealed a time-dependent decrease in cell death with increase in duration of OGD when using an MTT assay (upper left) or the Live–Dead cytotoxicity assay (upper right). B-35 cells were subjected to glutamate treatment for 1 h followed by 24 h recovery and analyzed by the MTT (lower left) or the Live–Dead assay (lower right). Experiments were performed 3 or more times in triplicate. Values are represented as means ± SEM; * denotes significant difference from control.

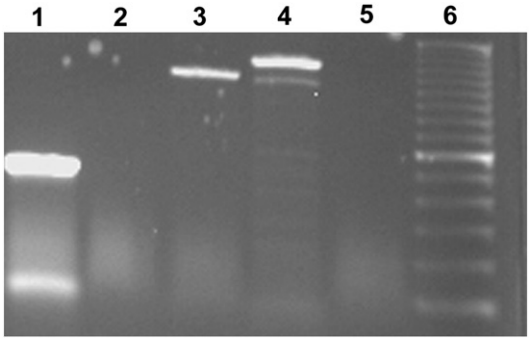

Fig. 5 – B35 cells express erbB receptor mRNA. RNA was isolated from B35 cells and erbB expression was examined by RT-PCR. GAPDH (lane 1), erbB1 (lane 2), erbB2 (lane 3), erbB3 (lane 4) and erbB4 (lane 5) PCR products along with DNA ladder (lane 6) were separated by agarose gel electrophoresis.

in the development novel neuroprotective strategies for ischemia and other neurological disorders.

4. Experimental procedure

4.1. *Cell cultures and oxygen glucose deprivation*

B35 rat neuroblastoma cells (ATCC, Manassas,VA) were cultured in Dulbecco modified eagle medium (DMEM), 10 U/ml penicillin, 10 mg/ml streptomycin, and 5% fetal bovine serum (FBS) at 37 °C in a 5% CO2 incubator. Cells were differentiated by the changing to a serum-free culture medium 3–5 days prior to treatment. Cultures were subjected to oxygen glucose deprivation (OGD) by incubation in a balanced salt solution (BSS: 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.01 mM glycine, and 10 mg/L phenol red) and placement in a hypoxic atmosphere of 1% O_2,

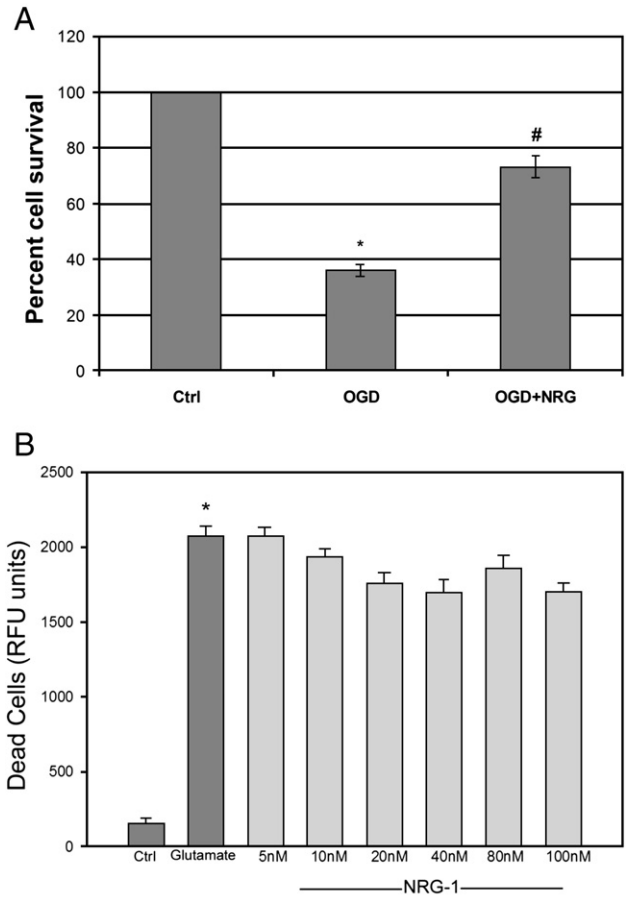

Fig. 6 – NRG-1 increases survival after OGD but not glutamate exposure. B-35 cells were subjected to 90 min of OGD 22.5 h of reoxygenation. NRG-1 treatment resulted in a two-fold increase in cell viability (A). When B-35 cells were incubated with 50 μM glutamate, NRG-1 did not protect neurons from glutamate excitotoxicity. Values are represented as means ± SEM; * denotes difference from control, # denotes difference from OGD ($n=5$; $p<0.05$).

5% CO_2. Cultures were subjected to OGD for 90 min. After OGD, cultures were treated with 10nM NRG-1 (NRG-1β EGF-like domain, R&D Systems, Minneapolis, Minnesota dissolved in 1% BSA/PBS) or vehicle and returned to normoxic conditions (reoxygenation) for 24 h. Control samples were placed in a balanced salt solution with 20 mM D-glucose.

4.2. *Glutamate excitotoxicity*

B-35 cells were washed in PBS solution and then incubated with 50 μM glutamate in Hanks BSS (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 1.0 mM NaH2PO4, 26.2 mM NaHCO3, 1.8 mM CaCl2, 0.01 mM glycine, and 20 mM glucose). After a 60 min exposure, medium was removed and cells washed in PBS and re-incubated in old conditioned medium for 24 h. Response of experimental cultures to glutamate excitotoxicity was compared to control cultures in different wells of the same plate where glutamate was not added. NRG-1 was added at the indicated concentrations one h prior to glutamate exposure.

4.3. *Immunocytochemistry*

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed three times in PBS buffer. Fixed cultures were blocked by incubation in PBS with 5% normal goat serum and 0.2% triton X-100 at room temperature for 1 h. Cultures were subsequently processed for immunocytochemical staining using primary antibodies against neuron specific enolase (NSE), neurofilament 200 (NF200), synaptophysin (SYN), choline acetyltransferase (ChAT), glutamic acid decarboxylase (GAD67), tyrosine hydroxylase (TH) and serotonin. Antibodies were diluted in blocking buffer then cultures were incubated with primary antibody for either 2 h at room temperature or overnight at 4 °C. Cells were washed four times with PBS, then incubated with fluorescent secondary antibodies for 1 h at room temperature or overnight at 4 °C. Cultures were mounted with Vectasheild containing DAPI for counterstaining. Cells were visualized using fluorescence confocal microscopy.

4.4. *Cell viability*

Cell viability was measured using the Molecular Probes Live/Dead Cell Viability/Cytotoxicity Assay Kit (Invitrogen, Carlsbad,CA). Calcein (live cell activity) was measured in either a fluorescent plate reader at excitation/emission 485/530 nm or via fluorescent microscopy using a fluorescein filter. EthD-1 (dead cell activity) was measured in either a fluorescent plate reader at excitation/emission 530/645 nm or via fluorescent microscopy using a Texas red filter. Live and dead cell values are shown as the mean ± SEM of assay results from three separate experiments performed in triplicate. Statistical analysis was carried out using the student t-test.

4.5. *Annexin V binding*

Cultures were subjected to OGD/reoxygenation and analyzed using an Annexin V Assay Kit (BD Pharmingen, San Diego, CA).

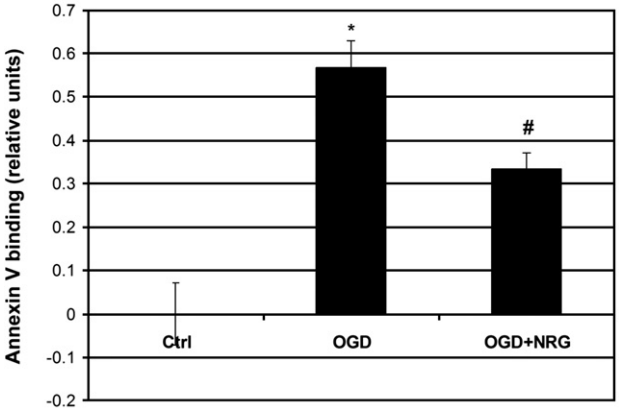

Fig. 7 – NRG-1 decreases annexin V labeling after OGD. OGD results in an increase in annexin V labeling of cells that is reduced by NRG-1. Values are represented as means ± SEM; * denotes difference from control, # denotes difference from OGD ($n=5$; $p<0.05$).

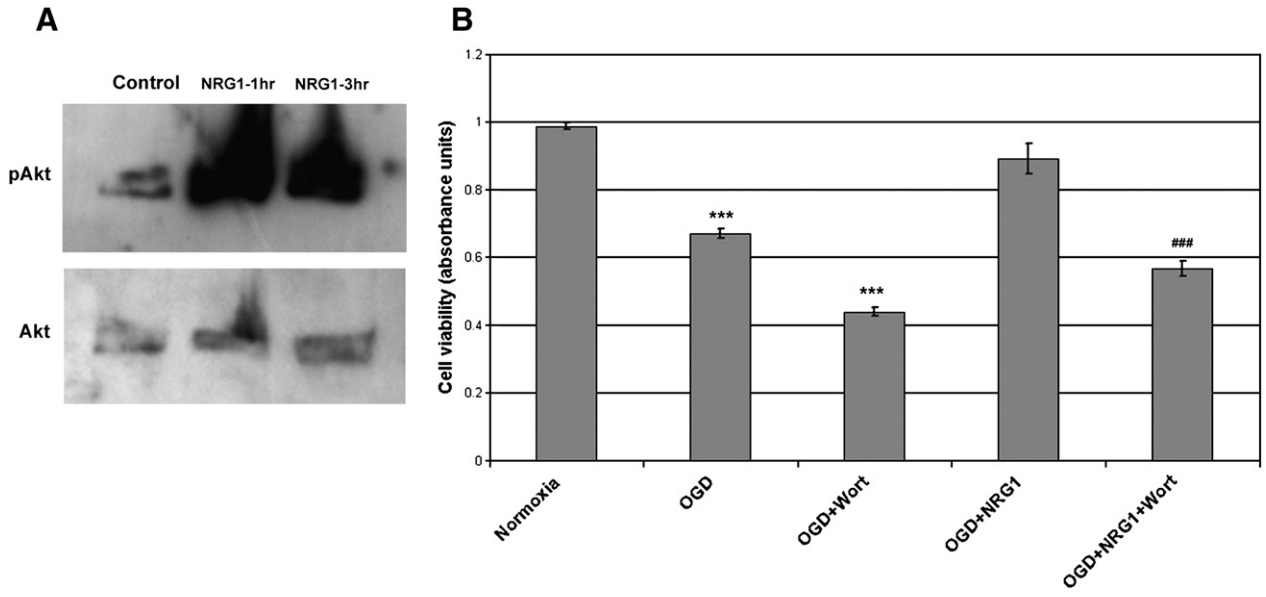

Fig. 8 – Neuroprotection by NRG-1 involved the PI3K/Akt pathway. Stimulation with NRG-1 resulted in a dramatic increase in pAkt levels while total Akt levels remained unchanged (A). Wortmannin, an inhibitor of PI3K, prevented NRG-1 from protecting neurons from OGD (B). Values are represented as means ± SEM; * denotes difference from control, ### denotes difference from OGD ($n=3$; $p<0.001$).**

Cultures were washed twice with PBS and annexin V binding was measured by fluorescent spectroscopy at excitation/emission 485/530 nm for annexin staining or by fluorescence microscopy. Relative fluorescent unit (RFU) values gathered by spectroscopy are presented as the mean ± SEM of assay results from three experiments. Statistical analysis was carried out using the student t-test.

4.6. *RNA isolation and RT-PCR*

Total RNA was isolated using the Ambion RNAqueous RNA isolation system (Austin, TX) according to the manufactures protocol. RT-PCR was used to amplify the isolated RNA in a Bio-Rad thermal cycler. To quantify mRNA expression primers for erbB2 (forward 5′-AGC TGG TGA CAC AGC TTA-3′; reverse 5′-TGG TTG GG ACT CTT GAC-3′); erbB3 (forward 5′-GAC CTA GAC CTA GAC TT-3′; reverse 5′-TCT GAT GAC TCT GAT GC-3′); erbB4 (forward 5′-CAT CTA CAC ATC CAG AAC A-3′; reverse 5′-AAA CAT CTC AGC CGT TGC A-3′); 'and GAPDH (forward 5′-GAA GGG CTC ATG ACC ACA GTC C-3′; reverse 5′-TCC ACC ACC CTG TTG CTG TAG CC-3′) (Sundaresan et al., 1999) were used for RT-PCR using the Qiagen (Valencia, CA) one-step kit (30 min at 50 °C, 15 min at 95 °C, then 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s with a 10 min final extension period at 72 °C). PCR products were loaded on 2% agarose gel, quantified using Kodak digital imaging software and expressed as a ratio of the control gene (GAPDH). Intensity values are means ± SEM of PCR results from three experiments.

4.7. *Protein isolation and western analysis*

Total protein was extracted from cells by lysing them with buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 1 mM sodium orthovandate, 1 mM phenyl methanesulfonyl fluoride, p 8.0 with 1:500 protease inhibitor cocktail) for 30 min at 4 °C. Harvested lysates were denatured with loading buffer (final concentrations: 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromophenol blue), resolved in SDS/5% polyacrylamide gels and transferred to polyvinyldifluoride (PVDF) membranes (Bio-Rad). Membranes were blocked with 3% nonfat dry milk in phosphate buffered saline-0.05% Tween 20 (PBST) and exposed to primary antibodies Akt and phospho-Akt (Cell Signaling Technology, Danvers, MA) that were diluted in blocking buffer overnight at 4 °C. Membranes were then incubated with secondary anti-rabbit HRP-linked antibodies and were visualized on X-ray film (Hyperfilm, Amersham Biosciences) using a chemiluminescence kit (New England Biolabs).

Acknowledgments

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