ProNGF Induced Neurodegeneration in Alzheimer's disease by the activation of Rho kinase

Jake Sustarich

Mentor-Dr. Geetha Thangiah

Department of Chemistry, Auburn University at Montgomery, Montgomery, AL 36117

Abstract

In Alzheimer's disease (AD) human hippocampal samples, the expression of pro-nerve growth factor (proNGF) was significantly increased compared to NGF level. NGF regulates cell survival and differentiation by binding TrkA and $p75^{NTR}$ receptors. ProNGF is the inactive precursor form of NGF, binds to $p75^{NTR}$ receptor and induces cell apoptosis. Here, we show that the PC12 cells stimulated with proNGF significantly enhanced the expression of $p75^{NTR}$ receptor. The proNGF stimulation also increased the activation of RhoA kinase and JNK apoptotic pathway. Interestingly, the activation of RhoA kinase and phosphorylation of JNK was also found to be increased in post-mortem human AD hippocampus compared to control, which might be due to increased expression of proNGF and $p75^{NTR}$ receptor. The addition of RhoA kinase inhibitor Y27632 not only blocked the RhoA kinase activity but also reduced the expression of $p75^{NTR}$ receptor induced by proNGF in PC12 cells. RhoA kinase inhibitor Y27632 also inhibited the proNGF induced neuronal death by abrogating the activation of JNK. These results suggest that overexpression of proNGF in AD enhances activation of RhoA thereby leading to neuronal cell death.

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder with progressive brain cell death that happens over a course of time. AD is characterized by a progressive loss of cognitive and behavioral abilities. Extracellular senile plaques and intracellular neurofibrillary tangles are hallmarks of AD (Selkoe 2001). Plaques are deposits of a protein fragment called beta-amyloid that builds up in the spaces between nerve cells. Tangles are twisted fibers of neurofibrillary protein called tau that accumulates inside the neuronal cells. Researchers aim to analyze the molecular mechanisms underlying AD pathogenesis; however, the therapeutic options available to treat this disease are inadequate.

Nerve growth factor (NGF) regulates the survival, maturation, differentiation, and maintenance of developing neurons (Levi-Montalcini 1987). NGF is a ~13 kDa peptide and is part of the neurotrophin family of structurally related neurotrophins such as brain derived neurotrophic factors, neurotrophin-3, and neurotrophin-4. ProNGF is the precursor form of NGF, which is cleaved by the matrix metalloproteinase-7 (MMP-7) into mature NGF (Hempstead 2009). NGF can bind to both a high-affinity tyrosine kinase Trk receptor and a low affinity $p75^{NTR}$ receptor (Meakin and Shooter 1992; Greene and Kaplan 1995). Tropomyosin receptor kinase A (TrkA) is a single transmembrane-spanning protein that belongs to the super family of tyrosine kinase (RTK) receptors, which regulate synaptic strength and plasticity in the mammalian nervous system (Huang and Reichardt 2003). Upon binding of NGF to TrkA, the receptor undergoes dimerization, autophosphorylation (Friedman and Greene 1999), polyubiquitination (Geetha *et al.,* 2005a), followed by internalization of TrkA into the signaling vesicles (Grimes *et al.,* 1997; Riccio *et al.,* 1997). TrkA activation leads to activation of Ras/MAPK and the PI3K/Akt pathways, thereby resulting in neuronal survival and differentiation.

$p75^{NTR}$ is a member of the tumor necrosis factor (TNF) super family of receptors

(Friedman & Greene 1999, Khursigara *et al.,* 2001, Geetha *et al.,* 2005b). $p75^{NTR}$ signaling pathways leads to cellular apoptosis, cell survival, differentiation, neurite outgrowth (Chen *et al.,* 2009, Mamidipudi & Wooten 2002, Harrington *et al.,* 2002), Schwann cell myelination, and sensory neuron development (Powell *et al.,* 2009, Khursigara *et al.,* 2001).

Recently, we found that in AD brain the MMP-7 which cleaves proNGF was reduced which caused increased accumulation of proNGF and decreased the NGF level (Chen *et al.,* 2015). The accumulation of proNGF in patients with Alzheimer's disease may play a role to induce apoptosis (Pedraza *et al.,* 2005, Lee *et al.,* 2001). In this study, we determined that the association of proNGF with $p75^{NTR}$ induces cell apoptosis via downstream activation of RhoA Kinase. RhoA is a small GTP-binding protein that acts as a molecular switch to play either a pro-death or pro-survival role in the nervous system. RhoA activation by proNGF directly induce neuronal death by activation of c-Jun N-terminal kinase (JNK). RhoA Kinase inhibitor Y-27632 has been shown to promote neurite growth in rat pheochromocytoma PC12 cells by reducing proNGF induced activation of JNK.

Materials and methods

Reagents and Antibodies

Anti-$p75^{NTR}$ was purchased from Promega (Madison, WI). Anti-RhoA and agarose conjugated rhotekin-RBD were purchased from Millipore (Billerica, MA). Phospho-p38MAPK and non-phospho p38 MAPK antibodies, JNK and phospho JNK, cleaved caspase-3 antibodies were purchased from Cell signaling (Danvers, MA). Cleaved PARP antibody was obtained from BD Bioscience Pharmingen (San Diego, CA). ProNGF was obtained from Alomon (Israel), NGF from Bioproducts for science (Indianapolis, IN), and Y-27632 (Rho kinase inhibitor) was purchased from Cayman Chemical Company (Ann Arbor, MI). Enhanced chemiluminescence was from Thermo Scientific (Waltham, MA) and all other reagents were obtained from Sigma-Aldrich.

Brain tissue

The human brain tissues used in this study were obtained from Emory University Alzheimer's Disease Center Brain Bank (Atlanta, GA, USA). Frozen samples of hippocampus from six AD cases aged 58 - 90 (mean = 69) and 6 control subjects aged 59 - 94 (mean = 70) were used for this study. The same brain tissues were used in Chen *et al.,* 2015.

Cell Culture

PC12 rat pheochromocytoma cells (1mL) were cultured in Dulbecco's modified Eagle's media supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and antibiotics (100 units/mL; streptomycin and penicillin) (9mL) on 100 mm plates coated with 150 µl Type I collagen. Cells were incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2 and 95% air. Cell culture medium was changed once a week. The cells were treated with proNGF (50 ng/ml) or NGF (50 ng/ml) in starved media overnight at 37^{o}C before cell lysis. To study Y-27632 (ROCK inhibitor) effects on proNGF stimulation, cells were treated with proNGF (50 ng/ml) in the presence or absence of Y-27632 (1 µM) overnight at 37^{o}C before cell lysis.

Western blotting analysis

At end of treatments cell were lysed with HEPES lysis buffer (50 mM HEPES [pH 7.6], 150 mM NaCl, 20 mM sodium pyrophosphate, 10 mM NaF, 20 mM beta-glycerophosphate, 1% Triton, 10 ug/ml leupeptin, 10 µg/ml aprotinin, 1 mM Na_3VO_4, and 1 mM PMSF). The protein concentrations were analyzed using the Bradford procedure (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard for all samples. The samples were boiled in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and resolved on SDS-PAGE gels, transferred onto polyvinylidene difluoride membrane, and analyzed by western blotting with appropriate antibodies.

RhoA kinase activity

RhoA kinase activity was determined by pull down assay. Brain homogenates or PC12 cell lysates were incubated with agarose conjugated rhotekin RBD agarose beads for 45 min at 4^0C and washed three times with lysis buffer. The beads were boiled with SDS-PAGE sample buffer to release active RhoA. Bound RhoA was detected by Western bloting with anti-RhoA antibody.

Results and Discussion

Our previous studies showed that postmortem AD human hippocampal samples have increase in expression of proNGF and $p75^{NTR}$ receptor compared to control samples (Chen *et al.,* 2015). ProNGF is known to promote neuronal apoptosis because of its high affinity to bind $p75^{NTR}$ (Casaccia-Bonnefil *et al.,* 1996; von Bartheld 1998). The effect of proNGF signalling, will be dependent upon the expression levels of its receptors (Fahnestock *et al.,* 2004; Masoudi *et al.,* 2009). Therefore, we evaluated whether proNGF induces the increase in expression of $p75^{NTR}$. PC12 cells treated with vehicle, proNGF and NGF were Western blotted with $p75^{NTR}$ and actin antibodies. Figure 1A, shows that cells treated with proNGF upregualed the expression of $p75^{NTR}$ compared to control and NGF treated cells. Actin levels were determined as positive control to check equal loading of all the samples. Rho family members are essential regulators of neuronal survival in the nervous system (Linseman and Loucks 2008). Therefore, we examined the activation of RhoA kinase pathway implicated in neuronal death. PC12 cells treated with vehicle, proNGF and NGF were subjected to pull down assay and Western blotted with Rho antibody. As

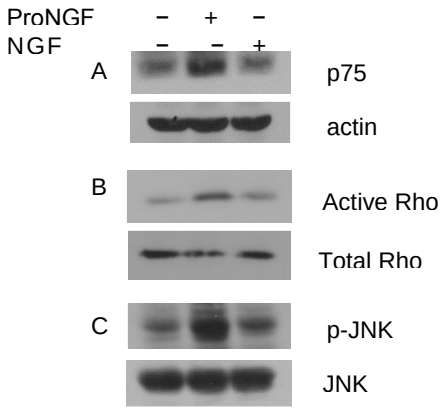

Fig.1 **Pro-NGF increased the expression of $p7^{5NTR}$ along with activation of Rho and JNK kinase.** PC12 cells were treated with pro-NGF (50 ng/mL) or NGF (50 ng/mL) overnight. The cells were lysed and (A) western blotted with anti-p75, anti-actin, (B) lysates were subjected to pull-down assay with agarose conjugated rhotekin-RBD followed by western blot with Rho antibody, (C) lysates were western blotted with phospho and non-phospho-JNK antibodies.

shown in figure 1B, activation of Rho kinase was increased in proNGF stimulated cells compared to control and NGF. The total Rho was equal is all the cell lysates. In addition to this we also determined the downstream target Jun amino-terminal kinases (JNK), as its activation leads to neuronal cell death. PC12 cell lysates stimulated with proNGF or NGF were Western blotted with phospho JNK and non-phospho JNK antibodies. The phosphorylation of JNK was increased by NGF than control or NGF treated cells (figure 1C). The level of non-phospho JNK is same in all the lysates. These results suggest that proNGF stimulation leads to increase in expression of $p75^{NTR}$, which in turn induces the activation of Rho kinase and JNK pathway thereby leading to neuronal death.

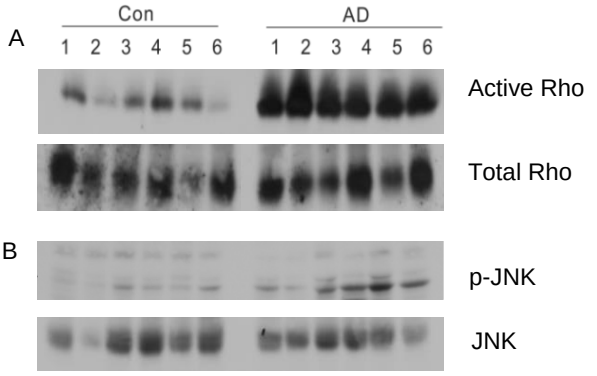

Fig. 2. **Increased activation of Rho and JNK pathway in hippocampal tissues of AD brain.** (A) Homogenates of postmortem normal and AD human hippocampal tissues were subjected to pull-down assay with agarose conjugated rhotekin-RBD to detect active Rho, (B) Homogenates were western blotted with phospho and non-phospho-JNK antibodies.

Next, we examined whether the RhoA kinase activity and the downstream signaling JNK was activated in AD brain since proNGF and $p75^{NTR}$ was increased in those samples. We used 6 postmortem control aged human hippocampal and 6 postmortem AD human hippocampal samples. The tissues were homogenized, and activation of RhoA was detected by pull down assay. Figure 2A suggests that RhoA kinase was activated in AD and not in control brain hippocampus. The expression of total Rho was equal in control and AD brain homogenates as shown by Western blot. RhoA activation induces the phosphorylation of JNK pathway, hence we determined the phosphorylation of JNK as well. Phosphorylation of JNK was increased in AD compared to control as shown by Western blotting with phospho-JNK antibody (figure 2B). The expression of JNK was equal in both control and AD hippocampus.

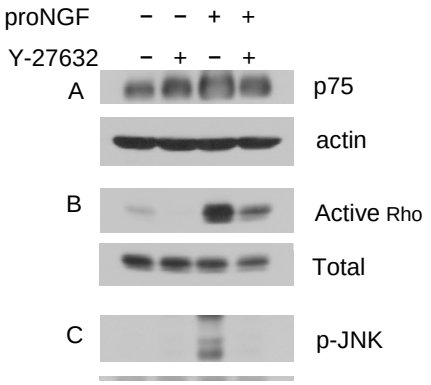

Fig. 3 **Inhibition of Rho activation blocked the JNK activation.** (A) PC12 cells were treated overnight with pro-NGF (50ng/mL) with or without Rho kinase inhibtor, Y-27632 (1uM). The cells were lysed and western blotted with anti-p75, anti-actin (B) pull-down assay showed Y-27632 reduced the activation of Rho induced by proNGF, (C) lysates western blotted with phospho and non-phospho-JNK antibodies.

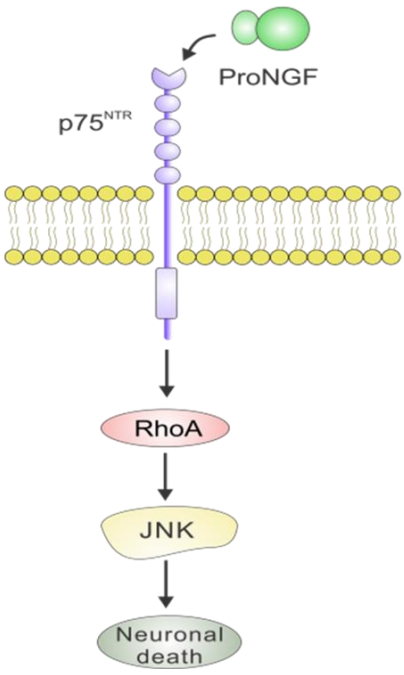

Fig. 4 **Schematic representation of proNGF signaling leading to neuronal death.**

RhoA is a small GTP-binding protein that acts as a molecular switch to play either a pro-death or pro-survival role in the nervous system. We want to determine, whether inhibition of RhoA activation will attenuate the neuronal death induced by proNGF. PC12 cells treated with proNGF with or without Rho kinase inhibitor, Y-27632. The cell lysates were Western blotted with $p75^{NTR}$ and actin antibodies (figure 3A). The expression of $p75^{NTR}$ was reduced by the inhibitor. Activation of RhoA was also detected by pull down assay. Figure 3B suggests that RhoA kinase activity was reduced by the Y-27632. The RhoA Kinase inhibitor Y-27632 also reduced proNGF induced activation of JNK (figure 3C).

The main findings of this study is accumulation of proNGF leads to neuronal death in AD, by increasing the expression of $p75^{NTR}$, which in turn activates Rho kinase and phosphorylates JNK leading to neuronal death (figure 4). These results demonstrate a novel signaling pathway by proNGF leading to neurodegeneration through $p75^{NTR}$ and Rho activation leading to neuronal death by activating JNK pathway.

Conclusion

In Alzheimer's Disease hippocampus the expression of proNGF, $p75^{NTR}$, activation of Rho and JNK is increased compared to control brain. PC12 cells stimulated with proNGF increased the expression of $p75^{NTR}$, Rho and JNK activation. Inhibition of Rho activation reduced the expression of $p75^{NTR}$, activation of Rho and JNK apoptosis.

References

von Bartheld, C.S. (1998) Neurotrophins in the developing and regenerating visual system. *Histol Histopathol* **13,** 437–459.

Casaccia-Bonnefil, P., Carter, B.D., Dobrowsky, R.T., Chao, M.V. (1996) Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. *Nature* **383,** 716–719.

Chen, Y., Zeng, J., Cen, L., Chen, Y., Wang, X., Yao, G., Wang, W., Qi, W. and Kong, K. (2009) Multiple roles of the p75 neurotrophin receptor in the nervous system. *Journal of international medical research,* **37,** 281-288.

Chen, Z., Geetha, T., Gearing, M. and Babu, J.R. (2015) Amyloid b-abrogated TrkA ubiquitination in PC12 cells analogous to Alzheimer's disease. *Journal of Neurochemistry,* 133, 919-925.

Fahnestock, M., Yu, G., Michalski, B. et al (2004) The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor. *J Neurochem* **89,** 581–592.

Friedman, W. J. and Greene, L. A. (1999) Neurotrophin signaling via Trks and p75. *Exp Cell Res,* **253,** 131-142.

Geetha, T., Jiang, J. and Wooten, M. W. (2005a) Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. *Molecular Cell,* **20,** 301-312.

Geetha, T., Kenchappa, R. S., Wooten, M. W. and Carter, B. D. (2005b) TRAF6-mediated ubiquitination regulates nuclear translocation of NRIF, the p75 receptor interactor. *EMBO J,* **24,** 3859-3868.

Greene, L. A. and Kaplan, D. R. (1995) Early events in neurotrophin signalling via Trk and p75 receptors. *Curr Opin Neurobiol,* **5,** 579-587.

Grimes, M. L., Beattie, E. and Mobley, W. C. (1997) A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA. *Proc Natl Acad Sci U S A,* **94,** 9909-9914.

Harrington, A. W., Kim, J. Y. and Yoon, S. O. (2002) Activation of Rac GTPase

by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. *J Neurosci,* **22,** 156-166.

Hempstead, B. L. (2009) Commentary: Regulating proNGF action: multiple targets for therapeutic intervention. *Neurotoxicity research,* **16,** 255-260.

Huang, E. J. and Reichardt, L. F. (2003) Trk receptors: roles in neuronal signal transduction. *Annual review of Biochemistry,* **72,** 609-642.

Khursigara, G., Bertin, J., Yano, H., Moffett, H., DiStefano, P. S. and Chao, M. V. (2001) A prosurvival function for the p75 receptor death domain mediated via the caspase recruitment domain receptor-interacting protein 2. *J Neurosci,* **21,** 5854-5863.

Lee, R., Kermani, P., Teng, K. K. and Hempstead, B. L. (2001) Regulation of cell survival by secreted proneurotrophins. *Science,* **294,** 1945-1948.

Levi-Montalcini, R. (1987) The nerve growth factor 35 years later. *Science,* **237,** 1154-1162.

Linseman, D,A,, Loucks, F.A. (2008) Diverse roles of Rho family GTPases in neuronal development, survival, and death. *Front Biosci* **13,** 657–676.

Mamidipudi, V. and Wooten, M. W. (2002) Dual role for p75(NTR) signaling in survival and cell death: can intracellular mediators provide an explanation? *J Neurosci Res,* **68,** 373-384.

Masoudi, R., Ioannou, M.S., Coughlin, M.D. et al (2009) Biological activity of nerve growth factor precursor is dependent upon relative levels of its receptors. *J Biol Chem* **284,** 18424–18433.

Meakin, S. O. and Shooter, E. M. (1992) The nerve growth factor family of receptors. *Trends Neurosci,* **15,** 323-331.

Montine, T. J., Phelps, C. H., Beach, T. G. et al. (2012) National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease: a practical approach. *Acta Neuropathol,* **123,** 1-11.

Pedraza, C. E., Podlesniy, P., Vidal, N., Arevalo, J. C., Lee, R., Hempstead, B., Ferrer, I., Iglesias, M. and Espinet, C. (2005) Pro-NGF isolated from the human brain affected by Alzheimer's disease induces neuronal apoptosis mediated by p75NTR. *Am J Pathol,* **166,** 533-543.

Powell, J. C., Twomey, C., Jain, R. and McCarthy, J. V. (2009) Association between Presenilin-1 and TRAF6 modulates regulated intramembrane proteolysis of the p75NTR neurotrophin receptor. *J Neurochem,* **108,** 216-230.

Riccio, A., Pierchala, B. A., Ciarallo, C. L. and Ginty, D. D. (1997) An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons. *Science,* **277,** 1097-1100.

Selkoe, D.J. (2001) Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev,* **81,** 741–766.

Acknowledgments

This work was supported by the Faculty Grant-in aid to GT. We thank Dr. Marla Gearing from Emory University for providing us with the brain samples. Also thank Nutrition Department at Auburn University for providing us the facilities for doing these experiments.

ProNGF Induced Neurodegeneration in Alzheimer's disease by the activation of Rho kinase

by
Jake Sustarich

An Undergraduate Thesis Submitted to
The University Honors Program
Auburn University at Montgomery

In partial fulfillment of the requirements for the degree of
Bachelor of Science in Chemistry

_____ April 17th, 2017
Geetha Thangiah, PhD

_____ April 17th, 2017
Daniel Kim, PhD

_____ April 17th, 2017
Matthew Carey Jordan, Director
University Honors Program

I understand that my project will become part of the permanent collection of the Auburn University at Montgomery Library, and will become part of the University Honors Program collection. My signature below authorizes release of my project and thesis to any reader upon request.

_____ April 17th, 2017
Jake Sustarich