Pro-Nerve Growth Factor Induced RhoA Kinase Activation in PC12 Cells

by

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Abstract

Alzheimer's Disease (AD) is characterized by neurodegeneration and progressive decline in memory, cognition and independence. Nerve Growth Factor (NGF) is the neurotrophin responsible for mediating neuronal survival and differentiation by binding transmembrane receptors, tropomyosin receptor kinase A (TrkA) and p75 neurotrophin receptor ($p75^{NTR}$). Active NGF is cleaved from its precursor, pro-nerve growth factor (proNGF), by the extracellular enzyme matrix metalloproteinase 7 (MMP-7). ProNGF favorably binds $p75^{NTR}$ receptor to induce neuronal apoptosis through RhoA Kinase activation. RhoA is a key GTP-binding protein that functions as a molecular switch between pro-survival and pro-apoptotic responses in the nervous system. RhoA activation by proNGF directly induces phosphorylation of c-Jun N-terminal kinase (JNK) and protein 38 mitogen-activated protein kinase (p38 MAPK) for apoptosis. Interestingly, Rho kinase inhibitor Y-27632 has been shown to mitigate proNGF induced activation of the JNK/p38 MAPK pathway and promote neurite outgrowth in rat pheochromocytoma (PC12) cells. Previously, our lab showed hippocampal brain tissue of AD patients expressed higher levels of proNGF and the receptor $p75^{NTR}$ as compared to age-matched control brain samples. Following these findings, investigation of $p75^{NTR}$ activity in proNGF enriched PC12 cells has revealed significant elevation in $p75^{NTR}$ receptor expression, RhoA kinase activity, and JNK/p38 MAPK phosphorylation. The addition of Rho kinase inhibitor Y27632 resulted in the reduction of $p75^{NTR}$ expression and subsequent RhoA activation of JNK/p38 MAPK apoptosis. These results suggest that overexpressed proNGF in AD promotes neuronal death through $p75^{NTR}$ mediated RhoA activation.

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CHAPTER 1: INTRODUCTION

Advancements in healthcare and technology in the twenty-first century have led to a significant increase in life expectancy and thus a rapid rise in the aging population.[1-4] In 2015, individuals over the age of 60 accounted for 900 million of the world's population and this number is expected to grow exponentially in the next several decades.[2,3] This global epidemic is no longer confined to high income countries, decline of mortality from infectious diseases in developing nations and the regression of fertility across the world have ushered the unparalleled rise in population aging.[1,4] Enhanced longevity has also resulted in greater incidence of chronic diseases and prevalence of noncommunicable diseases such as diabetes and dementia.[1,3,4] Dementia is the major neurological disorder observed in the elderly and presents the greatest origin of disability, dependency, institutionalization, and death among this age group.[2,4] As reported by Alzheimer's Disease International, 47 million people are living with dementia and this figure is estimated to increase to 145 million by the year 2050.[2,3]

The alarming prevalence and economic burden of dementia has gained the neurological disorder global priority.[2,3] Alzheimer's Disease (AD) accounts for 70-80% of all dementias and is responsible for increased morbidity and mortality of the elderly.[4,5] Enhanced degeneration of neurons results in premature loss of memory, cognition, and physical abilities.[6,7] AD is often diagnosed in the advanced stages of cognitive impairment and is promptly followed by augmented disability and dependence.[8] Loss of independence was found to correlate with decreased quality of life and increased burden on caregivers and the healthcare system.[5,8,9] AD also corresponds with high frequency of infections, malnutrition, dehydration, and fractures.[10] Thus, increased incidence of age-related diseases, complications, and co-morbidities are associated with disease progression.[10,11] Relative life expectancy of AD patients is 2 to 5 years

after diagnosis.[5] The debilitating cognitive impairment and occurrence of life-threatening complications support the need to advance our understanding of AD pathology to mitigate the disease and improve the quality of life throughout aging.

Despite early investigations into global prevalence, recent studies found dementia to be equally as prevalent in low-to-middle income countries as that of high-income nations.[1] Approximately 58% of all individuals diagnosed with dementia currently reside in low income countries with an expected growth of 10% by 2050.[1,3] Future projections estimate the steepest rise in incidence rate to occur in developing nations.[1,2] As most countries have inadequate healthcare systems and predominance of AD is specific to the aging population, it is crucial to advance research endeavors and address modifiable risk factors for Alzheimer's dementia to combat the increasing global prevalence of the disease.[1,2]

The sixth leading cause of death in the United States is Alzheimer's dementia[12]. As reported by the Alzheimer's Association, one out of ten Americans age 65 or older are diagnosed with AD.[13] The risk of manifestation advances with age and as the older adult population continues to multiply future prevalence of AD is estimated to double by the year 2050.[14] In 2016, the United States spent an unprecedented $236 billion on medical costs of AD, nearly a 9% increase from 2010.[11,15] Aside from age and genetics, ethnicity and gender have been shown to influence AD manifestation.[4,16,17] Medicare records reveal the incidence of AD in African-Americans and Hispanics is much greater than that of the white population, reporting diagnoses of 9.4% and 11.5% as compared to 6.9%, respectively.[11] Gender assessments found the prevalence of AD to be greater in women than men, exposing women as two-thirds of the AD population in the U.S.[13,17,18]

As aging is a normal and inevitable process of human life the rate of occurrence is related to regional concentration of the elderly population. Prevalence of AD in each state varies, although each state is estimated to experience a 14% increase by 2025.[11,13] Increasing frailty, oxidative stress, and physiological decline expose the entire aging population to the threat of AD.[13,19] Discrimination in AD incidence among ethnicities and gender are attributed to rates of poverty, level of education, and genetic differences.[20,21] While the strongest predictors of AD remain to be age, genetics, and familial history, modifiable risk factors have gained interest in recent years.[13,22] Associated risk factors such as cardiovascular disease, education, physical inactivity, obesity, and diabetes can be modified to reduce the risk of AD.[11,23] AD remains underreported due to the evolving nature of diagnosing criteria and absence of early detection diagnostics. The lack of detection prior to clinical manifestation makes it essential to raise awareness and expand our understanding of contributing risk factors.[18,19]

AD is the most common neurodegenerative disease, accounting for the majority of worldwide dementia cases.[24,25] AD is a multifarious, age-related, neurological disorder characterized by the irreversible and progressive degradation of neurons resulting in cognitive and behavioral deficits.[26] AD consists of three major clinical stages- mild, moderate, and severe.[26] The initial stage presents as short-term memory loss followed by neuropsychiatric manifestations which define the onset of the second stage.[26] The final stage is characterized by the loss of physical function including muscle rigidity.[24] Each phase is a direct result of cellular damage, neurotransmitter failure, and neuronal death caused by neuropathological changes in the brain.[27]

Histopathological hallmarks of AD are characterized as intercellular neurofibrillary tangles (NFT) and extracellular amyloid plaques.[26] Amyloid plaques are the product of amyloid-

βeta (Aβ) peptide cleavage from transmembrane amyloid precursor protein molecules (APP).[28] Aβ aggregations are also known as senile plaques and have been associated with localized inflammation, neuronal injury, and death.[27,29] NFTs are the result of abnormal phosphorylation of tau proteins on axonal microtubules.[30] NFTs have been found in the hippocampus during early AD development and parallel neuronal loss with disease progression.[31,32] Amplified accumulation and poor clearance of both proteinaceous aggregates attribute to advancement of cognitive decline.[33] Due to prominent loss of cholinergic neurons in the basal forebrain, several therapies are focused on neurotransmitter systems to maximize activity of remaining neurons.[24,25] As present-day therapies target the advanced aspects of AD, they fail to halt progression of the disease and provide only marginal therapeutic benefits.[24-28]

Neurotrophins are a family of growth factors and cytokines that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins (NT)-3 and -4.[34] Neurotrophins are synthesized as proneurotrophins that are cleaved by proteases into their mature forms.[35] Active neurotrophins and their precursors can stimulate growth, development, survival, and plasticity of neurons in the peripheral and central nervous systems.[34,36] During development, neurons are overproduced and undergo two stages of apoptosis in which survival is determined by the quantity of neurotrophic support.[34,36,37]Derangements in neurotrophin signaling have been found to play a role in AD pathology.[36] The survival, differentiation, and apoptosis of neurons is mediated by neurotrophin activation of receptor systems.[34] All proneurotrophins are able to bind p75 neurotrophin receptor ($p75^{NTR}$), while each mature neurotrophin selectively activates a member of the receptor tyrosine kinase family (TrkA-C).[38] Docking of neurotrophic factors to their respective receptors initiates intracellular cascades that modulate pro-survival or pro-apoptotic responses.[37,38]

NGF is a growth factor known to promote survival, maturation, and differentiation of developing neurons. NGF can bind both $p75^{NTR}$ receptor and TrkA receptor of the Trk gene family.[38-40] Throughout life, various cell types in the CNS, PNS, and immune system secrete NGF and its receptors, signifying NGF modulation is essential in nervous system health.[39] Basal forebrain expression of $p75^{NTR}$ and TrkA receptors is upregulated in the presence of NGF. Since the loss of cholinergic neurons in this region account for the significant memory decline in AD, presence of NGF is necessary to promote survival.[39,41,42] Although clinical trials involving cranial injection of NGF report restoration of neuron growth and improvement of cognitive decline, NGF alone is not curative.[41]

Proneurotrophins are precursors for neurotrophins and are the predominate form found in neurons throughout life.[35,43] Pro-nerve growth factor (proNGF) is cleaved by matrix metalloproteinase 7 (MMP-7) to form the mature NGF neurotrophin.[43] ProNGF is also able to impose trophic effects through receptor binding. Like NGF, proNGF serves as a ligand for $p75^{NTR}$, conversely it binds with greater affinity than NGF and induces apoptosis.[43,44] This opposing effect caused by the same receptor system suggest the balance of proneurotrophins and secreted neurotrophins is critical to neuronal fate during both development and aging.[44] Previously, our lab discovered that the hippocampal tissue of AD patients expressed far greater levels of proNGF as compared to NGF.[45] The accumulation of proNGF has been found to positively correlate with AD pathology.[46,47]

TrkA is a cell-surface transmembrane receptor that belongs to the superfamily of tyrosine kinase receptors.[39] The Trk receptor family is involved in mediating synaptic strength and plasticity of neurons and is comprised of three members: TrkA, TrkB, and TrkC. Each receptor displays different binding preference to neurotrophins; TrkA favors NGF while TrkB and TrkC

show preference for BDNF and NT-3, respectively.[48,49] Ligand binding causes TrkA to dimerize, autophosphorylate, and activate downstream signaling proteins including phospholipase C-γ and P13K/Akt, ERK/MAPK cascades.[47-49] Polyubiquitination of TrkA mediates its internalization into signaling vesicles and leads to subsequent cascade signaling of P13K/Akt and ERK/MAPK pathways. These signals intercept nuclear and mitochondrial death programs, resulting in neuronal survival and differentiation.[48,50,51] TrkA polyubiquitination is dependent upon interaction with ubiquitin E3 ligase tumor necrosis factor receptor-associated factor 6 (TRAF6) and scaffolding protein p62.[52] Our lab recently discovered this interaction to be blocked in the presence of Aβ. Moreover, TrkA was found to be nitrosylated rather than phosphorylated in hippocampal tissue of AD brain samples, suggesting AD interferes with TrkA polyubiquitination.[45]

$p75^{NTR}$ is a single-chain transmembrane receptor in the tumor necrosis factor (TNF) receptor superfamily and is widely expressed in the nervous system, including sensory, sympathetic, and hippocampal neurons.[40,53,54] Although $p75^{NTR}$ is known to induce apoptotic signaling, structural differences in its intracellular death domain separate it from other TNF receptors.[53,55,56] The diverse ability to bind both mature neurotrophins and their precursors allows $p75^{NTR}$ to mediate development, survival, and maintenance of the nervous system.[40,54-56] $p75^{NTR}$ activation stimulates several downstream signaling pathways including, Ras/ERK, nuclear factor-kappa B ($NF\text{-}_kB$), Jun N-terminal Kinase (JNK) and caspases. $p75^{NTR}$ does not exhibit intrinsic catalytic properties, thus it depends on interacting proteins such as TRAF6, NRIF, Src-1, and Rho to transmit signals to responder pathways.[54-56] NGF binding causes $p75^{NTR}$ to complex with TrkA receptor by involvement of TRAF6 to enhance survival signaling.[55-57] In the absence of NGF or in association with proNGF, $p75^{NTR}$ does not associate with TrkA and

induces apoptosis through JNK activation.[45,56] Several studies reveal $p75^{NTR}$ expression is dramatically increased in response to neuron injury or disease.[40,45,46] Furthermore, knockout (KO) of $p75^{NTR}$ proved to reduce damage and delay disease progression in neurodegenerative disease models.[58,59]

RhoA is an isoform of the Rho GTPase family that bind Rho kinase proteins (ROCKs) to activate phosphorylation of various target substrates.[60] ROCKs belong to the serine/threonine kinase family and are important downstream effectors of growth factor signaling.[61] Two isoforms of ROCK have been identified as RhoA GTP-binding proteins: ROCK1 and ROCK2.[61,62] Both isoforms contain a single Rho-binding-domain (RBD) to which RhoA, RhoB, and RhoC are able to bind.[63] While some GTPase molecules promote neurite growth, the activation of ROCK by RhoA leads to outgrowth arrest.[60] Modulation of the RhoA/ROCK pathway is believed to have potential therapeutic applicability in a wide variety of CNS pathologies.[61,62] ATP-competitive ROCK inhibitors such as Y-27632, have been shown to promote axonal regeneration and neurotrophin induced neurite outgrowth in PC12 cells.[61-63] Recent findings implicate ligand-dependent $p75^{NTR}$ mediates RhoA activity in axon extension.[64]

The goal of this study was to determine whether proNGF-induced $p75^{NTR}$ expression and subsequent JNK/p38MAPK apoptotic signaling are mitigated by Rho inhibition in PC12 cells. Results of this research will advance understanding of $p75^{NTR}$ mediated death signaling in neurons and may be implemented in the development of future therapeutic treatments of AD.

Objective and Hypothesis

Our first objective was to determine expression of $p75^{NTR}$ and Rho activity in proNGF treated PC12 cells. Second, we assessed if the JNK/p38MAPK apoptotic pathway is phosphorylated and activated in the presence of proNGF. Third, we analyzed if Rho inhibitor Y-27632 represses proNGF induced $p75^{NTR}$ expression and Rho activation. Fourth, we investigated if phosphorylation of JNK/p38MAPK apoptotic pathway was affected by Rho inhibitor Y-27632. We hypothesized that proNGF-induced expression of $p75^{NTR}$ and subsequent activation of JNK/p38MAPK mediated apoptosis can be mitigated through Rho inhibition.

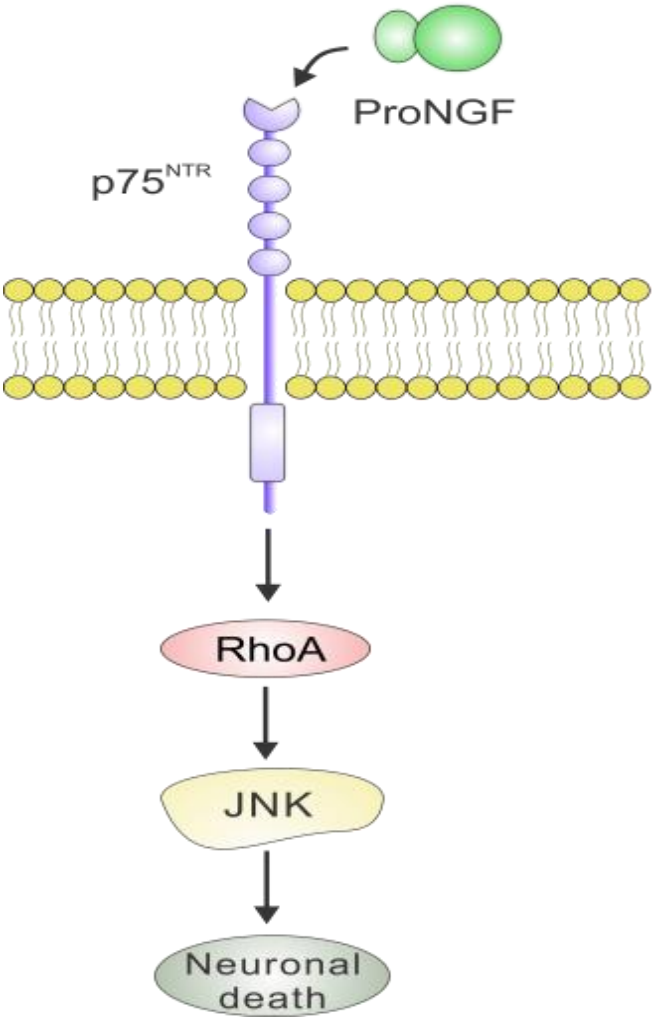

Figure 1. Schematic representation of hypothesized role of proNGF induced apoptotic signaling in neuronal tissue. Upon proNGF stimulation $p75^{NTR}$ enlists the help of RhoA to activate Rho Kinase phosphorylation of JNK/p38 MAPK signaling cascade. $p75^{NTR}$ induced JNK/p38 MAPK activation results in neuronal apoptosis.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Alzheimer's Disease

Alzheimer's Disease (AD) is clinically characterized as a disease of the elderly in which there is a slow, progressive decline in memory, cognitive ability, and physical function as a result of accelerated neuronal degeneration.[65] AD is the most common form of dementia and is classically defined by the build-up of extracellular amyloid-beta plaques (Aβ) and intracellular neurofibrillary tangles (NFT).[65] Neuronal damage and destruction is caused by accumulated Aβ and NFTs. Aβ aggregates disrupt synaptic signaling and induce neurotoxicity leading to degeneration and cognitive deficit.[65-67] NFTs are found in the hippocampus during early-stages of AD, the extent and distribution of this histopathological feature correlates with the degree and duration of the disease.[66] While genetic factors play a key role, mutations in genes APP, presenilin 1 (PS1), and presenilin 2 (PS2) are rare and account for less than 1% of all AD diagnoses.[67] Genetic mutations accelerate production of Aβ resulting in the development of AD before the age of 60, known as early-onset familial AD (FAD).[67,68] Late-onset AD (LOAD) or sporadic occurrence, presents after the age of 65 and accounts for the majority of AD cases.[68] Inefficient clearance of Aβ is the leading cause of LOAD, however presence of genetic risk factor ε4 allele (APOE4) can also contribute to LOAD pathogenesis.[67,69] In addition to aging and genetics, cardiovascular and lifestyle risk factors such as hypertension, cholesterol, diabetes, education, and smoking have been known to influence AD manifestation.[70] Moreover, recent studies suggest that imbalance of neurotrophic growth factors is yet another component of neurodegenerative disorders, specifically nerve growth factor (NGF) and its precursor pro-nerve growth factor (proNGF).[46,71] Elevated concentrations of proNGF as compared to NGF have been described in AD brain samples.[42,46] As cholinergic neurons are critically dependent upon NGF

for survival, loss of NGF presence results in the degradation of cholinergic neurons characteristic of AD.[72]

AD is typically not a direct cause of death, thus patients tend to survive well into the stages of severe impairment until an age-related comorbidity or infection such as pneumonia lead to death.[66] Early diagnosis is critical to improving advancement of disease and the quality of life patients will experience. Unfortunately, the current diagnosing criteria is limited and on-going, leaving many undiagnosed.[73] Current treatment options are minimal and only marginally effective. At present, two classes of drugs have been approved for treatment of AD: Acetylcholinesterase (AChE) inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists. Both treatment options target symptoms of LOAD and work to regulate levels of glutamate neurotransmitter.[74]

2.2 Alzheimer's Epidemiology

In 2010, global prevalence of dementia reached 35.6 million people with 18 million cases attributed to AD.[74,75] It has been estimated that by the year 2040, 80 million people will be diagnosed with AD worldwide.[76,77] Although 58% of all people with living with dementia reside in low-income countries, North America and Western Europe lead the world in prevalence, reporting incidence rates of 6.9% and 6.2%, respectively.[75,77] However, prevalence in Asia, Africa, and Latin America is expected to grow by 107-146% between the years 2010 and 2030.[75] For all populations incidence of AD advances exponentially with age, though in western societies early-century born individuals exhibit greater risk than those born later in the century.[77]

As of 2016, 5.4 million Americans have been diagnosed with AD. Fueled by the aging baby boomer generation, this number is expected to grow to 13.8 million by the year 2050.[11] AD is the sixth leading cause of death in the U.S and accounted for 32% of all deaths among older

adults in 2010.[11,78] In 2016, AD cost the nation $236 billion in medical expenses with an additional value of $221 billion in unpaid care.[11] Coinciding with global prevalence, more women than men are affected by AD in the U.S. (16% women, 11% men).[11,75] African Americans and Caribbean Hispanic Americans have a 2.6-3.6% higher incidence rate than other ethnic minorities and non-Hispanic whites.[79] Though occurrence in each state is expected to increase by 14% by the year 2025, the west and southeast regions of the U.S. are projected to see the largest percent growths.[11]

2.3 Amyloid-Beta

Amyloid-beta (Aβ) is a histological hallmark of AD and is generated by proteolytic cleavage of the amyloid precursor protein (APP). The presence of Aβ was found to accelerate age-related neurodegeneration and cognitive decline, exposing the peptide as a driving force in AD pathogenesis.[80,81] Chromosome 21 homes the gene encoding APP.[82] Proteolysis of APP results in splitting of its carboxyl terminus by a group of enzymes: α-, β- and γ-secretases.[68] The sequence by which the protein is cleaved determines whether the product will be amyloidogenic or non-amyloidgenic.[83] The three α-secretases involved in APP processing belong to the ADAM ("A Disintegrin and Metalloproteinase") protease family. These enzymes cleave APP 83 residues away from the C-terminal.[68] The β-secretases consist of BACE1 and BACE2 and cleave APP 99 residues from the C-terminal.[83] Lastly, the γ-secretase is an enzyme complex comprised of two catalytic sites, presenilin 1 (PS1) and presenilin 2 (PS2). This complex cuts APP in two distinct locations, the first to form a 50-amino acid peptide and the second at approximately 60 residues from the C-terminal.[68,83]

The processing of APP can be divided into a non-amyloidogenic pathway and an amyloidogenic pathway.[84] The non-amyloidogenic pathway is the sequential processing by α-

and γ -secretases resulting in a large N-terminal peptide called soluble APPα (sAPPα) and a short peptide called P3.[84,85] The amyloidogenic pathway is an alternative cleavage pathway for APP which leads to Aβ generation.[83] Initial proteolysis by β-secretase results in the release of an N-terminal peptide, soluble APPβ (sAPPβ), leaving a smaller C-terminal stub within the membrane.[68] The newly liberated sAPPβ undergoes subsequent extracellular cleavage by γ-secretase yielding two Aβ variants.[83] The majority of the Aβ peptides are generated as the 40-residue variant (Aβ40), however a small proportion are 42 residues in length (Aβ42). Unlike Aβ40, the Aβ42 variant is hydrophobic and more likely to aggregate.[86] Gene mutations known to cause FAD derail Aβ metabolism and result in Aβ accumulation.[87]

Aβ degradation is mediated by neprilysin (NEP) and insulin degrading enzyme (IDE).[68] Ineffective clearance results in Aβ accumulation leading to a variety of toxic mechanisms, including oxidative stress, mitochondrial diffusion, and excitotoxicity through neurotransmitter receptors.[88] Interestingly, in cases of LOAD, Aβ overproduction is also accompanied by IDE downregulation[89]. This finding suggests that amyloidosis is not a toxic accident, but rather may be part of a coordinated cell response to an upstream event. Such observations further implicate an intimate relationship between insulin resistance and development of AD [89]

2.4 Tau microtubules

Neurofibrillary tangles (NFTs) are the second histological hallmark of AD. NFTs consist of hyperphosphorylated tau proteins that aggregate inside neurons.[90] Tau proteins are key components in microtubule stabilization; they support structure, internal architecture, and are vital to axonal transport.[90,91] During the course of AD, tau proteins become hyperphosphorylated, detach from the microtubules, and accumulate intracellularly as paired helical filaments

(PHF).[91,92] The presence of ever-growing tau inclusions in addition to the unraveling of microtubules lead to impaired neuron function long before neuronal death is observed.[91]

The Tau gene is located on chromosome 17. Alternative splicing of its exons give rise to six isoforms, each ranging between 352 and 441 residues with multiple phosphorylation sites.[71,91] Tau proteins bind microtubules through the microtubule-binding domain located in the C-terminus of the protein.[93] Binding of tau to microtubules depend on the protein's state of phosphorylation.[30] Non-phosphorylated tau have great affinity for tubulin and promote polymerization and assembly. Whereas phosphorylated tau are less likely to bind microtubules, thus do not promote assembly.[71] Proline-directed kinases such as mitogen-activated protein kinase (MAPK), Tau-tubulin kinase, and cyclin-dependent kinase, phosphorylate tau at Ser/Thr-pro sites.[30,94] Stress-activated protein kinases are not proline-directed and attack non-Ser/Thr-pro sites of tau. Glycogen synthase kinase-3β (GSK-3β) is able to phosphorylate both non-Ser/Thr-pro and Ser/Thr-pro sites of tau.[94] Mutations of the Tau gene can also result in abnormal phosphorylation and cause a dementia that is pathologically similar to AD.[91,93]

The deposition of tangles occurs in a hierarchical fashion from the entorhinal cortex to the hippocampus, correlating with the observed cognitive decline in AD.[92] Tau tangles invade the hippocampus during early-stages of AD. Once tau reaches the hippocampus Aβ plaques can also appear, followed by progression of tau into the basal forebrain.[71,92] Taupathology correlates with Aβ deposition in the development of AD, however there is no spatiotemporal overlap. In AD, the neocortical regions are the first to develop Aβ deposits and the last to see NFTs, whereas the reverse is true for hippocampal regions.[94] Taken together, the Aβ/Tau mismatch seen in AD indicates that neurodegeneration and cognitive decline is not the direct result of Aβ neurotoxicity alone.[94]

2.5 Role of Neurotrophins

Neurotrophins are a broad group of peptide growth factors and cytokines that target neurons of the peripheral (PNS) and central nervous systems (CNS).[34] The mammalian neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT) -3 and -4.[95] Neurotrophic factors are essential during embryonic development and throughout life.[37] Knockout mice for NGF, BDNF, and NT displayed severe neural defects and subsequent death.[71] These neurotrophins activate a plethora of cell signaling pathways by binding two transmembrane receptor types, the tyrosine receptor kinase family (TrkA, TrkB, and TrkC) and the p75 neurotrophin receptor ($p75^{NTR}$). NGF selectively binds TrkA, while BDNF and NT-4 bind TrkB, and NT-3 shows preference for TrkC.[48] As they are often produced in sites outside of their action site, neurotrophic factors rely on functional axon transport to reach their target receptors for proper signaling.[71]

Mature, biologically active neurotrophins are initially synthesized as 32–34kDa precursors (proneurotrophins) containing a signal sequence, glycosylation sites, and paired basic amino acids. Serine protease, furin and other prohormone convertases cleave precursors at dibasic amino acid sites to liberate biologically active 12-14kDa neurotrophin homodimers.[96,97] Under normal physiological conditions, neurotrophins are expressed in low concentrations and thus proneurotrophins are the predominant form.[98] The prodomain of neurotrophins function as intracellular chaperones to facilitated oxidative folding of the mature domain and sorting of neurotrophins to secretory pathways.[99-101] However, recent sequence analysis revealed highly conserved regions within the prodomain sequence, suggesting ability to mediate additional biological actions.[97] Unlike neurotrophins, proneurotrophins only serve as ligands for $p75^{NTR}$.[102] The primary proneurotrophin involved in modulating neural death is proNGF.[43] While NGF is

able to promote pro-survival responses through both TrkA and $p75^{NTR}$ receptors, proNGF can only bind $p75^{NTR}$ and induce pro-apoptotic signaling.[97] Moreover, the role of neurotrophin signaling can be modulated by $p75^{NTR}$ and Trk receptor activity.[103]

2.6 Nerve Growth Factor (NGF)

NGF is a neurotrophic stimulant of neuronal growth and development during neurogenesis and continued neuron survival through adult life.[57] The gene encoding two NGF variants is located on human chromosome 1.[104,105] NGF is a non-covalent homodimer produced by proteolytic cleavage of precursor proNGF.[106,107] Biologically active NGF, known as β-NGF, is a 13.2kDa molecule comprised of three polypeptide structures including a twisted beta sheet, a cysteine-knot motif, and three beta-hairpin loops.[39] The cysteine-knot motif is characterized by three disulfide bond structures, two disulfide bridges form a loop that is penetrated by a third disulfide bridge.[98,108] Receptor specificity is dictated by the sequence variances embedded in the loop domains of the molecule.[107] Both the N-terminus and the beta-hairpin loops of mature NGF are important for high-affinity binding to TrkA.[109,110]

During development, NGF is produced by nonneuronal target cells such as keratinocytes, smooth muscle cells, and cells of various endocrine organs.[39] It was found that most of these cell types continue to modulate NGF production into adult life or in response to stimuli like inflammation and injury. Trophic properties of NGF have been recognized in sympathetic, sensory, and cholinergic neurons.[34,57,111] NGF expression is directed by cholinergic innervation from the basal forebrain and by hippocampal NMDA receptors.[112,113] Absence of NGF in cholinergic neurons results in cell shrinking and downregulation of transmitter-associated enzymes.[71] High affinity neurotrophin receptor TrkA and low affinity $p75^{NTR}$ receptor mediate NGF induced signaling.[57]

NGF activation of TrkA receptor was initially observed in pheochromocytoma-derived PC12 cell lines.[114] Since then PC12 cells have been highly useful in NGF signal transduction studies.[115] PC12 cell lines of rat pheochromocytoma cells contain catecholamine stores and many other secretory products comparable to human pheochromocytomas.[116] This cell line was found to be sensitive to NGF and undergo neuronal differentiation, survival, hypertrophy, and regulation of various biochemical markers.[50,116] Thus, PC12 is a primary cell model for neurotrophin signaling investigations.[115]

2.7 TrkA Receptor

Trk describes a transmembrane polypeptide chain belonging to the tyrosine kinase receptor (Trk) superfamily.[117] TrkA is one of three Trk receptor variants encoded by a gene on human chromosome 1.[39] During development, expression of TrkA is restricted to sensory and sympathetic neurons of the PNS and cholinergic neurons of the basal forebrain.[118,119] Trk receptors comprise of three extracellular leucine-rich motifs, flanked by two cysteine-rich clusters, followed by two immunoglobulin(Ig)-like domains that are proximal to the membrane.[48,120] The major ligand binding sites of Trk receptors have been localized to the Ig2 domain.[121,122] Each Trk receptor displays neurotrophin selectivity. TrkA serves as a high affinity receptor for NGF, TrkB serves as a receptor for BDNF, and TrkC functions as a receptor for NT-3.[120] However, NT-3 is also a ligand for $TrkA_{II}$.[123] $TrkA_{II}$ is an isoform of TrkA, distinguished only by a 6-amino acid insert near the extracellular domain.[124] This inclusion causes the conformational difference that enhances activation by NT-3 over NGF.[125] NGF secures the Ig2 domain of TrkA by two distinct patches.[117,126] The N-terminus of NGF serves as the second patch and is comprised of a unique residue sequence that specifies TrkA binding.[126] TrkA signaling induced by NGF elicits phosphorylation of pro-survival substrates resulting in growth

or continued survival.[120] TrkA receptor is neuroprotective in several cell lines and was found to mitigate Aβ accumulation by reducing β-cleavage of APP.[102,127] Expression of TrkA in cholinergic neurons of the basal forebrain is greatly reduced in AD.[128,129]

2.7.1 TrkA-NGF signaling

Binding of NGF to TrkA initiates a series of events characteristic to receptor kinase signaling.[49] Stimulated TrkA undergoes dimerization and autophosphorylation of tyrosine residues in the activation loop leading to kinase activity activation and subsequent trans-autophosphorylation of tyrosine residues outside the loop.[50,130] These activated residues serve as binding sites for adaptor proteins, the consequent phosphorylation and activation of these signaling molecules results in downstream signaling cascades.[131,132] This NGF induced tyrosine phosphorylation is rapid, highly specific, and dose-depended.[114] TrkA mediated signaling cascades include the following pathways: Ras-Erk-MAPK, PI3K/Akt, SNT proteins (suc-associated neurotrophic factor-induced tyrosine-phosphorylated target), and phospholipase C γ-1 (PLCγ-1).[130,131] Its previously been indicated that the NGF-TrkA complex can also be internalized into vesicles to propagate neurotrophin signaling responses.[133,134] Recent studies attribute this variance to the receptor's ability to mediate distinct NGF trophic effects. Modulation of TrkA endocytosis in PC12 cells revealed that survival is primarily mediated by membrane-bound TrkA via prolonged activation of the P13K/Akt pathway. Whereas, differentiation is directly stimulated by internalized active TrkA vesicles.[50]

Although initial on-rate for NGF binding to TrkA is slow, the rate of TrkA dissociation is even slower, signifying the strong binding affinity for NGF.[135,136] Interestingly, co-expression of $p75^{NTR}$ results in a 25-fold increase in on-rate binding between NGF and TrkA.[136] While expression of TrkA alone is sufficient to elicit NGF signaling, extensive studies show co-

expression of $p75^{NTR}$ results in greater sensitivity, selectivity and enhanced neurotrophin mediated effects.[48] However, these effects are highly depended upon relative receptor expression.[120] NGF binding to $p75^{NTR}$ promotes TrkA/$p75^{NTR}$ complex formation through $p75^{NTR}$ activated p62 scaffold and subsequent TRAF6 recruitment by TrkA.[52] Meanwhile, activation of TrkA by NGF leads to suppression of $p75^{NTR}$ mediated apoptotic cascades such as c-Jun Kinase (JNK) and RhoA.[48,103] In addition, peroxynitrite, the reactive oxygen species implicated in AD, was found to inhibit NGF signaling by forming nitrotyrosine residues on TrkA rendering the receptor inactive and contributing to AD pathology.[137]

2.8 Pro-Nerve Growth Factor (proNGF)

Pro-nerve growth factor (proNGF) is the precursor for biologically active NGF. ProNGF is the chief product of processed, NGF-gene-derived pre-pro-protein.[138] ProNGF is 30kDa molecule comprised of a 103 amino acid pro-peptide covalently bound to the mature 118 amino acid NGF peptide[108,138] The pro-peptide of proNGF lacks cysteine residues and is known to function as a chaperone to facilitate proper maturation and oxidative folding of the mature moiety.[101,108] In addition, recent studies demonstrate that the pro-peptide also confers apoptotic activity to the molecule.[97] Thus, once thought to be inactive, proNGF is an active homodimer released by cells to elicit cellular apoptosis.[139,140]

Intracellular proNGF is cleaved by furin in the trans-Golgi network and released by the constitutive pathway.[43,108] Dibasic and tetrabasic cleavage sites flank the sequence for mature NGF, while additional processing at glycosylation sites generates high-molecular-weight peptides and intermediates whose biological functions have not yet been defined.[109] Since cell-type-specific secretion of growth factors occurs as a mixture of proNGF and NGF, proNGF can be found in the extracellular matrix.[43] In addition to dibasic cleavage sites, proNGF contains

consensus sites for extracellular protease enzymes: plasmin and matrix metalloproteases (MMP-2,3,7,9).[97] In vitro studies reveal overexpressed proNGF cleaved by plasmin generates the mature 13kDa molecule, while cleavage by MMP-7 results in a 17kDa intermediate.[97,138] As extensive studies reveal elevation of proNGF in the CNS following damage or disease (AD), the activity of these extracellular proteases may be crucial in defining pro-apoptotic and pro-survival signaling.[43,46,141]

2.9 p75 neurotrophin receptor ($p75^{NTR}$)

Neurotrophin receptor p75 ($p75^{NTR}$) is a single pass transmembrane protein in the tumor necrosis factor receptor (TNFR) family.[59] $p75^{NTR}$ is a glycoprotein receptor that exists as a disulfide-linked dimer with the molecular weight of 75kDa.[142,143] The extracellular domain of $p75^{NTR}$ is characterized by a cysteine-rich structure that forms four ligand-binding sites.[144] The intracellular structure of $p75^{NTR}$ is comprised of a chopper domain and a globular TNFR-like death domain.[53,145] Unlike the type II death domains found in other TNFR members, the type I death domain of $p75^{NTR}$ does not self-associate and therefore does not signal death through the same mechanisms as its family members.[53,55,146] The third and fourth cysteine repeats of the $p75^{NTR}$ extracellular domain serve as binding sites for all neurotrophins, although greater affinity is shown for proNGF. This binding flexibility implicates $p75^{NTR}$ in an array of diverse cellular functions including survival, apoptosis, and axonal growth.[147-149] The facilitation of these various functions depend on the binding ligand, cellular context, and association of coreceptors.[150,151] During nervous system development, $p75^{NTR}$ is essential for synapse strengthening and neuron selection.[54] With the exception of cholinergic neurons, its widespread expression in the CNS and PNS is highly downregulated following synaptogenesis, however re-

expression has been observed in the adult brain in response to neurotrama, inflammation, and neurodegenerative disease.[152-154]

Although the molecular mechanism of $p75^{NTR}$ activation has not been fully elucidated, studies indicate that ligand-binding may prompt a conformational change in the $p75^{NTR}$ dimer resulting in rearrangement of its disulfide-linked subunits and subsequent recruitment of interacting proteins.[155] Activation of $p75^{NTR}$ triggers several downstream signaling events, including NF-kB, small GTP binding protein Rac, c-Jun N-terminal kinase (JNK), and caspases.[132,149,156] As $p75^{NTR}$ lacks intrinsic enzymatic activity, signal transduction to these downstream effectors relies on the interaction of several intracellular binding proteins such as NADE (neurotrophin associated cell death executor), NRIF (neurotrophin receptor interacting factors), TRAF6, NRAGE (neurotrophin receptor-interacting MAGE homologue), SC-1 (Schwann cell 1) and RhoA.[157-159]

In addition to neurotrophin stimulation and coreceptor formation, $p75^{NTR}$ is susceptible to metalloprotease and γ-secretase cleavage.[160] The cleaved fragments of $p75^{NTR}$ are able to propagate apoptotic signaling and have also been implicated in AD.[161,162] As $p75^{NTR}$ is chiefly expressed in cholinergic neurons of the adult brain, several studies have suggested that the neuronal loss observed in AD patients is $p75^{NTR}$ dependent.[163-165] Furthermore, Aβ was observed to cause $p75^{NTR}$ mediated neurotoxicity in several cell lines.[144,162,164]

2.9.1 $p75^{NTR}$-proNGF signaling

It was recently discovered that proNGF-dependent death signaling via $p75^{NTR}$, requires sortilin as coreceptor.[139,166] Upon binding, proNGF forms a heterotrimeric signaling complex with $p75^{NTR}$ and sortilin and activates $p75^{NTR}$ dependent apoptosis through the intrinsic caspase pathway, requiring phosphorylation of JNK and activation of caspases-9, -6, and -3.[167,168]

Although poorly understood, it is believed that $p75^{NTR}$ activation by proNGF promotes sequential cleavage of $p75^{NTR}$ by metalloproteases and α- and γ- secretases resulting in the release of its intracellular domain.[165,168] The cleavage of $p75^{NTR}$ is necessary for subsequent ubiquitination and nuclear translocation of NRIF, phosphorylation of JNK, phosphorylation and oligomerization of the BH3-domain-only family member (Bad), and activation of caspases 9, 6, and 3.[168-170]

In addition to NRIF, $p75^{NTR}$ binding protein, NRAGE, was found to promote JNK phosphorylation and subsequent caspase activation.[171] Recent investigations demonstrated that proNGF stimulation increases the association between NRIF and NRAGE, indicating several 75^{NTR}-binding proteins may be involved or complexed to induce apoptotic signaling.[168] In oligodendrocytes, proNGF binding to $p75^{NTR}$ led to activation of pro-apoptotic JNK cascade via activation of RhoA, a small GTPase.[172] Both proNGF and $p75^{NTR}$ have been implicated in mediating neuronal apoptosis following neuron injury and disease.[166,173] Furthermore, it has been shown that in AD, levels of proNGF are increased in a stage-dependent manner resulting in neuronal death of the hippocampal, cortical, and basal forebrain cholinergic neurons via $p75^{NTR}$ death signaling[174,175]

2.10 RhoA GTPase

RhoA GTPase belongs to the Rho GTPase subfamily of the Ras superfamily of GTPases that participate in the control of important physiologic functions, including cell contraction, migration, proliferation, adhesion, and inflammation.[63,176] In contrast to other members of the Ras superfamily, RhoA activation does not promote neuronal survival and instead elicits neuronal apoptosis.[177] RhoA has a molecular weight of approximately 24kDa and like all members of the Ras superfamily, RhoA controls signaling pathways by switching between a

biochemically inactive (GDP-bound) and an active (GTP-bound) form.[178,179] The general structure of Rho GTPases consists of several core components, including a shared G-domain fold, made of a six-stranded β-sheet surrounded by α-helices.[178] RhoA contains two "switch" segments that mediate the conformational change between active and inactive forms in response to GTP, denoted by switch I (residues 28-44) and switch II (residues 62-69).[180] The area between the switch segments contains hypervariable regions that distinguish each Rho family isoform.[176] The carboxyl terminal (C-terminal) of Rho proteins is essential for posttranslational modifications such as proteolysis and methylation.[181]

2.10.1 Rho-associated Protein Kinases (ROCKs)

ROCK is a serine/threonine protein kinase that was identified as downstream effector of Rho.[62] ROCK is a Rho GTP-binding protein with a molecular weight of approximately 160kDa.[182] There are two identified isoforms of ROCK encoded by two different genes: ROCK1 and ROCK2. The gene encoding ROCK1 is located on chromosome 18, while ROCK2 is encoded by a gene on chromosome 2.[63,183] ROCKs consist of three major domains: a RhoA binding domain (RBD) situated in a coiled-coil region, a kinase domain that is responsible for its catalytic activity, and a cysteine-rich domain in the C-terminus thought to participate in protein localization.[184,185] The C-terminal of ROCK also regulates its kinase activity. This C-terminal folds back into the kinase domain resulting in an autoinhibitory loop to maintain ROCK inactive. Binding of GTP-bound Rho to RBD site liberates the kinase domain and results in ROCK activation.[63,186] Despite similarity in protein sequences, the two ROCK isoforms have significant differences regarding tissue distribution and subcellular location.[179,187] ROCK1 is greatly expressed in non-neuronal tissues such as lung and liver, whereas ROCK2 is expressed in

neuronal tissues, brain specifically.[63,179] Animal studies revealed ROCK2 expression in the brain and the spinal cord increases with age.[188,189]

2.10.2 $p75^{NTR}$-RhoA Signaling

RhoA was identified as the $p75^{NTR}$ interacting protein involved in actin assembly.[190] Evidence indicated that RhoA activity is mediated by the α-helices in the cytoplasmic domain of $p75^{NTR}$. These early studies reported that while $p75^{NTR}$ is able to activate RhoA, neurotrophin binding prohibited RhoA activation and instead stimulated neurite elongation.[190,191] Further investigates reveal that RhoA activation is dependent upon direct interaction with the intracellular domain of $p75^{NTR}$, however, neurotrophins stabilize RhoA in its inactive form preventing activation.[191] RhoA bound to GDP (inactive) interacts with Rho-GDI, an intracellular shuttle that inhibits nucleotide dissociation and prevents Rho proteins from being activated and translocated to the membrane.[192] Thus, it has been proposed that $p75^{NTR}$ displaces the inactive GDP-bound RhoA from Rho-GDI.[191] Furthermore, it was discovered that "shedding" or cleavage of $p75^{NTR}$ liberates the intracellular domain fragment of $p75^{NTR}$ which causes the displacement of RhoA from Rho-GDI, thus activating RhoA and inhibiting neurite outgrowth.[193] As neurotrophins do not induce $p75^{NTR}$ cleavage, NGF binding to $p75^{NTR}$ does not elicit RhoA activation.[161,194] RhoA activation has been deemed necessary for JNK activation and apoptosis induced by $p75^{NTR}$.[172]

2.10.3 ROCK inhibition

Recent studies implicate ROCK2 involvement in propagating downstream effects such as axonal degeneration and apoptosis.[195] ROCK2 was found to limit axonal growth after CNS trauma and so it has been identified as a potential therapeutic target in neurodegenerative diseases.[189,195] The expression of RhoA/ROCK pathway is induced by neuron injury and is often

found in lesions of the CNS.[179] Use of ROCK inhibitors has shown to be promising in restoration of neurite growth after injury. Systemic and localized administration of ROCK inhibitor Y-27632, in both mouse and rat spinal-cord-injury models proved to restore function and accelerate recovery. Use of this inhibitor was also found to increase axonal regeneration following neuronal insult.[195-198] Furthermore, as RhoA was discovered to promote $A\beta_{42}$ production, administration of Y-27632 was shown to be effective in lowering $A\beta_{42}$ levels in both cell cultures and in PDAPP transgenic mice.[199]

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CHAPTER 3: Pro-Nerve Growth Factor Induced RhoA Kinase Activation in PC12 cells

3.1 Introduction

Alzheimer's disease (AD) is an age-related, neurological disorder characterized by irreversible and progressive neurodegeneration leading to cognitive and behavioral deficits.[1] Nerve growth factor (NGF) is a neurotrophin that regulates survival, maturation, and differentiation of neurons.[2] Pro-nerve growth factor (proNGF) is the precursor form of NGF, which is cleaved by the matrix metalloproteinase-7 (MMP-7) into NGF.[3] In contrast to mature NGF, proNGF selectively binds neurotrophin receptor p75 ($p75^{NTR}$) to elicit pro-apoptotic responses.[4,5] Peroxynitrite, a reactive oxygen species found to mediate oxidative damage in AD brain tissues, impairs the activity of MMP-7 and leads to proNGF accumulation.[6-8] Both, the concentration of proNGF/NGF and expression of their relative receptors, determine the balance between cell survival and cell death in the nervous system.[9,10]

$p75^{NTR}$ is single-chain transmembrane receptor of the TNF family that is best known for mediating neural cell death during development as well as in the adult brain following injury and disease.[11-14] $p75^{NTR}$ favorably binds proNGF and forms a heterotrimeric signaling complex with sortilin.[15]Activation and cleavage of $p75^{NTR}$ leads to ubiquitination and nuclear translocation of NRIF, phosphorylation of JNK, phosphorylation and oligomerization of the BH3-domain-only family member (Bad), and activation of caspases 9, 6, and 3.[16-19] As $p75^{NTR}$ has no intrinsic enzymatic activity it depends on several interacting proteins to propagate downstream signaling cascades. [20-22]

RhoA GTPase was discovered to be a $p75^{NTR}$ interacting protein and a necessary component of JNK activation in $p75^{NTR}$ mediated apoptosis.[23,24] RhoA is a major small GTPase that acts as a molecular switch between pro-survival and pro-death responses to neurotrophins

and neurodegenerative insults.[25] $p75^{NTR}$ activation of RhoA results in neuronal death via activation of JNK and p38MAPK signaling cascade.[26-29] Modulation of the RhoA/ROCK pathway is believed to have potential therapeutic applicability in a wide variety of CNS pathologies.[30,31] ROCK inhibitor Y-27632, has shown to promote axonal regeneration and neurite outgrowth in several cell lines.[30,32,33]

Previous research in our lab found the expression of proNGF was significantly increased and NGF level was decreased in AD human hippocampal brain tissue when compared to control.[34] We also discovered that the AD human hippocampal samples had decreased MMP-7 expression as compared with controls.[34] As MMP-7 cleaves proNGF into mature NGF, this impairment may augment the accumulation of proNGF in AD condition. In addition, $p75^{NTR}$ expression was observed to be greater in AD as compared to control.[34] ProNGF has great affinity to bind $p75^{NTR}$ and induce apoptosis via activation of JNK pathway.[35] In this study, we aimed to elucidate the molecular events by which proNGF contributes to AD-induced neurodegeneration by mimicking overexpression of proNGF in PC12 cell models. In particular, we examined the role of RhoA kinase activation as a downstream signaling pathway in response to proNGF. We demonstrate that inhibition of RhoA kinase reduced $p75^{NTR}$ expression, JNK phosphorylation, and restored cell viability.

3.2 Materials and Methods

3.2.1 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.

3.2.2 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 Zheng *et al.,* 2015.[34]

3.2.3 Cell Culture

PC12 rat pheochromocytoma cells (1mL) were cultured in Dulbecco's modified Eagle's media supplemented (DMEM) with 10% heat-inactivated horse serum, 5% fetal bovine serum, and antibiotics (100 units/mL; streptomycin and penicillin) (9mL) on 100mm plates coated with150 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.

3.2.4 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.

3.2.5 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.

3.2.6 Cell Viability

PC12 cells were treated with proNGF (50 ng/ml) or NGF (50 ng/ml) in starved media for 24 h. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (MTT) assay. MTT is a tetrazolium salt undergoes reduction by metabolically viable cells to a colored, formazan salt. The MTT assay was analyzed with the CellTiter96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI) according to the manufacturer's protocol. The results are represented as percentage of surviving neurons relative to control values (untreated serum-deprived cells, 100%).

3.2.7 Statistical analysis

All quantitative data is expressed as mean ± standard errors of the mean (SEM). Differences among groups were evaluated by ANOVA followed by Tukey-Kramer Multiple

comparison test (Graphpad Prism). Statistical significance was defined as $P<0.0001$.

3.3 Results

3.3.1 ProNGF increased expression of $p75^{NTR}$ and activation of Rho

The effect of proNGF signaling is dependent upon the expression levels of $p75^{NTR}$. Our lab has previously noted that AD hippocampal brain tissue expressed greater levels of $p75^{NTR}$ compared to control.[34] Therefore, as the first step, we evaluated whether proNGF stimulation can increase the expression of $p75^{NTR}$. PC12 cells were treated overnight with vehicle, proNGF or NGF. The cells were lysed and Western blotted with $p75^{NTR}$ and actin antibodies. Figure 2A, shows that cells treated with proNGF upregulated 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. $p75^{NTR}$ lacks intrinsic enzymatic activity and thus relies on several binding proteins for signal transduction to secondary messengers.[36] RhoA GTPase was identified as such a protein.[24] Therefore, we examined the activation of RhoA kinase pathway implicated in neuronal death. PC12 cells treated with vehicle, proNGF or NGF were subjected to pull down assay and Western blotted with Rho antibody. As shown in figure 2B, activation of Rho kinase was increased in proNGF stimulated cells compared to control and NGF. The expression of total Rho was equal in all the cell lysates. In addition to this, we also examined the Rho kinase activity in AD disease brain since proNGF and $p75^{NTR}$ was increased in those tissues. We used 6 postmortem control aged human hippocampal and 6 postmortem AD human hippocampal samples.[34] The activation of Rho kinase activity was determined by pull down assay in the tissue homogenates. As shown in figure 2C, the Rho 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.

3.3.2 ProNGF induced activation of JNK, p38 MAPK pathway and expression of apoptotic markers in PC12 cells

As both levels of $p75^{NTR}$ and RhoA kinase activity were elevated in proNGF treated cell cultures, we investigated the activation of p38/JNK MAPK as a common signaling pathway implicated in neuronal death. Overexpression of $p75^{NTR}$ has been shown to constitutively activate RhoA resulting in neuronal death via activation of p38/JNK MAPK pathways.[29,37] PC12 cell lysates stimulated overnight with vehicle, proNGF or NGF. The cell lysates were Western blotted with JNK/p38 MAPK or its specific-phospho antibodies. The results suggest that phosphorylation of JNK and p38 MAPK was increased by proNGF than control or NGF treated cells (Fig. 3A and B). The same lysates were also analyzed for apoptotic markers such as cleaved PARP and caspase-3. The expression level of cleaved PARP and caspase-3 was increased in cells treated with proNGF compared with control or NGF treated cells. (Fig. 3C).

3.3.3 Inhibition of Rho activation reduced the expression of $p75^{NTR}$ in PC12 cells

The expression of $p75^{NTR}$ is increased in AD and by overexpression of proNGF (Fig. 2A). The increased expression of proNGF and $p75^{NTR}$ in turn activates RhoA kinase (Fig. 2B and C). We further want to determine whether inhibiting Rho kinase activity will reduce the expression of $p75^{NTR}$. We pretreated the PC12 cells overnight with proNGF, Rho kinase inhibitor, Y-27632 or both. The cell lysates were Western blotted with $p75^{NTR}$ and actin antibodies (Fig. 4A). The expression of $p75^{NTR}$ was reduced by attenuating the Rho kinase activity. Activation of Rho kinase in these same lysates was also detected by pull down assay. Figure 3b, suggests that RhoA kinase activity was reduced by the addition of Y-27632. This clearly explains that Rho kinase activity causes the increase in the expression of $p75^{NTR}$.

3.3.4 Inhibition of Rho activation reduced the activation of JNK, p38 MAPK and apoptotic markers expression

Next, we determined whether attenuating the Rho kinase activity will block the phosphorylation of JNK and p38 MAPK. The cell lysates treated with either proNGF, Y-27632 or both were Western blotted with phospho and non-phospho antibodies of JNK and p38 MAPK antibodies. The Rho kinase inhibitor Y-27632 reduced the activation of JNK and p38 MAPK compared to proNGF alone treated cells (Fig. 5A and B). In addition to this the expression of cleaved-PARP and caspase-3 was also reduced by the Rho kinase inhibitor as shown in figure 5C.

3.3.5 ProNGF induced death was blocked by inhibiting Rho activation in PC12 cells

ProNGF binding to $p75^{NTR}$ elicits neuron apoptosis via activation of JNK pathway.[17] We found overexpression of proNGF induced expression of $p75^{NTR}$ and subsequent p38/JNK MAPK pathway via RhoA activation (Fig.2-3). Rho inhibitor Y-27632 was found to reduce expression of $p75^{NTR}$ and mitigate activation of p38/JNK MAPK (Fig.4-5). We wanted to determine whether proNGF leads to cell death by inducing Rho kinase activity. PC12 cells are grown normally in DMEM containing serum, however deprivation of serum for 24 h induces cell death. (A) PC12 cells were treated with proNGF (50ng/mL) or NGF (50 ng/mL) overnight. (B) PC12 cells were treated overnight with either proNGF (50 ng/mL) or Rho kinase inhibitor, Y-27632 (1 μM) or both. Cell viability was measured biochemically using the MTT assay. NGF treatment protected PC12 cells from death on serum deprivation, whereas proNGF significantly increases cell death (Fig. 6A). Addition of Rho kinase inhibitor Y-27632 along with proNGF protected serum-starved PC12 cells from cell death (Fig. 6B). Taken together, these findings suggest that proNGF induces cell death through activation of Rho kinase.

3.4 Discussion

In AD, the levels of proNGF are increased in a stage-dependent manner correlating with neuronal death of the hippocampal, cortical, and basal forebrain cholinergic neurons.[38,39] Previously our lab found the expression of proNGF was greater than NGF in AD human hippocampal brain samples. Furthermore, the expression of the proNGF converting enzyme, MMP-7, was reduced, resulting in proNGF accumulation.[34] Although it has been documented that proNGF can promote neuronal apoptosis through binding $p75^{NTR}$, the exact molecular events by which proNGF mediates its apoptotic action is not fully understood.[4,9]

Our intention was to expand on previous findings by investigating the molecular events by which proNGF contributes to AD-induced neurodegeneration. $p75^{NTR}$ serves as a high affinity receptor for proNGF and elicits apoptosis via JNK activation.[40] We mimicked AD overexpression of proNGF in PC12 cell models and analyzed activity of downstream signaling proteins. In particular, we examined the role of RhoA kinase activation as a downstream signaling pathway in response to proNGF.

PC12 cells treated with proNGF exhibited increased expression of $p75^{NTR}$ and activity of RhoA as compared to those treated with NGF or control. This is in agreement with previous reports that $p75^{NTR}$ constitutively activates RhoA.[27,37] RhoA activity was also found to be greater in AD human hippocampal samples as compared to age-matched healthy control samples. Thus, proNGF stimulation leads to over expression of $p75^{NTR}$ resulting in increased RhoA activity in vitro and in vivo. In addition, phosphorylation and activation of JNK, p38 MAPK, and apoptotic markers PARP, and caspase-3 was significantly increased in proNGF treated cell cultures. Together, these results indicate proNGF stimulation of $p75^{NTR}$ elicits death signaling by way of RhoA activation and p38/JNK MAPK cascade. As RhoA activates the p38/JNK MAPK pro-

apoptotic pathway, inhibition of Rho kinase may be neuroprotective.[33] Indeed, PC12 cells treated with proNGF in the presence of Rho inhibitor Y-27632 showed decreased expression of $p75^{NTR}$ as compared to cells treated with proNGF alone. Furthermore, inhibition of RhoA in proNGF treated cells mitigated phosphorylation and activation of JNK, p38 MAPK, PARP, and caspase-3. MTT assays of cell survival under experimental treatments revealed overexpression of proNGF led to augmented cell death. However, administration of Rho inhibitor Y-27632 mitigated proNGF effects and restored cell survival.

In this study, we found that Rho kinase inhibitor reduced the expression of $p75^{NTR}$ which was increased by proNGF (Fig. 4A), The activation of downstream signaling JNK and p38MAPK was also inhibited by Rho kinase inhibitor and attenuated the cell death induced by proNGF in PC12 (Fig. 5 and 6). These findings suggest that inhibition of activation of Rho kinase can reverse the proNGF induced cell death through $p75^{NTR}$ in Alzheimer's disease.

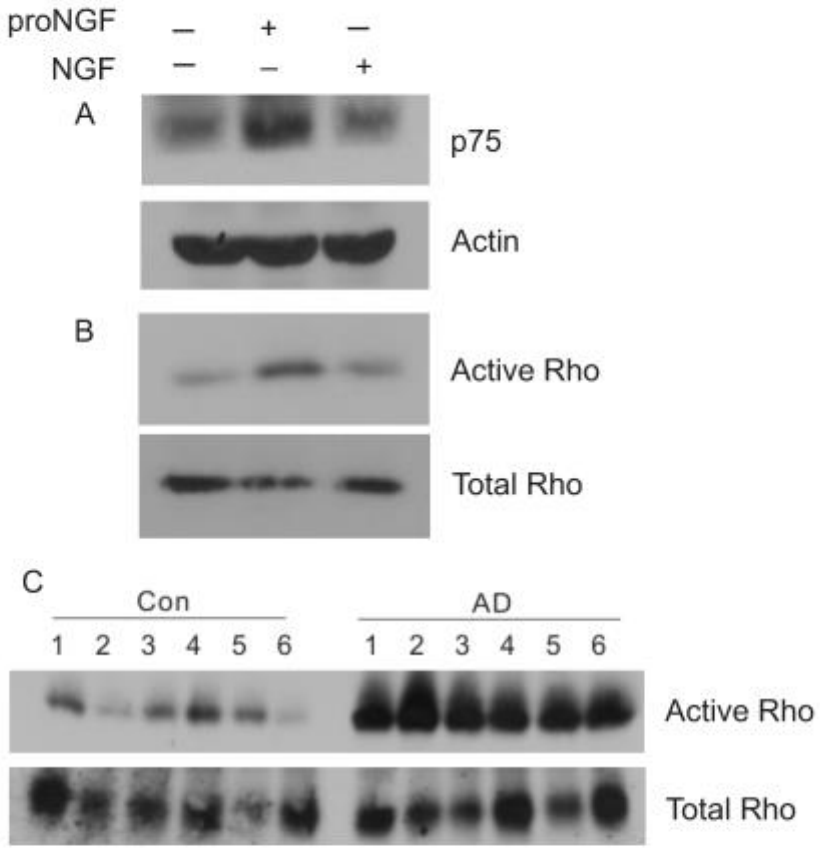

Fig. 2 ProNGF increased the expression of $p75^{NTR}$ and activation of Rho. PC12 cells were treated with pro-NGF (50 ng/mL) or NGF (50 ng/mL) overnight. The cells were lysed and (A) western blotted with anti-p75, anti-actin, (B) lysates were subjected to pull-down assay with agarose conjugated rhotekin-RBD followed by western blot with Rho antibody. (C) Homogenates of postmortem control age matched and AD human hippocampal tissues were subjected to pull-down assay with agarose conjugated rhotekin-RBD to detect active Rho in human hippocampal tissues.

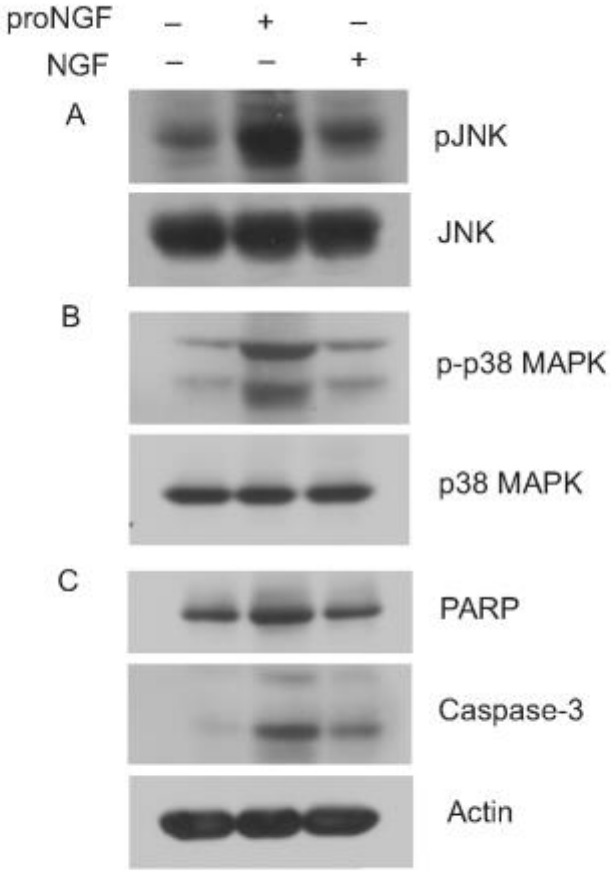

Fig. 3 ProNGF induced activation of JNK, p38 MAPK pathway and expression of apoptotic markers in PC12 cells. PC12 cells were treated with proNGF (50 ng/mL) or NGF (50 ng/mL) overnight. The cells were lysed and western blotted with (A) phospho and non-phospho-JNK antibodies, (B) phospho and non-phospho-p38MAPK antibodies, and (C) PARP, caspase-3, actin antibodies.

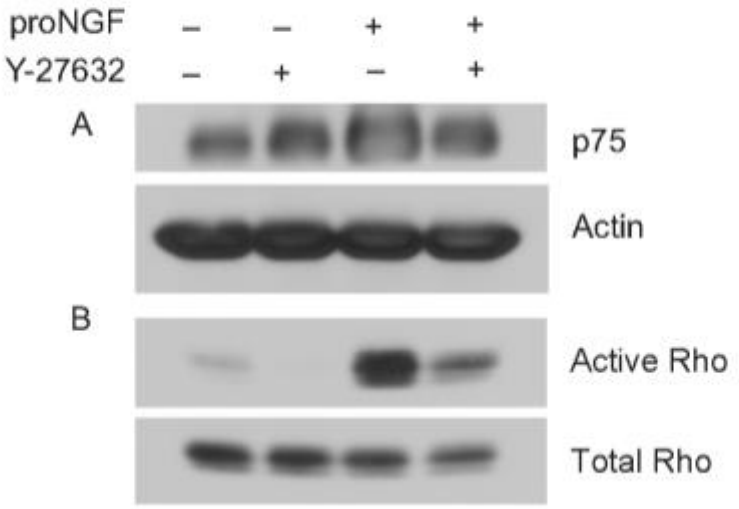

Fig. 4 Inhibition of Rho activation reduced the expression of p75^{NTR} in PC12 cells. PC12 cells were treated overnight with either proNGF (50 ng/mL) or Rho kinase inhibitor, Y-27632 (1 μM) or both. The cells were lysed and (A) western blotted with anti-p75NTR, anti-actin (B) pull-down assay with agarose conjugated rhotekin-RBD to detect the activation of Rho.

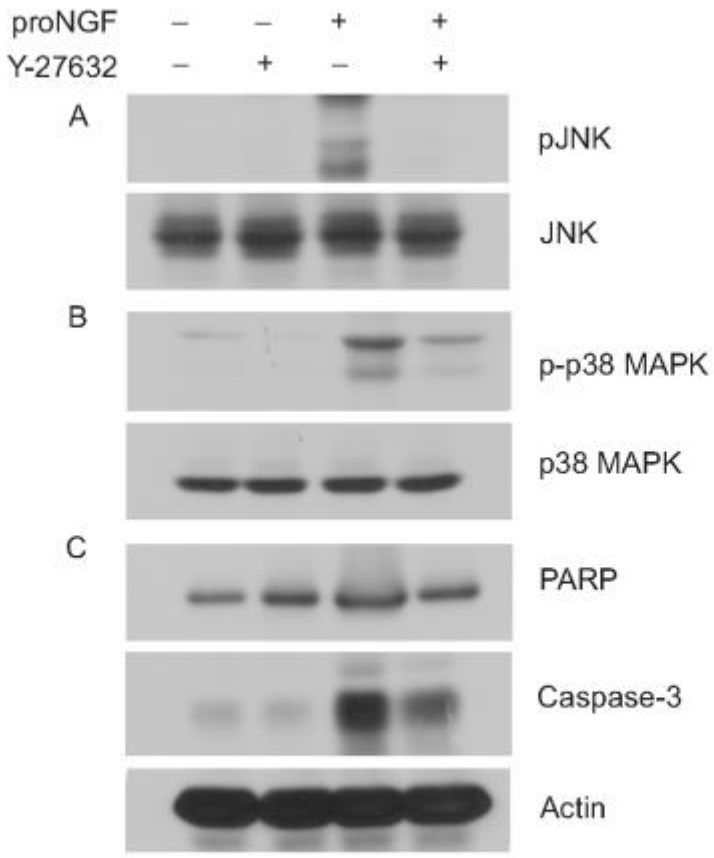

Fig. 5 Inhibition of Rho activation reduced the activation of JNK, p38 MAPK and apoptotic markers expression. PC12 cells were treated overnight with either proNGF (50 ng/mL) or Rho kinase inhibitor, Y-27632 (1 μM) or both. The cells were lysed and western blotted with (A) phospho and non-phospho-JNK antibodies, (B) phospho and non-phospho-p38MAPK antibodies, and (C) PARP and caspase-3 antibodies.

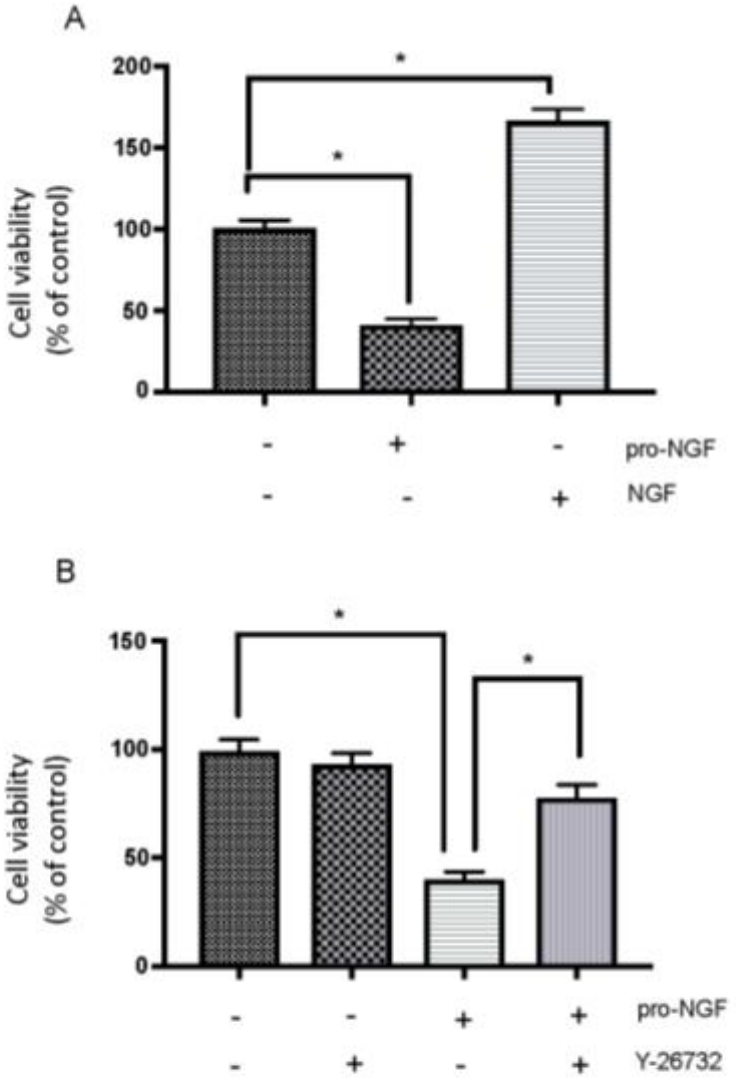

Fig. 6 ProNGF induced neuronal death was blocked by inhibiting Rho activation in PC12 cells. (A) PC12 cells were treated with proNGF (50 ng/mL) or NGF (50 ng/mL) for 24 h. (B) PC12 cells were treated with either proNGF (50 ng/mL) or Rho kinase inhibitor, Y-27632 (1 mM) or both. Cell viability was measured biochemically using the MTT assay. The mean and standard deviation of three experiments is shown ($P<0.0001$).

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