The Role of Polyubiquitination in Nerve Growth Factor Signaling and its Alteration in Alzheimer's Disease

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 6, 2016

Keywords: Alzheimer's disease, ubiquitination, NGF, aymolid beta, TrkA, $p75^{NTR}$

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Abstract

Nerve growth factor (NGF) is a neurotrophin regulates survival and differentiation of neurons. To execute its antiapoptotic function, NGF binds to selective receptor TrkA and non-selective receptor $p75^{NTR}$, leading to TrkA dimerization and autophosphorylation, which recruits downstream signaling proteins, including phospholipase C-1, Shc, FRS2 and phosphoinositol 3-kinase (PI3K)/Akt. Here we demonstrate that NGF stimulation leads to both $p75^{NTR}$ and TrkA polyubiquitination resulting in neuronal cell survival. We also show that both $p75^{NTR}$ and TrkA undergo TRAF6-mediated polyubiquitination with Lys-63(K63) ubiquitin chains on NGF stimulation, which is required for activation of downstream cell signaling such as PI3K/Akt pathway and mitogen-activated protein kinase (ERK/MAPK) pathway. TRAF6, an ubiquitin E3 ligase, interacts with scaffold protein p62 on NGF stimulation and promotes polyubiquitination of $p75^{NTR}$ and TrkA.

Amyloid beta (Aβ) protein is the primary proteinaceous deposit found in the brains of patients with Alzheimer's disease (AD). Evidence suggests that Aβ plays a central role in the development of AD pathology. We revealed that both $p75^{NTR}$ and TrkA polyubiquitinations are impaired in the presence of Aβ *in vitro* or in AD brains. Interestingly, the nitrotyrosylation of TrkA was increased in AD hippocampus and this might be an explanation for reduced phosphotyrosylation and ubiquitination of TrkA. Furthermore, In AD brain, the matrix metalloproteinase-7 (MMP-7), which cleaves

proNGF to produce mature NGF, was also reduced, thereby leading to the imbalance between pro-NGF and NGF and eventually activating cell apoptosis.

Acknowledgments

I would like to express my deepest gratitude is to my advisor and my committee chair, Dr. Ramesh Jegenathan for his patient guidance, motivation and immense knowledge. His continued support helped me overcome many crisis situations and finish this dissertation.

Besides my advisor, I would like to thank the rest of my committee: Dr. Douglas White, Dr. Kevin Huggins and Dr. Heidi Kluess, for their encouragement and insightful comments.

I am also thankful to Dr. Geetha Thangiah for guiding me sort out the technique details of my work and the long discussion of experimental design with me.

I thank my fellow labmates in for the stimulating discussions and their advice on my Ph.D. study.

Last but not the least, I would like to thank my family: my wife and my parents and to my other relatives for supporting me spiritually throughout writing this dissertation and my life in general.

Reference Style

This document is referenced using the citation style of the *Journal of Biological Chemistry*.

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CHAPTER 1: INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder and cause of dementia (1), affecting millions of Americans. Clinical manifestations of AD begin with difficulties in short-term memory and visual-spatial confusion. Memory loss becomes more severe over time and eventually causes reduced intellectual capacity and cognitive dysfunction. Eventually, people with AD become unable to maintain their personal independence (2-4).

AD pathology is characterized by an accumulation of misfolded proteins, inflammatory changes, and oxidative damage that leads to loss of neurons and synapses (5). Two hallmarks detected in AD brains are extracellular senile plaques caused by the accumulation of Amyloid-β (Aβ) and intracellular neurofibrillary tangles (NFT) caused by the accumulation of hyperphosphorylated tau.

Amyloid β protein (Aβ) is a 39-43 amino acids peptide derived by the β- and γ- secretase cleavage of amyloid precursor protein (6). Aβ aggregates are the major component of senile plaques found in the patients of AD (7). Those redundant Aβ arise from two ways: overproduction of the Aβ due to gene mutation causes its accumulation thereby leads to early-onset familial AD (EOFAD) (6); Failure to remove Aβ from the brain causes late-onset sporadic AD (LOSAD) (8). This might be due to diminished ability of microglial cells to clear Aβ, impairment of neprilysin and insulysin Aβ degrading proteases, and diminished perivascular and vascular drainage (8-12).

Nerve growth factor (NGF) is a neurotrophin that regulates survival, differentiation and maintenance of sensory and sympathetic neurons (13). Supply of NGF displays multiple physiological actions in the central nervous system (CNS). It is also a key neurotrophin and its dysregulation could be involved in various neurodegenerative diseases such as AD. NGF can bind to two classes of cell-surface receptors, namely a high-affinity tyrosine kinase TrkA receptor and a low-affinity $p75^{NTR}$ receptor (14,15).

TrkA is a single transmembrane-spanning protein which belongs to the super family of tyrosine kinase (RTK) receptors, that regulate synaptic strength and plasticity in the mammalian nervous system (16). Upon selective binding of NGF to TrkA, the receptor undergoes a series of events, including dimerization, autophosphorylation (17), polyubiquitination (18), followed by internalization of the receptor to signaling vesicles (19,20), which elicits many of the classical neurotrophic actions (21). TrkA activation leads to subsequent cascade signaling such as Ras/MAPK and the PI3K pathways, which act to intercept nuclear and mitochondrial cell-death programs, resulting in neuronal survival and differentiation.

Another one, non-selective p75 neurotrophin receptor ($p75^{NTR}$), is expressed in basal forebrain cholinergic neurons that undergo degeneration in AD (22). $p75^{NTR}$ has multiple roles in neurons.

The ligands for $p75^{NTR}$ are NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). However, recent evidence suggest that $p75^{NTR}$ is not only a receptor for mature neurotrophin, but a high affinity receptor of proneurotrophins. Given that binding of proneurotrophin to $p75^{NTR}$ leads to apoptosis, an emerging concept is that the two distinct receptor classes, Trk and $p75^{NTR}$, preferentially bind mature and

proneurotrophins (neurotrophin precursors), respectively, to elicit opposing biological responses (23), which is consistent with our recent finding that binding of NGF to $p75^{NTR}$ mediates neuronal cell survival (24), whereas the $p75^{NTR}$ binds to Aβ (25-27) will mediate Aβ-induced neuronal death (28-30). Interestingly, polyubiquitination of $p75^{NTR}$ undergoes polyubiquitination by interacting to tumor necrosis factor receptor-associated factor 6 (TRAF6) upon NGF stimulation (24), which is shown to be important to execute its neuronal protective effect.

Protein ubiquitination is one of the most important posttranslational modifications involved in a variety of biological functions, including DNA repair, endocytosis, transcription, degradation and altering protein location (31). Ubiquitination is accomplished by a three-step enzymatic cascade. First, ubiquitin (Ub) is activated by E1 (activating enzyme) to form a high energy thioester bond using ATP. Then the activated Ub is transferred to a member of the E2 family (conjugating enzyme). E3 (ligase) mediates the activated Ub onto a lysine residue of the substrate (32). Once the first Ub is conjugated, other Ubs can be added on any of seven lysine residues of the first Ub, forming a polyUb chain. The different types of Ub chains lead to a board functions (33). It is known that Lys48 (K48)-linked polyubiquitination chain target substrates for proteasome-mediated degradation, whereas the K63-linked ubiquitination plays an important role in protein trafficking and cell signaling activation.

The Ub type is determined by E3 ligase. TRAF6, an E3 ligase, specifically mediate K63-linked polyubiquitination which can regulate a variety of cell signaling activation such as NF-κB signaling (34), mTOR signaling (35) and Akt signaling (36). During this process, p62 is found to interact with TRAF6 as a scaffold protein (34). p62 also induces self-

polyubiquitination of TRAF6 and oligomerization thereby increasing the activity of TRAF6 (37). TRAF6/p62 ubiquitinates several substrates and leads to various cellular functions: ubiquitination of TrkA, improving cell survival and differentiation (18); activation of NRIF, leading to apoptosis (38); activation of Unc-51-like kinase 1/2 regulating filopodia extension and axon branching in sensory neurons (39); and initiation of proteasomal degradation of tau (40).

A large number of studies reported that decreased NGF signaling leads to loss of neuronal markers and shrinkage in AD (41). Considering the function of K63-linked ubiquitination in cell signaling, the purpose of this study was to determine the role of ubiquitination in NGF signaling, especially in AD condition.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Alzheimer's disease

2.1.1 Dementia and Alzheimer's disease

Dementia is classified as a neurocognitive disorder that describes a wide range of symptoms associated with a decline in memory and other cognitive functions severe enough to interfere with daily activities (42). Various cognitive abilities can be impaired with dementia, including memory, language, reasoning, decision making, visuospatial function, attention, and orientation. In individuals with dementia, cognitive impairments are often accompanied by changes in personality, emotional regulation, and social behaviors. Importantly, the cognitive and behavioral changes that occur with dementia interfere with work, social activities, and relationships and impair a person's ability to perform routine daily activities (e.g., driving, shopping, housekeeping, cooking, managing finances, and personal care).

There are several causes of dementia, including neurodegenerative and/or vascular processes in the brain. Alzheimer's disease (AD) is the most common neurodegenerative disorder and cause of dementia (1), accounting for an estimated 60% to 80% of cases in the United States (43).

Clinical manifestations of AD begin with short-term memory loss and visual-spatial confusion. Memory loss becomes more severe over time and eventually causes reduced intellectual capacity and cognitive dysfunction. Eventually, people with AD become unable to maintain their personal independence (2-4).

2.1.2 Epidemiology

AD is a critical public health issue in the United States and many other countries around the world, with a significant health, social, and financial burden on society.

An estimated 5.3 million Americans of all ages have AD or about one new case every 67 second, including an estimated 5.1 million people age 65 and older (43). In the United States, AD is the fifth leading cause of death among older adults, and 17.9 billion hours of informal assistance were provided to people with AD and other dementias, which is approximately valued at $217.7 billion.

Because of the increasing number of people age 65 and older in the United States, particularly the oldest-old, the annual number of new cases of AD and other dementias is projected to double by 2050. This translates to approximately 65 million Americans may have AD by 2030 (13.8 million by 2050) (43,44).

Like other common chronic diseases, several risk factors are associated to AD development and progression except the rare cases of AD caused by genetic mutations. Age is the greatest risk factor for the development of AD. Although people younger than 65 can also develop the disease which is known as early-onset familial AD (EOFAD), the majority of cases (95% of all cases) are aged 65 or older which is known as late-onset sporadic AD (LOSAD). The incidence of the disease doubles every 5 year from age 65 (5).

Another important risk factor is gender. The prevalence of AD is higher among females, reflecting the longer life expectancy of women (45).

Other factors include: Lower educational attainment has been associated with increased risk of AD dementia. Some researchers believe that education builds a cognitive reserve that enables individuals to better compensate for changes in the brain (46). People with a history of diabetes, hypertension, obesity, and smoking have a substantially elevated risk of AD (47). Family history of AD is not necessary for an individual to develop the disease. However, people who have first-degree relatives with AD are at higher risk to have AD (48).

2.1.3 Genetics of AD

The cause of most Alzhemer's cases is still unknown except of only around 0.1% of the cases resulted from familial forms of autosomal dominant inheritance (49). Most individuals with EOFADs develop as a result of mutations to any of three specific genes: *presenilin 1* (*PSEN1*), *presenilin 2* (*PSEN2*) and *amyloid precursor protein* (*APP*) (50,51). APP is a transmembrane protein that influences β-catenin regulation. β-catenin anchors proteins to the actin cytoskeleton and plays an essential role in cell-to-cell adhesion and in Wnt signaling (52). APP is cleaved via γ-secretase-mediated processes and PSEN1 and PSEN2, producing neurotoxic Aβ which is main component of the senile plaques found in the brains of AD.

The genetics of LOSAD are more complex and less well understood. Apolipoprotein E (APOE) is a class of apolipoprotein which is essential for the normal catabolism of triglyceride-rich lipoprotein constituents and modify the γ-secretase activity. It is known that people carried APOE gene ε4 allele have a three-fold higher risk of developing AD than those without ε4 allele (48,53,54). The products of other genes such as *insulin-*

degrading enzyme (*IDE*) and *ubiquilin-1*(*UBQLN1*) may be involved in Aβ degradation and intracellular trafficking of APP, respectively (54).

2.1.4 Neuropathology of AD

The histopathology of AD is characterized by an accumulation of misfolded proteins, inflammatory changes, and oxidative damage that leads to loss of neurons and synapses (5). Today, people have observed the neuropathological features of typical AD i.e. extracellular deposits or amyloid plaques caused by the accumulation of Aβ (55) and intracellular neurofibrillary tangles (NFT) caused by the accumulation of hyperphosphorylated tau (56). Several hypotheses have been put forward on the basis of the various causative factors, including Aβ hypothesis, tau hypothesis, cholinergic hypothesis and inflammation hypothesis (57). Because amyloid plaques are a unique feature of AD, whereas NFTs are found in other neurodegenerative conditions, Aβ hypothesis has been the basis for most work on the pathogenesis of AD over the years (58). However, although Aβ hypothesis has been strongly supported by data from EOFAD, it does not account for more complex sporadic AD well and has so far failed clinically (59).

2.1.4.1 Aβ hypothesis

Aβ is a multifunctional peptide with significant non-pathologic activity (60). It is the primary component of amyloid plaques associated with AD, and the primary component of senile plaque protein deposits found in the brains of patients with AD (7). The accumulation of Aβ aggregates is believed to be fundamental to the development of neurodegenerative pathology and to contribute to the progression of AD.

According to Aβ hypothesis, the APP undergoes normal cleavage by α-secretase and aberrantly processed by β- and γ-secretase. The latter cleavage results a 36-43 amino acid

peptide - Aβ production (61). First, β-secretase (β-site-APP-cleaving-enzyme, also called BACE) cleave the ectodomain of APP, resulting in the shedding of APPsβ. γ-Secretase subsequently cleaves the transmembrane domain of the APP carboxy-terminal fragments (β-CTF), generating a number of isoforms of Aβ peptides into extracellular environment (62).

As a consequence, Aβ peptides spontaneously self-aggregate into multiple coexisting physical forms such as soluble oligomers or coalesce to grow into fibrils, forming insoluble β-pleated sheets and are eventually deposited in diffuse senile plaques. The major isoforms of Aβ are $A\beta_{40}$ and $A\beta_{42}$. The only difference between them is $A\beta_{42}$ has 2 extra residues at C-terminus.

Monomers of $A\beta_{40}$ are much more prevalent than aggregation-prone and damaging $A\beta_{42}$ species. The concentration of $A\beta_{40}$ in cerebral spinal fluid has been found to be several-fold more than that of $A\beta_{42}$. However, $A\beta_{42}$ is more toxic and forms fibrils significantly faster than $A\beta_{40}$ (63). Also, $A\beta_{42}$ aggregates were the major component of amyloid plaques in AD brains while $A\beta_{40}$ is less detected (64). Recent study suggest that both $A\beta_{42}$ and $A\beta_{40}$ form interlaced amyloid fibrils *in vitro* process (65). These evidence indicate unrecognized mechanisms that lead to amyloid plaques at the extracellular space.

It has been shown that Aβ oligomers induce a series of pathological changes in brain such as oxidative damage, tau hyperphosphorylation, toxic effects on synapses and mitochondria (57) (66). However, it is difficult to clarify the oligomer's effect with the long phase of human AD. Furthermore, the specific species of Aβ which mediate downstream pathology remain unclear (67). While the direct neurotoxicity of Aβ oligomers

in vivo is still unknown, these species have been shown to directly initiate tau phosphorylation both *in vitro* and *in vivo* (68,69).

2.1.4.2 Tau pathology

Tau is a highly soluble microtubule-associated protein (MAP) found in neurons. The function of tau is to stabilize the microtubule network through its C-terminal 3 or 4 microtubule-binding domains (MBDs) (70). In nerve cells, tau is concentrated in axons (71). Six tau isoforms are expressed in adult human brain. These are generated by alternative splicing of the microtubule-associated protein tau (MAPT) gene (71).

Tau undergoes various post-translational modifications, including hyperphosphorylation, glycosylation, ubiquitination, glycation, polyamination, nitration, and proteolysis (72,73). Phosphorylation is one of the most important modifications because the biological activity of tau in promoting assembly and stability of microtubules is regulated by its degree of phosphorylation (74). Hyperphosphorylation of tau reduces its microtubule assembly activity and affinity to microbutules (75,76). In AD, tau is abnormally hyperphosphorylated, insoluble and self-associated as intraneuronal tangles of paired helical filaments (PHF). Although the mechanism of initiation of tau fibrillization in pathological conditions remains unclear, hexapeptide motifs of tau can function as a core to form β-sheet structures and induce PHF formation (77).

Evidence from young adults and fetal brains showed that tau is phosphorylated at some sites as those known to occur in PHF-tau (78). However, the level of phosphorylation at the sites is less than 5% of that in AD tau (79). Furthermore, hyperphosphorylation of tau may precede its accumulation in AD neurons. The mechanism of the formation of

hyperphosphorylation of tau might be due to a conformational changes in tau in the AD brain, which make it more suitable for phosphorylation and less possible for dephosphorylation (80).

Some kinases may be involved in this hyperphosphorylation, including glycogen synthase 3β (GSK3β), cyclin-dependent kinase 5 (CDK5), protein kinase A (PKA), and extracellular signal-regulated kinase 2 (ERK2). Reductions in both the expression and activity of PP2A have also been reported in the brains of patients with AD (81), which may be involved in dephosphorylation of tau. In AD, tau might first misfold, and the resulting conformational change could lead to a better substrate for kinases but a conformation that inhibits the phosphatases. Accumulated hyperphosphorylated tau leads to intraneuronal filamentous inclusions termed PHFs that are the main component of NFTs.

Ubiquitination is another important post-translational modification of tau. One of the hexapeptide motifs of tau includes the ubiquitination site (Lys-311), suggesting a possible link between tau aggregation and tau degradation through ubiquitination. In addition to hexapeptide motifs of tau, other components of NFTs are ubiquitin and APOE proteins. Tau also undergoes a stepwise fragmentation to generate cleaved tau molecules that have pro-aggregation properties and can induce neurodegeneration (82). Dolan *et al.,* found that these truncated forms of tau can be cleared by the autophagy system rather than Ubiquitin-Proteasome System (UPS), which is usually responsible for the degradation of full-length tau (83). However, Grune *et al.,* found that degradation of tau protein can be catalyzed by the ATP/ubiquitin-independent 20S proteasome (84).

The UPS is involved in tau turnover. Keck *et al.,* showed that the 20S proteasome co-precipitated with tau aggregates, and that this is accompanied by a decrease in proteasome

activity (85). Additional evidence confirms that tau can be degraded by the proteasome. Proteasome inhibition in cell culture abrogates tau degradation (86,87). Moreover, tau was degraded after incubation with the 20S proteasome in vitro (88). Evidence suggests a relationship between proteasome activity and tau accumulation in AD. The inhibition of the proteasome may explain the overall increase in ubiquitinated proteins in AD. First, ubiquitin binds tau at Lys-254, Lys-311, and Lys-353, as an early event in the development of AD (89). Next, additional ubiquitin molecules bind to those at Lys-6, 11, and 48. Binding at Lys-11 and 48 are known to target the protein to the UPS, although Lys-6 inhibits ubiquitin-dependent protein degradation and favors the formation of PHF over tau clearance (89).

Tau also affects the UPS activity. In AD brains, the endogenous proteasome activity is reduced (90,91). However, this reduction is neither associated with the diminished proteasome proteins nor the amount of hyperphosphorylated tau. The inhibitory binding of PHF-tau to proteasomes contributes to this reduction (85). In AD synapses, the accumulation of hyperphosphorylated tau oligomers is negatively correlated to valosin-containing protein, which is a chaperone that delivers substrates to proteasomes. However, this correlation does not exist with Aβ accumulation, suggesting tau hyperphosphorylation and the UPS dysfunction may progress together (92).

Studies have shown that CDK5 and GSK3 phosphorylates tau which in turn causes tau to interact with the carboxy-terminus of heat shock protein 70-interacting protein (CHIP), an E3 ubiquitin ligase (93,94). In CHIP knockout mice, soluble phosphorylated tau was found to be accumulated in the brain (95). In addition, a cellular kinase (AKT) and microtubule-affinity-regulating kinase (PAR1/MARK2) interact directly and together with hsp90 to

regulate tau degradation (96). Although hsp70 degrades tau regardless of its phosphorylation state, it does not affect the spatial memory of rats (93). BAG-1 is a binding partner of hsp70 that when overexpressed inhibits the degradation of tau and results in the accumulation of tau in neurons and contributes to the formation of NFTs (86).

2.2 NGF receptors and its signaling pathways

2.2.1 Neurotrophin family

Neurotrophins are central to many facets of CNS function, promoting proliferation, differentiation and survival of neurons and glia (97). In the mammalian brain, four neurotrophins have been identified: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5). These closely related molecules activate various cell signaling pathways by binding to two distinct classes of transmembrane receptor: the p75 neurotrophin receptor ($p75^{NTR}$) and the Trk family of receptor tyrosine kinases, which includes TrkA, TrkB and TrkC (98). While they all bind to $p75^{NTR}$ receptor with a non-selective, small affinity, specific Trk receptor selectively binds a different neurotrophin with a high affinity.

2.2.2 NGF

NGF was first discovered in the 1950s by Levi-Montalcini and Hamburger (99) due to its ability to promoted the growth of nearby sensory and sympathetic ganglia (100). More studies discover that NGF is essential for developing neurons in the peripheral nervous system (PNS) and survival of cholinergic neurons in the CNS depending on target-derived trophic factors retrogradely transported to innervating neurons (101,102).

NGF is ~13kDa peptide, sharing very similar higher-order protein structures and exhibit about 50% amino acid sequence homology with other neurotrophins (103), suggesting their potent evolutionary importance in nervous system regulation (104). Moreover, NGF appears to be highly conserved across the entire vertebrate lineage (105).

Like other secreted proteins, neurotrophins are derived from precursors, proneurotrophins, which are proteolytically cleaved to produce mature proteins. NGF is synthesized and secreted by glia or microglia (106) as a precursor (proNGF) (107). It is then cleaved intracellularly by furin and extracellularly by matrix metalloproteinase-7 (MMP-7), generating the mature, active mature form NGF (108). In the CNS, NGF is a key neurotrophin and its dysregulation could be involved in various neuronal degeneration diseases such as AD and multiple sclerosis [23, 24]. Due to its antiapoptotic role, clinical studies have been applied to investigate the effect in the treatment of AD. According to results from a phase I clinical trial, published recently in JAMA Neurology, the degenerating neurons retained an ability to sense and respond to NGF resulting in a neuronal growth (109). Because the majority of clinical trials in AD have focused on Aβ pathology, and the results have been largely disappointing, NGF could provide an alternative or complementary strategy to slow down the development of AD.

2.2.3 NGF signaling

NGF exerts the trophic and survival actions through activation of 2 types of receptors: high affinity receptor tyrosin kinase A (TrkA) which only binds to NGF and low

affinity, non-selective p75 neurotrophin receptor ($p75^{NTR}$) which can bind all members of neurotrophins family (17).

NGF binds to TrkA, leading to receptor dimerization and autophosphorylation of tyrosine kinase segment (110), which in turn recruit and activate phosphatidylinositol 3-kinase (PI3K) on plasma membrane, subsequently leading to the production of phosphoinositide 3,4,5-trisphophate (PIP3) and membrane translocation of the serine/threonine-protein kinases Akt, and eventually Akt is activated after receiving phosphorylation by PDK on the membrane. Despite other downstream targets, PI3K/Akt signaling pathway is particularly important for neuronal survival.

Another NGF signaling pathway is initiated through recruitment and phosphorylation of Shc, which leads to activation of Ras-mitogen activated protein kinase (MAPK).

Ras is a membrane-associated G protein; the active Ras phosphorylates the protooncogene Raf, which in turn activates MAPK kinase (MEK) and the latter one activates extracellular signal-regulated kinase 1/2 (ERK1/2) (111). Active ERK1/2 may enter into nucleus and regulate the activity of many transcription factors; it can also phosphorylate ribosomal S6 kinase (S6K) through mTORC1, affecting the regulation of the expression of NGF-inducible genes and, thus, contributing to neuronal differentiation or neurite outgrowth (112).

Besides the two pathways mentioned above, TrkA activation also mediate neuronal survival and outgrowth through phospholipase C gamma1 (PLCγ1) (113), which activate PKC signaling pathway and is thus involved in antimitogenic/mitogenic signaling. The Schematic representation of NGF signaling is in Fig. 1 below.

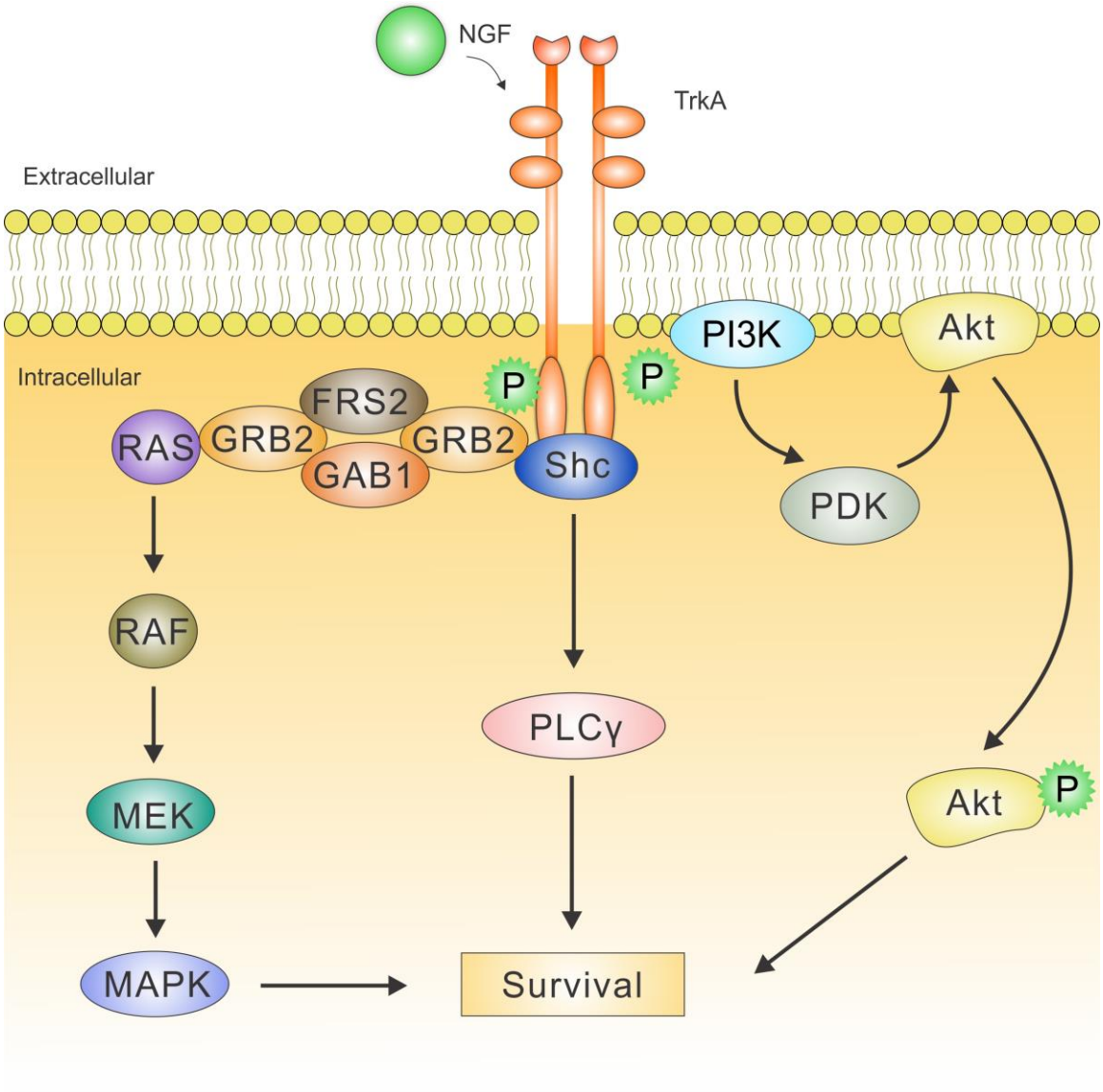


Fig. 1. Signaling Pathways activated by NGF. NGF binding to TrkA mediates neuronal survival through PI3K/Akt, Ras/MAPK and PLCγ downstream signaling.

Unlike the TrkA is mainly expressed at neurons responsive to NGF such as sympathetic neurons and basal forebrain cholinergic neurons, $p75^{NTR}$ is more widely distributed. $p75^{NTR}$ has more complicated role. On one side, it is a positive modulator of NGF/TrkA

complex (114) through forming a heterodimer co-receptor with TrkA, which provide higher affinity and specificity with NGF (17). On the other side, in the absence of TrkA receptors, $p75^{NTR}$ may form co-receptor with sortilin to initiate apoptosis in response to unprocessed proNGF (115). ProNGF also exhibits neurotrophic activity by stimulating TrkA to a lesser degree than NGF (116), suggesting that the proNGF: NGF and $p75^{NTR}$: TrkA ratios may play an important role to determine that either apoptotic or neurotrophic effects is predominated (117). Consequently, the particular role of $p75^{NTR}$ may depend on cellular contexts to a large extent.

The major pathways activated by $p75^{NTR}$ are the apoptotic c-Jun N-terminal kinase (JNK) signaling cascade, antiapoptotic nuclear factor κB (NF-κB) signaling and ceramide generation (100). $p75^{NTR}$ activates JNK through activation of Rac GTPase. JNKs stimulate the expression of proapoptotic genes via the transactivation of specific transcription factors (118), leading to programmed cell death. However, binding of NGF to $p75^{NTR}$ activates NF-κB, enhancing the survival response of developing sensory neurons to nerve growth factor (119). NF-κB is a nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of cell survival.

2.3 Ubiquitination

2.3.1 Stages of Ubiquitination

Protein ubiquitination was discovered in the early 1980s as a post-translational modification. Ubiquitin is attached via its C-terminal glycine residue to the ε-amino group of a substrate lysine residue. This reaction is catalyzed by sequential three-step enzymatic

cascades: activating (E1), conjugating (E2), and ligating (E3) enzymes (120,121). The initial step is that ubiquitin, a small protein of 76 amino acids, is activated by E1 to form an ubiquitin-E1 thiol ester bond. Next, E2 replaces E1 to conjugate the activated ubiquitin, and associates with a specific E3 ubiquitin ligase. The E3 is key in this process because it ligates ubiquitin to a lysine (Lys) residue in a given specific protein substrate.

2.3.2 Ubiquitin chain types

The ubiquitin modification exists as three general forms: mono-ubiquitination, multi-mono-ubiquitination and polyubiquitination. It has been shown that (multi-)mono-ubiquitination of cell-surface receptors triggers their internalization and subsequent degradation in lysosomes, or recycling to the cell surface (122). Furthermore, unlike other post-translational modification, ubiquitination has additional layer of complexity: ubiquitin contains seven lysine residues itself (Lys-6, Lys-11, Lys-27, Lys-29, Lys-33, Lys-48 and Lys-63). All of them can serve as acceptors of further ubiquitin molecules, leading to different ubiquitin chains or called polyubiquitination. The last decade a huge numbers of papers worked on function of Lys-48 and Lys-63 ubiquitin chain, whereas only few papers reported other chain types. To date, it remains unclear whether polyubiquitin chains with Lys-6, Lys-11, Lys-27, Lys-29, and Lys-33 linkages have biological functions although they have also been found in the cells (123,124).

2.3.3 Lys-48 Ubiquitin chain

While additional molecules of ubiquitin bind to Lys-48 of the first ubiquitin to mark the substrate, polyubiquitination is recognized by the 26S proteasome, which leads to degradation of the substrate by 26S proteasome (120). The proteasome is a multi-subunit, barrel-shaped complex. It exists as different oligomeric assemblies such as 20S and 26S

complexes. The primary difference between them is that the 26S assembly degrades polyubiquitinated proteins in an ATP-dependent manner. The 20S assembly degrades non-ubiquitinated proteins and peptides without expenditure of ATP (120,125). The 26S proteasome comprises the 20S catalytic core assembly and two 19S regulatory assemblies (126). The ubiquitin proteasome system (UPS) is one of the major pathways involved in clearance of proteins in mammalian cells, and plays an essential role in protein hemostasis.

2.3.4 Lys-63 Ubiquitin chain

Lys-63-linked chain is the second-most abundant type, accounting for 17% of all linkages. An increasing number of evidence on Lys-63 polyubiquitin chains has now identified important non-degradative roles, including substrate endocytosis, DNA-damage repair and promoting cell signaling transduction (127). It has now become clear that Lys-63 ubiquitin chain mediate internalization of signaling receptors (128-130) such as TrkA (18) or have a role in intracellular signaling via NF-κB (131).

2.3.5 TRAF6

The E3s has two major types, one containing a homologous to the HECT domain and the other containing RING-like domain (132). Most of E3s recognize target proteins for K48-linked ubiquitination. However, a few E3s such as tumor necrosis factor receptor-associated factor 6 (TRAF6) tagged proteins for K63-linked chain (132). TRAF6 is a crucial signaling mediator involving in innate immune response and regulating a series of cell signaling events (133,134). TRAF6 comprise an N-terminal zinc-binding domain, specifically a RING finger followed by five zinc fingers and a highly conserved C-terminal domain. The RING finger domain is the binding site of E2 enzymes and is essential for the E3 ligase activity.

TRAF6 has been implicated in directing diverse cell signaling receptors, including the TNF receptor superfamily (TNFRSF) (135), IL-1R/Toll-like receptor superfamily (IL-1R/TLRSF) (136), and TGFβR (137). Furthermore, TRAF6-induced K63-linked ubiquitination is required for downstream signaling activation of PI3K, MAPK cascade as well as other transcription factor families NF-κB, NFAT and IRF.(138,139).

2.3.6 Sequestosome 1/p62

Sequestosome 1/p62 is a ubiquitin binding protein involved in cell signaling and is found in most NFTs (140). It was first described as the interacting protein of the atypical PKCζ (141) and was subsequently found to serves as a scaffold for several signaling proteins.

p62 contains an ubiquitin like domain (UBL) at its N-terminus, ubiquitin associated domain (UBA) at the C-terminus and a TRAF6 binding domain. TRAF6-mediated ubiquitination requires p62 interaction to form TRAF6/p62 complex, which is essential in a diversity of signaling activation (35,37,142). Overexpression of p62/TRAF6 restored $p75^{NTR}$ polyubiquitination upon Aβ/NGF treatment (143). Polyubiquitination of $p75^{NTR}$ by p62/TRAF6 directs neuronal cell survival (144). The impairment of the association of p62/TRAF6 and $p75^{NTR}$ lead to cell death, which is consistent with that

It has been shown that loss of p62 induced Alzheimer's like phenotype in mice (145), indicating its importance in AD development. p62 has affinity for multi-Ub chains and may serve as a receptor to bind ubiquitinated proteins (146) and to shuttle the proteins to proteasome for degradation (147). For instance, p62 shuttles polyubiquitinated tau to proteasome for degradation (40).

References

1. Selkoe, D. J. (1991) The molecular pathology of Alzheimer's disease. *Neuron* **6**, 487-498
2. Lambon Ralph, M. A., Patterson, K., Graham, N., Dawson, K., and Hodges, J. R. (2003) Homogeneity and heterogeneity in mild cognitive impairment and Alzheimer's disease: a cross-sectional and longitudinal study of 55 cases. *Brain : a journal of neurology* **126**, 2350-2362
3. Artero, S., Tierney, M. C., Touchon, J., and Ritchie, K. (2003) Prediction of transition from cognitive impairment to senile dementia: a prospective, longitudinal study. *Acta Psychiatr Scand* **107**, 390-393
4. Mattson, M. P. (2004) Pathways towards and away from Alzheimer's disease. *Nature* **430**, 631-639
5. Querfurth, H. W., and LaFerla, F. M. (2010) Alzheimer's disease. *N Engl J Med* **362**, 329-344
6. Sisodia, S. S., and Tanzi, R. E. (2007) *Alzheimer's Disease: Advances in Genetics, Molecular and Cellular Biology*, Springer Science + Business Media, New York
7. Selkoe, D. J. (2004) Cell biology of protein misfolding: the examples of Alzheimer's and Parkinson's diseases. *Nat Cell Biol* **6**, 1054-1061
8. Whitfield, J. F. (2007) The road to LOAD: late-onset Alzheimer's disease and a possible way to block it. *Expert Opin Ther Targets* **11**, 1257-1260

9. Deane, R., and Zlokovic, B. V. (2007) Role of the blood-brain barrier in the pathogenesis of Alzheimer's disease. *Curr Alzheimer Res* **4**, 191-197

10. Huang, S. M., Mouri, A., Kokubo, H., Nakajima, R., Suemoto, T., Higuchi, M., Staufenbiel, M., Noda, Y., Yamaguchi, H., Nabeshima, T., Saido, T. C., and Iwata, N. (2006) Neprilysin-sensitive synapse-associated amyloid-beta peptide oligomers impair neuronal plasticity and cognitive function. *J Biol Chem* **281**, 17941-17951

11. Marques, M. A., Kulstad, J. J., Savard, C. E., Green, P. S., Lee, S. P., Craft, S., Watson, G. S., and Cook, D. G. (2009) Peripheral amyloid-beta levels regulate amyloid-beta clearance from the central nervous system. *J Alzheimers Dis* **16**, 325-329

12. Nicoll, J. A., Yamada, M., Frackowiak, J., Mazur-Kolecka, B., and Weller, R. O. (2004) Cerebral amyloid angiopathy plays a direct role in the pathogenesis of Alzheimer's disease. Pro-CAA position statement. *Neurobiol Aging* **25**, 589-597; discussion 603-584

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

14. Meakin, S. O., and Shooter, E. M. (1992) The Nerve Growth-Factor Family of Receptors. *Trends Neurosci* **15**, 323-331

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

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

17. Friedman, W. J., and Greene, L. A. (1999) Neurotrophin signaling via Trks and p75.

Exp Cell Res **253**, 131-142

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

19. 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

20. 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

21. Loeb, D. M., Maragos, J., Martin-Zanca, D., Chao, M. V., Parada, L. F., and Greene, L. A. (1991) The trk proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. *Cell* **66**, 961-966

22. Dechant, G., and Barde, Y. A. (2002) The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat Neurosci* **5**, 1131-1136

23. Lu, B., Pang, P. T., and Woo, N. H. (2005) The yin and yang of neurotrophin action. *Nat Rev Neurosci* **6**, 603-614

24. Geetha, T., Zheng, C., Unroe, B., Sycheva, M., Kluess, H., and Babu, J. R. (2012) Polyubiquitination of the neurotrophin receptor p75 directs neuronal cell survival. *Biochem Biophys Res Commun*

25. Yaar, M., Zhai, S., Pilch, P. F., Doyle, S. M., Eisenhauer, P. B., Fine, R. E., and Gilchrest, B. A. (1997) Binding of beta-amyloid to the p75 neurotrophin receptor

induces apoptosis. A possible mechanism for Alzheimer's disease. *J Clin Invest* **100**, 2333-2340

26. Yaar, M., Zhai, S., Fine, R. E., Eisenhauer, P. B., Arble, B. L., Stewart, K. B., and Gilchrest, B. A. (2002) Amyloid beta binds trimers as well as monomers of the 75-kDa neurotrophin receptor and activates receptor signaling. *J Biol Chem* **277**, 7720-7725

27. Kuner, P., Schubenel, R., and Hertel, C. (1998) Beta-amyloid binds to p57NTR and activates NFkappaB in human neuroblastoma cells. *J Neurosci Res* **54**, 798-804

28. Coulson, E. J. (2006) Does the p75 neurotrophin receptor mediate Abeta-induced toxicity in Alzheimer's disease? *J Neurochem* **98**, 654-660

29. Yaar, M., Zhai, S., Panova, I., Fine, R. E., Eisenhauer, P. B., Blusztajn, J. K., Lopez-Coviella, I., and Gilchrest, B. A. (2007) A cyclic peptide that binds p75(NTR) protects neurones from beta amyloid (1-40)-induced cell death. *Neuropathol Appl Neurobiol* **33**, 533-543

30. Rabizadeh, S., Bitler, C. M., Butcher, L. L., and Bredesen, D. E. (1994) Expression of the low-affinity nerve growth factor receptor enhances beta-amyloid peptide toxicity. *Proc Natl Acad Sci U S A* **91**, 10703-10706

31. Hicke, L., Schubert, H. L., and Hill, C. P. (2005) Ubiquitin-binding domains. *Nat Rev Mol Cell Biol* **6**, 610-621

32. Laney, J. D., and Hochstrasser, M. (1999) Substrate targeting in the ubiquitin system. *Cell* **97**, 427-430

33. Liu, F., and Walters, K. J. (2010) Multitasking with ubiquitin through multivalent interactions. *Trends Biochem Sci* **35**, 352-360

34. Sanz, L., Diaz-Meco, M. T., Nakano, H., and Moscat, J. (2000) The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. *EMBO J* **19**, 1576-1586

35. Linares, J. F., Duran, A., Yajima, T., Pasparakis, M., Moscat, J., and Diaz-Meco, M. T. (2013) K63 polyubiquitination and activation of mTOR by the p62-TRAF6 complex in nutrient-activated cells. *Mol Cell* **51**, 283-296

36. Yang, W. L., Wang, J., Chan, C. H., Lee, S. W., Campos, A. D., Lamothe, B., Hur, L., Grabiner, B. C., Lin, X., Darnay, B. G., and Lin, H. K. (2009) The E3 ligase TRAF6 regulates Akt ubiquitination and activation. *Science* **325**, 1134-1138

37. Wooten, M. W., Geetha, T., Seibenhener, M. L., Babu, J. R., Diaz-Meco, M. T., and Moscat, J. (2005) The p62 scaffold regulates nerve growth factor-induced NF-kappaB activation by influencing TRAF6 polyubiquitination. *J Biol Chem* **280**, 35625-35629

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

39. Zhou, X., Babu, J. R., da Silva, S., Shu, Q., Graef, I. A., Oliver, T., Tomoda, T., Tani, T., Wooten, M. W., and Wang, F. (2007) Unc-51-like kinase 1/2-mediated endocytic processes regulate filopodia extension and branching of sensory axons. *Proc Natl Acad Sci U S A* **104**, 5842-5847

40. Babu, J. R., Geetha, T., and Wooten, M. W. (2005) Sequestosome 1/p62 shuttles polyubiquitinated tau for proteasomal degradation. *J Neurochem* **94**, 192-203

41. Scott, S. A., and Crutcher, K. A. (1994) Nerve growth factor and Alzheimer's

disease. *Rev Neurosci* **5**, 179-211

42. Chare, L., Hodges, J. R., Leyton, C. E., McGinley, C., Tan, R. H., Kril, J. J., and Halliday, G. M. (2014) New criteria for frontotemporal dementia syndromes: clinical and pathological diagnostic implications. *J Neurol Neurosurg Psychiatry* **85**, 865-870

43. Hebert, L. E., Weuve, J., Scherr, P. A., and Evans, D. A. (2013) Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. *Neurology* **80**, 1778-1783

44. Prince, M., Bryce, R., Albanese, E., Wimo, A., Ribeiro, W., and Ferri, C. P. (2013) The global prevalence of dementia: a systematic review and metaanalysis. *Alzheimers Dement* **9**, 63-75 e62

45. Hebert, L. E., Scherr, P. A., McCann, J. J., Beckett, L. A., and Evans, D. A. (2001) Is the risk of developing Alzheimer's disease greater for women than for men? *Am J Epidemiol* **153**, 132-136

46. Stern, Y. (2012) Cognitive reserve in ageing and Alzheimer's disease. *Lancet Neurol* **11**, 1006-1012

47. Barnes, D. E., and Yaffe, K. (2011) The projected effect of risk factor reduction on Alzheimer's disease prevalence. *Lancet Neurol* **10**, 819-828

48. Loy, C. T., Schofield, P. R., Turner, A. M., and Kwok, J. B. (2014) Genetics of dementia. *Lancet* **383**, 828-840

49. Blennow, K., de Leon, M. J., and Zetterberg, H. (2006) Alzheimer's disease. *Lancet* **368**, 387-403

50. Goldman, J. S., Hahn, S. E., Catania, J. W., LaRusse-Eckert, S., Butson, M. B.,

Rumbaugh, M., Strecker, M. N., Roberts, J. S., Burke, W., Mayeux, R., Bird, T., American College of Medical, G., and the National Society of Genetic, C. (2011) Genetic counseling and testing for Alzheimer disease: joint practice guidelines of the American College of Medical Genetics and the National Society of Genetic Counselors. *Genet Med* **13**, 597-605

51. Waring, S. C., and Rosenberg, R. N. (2008) Genome-wide association studies in Alzheimer disease. *Arch Neurol* **65**, 329-334

52. Chen, Y., and Bodles, A. M. (2007) Amyloid precursor protein modulates beta-catenin degradation. *Journal of neuroinflammation* **4**, 29

53. Holtzman, D. M., Herz, J., and Bu, G. (2012) Apolipoprotein E and apolipoprotein E receptors: normal biology and roles in Alzheimer disease. *Cold Spring Harb Perspect Med* **2**, a006312

54. Minati, L., Edginton, T., Bruzzone, M. G., and Giaccone, G. (2009) Current concepts in Alzheimer's disease: a multidisciplinary review. *American journal of Alzheimer's disease and other dementias* **24**, 95-121

55. Glenner, G. G., and Wong, C. W. (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun* **120**, 885-890

56. Iqbal, K., Alonso Adel, C., Chen, S., Chohan, M. O., El-Akkad, E., Gong, C. X., Khatoon, S., Li, B., Liu, F., Rahman, A., Tanimukai, H., and Grundke-Iqbal, I. (2005) Tau pathology in Alzheimer disease and other tauopathies. *Biochim Biophys Acta* **1739**, 198-210

57. Kurz, A., and Perneczky, R. (2011) Novel insights for the treatment of Alzheimer's

disease. *Prog Neuropsychopharmacol Biol Psychiatry* **35**, 373-379

58. Hardy, J. (2009) The amyloid hypothesis for Alzheimer's disease: a critical reappraisal. *J Neurochem* **110**, 1129-1134

59. Morris, G. P., Clark, I. A., and Vissel, B. (2014) Inconsistencies and controversies surrounding the amyloid hypothesis of Alzheimer's disease. *Acta Neuropathol Commun* **2**, 135

60. Lahiri, D. K., and Maloney, B. (2010) Beyond the signaling effect role of amyloid-ss42 on the processing of APP, and its clinical implications. *Exp Neurol* **225**, 51-54

61. Selkoe, D. J. (1997) Alzheimer's disease: genotypes, phenotypes, and treatments. *Science* **275**, 630-631

62. Sisodia, S. S., and St George-Hyslop, P. H. (2002) gamma-Secretase, Notch, Abeta and Alzheimer's disease: where do the presenilins fit in? *Nat Rev Neurosci* **3**, 281-290

63. Jarrett, J. T., Berger, E. P., and Lansbury, P. T., Jr. (1993) The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* **32**, 4693-4697

64. Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N., and Ihara, Y. (1994) Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43). *Neuron* **13**, 45-53

65. Gu, L., and Guo, Z. (2013) Alzheimer's Abeta42 and Abeta40 peptides form interlaced amyloid fibrils. *J Neurochem* **126**, 305-311

66. Tomiyama, T., Matsuyama, S., Iso, H., Umeda, T., Takuma, H., Ohnishi, K., Ishibashi, K., Teraoka, R., Sakama, N., Yamashita, T., Nishitsuji, K., Ito, K., Shimada, H., Lambert, M. P., Klein, W. L., and Mori, H. (2010) A mouse model of amyloid beta oligomers: their contribution to synaptic alteration, abnormal tau phosphorylation, glial activation, and neuronal loss in vivo. *J Neurosci* **30**, 4845-4856

67. Lesne, S., Koh, M. T., Kotilinek, L., Kayed, R., Glabe, C. G., Yang, A., Gallagher, M., and Ashe, K. H. (2006) A specific amyloid-beta protein assembly in the brain impairs memory. *Nature* **440**, 352-357

68. Zhang, Y., Lu, L., Jia, J., Jia, L., Geula, C., Pei, J., Xu, Z., Qin, W., Liu, R., Li, D., and Pan, N. (2014) A lifespan observation of a novel mouse model: in vivo evidence supports abeta oligomer hypothesis. *PLoS One* **9**, e85885

69. Ma, Q. L., Yang, F., Rosario, E. R., Ubeda, O. J., Beech, W., Gant, D. J., Chen, P. P., Hudspeth, B., Chen, C., Zhao, Y., Vinters, H. V., Frautschy, S. A., and Cole, G. M. (2009) Beta-amyloid oligomers induce phosphorylation of tau and inactivation of insulin receptor substrate via c-Jun N-terminal kinase signaling: suppression by omega-3 fatty acids and curcumin. *J Neurosci* **29**, 9078-9089

70. Lee, M. J., Lee, J. H., and Rubinsztein, D. C. (2013) Tau degradation: the ubiquitin-proteasome system versus the autophagy-lysosome system. *Prog Neurobiol* **105**, 49-59

71. Goedert, M., Spillantini, M. G., Jakes, R., Rutherford, D., and Crowther, R. A. (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron* **3**, 519-

526

72. Martin, L., Latypova, X., and Terro, F. (2011) Post-translational modifications of tau protein: implications for Alzheimer's disease. *Neurochem Int* **58**, 458-471

73. Hernandez, F., and Avila, J. (2007) Tauopathies. *Cell Mol Life Sci* **64**, 2219-2233

74. Kopke, E., Tung, Y. C., Shaikh, S., Alonso, A. C., Iqbal, K., and Grundke-Iqbal, I. (1993) Microtubule-associated protein tau. Abnormal phosphorylation of a non-paired helical filament pool in Alzheimer disease. *J Biol Chem* **268**, 24374-24384

75. Alonso, A. C., Zaidi, T., Grundke-Iqbal, I., and Iqbal, K. (1994) Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proc Natl Acad Sci U S A* **91**, 5562-5566

76. Yoshida, H., and Ihara, Y. (1993) Tau in paired helical filaments is functionally distinct from fetal tau: assembly incompetence of paired helical filament-tau. *J Neurochem* **61**, 1183-1186

77. von Bergen, M., Friedhoff, P., Biernat, J., Heberle, J., Mandelkow, E. M., and Mandelkow, E. (2000) Assembly of tau protein into Alzheimer paired helical filaments depends on a local sequence motif ((306)VQIVYK(311)) forming beta structure. *Proc Natl Acad Sci U S A* **97**, 5129-5134

78. Garver, T. D., Harris, K. A., Lehman, R. A., Lee, V. M., Trojanowski, J. Q., and Billingsley, M. L. (1994) Tau phosphorylation in human, primate, and rat brain: evidence that a pool of tau is highly phosphorylated in vivo and is rapidly dephosphorylated in vitro. *J Neurochem* **63**, 2279-2287

79. Goedert, M. (1996) Tau protein and the neurofibrillary pathology of Alzheimer's disease. *Ann N Y Acad Sci* **777**, 121-131

80. Jicha, G. A., Berenfeld, B., and Davies, P. (1999) Sequence requirements for formation of conformational variants of tau similar to those found in Alzheimer's disease. *J Neurosci Res* **55**, 713-723

81. Vogelsberg-Ragaglia, V., Schuck, T., Trojanowski, J. Q., and Lee, V. M. (2001) PP2A mRNA expression is quantitatively decreased in Alzheimer's disease hippocampus. *Exp Neurol* **168**, 402-412

82. Wang, Y., Garg, S., Mandelkow, E. M., and Mandelkow, E. (2010) Proteolytic processing of tau. *Biochem Soc Trans* **38**, 955-961

83. Dolan, P. J., and Johnson, G. V. (2010) A caspase cleaved form of tau is preferentially degraded through the autophagy pathway. *J Biol Chem* **285**, 21978-21987

84. Grune, T., Botzen, D., Engels, M., Voss, P., Kaiser, B., Jung, T., Grimm, S., Ermak, G., and Davies, K. J. (2010) Tau protein degradation is catalyzed by the ATP/ubiquitin-independent 20S proteasome under normal cell conditions. *Arch Biochem Biophys* **500**, 181-188

85. Keck, S., Nitsch, R., Grune, T., and Ullrich, O. (2003) Proteasome inhibition by paired helical filament-tau in brains of patients with Alzheimer's disease. *J Neurochem* **85**, 115-122

86. Tseng, B. P., Green, K. N., Chan, J. L., Blurton-Jones, M., and LaFerla, F. M. (2008) Abeta inhibits the proteasome and enhances amyloid and tau accumulation. *Neurobiology of aging* **29**, 1607-1618

87. David, D. C., Layfield, R., Serpell, L., Narain, Y., Goedert, M., and Spillantini, M. G. (2002) Proteasomal degradation of tau protein. *Journal of neurochemistry* **83**,

176-185

88. Cardozo, C., and Michaud, C. (2002) Proteasome-mediated degradation of tau proteins occurs independently of the chymotrypsin-like activity by a nonprocessive pathway. *Archives of biochemistry and biophysics* **408**, 103-110

89. Cripps, D., Thomas, S. N., Jeng, Y., Yang, F., Davies, P., and Yang, A. J. (2006) Alzheimer disease-specific conformation of hyperphosphorylated paired helical filament-Tau is polyubiquitinated through Lys-48, Lys-11, and Lys-6 ubiquitin conjugation. *J Biol Chem* **281**, 10825-10838

90. Keller, J. N., Hanni, K. B., and Markesbery, W. R. (2000) Impaired proteasome function in Alzheimer's disease. *J Neurochem* **75**, 436-439

91. Lopez Salon, M., Morelli, L., Castano, E. M., Soto, E. F., and Pasquini, J. M. (2000) Defective ubiquitination of cerebral proteins in Alzheimer's disease. *J Neurosci Res* **62**, 302-310

92. Tai, H. C., Serrano-Pozo, A., Hashimoto, T., Frosch, M. P., Spires-Jones, T. L., and Hyman, B. T. (2012) The synaptic accumulation of hyperphosphorylated tau oligomers in Alzheimer disease is associated with dysfunction of the ubiquitin-proteasome system. *Am J Pathol* **181**, 1426-1435

93. Petrucelli, L., Dickson, D., Kehoe, K., Taylor, J., Snyder, H., Grover, A., De Lucia, M., McGowan, E., Lewis, J., Prihar, G., Kim, J., Dillmann, W. H., Browne, S. E., Hall, A., Voellmy, R., Tsuboi, Y., Dawson, T. M., Wolozin, B., Hardy, J., and Hutton, M. (2004) CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation. *Human molecular genetics* **13**, 703-714

94. Shimura, H., Schwartz, D., Gygi, S. P., and Kosik, K. S. (2004) CHIP-Hsc70

complex ubiquitinates phosphorylated tau and enhances cell survival. *The Journal of biological chemistry* **279**, 4869-4876

95. Dickey, C. A., Yue, M., Lin, W. L., Dickson, D. W., Dunmore, J. H., Lee, W. C., Zehr, C., West, G., Cao, S., Clark, A. M., Caldwell, G. A., Caldwell, K. A., Eckman, C., Patterson, C., Hutton, M., and Petrucelli, L. (2006) Deletion of the ubiquitin ligase CHIP leads to the accumulation, but not the aggregation, of both endogenous phospho- and caspase-3-cleaved tau species. *J Neurosci* **26**, 6985-6996

96. Layfield, R., Alban, A., Mayer, R. J., and Lowe, J. (2001) The ubiquitin protein catabolic disorders. *Neuropathology and applied neurobiology* **27**, 171-179

97. Huang, E. J., and Reichardt, L. F. (2001) Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci* **24**, 677-736

98. Chao, M. V. (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat Rev Neurosci* **4**, 299-309

99. Levi-Montalcini, R., and Hamburger, V. (1951) Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *The Journal of experimental zoology* **116**, 321-361

100. Levi-Montalcini, R. (1987) The nerve growth factor: thirty-five years later. *EMBO J* **6**, 1145-1154

101. Heymach, J. V., Jr., and Barres, B. A. (1997) Neurotrophins moving forward. *Nature* **389**, 789, 791

102. Ehlers, M. D., Kaplan, D. R., Price, D. L., and Koliatsos, V. E. (1995) NGF-stimulated retrograde transport of trkA in the mammalian nervous system. *J Cell Biol* **130**, 149-156

103. Suter, U., Angst, C., Tien, C. L., Drinkwater, C. C., Lindsay, R. M., and Shooter, E. M. (1992) NGF/BDNF chimeric proteins: analysis of neurotrophin specificity by homolog-scanning mutagenesis. *J Neurosci* **12**, 306-318

104. Lindsay, R. M., Wiegand, S. J., Altar, C. A., and DiStefano, P. S. (1994) Neurotrophic factors: from molecule to man. *Trends Neurosci* **17**, 182-190

105. Hallbook, F. (1999) Evolution of the vertebrate neurotrophin and Trk receptor gene families. *Curr Opin Neurobiol* **9**, 616-621

106. Yune, T. Y., Lee, J. Y., Jung, G. Y., Kim, S. J., Jiang, M. H., Kim, Y. C., Oh, Y. J., Markelonis, G. J., and Oh, T. H. (2007) Minocycline alleviates death of oligodendrocytes by inhibiting pro-nerve growth factor production in microglia after spinal cord injury. *J Neurosci* **27**, 7751-7761

107. Scott, J., Selby, M., Urdea, M., Quiroga, M., Bell, G. I., and Rutter, W. J. (1983) Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. *Nature* **302**, 538-540

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

109. Tuszynski, M. H., Yang, J. H., Barba, D., U, H. S., Bakay, R. A., Pay, M. M., Masliah, E., Conner, J. M., Kobalka, P., Roy, S., and Nagahara, A. H. (2015) Nerve Growth Factor Gene Therapy: Activation of Neuronal Responses in Alzheimer Disease. *JAMA Neurol* **72**, 1139-1147

110. Kaplan, D. R., and Miller, F. D. (2000) Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol* **10**, 381-391

111. Wang, Z., Liu, Y., Takahashi, M., Van Hook, K., Kampa-Schittenhelm, K. M.,

Sheppard, B. C., Sears, R. C., Stork, P. J., and Lopez, C. D. (2013) N terminus of ASPP2 binds to Ras and enhances Ras/Raf/MEK/ERK activation to promote oncogene-induced senescence. *Proc Natl Acad Sci U S A* **110**, 312-317

112. Cheng, H. C., Shih, H. M., and Chern, Y. (2002) Essential role of cAMP-response element-binding protein activation by A2A adenosine receptors in rescuing the nerve growth factor-induced neurite outgrowth impaired by blockage of the MAPK cascade. *J Biol Chem* **277**, 33930-33942

113. Chao, M. V., Rajagopal, R., and Lee, F. S. (2006) Neurotrophin signalling in health and disease. *Clin Sci (Lond)* **110**, 167-173

114. Esposito, D., Patel, P., Stephens, R. M., Perez, P., Chao, M. V., Kaplan, D. R., and Hempstead, B. L. (2001) The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to nerve growth factor. *J Biol Chem* **276**, 32687-32695

115. Nykjaer, A., Lee, R., Teng, K. K., Jansen, P., Madsen, P., Nielsen, M. S., Jacobsen, C., Kliemannel, M., Schwarz, E., Willnow, T. E., Hempstead, B. L., and Petersen, C. M. (2004) Sortilin is essential for proNGF-induced neuronal cell death. *Nature* **427**, 843-848

116. Fahnestock, M., Yu, G., Michalski, B., Mathew, S., Colquhoun, A., Ross, G. M., and Coughlin, M. D. (2004) The nerve growth factor precursor proNGF exhibits neurotrophic activity but is less active than mature nerve growth factor. *J Neurochem* **89**, 581-592

117. Clewes, O., Fahey, M. S., Tyler, S. J., Watson, J. J., Seok, H., Catania, C., Cho, K., Dawbarn, D., and Allen, S. J. (2008) Human ProNGF: biological effects and

binding profiles at TrkA, P75NTR and sortilin. *J Neurochem* **107**, 1124-1135

118. Liu, J., and Lin, A. (2005) Role of JNK activation in apoptosis: a double-edged sword. *Cell Res* **15**, 36-42

119. Hamanoue, M., Middleton, G., Wyatt, S., Jaffray, E., Hay, R. T., and Davies, A. M. (1999) p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. *Mol Cell Neurosci* **14**, 28-40

120. Glickman, M. H., and Ciechanover, A. (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiological reviews* **82**, 373-428

121. Pickart, C. M. (2004) Back to the future with ubiquitin. *Cell* **116**, 181-190

122. Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003) Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat Cell Biol* **5**, 461-466

123. Komander, D. (2009) The emerging complexity of protein ubiquitination. *Biochemical Society transactions* **37**, 937-953

124. Ye, Y., and Rape, M. (2009) Building ubiquitin chains: E2 enzymes at work. *Nature reviews. Molecular cell biology* **10**, 755-764

125. Nandi, D., Tahiliani, P., Kumar, A., and Chandu, D. (2006) The ubiquitin-proteasome system. *J Biosci* **31**, 137-155

126. Chondrogianni, N., and Gonos, E. S. (2012) Structure and function of the ubiquitin-proteasome system: modulation of components. *Progress in molecular biology and translational science* **109**, 41-74

127. Chen, Z. J., and Sun, L. J. (2009) Nonproteolytic functions of ubiquitin in cell

signaling. *Mol Cell* **33**, 275-286

128. Kamsteeg, E. J., Hendriks, G., Boone, M., Konings, I. B., Oorschot, V., van der Sluijs, P., Klumperman, J., and Deen, P. M. (2006) Short-chain ubiquitination mediates the regulated endocytosis of the aquaporin-2 water channel. *Proc Natl Acad Sci U S A* **103**, 18344-18349

129. Duncan, L. M., Piper, S., Dodd, R. B., Saville, M. K., Sanderson, C. M., Luzio, J. P., and Lehner, P. J. (2006) Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. *EMBO J* **25**, 1635-1645

130. Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S., and Sorkin, A. (2006) Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. *Mol Cell* **21**, 737-748

131. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z. J. (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103**, 351-361

132. Bhoj, V. G., and Chen, Z. J. (2009) Ubiquitylation in innate and adaptive immunity. *Nature* **458**, 430-437

133. Chen, Z. J. (2005) Ubiquitin signalling in the NF-kappaB pathway. *Nat Cell Biol* **7**, 758-765

134. Yamashita, M., Fatyol, K., Jin, C., Wang, X., Liu, Z., and Zhang, Y. E. (2008) TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta. *Mol Cell* **31**, 918-924

135. Maezawa, Y., Nakajima, H., Suzuki, K., Tamachi, T., Ikeda, K., Inoue, J., Saito, Y.,

and Iwamoto, I. (2006) Involvement of TNF receptor-associated factor 6 in IL-25 receptor signaling. *J Immunol* **176**, 1013-1018

136. Abbott, D. W., Yang, Y., Hutti, J. E., Madhavarapu, S., Kelliher, M. A., and Cantley, L. C. (2007) Coordinated regulation of Toll-like receptor and NOD2 signaling by K63-linked polyubiquitin chains. *Mol Cell Biol* **27**, 6012-6025

137. Sorrentino, A., Thakur, N., Grimsby, S., Marcusson, A., von Bulow, V., Schuster, N., Zhang, S., Heldin, C. H., and Landstrom, M. (2008) The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. *Nat Cell Biol* **10**, 1199-1207

138. Wu, H., and Arron, J. R. (2003) TRAF6, a molecular bridge spanning adaptive immunity, innate immunity and osteoimmunology. *Bioessays* **25**, 1096-1105

139. Takaoka, A., Yanai, H., Kondo, S., Duncan, G., Negishi, H., Mizutani, T., Kano, S., Honda, K., Ohba, Y., Mak, T. W., and Taniguchi, T. (2005) Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* **434**, 243-249

140. Kuusisto, E., Salminen, A., and Alafuzoff, I. (2002) Early accumulation of p62 in neurofibrillary tangles in Alzheimer's disease: possible role in tangle formation. *Neuropathol Appl Neurobiol* **28**, 228-237

141. Sanchez, P., De Carcer, G., Sandoval, I. V., Moscat, J., and Diaz-Meco, M. T. (1998) Localization of atypical protein kinase C isoforms into lysosome-targeted endosomes through interaction with p62. *Mol Cell Biol* **18**, 3069-3080

142. Zotti, T., Scudiero, I., Settembre, P., Ferravante, A., Mazzone, P., D'Andrea, L., Reale, C., Vito, P., and Stilo, R. (2014) TRAF6-mediated ubiquitination of NEMO

requires p62/sequestosome-1. *Mol Immunol* **58**, 27-31

143. Geetha, T., Zheng, C., McGregor, W. C., Douglas White, B., Diaz-Meco, M. T., Moscat, J., and Babu, J. R. (2012) TRAF6 and p62 inhibit amyloid beta-induced neuronal death through p75 neurotrophin receptor. *Neurochem Int* **61**, 1289-1293

144. Geetha, T., Zheng, C., Unroe, B., Sycheva, M., Kluess, H., and Babu, J. R. (2012) Polyubiquitination of the neurotrophin receptor p75 directs neuronal cell survival. *Biochem Biophys Res Commun* **421**, 286-290

145. Ramesh Babu, J., Lamar Seibenhener, M., Peng, J., Strom, A. L., Kemppainen, R., Cox, N., Zhu, H., Wooten, M. C., Diaz-Meco, M. T., Moscat, J., and Wooten, M. W. (2008) Genetic inactivation of p62 leads to accumulation of hyperphosphorylated tau and neurodegeneration. *J Neurochem* **106**, 107-120

146. Shin, J. (1998) P62 and the sequestosome, a novel mechanism for protein metabolism. *Arch Pharm Res* **21**, 629-633

147. Seibenhener, M. L., Babu, J. R., Geetha, T., Wong, H. C., Krishna, N. R., and Wooten, M. W. (2004) Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. *Mol Cell Biol* **24**, 8055-8068

Chapter 3: Polyubiquitination of the neurotrophin receptor p75 directs neuronal cell survival

3.1 Abstract

Specific binding of nerve growth factor (NGF) to p75 neurotrophin receptor ($p75^{NTR}$) leads to $p75^{NTR}$ polyubiquitination and its subsequent interaction with TRAF6 resulting in neuronal cell survival. However, when the binding of NGF to $p75^{NTR}$ was blocked with p75 antiserum, $p75^{NTR}$ polyubiquitination and neuronal cell survival were impaired. Results showed that tyrosine phosphorylation of $p75^{NTR}$ increased the polyubiquitination of $p75^{NTR}$ and contributed to the observed apparent neuroprotective effects. Similar to $p75^{NTR}$ polyubiquitination, interaction of TRAF6 with $p75^{NTR}$ was NGF/tyrosine phosphorylation dependent suggesting that TRAF6 might function as an E3 ubiquitin ligase. In sum, the results show that specific binding of NGF to $p75^{NTR}$ mediates neuronal cell survival.

3.2 Introduction

NGF (nerve growth factor) is a neurotrophin that regulates survival, differentiation and maintenance of sensory and sympathetic neurons (1). Neurotrophins can bind to two classes of cell-surface receptors, namely a high-affinity tyrosine kinase Trk receptor and a low-affinity $p75^{NTR}$ receptor (2,3). $p75^{NTR}$ is a member of the tumor necrosis factor (TNF) super family of receptors (4-6), and is involved in cellular apoptosis, cell survival, differentiation, neurite outgrowth (7-9), Schwann cell myelination and development (5,10).

$p75^{NTR}$ contains four ligand-binding pockets in cysteine-rich extracellular domain and the intracellular domain possess the chopper domain and TNFR-like death domain (11). $p75^{NTR}$ has no intrinsic enzymatic activity, however NGF leads to tyrosine phosphorylation of $p75^{NTR}$ and activates MAPK pathway (12). The tyrosine phosphorylation of $p75^{NTR}$ can also be induced by the phosphatase inhibitor pervanadate (13).

$p75^{NTR}$ regulates both neuronal survival and apoptosis (5,14,15). $p75^{NTR}$ can bind to several interacting proteins involved in its functions. The neurotrophin receptor interacting factor (NRIF), $p75^{NTR}$-associated death executer (NADE), and neurotrophin receptor interacting MAGE homologue (NRAGE) proteins bind to different regions of the $p75^{NTR}$ cytoplasmic domain and are involved in the induction of apoptosis (16-18). $p75^{NTR}$ promotes the activation of sphingomyelinase and ceramide production that function to activate the JNK kinase (19,20). Interaction of TRAF6 with $p75^{NTR}$ enhances the cell survival (14,21). However, the mechanism to describe how TRAF6 induces cell survival is unknown.

Ubiquitin is a 76 amino acid polypeptide; covalently bind through an isopeptide bond between its C-terminal glycine and the lysine residues of the substrate proteins. Three enzymes are required for ubiquitination: E1 (ubiquitin-activating enzyme), which forms a high-energy thioester bond with the C-terminus of ubiquitin in an ATP-dependent reaction. The second step involves the transfer of the activated ubiquitin to the cysteine residue in one of 22 known E2 (ubiquitin-conjugating enzyme) enzymes. In the last step, E3 (ubiquitin ligase) enzymes mediate the formation of an isopeptide bond with the lysine residue in the substrate proteins (22). Substrates can be mono-ubiquitinated (23) (24,25) or polyubiquitinated, (26-28) linked through each of the seven lysine residues present in ubiquitin (Lys^{6}, Lys^{11}, Lys^{27}, Lys^{29}, Lys^{33}, Lys^{48} and Lys^{63}) (29). The function of Lys^{48}-

linked ubiquitin chains is to target proteins for proteasomal degradation, whereas Lys^{63}-linked ubiquitin chains play a role in endocytosis, protein sorting and receptor trafficking (30). Previously, it has been shown that $p75^{NTR}$ interacts with TRAF6 (14). TRAF6 is an E3 RING ubiquitin ligase (31) directing the synthesis of Lys63-linked polyubiquitin chains (32) and is itself activated by Lys63-linked polyubiquitination (32). Moreover, $p75^{NTR}$ signaling is deficient in traf6-/- mice (15)(Yeiser et al., 2004), which suggests that $p75^{NTR}$/TRAF6 may play a role in the regulation of $p75^{NTR}$ signaling. This prompted us to examine whether NGF binding and tyrosine phosphorylation of $p75^{NTR}$ could stimulate $p75^{NTR}$ polyubiquitination. Herein, we demonstrate that $p75^{NTR}$ is polyubiquitinated and leads to neuroprotection.

3.3 Materials and Methods

3.3.1 Materials

The rabbit $p75^{NTR}$ and ubiquitin antibodies were purchased from Santa Cruz Biotechnology (La Jolla, CA). $p75^{NTR}$ antibody was purchased from Millipore (Billerica, MA) and TRAF6 rabbit antibody for western blotting was obtained from Abcam (Cambridge, MA). Anti-phosphotyrosine (PY20) was purchased from BD Transduction Laboratories (San Diego, CA). Nerve growth factor (NGF) (2.5S) was from Bioproducts for Science (Indianapolis, IN). $p75^{NTR}$ antiserum (antibody 9561) directed against the third and fourth cysteine-rich repeats of the extracellular domain of mouse $p75^{NTR}$ was generously provided by Dr. Moses Chao, New York University School of Medicine.

3.3.2 Cell Culture

The mouse hippocampal cell line, HT22 cells was a generous gift from Dr. David Schubert (The Salk Institute, La Jolla, CA) (33). The cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplied with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) as previously described (34). Cells were incubated in a 5% CO_2 atmosphere at 37^0C. The cells were lysed with triton lysis buffer to detect protein-protein interactions (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin and aprotinin) or SDS lysis buffer to detect covalent interaction of ubiquitin and $p75^{NTR}$ (Triton lysis buffer containing 1% SDS) (35). Protein was estimated by Bradford procedure (Bio-Rad) and with bovine serum albumin (BSA) as a standard for all samples except those containing SDS, which were estimated by DC assay (Bio-Rad).

3.3.3 Immunoprecipitation and Western Blotting Analysis

Cell lysates (1 mg) were diluted in lysis buffer and incubated with 4 μg of primary antibody at 4°C for 3 h. The immunoprecipitates were collected with agarose-coupled secondary antibody for 2 h at 4°C and then were washed three times with lysis buffer. Samples were boiled in sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and resolved on 7.5%–12% SDS-PAGE, transferred onto nitrocellulose membranes, and analyzed by Western blotting with the appropriate antibodies.

3.3.4 Cell Survival Assay

Cell survival was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HT22 cells were seeded in six wells plate in DMEM containing 10% FBS. The next day, cells were serum-starved and treated according to the experimental design. The cells were incubated in the presence of MTT and converted to a

water-soluble formazan by dehydrogenase enzyme found in metabolically active cells. The quantity of formazon product was determined by spectrophotometry at 490 nm. The results are shown as the percentage of the controls specified in the experiment.

3.4 Results and discussion

3.4.1 NGF Induces the Ubiquitination of $p75^{NTR}$

We sought to explore whether NGF might induce $p75^{NTR}$ receptor ubiquitination. HT22 cells were treated with 100 ng/ml NGF for different times and the $p75^{NTR}$ ubiquitination was determined by immunoprecipitation with $p75^{NTR}$ antibody and western blotting for anti-ubiquitin and anti- $p75^{NTR}$. NGF treatment for 10 and 20 min resulted in maximum $p75^{NTR}$ polyubiquitination (2A); although, polyubiquitination was sustained above basal levels in the presence of NGF. Antiserum (antibody 9561) directed against the third and fourth cysteine-rich repeats of the extracellular domain of mouse $p75^{NTR}$ inhibit NGF neurotrophic activity by interfering with NGF- $p75^{NTR}$ interaction (36). HT22 cells were treated with $p75^{NTR}$ antiserum and control non-immune sera (final dilutions of 1:100) in the presence or absence of NGF for 10 min. $p75^{NTR}$ ubiquitination was examined in these treated cells. Therein, we observed that $p75^{NTR}$ antiserum abrogated the $p75^{NTR}$ polyubiquitination (Fig. 2B). These results indicate that $p75^{NTR}$ is ubiquitinated through a pathway that is activated by NGF binding to the $p75^{NTR}$ receptor.

3.4.2 $p75^{NTR}$ blocking antibody impairs the interaction of $p75^{NTR}$ with TRAF6

TRAF6 functions as ubiquitin ligase and catalyzes the formation of noncanonical K63-linked polyubiquitin chains (32). Because TRAF6 has been shown to interact with $p75^{NTR}$

receptor (14,37), we sought to determine if the interaction could be recapitulated *in vivo* in HT22 cell line. These cells were stimulated with NGF followed by immunoprecipitation of $p75^{NTR}$ and blotting for $p75^{NTR}$ and TRAF6. Lysates were also Western blotted for $p75^{NTR}$ and TRAF6. As shown in Fig. 3A, p75 and TRAF6 showed maximum co-interaction at 10 and 20 min of NGF stimulation, and the co-association was transient. Interestingly, we observed that $p75^{NTR}$ and TRAF6 existed as a complex at the same time point of $p75^{NTR}$ ubiquitination. These results indicate that TRAF6 interaction may lead to $p75^{NTR}$ ubiquitination. Next, we evaluated the contribution of NGF involved in the interaction of $p75^{NTR}$ and TRAF6. $p75^{NTR}$ antiserum that blocks the binding of NGF with $p75^{NTR}$ receptor was treated to HT22 cells in the presence or absence of NGF for 10 min. $p75^{NTR}$ receptor was immunoprecipitated followed by Western blotting with anti- $p75^{NTR}$ and anti-TRAF6 (Fig. 3B). In control cells, $p75^{NTR}$ interacts with TRAF6 on NGF treatment. When the cells were treated with $p75^{NTR}$ blocking antibody along with NGF, the co-interaction of $p75^{NTR}$ with TRAF6 was impaired as shown in Fig. 3B. The results parallel with the impairment of $p75^{NTR}$ ubiquitination by the $p75^{NTR}$ blocking antibody (Fig. 2B). These findings further support a model whereby TRAF6 interaction is necessary for $p75^{NTR}$ ubiquitination.

3.4.3 Tyrosine phosphorylation of $p75^{NTR}$ leads to its ubiquitination and TRAF6 interaction.

Next we analyzed whether $p75^{NTR}$ tyrosine phosphorylation would lead to its ubiquitination. The tyrosine phosphorylation of $p75^{NTR}$ was induced by the phosphatase inhibitor pervanadate (13). HT22 cells were treated with increasing concentration of pervanadate for

10 min, and the cell lysates were immunoprecipitated with anti- $p75^{NTR}$ and Western blotted with anti-Ub and anti- $p75^{NTR}$. Pervanadate (20 and 40 μM) treatment increased the $p75^{NTR}$ ubiquitination as shown in figure 3A. In addition, we also determined whether increase in tyrosine phosphorylation of $p75^{NTR}$ will enhance the interaction of TRAF6 with the $p75^{NTR}$. The pervanadate treatment cells were immunoprecipitated with $p75^{NTR}$ antibody and analyzed for the co-interaction of $p75^{NTR}$ with TRAF6 by Western blotting. As the pervanadate concentration increases the tyrosine phosphorylation of $p75^{NTR}$ increases as shown by Western blotting with phospho-tyrosine (PY20) antibody. The co-interaction of TRAF6 with $p75^{NTR}$ increases as the phosphorylation of $p75^{NTR}$ increases (Fig. 4B). The cell extracts were also blotted with anti- $p75^{NTR}$ and anti-TRAF6 to check the expression of the proteins (Fig. 4C). These results suggest that the $p75^{NTR}$ ubiquitination and its interaction with TRAF6 is dependent upon the tyrosine phosphorylation of $p75^{NTR}$.

3.4.4 $p75^{NTR}$ ubiquitination is a neuronal survival factor

$p75^{NTR}$ can lead to neuronal cell survival or cell death (8,11). To investigate the role of $p75^{NTR}$ ubiquitination and TRAF6 interaction, we determined whether NGF binding to $p75^{NTR}$ is involved in neuroprotection (Fig 4A). Under normal condition HT22 cells are grown in DMEM containing 10% FBS. HT22 cells on serum-withdrawal for 24 h induced-cell death. However, NGF stimulation induced neuroprotective activity to save HT22 neurons from serum withdrawal induced-cell death. When the NGF binding specifically to the $p75^{NTR}$ was blocked by adding $p75^{NTR}$ antiserum, the neuroprotective activity of NGF was abolished as shown in figure 4A. Additionally, increase in the tyrosine

phosphorylation of $p75^{NTR}$ enhances the $p75^{NTR}$ ubiquitination and TRAF6 interaction (Fig. 4). Based on these observations we set out to investigate whether tyrosine phosphorylation of $p75^{NTR}$ enhances the neuroprotective activity. Tyrosine phosphorylation of $p75^{NTR}$ was induced by addition of increasing concentration of pervanadate to the serum-withdrawal media and cell death was monitored biochemically by MTT assay (Fig. 5B). We discovered that the addition of pervanadate provided protection for HT22 cells against serum-withdrawal cell death. Also, as the concentration of pervanadate increases, it enhanced the ability of the cells to survive which is in parallel to the tyrosine phosphorylation of $p75^{NTR}$. Collectively, these results explain that NGF and pervanadate enhances the $p75^{NTR}$ ubiquitination and TRAF6 interaction also increases the neuroprotection.

In the present study, we provide evidence that NGF induced the $p75^{NTR}$ polyubqiuitination and interaction with TRAF6 which regulates neuronal cell survival. NGF is known to bind both Trk receptor and $p75^{NTR}$ (35,38). To confirm that NGF binding specifically to $p75^{NTR}$ can lead to its polyubiquitination and neuroprotection, we blocked the binding of NGF to $p75^{NTR}$ by using p75 antiserum which will block the binding of NGF only to $p75^{NTR}$ and not the Trk receptor (36). Interaction of TRAF6 with $p75^{NTR}$ is also NGF dependent similar to its polyubiquitination, suggesting that TRAF6 might function as E3 ubiquitin ligase. Our results are in parallel to Powell et al., (10), who suggested that $p75^{NTR}$ is a substrate for TRAF6-mediated ubiquitination. We also show that tyrosine phosphorylation of $p75^{NTR}$ increases the polyubiquitination of $p75^{NTR}$ and TRAF6 interaction. Tyrosine phosphorylation of $p75^{NTR}$ also leads to neuroprotection. These observations indicate the

signaling cascade can be represented as follows: NGF → $p75^{NTR}$ stimulation → tyrosine phosphorylation/$p75^{NTR}$ polyubiquitination/TRAF6 interaction → neuronal survival.

3.5 Figure Legends

Fig. 2. NGF stimulates $p75^{NTR}$ polyubiquitination. (A) HT-22 cells were treated with 100 ng/ml of NGF for 0, 5, 10, 20, 30 and 60 min at 37°C, lysed, and ubiquitination was examined by immunoprecipitation (IP) with anti- $p75^{NTR}$, followed by Western blot (WB) analysis with anti-ubiquitin and anti-$p75^{NTR}$. (B) HT-22 cells were treated with $p75^{NTR}$ antiserum ($p75^{NTR}$ blocking antibody) that is directed against the extracellular domain of $p75^{NTR}$ (1:100 final dilution) along with or without 100 ng/ml of NGF for 10 min. Ubiquitination was examined by immunoprecipitation (IP) with anti- $p75^{NTR}$ followed by Western blot (WB) analysis with anti-ubiquitin and anti- $p75^{NTR}$.

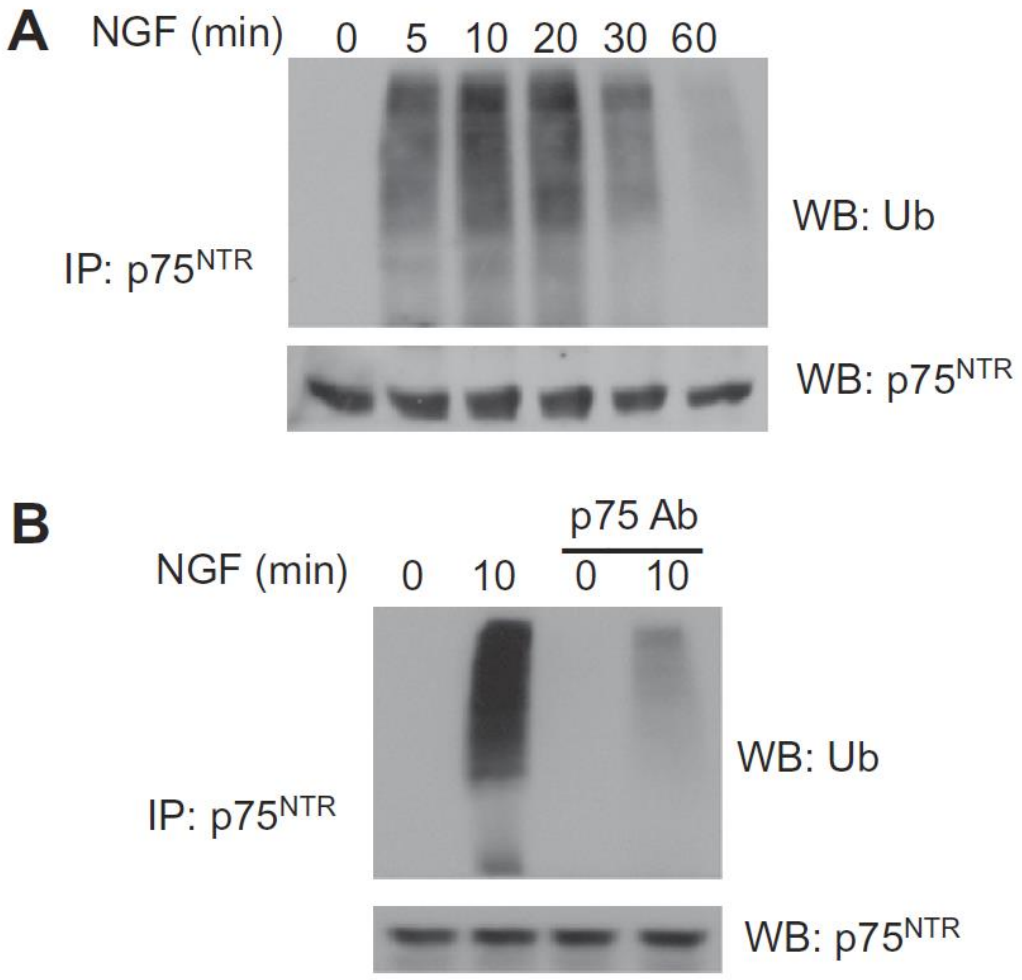
Fig. 3. $p75^{NTR}$ interacts with TRAF6 on NGF stimulation. (A) HT-22 cells were stimulated with NGF (100 ng/ml) for or 0, 5, 10, 20, 30 and 60 min. The cells were immunoprecipitated (IP) with anti-p75 and Western blotted (WB) with $p75^{NTR}$ and TRAF6 antibody. (B) HT-22 cells were treated with or without $p75^{NTR}$ blocking antibody followed by NGF stimulation for 0 and 10 min. The lysate was immunoprecipitated (IP) with anti-$p75^{NTR}$ and Western blotted (WB) with antibody for $p75^{NTR}$ and TRAF6. The cell lysate was also blotted with the same antibodies to verify the expression.

Fig. 4. Phosphorylation of $p75^{NTR}$ enhances the $p75^{NTR}$ polyubiquitination. (A) HT-22 cells were treated with increasing concentration of pervanadate and immunoprecipitated (IP) with anti- $p75^{NTR}$, followed by Western blot (WB) analysis with anti-ubiquitin and anti-

$p75^{NTR}$. (B) Pervanadate treated HT-22 cells were immunoprecipitated (IP) with anti-$p75^{NTR}$ and Western blotted (WB) with $p75^{NTR}$ and TRAF6 antibody. (C) For input, the cell lysate was also blotted with $p75^{NTR}$ and TRAF6 antibody.

Fig. 5. NGF and pervanadate enhances the cell survival. (A) Neuronal HT22 cells were serum-starved and incubated with NGF (50 ng/ml) for 24 h in the presence or absence of p75 blocking antibody. (B) HT22 cells were incubated with increasing concentration of pervanadate in serum-starved media for 24 h. Cell death was assessed biochemically by the MTT assay. The mean and S.D. of three experiments is shown.

Fig. 2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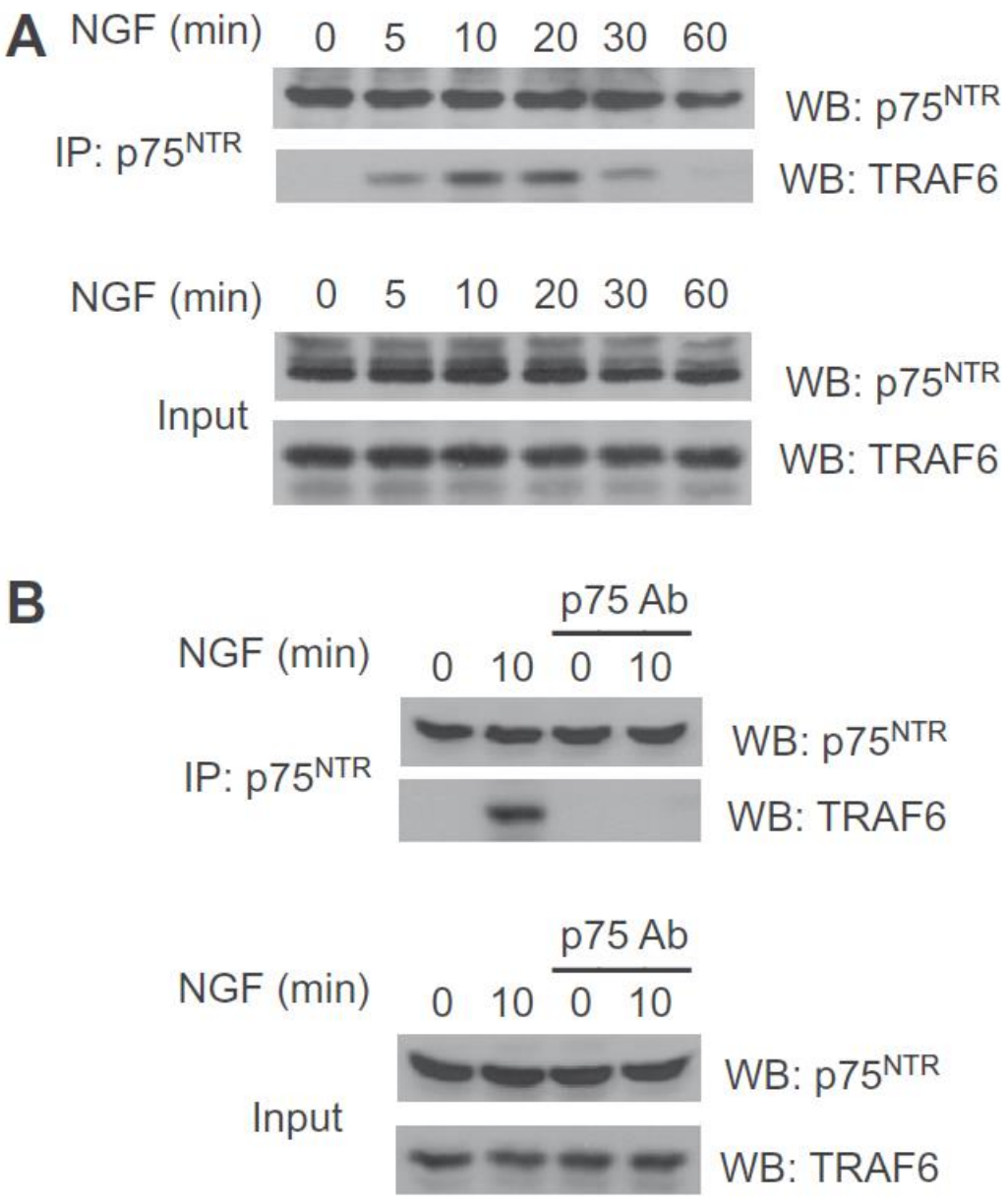
IP: p75NTR
WB: Ub
WB: p75NTR

Fig. 3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WB: p75NTR
WB: TRAF6
NGF (min)
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WB: TRAF6
B
p75 Ab
NGF (min)
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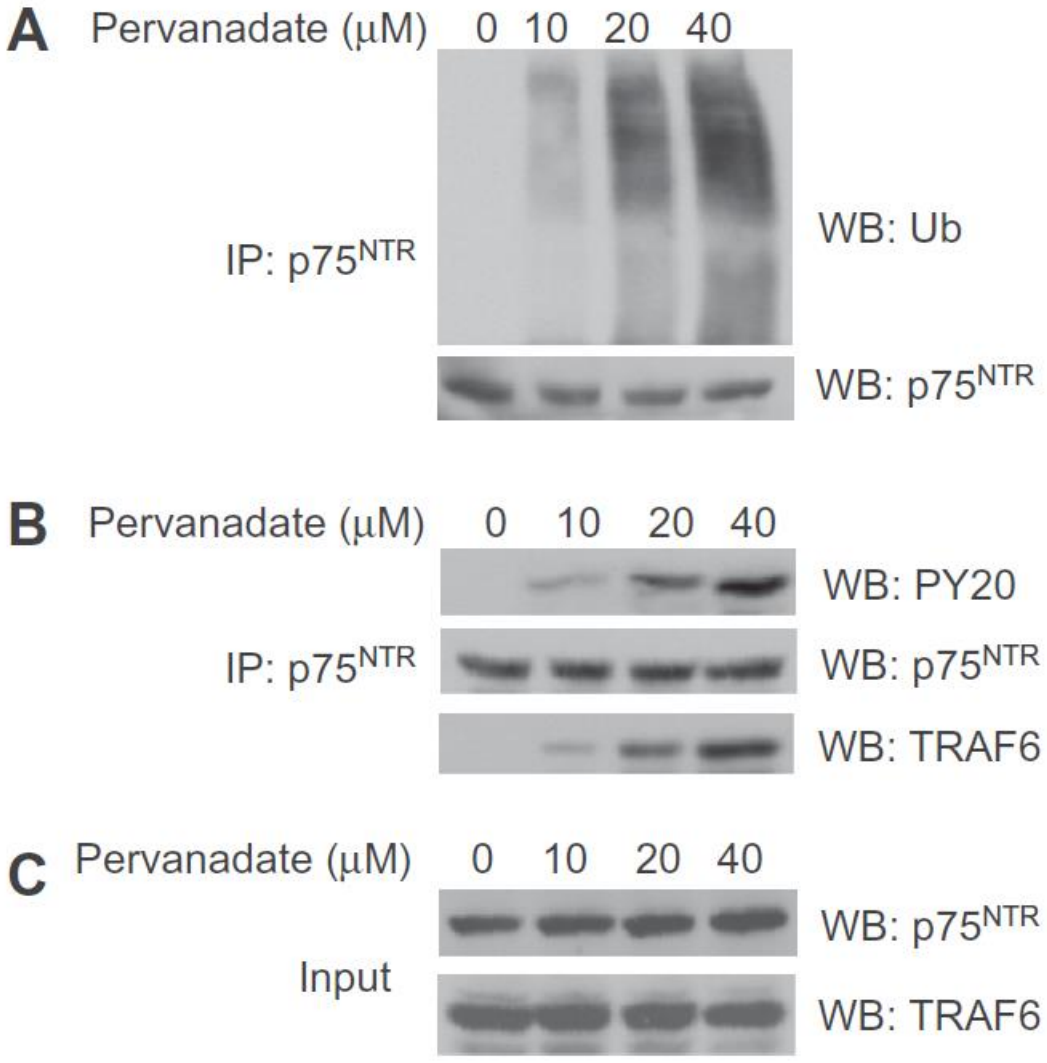
WB: TRAF6

Fig. 4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WB: p75NTR
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0 10 20 40
WB: PY20
IP: p75NTR
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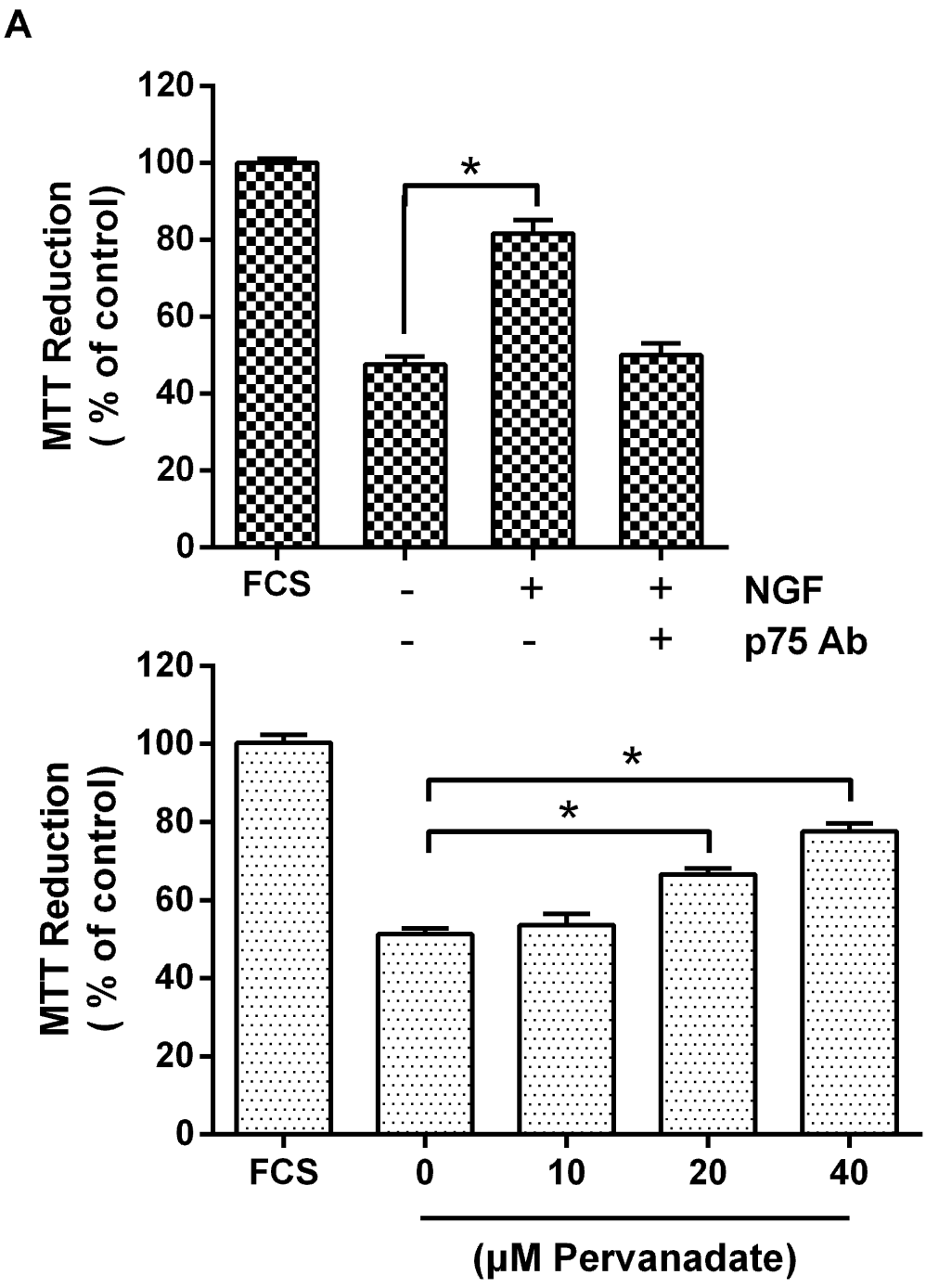
WB: p75NTR
Input
WB: TRAF6

Fig. 5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MTT Reduction
(% of control)
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100
80
60
40
20
0
*
FCS
-
+
+
NGF
-
-
+
p75 Ab
MTT Reduction
(% of control)
120
100
80
60
40
20
0
*
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FCS
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10
20
40
(μM Pervanadate)

Chapter 4: TRAF6 and p62 inhibit amyloid β-induced neuronal death through p75 neurotrophin receptor.

4.1 Abstract

Amyloid β (Aβ) aggregates are the primary component of senile plaques in Alzheimer disease (AD) patient's brain. Aβ is known to bind p75 neurotrophin receptor ($p75^{NTR}$) and mediates Aβ -induced neuronal death. Recently, we showed that NGF leads to $p75^{NTR}$ polyubiquitination, which promotes neuronal cell survival. Here, we demonstrate that Aβ stimulation impaired the $p75^{NTR}$ polyubiquitination. TRAF6 and p62 are required for polyubiquitination of $p75^{NTR}$ on NGF stimulation. Interestingly, we found that overexpression of TRAF6/p62 restored $p75^{NTR}$ polyubiquitination upon Aβ/NGF treatment. Aβ significantly reduced NF-κB activity by attenuating the interaction of $p75^{NTR}$ with IKKβ. $p75^{NTR}$ increased NF-κB activity by recruiting TRAF6/p62, which thereby mediated cell survival. These findings indicate that TRAF6/p62 abrogated the Ab-mediated inhibition of $p75^{NTR}$ polyubiquitination and restored neuronal cell survival.

4.2 Introduction

Amyloid β protein (Aβ) is a 39-43 amino acids peptide derived by the β- and γ- secretase cleavage of amyloid precursor protein (39). Aβ aggregates are the major component of senile plaques found in the patients of Alzheimer's disease (AD) (40). Overproduction of the Aβ causes its accumulation thereby leads to early-onset familial AD (EOFAD) (39). Failure to remove Aβ from the brain causes late-onset AD (LOAD) (41). This might be

due to diminished ability of microglial cells to clear Aβ, impairment of neprilysin and insulysin Aβ degrading proteases, and diminished perivascular and vascular drainage (41-45).

p75 neurotrophin receptor ($p75^{NTR}$) is expressed in basal forebrain cholinergic neurons that undergo degeneration in AD (46). The ligands for $p75^{NTR}$ are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). The $p75^{NTR}$ can also bind to Aβ (47-49) and mediates Aβ-induced neuronal death (50-52). Recently we showed that binding of NGF to $p75^{NTR}$ mediates neuronal cell survival (53). Polyubiquitination of $p75^{NTR}$ and interaction of tumor necrosis factor receptor-associated factor 6 (TRAF6) with $p75^{NTR}$ was NGF dependent (53). TRAF6 functions as an ubiquitin ligase (31) and polyubiquitinates several substrates (32). The ligase activity of TRAF6 is dependent upon its polyubiquitination and oligomerization (54,55). p62 is found to interact with TRAF6 (56). p62 induces polyubiquitination of TRAF6 and oligomerization thereby increasing the activity of TRAF6 (57). TRAF6/p62 ubiquitinates several substrates and leads to various cellular functions: ubiquitination of TrkA, leading to survival and differentiation (35); activation of NRIF, leading to apoptosis (6); activation of Unc-51-like kinase 1/2 regulating filopodia extension and axon branching in sensory neurons (58); and initiation of proteasomal degradation of tau (59).

NF-κB activation requires the phosphorylation of IκBα prior to its degradation by the IκB kinase (IKK) thereby leads to nuclear translocation of NF-κB. IκBα is found to be tyrosine phosphorylated (60) and degraded by NGF (61), however 800 nM of Aβ blocked the phosphorylation and degradation of IκBα (61). Activation of NF-κB by NGF is predominantly through the $p75^{NTR}$ receptor (62). Interaction of TRAF6 with $p75^{NTR}$

enhances the NF-κB activation (63). p62 functions as a scaffold for the activation of NF-κB by NGF (64). In this study we demonstrate that Aβ impaired the $p75^{NTR}$ polyubiquitination, interaction of TRAF6 and p62 with $p75^{NTR}$ and NF-κB activation induced by NGF. However, overexpression of TRAF6/p62 restored the $p75^{NTR}$ polyubiquitination and NF-κB activation on Aβ/NGF treatment.

4.3 Materials and methods

4.3.1 Antibodies and reagents

$p75^{NTR}$ antibody was purchased from Millipore (Billerica, MA) and rabbit TRAF6 antibody for Western blotting was obtained from Abcam (Cambridge, MA). Rabbit $p75^{NTR}$ and p62, Oct A, ubiquitin antibodies were purchased from Santa Cruz Biotechnology (La Jolla, CA). IKKβ antibody was from Cell signaling (Danvers, MA). NGF (2.5S) was obtained from Bioproducts for Science (Indianapolis, IN) Anti-rabbit IgG and anti-mouse IgG-HRP linked secondary antibodies were from GE Healthcare UK Ltd., and Enhanced Chemiluminescence (ECL) was from Thermo Scientific, IL. Amyloid β fragment (1-40), protein A sepharose beads and all other reagents were obtained from Sigma-Aldrich Co. (St Louis, MO).

4.3.2 Cell culture

The mouse hippocampal cell line HT-22 was a generous gift from Dr. David Schubert (The Salk Institute, La Jolla, CA) (33). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplied with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) as previously described (34). Cells were incubated in a 5% CO_2 atmosphere at 37ºC. HT-22 cells were transfected with using the cationic lipid method by

using Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA). The cells were deprived of serum in culture medium overnight at 37ºC before cell lysis.

4.3.3 Amyloid β fragment (1-40)

The amino acid sequence of Aβ 1-40 is the human sequence (Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-OH).

The lyophilized peptide was initially dissolved in water (6 mg/ml). For maximal biological activity, it was further diluted with calcium-free PBS to 1 mg/ml and incubated at 37°C for 4 days before adding to the culture media at the final desired concentration.

4.3.4 Immunoprecipitation and Western blotting analysis

Cells were stimulated with NGF (100 ng/ml) for 10 min or with 10 or 20 μM amyloid β (1-40) for 24 h at 37°C. To detect protein-protein interactions, the cells were lysed with Triton lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin and aprotinin) or SDS lysis buffer (Triton lysis buffer containing 1% SDS) to detect covalent interaction of ubiquitin and $p75^{NTR}$ (Geetha et al., 2005a). Protein concentrations were estimated using the Bradford procedure (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard for all samples except for those that contained SDS where the DC assay was used (Bio-Rad, Hercules, CA). The cell lysates were incubated with 4 μg of primary antibody at 4°C for 3 h. The immunoprecipitates were collected with agarose-coupled secondary antibody for 2 h at 4°C and then were washed three times with lysis buffer. Samples were boiled in

sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and resolved on 7.5%–12% SDS-PAGE gels, transferred onto nitrocellulose membranes, and analyzed by Western blotting with the appropriate antibodies.

4.3.5 Measurement of NF-κB activity

HT-22 cells were transfected with TRAF6 and p62 or TRAF6ΔR and ASp62, which was followed by addition of 10 μM of amyloid β (1-40) for 24 h with or without 100 ng/ml NGF for 1 h. Nuclear extracts were prepared from the cells using a nuclear extract kit (Marlingen Biosciences Inc., Ijamsville, MD), and NF-κB activity was measured in the nuclear extracts using a NF-κB transcription factor microplate assay (Marlingen Biosciences Inc. Ijamsville, MD) according to the manufacturer's protocol.

4.3.6 Measurement of cell viability

HT-22 cells were transfected with TRAF6 and p62 or TRAF6ΔR and ASp62, which was followed by serum-deprivation and addition of 10 μM of amyloid β (1-40) with or without 50 ng/ml NGF for 24 h. Cell viability was assessed by the 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (MTT) assay. MTT is a tetrazolium salt that is reduced by metabolically viable cells to a colored, formazan salt. The MTT assay was performed 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%).

4.4 Results and discussion

4.4.1 Amyloid β inhibits the NGF-induced polyubiquitination of $p75^{NTR}$

We previously showed that NGF induces $p75^{NTR}$ polyubiquitination and leads to neuronal cell survival (53). Aβ is known to bind $p75^{NTR}$ and induce cell death (50-52); therefore, we sought to investigate whether Aβ would block the polyubiquitination of $p75^{NTR}$. HT-22 cells were treated either with 100 ng/ml NGF alone for 10 min or with the addition of 10 or 20 μM amyloid β (1-40) for 24 h at 37°C or both. The $p75^{NTR}$ polyubiquitination was determined by immunoprecipitation with $p75^{NTR}$ antibody and Western blotting with anti-ubiquitin and anti-$p75^{NTR}$. NGF treatment resulted in a strong signal indicating $p75^{NTR}$ polyubiquitination. Aβ treatment diminished $p75^{NTR}$ polyubiquitination in the absence or presence of NGF (Fig. 6). Interestingly, the $p75^{NTR}$ polyubiquitination induced by NGF was prevented by Aβ.

4.4.2 Amyloid β impairs the interaction of $p75^{NTR}$ with TRAF6 and p62

NGF stimulation promotes the interaction of $p75^{NTR}$ with TRAF6 and may lead to $p75^{NTR}$ ubiquitination (53). TRAF6 in turn binds to p62 through its TRAF6 interacting domain (56,64). Since Aβ blocked the $p75^{NTR}$ polyubiquitination, we hypothesized that the binding of Aβ to $p75^{NTR}$ would also impair the interaction of $p75^{NTR}$ with TRAF6 and p62. HT-22 cells were treated with 10 or 20 μM amyloid β (1-40) for 24 h with or without NGF for 10 min. The cells were lysed in Triton lysis buffer and immunoprecipitated with $p75^{NTR}$ and Western blotted with $p75^{NTR}$, TRAF6 and p62 antibodies. NGF stimulation resulted in a substantial increase in the interaction of both TRAF6 and p62 with $p75^{NTR}$ (Fig. 7A), which is expected when $p75^{NTR}$ is polyubiquitinated. However, the interaction of $p75^{NTR}$ with

TRAF6 and p62 was abrogated when HT-22 cells were treated with amyloid β (1-40) alone or with NGF (Fig. 7A). These results corroborate the impaired $p75^{NTR}$ ubiquitination caused by Aβ shown in Figure 1. Reciprocal immunoprecipitation with anti-TRAF6 or anti-p62 was performed and similar results were obtained as shown in Figure 6B and 6C. The cell lysates were also Western blotted for the presence of $p75^{NTR}$, TRAF6 and p62 (Fig. 7D).

4.4.3 $p75^{NTR}$ polyubiquitination was enhanced by TRAF6/p62 overexpression

TRAF6 is an E3 RING ubiquitin ligase that mediates the transfer of ubiquitin (31) and directs synthesis of noncanonical K63-linked polyubiquitin chains (32). TRAF6 requires p62 for both oligomerization and polyubiquitination, and thereby enhances the E3 ubiquitin ligase activity of TRAF6 (57). To verify whether TRAF6 and p62 are essential for $p75^{NTR}$ polyubiquitination upon NGF stimulation, HT-22 cells were transfected with a RING finger deletion mutant of TRAF6 (Flag-TRAF6ΔR) or antisense p62 (ASp62), which depletes the endogenous p62. Analysis of anti-$p75^{NTR}$ immunoprecipitates revealed that TRAF6ΔR and ASp62 blocked the NGF-induced polyubiquitination of $p75^{NTR}$ (Fig. 8A). The lysates were also Western blotted with p62 and Oct-A antibody to check the protein expression of the plasmids. These findings revealed that $p75^{NTR}$ polyubiquitination is TRAF6/p62 dependent.

Additional evidence that TRAF6 and p62 were essential for $p75^{NTR}$ polyubiquitination was obtained by overexpression of TRAF6 and p62 to restore the $p75^{NTR}$ polyubiquitination upon Aβ/NGF treatment (Fig. 8B). HT-22 cells were treated with 20 μM amyloid β (1-40)

for 24 h followed by stimulation with 100 ng/ml NGF for 10 min. In lane 5, the cells were transfected with Flag-tagged WT-TRAF6 and WT-p62 and treated with Aβ/NGF. In lane 6, cells were transfected with Flag-tagged TRAF6ΔR and ASp62 and treated with Aβ/NGF. Cells were lysed in SDS lysis buffer and immunoprecipitated (IP) with $p75^{NTR}$ and Western blotted with ubiquitin or $p75^{NTR}$ antibody. NGF stimulation led to $p75^{NTR}$ polyubiquitination, but this was impaired by Aβ pretreatment as shown in Figure 7B (lane 2 vs lane 4). Transfection of the cells with WT TRAF6 and p62 restored the $p75^{NTR}$ polyubiquitination otherwise inhibited by Aβ (Fig. 8B, lane 5), but transfection of the cells with the TRAF6 mutant delta RING finger and ASp62 did not (Fig. 8B, lane 6). Thus, while Aβ alone prevents polyubiquitination of $p75^{NTR}$, the overexpression of TRAF6 and p62 can remediate the problem.

4.4.4 TRAF6/p62 potentiates NGF-induced NF-κB activity

Aβ has been found to induce neuronal cell death through $p75^{NTR}$ (65). Upon stimulation with NGF, TRAF6 binds to $p75^{NTR}$ and leads to NF-κB activation (63). ASp62 increased p75-mediated cell death and decreased NGF-induced NF-κB activation (64). To determine how TRAF6/p62 contributes to the activity of NF-κB, HT-22 cells were transfected with WT-TRAF6 and p62 or with TRAF6ΔR and ASp62 and then treated with Aβ in presence or absence of NGF. Figure 4A shows that Aβ treatment in presence or absence of NGF reduced NF-κB activity. Conversely, transfection with WT-TRAF6 and p62 followed by Aβ/NGF treatment enhanced the activity of NF-κB. However, TRAF6ΔR and ASp62 did not support NF-κB activity (Fig. 9A). These results indicate that the interaction of TRAF6 and p62 with $p75^{NTR}$ is essential for the activity of NF-κB.

Two IκB kinases (IKK) have been identified (IKKα and IKKβ) to phosphorylate IκBα (66-69). NGF leads to the activation of both IKKα and IKKβ (70). It has been previously shown that ASp62 impairs NGF-stimulated activation of IKKβ (64). To examine if TRAF6/p62 would enhance the interaction of $p75^{NTR}$ with IKKβ upon Aβ/NGF treatment, HT-22 cells were treated with 10 μM Aβ with or without NGF. Cells were transfected with Flag-tagged WT-TRAF6 and p62 (Fig. 9B, lane 5) or with Flag-TRAF6ΔR and ASp62 (Fig. 9B, lane 6) and treated with Aβ/NGF. The cells were lysed and $p75^{NTR}$ was immunoprecipitated and Western blotted with anti-IKKβ or anti- $p75^{NTR}$. NGF stimulation led to the interaction of IKKβ with $p75^{NTR}$ (Fig. 9B, lane 2), whereas the Aβ treatment with or without NGF blocked that interaction (Fig. 9, lane 3 and 4). Overexpression of WT-TRAF6 and p62 restored the recruitment of IKKβ with $p75^{NTR}$ that was otherwise impaired by Aβ (Fig. 9B, lane 5), but overexpression of TRAF6ΔR and ASp62 did not (Fig. 9B, lane 6). These results demonstrate that interaction of IKKβ with $p75^{NTR}$ upon NGF stimulation requires TRAF6 and p62.

NGF binding to $p75^{NTR}$ increases the neuronal survival (71) and Aβ increases the neuronal death (65). As TRAF6 and p62 are essential for NF-κB activation, we sought to determine whether TRAF6 and p62 promotes neuronal cell survival using the MTT assay. HT-22 neuronal cells are grown normally in DMEM containing 10% FBS, however deprivation of serum for 24 h induces cell death. NGF treatment protected HT-22 cells from death on serum deprivation, whereas Aβ significantly increased cell death (Fig. 9C). Overexpression of WT-TRAF6 and p62 followed by Aβ/NGF treatment protected serum-starved HT-22 cells significantly from cell death, whereas TRAF6ΔR and ASp62 were not

neuroprotective (Fig. 9C). Taken together, these findings underscore the requirement for TRAF6 and p62 for $p75^{NTR}$-mediated NF-κB activation and survival signaling (Fig. 9).

4.5 Discussion

In conclusion, the findings reported here reveal that Aβ impairs 1) $p75^{NTR}$ polyubiquitination, 2) the interaction of $p75^{NTR}$ with TRAF6 and p62, 3) the NF-κB activity and neuronal survival otherwise induced by NGF. TRAF6 and p62 are essential for $p75^{NTR}$ polyubiquitination upon NGF stimulation. Overexpression of TRAF6/p62 restored the $p75^{NTR}$ polyubiquitination upon Aβ/NGF treatment. $p75^{NTR}$ activates NF-κB by recruiting TRAF6/p62 to mediate cell survival (62). Aβ reduced the NF-κB activity by blocking the interaction of $p75^{NTR}$ and IKKβ, but overexpression of WT-TRAF6 and p62 followed by Aβ/NGF treatment increased the NF-κB activity and neuronal survival. These findings suggest that TRAF6/p62 possesses the unique ability to reverse Aβ mediated inhibition of $p75^{NTR}$ polyubiquitination, NF-κB activity and neuronal survival.

4.6 Figure Legends

Fig. 6 Amyloid β abrogates the NGF induced $p75^{NTR}$ polyubiquitination. HT-22 cells were stimulated with NGF (100 ng/ml) for 10 min or 1 or 2 μM of amyloid β (1-40) for 1 h at 37°C or both. Cells were lysed and ubiquitination was examined by immunoprecipitation (IP) with anti-$p75^{NTR}$ followed by Western blot (WB) analysis with anti-ubiquitin (Ub) and anti- $p75^{NTR}$.

Fig. 7 Amyloid β blocks the interaction of TRAF6 and p62 with $p75^{NTR}$. HT-22 cells were treated with 1 or 2 μM of amyloid β (1-40) for 1 h along with or without NGF (100 ng/ml) for 10 min. The interaction of anti-$p75^{NTR}$ was examined by immunoprecipitation (IP) with (A) anti-$p75^{NTR}$ (B), TRAF6 and (C) p62 followed by Western blot (WB) analysis with anti-$p75^{NTR}$, anti-TRAF6 and anti-p62. (D) The same lysates were also Western blotted with the $p75^{NTR}$, TRAF6 and p62 antibodies.

Fig. 8 $p75^{NTR}$ ubiquitination was impaired by Aβ but is enhanced by TRAF6/p62 overexpression. (A) HT-22 cells were transfected either with Flag-TRAF6ΔR or ASp62 and treated with or without 100 ng/ml of NGF for 10 min. The cells were lysed in SDS lysis buffer and the ubiquitination of $p75^{NTR}$ was determined by immunoprecipitation (IP) of $p75^{NTR}$ followed by immunoblotting with anti-ubiquitin and anti-$p75^{NTR}$. The lysates were Western blotted with p62 and Oct A antibody to check their expression. (B) HT-22 cells were treated with 2 μM of amyloid β (1-40) for 1 h followed by stimulation with 100 ng/ml of NGF for 10 min. In lane 5 the cells were transfected with Flag-WT TRAF6 and p62 and lane 6 with Flag-TRAF6ΔR and ASp62 followed by Aβ/NGF treatment. Lysates were immunoprecipitated (IP) with $p75^{NTR}$ and immunoblotted with ubiquitin or $p75^{NTR}$ antibody.

Fig. 9 Amyloid β blocks the NGF-induced NF-κB activation but TRAF6/p62 potentiates NF-κB activation. (A) NF-κB activation was measured in control/NGF, cells treated with Aβ/NGF, overexpressed with TRAF6/p62 or TRAF6ΔR/ASp62 followed by Aβ/NGF treatment (*, $p < 0.001$). (B) HT-22 cells were treated with 2 μM of amyloid β (1-40) for 1

h along with or without NGF for 10 min. In lane 5 the cells were transfected with Flag-WT TRAF6 and p62 and lane 6 with Flag-TRAF6ΔR and ASp62 followed by Aβ/NGF treatment. $p75^{NTR}$ was immunoprecipitated (IP) and immunoblotted with IKKβ or $p75^{NTR}$ antibody. The lysates were Western blotted with the IKKβ and $p75^{NTR}$ antibodies.

Fig. 6

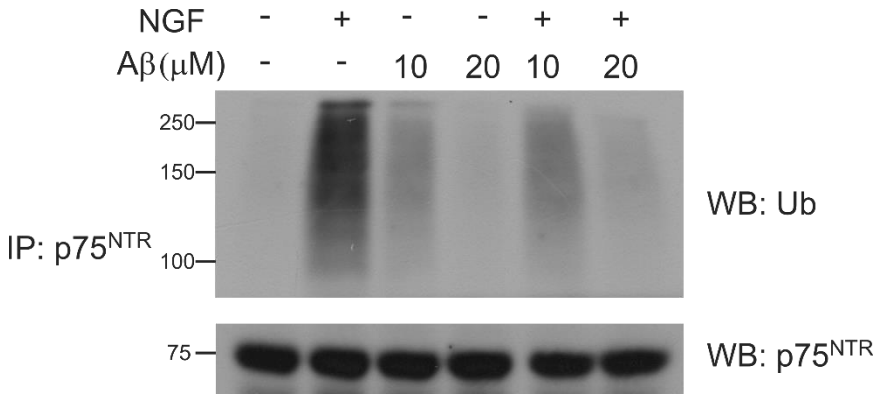
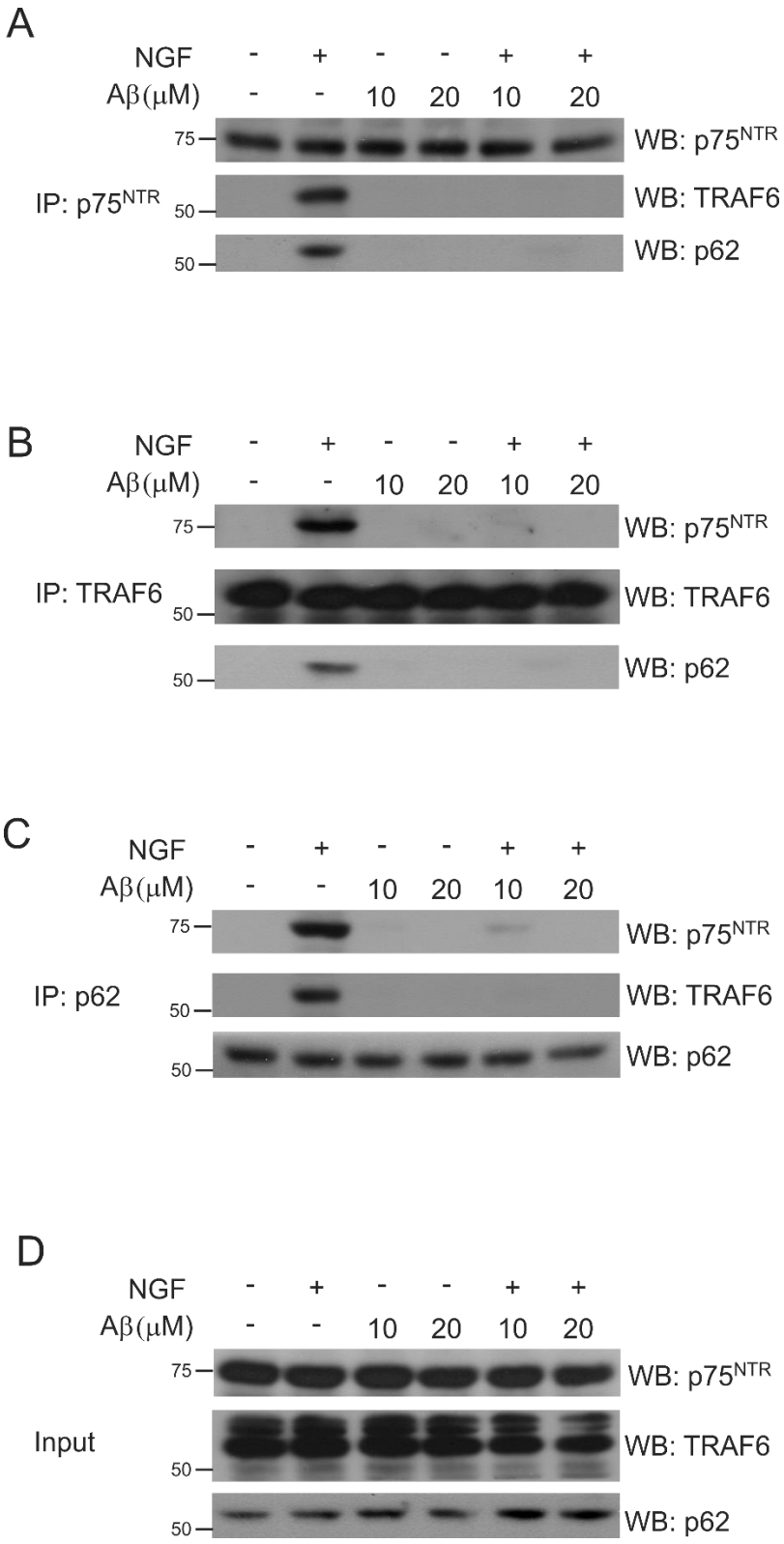


Fig. 7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WB: p75NTR
IP: p75NTR
50
WB: TRAF6
50
WB: p62
B
NGF - + - - + +
Aβ(μM) - - 10 20 10 20
75
WB: p75NTR
IP: TRAF6
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C
NGF - + - - + +
Aβ(μM) - - 10 20 10 20
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WB: p75NTR
IP: p62
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WB: TRAF6
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WB: p62
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NGF - + - - + +
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WB: p75NTR
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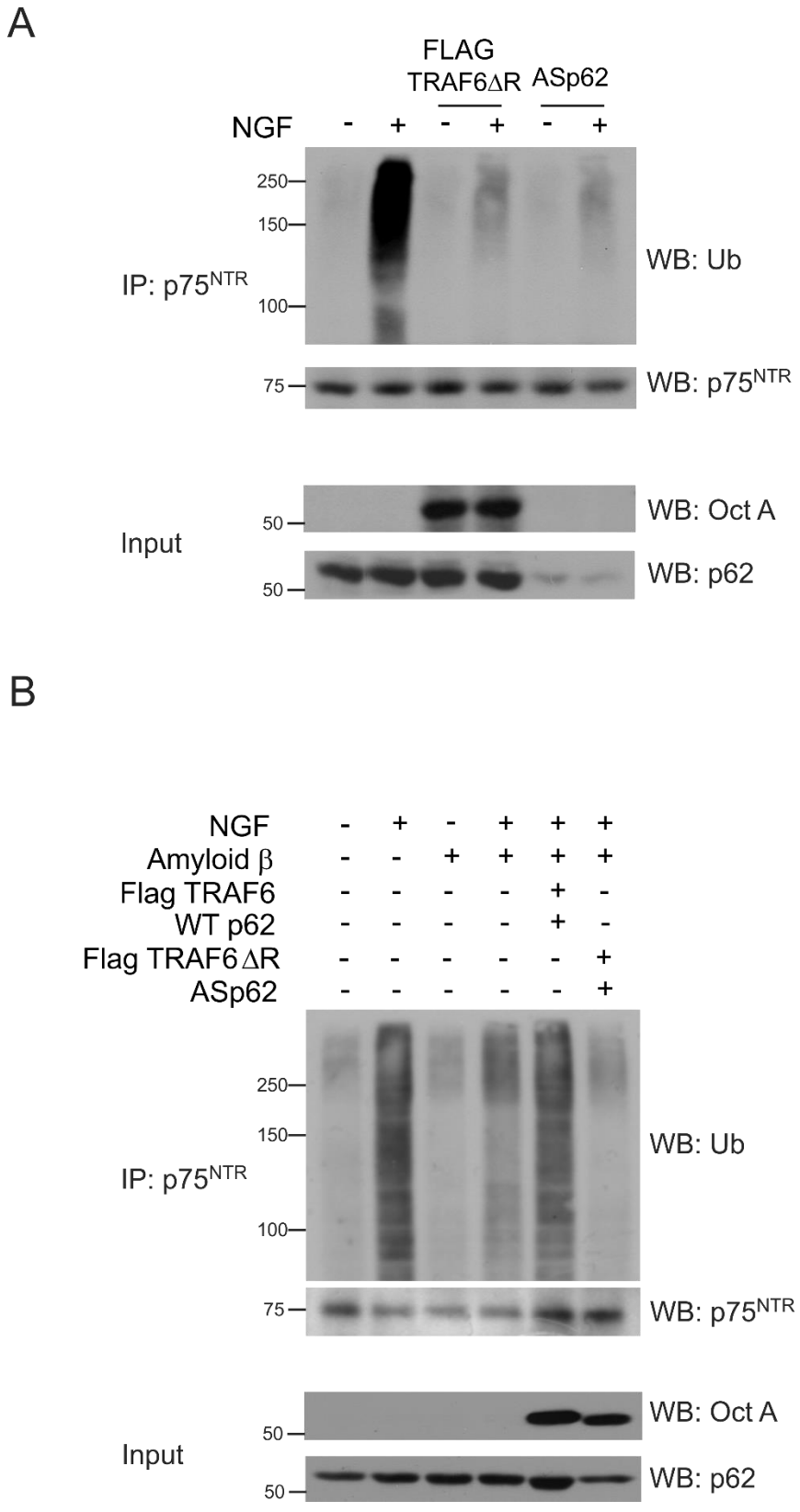
WB: p62

Fig. 8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TRAF6ΔR
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NGF - + - + - +
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150
100
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50
WB: Oct A
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WB: p62
B
NGF - + - + + +
Amyloid β - - + + + +
Flag TRAF6 - - - - + -
WT p62 - - - - + -
Flag TRAF6 ΔR - - - - - +
ASp62 - - - - - +
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IP: p75NTR
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WB: p75NTR
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WB: Oct A
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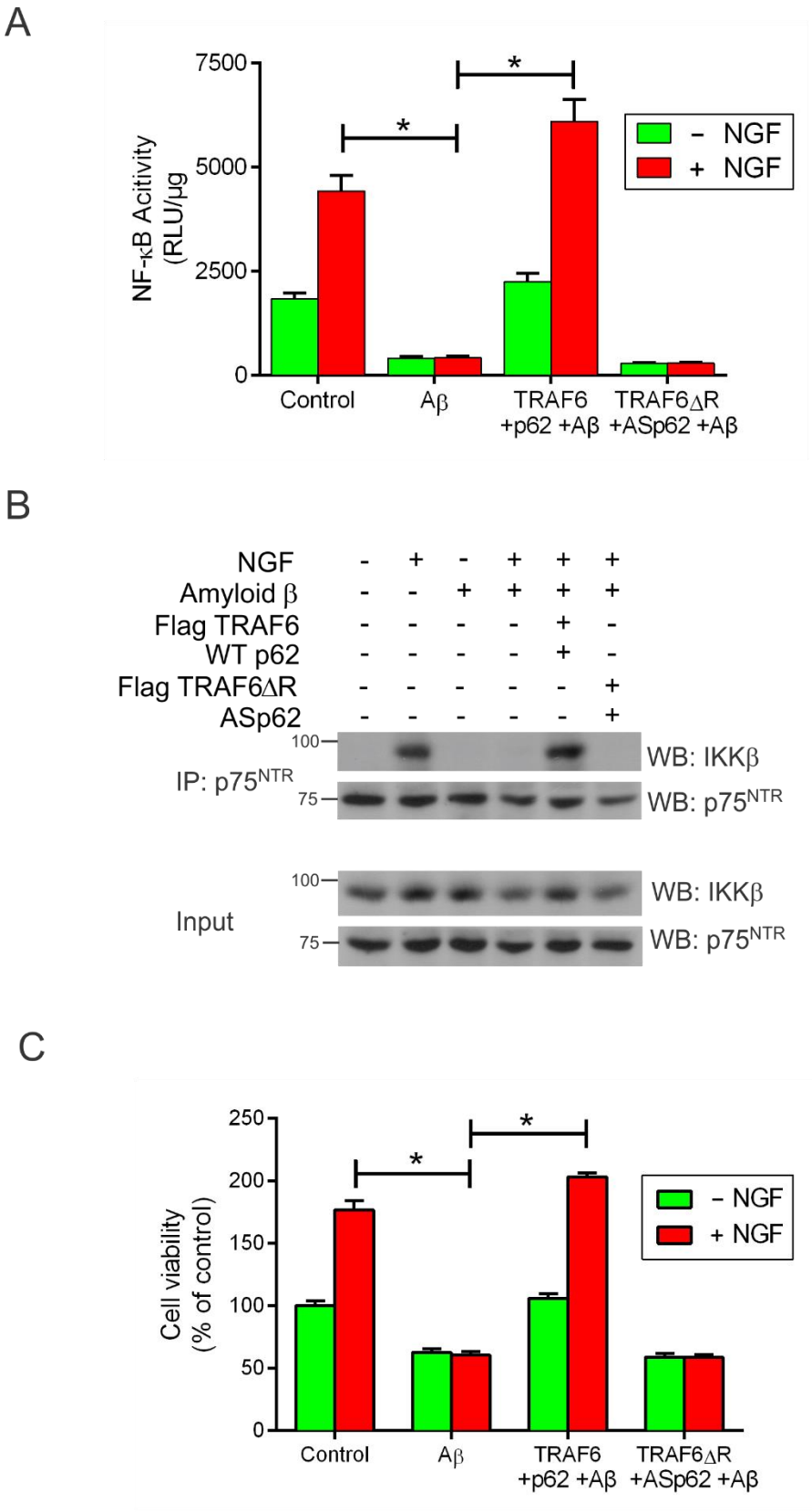
WB: p62

Fig. 9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7500
5000
2500
0
*
*
– NGF
+ NGF
Control
Aβ
TRAF6 +p62 +Aβ
TRAF6ΔR +ASp62 +Aβ
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NGF
Amyloid β
Flag TRAF6
WT p62
Flag TRAF6ΔR
ASp62
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75
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WB: p75NTR
C
Cell viability (% of control)
250
200
150
100
50
0
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– NGF
+ NGF
Control
Aβ
TRAF6 +p62 +Aβ
TRAF6ΔR +ASp62 +Aβ

Chapter 5: Amyloid β-abrogated TrkA ubiquitination in PC12 cells analogous to Alzheimer's disease

5.1 Abstract

Amyloid β (Aβ) protein is the primary proteinaceous deposit found in the brains of patients with Alzheimer's disease (AD). Evidence suggests that Aβ plays a central role in the development of AD pathology. Here, we show that Aβ impairs TrkA ubiquitination, phosphorylation and association of TrkA from $p75^{NTR}$ and p62/TRAF6 complex induced by NGF in PC12 cells. The ubiquitination and tyrosine phosphorylation of TrkA was also found to be impaired in postmortem human AD hippocampus compared to control. Interestingly the nitrotyrosylation of TrkA was increased in AD hippocampus and this explains why the phosphotyrosylation and ubiquitination of TrkA was impaired. In AD brain the production of matrix metalloproteinase-7 (MMP-7) which cleaves proNGF was reduced, thereby leading to accumulation of proNGF and decreasing the level of active NGF. TrkA signaling events, including Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways, are deactivated with Aβ and in human AD hippocampus. Findings show that Aβ blocks the TrkA ubiquitination and downstream signaling similar to AD hippocampus.

5.2 Introduction

Amyloid β protein (Aβ) is a peptide (36–43 amino acids) processed from amyloid precursor protein (APP) cleavage by the β- and γ-secretase (72). Aβ is a multifunctional peptide,

usually producing in all neurons with significant non-pathological activity (73). However, its aggregates are believed to be the principal component of senile plaques, which is found in the brains of patients with Alzheimer's disease (AD) (40). Increase of Aβ production has been found in all three types of familial AD (APP, presenilin-1 or presenilin-2 mutations) (74). In AD, Aβ accumulation leads to a high diversity of toxic mechanisms, including oxidative stress, mitochondrial diffusion, excitotoxicity through interactions with neurotransmitters receptors (75).

Nerve growth factor (NGF) is a ~13kDa peptide and is part of the neurotrophin family of molecules, including brain-derived neurotrophic factors (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (76). NGF is synthesized as a precursor form proNGF, that is cleaved to release C-terminal mature NGF, extracellulary by the matrix metalloproteinase-7 (MMP-7) (77). NGF regulates the survival, maturation, differentiation, and maintenance of developing neurons (1). NGF can bind to both a high-affinity tyrosine kinase Trk receptor and a low-affinity $p75^{NTR}$ receptor (3,78). TrkA is a single transmembrane-spanning protein which belongs to the super family of tyrosine kinase (RTK) receptors, that regulate synaptic strength and plasticity in the mammalian nervous system (79). Upon binding of NGF to TrkA , the receptor undergoes a series of events, including dimerization, autophosphorylation (4), polyubiquitination (35), followed by internalization of the receptor to signaling vesicles (80,81), which elicits many of the classical neurotrophic actions (82). TrkA activation leads to subsequent cascade signaling such as Ras/MAPK and the PI3K pathways, which act to intercept nuclear and mitochondrial cell-death programs, resulting in neuronal survival and differention.

$p75^{NTR}$ is a member of the tumor necrosis factor (TNF) super family of receptors (4,6,83), and is widely expressed in the nervous system and many peripheral tissues (74,84). $p75^{NTR}$ is involved in multiple intracellular signaling pathways to execute a diversity of biologic functions, including cellular apoptosis, cell survival, differentiation, neurite outgrowth (85-87), Schwann cell myelination, and sensory neuron development (10,83).

$p75^{NTR}$ is polyubiquitinated upon NGF stimulation, which requires interaction of ubiquitin E3 ligase TRAF6 (63) and scaffolding protein p62 (56,71). This modification was found to be disturbed by Aβ stimulation (88). TrkA forms a complex with $p75^{NTR}$ through p62/TRAF6 complex (64). $p75^{NTR}$ and p62/TRAF6 complex are necessary for TrkA polyubiquitination and critical for downstream signaling (35). Herein, we demonstrate that Aβ and AD conditions impairs the tyrosine phosphorylation, ubiquitination of TrkA and its interaction with $p75^{NTR}$ through p62/TRAF6 thereby leadings to inactivation of TrkA signaling pathway.

5.3 Materials and methods

5.3.1 Antibodies and reagents

Anti-$p75^{NTR}$ was purchased from abcam (Cambridge, MA). NGF, MMP-7, p62, TrkA, ubiquitin, p-Tyr (PY99) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TRAF6 was purchased from Abcam (Cambridge, MA), anti-proNGF and anti-nitrotyrosine from Millipore (Denvers, MA). Akt, Phospho-Akt (Ser-473), MAPK, phospho-MAPK antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-rabbit IgG and anti-mouse IgG-HRP linked secondary antibodies were from GE

Healthcare UK Ltd., and enhanced chemiluminescence (ECL) was from Thermo Scientific. Protein A-Sepharose beads and all other reagents were obtained from Sigma-Aldrich.

5.3.2 Cell culture

PC12 rat pheochromocytoma cells were maintained in Dulbecco's modified Eagle's media (DMEM) with 10% heat-inactivated horse serum, 5% fetal bovine serum, and antibiotics (100 units/ml; streptomycin and penicillin). Cells were incubated in a humidified atmosphere containing 5% CO_2 and 95% air at 37^{o}C. The cells were deprived of serum in culture medium overnight at 37^{o}C before cell lysis.

5.3.3 Human 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 AD cases met NIA-Reagan criteria (89) for the neuropathologic diagnosis of AD and NIA-AA criteria (90) for a high level of AD neuropathologic change. Controls had no clinical history of neurological illness and no significant neurodegenerative changes at autopsy.

5.3.4 Amyloid beta fragment (1–40)

The amino acid sequence of Aβ 1–40 is the human sequence (Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-OH). The lyophilized

peptide was initially dissolved in water (6 mg/ml). For maximal biological activity, it was further diluted with calcium-free PBS to 1 mg/ml and incubated at 37°C for 4 days before being added to the culture media at the final desired concentration.

5.3.5 Immunoprecipitation and Western blotting analysis

PC12 cells were treated with 10 μM amyloid β (1–40) for 24 h followed with or without NGF (100 ng/ml) for 10 min at 37°C. The cells were lysed with Triton lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin and aprotinin) to detect protein–protein interactions, or SDS lysis buffer (Triton lysis buffer containing 1% SDS) to detect covalent interaction of ubiquitin and TrkA (Geetha et al., 2005a). Protein concentrations were estimated using the Bradford procedure (Bio-Rad, Hercules, CA) except for those that contained SDS where the DC assay was used (Bio-Rad, Hercules, CA). The cell lysates were incubated with 4 μg of primary antibody at 4°C for 3 h. The immunoprecipitates were collected with agarose-coupled secondary antibody for 2 h at 4°C and then were washed 3 times with lysis buffer. Samples were boiled in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and resolved on SDS–PAGE gels, transferred onto nitrocellulose membranes, and analyzed by Western blotting with the appropriate antibodies.

5.3.6 Statistical Analysis

For all experiments at least three replicate were analyzed. All quantitative data were expressed as means ± standard errors of the mean (SEM) and analyzed using unpaired

Student's t-test as indicated (Graphpad Prism). Differences with statistical significance are denoted by $P < 0.001$.

5.4 Results

TrkA receptor is known to be polyubiquitinated by NGF through $p75^{NTR}$ receptor (35). We recently found that Aβ treatment impaired the $p75^{NTR}$ polyubiquitination (88). Since $p75^{NTR}$ is a modulator of TrkA (91,92), we sought to investigate whether Aβ would affect NGF-dependent polyubiquitination of TrkA. PC12 cells were treated either with 100 ng/ml NGF alone for 10 min or with the addition of 10 μM amyloid β (1–40) for 24 h at 37°C. The TrkA polyubiquitination was examined using immunoprecipitation (IP) with the TrkA antibody and Western blotting with anti-ubiquitin and anti-TrkA. NGF treatment resulted in a significant enhancement in TrkA polyubiquitination. Nevertheless, Aβ treatment reduced TrkA polyubiquitination induced by NGF treatment. (Fig. 10A). Binding of the NGF to TrkA induces its autophosphorylation and activation of signaling events that regulate the maintenance, differentiation, and survival, of neurons. We also detected tyrosine phosphorylation of TrkA in the presence of Aβ. The TrkA was immunoprecipitated and Western blotted with anti-PY99, a phospho tyrosine antibody and anti-TrkA. We found that the presence of Aβ caused a substantial decrease in TrkA autophosphorylation along with the treatment of NGF (Fig. 10B). Therefore, Aβ not only obstructed TrkA polyubiquitination but also blocked its tyrosine phosphorylation.

TrkA polyubiquitination is dependent upon its interaction with TRAF6/p62 (35). Since Aβ blocked the polyubiquitination of TrkA, we hypothesized that Aβ would also impair the interaction of TrkA with TRAF6 and p62. PC12 cells were treated with Aβ in the presence

or absence of NGF, followed by immunoprecipitation of TrkA and Western blotting with p62 and TRAF6. Aβ treatment blocked the interaction of TrkA with p62 and TRAF6 in presence of NGF (Fig. 11A). In our previous studies we have shown that Aβ blocked the interaction of $p75^{NTR}$ with TRAF6 and p62 (88). TrkA polyubiquitination is induced by NGF binding to the $p75^{NTR}$ receptor (35). Since the interaction of TrkA with $p75^{NTR}$ receptor is essential for TrkA polyubiquitination, we also checked the interaction in presence of Aβ. Interestingly, interaction of TrkA and $p75^{NTR}$ was also reduced in the presence of Aβ along with NGF (Fig. 11B). This evidence is consistent with the result shown in the Figure 1 where Aβ treatment attenuated ubiquitination of TrkA.

We also examined the polyubiquitination of TrkA in homogenates recovered from 6 postmortem control aged human hippocampal and 6 postmortem AD human hippocampal samples. The tissues were homogenized, immunoprecipitated with TrkA, and subsequently examined by Western blotting with specific antibodies as indicated in the corresponding figures. TrkA polyubiquitination (Fig. 12A) was reduced in AD human hippocampal samples relative to normal aged human hippocampal samples. The tyrosine phosphorylation and nitrosylation of TrkA was also determined in these samples by imuunoprecipitating the TrkA and western blotting with phosphotyrosine, nitrotyrosine and TrkA antibody. Interestingly, the TrkA tyrosine phosphorylation was decreased and nitrosylation was increased in AD human hippocampal samples compared to control (Fig. 12B). This suggests that AD may lead to posttranslational modification of TrkA by nitrating the tyrosine amino acids instead of phosphorylation thereby affecting the TrkA polyubiquitination.

As TrkA tyrosine nitration was enhanced in AD samples, it prompted us to check the level of proNGF and NGF in control and AD human hippocampal samples. The expression of proNGF was significantly increased and NGF level was decreased in AD human hippocampal samples compared to control (Fig. 13A). proNGF can be proteolytically cleaved by extracellular MMP-7 into mature NGF, hence we determined the level of MMP-7 in control and AD human hippocampal samples. The AD human hippocampal samples had decreased level of expression of MMP-7 compared with controls, which may lead to the accumulation of proNGF in AD condition. (Fig. 13A and B). The inactive proNGF has higher affinity to bind $p75^{NTR}$ and can induce apoptosis (93,94).

NGF stimulation leads TrkA to form a complex with p62, TRAF6 and $p75^{NTR}$ (6,64). First, we sought to determine if the interaction of TrkA with p62 and TRAF6 could be recapitulated in human hippocampal samples. Compared with normal aged hippocampal samples, the interaction of TrkA with p62 and TRAF6 was impaired in AD human hippocampal samples (Fig. 14A). To determine the expression of p62 and TRAF6, the homogenates from control aged and AD human hippocampal samples were western blotted with anti-p62 and anti-TRAF6. The expression levels of p62 and TRAF6 was greater in control than in AD hippocampal samples (Fig. 14A). Du et al., 2009 have shown decrease in level of p62 in AD human brain and in 3xTg-AD mice in comparison to control. The interaction of TrkA with p62 and TRAF6 might be impaired in AD due to the reduced expression levels. The interaction of TrkA with $p75^{NTR}$ was also found to be impaired in AD hippocampal samples, even though the expression of $p75^{NTR}$ is greater in AD compared to control (Fig. 14B). The expression of $p75^{NTR}$ was found to be enhanced in 12-15 month old 3xTg-AD mice (Chakravarthy et al., 2010) and in AD hippocampal brains compared

to control (Chakravarthy et al., 2012). Aβ treatment and AD human hippocampal samples impaired the ubiquitination of TrkA, and the association of TrkA with p62, TRAF6, and $p75^{NTR}$. NGF has a high affinity for binding TrkA by its association with $p75^{NTR}$ (35,95). Therefore, Aβ may sustain low-affinity binding of NGF to TrkA and attenuate downstream signaling pathway. The decrease in active NGF may impair TrkA polyubiquitination and TrkA receptor-mediated signaling in hippocampal samples obtained from AD patients.

TrkA receptor activation is essential for cell survival and differentiation. The activation of the Ras/MAPK pathway is essential for the neurotrophin-induced differentiation of neuronal and neuroblastoma cells (96). The PI3K/Akt pathway signaling is critical for both facilitation of neurotrophin-induced cell survival and regulation of vesicular trafficking (96). To examine the effects of Aβ on receptor signaling, we studied Akt and MAPK as downstream targets. PC12 cells were treated either with 10 μM amyloid β (1–40) for 24 h or with the addition of 100 ng/ml NGF for 10 min at 37°C. The cells were lysed and then immunoblotted with Akt/MAPK or its specific-phospho antibodies. The results suggest that phospho-Akt and phospho-MAPK, as indicators of functional Akt and MAPK, were indeed blocked by Aβ in PC12 cells (Fig. 14A and B). In addition, phosphorylation of Akt and MAPK were also diminished in AD human hippocampal samples as compared with normal human hippocampal samples (Fig. 16A and B). These results suggest that active NGF, tyrosine phosphorylation and polyubiquitination of TrkA is essential for downstream signaling.

5.5 Discussion

The emergencing evidence suggests that polyubiquitination participates in activation of signaling events such as TrkA signaling cascade (35,97). Furthermore, Aβ is capable of activation of several signaling pathways involved in neuronal damage observed in the AD. However, the relationship between Aβ and TrkA receptor is still not fully understood. Our findings reveal that Aβ abrogates the tyrosine phosphorylation and polyubiquitination of TrkA induced by NGF in PC12 cells and human hippocampal tissues. Tyrosine phosphorylation of TrkA is essential for its polyubiquitination. This evidence correlates with our previous study that showed K252a, an inhibitor of TrkA tyrosine kinase activity, reduced TrkA polyubiquitination (35).

The p62 and TRAF6 complex serves as a link to connect the NGF receptors TrkA and $p75^{NTR}$ (64). Our findings reveal that Aβ abrogated the complex formation of TrkA, p62, and TRAF6 with $p75^{NTR}$, and activation of downstream signaling in PC12 cells. These signaling events are also found to be abrogated in AD human hippocampal tissues due to the inability to induce active NGF due to reduction in MMP-7. The increased expression of $p75^{NTR}$ in AD hippocampal samples might be due to increased accumulation of Aβ in AD (Chakravarthy et al., 2012), thereby promoting neuronal cell apoptosis through binding to proneurotrophins such as proNGF which is increased in AD. On the other side, decrease in NGF leads to nitrotyrosylation of TrkA in AD, thereby impairing its phosphorylation, ubiquitination and signaling (Fig. 17). The level of pro-NGF and NGF determines the balance between cell survival and cell death (98). In AD, Aβ accumulation eventually lead to increased proNGF/NGF ratio and excess $p75^{NTR}$, facilitating neuronal cell death. Consistently, we detected less activation of both Akt and MAPK in Aβ-treated PC12 cells

and AD human hippocampal tissues. However, in cultured rat hippocampal neurons, treatment of Aβ (25-35) for 30 min induces about 5 fold higher expression of TrkA and more NGF release along with the activation of the TrkA pathway (99). It seems that these cells strive to excessively activate TrkA signaling against toxic effect resulted from short time exposure to Aβ.

5.6 Figure Legends

Fig. 10. NGF-dependent ubiquitination and autophosphorylation of TrkA was attenuated with Aβ. (A) PC12 cells were treated with 10 μM amyloid β (1-40) for 24 h followed with or without NGF (100 ng/ml) for 10 min at 37℃. Cells were lysed and ubiquitination was examined by immunoprecipitation (IP) with anti-TrkA followed by Western blot with anti-ubiquitin (Ub) and anti-TrkA. (B) Above cell lysates were also immunoprecipitated with anti-TrkA and Western blotted with anti-PY99 and anti-TrkA.

Fig. 11. Interaction of TrkA with $p75^{NTR}$, p62 and TRAF6 was interrupted with Aβ. (A) PC12 cells were stimulated with Aβ and NGF similar to Figure 1. Cell lysates were immunoprecipitated with anti-TrkA and Western blotted with anti-TrkA, anti-p62, and anti-TRAF6. (B) TrkA immunoprecipitates were also Western blotted with anti-$p75^{NTR}$.

Fig. 12. TrkA ubiquitination is reduced in AD human hippocampal tissues. (A) Tissue proteins were prepared from 6 postmortem control aged and 6 AD human hippocampal samples. The homogenates were immunoprecipitated with anti-TrkA, and Western blotted

with anti-Ub and anti-TrkA. (B) as above, TrkA immunoprecipitates were blotted with anti-PY99 (phosphotyrosine), anti-NY (nitrotyrosine) and anti-TrkA.

Fig. 13. Imbalance of proNGF and NGF in AD human hippocampal samples. (A) Control and AD human hippocampal tissue homogenates were Western blotted with anti-proNGF, anti-NGF and anti-MMP-7. (B) Bar graph quantifying the western blot of proNGF, NGF and MMP-7 shown in A. Control was compared to AD human hippocampus samples, $p < 0.001$.

Fig. 14. Interaction between TrkA and $p75^{NTR}$, p62 and TRAF6 were abated in AD human hippocampal tissues. (A) Postmortem control aged and AD human hippocampal tissue homogenates were immunoprecipitated with TrkA and Western blotted with anti-p62, anti-TRAF6 and anti-TrkA. (B) the above TrkA immunoprecipitates were blotted with anti-$p75^{NTR}$ and anti-TrkA.

Fig. 15. Phosphorylation of Akt and MAPK was blocked with Aβ. (A) PC12 cells were stimulated with NGF or amyloid β (1-40) as described before. Cell lysates were separated on SDS-PAGE followed by Western blot with phospho-Akt (S473) and non-phospho-Akt antibodies. (B) Above cell lysates were examined with phospho-MAPK and total MAPK antibodies.

Fig. 16. Phosphorylation of Akt and MAPK was inhibited in AD human hippocampal tissues. (A) Homogenates of postmortem normal aged and AD human hippocampal tissues

were Western blotted with phospho-Akt (S473) and non-phospho-Akt antibodies. (B) Homogenates were also blotted with phospho and non-phospho-MAPK antibodies.

Fig. 17. Schematic representation of NGF signaling in normal and Alzheimer's disease. In normal condition NGF binds to TrkA leading to its phosphorylation, polyubiquitination and complex formation with TRAF6/p62, $p75^{NTR}$ resulting in cell survival and differentiation. In Alzheimer's disease proNGF is accumulated leading to TrkA nitrosylation, thereby impairing its polyubiquitination and interaction with TRAF6/p62, $p75^{NTR}$. The accumulated proNGF binds to $p75^{NTR}$ thereby inducing apoptosis.

Fig. 10

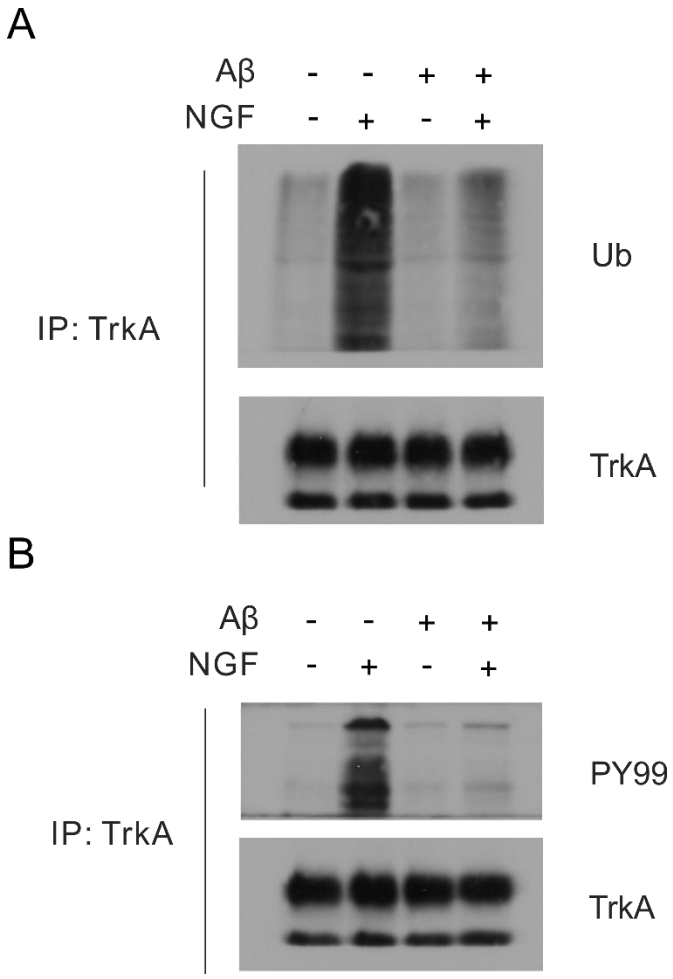
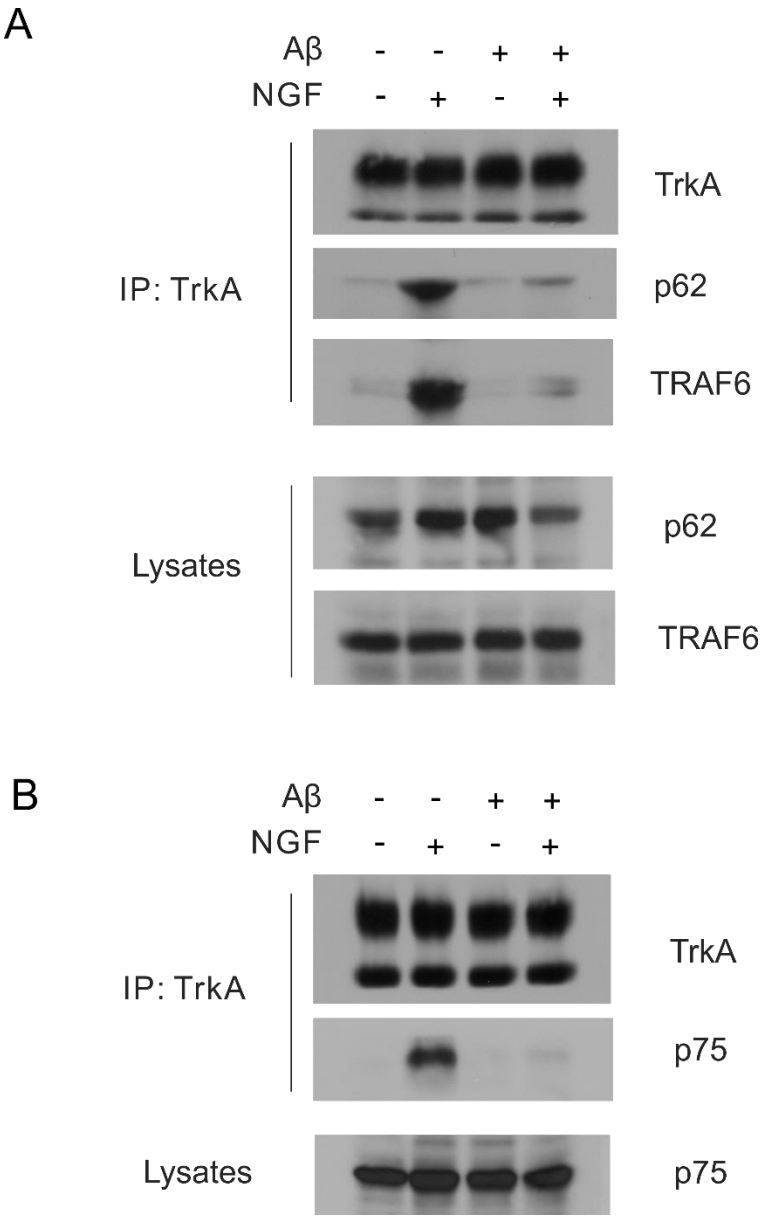


Fig. 11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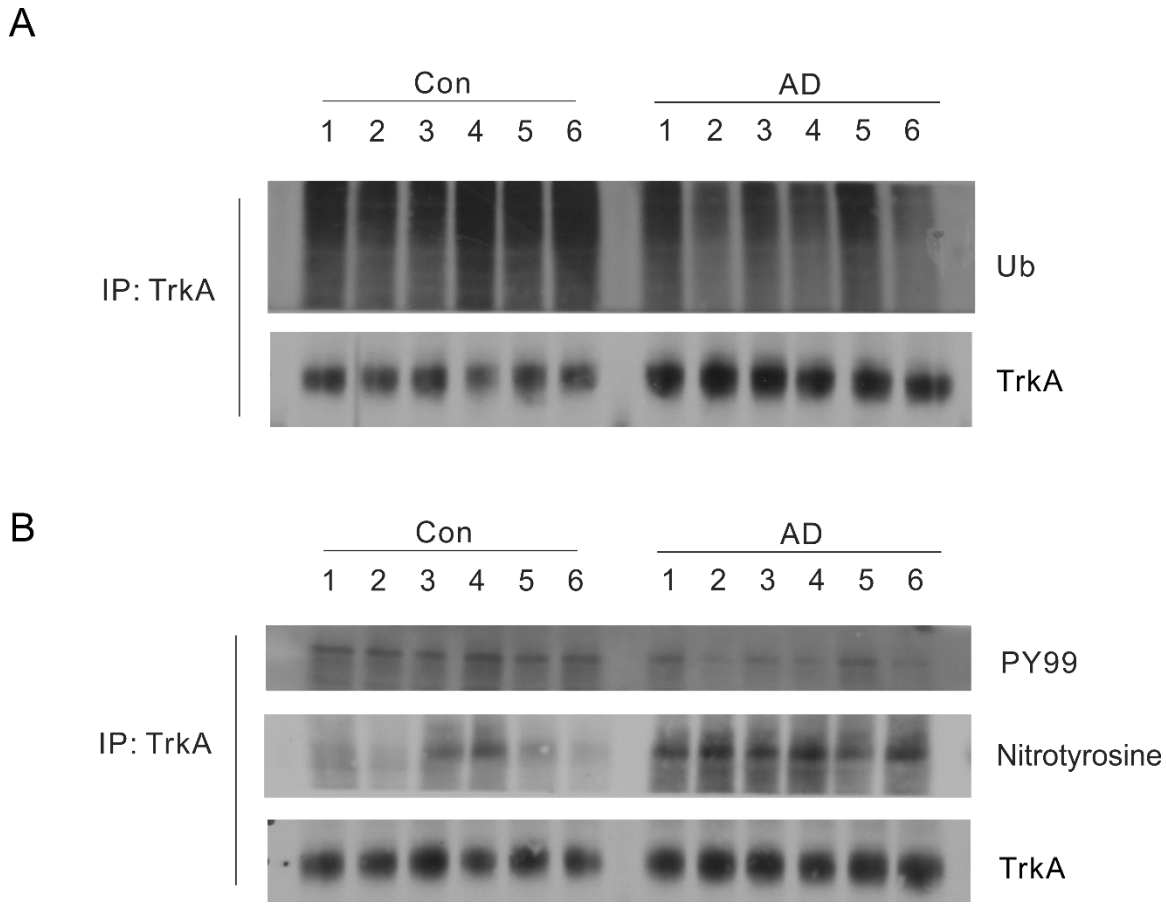
TrkA
p75
Lysates
p75

Fig. 12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TrkA
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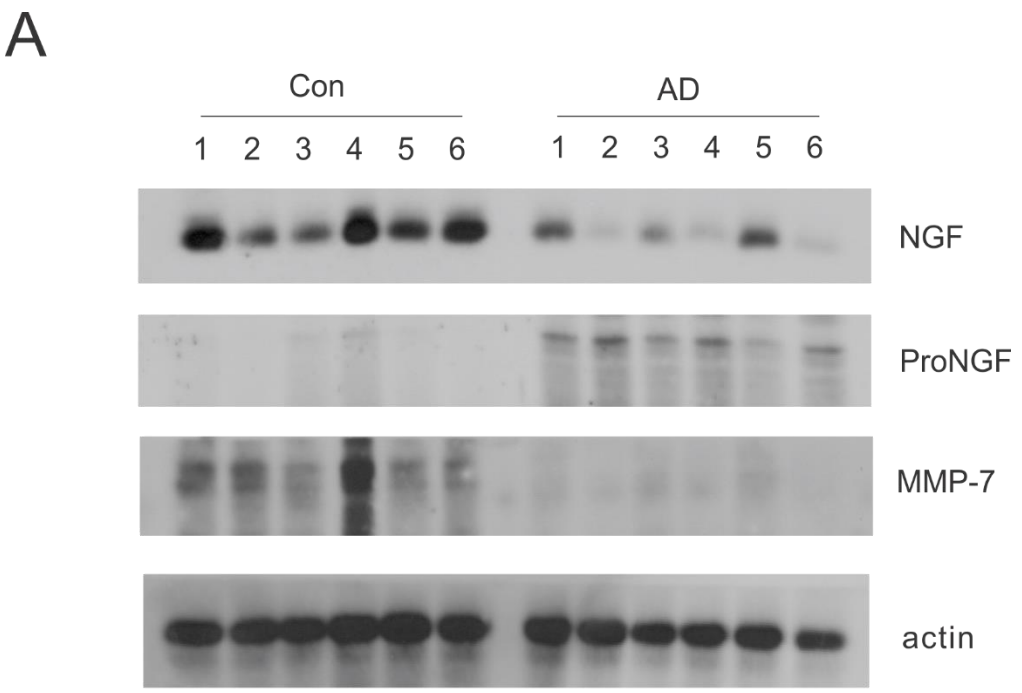
PY99
Nitrotyrosine
TrkA

Fig. 13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ProNGF
MMP-7

Fig. 14

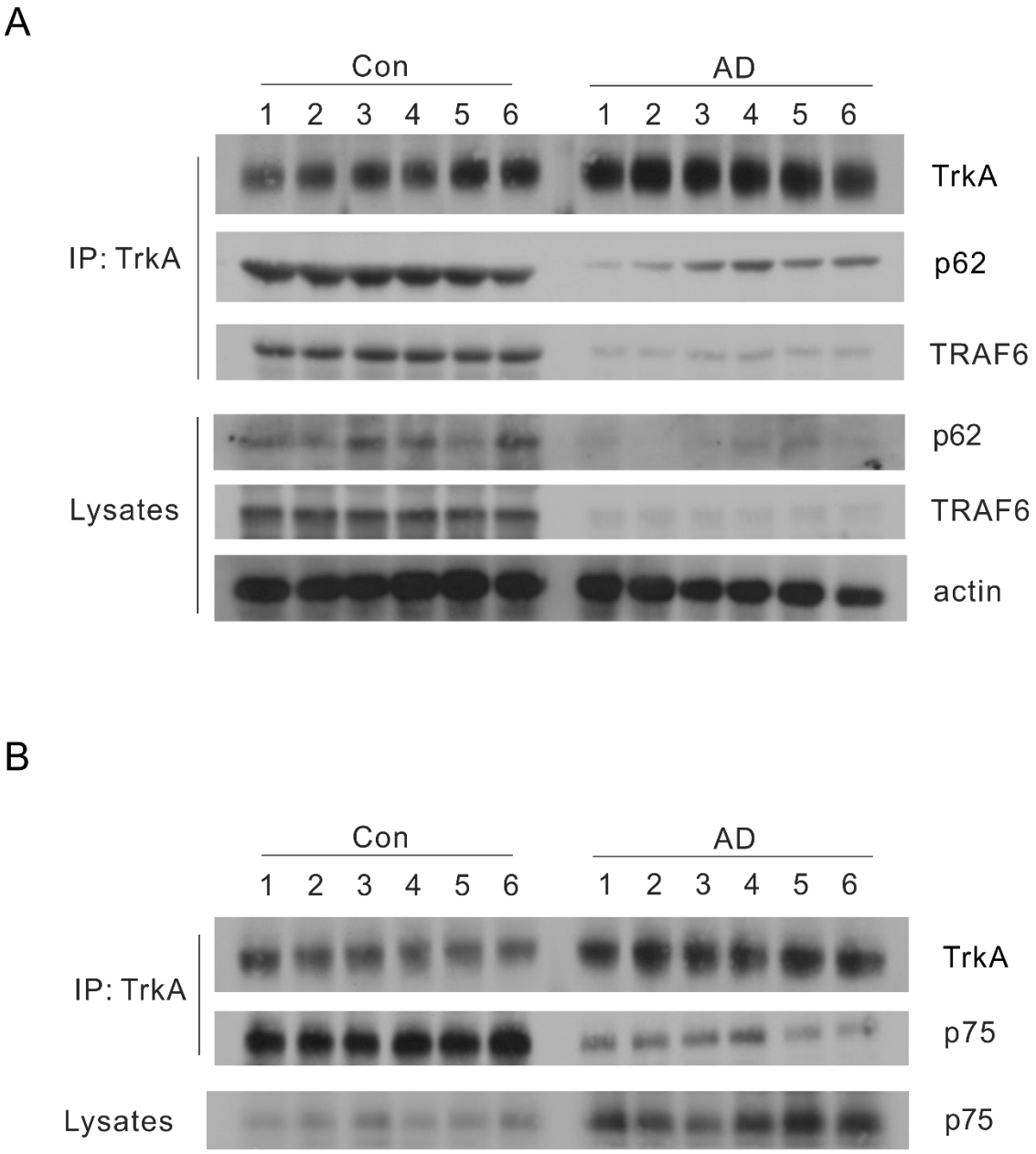
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Fig. 15

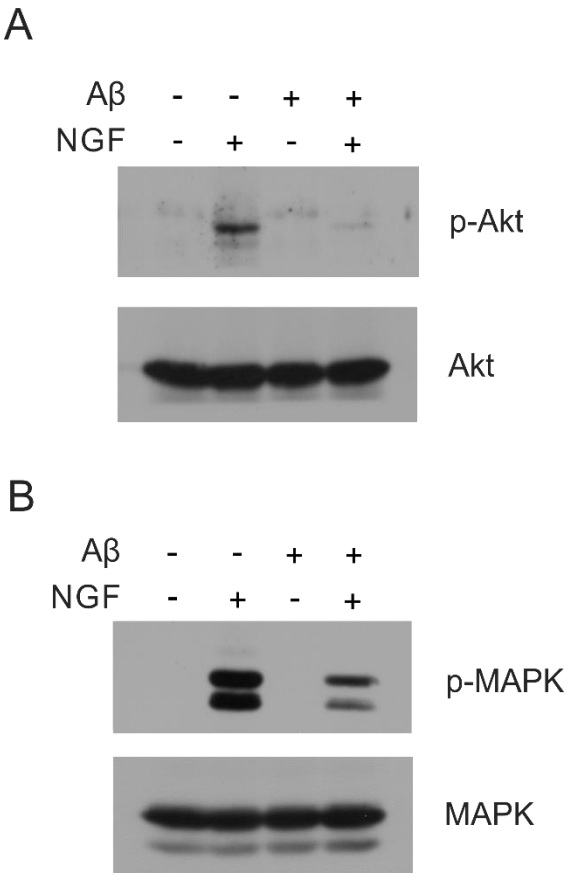
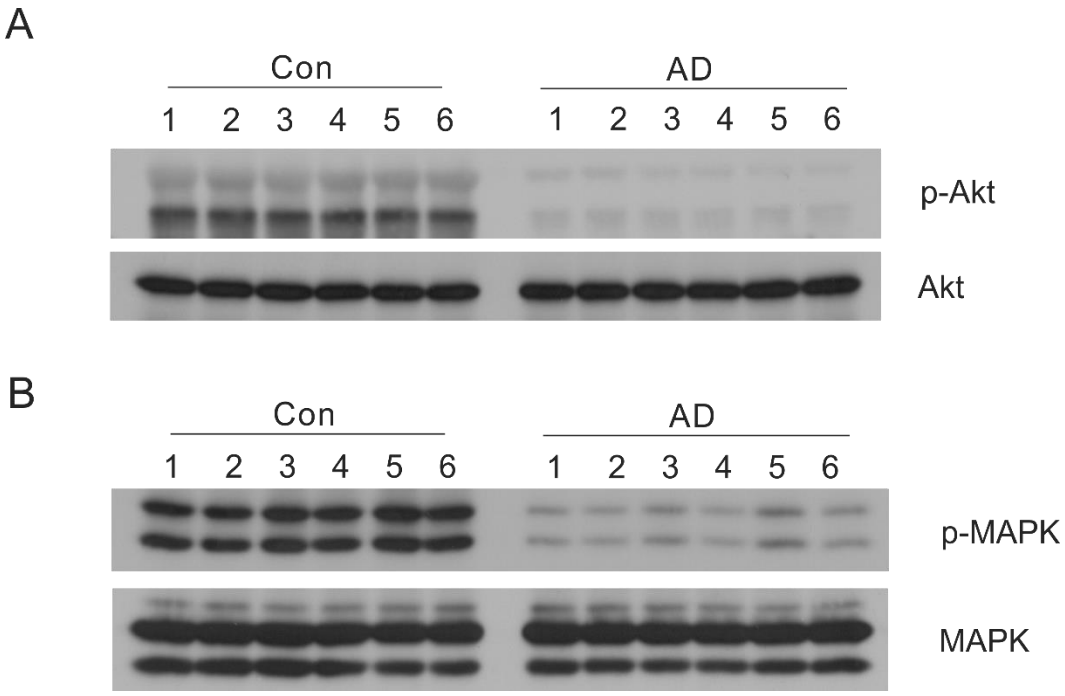


Fig. 16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B
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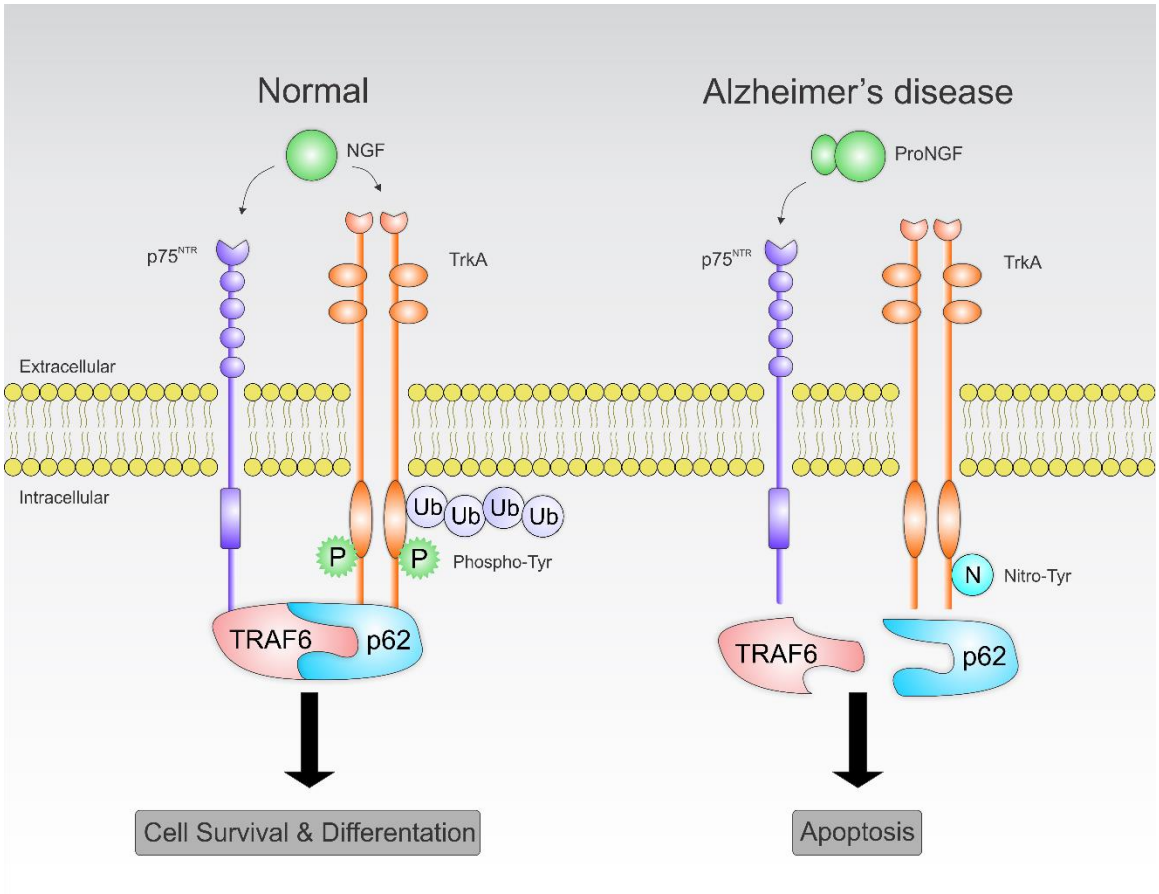
MAPK

Fig. 17


Normal
Alzheimer's disease
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ProNGF
p75NTR
TrkA
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TrkA
Extracellular
Intracellular
Ub
Ub
Ub
Ub
P
P
Phospho-Tyr
N
Nitro-Tyr
TRAF6
p62
TRAF6
p62
Cell Survival & Differentation
Apoptosis

References

1. Levi-Montalcini, R. (1987) The nerve growth factor 35 years later. *Science* **237**, 1154-1162
2. Meakin, S. O., and Shooter, E. M. (1992) The Nerve Growth-Factor Family of Receptors. *Trends Neurosci* **15**, 323-331
3. Greene, L. A., and Kaplan, D. R. (1995) Early events in neurotrophin signalling via Trk and p75 receptors. *Curr Opin Neurobiol* **5**, 579-587
4. Friedman, W. J., and Greene, L. A. (1999) Neurotrophin signaling via Trks and p75. *Exp Cell Res* **253**, 131-142
5. 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
6. Geetha, T., Kenchappa, R. S., Wooten, M. W., and Carter, B. D. (2005) TRAF6-mediated ubiquitination regulates nuclear translocation of NRIF, the p75 receptor interactor. *EMBO J* **24**, 3859-3868
7. Chen, Y., Zeng, J., Cen, L., Wang, X., Yao, G., Wang, W., Qi, W., and Kong, K. (2009) Multiple roles of the p75 neurotrophin receptor in the nervous system. *J Int Med Res* **37**, 281-288
8. 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

9. 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

10. 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

11. Diarra, A., Geetha, T., Potter, P., and Babu, J. R. (2009) Signaling of the neurotrophin receptor p75 in relation to Alzheimer's disease. *Biochem Biophys Res Commun* **390**, 352-356

12. Susen, K., Heumann, R., and Blochl, A. (1999) Nerve growth factor stimulates MAPK via the low affinity receptor p75(LNTR). *FEBS Lett* **463**, 231-234

13. Ohrt, T., Mancini, A., Tamura, T., and Niedenthal, R. (2004) c-Cbl binds to tyrosine-phosphorylated neurotrophin receptor p75 and induces its ubiquitination. *Cell Signal* **16**, 1291-1298

14. Khursigara, G., Orlinick, J. R., and Chao, M. V. (1999) Association of the p75 neurotrophin receptor with TRAF6. *J Biol Chem* **274**, 2597-2600

15. Yeiser, E. C., Rutkoski, N. J., Naito, A., Inoue, J., and Carter, B. D. (2004) Neurotrophin signaling through the p75 receptor is deficient in traf6-/- mice. *J Neurosci* **24**, 10521-10529

16. Salehi, A. H., Roux, P. P., Kubu, C. J., Zeindler, C., Bhakar, A., Tannis, L. L., Verdi, J. M., and Barker, P. A. (2000) NRAGE, a novel MAGE protein, interacts with the

p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis. *Neuron* **27**, 279-288

17. Casademunt, E., Carter, B. D., Benzel, I., Frade, J. M., Dechant, G., and Barde, Y. A. (1999) The zinc finger protein NRIF interacts with the neurotrophin receptor p75(NTR) and participates in programmed cell death. *EMBO J* **18**, 6050-6061
18. Mukai, J., Hachiya, T., Shoji-Hoshino, S., Kimura, M. T., Nadano, D., Suvanto, P., Hanaoka, T., Li, Y., Irie, S., Greene, L. A., and Sato, T. A. (2000) NADE, a p75NTR-associated cell death executor, is involved in signal transduction mediated by the common neurotrophin receptor p75NTR. *J Biol Chem* **275**, 17566-17570
19. Dobrowsky, R. T., Werner, M. H., Castellino, A. M., Chao, M. V., and Hannun, Y. A. (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. *Science* **265**, 1596-1599
20. Dobrowsky, R. T., Jenkins, G. M., and Hannun, Y. A. (1995) Neurotrophins induce sphingomyelin hydrolysis. Modulation by co-expression of p75NTR with Trk receptors. *J Biol Chem* **270**, 22135-22142
21. Kong, H., Kim, A. H., Orlinick, J. R., and Chao, M. V. (1999) A comparison of the cytoplasmic domains of the Fas receptor and the p75 neurotrophin receptor. *Cell Death Differ* **6**, 1133-1142
22. Laney, J. D., and Hochstrasser, M. (1999) Substrate targeting in the ubiquitin system. *Cell* **97**, 427-430
23. Terrell, J., Shih, S., Dunn, R., and Hicke, L. (1998) A function for monoubiquitination in the internalization of a G protein-coupled receptor. *Mol Cell* **1**, 193-202

24. Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003) Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat Cell Biol* **5**, 461-466

25. Mosesson, Y., Shtiegman, K., Katz, M., Zwang, Y., Vereb, G., Szollosi, J., and Yarden, Y. (2003) Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. *J Biol Chem* **278**, 21323-21326

26. Govers, R., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1997) Linkage of the ubiquitin-conjugating system and the endocytic pathway in ligand-induced internalization of the growth hormone receptor. *EMBO J* **16**, 4851-4858

27. Roth, A. F., and Davis, N. G. (1996) Ubiquitination of the yeast a-factor receptor. *J Cell Biol* **134**, 661-674

28. Kolling, R., and Losko, S. (1997) The linker region of the ABC-transporter Ste6 mediates ubiquitination and fast turnover of the protein. *EMBO J* **16**, 2251-2261

29. Peng, J., Schwartz, D., Elias, J. E., Thoreen, C. C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S. P. (2003) A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol* **21**, 921-926

30. Madura, K. (2002) The ubiquitin-associated (UBA) domain: on the path from prudence to prurience. *Cell Cycle* **1**, 235-244

31. Joazeiro, C. A., and Weissman, A. M. (2000) RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **102**, 549-552

32. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z. J. (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique

polyubiquitin chain. *Cell* **103**, 351-361

33. Li, Y., Maher, P., and Schubert, D. (1997) Requirement for cGMP in nerve cell death caused by glutathione depletion. *J Cell Biol* **139**, 1317-1324

34. Suo, Z., Wu, M., Citron, B. A., Palazzo, R. E., and Festoff, B. W. (2003) Rapid tau aggregation and delayed hippocampal neuronal death induced by persistent thrombin signaling. *J Biol Chem* **278**, 37681-37689

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

36. Huber, L. J., and Chao, M. V. (1995) Mesenchymal and neuronal cell expression of the p75 neurotrophin receptor gene occur by different mechanisms. *Dev Biol* **167**, 227-238

37. Wooten, M. W., Vandenplas, M. L., Seibenhener, M. L., Geetha, T., and Diaz-Meco, M. T. (2001) Nerve growth factor stimulates multisite tyrosine phosphorylation and activation of the atypical protein kinase C's via a src kinase pathway. *Mol Cell Biol* **21**, 8414-8427

38. Chao, M. V. (1994) The p75 neurotrophin receptor. *J Neurobiol* **25**, 1373-1385

39. Sisodia, S. S., and Tanzi, R. E. (2007) *Alzheimer's Disease: Advances in Genetics, Molecular and Cellular Biology*, Springer Science + Business Media, New York

40. Selkoe, D. J. (2004) Cell biology of protein misfolding: the examples of Alzheimer's and Parkinson's diseases. *Nat Cell Biol* **6**, 1054-1061

41. Whitfield, J. F. (2007) The road to LOAD: late-onset Alzheimer's disease and a possible way to block it. *Expert Opin Ther Targets* **11**, 1257-1260

42. Deane, R., and Zlokovic, B. V. (2007) Role of the blood-brain barrier in the pathogenesis of Alzheimer's disease. *Curr Alzheimer Res* **4**, 191-197

43. Huang, S. M., Mouri, A., Kokubo, H., Nakajima, R., Suemoto, T., Higuchi, M., Staufenbiel, M., Noda, Y., Yamaguchi, H., Nabeshima, T., Saido, T. C., and Iwata, N. (2006) Neprilysin-sensitive synapse-associated amyloid-beta peptide oligomers impair neuronal plasticity and cognitive function. *J Biol Chem* **281**, 17941-17951

44. Marques, M. A., Kulstad, J. J., Savard, C. E., Green, P. S., Lee, S. P., Craft, S., Watson, G. S., and Cook, D. G. (2009) Peripheral amyloid-beta levels regulate amyloid-beta clearance from the central nervous system. *J Alzheimers Dis* **16**, 325-329

45. Nicoll, J. A., Yamada, M., Frackowiak, J., Mazur-Kolecka, B., and Weller, R. O. (2004) Cerebral amyloid angiopathy plays a direct role in the pathogenesis of Alzheimer's disease. Pro-CAA position statement. *Neurobiol Aging* **25**, 589-597; discussion 603-584

46. Dechant, G., and Barde, Y. A. (2002) The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat Neurosci* **5**, 1131-1136

47. Yaar, M., Zhai, S., Pilch, P. F., Doyle, S. M., Eisenhauer, P. B., Fine, R. E., and Gilchrest, B. A. (1997) Binding of beta-amyloid to the p75 neurotrophin receptor induces apoptosis. A possible mechanism for Alzheimer's disease. *J Clin Invest* **100**, 2333-2340

48. Yaar, M., Zhai, S., Fine, R. E., Eisenhauer, P. B., Arble, B. L., Stewart, K. B., and Gilchrest, B. A. (2002) Amyloid beta binds trimers as well as monomers of the 75-

kDa neurotrophin receptor and activates receptor signaling. *J Biol Chem* **277**, 7720-7725

49. Kuner, P., Schubenel, R., and Hertel, C. (1998) Beta-amyloid binds to p57NTR and activates NFkappaB in human neuroblastoma cells. *J Neurosci Res* **54**, 798-804

50. Coulson, E. J. (2006) Does the p75 neurotrophin receptor mediate Abeta-induced toxicity in Alzheimer's disease? *J Neurochem* **98**, 654-660

51. Yaar, M., Zhai, S., Panova, I., Fine, R. E., Eisenhauer, P. B., Blusztajn, J. K., Lopez-Coviella, I., and Gilchrest, B. A. (2007) A cyclic peptide that binds p75(NTR) protects neurones from beta amyloid (1-40)-induced cell death. *Neuropathol Appl Neurobiol* **33**, 533-543

52. Rabizadeh, S., Bitler, C. M., Butcher, L. L., and Bredesen, D. E. (1994) Expression of the low-affinity nerve growth factor receptor enhances beta-amyloid peptide toxicity. *Proc Natl Acad Sci U S A* **91**, 10703-10706

53. Geetha, T., Zheng, C., Unroe, B., Sycheva, M., Kluess, H., and Babu, J. R. (2012) Polyubiquitination of the neurotrophin receptor p75 directs neuronal cell survival. *Biochem Biophys Res Commun*

54. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**, 346-351

55. Ea, C. K., Sun, L., Inoue, J., and Chen, Z. J. (2004) TIFA activates IkappaB kinase (IKK) by promoting oligomerization and ubiquitination of TRAF6. *Proc Natl Acad Sci U S A* **101**, 15318-15323

56. Sanz, L., Diaz-Meco, M. T., Nakano, H., and Moscat, J. (2000) The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6

pathway. *EMBO J* **19**, 1576-1586

57. Wooten, M. W., Geetha, T., Seibenhener, M. L., Babu, J. R., Diaz-Meco, M. T., and Moscat, J. (2005) The p62 scaffold regulates nerve growth factor-induced NF-kappaB activation by influencing TRAF6 polyubiquitination. *J Biol Chem* **280**, 35625-35629

58. Zhou, X., Babu, J. R., da Silva, S., Shu, Q., Graef, I. A., Oliver, T., Tomoda, T., Tani, T., Wooten, M. W., and Wang, F. (2007) Unc-51-like kinase 1/2-mediated endocytic processes regulate filopodia extension and branching of sensory axons. *Proc Natl Acad Sci U S A* **104**, 5842-5847

59. Babu, J. R., Geetha, T., and Wooten, M. W. (2005) Sequestosome 1/p62 shuttles polyubiquitinated tau for proteasomal degradation. *J Neurochem* **94**, 192-203

60. Bui, N. T., Livolsi, A., Peyron, J. F., and Prehn, J. H. (2001) Activation of nuclear factor kappaB and Bcl-x survival gene expression by nerve growth factor requires tyrosine phosphorylation of IkappaBalpha. *J Cell Biol* **152**, 753-764

61. Arevalo, M. A., Roldan, P. M., Chacon, P. J., and Rodriguez-Tebar, A. (2009) Amyloid beta serves as an NGF-like neurotrophic factor or acts as a NGF antagonist depending on its concentration. *J Neurochem* **111**, 1425-1433

62. Mamidipudi, V., Li, X., and Wooten, M. W. (2002) Identification of interleukin 1 receptor-associated kinase as a conserved component in the p75-neurotrophin receptor activation of nuclear factor-kappa B. *J Biol Chem* **277**, 28010-28018

63. Khursigara, G., Orlinick, J. R., and Chao, M. V. (1999) Association of the p75 neurotrophin receptor with TRAF6. *J Biol Chem* **274**, 2597-2600

64. Wooten, M. W., Seibenhener, M. L., Mamidipudi, V., Diaz-Meco, M. T., Barker, P.

A., and Moscat, J. (2001) The atypical protein kinase C-interacting protein p62 is a scaffold for NF-kappaB activation by nerve growth factor. *J Biol Chem* **276**, 7709-7712

65. Sotthibundhu, A., Sykes, A. M., Fox, B., Underwood, C. K., Thangnipon, W., and Coulson, E. J. (2008) Beta-amyloid(1-42) induces neuronal death through the p75 neurotrophin receptor. *J Neurosci* **28**, 3941-3946

66. Mercurio, E., Pellegrini, M., and Mierke, D. F. (1997) Structure, dynamics, and topological orientation of the polyether, ionophore antibiotic monensin, in a micellar environment. *Biopolymers* **42**, 759-769

67. Karin, M. (1999) The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. *J Biol Chem* **274**, 27339-27342

68. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature* **388**, 548-554

69. Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z., and Rothe, M. (1997) Identification and characterization of an IkappaB kinase. *Cell* **90**, 373-383

70. Foehr, E. D., Lin, X., O'Mahony, A., Geleziunas, R., Bradshaw, R. A., and Greene, W. C. (2000) NF-kappa B signaling promotes both cell survival and neurite process formation in nerve growth factor-stimulated PC12 cells. *J Neurosci* **20**, 7556-7563

71. Geetha, T., Zheng, C., Unroe, B., Sycheva, M., Kluess, H., and Babu, J. R. (2012) Polyubiquitination of the neurotrophin receptor p75 directs neuronal cell survival. *Biochem Biophys Res Commun* **421**, 286-290

72. Selkoe, D. J. (1997) Alzheimer's disease: genotypes, phenotypes, and treatments.

Science **275**, 630-631

73. Lahiri, D. K., and Maloney, B. (2010) Beyond the signaling effect role of amyloid-ss42 on the processing of APP, and its clinical implications. *Exp Neurol* **225**, 51-54

74. Nathalie, P., and Jean-Noel, O. (2008) Processing of amyloid precursor protein and amyloid peptide neurotoxicity. *Curr Alzheimer Res* **5**, 92-99

75. Verdier, Y., and Penke, B. (2004) Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease. *Curr Protein Pept Sci* **5**, 19-31

76. Ibanez, C. F. (1994) Structure-function relationships in the neurotrophin family. *J Neurobiol* **25**, 1349-1361

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

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

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

80. 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

81. 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

82. Loeb, D. M., Maragos, J., Martin-Zanca, D., Chao, M. V., Parada, L. F., and Greene,

L. A. (1991) The trk proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. *Cell* **66**, 961-966

83. 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

84. Peeraully, M. R., Jenkins, J. R., and Trayhurn, P. (2004) NGF gene expression and secretion in white adipose tissue: regulation in 3T3-L1 adipocytes by hormones and inflammatory cytokines. *Am J Physiol Endocrinol Metab* **287**, E331-339

85. 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. *The Journal of international medical research* **37**, 281-288

86. 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

87. 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

88. Geetha, T., Zheng, C., McGregor, W. C., Douglas White, B., Diaz-Meco, M. T., Moscat, J., and Babu, J. R. (2012) TRAF6 and p62 inhibit amyloid beta-induced neuronal death through p75 neurotrophin receptor. *Neurochem Int* **61**, 1289-1293

89. (1997) Consensus recommendations for the postmortem diagnosis of Alzheimer's disease. The National Institute on Aging, and Reagan Institute Working Group on

Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease. *Neurobiol Aging* **18**, S1-2

90. Montine, T. J., Phelps, C. H., Beach, T. G., Bigio, E. H., Cairns, N. J., Dickson, D. W., Duyckaerts, C., Frosch, M. P., Masliah, E., Mirra, S. S., Nelson, P. T., Schneider, J. A., Thal, D. R., Trojanowski, J. Q., Vinters, H. V., Hyman, B. T., National Institute on, A., and Alzheimer's, A. (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

91. Casaccia-Bonnefil, P., Gu, C., Khursigara, G., and Chao, M. V. (1999) p75 neurotrophin receptor as a modulator of survival and death decisions. *Microsc Res Tech* **45**, 217-224

92. Barker, P. A. (2004) p75NTR is positively promiscuous: novel partners and new insights. *Neuron* **42**, 529-533

93. 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

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

95. Chao, M. V. (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat Rev Neurosci* **4**, 299-309

96. Segal, R. A. (2003) Selectivity in neurotrophin signaling: theme and variations. *Annual review of neuroscience* **26**, 299-330

97. Chen, Z. J. (2005) Ubiquitin signalling in the NF-kappaB pathway. *Nat Cell Biol* **7**, 758-765

98. Chao, M. V., and Bothwell, M. (2002) Neurotrophins: to cleave or not to cleave. *Neuron* **33**, 9-12

99. Bulbarelli, A., Lonati, E., Cazzaniga, E., Re, F., Sesana, S., Barisani, D., Sancini, G., Mutoh, T., and Masserini, M. (2009) TrkA pathway activation induced by amyloid-beta (Abeta). *Mol Cell Neurosci* **40**, 365-373

CHAPTER 6: SUMMARY AND CONCLUSION

NGF promotes neuronal survival and differentiation through binding to its receptors $p75^{NTR}$ and TrkA and subsequently activating downstream signaling cascades. Impairment of NGF signaling activation has been shown in AD. The emerging evidence suggests that polyubiquitination participates in activation of signaling events.

Our findings reveal that ubiquitination plays an important role in NGF signaling activation. NGF can induce the $p75^{NTR}$ polyubiquitination, regulating neuronal cell survival. TrkA receptor is also polyubiquitinated upon NGF stimulation. Both of these ubiquitination are catalyzed by E3 ligase TRAF6 with scaffold protein p62, which are required for signaling transduction. Furthermore, Aβ is capable of activation of several signaling pathways involved in neuronal damage observed in the AD. Our findings reveal that Aβ abrogates the tyrosine phosphorylation and polyubiquitination of TrkA induced by NGF in PC12 cells as well as human hippocampal tissues.

The TRAF6/p62 complex serves as a link to connect the NGF receptors TrkA and $p75^{NTR}$. Our findings reveal that Aβ spontaneously blocked the complex formation of TrkA, p62, and TRAF6 with $p75^{NTR}$, and activation of downstream signaling *in vitro*. These signaling events are also found to be abrogated in AD human hippocampal tissues due to the inability to induce active NGF due to reduction in MMP-7. Furthermore, we observed that decrease in NGF leads to nitrotyrosylation of TrkA in AD, thereby impairing its phosphorylation, ubiquitination and signaling. As a result of MMP-7 reduciton, increased proNGF/NGF ratio and excess $p75^{NTR}$ facilitating neuronal cell death, which is consistent with less

activation of both Akt and MAPK in Aβ-treated *in vitro* and AD human hippocampal tissues. Taken together, all of these evidence suggest the importance of ubiquitination in NGF signaling and provide better understanding to AD pathological process at molecular level.