# BIOLOGICAL ACTIVITY OF A NEUROTROPHIN PRECURSOR AND MECHANISM OF NEUROTROPHIN DYSREGULATION IN NEURODEGENERATIVE DISEASES

# BIOLOGICAL ACTIVITY OF A NEUROTROPHIN PRECURSOR AND MECHANISM OF NEUROTROPHIN DYSREGULATION IN NEURODEGENERATIVE DISEASES

BY

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**ABSTRACT:**

Neurotrophins, such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), are key factors in neuronal survival and function. In Alzheimer's disease (AD), there is a change in the normal level of these neurotrophins and their precursors (proNGF and proBDNF). The mechanism/s underlying changes in the levels of these neurotrophins in AD is not fully understood.

According to the amyloid cascade hypothesis, amyloid-β is the original insult in AD and tau pathology is a downstream event. Amyloid-β interferes with axonal transport and reduces BDNF levels. However, it is not clear if amyloid-β affects neurotrophin levels directly or through tau hyperphosphorylation.

If tau is responsible for changes in the level of neurotrophins in AD, we expect to observe the same alteration in neurotrophin levels in other diseases with tau dysfunction such as tauopathies. We tested the levels of BDNF mRNA and proNGF protein in subjects with tauopathies. We observed significant decrease in BDNF mRNA levels in subjects with Corticobasal degeneration. Our result suggests that BDNF may be down-regulated by tau hyperphosphorylation.

Moreover, we showed that there was a significant increase in the level of proNGF in Pick's disease (PiD). Interestingly, AD and PiD share common tau modifications. Our result demonstrates a role for tau dysfunction in changes in the level of proNGF. Therefore, study of the levels of NGF and BDNF in non-AD tauopathies has shed light on the mechanisms underlying neurotrophin dysregulation in AD.

How do increased levels of proNGF impact the brain in AD or PiD? Is neuronal degeneration in AD or PiD due to the lack of neurotrophic support of proNGF or do increased levels of apoptotic proNGF cause neurodegeneration? Lee et al. (2001b) and Fahnestock et al. (2004a) produced two different cleavage-resistant proNGFs with opposite activities (apoptotic versus neurotrophic). Structural and procedural differences between the two cleavage-

resistant proNGFs and different bioassays can cause opposite activities. We showed that proNGF from Lee's lab was neurotrophic when it was expressed in the expression system used by Fahnestock et al. or when it was purified. ProNGF expressed in a different expression system was also neurotrophic. ProNGF was neurotrophic in all bioassays except the serum withdrawal assay. We conclude that proNGF is normally neurotrophic but may be apoptotic when cell survival is already compromised. We propose that in AD, cells undergo degeneration due to the lack of neurotrophic support of proNGF (impaired transport). Moreover, TrkA is downregulated in AD which compromises cell survival and may lead to apoptosis induced by increased levels of proNGF.

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## ABBREVIATIONS:

| | |
|---|---|
| 3R-tau | 3 repeat tau |
| 4R-tau | 4 repeat tau |
| ACh | Acetylcholine |
| AD | Alzheimer's disease |
| ADDLs | Amyloid-beta derived diffusible ligands |
| ALS | Amyotrophic lateral sclerosis |
| ApoE4 | Apolipoprotein E4 |
| APP | Amyloid precursor protein |
| Aβ | Amyloid-beta peptide |
| BAD | Bcl-XL/Bcl-2-associated death promoter |
| BDA | Biotinylated dextran amine |
| BDNF | Brain derived neurotrophic factor |
| BFCNs | Basal forebrain cholinergic neurons |
| BSA | Bovine serum albumin |
| CBD | Corticobasal degeneration |
| CBS | Corticobasal syndrome |
| cDNA | Complementary deoxyribonucleic acid |
| ChAT | Choline acetyl transferase |
| C-terminus | Carboxy terminus |
| cdk5 | Cyclin-dependent kinase-5 |
| CNS | Central nervous system |
| CREB | Cyclic AMP response element binding protein |
| dNTPs | Deoxynucleotide triphosphates |
| E.coli | Escherichia coli |
| EDTA | Ethylene diamine tetra acetic acid |
| EGTA | Ethylene glycol-bis (b-aminoethyl ether) N,N,N',N'-tetraacetic acid |
| Erk | External signal-regulated kinase |
| FI | Furin inhibitor |
| FTDP-17 | Frontotemporal dementia and parkinsonism linked to chromosome 17 |
| GSK-3β | Glycogen synthase kinase-3β |
| HEK | Human embryonic kidney |
| HRP | Horseradish proxidase |
| JNK1 | C-Jun N-terminal kinase |
| LB | Luria Bertani |
| LTP | Long term potentiation |
| MAP | Microtubule-associated protein |
| MAPK | Mitogen-activated protein kinase |
| MMP-7 | Matrix metalloprotease-7 |
| mRNA | messenger ribonucleic acid |
| MT | Microtubule |
| NBM | Nucleus basalis of Meynert |

| | |
|---|---|
| NGF | Nerve growth factor |
| NT-3 | Neurotrophin-3 |
| NT-4/5 | Neurotrophin-4/5 |
| NFTs | Neurofibrillary tangles |
| NF-KB | Nuclear factor kappa B |
| N-terminus | Amino terminus |
| NTR | Neurotrophin Receptor |
| PBS | Phosphate buffered saline |
| PC12 | Pheochromocytoma |
| PD | Parkinson's disease |
| Pen/Strep | Penicillin /Streptomycin |
| PFA | Paraformaldehyde |
| PFU | Plaque forming units |
| PHFs | Paired helical filaments |
| PI-3 kinase | Phosphatidylinositol- 3-kinase |
| PiB | Pick's bodies |
| PiD | Pick's disease |
| PKA | Protein kinase A |
| PLC | Phospholipase C |
| PMSF | Phenylmethylsulphonyl fluoride |
| proBDNF | pro brain derived neurotrophic factor |
| proNGF | pro nerve growth factor |
| PrP | Protease-resistant prion protein |
| PSP | Progressive supranuclear palsy |
| PVDF | Polyvinylidene fluoride |
| qPCR | Quantitative real-time polymerase chain reaction |
| rRNA | Ribosomal ribonucleic acid |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SCG | Superior cervical ganglion |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| siRNA | Small interfering RNA |
| SOD1 | Superoxide-dismutase-1 |
| SP | Senile plaques |
| Tg | Transgenic |
| Trk | Tropomyosin related kinase |
| TUNEL | Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling |
| WT | Wild type |

# CHAPTER 1:
# INTRODUCTION:

## *1.1: Neurodegenerative diseases:*

Nearly all neurodegenerative diseases manifest clinically in late life. As life expectancy has increased in developed countries, neurodegenerative diseases have become more common and they account for a significant and increasing number of mortalities. Therefore, more studies are needed to elucidate the mechanisms involved in these diseases in order to develop essential and constructive therapeutic approaches.

Most, if not all, age-related neurodegenerative diseases share a common pathological lesion which is aggregation and deposition of misfolded proteins in different areas of the central nervous system (CNS). Although most cases of neurodegenerative diseases are sporadic, there are some cases affected by mutations in the specific genes leading to protein aggregation and deposition. These proteins form insoluble filamentous inclusions which interfere with the normal function of neurons leading to neuronal degeneration (Skovronsky et al., 2006).

Depending on the type of aggregated protein and which area of the CNS is involved, different clinical manifestations and complications such as progressive cognitive and/or motor impairment are expected. For instance, in Alzheimer's disease (AD), aggregation of amyloid-β and hyperphosphorylated tau in cortical and subcortical areas of the brain causes dementia. In Parkinson's disease (PD), α-synuclein aggregation in the substantia nigra leads to movement dysfunction. Table 1.1 summarizes a list of common neurodegenerative diseases involving aggregation of specific proteins (Skovronsky et al., 2006).

| Disease | Microscopic lesion | Location | Aggregated protein |
|---|---|---|---|
| Alzheimer's disease | Amyloid plaque<br>Neurofibrillary tangle Lewy bodies (seen in Lewy body variant) | Extracellular<br>Intracytoplasmic (neurons)<br>Intracytoplasmic (neurons) | Amyloid-β<br>Tau<br>α-synuclein |
| Amyotrophic lateral sclerosis | Hyaline inclusions | Intracytoplasmic (neurons) | Superoxide dismutase-1 (SOD1) |
| Corticobasal degeneration | Tau positive inclusions | Intracytoplasmic (neurons, oligodendroglia and astrocytes) | Tau |
| Progressive supranuclear palsy | Tau positive inclusions | Intracytoplasmic (neurons, oligodendroglia and astrocytes) | Tau |
| Dementia with Lewy bodies | Lewy bodies | Intracytoplasmic (neurons) | α-synuclein |
| Huntington's disease | Neuronal inclusions | Intranuclear (neurons) | Huntingtin (containing Polyglutamine repeat expansion) |
| Multiple system atrophy | Glial cytoplasmic Inclusions | Intracytoplasmic (oligodendroglia) | α-synuclein |
| Parkinson's disease | Lewy bodies | Intracytoplasmic (neurons) | α-synuclein |
| Pick's disease | Tau positive inclusions | Intracytoplasmic (neurons) | Tau |

| Prion diseases | Prion plaques | Extracellular | Protease-resistant prion protein (PrP) |
|---|---|---|---|
| Spinocerebellar ataxia | Neuronal inclusions | Intranuclear (neurons) | Ataxin (containing polyglutamine repeat expansion) |

**Table 1.1: Summary of common neurodegenerative diseases involving deposition of aggregated proteins** (Skovronsky et al., 2006).

### *1.2. Tauopathies:*

As can be seen from table 1.1, among these neurodegenerative disorders, some share a common aggregated protein. For example, tau dysfunction and aggregation is the pathological hallmark of a group of neurodegenerative diseases termed tauopathies. Some tauopathies with well-defined tau pathology are Alzheimer's disease, Pick's disease (PiD), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD). All of these disorders share the common feature of intracellular deposition of tau in neurons or glial cells followed by neurofibrillary tangle (NFT) formation in the brain. As AD is the only tauopathy with both tau dysfunction and amyloid-β pathology, tauopathies other than AD are called non-AD tauopathies.

Tauopathies are very heterogeneous. They may lead to dementia with cognitive impairment and behavioral abnormalities and/or cause movement disorders probably depending on the different areas of the brain affected by the tau inclusions. AD is the most common form of degenerative dementia and there are an increasing number of people diagnosed with AD each year (Hebert et al., 2003). Due to these facts, AD is the most well studied tauopathy compared to non-AD tauopathies which are rare. There are a large number of studies and investigations carried out on AD pathology and therapeutic approaches, and major progress has been made in this regard, but there is still a lot to be discovered.

Although there are various clinical manifestations for different tauopathies, the many links between PSP, CBD, PiD and AD, such as aggregation of hyperphosphorylated tau, suggest that a breakthrough in one disorder may provide important insights into the other disorders. Studies of non-AD tauopathies may shed light on the mechanism involved in pathological events in AD related to tau dysfunction.

### *1.3. Tau:*

Tau is a microtubule-associated protein (MAP) that is associated with both axonal and dendritic microtubules in neurons and is found in astrocytes and oligodendrocytes as well (reviewed by Yoshida, 2006). The human tau gene is located on chromosome 17q21 and includes 16 exons. Through alternative mRNA splicing of exons 2, 3, and 10, six tau isoforms are generated in the CNS. The acidic amino-terminus (N-terminus) of tau protein is followed by a basic proline-rich region and a highly conserved motif of tubulin binding domain repeats at the carboxy-terminus (C-terminus) (reviewed in Ballatore et al., 2007). Three different isoforms (0N, 1N, and 2N) are formed due to the presence or absence of a 29 amino acid region at the N-terminus of the tau molecule. Each of these isoforms is divided into two types depending on the number of tubulin binding repeats [3 repeats (3R-tau) or 4 repeats (4R-tau)] at the C-terminus of the tau protein (Figure 1.1)

Tau is involved in assembly (Hirokawa, 1994, Mandelkow and Mandelkow, 1995) and organization of the microtubule (MT) cytoskeleton (Barlow et al., 1994, Matus, 1994). There are two ways by which tau controls MT polymerization and stability: 1.) through tau phosphorylation; and 2.) via a ratio of tau isoforms.

1.) Phosphorylation is one of the most important post translational modifications of tau, as it regulates the ability of tau to bind to MTs (Lindwall and Cole, 1984). The regions of tau which flank the tubulin-binding domains have several phosphorylation sites on serine and threonine residues. An increase in tau phosphorylation leads to tau detachment from MTs and causes MT depolymerization and instability. Moreover, hyperphosphorylated tau is prone to aggregation and formation of large tangles of filaments.

2.) 3R-tau is coded by isoforms lacking exon 10 while 4R-tau is produced by isoforms including exon 10. 4R-tau isoforms stabilize MTs better. In normal brain, levels of 3R-tau and 4R-tau are equal. However, in tauopathies this ratio is altered (either more 3R or 4R) (reviewed by Williams, 2006). A change in the

ratio of these isoforms (more 3R-tau) increases the proportion of unbound tau available for aggregation (reviewed by Tsuboi, 2006).

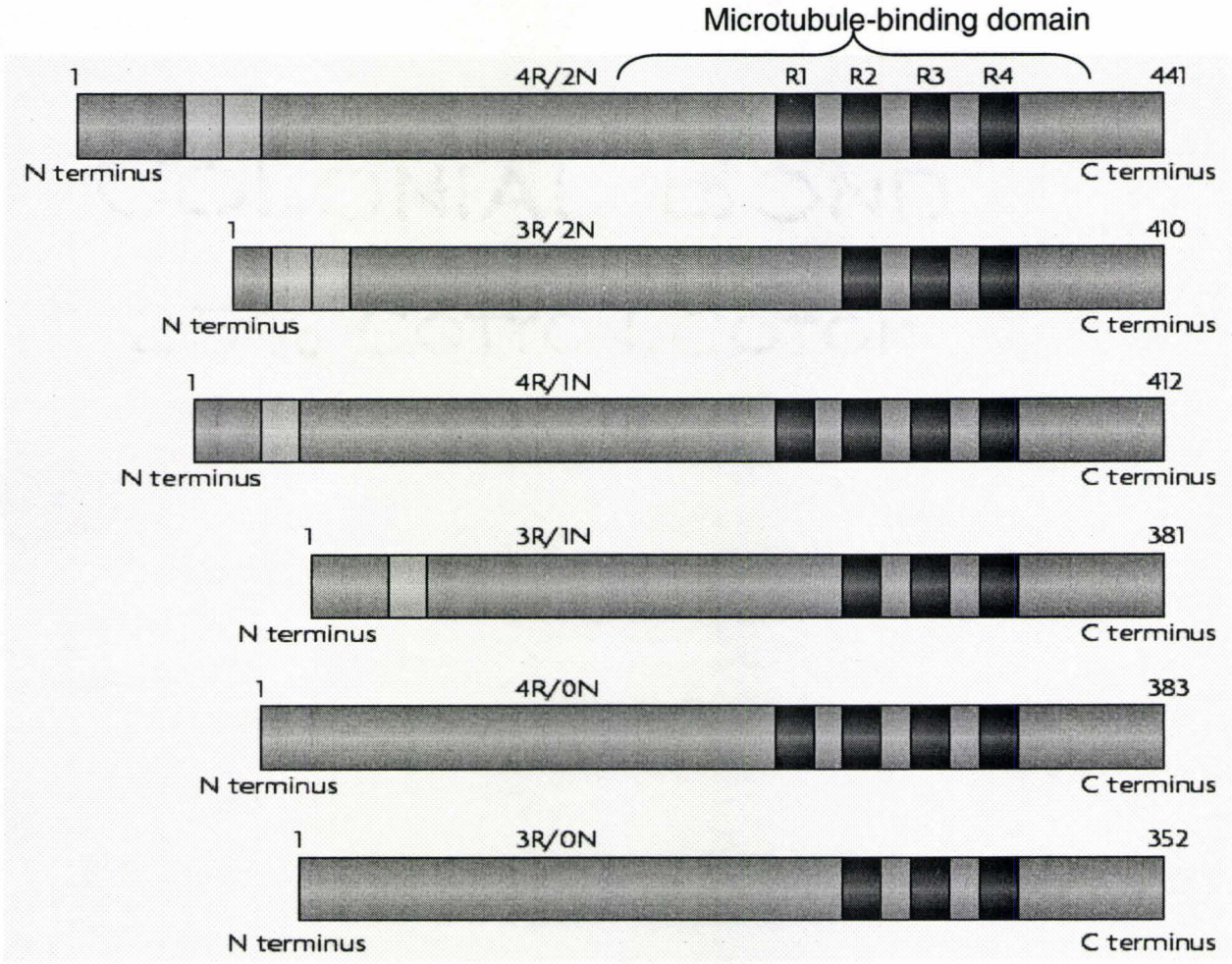



**Figure 1.1: six different tau isoforms are produced in the central nervous system through alternative mRNA splicing** (Ballatore et al., *Nature Reviews Neuroscience,* 2007).

Ample evidence demonstrates that mutations in the tau gene are sufficient to cause a dementia termed Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), and prove the role of tau protein in tauopathies (Spillantini et al., 1998, Poorkaj et al., 1998, Hutton et al., 1998). More than 40 different mutations have been identified in the tau gene. Most mutations that

occur in the coding area of the tau gene are in the MT binding region of this gene. These mutations affect the ability of tau to bind to MTs. Some mutations change the phosphorylation state of tau by enhancing tau phosphorylation or reducing tau dephosphorylation. These conditions may disable tau from binding to MTs. Splicing of exon 10 is affected by mutations occurring in non-coding regions (Lee et al., 2001c) which alter the ratio of 3R-tau and 4R-tau. Some other mutations make tau molecules prone to aggregation. Therefore, different type of tau pathology may be observed depending on where mutations occur on the gene.

Tau dysfunction and aggregation in tauopathies can contribute to neuronal degeneration either by loss of normal tau function (leading to dysfunctional cytoskeleton) or by gain of pathological function [(blocking cellular transport by neurofibrillary tangles (NFTs)]. Both mechanisms are thought to affect axonal transport (retrograde and anterograde) in neurons.

***1.4. Non-AD tauopathies:***

There are several diseases exhibiting tau dysfunction that do not show the amyloid-β deposition seen in AD. Depending on the regional distribution of tau aggregation, clinical symptoms may vary in different tauopathies. Most of them lead to dementia while some show more motor dysfunction. Although all tauopathies are recognized via tau aggregation in different areas of CNS, the aggregation has its own morphological and biochemical signature in each tauopathy. Therefore, various tauopathies are categorized according to the type of tau inclusion and also the area of the brain where aggregation occurs. Figure 1.2 shows various types of tau inclusions in different tauopathies.

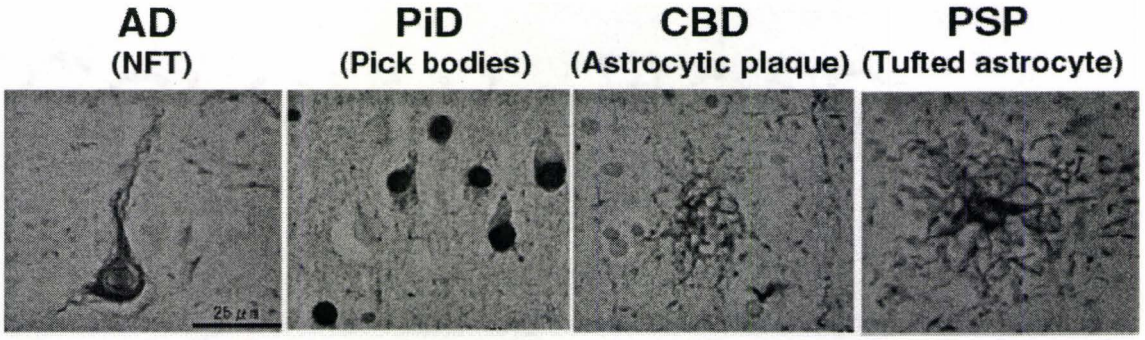



**Figure1.2. Each tauopathy shows a specific type of tau aggregation** (Hasegawa, *Neuropathology*, 2006).

### *1.4.1. Pick's Disease (PiD):*

Pick's disease was first described in 1892 by Arnold Pick and for many years it was believed that this disease was a form of AD. The age of onset is 60-65. PiD, AD and senile dementia are together considered as Alzheimer-type dementia as they share common behavioral impairment and they show similarity in both macroscopic and microscopic pathology (reviewed in Marcinkowski, 1996).

In terms of clinical features, PiD accounts for 0.2-4% of dementia which makes it 3-5 times less common compared to AD (reviewed in Frederick, 2006). Dementia in PiD is associated with changes in personality and language disturbances in the early stages of the disease which is less common in AD. While there may be impairment in executive functions such as organization and planning, in PiD there is no change in visual-spatial functions (reviewed in Frederick, 2006).

Focal frontotemporal atrophy is the macroscopic hallmark of PiD. This atrophy is asymmetric (Figure 1.3) in 60% of cases and the left hemisphere is more affected than the right hemisphere (reviewed in Frederick, 2006). Parietal cortex has also been reported to be affected in some cases of PiD (reviewed by Tsuchiya et al., 2001). Other affected areas include the entorhinal cortex,

pyramidal cells of the hippocampus, and granule cells of the dentate gyrus (reviewed in Yoshida, 2006).

Pick's cells and Pick's bodies (PiB) are microscopic pathological hallmarks of PiD. Pick's cells are large, swollen, ballooned neurons. These cells are argyrophilic, with vacuolated cytoplasm and no Nissl substance. PiB are cytoplasmic spherical inclusions in neurons, and these inclusions are positive for tau immunoreactivity (reviewed in Frederick, 2006). It has been shown that 3R-tau is the predominant isoform of tau in the brain of PiD patients (reviewed in Janus, 2008). Compared to PSP and CBD, tau immunoreactivity in astrocytes and oligodendroglia in PiD has been less characterized (reviewed by Yoshida, 2006).

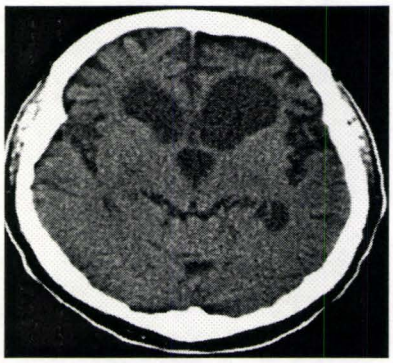


**Figure 1.3: Focal atrophy of frontotemporal lobes in PiD which is greater in the left hemisphere** (on the viewer's right) (reviewed in Frederick, *Arch Pathol Lab Med,* 2006).

### *1.4.2. Progressive Supranuclear Palsy (PSP):*

A progressive symmetric akinetic-rigid syndrome with prominent supranuclear gaze palsy and gait impairment was described in some patients by Steele et al. and was called PSP (Steele et al., 1964). Loss of balance and falling are the first symptoms of PSP together with personality changes and movement impairment, while dementia is among one of the later symptoms of this disease. Poor learning but not recall impairment have been reportedly observed in PSP

(reviewed in Boeve, 2007). A specific genetic polymorphism (H1 haplotype) in the tau gene is highly associated with PSP (Pittman et al., 2005).

Some suspected PSP patients show pathological symptoms other than those mentioned above. They are called progressive supranuclear palsy syndrome which includes PSP. Frontosubcortical gray and white matter undergo the most atrophy in this group of disorders (reviewed in Boeve, 2007). Basal ganglia, midbrain of the brain stem (where supra nuclear eye movement is located), the dentate nucleus of the cerebellum, cerebral cortex and spinal cord are involved in PSP.

Neurofibrillar inclusions in PSP are globose type and are associated with widespread neuronal degeneration. A concentric arrangement of fine branching fibers in tuft-shaped astrocytes is a distinctive feature of PSP (reviewed in Yoshida, 2006 and Williams, 2006). 4R-tau is the predominant isoform of tau in PSP brain (reviewed in Janus, 2008).

***1.4.3. Corticobasal degeneration (CBD):***

In 1967, a progressive asymmetric akinetic-rigid syndrome with apraxia was described by Rebeiz et al., (1967) which was later termed corticobasal degeneration (CBD). Asymmetric motor dysfunction is the first symptom of CBD. This disease can cause some cognitive dysfunction at later stages of the disease. There is a highly significant association of H1 haplotype of the tau gene with CBD (Pittman et al., 2005). Parietofrontal cortex degeneration in a focal or asymmetric manner has been reported in a group of diseases called corticobasal syndrome (CBS) which includes CBD. CBD is associated with atrophy and neuronal loss in cerebral cortex and subcortical area such as the striatum and substantia nigra (Boeve, 2007).

A microscopic hallmark of CBD is the presence of a corona-like arrangement and fuzzy short processes of tau aggregates in astrocytic plaques. The presence of ballooned neurons is a distinctive feature of CBD which is similar to Pick cells in PiD (reviewed in Yoshida, 2006 and Williams, 2006). CBD,

similar to PSP, is a 4R-tauopathy in which 4R-tau is predominant (reviewed in Janus 2008).

### *1.5. Alzheimer's disease:*

Alois Alzheimer, in 1906, described a dementia which was associated with senile plaques (SP) and NFTs in the brain. Later, it was demonstrated that amyloid-β is the main component of SP (Glenner and Wong, 1984) and tau is the major component of NFT (Grundke-Iqbal et al., 1986). There is progressive extracellular deposition of amyloid-β peptide (Aβ) in senile plaques (Whitehouse et al., 1982). Degeneration of the cholinergic neurons in basal forebrain is another major hallmark of AD (reviewed in Auld et al., 2002).

#### *1.5.1. Amyloid-β deposition:*

Aβ (a peptide of 39-43 residues) is produced by β and γ secretase cleavage of the amyloid precursor protein (APP) (Seubert et al., 1993, Selkoe and Wolfe, 2000). APP is a transmembrane glycoprotein and its gene is located on chromosome 21. There are two pathways involved in APP processing: the non-amyloidogenic and amyloidogenic pathways. If APP is processed by α-secretase which cuts APP within the amyloid-β domain, formation of Aβ is prevented and soluble APP is produced. This pathway is called the non-amyloidogenic pathway. The amyloidogenic pathway leads to amyloid-β formation by β and γ secretase cleavage (reviewed in Schindowski et al., 2008). Aβ accumulation and deposition have been reported to play a significant role in AD pathology. Although the mechanism by which Aβ contributes to AD pathology is not clearly known, free radical generation, oxidative stress, inflammation and neurotrophin deprivation may account for Aβ toxicity in AD (reviewed in Schindowski et al., 2008, Garzon and Fahnestock, 2007).

#### *1.5.2. Neurofibrillary tangles:*

Intracellular NFTs contain paired-helical filaments (PHFs) of abnormally phosphorylated tau in the brain of AD patients. Hyperphosphorylated tau is observed along neurites as neuropil threads which later progress to the soma. In

some types of tauopathies such as PSP and CBD, tau dysfunction can be observed in non-neuronal cells such as astrocytes, as well. Tau hyperphosphorylation can occur long before amyloid-β deposition in AD. In fact, normal aging is also associated with hyperphosphorylated tau which is present from age 75. Tau pathology starts from the transentorhinal cortex and expands to entorhinal cortex and hippocampus (Braak & Braak, 1991, Delacourte et al., 2002). Amyloid-β deposition may occur at this stage. Later, basal forebrain and other areas of the cortex will be involved. If both tau aggregation and amyloid-β accumulation are present, the disease is considered Alzheimer's disease.

***1.5.3. Basal forebrain cholinergic neuron degeneration:***

Another important pathological event in AD is neuronal degeneration and loss of function in specific regions of the brain. Cerebral cortex is the predominant area affected by AD. However, subcortical areas, such as the basal forebrain, are also involved in this neurodegenerative disease. Basal forebrain cholinergic neurons (BFCN) innervate several regions of the brain such as hippocampus and cortex to provide cholinergic support for those areas. Meanwhile, the hippocampus and cortex provide neurotrophic support for BFCN via retrograde transport (reviewed in Salehi et al., 2004). Loss of cholinergic markers in hippocampus, cortex, and basal forebrain in AD is associated with severe atrophy in BFCNs in early stages of AD (Auld et al., 2002, Geula et al., 1994, Giacobini, 2003). BFCNs contribute to memory formation and attention (Arciniegas, 2003) and their degeneration and loss correlate strongly with the degree of dementia in AD (Coyle et al., 1983).

BFCNs are dependent on neurotrophins for their survival, function, and phenotypic maintenance (Hefti, 1986, Alderson et al., 1990). In AD, atrophy of BFCNs is associated with changes in the levels of their supportive neurotrophins or their receptors. This impaired neurotrophic support has been shown to contribute to cholinergic system dysfunction (reviewed in Auld et al., 2002).

### *1.6. Neurotrophins:*

Neurotrophins are a group of structurally related dimeric proteins from the neurotrophic factor family. Nerve growth factor, NGF, isolated from male mouse submandibular gland, was the first member of the neurotrophin family to be discovered. It was found to provide trophic support for sensory and sympathetic neurons by Levi-Montalcini in the early 1950s (Levi-Montalcini and Hamburger, 1951). Following that, brain-derived neurotrophic factor (BDNF) was identified in 1982 and purified later from pig brain (Barde et al., 1982). Other neurotrophins including neurotrophin-3 (Maisonpierre et al., 1990) and neurotrophin-4/5 (Hallbook et al., 1991) were described later. The mature form of neurotrophins consists of approximately 120 amino acids, only 28 residues of which vary between each neurotrophin (reviewed in McDonald and Chao, 1995). Because of this similarity, neurotrophins have similar conformations and are able to form heterodimers with each other. All neurotrophins share a common three dimensional structure composed of two pairs of antiparallel β-strands and a cysteine knot motif composed of 6 cysteine residues (Holland et al., 1994).

Neurotrophins regulate neuronal survival, neurite outgrowth and differentiation in the peripheral and central nervous systems (reviewed in Huang and Reichardt, 2001). Each neurotrophin supports specific populations of neurons depending on which receptors are expressed by these neurons. During development, innervating neurons are dependent on neurotrophic factors expressed by target tissues. Neurons which do not receive sufficient amounts of neurotrophic factors undergo apoptosis. Neurotrophins are essential for survival not only during development but also in adulthood. They have roles in neuronal maintenance, synaptic plasticity (Kang et al., 1995, Patterson et al., 1996), learning and memory in adults (Linnarsson et al., 1997, Minichiello et al., 1999).

Neurotrophins act by binding to two types of cell surface receptors: 1) tropomyosin-related kinase (Trk) of the receptor tyrosine kinase family (TrkA to NGF and NT3, TrkB to BDNF, NT3, NT4/5, and TrkC to NT3); 2) A tumor

necrosis factor family receptor called p75 Neurotrophin Receptor (NTR), which binds all neurotrophins with almost equal affinity (reviewed in Huang and Reichardt, 2001). Neurotrophin binding causes Trk to dimerize, which leads to the phosphorylation of the tyrosine kinases located on the intracellular domain of the receptor. The intracellular kinase domain can trigger various signaling cascades (Figure 1.4.) such as Ras/ERK (extracellular signal–regulated kinase) protein kinase pathway, the phosphatidylinositol-3-kinase (PI-3 kinase)/Akt kinase pathway, and phospholipase C (PLC)-γ1 (reviewed in Reichardt, 2006). Some of the signaling pathways triggered by neurotrophin binding to $p75^{NTR}$ are involved in NF-kB activation leading to cell survival and Jun kinase activation leading to programmed cell death (reviewed in Huang and Reichardt, 2001). These pathways will be discussed in more detail in section 1.6.1 and 1.6.2, when NGF and BDNF signaling pathways are reviewed.

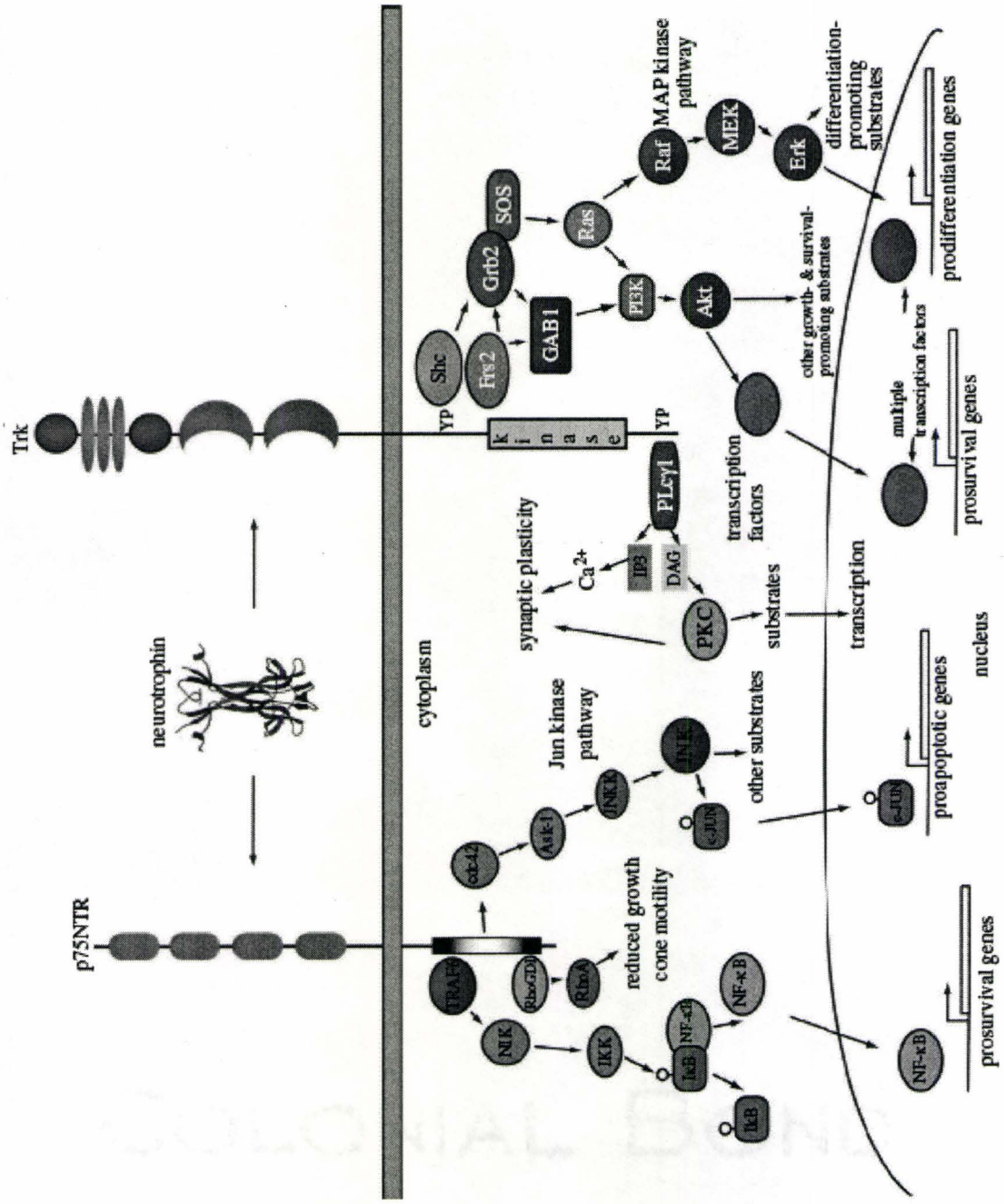



**Figure1.4. Different signaling pathways triggered by neurotrophins through their receptors** (Reichardt, *Philos Tran R Soc Lond B Biol Sci. 2006)*

### *1.6.1. Nerve growth factor:*

Purified NGF from mouse submandibular gland, where NGF is abundant, is a multimeric protein composed of α, β, and γ subunits (α2βγ2). The β subunit is associated with the biological activity of NGF and the γ subunit is involved in β subunit processing. The gene encoding the β subunit is located on chromosome 1p22 in human (Francke et al., 1983) and chromosome 3 in mouse and is about 45 kilobases in length (Ullrich et al., 1983).

There are four messenger ribonucleic acid (mRNA) transcripts produced through two separate promoters and alternative splicing of four exons in the NGF gene (Selby et al., 1987, Racke et al., 1996). Two major transcripts are translated from initiation sites -187 and -121 to form 34 and 27 kDa preproNGF, respectively (Darling et al., 1983). In the endoplasmic reticulum, the signal peptide is removed to produce 32 and 25 kDa pro nerve growth factor (proNGF). ProNGF may undergo post-translational modification and protease cleavage to yield 13kDa mature NGF (Greene et al., 1968, Edwards et al., 1988b). Mature NGF is also known as β-NGF or 2.5S NGF depending on the isolation procedure (Mobley et al., 1976).

Furin, a membrane-associated endoprotease located in the Golgi apparatus, is one of the prohormone convertases able to process proNGF at its amino-terminus to produce the mature form of NGF (Bresnahan et al. 1990, Seidah et al., 1996). Other prohormone convertases such as plasmin and matrix metalloprotease-7 (MMP-7) also process the NGF precursor (Lee et al., 2001b). Throughout NGF processing, several intermediates are produced other than proNGF (32kDa) and mature NGF (13kDa) such as 27-29 kDa, 22-24 kDa, and 18-19 kDa intermediates (reviewed in Fahnestock et al., 2004b). Biological activities of these intermediates are not known.

Mature NGF forms a non-covalent homodimer (Ullrich et al., 1983) and binds with high affinity ($k_d \approx 10^{-11}$ M) to TrkA and with low affinity ($k_d \approx 10^{-9}$ M) to the common neurotrophin receptor $p75^{NTR}$ (reviewed in Bible and Barde 2000,

Lee et al., 2001a). NGF is retrogradely transported from nerve terminals to soma. Both TrkA and $p75^{NTR}$ are involved in the retrograde transport of NGF (reviewed in Kramer et al., 1999). NGF promotes cell survival in cells expressing TrkA through activation of the Ras/PI-3 kinase/AKT pathway (Kaplan and Miller, 2000) and the Ras/Mitogen-activated protein kinase (MAPK) pathway (reviewed in Grewal et al., 1999).

$P75^{NTR}$ plays diverse roles ranging from cell survival to cell death, depending on the cellular context in which it is expressed. Through activation of the NF-κB pathway, $p75^{NTR}$ can contribute to cell survival in sensory neurons (Hamanoue et al., 1999).This receptor can be involved in axonal growth via regulation of Rho activity (Yamashita et al., 1999). This receptor can also interact with Trks and enhance the neurotrophin affinity (at low concentration of ligand) and specifity of binding to Trk (Dechant. 2001, Roux and Barker, 2002, Huang and Reichardt, 2003). On the other hand, high levels of $p75^{NTR}$ expression can induce apoptosis when there are low levels of Trk or Trk is absent (Aloyz et al., 1998, Yoon et al., 1998). The apoptosis occurs through increased ceramide (Brann et al., 2002), activation of c-Jun N-terminal kinase (JNK1), and p53 (Aloyz et al., 1998, Friedman, 2000). Recently, it has been shown that $p75^{NTR}$ requires a co-receptor called sortilin to induce cell death in primary neurons (Nykjaer et al., 2004).

Several factors affect the signaling pathways activated by neurotrophins. Neurotrophins binding to different variants of Trk receptors promote different signals. Trk internalization occurs following binding to neurotrophins. Depending on the Trk internalization mechanisms, various pathways may be activated. Trk internalization through micropinocytosis leads to ERK5 activation while ERK1/2 activation occurs following clathrin-mediated endocytosis of Trk. The ratio of Trk to $p75^{NTR}$ also plays an important role in triggering distinct signaling pathways (death or survival) in response to neurotrophins. Moreover, $p75^{NTR}$ binding to

other receptors such as sortilin determine distinct signaling pathways promoted by neurotrophins (reviewed in Bronfman et al., 2007).

It has been shown that NGF is able to regulate its own receptors. Both TrkA and $p75^{NTR}$ mRNA and protein levels are upregulated in Pheochromocytoma (PC12) cells in response to NGF (Zhou et al., 1995). NGF induces expression of these receptors in basal forebrain cholinergic neurons as well (Holtzman et al., 1992).

ProNGF, not NGF, is the predominant form of NGF in the CNS (Fahnestock et al., 2001). ProNGF activates TrkA and induces neuronal survival and differentiation (Fahnestock et al., 2004a). However, there are studies which show apoptotic activity for this precursor through $p75^{NTR}$ and sortilin binding (Lee et al., 2001b, Nykjaer et al., 2004). They showed that proNGF's affinity to bind to $p75^{NTR}$ is five times greater than mature NGF (Lee et al., 2001a).

The levels of NGF mRNA are greatest in hippocampus and neocortex of adult brain. NGF receptors (both TrkA and $p75^{NTR}$) are detected in basal forebrain, caudate-putamen, medulla oblongata, ventral cochlear nucleus and the dorsal nucleus of the lateral lemniscus (reviewed in Krewson et al., 1995). Some neurons such as cholinergic neurons in basal forebrain are dependent on NGF for their survival and proper functions involved in learning and memory.

***1.6.2. Brain-derived neurotrophic factor:***

The gene encoding BDNF is located on chromosome 11p in human. This gene (70 kb) includes eleven exons of which one 3' exon encodes the BDNF protein (reviewed in Pruunsild et al., 2007). Through alternative splicing 10 different transcripts are produced. While transcripts containing exons I, II and IV (exon III according to the old nomenclature) are predominant in the brain, transcript V (exon IV according to the old nomenclature) is expressed more in the lung and heart (reviewed in Binder and Scharfman, 2004).

BDNF, the same as NGF, is produced as a precursor called proBDNF (36kDa) which can be processed to the mature form (14kDa) either intracellularly

by furin or extracellularly in the synaptic cleft by tissue plasminogen activator via plasmin (Lu et al., 2005). The non-covalent homodimer of mature BDNF binds to TrkB leading to receptor dimerization and autophosphorylation of its intracellular kinase domain. This kinase domain can activate several downstream neuroprotective signaling pathways such as the Ras-MAPK cascade and phosphorylation of cyclic AMP-response element binding protein (CREB) (Shieh et al., 1998, Tao et al., 1998). There are at least 3 different TrkB isoforms produced through RNA splicing. Of the 3 isoforms, one contains the full-length tyrosine kinase receptor, while the other two are truncated, lacking tyrosine kinase domains. Both truncated receptors have inhibitory effects on the signaling pathways triggered by the full-length receptor (reviewed in Lipsky and Marini, 2007). The truncated receptors play several roles independent of full-length TrkB, including growth and development. BDNF, like all neurotrophins, binds to $p75^{NTR}$ with a low affinity and can induce apoptosis (reviewed in Binder and Scharfman, 2004).

Like BDNF, proBDNF (BDNF precursor) is able to bind to TrkB to induce neuroprotective signals (Fayard et al., 2005). It has been shown however, that this protein also induces apoptosis through a receptor complex of p75 and sortilin (Teng et al., 2005).

BDNF and TrkB, at both mRNA and protein levels, are widespread throughout the central nervous system and highly expressed in the hippocampus. This neurotrophin is involved in survival and growth of a variety of neurons such as hippocampal neurons, cortical neurons, dorsal root ganglion cells, and certain peripheral sensory neurons such as nodose ganglia. Lack of BDNF, unlike NGF, in homozygous (-/-) transgenic mice does not affect motor or sympathetic neurons (reviewed in Binder and Scharfman, 2004).

BDNF plays an important role not only in neuronal survival (Yuan and Yanker, 2000), but also in synaptic plasticity (McAllister et al., 1999), learning, and memory formation (Lu and Chow, 1999 and Yamada et al., 2002). It has

been shown that BDNF enhances action potential frequency and excitatory synaptic activities of cultured neurons (reviewed by Arancio and Chao, 2007).

***1.7: Neurotrophins in neurodegenerative diseases:***

Lack of either neurotrophin can lead to serious complications. Transgenic mice with either the neurotrophin or its receptor deficient show peripheral and motor neuron dysfunctions (Snider, 1994). NGF deficit in transgenic mice called AD11 causes visual recognition and spatial memory impairment which can be improved upon NGF administration (De Rosa et al., 2005). Homozygous transgenic mice lacking BDNF do not live longer than 3 weeks which is not enough time to determine the potential role of BDNF in behavioral tasks. Heterozygous BDNF knockout mice (+/-) with reduced levels of BDNF exhibit long term potentiation (LTP) deficits (Patterson et al., 1996) and impaired spatial learning (Linnarsson et al., 1997). Application of BDNF to heterozygous BDNF knockout mice hippocampal slices improved synaptic transmission and LTP (Patterson et al., 1996, Korte et al., 1996).

In human, alterations in neurotrophin levels (mRNA or protein), function, their receptors, or their trafficking can damage neurons leading to gradual neuronal degeneration (reviewed in Kruttgen et al., 2003). For instance, imbalance in NGF or BDNF levels or their receptors has been reported in some neurodegenerative diseases such as Huntington's disease (HD) (Zuccato et al., 2001), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and Alzheimer's disease (AD) (reviewed in Kruttgen et al., 2003). Specific neurons such as striatal neurons in HD, cholinergic neurons in AD, dopaminergic neurons in PD and motor neurons in ALS undergo degeneration (reviewed in Kruttgen et al., 2003). Although neurotrophin levels and their receptors have been studied widely in AD, there are few studies of neurotrophin roles in tauopathies other than AD.

### *1.8. Neurotrophins in Alzheimer's disease:*

#### *1.8.1 BDNF in Alzheimer's disease:*

In AD brain, BDNF mRNA decreases in hippocampus (Philips et al. 1991 and Murray et al., 1994), frontal cortex (Soontornniyomkij et al., 1999), and parietal cortex (Holsinger et al., 2000). Moreover, reduced BDNF protein levels have been demonstrated in the entorhinal cortex (Narisawa-Saito et al., 1996), temporal cortex and hippocampus (Connor et al., 1997), frontal cortex (Ferrer et al., 1999), and parietal cortex (Peng et al., 2005) of AD patients. In addition, a specific genetic polymorphism in the BDNF gene is associated with sporadic AD (Kunugi et al., 2001 and Ventriglia et al., 2002).

Interestingly, Peng et al. (2005) showed that there is a correlation between the severity of cognitive impairment and a reduced level of BDNF protein in AD brain. This finding supports the role of BDNF reduction in cognitive dysfunction and memory deficits in AD. However, the mechanism underlying BDNF down-regulation in AD is not fully understood.

As was already mentioned, AD is not primarily a tauopathy. Another hallmark of AD is Aβ deposition in the brain. According to the amyloid cascade hypothesis, Aβ is the main culprit in AD pathology (Selkoe, 1994) while tau hyperphosphorylation is downstream of Aβ in this pathway. Supporting this idea, there is increasing evidence showing that Aβ is involved in tau pathology. Glycogen synthase kinase 3 (GSK-3β) is a major enzyme in tau phosphorylation. It has been shown that Aβ causes neuronal death through GSK-3β and tau hyperphosphorylation (reviewed in Takashima, 2006). Reduced GSK-3β activation and tau phosphorylation have been reported *in vivo* and *in vitro* after treatment with an antibody against Aβ (Ma et al., 2006). Interestingly, it has been shown that Aβ is involved in BDNF dysregulation. Soluble aggregated Aβ reduces BDNF mRNA levels in human neuroblastoma cells. This study showed that two transcripts of BDNF (IV and V) significantly decrease after treatment with Aβ in those cells (Garzon & Fahnestock, 2007). In human cortical tissue,

transcript IV is expressed the most among all BDNF transcripts and decreases in AD (Garzon et al., 2002). It may be concluded that Aβ is the cause of BDNF mRNA reduction in AD.

Altogether, these data suggest that Aβ may play a role in both tau dysfunction and BDNF down-regulation. However, the sequence of events and whether Aβ affects BDNF levels directly or through tau dysfunction is not clear. There is some evidence which may support the role of tau pathology in BDNF dysregulation. Chronic treatment with lithium (inactivating GSK-3β) increases BDNF expression in the brain. It is believed that lithium's neuroprotective effect is, to some extent, acting through GSK-3β inactivation leading to CREB activation and BDNF up-regulation (reviewed in Wada et al., 2005). Moreover, NFT formation, BDNF reduction, and neuronal degeneration all correlate well with cognitive dysfunction (Peng et al., 2005, Giannakopoulos et al., 2003). Tau hyperphosphorylation has been suggested to cause cognitive decline in transgenic mice overexpressing GSK-3β (Engel et al., 2007). Transgenic rats expressing human truncated tau show tau hyperphosphorylation and tangle formation which is associated with behavioral deficits in those animals (Hrnkova et al., 2007). Interestingly, it has been shown that cognitive impairment induced by Aβ in transgenic mice expressing human APP can be alleviated by reducing endogenous tau (Roberson et al., 2007). Finally, it has been reported that in tau transgenic animals with memory deficits and synaptic loss (Schindowski et al., 2006), BDNF mRNA and protein decrease in the cortex and hippocampus. This BDNF reduction correlates with tau dysfunction (Schindowski et al., 2007).

Taken together, all of these data suggest that tau dysfunction may be responsible for BDNF reduction and cognitive dysfunction in AD. However, whether tau dysfunction regulates BDNF decrease in non-AD tauopathies has not been investigated yet.

### *1.8.2. NGF in Alzheimer's disease:*

### *1.8.2. A. NGF and BFCN:*

It has been shown that certain memory and learning functions depend on a proper interaction between NGF and BFCNs (Gutierrez et al., 1997, Woolf. et al., 2001). NGF, as a target-derived neurotrophin, is synthesized and released in the hippocampus and cortex and binds to its receptor (TrkA) on the axonal terminals of BFCNs. NGF-TrkA complexes are internalized into endosomes, and retrogradely transported along microtubules to the cell bodies of BFCNs, and trigger the signaling pathways such as ERK pathway involved in cellular function and morphology maintenance (Sofroniew et al., 2001, Howe and Mobley, 2005). NGF increases transcription of choline acetyl transferase (ChAT), a neurotransmitter synthesizing enzyme, or enhances its mRNA stability (Sofroniew et al., 2001), and increases acetylcholine (ACh) release from BFCNs (Auld et al., 2002).

BFCN atrophy is associated with a decrease in NGF protein levels in cell bodies of these neurons (Albeck et al., 2003, Mufson et al., 1994 and 1995, Scott and Crutcher, 1994) and an increase in NGF protein in the cerebral cortex and hippocampus of AD patients and during aging (Scott and Crutcher, 1994, Scott et al., 1995, Fahnestock et al., 1996, Albeck et al., 2003, Peng et al., 2004). NGF mRNA levels in those tissues however, remain unchanged in the AD brain (Jette et al., 1994, Fahnestock et al., 1996). Therefore, it does not seem likely that there is a decrease in NGF synthesis in NGF-producing tissue. Moreover, studies using $^{125}$I-NGF or neuronal tracers have shown that retrograde transport of NGF is disrupted from hippocampus and cortex to the basal forebrain during aging (Cooper et al., 1994, Niewiadomska et al., 2003, 2005). Therefore, defects in axonal trafficking of NGF may account for the increase of NGF protein in target tissues and the lack of trophic support for BFCNs in AD.

### *1.8.2. B. Axonal transport of NGF and its interruption in AD:*

NGF axonal trafficking is supported by microtubules. Colchicine, which disrupts microtubule polymerization, blocks NGF movement along the axon (Hendry et al., 1974, Krah and Meller, 1999). MTs are one of the main cytoskeletal elements in all eukaryotic cells and, in particular, in neuronal cells, where they contribute to intracellular trafficking, cell division and cell morphogenesis (reviewed in Trinczek et al., 1999). To mediate these functions, microtubule-associated proteins (MAPs) play important roles.

There are two types of MAPs in neuronal cells which decorate MTs: fibrous MAPs, including tau and MAP2, and force producing MAPs, including kinesin and cytoplasmic dynein. Tau and MAP2 promote assembly (Hirokawa, 1994, Mandelkow and Mandelkow, 1995,) and organization of the MT cytoskeleton (Matus 1994, Barlow et al., 1994). Kinesin produces force towards the plus end of MTs *in vitro* and is involved in anterograde transport, while cytoplasmic dynein is a minus-end directed motor protein and contributes to retrograde transport (Hagiwara et al., 1994, Trinczek et al., 1999).

In normal cells, tau is phosphorylated by several kinases including GSK-3β and cyclin-dependent kinase-5 (cdk5). As was already mentioned, the areas of tau flanking the MT-binding domains of this protein have several phosphorylation sites. These sites play an important role in binding tau to MTs. Hyperphosphorylation in these areas (for example because of mutation) leads to tau detachment from MTs which causes MT depolymerization and instability.

In AD, tau is hyperphosphorylated or abnormally phosphorylated (reviewed in Salehi et al., 2003, Balaraman et al., 2006). Abnormal modification of tau leads to tau detachment from MTs and causes MT instability (Goode et al., 1997, Utton et al.,1997, Buee et al., 2000, Sassin et al., 2000). Moreover, hyperphosphorylated tau is prone to self aggregation, and following interaction between normal tau and abnormally hyperphosphorylated tau (p-tau), large tangles of filaments are formed (Alonso et al., 1996). The inability of tau, isolated

from PHF, to bind to MTs has been reported by Bramblett et al., (1993). Interestingly, tau isolated from PHF is able to bind to MT after dephosphorylation (Bramblett et al., 1993).

***1.8.2. C. Tau hyperphosphorylation and axonal transport impairment:***

Hyperphosphorylated tau may contribute to axonal transport disruption by making MTs unstable (Buee et al., 2000). Less axonal transport of the tracer biotinylated dextran amine (BDA) has been found in the temporal cortex, compared to prefrontal cortex, of AD postmortem material. Interestingly, in the same study, they showed that there are more severe pathological changes in the temporal cortex in AD in terms of NFT formation compared to prefrontal cortex (Dai et al., 2002). It was concluded that there is a correlation between severity of NFT and impaired axonal transport.

It has been reported that redistribution of phospho-tau (P-Thr 231 and P-Ser404) and the active form of GSK-3β from axons and dendrites to soma in aged rats coincide with impaired retrograde transport in those animals (Niewiadomska et al., 2005). They concluded that changes in tau distribution lead to cytoskeletal breakdown which can hamper retrograde transport. In young rats, tau 1 antibody detected non-phosphorylated tau in the septo-hippocampal pathways of BFCNs. In contrast, the somatodentritic compartment of BFCN showed immunoreactivity for tau 1 in old rats. This redistribution of tau 1 also coincides with impaired retrograde transport of Fluorogold (a neuronal tracer) in aged animals (Niewiadomska et al., 2003).

Transgenic mice overexpressing the shortest human tau isoform, fetal tau, show retarded fast axonal transport in the ventral roots of spinal cord. *In vitro*, impaired axonal transport of mitochondria and microfilaments has been reported in neurons overexpressing tau (Stamer et al., 2002). It has been demonstrated that in transgenic Drosophila overexpressing tau, there is vesicle aggregation, motor dysfunction and axonal transport defects. In these flies, co-expression of GSK-3β and tau increases the deficits in axonal transport while inhibition of GSK-

3β reverses this impairment (Mudher et al. 2004). In transgenic mice expressing human apolipoprotein E4 (ApoE4), synaptophysin, mitochondria and vesicles accumulate in the axons (Tesseur et al. 2000b), which is a sign of impaired axonal transport. Interestingly, ApoE enhances tau phosphorylation (Tesseur et al., 2000a). Impaired fast axonal transport of fluorescent dextran has been reported in transgenic mice overexpressing tau following intraneural injection of the dye (Kunzi et al., 2002). Axonal transport dysfunction in transgenic mice with R406W mutant tau has been reported (Zhang et al., 2004). Taken together, these data support the idea that an abnormality in tau function may account for impairment in retrograde transport observed in AD.

Other mechanisms of axonal transport disruption in AD and aged brain have also been suggested. For instance, physical blockade of pathways by aggregated tau or NFTs can interfere with the movement along the axon (for review, see Salehi et al., 2004). However, Drosophila overexpressing tau showed axonal defects with no tau aggregation (Mudher et al., 2004). Moreover, retrograde transport impairment in transgenic mice with trisomy of chromosome 16 was observed in the absence of any NFTs (Cooper et al., 2001). In addition, retarded axonal transport in transgenic mice expressing the shortest human tau isoform is reversed by treatment with a MT-binding drug. It may improve axonal transport by offsetting the disability of tau to stabilize MTs (Zhang et al., 2005). These data may support the effect of tau dysfunction (not physical blockade by NFTs) on the impairment of NGF transport. On the other hand, reduction in TrkA expression and immunoreactivity in AD have been reported (for review, see Siegel and Chauhan, 2000 and Mufson et al., 2007) which can also influence the transport of NGF. Whether tau dysfunction or TrkA reduction or both interferes with NGF transport in AD is not clear. Moreover, it has not yet been shown if retrograde transport of NGF is disrupted in any other tauopathies such as PiD, PSP, and CBD. Study of NGF levels in tauopathies other than AD may shed light on the role of tau in NGF transport impairment in AD.

### *1. 9. Biological activity of proneurotrophins:*

#### *1.9.1. Nerve growth factor precursor:*

NGF precursor (proNGF) is secreted by many tissues such as prostate cells, spermatids, sympathetic neurons, hair follicles, heart and spleen (Chen et al., 1997, Delsite and Djakiew, 1999, Yardley et al., 2000, Bierl et al., 2005). ProNGF is the predominant form of NGF in the CNS, in sympathetic and sensory ganglia and their peripheral targets, and in oral mucosal keratinocytes, while little or no mature NGF can be detected (Fahnestock et al., 2001, Smith et al., 2002, Bierl et al., 2005, Pedraza et al., 2005, Bierl and Isaacson, 2007, Hayashi et al., 2007). In the AD brain, retrograde transport of NGF from the cortex and hippocampus to BFCNs is reduced as these neurons degenerate, and NGF accumulates in the cortex and hippocampus. The NGF that accumulates in these tissues is proNGF, with no mature NGF detectable (Fahnestock et al., 2001, Pedraza et al., 2005). We show in Chapter 4 that levels of proNGF protein also increase in PiD brain. These data suggest that proNGF may exhibit biological activity besides its prodomain function in promoting protein folding and regulation of neurotrophin secretion. ProNGF may even be the biologically active form of NGF in the brain and other tissues.

Recently, many investigations have been conducted in order to discover the function of proNGF. It has been shown that proNGF also forms a dimer in solution (Rattenholl et al., 2001, Fahnestock et al., 2004b, Paoletti et al., 2006). Two structural models have been suggested for proNGF, a crab-like shape and a rod-like shape (Paoletti et al., 2006). In both shapes, the main part is a β-sheet secondary structure, similar to mature NGF, and two flexible arms (Figure 1.5). This protein, like its mature form, is able to bind to TrkA in cross-linking studies (Fahnestock et al., 2004a). It has been shown that this protein has more affinity to bind to $p75^{NTR}$ receptor compared to NGF (Lee et al., 2001b). Even though increasing numbers of studies examine proNGF biological activity, there are still contradictory reports regarding the role of this precursor.

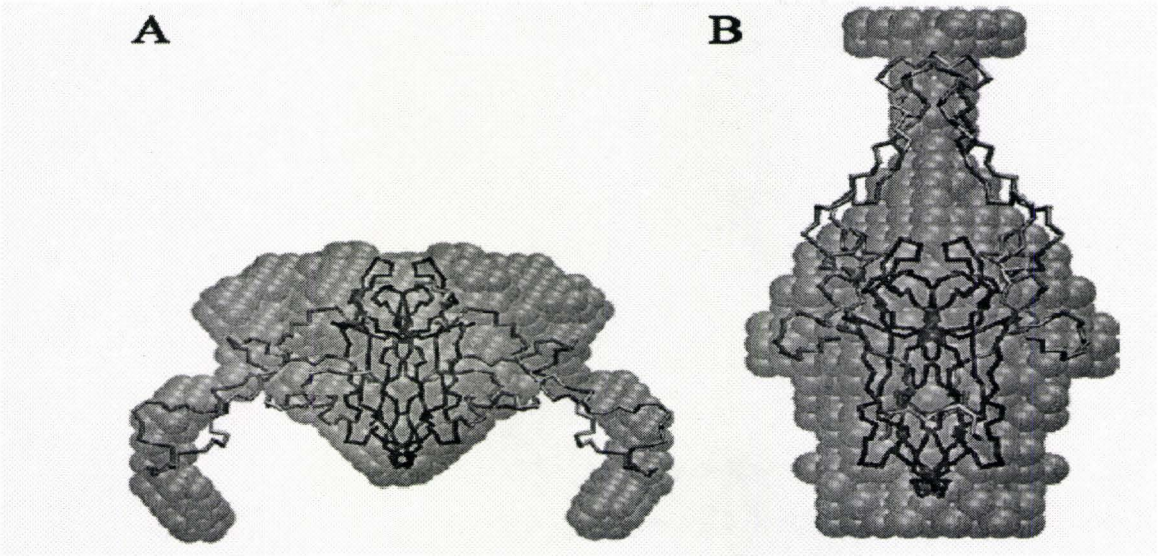



**Figure 1.5: Crab-like shape and rod-like shape of proNGF** (Paoletti et al., *Biochem Soc Trans.* 2006).

### *1.9.2. Controversy over the activity of proNGF:*

There are several reports demonstrating neurotrophic activity for either wild type (WT)-proNGF or partially cleavable proNGF (Saboori and Young, 1986, Lakshmanan et al., 1989, Suter et al., 1991, Hayashi et al., 2007, discussed in more detail in discussion chapter). *In vivo*, proNGF is very stable, as in many tissues proNGF is the predominant form of NGF. *In vitro*, proNGF is easily cleavable to its mature form. Therefore, in order to study the biological role of this protein *in vitro* without mature NGF interfering, a point mutation in the cleavage site for furin (a proprotein convertase) can minimize conversion of proNGF to mature NGF. The resulting recombinant, cleavage-resistant proNGFs exhibit either apoptotic activity or neurotrophic activity. Our lab produced a cleavage resistant proNGF mutated at a single amino acid which induced neurite outgrowth on both superior cervical ganglion (SCG) neurons and PC12 cells and showed TrkA binding and activation. This cleavage-resistant proNGF, Similar to mature NGF, did not induce apoptosis in SCG neurons (Fahnestock et al., 2004a).

On the other hand, a different cleavage resistant proNGF was not able to promote Trk A phosphorylation in PC12 cells or neurite outgrowth in the SCG bioassay. This protein displayed at least fivefold greater affinity to $p75^{NTR}$ than did mature NGF and it had at least 10 times more potential in inducing apoptotic cell death in SCG neurons compared to mature NGF (Lee et al., 2001b).

These two recombinant proteins are different in several aspects. Cleavage-resistant proNGF with apoptotic activity carries four separate amino acid substitutions. The first two substitutions at -1 and -2 (R-R to A-A) were designed to eliminate the prodomain cleavage site. The second two substitutions at 118 and 119 (R-R to A-A) were designed to eliminate a carboxyl terminal cleavage site that could release an added poly histidine tag. This proNGF was expressed in mammalian cells and purified using nickel column chromatography. However, cleavage-resistant proNGF with neurotrophic activity has one amino acid substitution at the -1 position (R to G) designed to eliminate the cleavage site for furin and carries no tags. This proNGF was expressed in insect cells using a baculovirus expression system. There are also differences in the bioassay methods used to test the two proteins. Each of these differences may affect the activity of the two proteins in such a way that one is apoptotic and the other is neurotrophic. Table 1.2 summarizes the differences between these two cleavage-resistant proNGFs.

| Type of proNGF Differences | proNGFhis (Lee et al) | proNGF (R-1G) (Fahnestock et al) |
|---|---|---|
| Amino acid substitution | K-R to A-A at -1, -2 positions; R-R to A-A at 118,119 positions | R to G at –1 position |
| His-tag | A His-tag at COOH terminus | No tag |
| Expression System | Mammalian expression system using 293 cells | Insect expression system using Sf9 cells and baculovirus |
| Method of Purification | Nickel column | No nickel column |

**Table 1.2. Neurotrophic proNGF is different from apoptotic proNGF in structure, purification method and expression system.**

### *1.10. The purpose of this Ph.D. thesis:*

#### *1.10.1. Mechanism of changes in neurotrophins in neurodegenerative diseases:*

Changes in the normal levels of neurotrophins such as NGF and BDNF have been reported in neurodegenerative diseases such as AD. These alterations occur early and show correlation with the impairment of cognitive function in AD patients (Peng et al., 2004, 2005).

In AD brain, BDNF shows reductions in both mRNA and protein levels in several areas involved in cognition and memory formation, and induction of BDNF can ameliorate the memory impairment caused by Aβ in transgenic animals.

In AD, a decrease in the level of NGF in BFCN is associated with an increase in the level of NGF in their target tissues such as the hippocampus and cortex. There is also a reduction in the NGF receptor (TrkA) in BFCNs which suggests that impairment in the retrograde transport of NGF may contribute to neurotrophic deprivation and BFCN degeneration. Providing NGF to those neurons as a therapeutic agent can save the cells from degeneration and improve the cognitive impairment (Bradbury, 2005).

Although involvement of neurotrophin dysregulation in AD pathology and the importance of neurotrophins in restoring memory dysfunction have been firmly investigated, the mechanism underlying the changes in neurotrophin levels in AD is not known. Whether Aβ or tau dysfunction or both are involved in these changes in neurotrophin levels in AD requires further investigation.

There is evidence indicating that Aβ is upstream of tau hyperphosphorylation and BDNF reduction during AD pathology. However, it is not clear whether Aβ affects BDNF levels directly or through GSK-3β and tau hyperphosphorylation. Cognitive defects in tau transgenic animals supports the idea that tau dysfunction may change the level of BDNF leading to memory deficits in those animals (Schindowski et al., 2007). Moreover, prevention of tau

hyperphosphorylation using lithium increases BDNF levels in the brain (Wada et al., 2005). Tau dysfunction has been shown to disrupt axonal transport. In AD, a tau abnormality may be responsible for impaired retrograde transport of NGF leading to elevated levels of this neurotrophin in the target tissues. Therefore, we expect to observe the same changes in the levels of neurotrophins in tauopathies other than AD if tau is responsible for those changes.

Studying the levels of NGF and BDNF in non-AD tauopathies could shed light on the mechanism underlying neurotrophin regulation in AD. We hypothesized that tau dysfunction in tauopathies may alter the normal levels of neurotrophic factors such as NGF and BDNF.

In Chapter 3, we address the question of whether BDNF mRNA levels decrease in non-AD tauopathies including PiD, CBD and PSP.

In Chapter 4, we determine whether the level of proNGF protein is increased in non-AD tauopathies.

### *1.10.2. Biological activity of proNGF:*

As has already been shown, the predominant form of NGF in the CNS is proNGF (Fahnestock et al. 2001). Moreover, the level of proNGF is increased in AD brain (Fahnestock et al., 2001, Pedraza et al., 2005). We will also show in Chapter 4 that the level of this protein increases in PiD. These data suggest that proNGF may mediate biological activity besides its function as a precursor. The role of proNGF is controversial and opposite activity (neurotrophic versus apoptotic activity) has been reported for proNGF (Lee et al., 2001b, Fahnestock et al., 2004a). If proNGF is apoptotic, increases in the level of this neurotrophin may account for the neuronal degeneration occurring in AD. However, if proNGF is neurotrophic, neuronal degeneration occurs in the AD brain because interruption in proNGF axonal transport by tau dysfunction and/or decreased TrkA reduces trophic support to BFCNs. The first part of this investigation (1.10.1) conducted on proNGF levels in tauopathies showed that some tauopathies (PiD) sharing the same tau modification as AD exhibit increased

levels of proNGF. This result supports the idea that tau dysfunction in AD may lead to accumulation of proNGF in target tissues, and neuronal degeneration happens due to lack of neurotrophic support (lack of proNGF). To provide more direct evidence regarding the bioactivity of proNGF, we conducted a series of bioassays in Chapter 5 to compare the two recombinant proNGFs with reportedly opposite activity. The goal of Chapter 5 was to determine whether any of the structural or production differences (Table 1.2) are responsible for generating the opposing biological activity for these two cleavage-resistant proNGFs.

# CHAPTER 2

# MATERIALS AND METHODS:

## *2.1. Human brain tissue samples*:

All human samples were provided by Dr. Virginia M.Y. Lee (University of Pennsylvania, USA). Parietal cortex of human postmortem brain from subjects with tauopathies including Pick's disease (PiD) (n=8), corticobasal degeneration (CBD) (n=12), progressive supranuclear palsy (PSP) (n=13), and from controls (n=12) were used for RNA isolation, and n=6 for all groups were used for protein isolation. Access to clinical and diagnostic evaluation for each disease was not possible as the tissues were obtained from various clinical centers. Characteristics of the subjects are shown in Table 2.1. Patients' demographic and raw data for normalized BDNF mRNA and normalized proNGF protein are shown in Table 2.2.

| | Clinical diagnosis | | | | | P-value* |
|---|---|---|---|---|---|---|
| | Normal N=12 | PiD N=8 | CBD N=12 | PSP N=13 | Total N=45 | |
| **Age (Years) at death:** | | | | | | |
| (Mean± SD) | 68.6±11.2 | 65.4±10.3 | 69.4±6.4 | 68.8±7.9 | 68.2±8.8 | 0.77 |
| (Range) | 36-81 | 42-76 | 56-80 | 48-79 | 36-81 | |
| **Number (%) of Males** | 5 (42%) | 5 (55%) | 5 (42%) | 5 (38%) | 20 (44%) | 0.72 |
| **Yield of mRNA (µg/mg)** | 0.35±0.15 | 0.38±0.1 | 0.32±0.13 | 0.34±0.08 | 0.34±0.1 | 0.73 |
| (Range) | 0.05-0.49 | 0.3-0.54 | 0.15-0.52 | 0.17-0.45 | 0.05-0.54 | |

| | Normal N=6 | PiD N=6 | CBD N=6 | PSP N=6 | Total N=24 | P-Value* |
|---|---|---|---|---|---|---|
| **Age (Years) at death:** | | | | | | |
| (Mean± SD) | 72.3±2.4 | 68.3±6.8 | 70.7±4.5 | 72.8±3.2 | 71±4.6 | 0.34 |
| (Range) | 69-75 | 59-76 | 62-74 | 70-79 | 59-79 | |
| **Number (%) of Males** | 3 (50%) | 4 (66%) | 3 (50%) | 2 (33%) | 12 (50%) | 0.72 |
| **Yield of protein (µg/mg)** | 44±5 | 40±5.8 | 42.5±6.6 | 42.3±5.3 | 42.2±5.5 | 0.69 |
| (Range) | 41-50 | 31-47 | 37-55 | 36-50 | 31-55 | |

**Table 2.1. Characteristics and yield of RNA extraction and protein extraction for tauopathy subjects.** * P-value was determined using one way ANOVA for age and yield and Pearson Chi-square for gender.

| | Patient ID | Age | Sex | Ratio (BDNF/β-actin) | Ratio (proNGF/β-actin) |
|---|---|---|---|---|---|
| PiD | 1308 | 72 | M | 0.001681 | 1.255209 |
| | 1548 | 74 | M | 0.002311 | 1.042326 |
| | 1621 | 76 | M | 0.001016 | 1.272563 |
| | 1650 | 62 | F | 0.016474 | 0.61027 |
| | 1697 | 59 | M | 0.025231 | 0.716994 |
| | 1436 | 67 | F | ..... | 0.928946 |
| | 1172 | 69 | F | 0.004064 | ..... |
| | 1743 | 42 | F | 0.007564 | ..... |
| | 1823 | 68 | M | 0.002159 | ..... |
| CBD | 1421 | 62 | F | 0.123043 | 0.73979 |
| | 1562 | 71 | F | 0.006198 | 0.994954 |
| | 1578 | 74 | M | 0.025849 | 0.959529 |
| | 1591 | 70 | F | 0.010331 | 0.626727 |
| | 1673 | 74 | M | 0.0018 | 0.54099 |
| | 1736 | 73 | M | 0.008206 | 0.651399 |
| | 1811 | 68 | F | 0.001212 | ..... |
| | 1845 | 56 | F | 0.002916 | ..... |
| | 1254 | 71 | M | 0.005928 | ..... |
| | 1795 | 71 | F | 0.001517 | ..... |
| | 1716 | 80 | F | 0.007486 | ..... |
| | 1786 | 63 | M | 0.00081 | ..... |
| PSP | 1529 | 70 | F | 0.004863 | 0.637011 |
| | 1539 | 79 | F | 0.006056 | 0.693117 |
| | 1546 | 73 | M | 0.015136 | 0.578304 |
| | 1634 | 72 | F | 0.010955 | 0.56179 |
| | 1635 | 71 | F | 0.013631 | 0.553892 |
| | 1762 | 72 | M | 0.013356 | 0.471867 |
| | 1863 | 64 | F | 0.025901 | ..... |
| | 1185 | 48 | M | 0.000715 | ..... |
| | 1900 | 64 | F | 0.014927 | ..... |
| | 1822 | 67 | M | 0.01829 | ..... |
| | 1913 | 71 | F | 0.002732 | ..... |
| | 1832 | 79 | F | 0.003742 | ..... |
| | 1600 | 64 | M | 0.007869 | ..... |
| Normal | 1288 | 72 | F | 0.021587 | 0.622537 |
| | 1317 | 74 | F | 0.003936 | 0.751419 |
| | 1423 | 70 | M | 0.015366 | 0.648902 |
| | 1444 | 74 | F | 0.198986 | 0.615164 |
| | 1449 | 69 | M | 0.05219 | 0.637184 |
| | 1087 | 75 | M | 0.030611 | 0.951045 |
| | 1962 | 72 | F | 0.002693 | ..... |
| | 1037 | 36 | F | 0.010073 | ..... |
| | 1754 | 67 | M | 0.003698 | ..... |
| | 1731 | 70 | F | 0.010374 | ..... |
| | 1299 | 81 | F | 0.010728 | ..... |
| | 1411 | 63 | M | 0.015888 | ..... |

**Table 2.2. Tauopathy patients' demographic and raw data for normalized BDNF mRNA and normalized proNGF protein.** M: Male, F: Female. Outlier's in CBD and normal subjects are in different color.

***2.2. Animal brain tissue samples:***

Brain tissue (cortex) from JNPL mice with a tau mutation corresponding to the human tauopathy FTDP-17 (P301L) were provided by Dr Stephen Ginsberg (Nathan Kline Institute, Orangeburg, NY, USA). These transgenic mice exhibit neurofibrillary tangles, motor disturbances and behavioral dysfunction such as limb weakness, weight loss, reduction in grooming and vocalization, and eye irritation (reviewed in Lewis et al., 2000).

The cortex and hippocampus of transgenic mice overexpressing GSK-3β were provided by Dr Jose J. Lucas (Madrid, Spain). The transgenic mouse design was previously described in Lucas et al. (2001). Briefly, the GSK-3β transgene was expressed under control of two promoters: a tetracycline-responsive promoter in order to conditionally overexpress the gene only during adulthood and prevent prenatal lethality, and a calcium/calmodulin kinase IIα promoter for restricted expression in the CNS. GSK-3β showed overexpression in both cortex and hippocampus while significant tau hyperphosphorylation (PHF) could be detected only in hippocampus but not in cortex. There was somatodendritic localization of hyperphosphorylated tau and marked cellular degeneration in the hippocampus of transgenic mice overexpressing GSK-3β which mimics AD pathology in hippocampus (Lucas et al., 2001).

Brain samples (cortex) of transgenic mice expressing the 4-repeat isoform of human tau (Thy-tau22 and Thy-tau30) were obtained from Dr Luc Buée (Inserm, Lille, France). These mice were generated as described in Schindowski et al., 2006. The 4R-human tau carries two mutations, G272V and P301S, under the Thy 1.2 promoter, which is specific for neurons and is activated postnatally. Hyperphosphorylated tau was detected in both cortex and hippocampus of 3-6 month animals. Spatial learning and memory impairment was also observed in the Thy-tau22 mice. Cell loss was confirmed in older ages at about 12 months. This mouse model, unlike most tau transgenic mice, did not show any motor dysfunction at younger or older ages (Schindowski et al., 2006). The Thy-tau30

mouse line showed more tau hyperphosphorylation in spinal cord compared to brain. Motor dysfunction was observed in the Thy-tau30 mice which made them a poor model for AD (Schindowski et al., 2006).

The cortex of Pick's disease mice (PiD mice) was obtained from Dr Jurgen Gotz's laboratory (University of Sydney, Australia). These transgenic mice overexpress a human tau mutation K369I, implicated in PiD (Neumann et al., 2001), under control of the Thy1.2 promoter. Tau overexpression was observed in the substantia nigra, cortex, hippocampus, and amygdala. They showed classical motor disturbances at age 4-6 weeks (reviewed in Janus, 2008). The characteristics of each transgenic mouse line are summarized in Table 2.3.

| | JNPL Tg(n=3), WT(n=4) | GSK-3β overexpressing mice, Tg (n=3), WT(n=3) | Thy-Tau22 Tg (n=7), WT(n=6) | Thy-Tau30 Tg (n=10), WT(n=3) | PiD Tg(n=3), WT(n=3) |
|---|---|---|---|---|---|
| Transgene | Tau | GSK-3β | 4R human tau | 4R human tau | tau |
| Mutation | P301L | ---------- | G272V, P301S | G272V, P301S | K369I |
| Age | 14-22 months | Unknown | 2-13 months | 2-13 months | 8-9 Months |
| Sex | Male | Male | Male | Male | Male |
| Cognitive impairment | N/D | Yes | Yes | N.D. | N.D. |
| Motor dysfunction | Yes | N.D. | No | Yes | Yes |
| Background | SW/DBA2/ C57Bl | C57Bl/6 | C57BL6/ CBA | C57BL6/ CBA | C57Bl/6 |
| Tissue | Cortex | Cortex/ hippocampus | Cortex | Cortex | Cortex |
| Application | Protein | Protein/RNA | RNA | RNA | Protein |
| Yield of protein (µg/mg) (Range) | 60.2 ± 9.3 (50.1-72.6) | 58.9 ± 6.4 Cx (48.3-67.3) 62.6 ± 2.8 Hipp (57.3-64.8) | ---------- | ---------- | 75.9 ± 6.4 (67.3-85.2) |
| Yield of RNA (µg/mg) (Range) | ---------- | 0.64 ± 0.13 (0.5-0.9) [Hipp Tg (n=4), WT(n=4)] | 0.81 ± 0.17 (0.63-1.2) | 0.84 ± 0.16 (0.5-1.1) | ---------- |

**Table 2.3: Characteristics and yield of RNA and protein for tau transgenic mice (Tg) and wild-type mice (WT).** Not determined (N.D.), Cortex (Cx), Hippocampus (Hipp).

### *2.3. BDNF mRNA quantification:*

#### *2.3.1. Sample preparation for RNA extraction:*

All tissues were stored at -80℃. When working with RNA, an RNase-free area is required as RNA is easily degraded by RNase. Hands, dust, contaminated laboratory, all can be the source of RNase contamination. Therefore, general precautions including using autoclaved instruments, filtered and RNase-free pipette tips and wiping down the pipettes and working area with RNase AWAY® (Bioproducts, Inc. Warrenton, OR, USA) were followed. Frozen tissues were weighed out in prechilled, sterile Eppendorf tubes. 45-100mg of parietal cortex tissue from human tauopathies brain, 7-15 mg cortical tissue from GSK-3β overexpressing mice, 25-70 mg of cortex from Thy-tau22 transgenic mice, and 30-60 mg of cortex from Thy-tau30 transgenic mice were used for RNA extraction.

#### *2.3.2. RNA isolation and DNase treatment:*

Tissues were sonicated in 1 ml of Trizol (Invitrogen, Burlington, Canada) per 100 mg of tissue using a sonicator, Sonic Dismembrator Model 100 (Fisher Scientific, Ottawa, ON, Canada), 3 times for 3 seconds each. Homogenates were chilled on ice for 3 seconds between each repeat to prevent RNA degradation. Debris was pelletted using an Eppendorf microcentrifuge 5415C (Brinkmann Instruments, Mississauga, ON, Canada) for 3 min at 5500 × g at 4ºC. Supernatants were transferred to a new Eppendorf tube and 200 µl chloroform (per 1 ml of Trizol) was added to the tube. After vigorous shaking for 15 seconds, samples were incubated for 3 min at room temperature and then were centrifuged at 9500 × g for 15 min. at 4ºC in the Eppendorf microcentrifuge. The upper aqueous phase including RNA was transferred to a fresh tube and RNA extraction and DNase treatment were followed using an RNeasy® minikit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's protocol. After the RNA was bound to the silica-gel-based membrane in the mini column, DNase I (total of 27 Kunitz units of DNase) was added to the column for

15 min at room temperature. After washing off the DNase, RNA was eluted from the RNeasy® Spin Column using 30 µl of RNase-free water and stored in the -80ºC freezer. The purity and yield of RNA was checked by spectrophotometry at 260/280 nm using a Beckman DU™ Series 60 Spectrophotometer (Beckman Instruments Inc, Fullerton, CA, USA). Briefly, 2 µl of RNA was diluted in 98 µl of distilled autoclaved water and its optical density was detected at 260 and 280 nm. RNA was run on an agarose gel (30-45 min) to check its quality and size distribution. 0.5 µg/ml of ethidium bromide (Sigma-Aldrich, Oakville, Canada) was added to a 1% w/v agarose gel to visualize RNA using a UV transilluminator. Two bands were detected for each RNA at 1.8 kb and 4.6kb corresponding to 18S and 28S eukaryotic ribosomal RNA (rRNA), respectively (Figure 2.1 A , B).

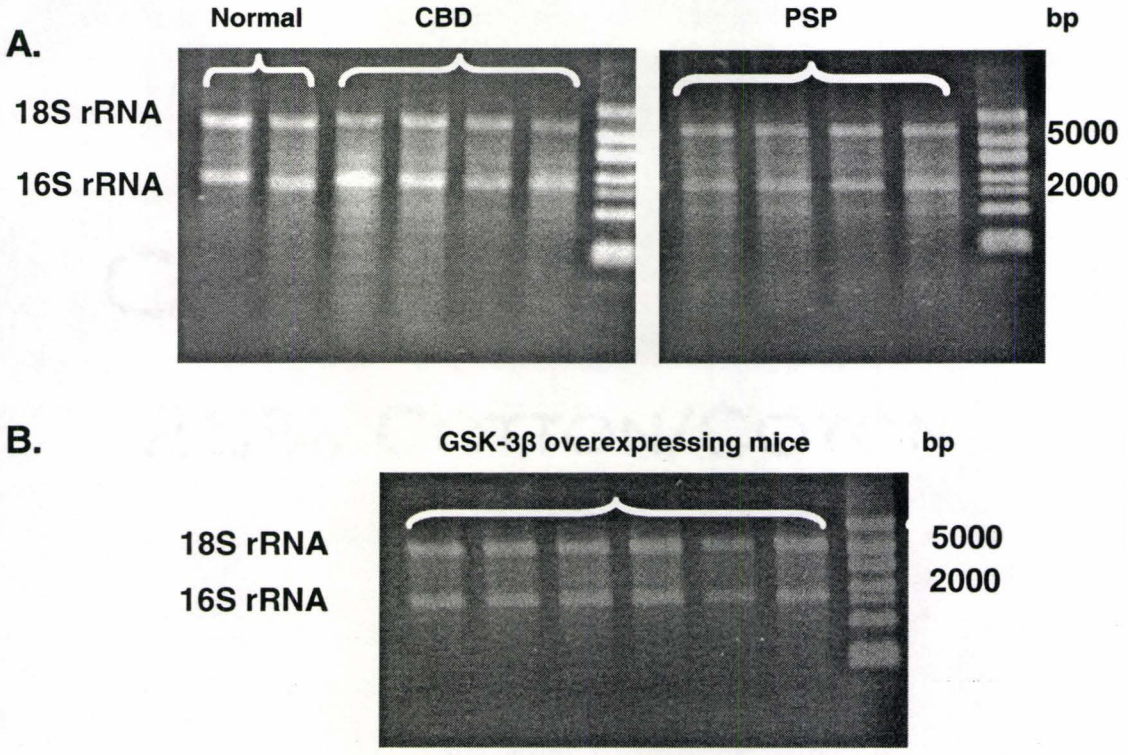



**Figure 2.1: Agarose gel electrophoresis for extracted RNA from tauopathy subjects showed that RNA was intact.** A. Representative photo of human tauopathy brain RNA. B. Representative photo of tau transgenic mice RNA.

### *2.3.3. Reverse transcription polymerase chain reaction (RT-PCR):*

1 µg of total RNA was reverse transcribed in a total volume of 20 µl using 200 units of Superscript™ II reverse transcriptase (M-MLV RT) (Invitrogen), 250 ng of random primers, 0.5 mM deoxynucleotide triphosphates (dNTPs), 1X first-strand buffer, 0.05mM dithiothreitol (DTT), and 2 units of RNaseOUT™. 1 µg of RNA was treated with water instead of the RT enzyme (No-RT) to serve as a control. Reactions were carried out for 10 min. at 25ºC, 50 min. at 42ºC, and 15 min. at 70ºC. All reagents at this step were provided from Invitrogen, Burlington, Canada.

### *2.3.4. Quantitative real-time polymerase chain reaction (qPCR):*

cDNA from 50 ng of RNA was incubated with 1X Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen), 300 nM forward and reverse primers (Mobix, McMaster University, Hamilton, Canada) and 30 nM ROX as a reference dye in the Stratagene MX3000P (Stratagene, La Jolla, CA, USA). As a negative (no-template) control, water (instead of RNA) was incubated with the same mixture used for cDNA. β-actin was used as a housekeeping gene to normalize the data. Primers for both BDNF and β-actin were designed in our lab (Forward primer for human BDNF: 5′-AAA CAT CCG AGG ACA AGG TG-3′, Reverse primer for human BDNF: 5′-AGA AGA GGA GGC TCG AAA GG-3′, Forward primer for human β-actin: 5′-CTC TTC CAG CCT TCC TTC-3′, Reverse primer for human β-actin: 5′-TGT TGG CGT ACA GGT CTT-3′, Forward primer for mouse BDNF: 5′-CAG CGG CAG ATA AAA AGA-3′, Reverse primer for mouse BDNF: 5′-TCA GTT GGC CTT TGG ATA-3′, Forward primer for mouse β-actin: 5′-CTG ACA GGA TGC AGA AGG-3′, Reverse primer for mouse β-actin: 5′-GAG TAC TTG CGC TCA GGA -3′). The product size for human BDNF was 249 bp and for β-actin was 85 bp. Mouse product sizes for BDNF and β-actin were 87 bp and 85 bp, respectively. Absolute quantification was used to determine the level of BDNF and β-actin.

For BDNF or β-actin standards, cDNA (complementary deoxyribonucleic acid) was amplified from 50 ng of reverse transcribed DNA product by regular PCR and using primers mentioned above and the following thermal profile: 94ºC for 2 min followed by 40 cycles of 95ºC for 15 s, 58ºC for 30 s and 72ºC for 30 s. PCR product was purified from an agarose gel using Qiagen MinElute™ gel extraction kit (Qiagen) according to the manufacturer's protocol. Purified product was quantified using spectrophotometry (A260/A280) using the following equation:

PCR product concentration (μg/μl) = (A260 × dilution factor × 50*)/1000.

*Optical density of 50 μg/ml of double-stranded DNA at A260 is equal to 1.

Concentration of PCR product was adjusted to 1ng/μl and copy number was calculated using following equation:

Copy number = [Concentration/ (product size (bp) × 660*)] × 6.022 $\times 10^{23}$•

*660 is the molecular mass for a double-stranded DNA base pair.

• Avogadro constant.

Six 10-fold dilutions of cDNA starting at $2.4 \times 10^6$ copies of human BDNF, $1.0 \times 10^7$ copies of human β-actin, $1.05 \times 10^7$ copies of mouse BDNF, and $1.1 \times 10^7$ copies of mouse β-actin were amplified to generate a standard curve. All samples and standards were run in triplicate. The thermal profile used for both β-actin and BDNF PCR was 2 min. at 50ºC and 2 min. at 95ºC followed by 40 cycles of 95ºC for 15 s, 58ºC for 30 s and 72ºC for 30 s. $R^2$ values were >0.998 and efficiencies were between 94 and 99% for all standard curves. A dissociation curve was run for each product after PCR amplification to confirm that no secondary product had formed. Stratagene MxPro™ software was used to calculate the mRNA levels as copies per ng of total RNA, and the levels of RNA were normalized as a ratio of BDNF/β-actin.

### *2.4. ProNGF protein quantification:*

#### *2.4.1. Sample preparation and protein extraction:*

Frozen tissues were weighed out in prechilled, sterile Eppendorf tubes. 40-90mg of parietal cortex tissue from tauopathies, 10-20mg cortical and hippocampal tissues from GSK-3β overexpressing mice, 20-60mg cortical tissue from JNPL mice, and 40-60mg of PiD transgenic mice were homogenized using a sonicator (Sonic Dismembrator Model 100, Fisher Scientific) in a 1:10 (tauopathies) 1:15 (JNPL and PiD mice), or 1:17 (GSK-3β overexpressing mice) ratio of tissue weight to volume of homogenization buffer [0.05 M Tris pH 7.5, 0.5% Tween 20, 10 mM ethylene diamine tetra acetic acid (EDTA), 2μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulphonyl fluoride (PMSF)]. Homogenates were kept on ice for 15 min and were centrifuged in the Eppendorf microcentrifuge for 15 min at 9500 × g at 4℃ to remove debris. Equal amounts of supernatants were assayed for total protein using the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). This assay is a colorimetric assay for protein concentration and shares the same basis with the known Lowry assay (Lowry et al., 1951).

#### *2.4.2. SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) and Western blotting using ECL:*

An equal amount of total protein from each sample was loaded on SDS-polyacrylamide gels. Proteins were separated by 12% SDS-PAGE at 80 volts for 30 min in the stacking gel and 120 volts for one hour in the resolving gel, transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Oakville, Canada) in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for 1.5 hours at 120 volts at 4°C, and blocked for 30 min. to 1 hour at room temperature in TBS-T (50 mM Tris, pH 8.0, 133 mM NaCl, 0.2% (v/v) Tween 20) with 5% (w/v) Carnation nonfat milk powder (Nestle, North York, Canada). Membranes were probed with primary antibody either against NGF (1:500 dilution) (affinity purified polyclonal rabbit anti-NGF, H-20, Santa Cruz

Biotechnology, CA, USA) or β-actin (1:10000) (mouse monoclonal anti-β-actin, Sigma, St Louis, MO, USA) overnight at 4°C. After washing 3 times in TBS-T (5 min. each wash), membranes were incubated in horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (for NGF) or sheep anti-mouse (for β-actin) IgG (1:5000) (Amersham) as a secondary antibody in TBS-T with 5% nonfat milk powder for 1 h at room temperature. Bands were visualized by ECL™ chemiluminescence (Amersham) on KODAK film (Kodak X-OMAT LS, Kodak, Vancouver, BC Canada). In human tauopathies, the same membrane used for proNGF Western blotting was reprobed with anti-β-actin to normalize the results. For mouse samples, separate gels were run for β-actin.

***2.4.3. SDS-PAGE and Western blotting using the Odyssey™ (infrared imaging) system:***

For one strain of transgenic mice (Pick's disease mice) and the cell lysates from the TrkA-MAPK activation assay (refer to section 2.5.10 in this chapter), Western blotting was carried out using the Odyssey™ system. The main difference between this system and previous Western blotting is its detection method (fluorescence instead of chemiluminescence). Gel percentage, times for running the gel and transferring proteins to the membrane were the same as mentioned above. The primary antibodies were H-20 for the PiD mice and affinity purified rabbit anti-NGF (MC51) for the cell lysates. Incubation and dilutions used were the same as before. However, the secondary antibody was IRDye® 680 conjugated goat polyclonal anti-rabbit IgG (LI-COR Biosciences, Lincoln, Nebraska, USA). The membrane was scanned at the appropriate channel (680nm wavelength) using an Odyssey infrared imaging system (LI-COR Biosciences).

***2.4.4. Antibody specifity:***

Rabbit anti-NGF (H-20) antibody (Santa Cruz) was blocked using 5 fold molar excess of H-20 peptide (Santa Cruz) for 2 hours at room temperature. 30 µg of homogenate from one PiD subject, one PSP subject and 0.5 ng of a

recombinant proNGF (all in duplicate) were run on a 12% SDS-PAGE gel. After transferring the proteins to the PVDF membrane, the membrane was cut into two halves in a way that each half included one set of the samples. One half was incubated with the blocked antibody and another half was treated with the regular antibody (H-20) which was kept at room temperature for 2 hours (the same as blocked antibody). The Western blots were continued as mentioned in section 2.4.2.

***2.4.5. Densitometry:***

Each Western blot contained a standard curve consisting of different amounts of total protein from a single sample common to all blots. The amount of protein for samples was chosen so that the intensity of the immunoreactive band fell within the linear range of the standard curve. The pixel value of the immunoreactive bands was determined by densitometery of films using an HP Scanjet scanner and Scion Image beta 4.01 software with local background subtracted. The standard curves were used to normalize pixel values between blots. Samples were analyzed 3 times in independent experiments. Western blots carried out by Odyssey system (LICOR) were analyzed using Odyssey software (LI-COR) measuring integrated intensity of a specified area and background subtracted using the median background method (refer to chapters 8 and 12 in user guide for Odyssey infrared imaging system, version 1.2). The sum of the intensity values for all pixels enclosed by a shape, multiplied by the area of the shape (counts/mm2) account for integrated intensity.

**2.4.6. Statistical analysis:**

For both Western blotting and real time PCR experiments, the average of 3 individual values per sample was used to calculate group means and standard errors of means, and between-group differences were analyzed by two-tailed Student's *t*-test (Excel 2000, Microsoft, Canada) or one-way analysis of variance (ANOVA) to determine the differences among more than two groups of subjects (SPSS 16.0.1 software). Tukey's test was used as a post-hoc test.

### *2.5. Expression of proNGFhis in a baculovirus–insect cell system:*

#### *2.5.1. Subcloning of proNGFhis into a baculovirus transfer vector:*

ProNGFhis cDNA in pcDNA3 (Figure 2.2A) (a transfer vector for mammalian expression systems) was obtained from Dr. B. Hempstead (Cornell Medical College, NY, NY). ProNGFhis was subcloned into the baculovirus transfer vector pVL1393 (Figure 2.2A) (BD Biosciences, Mississauga, Canada). Briefly, double restriction enzyme digestion with EcoR*I* and BamH*I* (Fermentas, Burlington, Canada) was carried out for proNGFhis in order to cut the proNGFhis out of pcDNA3 (Figure 2.2B). Baculovirus transfer vector (pVL1393) was also digested with the same enzymes to make the same sticky ends as the proNGFhis insert. Digestion was performed at 37°C for two hours using 1 unit of BamH*I* per 1 µg of DNA, 2 units of EcoR*I* per 1 µg of DNA, and 1X BamH*I* buffer (Fermentas). Both digested DNAs were run on a 0.8% agarose gel at 110 volts for about 45 min to one hour before extracting the DNA from the gel. Bands corresponding to proNGF or pVL1393 were cut from the gel and were extracted using a QIAquick® gel extraction kit (Qiagen, Mississauga, Canada). Ligation was carried out at 16°C overnight using T4 DNA ligase to insert proNGFhis into pVL1393 (Fermentas ligation kit). 2:1 molar ratio of insert DNA termini to vector DNA termini was used for ligation reaction [157.8 ng of proNGF (950bp) was added to 800 ng of pVL1393 (9632bp)].

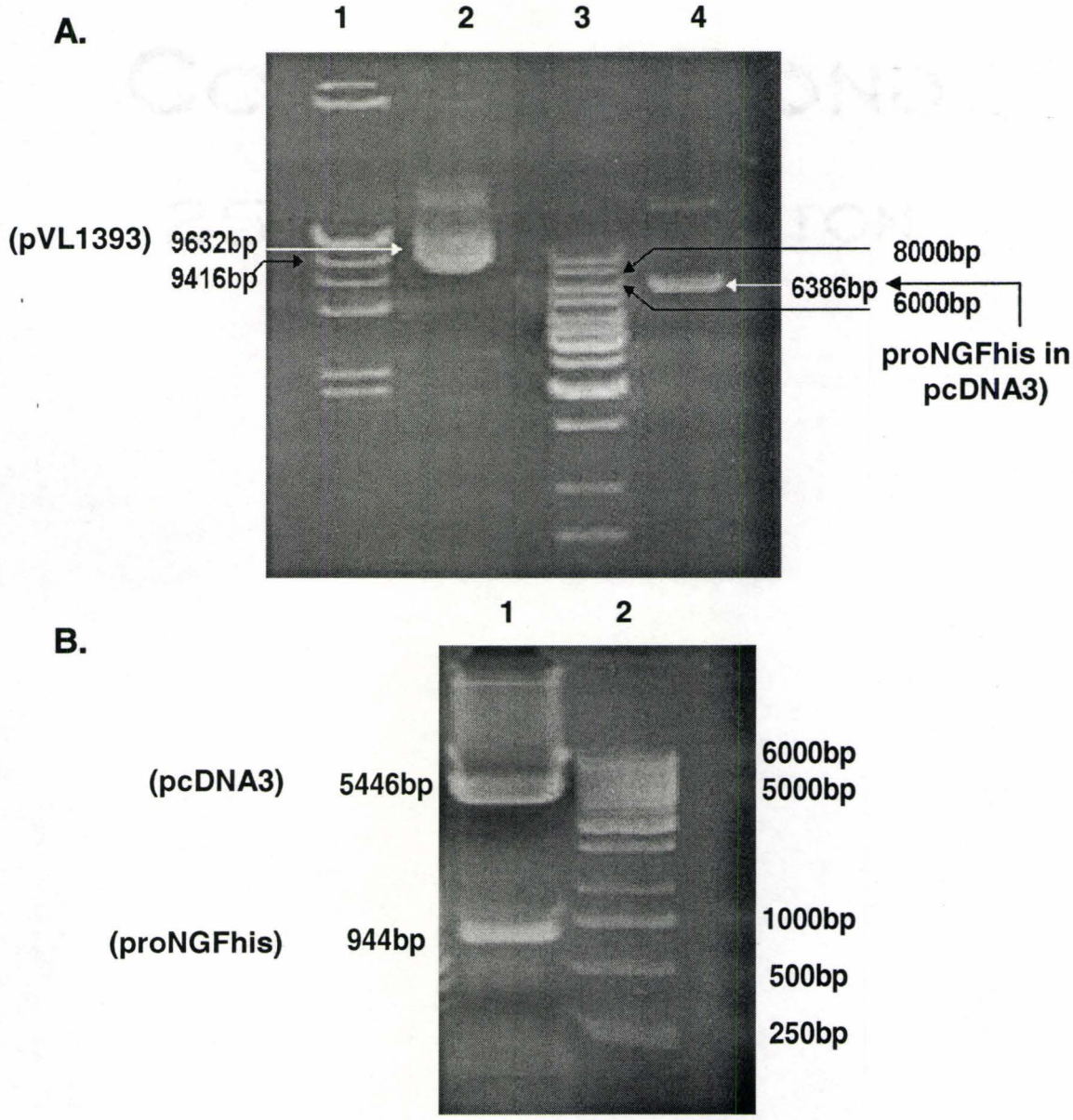



**Figure 2.2: A. Results of gel electrophoresis for pVL1393 and proNGFhis in pcDNA3 plasmid before digestion confirms the correct size for each plasmid.** 1. λ Hind*III* marker, 2. pVL1393 (9632bp), 3. 1kb Marker, 4. proNGFhis (994bp) in pcDNA3 (5446bp). B. Double restriction enzyme digestion (*EcoRI and* BamH*I)* cut the proNGFhis out of pcDNA3. 1. 944bp band represents proNGFhis and 5446bp band represents pcDNA3. 2. 1kb Marker.

### *2.5.2. Bacterial transformation and bacterial culture:*

ProNGFhis in pVL1393 was transformed into subcloning efficiency DH5α competent cells (Invitrogen) using heat shock bacterial transformation. Only transformed bacteria were able to form colonies in agar plates containing 50 µg/ml of ampicillin. Several colonies were picked and used for bacterial culture. Bacteria were cultured in Luria Bertani (LB) medium containing 100 µg/ml of ampicillin for 24 hours at 37°C.

### *2.5.3. Plasmid purification and sequencing:*

The plasmid was purified using alkaline lysis (SamBrook and Russell, 2001). Double restriction enzyme digestion (as mentioned above) was carried out to verify the size and direction of insert (Figure 2.3). The sequence (Figure 2.4) of the insert was confirmed by sequencing both strands (Mobix lab, McMaster University, Hamilton, Canada).

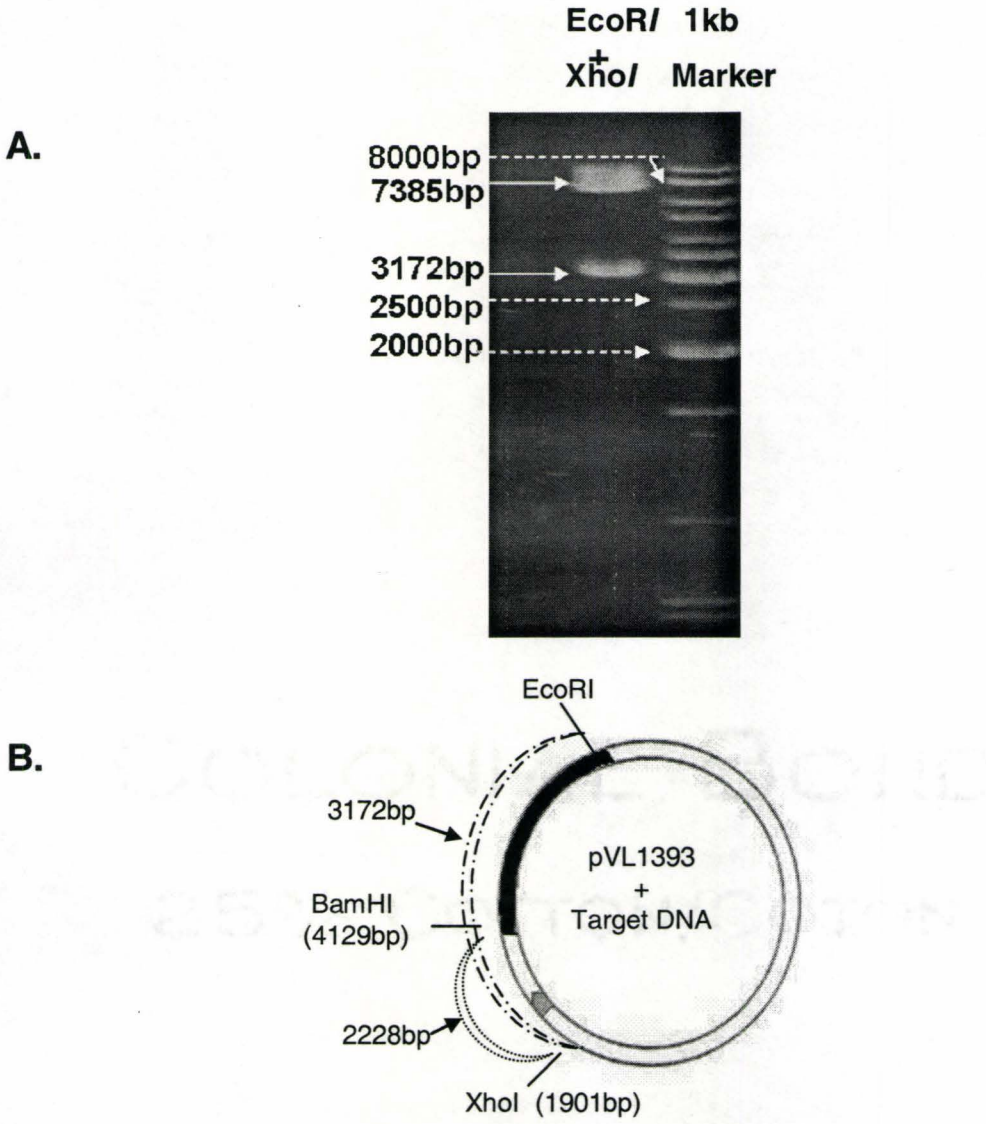



**Figure 2.3: Double restriction enzyme (EcoR*I and* Xho*I*) digestion confirms the correct direction for inserting proNGFhis into pVL1393.** A. Presence of two bands, one at 3172 bp position and another at 7385 bp position confirms the correct direction for the insert (wrong direction would also give two bands, one of which is 2228 bp). B. Schematic of the insert directions (2228 bp would be the result of wrong direction and 3172 bp is the fragment produced after double restriction enzyme digestion if the insert is in the correct direction).

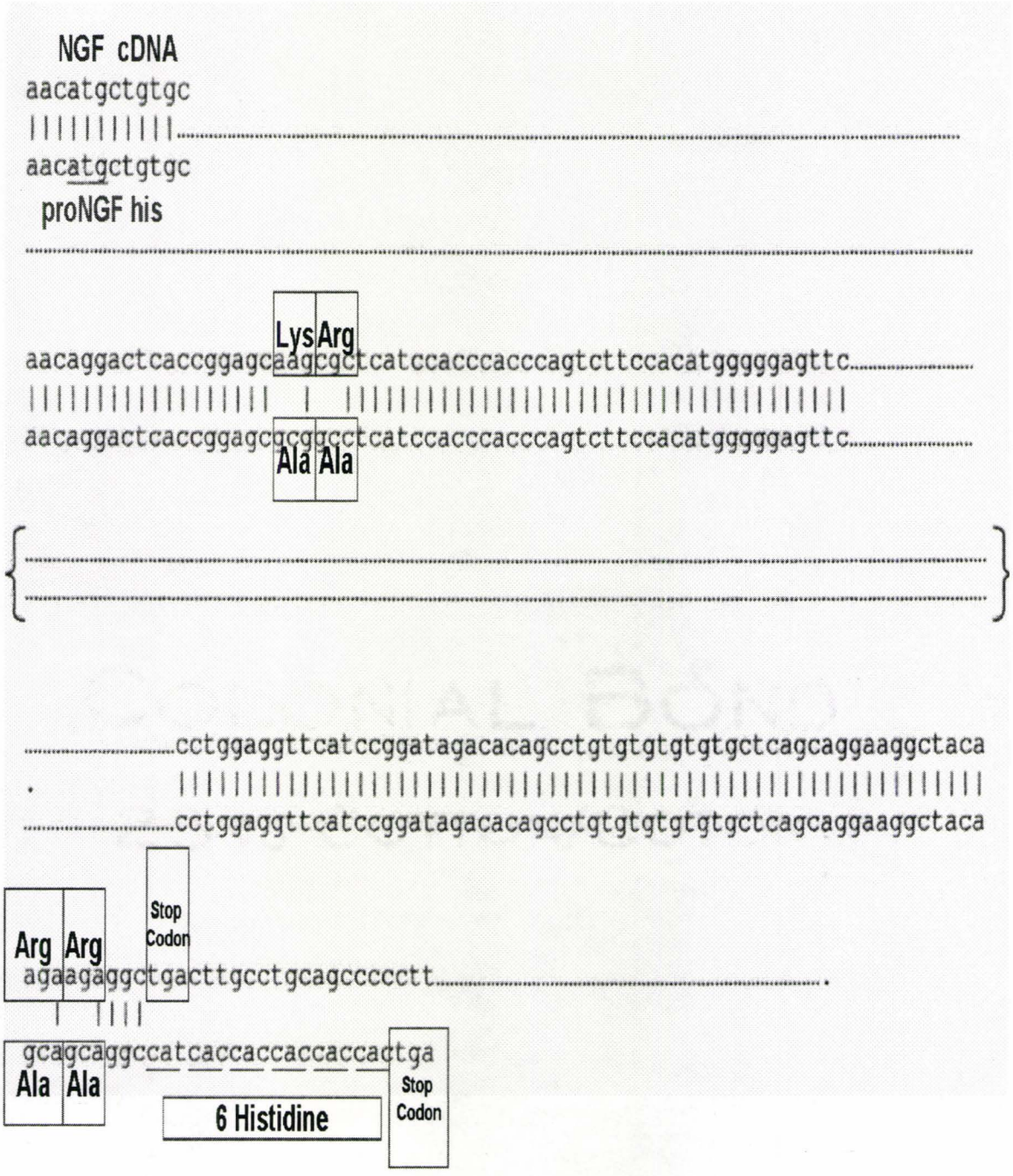



**Figure 2.4: Results of sequencing showed that proNGFhis is inserted into pVL1393 in the correct direction.** The sequence matched the proNGF sequence containing mutations and additions mentioned in Lee et al, (2001b).

### *2.5.4. Recombinant proNGFhis baculovirus, virus amplification and protein expression*:

Cotransfection of linearized baculovirus DNA (BD Biosciences, Mississauga, Canada) with pVL-proNGFhis into Sf9 insect cells (Invitrogen) was performed according to the manufacturer's instructions using a calcium precipitation kit (BD Biosciences). $2 \times 10^6$ cells were plated onto 60mm cell culture dishes (Falcon, BD Biosciences) to obtain 50-70% cell confluency in TNM-FH insect cell medium (BD Biosciences). A mixture of 0.5 µg of linearized baculovirus DNA, 2 µg of recombinant pVL-proNGFhis, and transfection buffer were added to the cells for 4 hours at 27°C. The transfection mixture was replaced with TNM-FH medium and the cells were incubated at 27°C. After 4 days, supernatant which includes recombinant virus was collected and stored at 4°C and used to infect more Sf9 cells for virus amplification.

In order to start the virus amplification from a single virus clone, recombinant proNGFhis baculovirus was plaque-purified by plaque assay. Briefly, $2 \times 10^6$ cells were plated onto 6-well plates (Falcon) for 1-2 hours at 27°C. Serial dilutions ($10^4$- $10^8$) of recombinant baculovirus in TNM-FH were added to the cells for 1 hour at 27°C to allow the infection to occur. Empty baculovirus was used as a positive control and cells grown only in medium were considered as a negative control. Following infection, the virus inoculum was replaced with 2 ml of 1% sterile agarose [Sea plaque, low melting point (Mandel, Cambrex Biosciences, East Rutherford, NJ, USA)] in TNM-FH and cells were incubated for 5 days at 27°C. 0.05% MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, Sigma-Aldrich] in 1% agarose was added as an overlay to the cells and incubated at 27°C for one more day. The MTT substrate is metabolized by live cells to a purple product, while dead cells are not able to metabolize the substrate and, in the area of dead cells (infected by the virus), form a white plaque. The plaque forming units (PFU)/ml of the original recombinant virus was calculated according to this formula: 1/dilution factor ×

number of plaques × 1/volume of inoculum/ well. A single plaque was picked up, dissolved in TNM-FH medium overnight and kept at 4°C for further virus amplification. For each amplification, Sf9 cells were incubated with virus for 3-5 days in Sf900 II serum free medium (Gibco, Burlington, Canada) and supernatant was collected when cells stopped dividing and started enlarging. In order to obtain a high titer of viral stock, four passages of virus amplification were carried out and the PFU of each passage was measured using the plaque assay. The fourth passage was used for protein expression with a multiplicity of infection of 0.5 (MOI=0.5).

In order to express proteins in a large volume, Sf9 cells were adjusted to grow in shaking culture. Wild type NGF (cleavable proNGF with no mutation and no histidine tag described in Fahnestock et al., 2004a), cleavage-resistant proNGF(R-1G) and wild type (empty) baculovirus (BD Biosciences) as controls were expressed in Sf9 cells at a MOI=0.5 in parallel with proNGFhis expression. All protein expressions were carried out in Sf900 serum free medium (Invitrogen Life Technologies) in a 27ºC shaking incubator at 120 rpm, and supernatants were harvested by centrifugation at 2000 rpm for 5 min on day 3 after infection. The highest amount of recombinant proNGF expressed by this method was over 100ng/ml (Concentration was determined by ELISA as described in Section 2.5.8).

### *2.5.5. Western blotting for wild-type NGF and both cleavage-resistant proNGFs:*

Western blotting using a primary antibody against the histidine tag (Cell Signaling Technology through New England Biolab LTD, Pickering, ON, Canada) was carried out for proNGFhis on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels according to the manufacturer's protocol. Western blotting was performed (as described in section 2.4.2.) using either affinity-purified rabbit anti-NGF IgG (MC51, a gift from Dr. Michael Coughlin, McMaster University) or a specific antibody against proNGF (a gift from Dr Xin-

Fu Zhou, Flinders University, Adelaide, Australia). The secondary antibody was horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (Amersham Biosciences, QC, Canada) for anti-NGF and anti-histidine tag primary antibodies, and HRP-conjugated donkey anti-sheep IgG (Sigma-Aldrich, Oakville, Canada) for the antibody against proNGF.

***2.5.6. Protein purification:***

Supernatant harvested from the protein expression was dialyzed (6000-8000Da cut-off) against 10 volumes of phosphate buffer (50 mM $NaPO_4$ and 0.5 M NaCl, pH=8) at 4ºC with three changes (twice for 3 hours and once overnight). Dialyzed material was bound to nickel beads (Invitrogen, Burlington, Canada) with shaking for 1 hour at room temperature. Beads were washed 4 times with washing buffer containing 20 mM imidazole in phosphate buffer pH=8, followed by elution with 250mM imidazole in phosphate buffer (50 mM $NaPO_4$ and 0.5 M NaCl, pH=8). Western blotting using antibodies against both the histidine tag and NGF confirmed the correct size for purified proNGFhis (34 kDa). The first three to four fractions (each fraction was about 1.5 ml) eluted from the beads were pooled. The yield for protein purification was about 30% (30% of total protein used for purification was recovered after purification).

***2.5.7. Sample preparation:***

Prior to use in neurite outgrowth assays or Trk activation assays, all samples were dialyzed against 10 volumes of RPMI 1640 (Invitrogen, Burlington, Canada) overnight at 4ºC with three changes (twice for 3 hours and once overnight). Concentrations were determined by NGF ELISA.

***2.5.8. NGF Enzyme-linked ImmunoSorbent assay (ELISA):***

A two-site sandwich ELISA was performed to determine the concentration of proNGFhis before bioassays. As a primary antibody, an affinity-purified rabbit polyclonal NGF antibody (MC-51) was diluted (0.2 µg/ml) in a coating buffer (50 mM $Na_2CO_3$-$NaHCO_3$, pH=9.6) and was used to cover wells in a Maxisorb 96-well ELISA plate (Nunc, Gibco Life Technologies). After 2 hours at 37°C, primary

antibody was replaced with blocking solution [1% (W/V) bovine serum albumin (BSA, Sigma) in coating buffer] for 30 min. After 3 times washing with washing buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, 0.1% (W/V) Triton X-100, 0.05% (W/V) $NaN_3$, pH= 7.0), serial dilutions of 2.5S NGF (as standard) and two dilutions (1/50, 1/500) of proNGFhis, (R-1G) proNGF, WT-NGF, and empty baculovirus were prepared. 100 µl of each dilution was added to each well, in triplicate. Samples were incubated at 4°C overnight in a humidified chamber. Dilutions were removed and wells were washed three times and then were incubated with secondary antibody (sheep anti-NGF antibody conjugated to β-galactosidase) (150 mU/ml) for 4 hours at 37°C. The secondary antibody was washed off using washing buffer (3 times) and substrate buffer (once) (100 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, 1% BSA, 0.1% (W/V) $NaN_3$, pH=7) and wells were incubated with the fluorescent substrate 4-methylumbelliferyl β-D galactoside (0.2 mg/ml in substrate buffer) (Sigma-Aldrich) at 37°C overnight. The reaction was stopped by 0.1 M glycine pH 10.3 and the CytoFluor II fluorescent microplate reader (Perseptive Biosciences, Framingham, MA, USA) was used to measure NGF-immunoreactive protein levels at 360/460 nM. Values were determined in pg/ml by comparison to a standard curve of purified 2.5S NGF at concentrations of 1000 pg/ml to 4 pg/ml.

***2.5.9. Neurite outgrowth bioassay in serum-free medium:***

Mouse superior cervical ganglia (SCG) from 1 day old pups were dissected and dissociated by Dr M.D. Coughlin as described before (Fahnestock et al., 2004a). Approximately 7000 SCG cells were cultured in collagen-coated 24-well plates in DMEM-F12 (Invitrogen, Burlington, Canada), 10% fetal bovine serum (FBS), $10^{-5}$ M uridine (Sigma-Aldrich) and $10^{-5}$ M 5-fluoro-2-deoxyuridine (Sigma-Aldrich) for two hours at 37°C and 5% $CO_2$. Attached cells were rinsed with DMEM-F12 and then incubated with 1-10 ng/ml (0.04-0.4 nM) of proNGFhis or 2.5S NGF diluted in serum-free medium [DMEM-F12, $10^{-5}$ M uridine, $10^{-5}$ M 5-fluoro-2-deoxyuridine, 0.5% penicillin/streptomycin (pen/strep) (Invitrogen) and

1% N2 supplement containing 1 mM human transferrin, 0.08 mM insulin, 0.002 mM progesterone, 10 mM putrescine, and 0.003 mM selenite (Invitrogen)] for 48 hours.

In addition, cells were treated with 0.2 nM of proNGFhis + 30 μM furin inhibitor [Decanoyl-RVKR-CMK (membrane permeable, Blakytny et al., 2004), Calbiochem, CA, USA] or 0.2 nM of 2.5S NGF + 30 μM furin inhibitor to determine whether the observed neurite outgrowth in cells treated with proNGF was from intracellular cleavage of proNGF by furin or from proNGF itself. After two days, cells with neurites twice the length of their cell body diameter were counted as neurite-bearing cells. At least 100 cells were counted per each well. Prism 3 (GraphPad Software 3.03, Inc, San Diego, CA, USA) was used to generate dose-response curves. For all experiments, including Western blotting, ELISA, and bioassay, 2.5 S NGF served as an internal standard and conditioned medium from insect cells infected with wild type (empty) baculovirus (WT baculovirus) was used as a negative control.

***2.5.10. TrkA-MAP kinase activation assay:***

$1\times10^6$ cells/well of rat pheochromocytoma (PC12) cells (ATCC) were plated in Cell+ 6-well plates (Sarstedt, Montreal, Canada) in RPMI 1640 containing 10% horse serum and 5% FBS for 24 hours, then medium was switched to RPMI with no serum for 24 hours. Serum-free medium was changed 2 hours before stimulation. Cells were stimulated by serial dilutions of wild type (cleavable) proNGF (WT NGF) (0.4-1 nM), proNGFhis (unpurified or purified) (0.4-1 nM), proNGF expressed in Escherichia coli (E.coli) (a generous gift from Dr David Dawbarn from Bristol University U.K) (1-200 nM), or 2.5S NGF (1-50 nM) for 5 min at 37°C and 5% $CO_2$. Cells were lysed using 150 μl/well of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylene glycol-bis (b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL each aprotinin, leupeptin, and pepstatin, 1 mM $Na_3VO_4$, and 1 mM NaF)

chilled on ice for 15 min. Lysates were centrifuged at 13,000 × g for 10 min at 4ºC. Supernatant was collected and total protein concentration was measured using the DC protein assay (BioRad Laboratories, Hercules, CA). 30 µg of total protein was loaded per lane on 10% SDS-PAGE gels for Western blotting using antibodies against phosphorylated p140TrkA (Cell Signalling Technology, Inc., Mississauga, Ontario, Canada), p44/42MAPK (anti-ERK1/2, Thr202/Tyr204; Cell Signalling Technology), total p44/42MAPK (Cell Signalling Technology), or total TrkA (Chemicon, Mississauga, Canada). All antibodies were used according to the manufacturer's protocols.

In some experiments as indicated, serum-free medium was supplemented with furin inhibitor for 2 hours before stimulation. Cells were stimulated with either proNGF in 30µM furin inhibitor or WT NGF in 30µM furin inhibitor for 5 min.

***2.5.11. Apoptosis assay*:**

Approximately 3000 dissociated SCG neurons from 1-day-old mouse pups were plated as described above for the neurite outgrowth assay in collagen-coated 96-well plates. After 24 hours, cells were rinsed with PBS and fixed in 4% paraformaldehyde (PFA) for 1 hour at room temperature. Cells were incubated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min. on ice and then were rinsed with PBS and incubated with 3% goat serum in 1% bovine serum albumin/phosphate buffered saline (BSA/PBS) as blocking solution for 30 min. at room temperature. After washing with PBS to remove blocking solution, cells were incubated with a 1:500 dilution (in 1% BSA/PBS) of neuronal anti-βIII-tubulin (TUJ1, Covance, CA, USA) overnight at room temperature. The primary antibody was rinsed away using PBS. Texas Red-labeled anti-mouse IgG (Cedarlane, Burlington, ON, Canada) was diluted 1:100 in TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) reaction mixture (Roche, Mississauga, Canada) and added to the cells for 1 hour at 37ºC. Cells were then rinsed with PBS and incubated with 1:10000 dilution (stock of 10 mg/ml in water) of Hoechst (bis Benzimide H33342

trihydrochloride, Sigma-Aldrich) in PBS for 5-15 min at room temperature. After washing, pictures of cells were taken using a high-resolution deconvolution widefield fluorescent microscope (Leica DMI 3000 B) at 20X magnification, and a Hamamatsu Orca ER-AG camera. The Acquisition Software used by this system was Volocity 4 and the filters were 377/50, 472/30, and 628/40 for Hoechst, TUNEL, and TUJ, respectively.

Cells stained with TUJ were considered as neurons. Neurons carrying outgrowth twice longer than the cell body diameter were counted [using a Leica DMI L fluorescent microscope (X- Cite® 120 Fluorescence illumination system) and 10X magnification] as neurons with outgrowth. At least 100 cells were counted for each plate, randomly.

***2.5.12. Primed apoptosis assay or NGF withdrawal assay:***

Dissociated SCG (prepared as above) were incubated in DMEM, 10% FBS, and 50 ng/ml (2 nM) 2.5S NGF for 6 days at 37°C and 5% $CO_2$. Medium containing 2.5S NGF was replaced every other day. The 2.5S NGF concentration was dropped to 10 ng/ml (0.4 nM) for 3 days. Cells were rinsed 5 times with medium without 2.5S NGF, and then cells were treated with 5-10 ng/ml (0.2-0.4 nM) of proNGFhis or 2.5S NGF diluted in serum-free medium (DMEM, $10^{-5}$ M uridine, $10^{-5}$ M 5-fluoro-2-deoxyuridine, 0.1% BSA, 0.5% pen/strep). After 32 hours, cells were rinsed and incubated with TUJ1 and TUNEL reaction mixture as described above.

***2.5.13. Serum deprivation assay using PC12 cells.***

$3\times10^4$ PC12 cells were plated in Cell+ 96 well plates (Sarstedt) in RPMI 1640 containing 1% pen/strep, 10% horse serum and 5% FBS for 48 hours. Then cells were rinsed with RPMI containing no serum to remove the serum. Cells were then incubated with 10 ng/ml (0.4 nM) of proNGFhis, WT NGF, 2.5 S NGF or medium alone. Dilutions were prepared using medium containing no serum, 1% pen/strep, 0.1% BSA, 0.5% N2 supplement, 30 µM aprotinin, 100 nM MMP-II inhibitor (Calbiochem), and 30 µM furin inhibitor (Calbiochem) for 48 hours. Cells

were fixed using PFA and stained with TUJ1 and Hoechst as described in Section 2.5.11.

***2.5.14. Determination of proNGF cleavage after each bioassay:***

For all bioassays including the neurite outgrowth assay, apoptosis assay, TrkA-MAPK activation assay, NGF or serum withdrawal assay, a sample of treatment media was run on a Western blot using antibody against NGF as described above to check the level of proNGF cleavage after each experiment. There was no or less than 1-2% cleavage in all bioassays reported here which confirms the role of unprocessed proNGF in each assay. In only one assay, the TrkA-MAPK activation assay + furin inhibitor, cell lysates were also run on Western bots using an NGF antibody to detect the level of proNGF cleavage inside the cells.

# CHAPTER 3

# RESULTS:

## *BDNF mRNA DECREASES IN CORTICOBASAL DEGENERATION*

It has been shown that BDNF is involved in learning and memory (Yamada et al., 2002) and in synaptic plasticity (Lu and Chow, 1999). BDNF is required for survival and function of neurons in several areas of the brain including hippocampus, cortex, and cholinergic basal forebrain (Ghosh et al., 1994, Gorski et al., 2003, Heldt et al., 2007). Levels of this neurotrophin are compromised in neurodegenerative diseases such as Alzheimer's disease. Reduction in BDNF levels correlates with cognitive impairment observed in this disease (Peng et al., 2005). Mechanism/s involved in the BDNF reduction are not known.

Aβ aggregation and tau hyperphosphorylation, as two major insults in AD, could underlie changes in the levels of BDNF. Aβ aggregation is involved in tau hyperphosphorylation (through GSK-3β activation) as an upstream factor in the cascade of events occurring in AD (Takashima, 2006, Ma et al., 2006). Moreover, it has been shown that an oligomeric form of Aβ is able to reduce BDNF mRNA levels in vitro (Tong et al., 2004, Garzon and Fahnestock, 2007). Therefore, aggregated amyloid can be responsible for changes in the level of BDNF in AD. However, it is not clear whether Aβ aggregation affects BDNF levels directly or through tau hyperphosphorylation. Tau dysfunction alone is enough to cause cognitive impairment (Spillantini et al., 1998). Moreover, lithium which prevents GSK-3β activation, increases BDNF levels in the brain. This evidence suggests that tau dysfunction may be involved in BDNF reduction in AD.

AD shares a common feature with a group of diseases named tauopathies (including PiD, CBD, and PSP) which exhibit tau abnormalities in certain areas of the brain. Most cases of tauopathies are associated with cognitive impairment and dementia either in early or late stages of the disease.

Moreover, no Aβ deposition has been reported in tauopathies. In order to study the role of tau abnormalities in the regulation of BDNF in AD and without amyloid-β interfering, tauopathies can be a good model. We hypothesize that tau dysfunction causes BDNF down-regulation in tauopathy. In this chapter, we compared the level of BDNF mRNA in tauopathies and normal brain. Real time qPCR was run on cDNA reverse transcribed from RNA extracted from parietal cortex of human tauopathy subjects. All samples were within the range of the standard curves for both BDNF and β-actin (Figure 3.1A, B). β-actin mRNA levels did not show any significant ($p=0.98$, one-way ANOVA) changes between groups studied in this research (Figure 3.2). Therefore, β-actin was used as a housekeeping gene to normalize the levels of BDNF mRNA for each sample (ratio of BDNF to β-actin). Results from dissociation curves for both BDNF and β-actin showed that no secondary products had formed (Figure 3.1C, D). There was a signal in the No-RT control for β-actin (Figure 3.1D) that appeared at lower temperatures. Because this signal appeared in later cycles of PCR and the number of copies was negligible in comparison to the copy number in the lowest standard, it was not a concern. One outlier from control and two outliers from CBD group were removed before statistical analysis (Figure 3.3).

As can be seen from Figure 3.4, there was a tendency for BDNF mRNA (normalized to β-actin) to decrease in all 3 groups of tauopathies (CBD, PiD, PSP) compared to control (normal brain). There was a significant effect of experimental groups ($p=0.05$, one-way ANOVA) after removing two outliers from the CBD group and one outlier from the control group (it was not significant before removing outliers, $p=0.3$, one-way ANOVA). Differences between groups were further analyzed using post hoc Tukey's test. Reduction in BDNF mRNA levels was not significant in either PiD ($p=0.2$) or PSP ($p=0.49$) compared to control. However, normalized BDNF mRNA levels showed a significant decrease in CBD ($p=0.04$) compared to control. There was a 71% (3.5-fold) decrease in

the ratio of BDNF to β-actin in CBD brain compared to control. This reduction was 53% or 2-fold in PiD and 34% or 1.5 fold in PSP.

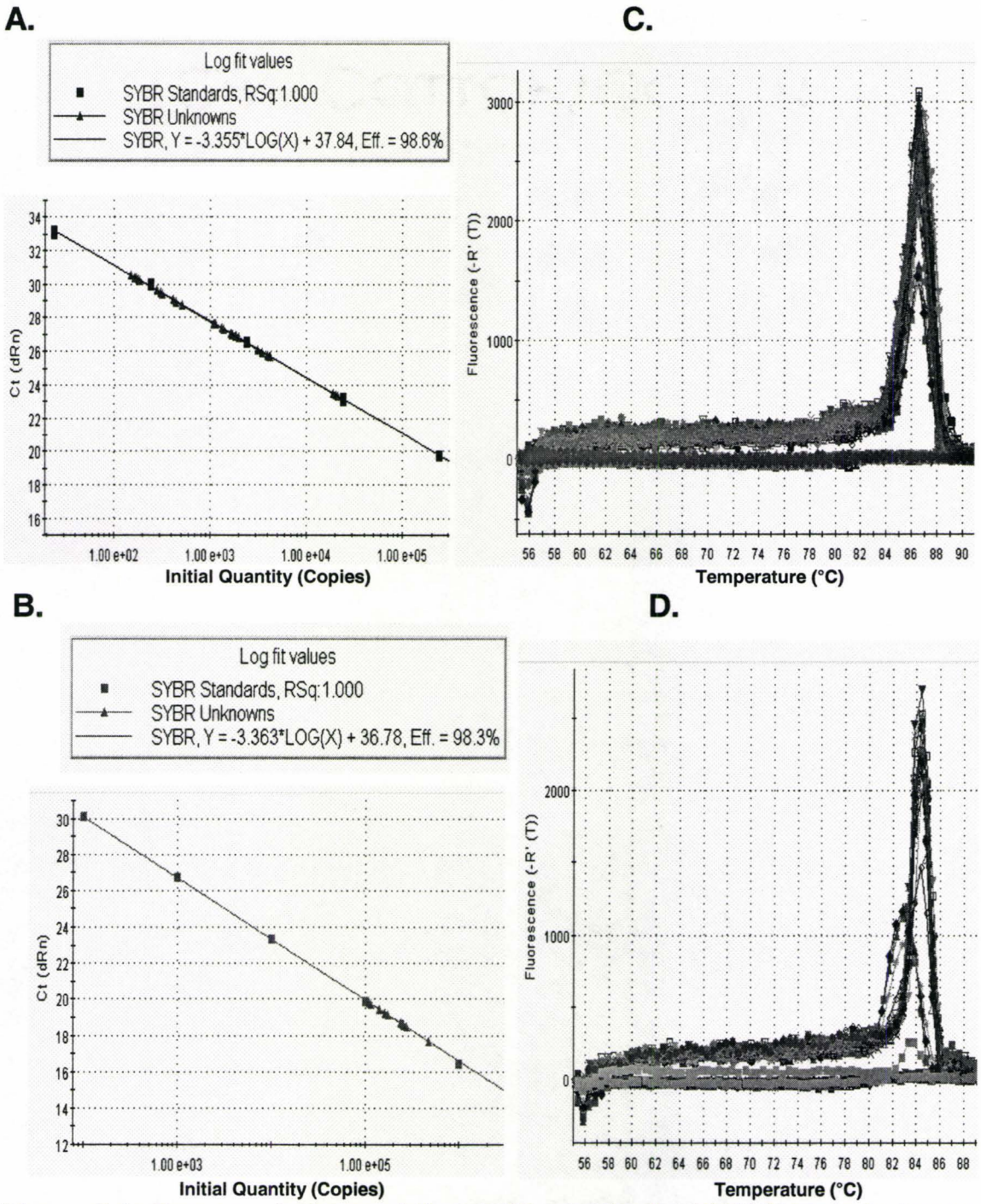



**Figure 3.1: Representative real time PCR graphs for BDNF levels in human tauopathies subjects.** A and B: $R^2$ values for standard curves for both BDNF and β-actin were >0.998 and efficiencies were between 94 and 99% and all samples were

within the range of the standard curve. Results from the dissociation curve for both BDNF (C) and β-actin (D) showed that no secondary products had formed.

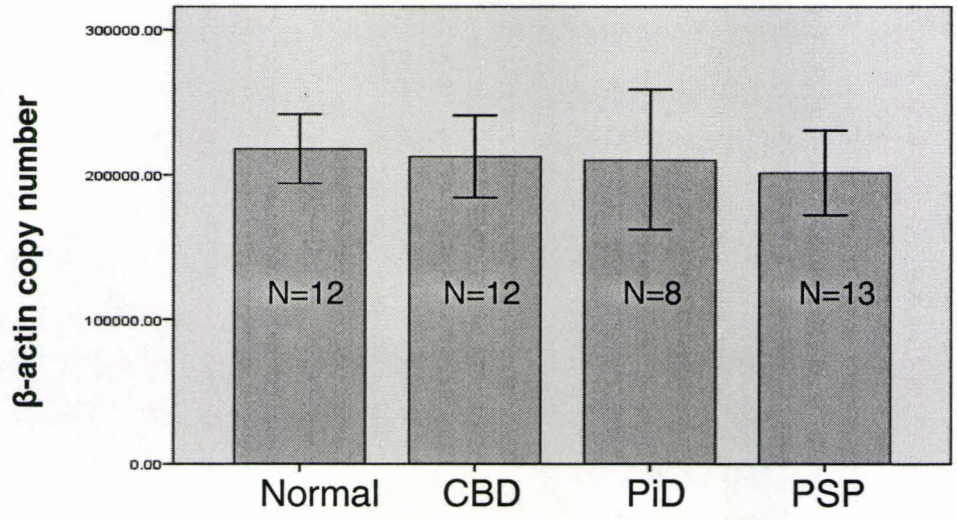



**Figure 3.2: No change in β-actin mRNA levels in tauopathy subjects.** There was no significant difference in β-actin mRNA levels between the groups. Therefore, this housekeeping gene (β-actin) was used to normalize the level of BDNF mRNA. All samples were analyzed in triplicate. Error bars represent standard error of the mean (S.E.M).

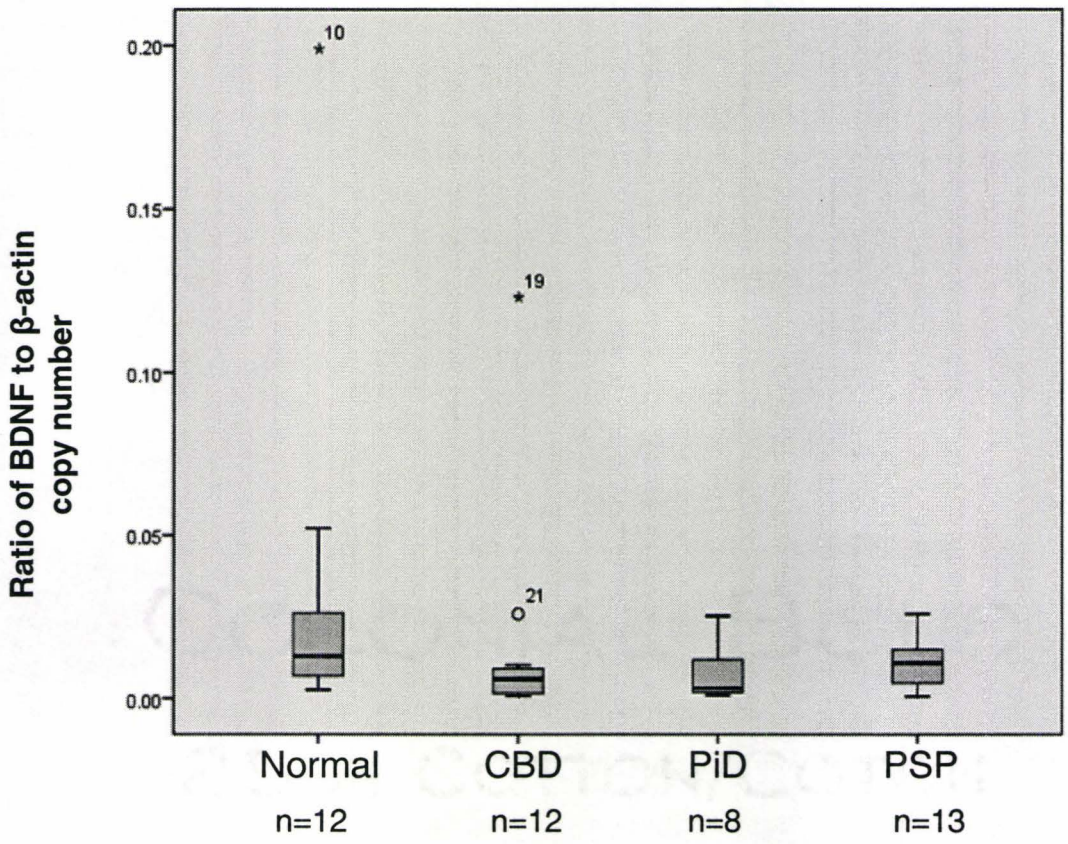



**Figure 3.3: Box plot of the ratio of BDNF mRNA to β-actin mRNA in tauopathies** (SPSS 16.0, SPSS Inc, Chicago, USA was used to determine the outliers). The first and last horizontal lines in each box (from the top) are the highest and lowest data values. The thick black line inside the box is the median. The top and bottom of the box are upper and lower quartiles. The upper quartile is the median of the first 50% of the data. The lower quartile is the median of the last 50% of the data. All samples were analyzed in triplicate.

°= Mild outlier: 1.5-3 IQR (interquartile range) above upper quartile.

*= Extreme outlier: 3 IQR above upper quartile.

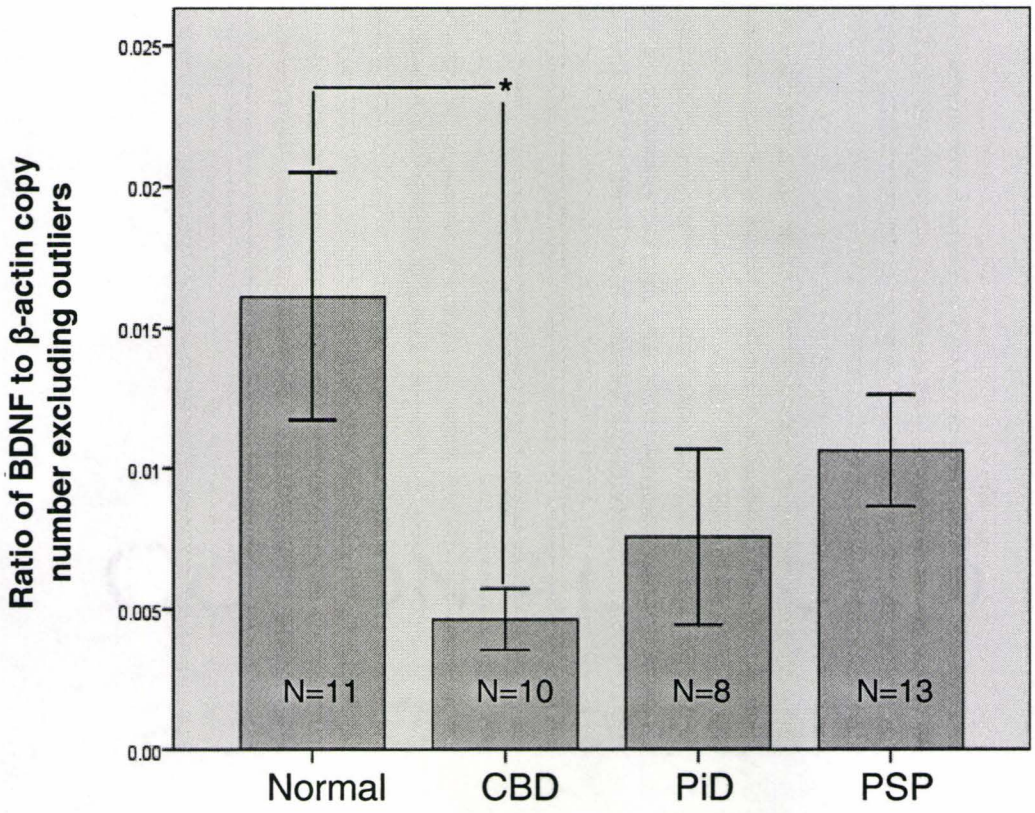



**Figure 3.4: Significant decrease in the normalized level of BDNF mRNA in CBD.** BDNF mRNA was normalized to β-actin. Outliers were removed from control and CBD groups. All samples were analyzed in triplicate. Error bars represent standard error of the mean (S.E.M).

*= Significant decrease ($p=0.04$, post hoc Tukey's test) was observed in the normalized level of BDNF mRNA compared to control.

### *3.2. Tau transgenic mice do not show BDNF reduction.*

Several lines of tau transgenic mice (cortex or hippocampus) were tested in this study to determine the level of BDNF mRNA in their brain. qPCR was run to measure the absolute amount of BDNF and β-actin in each transgenic line. All samples were within the range of the standard curves for both BDNF and β-actin (Figure 3.5 A, B). Results from the dissociation curve for both BDNF and β-actin showed that no secondary products had formed (Figure 3.5C, D).

#### *3.2.1. No significant decrease in the level of BDNF mRNA in hippocampus of transgenic mice overexpressing GSK-3β:*

Tau hyperphosphorylation was detected in hippocampus of transgenic mice overexpressing GSK-3β (Lucas et al., 2001). Therefore, we measured the level of BDNF mRNA in hippocampus of these transgenic mice. We could not detect any significant decrease in BDNF mRNA levels in hippocampus of transgenic mice overexpressing GSK-3β compared to wild type littermates (Student's *t*-test p=0.17) (Figure 3.6A). As β-actin indicated no difference (Student's *t*-test p=0.25) between wild type and transgenic mice (Figure 3.6B), levels of β-actin expression were used to normalize BDNF mRNA levels. After normalization against β-actin, there was still no difference between transgenic and wild type mice (Non-parametric Mann-Whitney test, p=0.88) (Figure 3.6C) (see discussion for more detail).

#### *3.2.2. Transgenic mice expressing human tau did not show any reduction in the BDNF levels.*

Two different lines (tau22 and tau30) of transgenic mice expressing 4R-mutated human tau were used to measure the level of BDNF in their cortices. Tau22 mice display tau hyperphosphorylation in hippocampus and cortex while tau30 mice show more tau dysfunction in spinal cord (Schindowski et al., 2006). β-actin levels did not show any difference in either strain [Student's *t*-test p= 0.49 for tau22 mice (Figure 3.7B) and p=0.45 for tau30 mice (Figure 3.8B)]. Therefore, β-actin was used as an internal control. No difference was detected for BDNF

mRNA levels [Student's *t*-test p=0.17 for tau22 mice (Figure 3.7A) and p=0.34 for tau30 mice (Figure 3.8A)] or normalized BDNF levels [Non-parametric Mann-Whitney test, p=0.29 for tau22 mice (Figure 3.7C) and p=0.9 for tau30 mice (Figure 3.8C)] (see discussion for more detail).

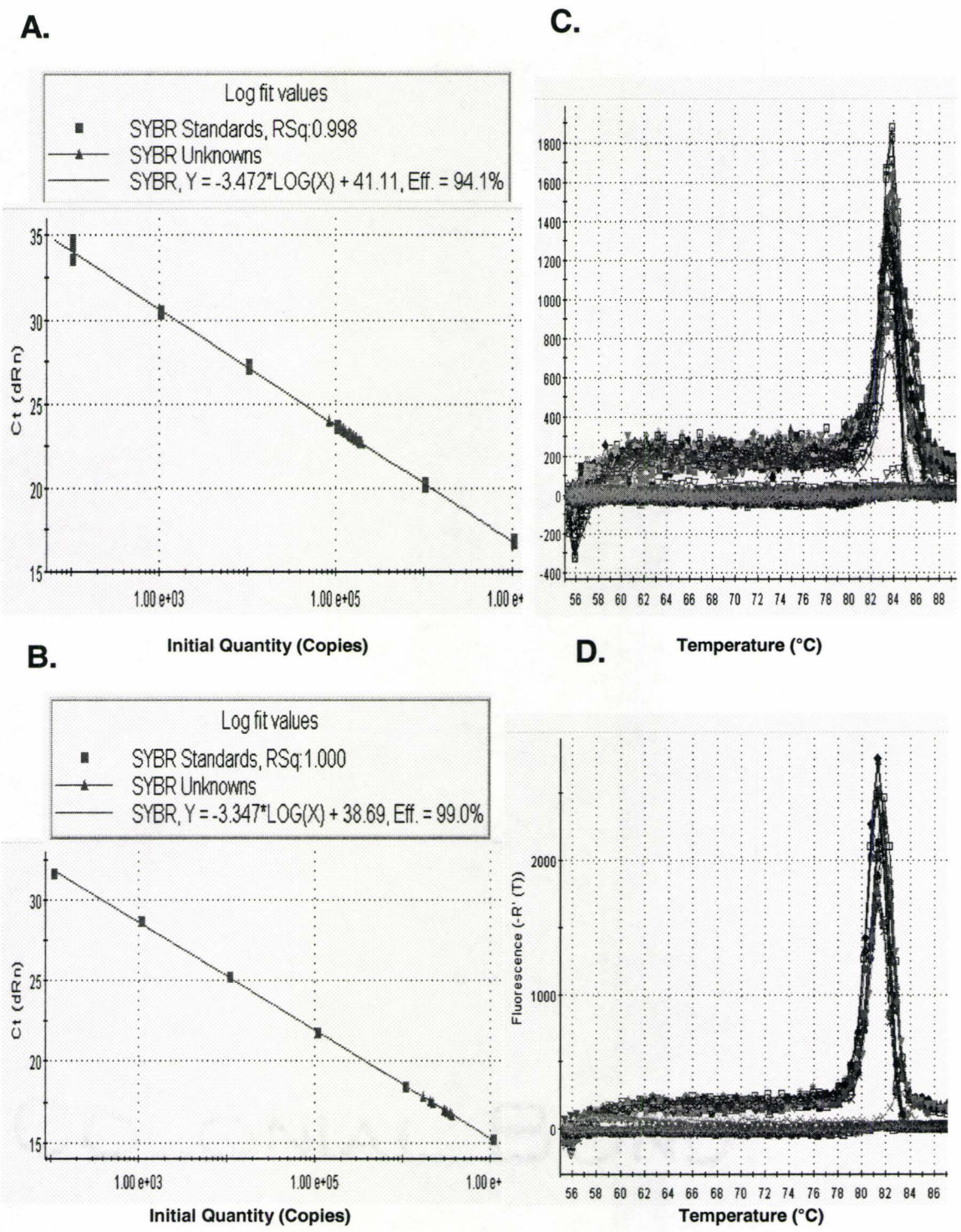



**Figure 3.5: Representative real time PCR graphs for BDNF levels in transgenic mice.** A and B: $R^2$ values for standard curves for both BDNF and β-actin

were >0.998 and efficiencies were between 94 and 99% and all samples were within the range of the standard curve. Results from the dissociation curve for both BDNF (C) and β-actin (D) showed that no secondary products had formed.

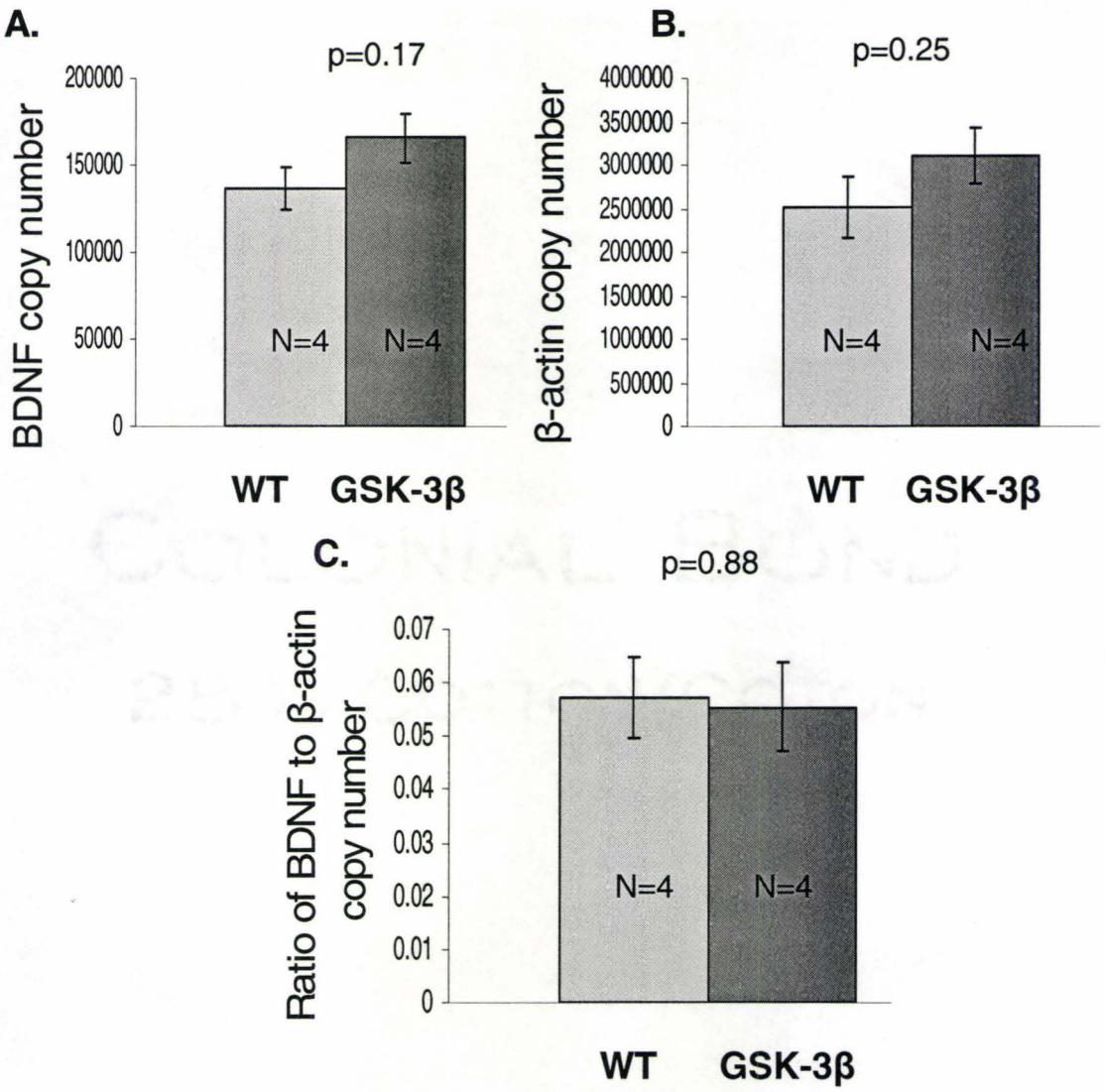



**Figure 3.6: No change in the level of BDNF RNA (normalized to β-actin) was detected in left hippocampus of transgenic mice overexpressing GSK-3β compared to wild type littermates.** A. BDNF mRNA copy number in GSK-3β and WT mice. B. As β-actin mRNA copy number did not show any difference between transgenic and WT mice, level of this housekeeping gene expression was used to normalize BDNF levels. C. Comparison of BDNF ratio to β-actin in GSK3β-overexpressing and WT mice. All samples were analyzed in triplicate. Error bars represent standard error of the mean (S.E.M).

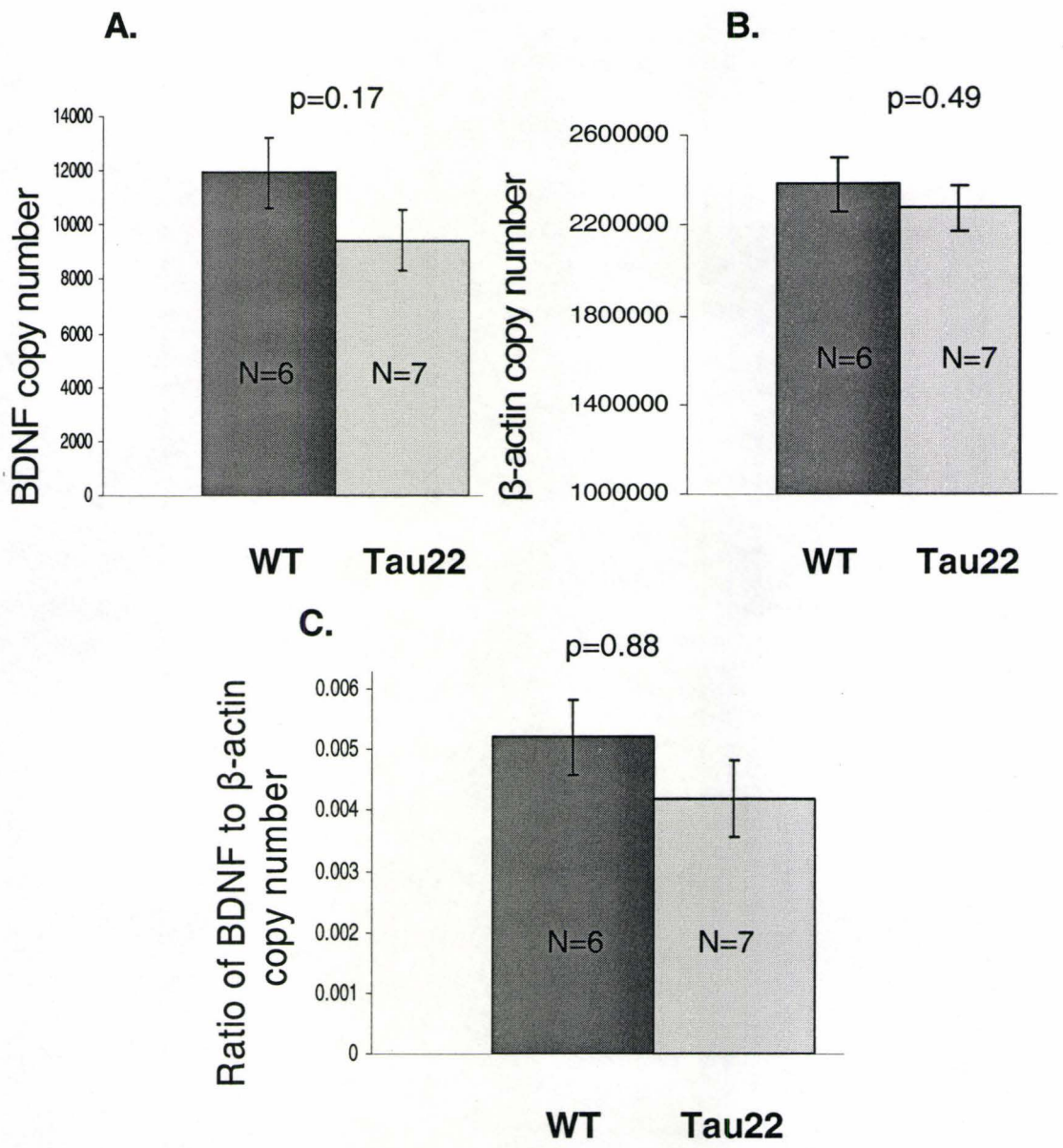



**Figure 3.7: Level of BDNF mRNA (normalized to β-actin) indicated no significant reduction in cortex of transgenic mice expressing human tau gene compared to wild type littermates.** A. BDNF mRNA copy number in tau22 and WT mice. B. As β-actin mRNA copy number did not show any difference between transgenic and WT mice, this housekeeping gene was used to normalize BDNF levels. C. Ratio of BDNF to β-actin was compared in tau22 and WT mice. All samples were analyzed in triplicate. Error bars represent standard error of the mean (S.E.M).

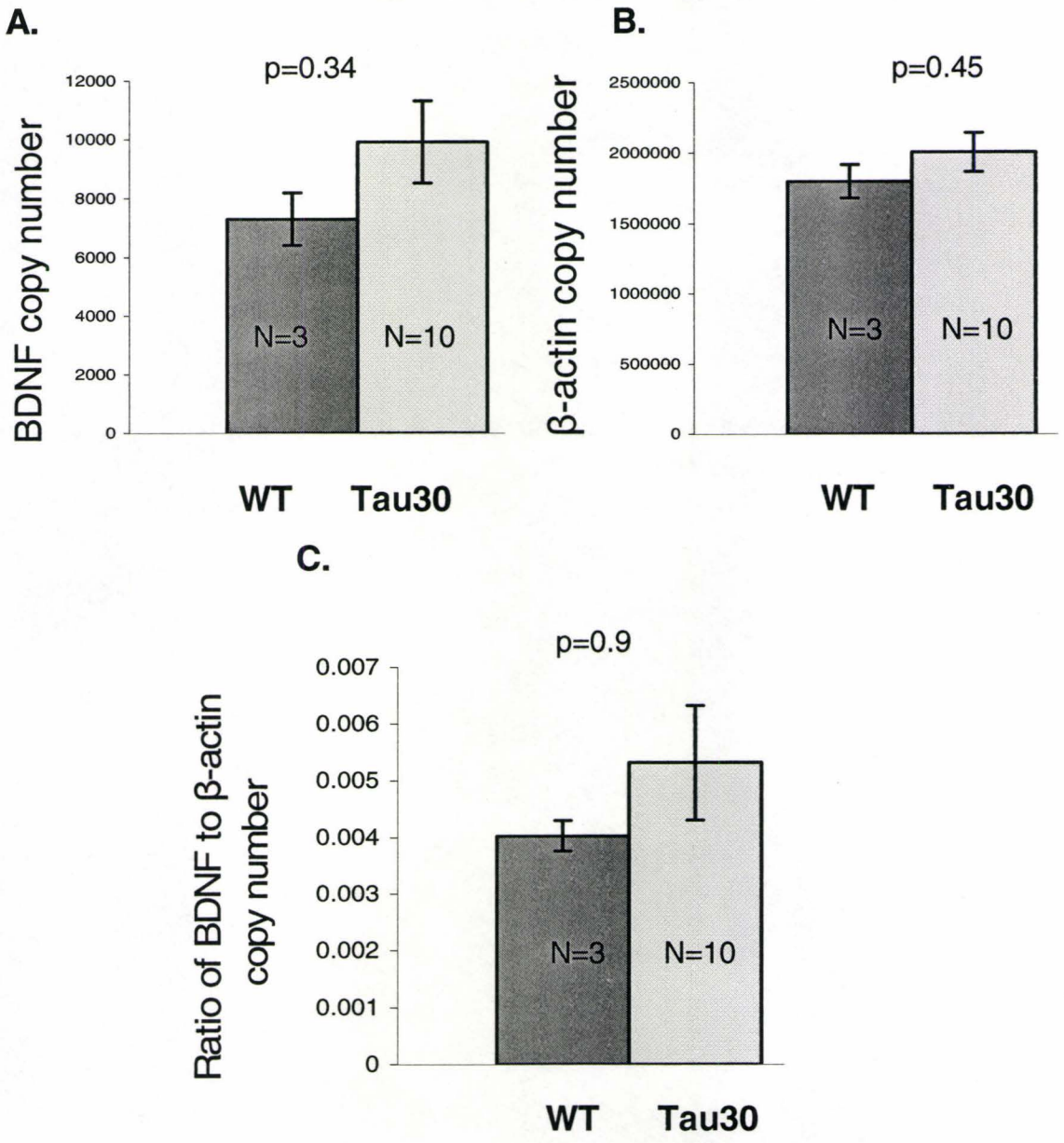



**Figure 3.8: There was no change in the level of BDNF mRNA (normalized to β-actin) in cortex of transgenic mice expressing human 4R-tau (tau30) compared to wild type littermates.** A. BDNF mRNA copy number in tau30 and WT mice. B. β-actin mRNA copy number was used to normalize BDNF levels as it did not show any difference between transgenic and WT mice. C. Comparison of BDNF ratio to β-actin between tau30 and WT littermates. All samples were analyzed in triplicate. Error bars represent standard error of the mean (S.E.M).

## SUMMARY (CHAPTER 3)

In this chapter, we have shown that BDNF mRNA levels exhibited a significant reduction in the parietal cortex of CBD compared to normal brain. PiD and PSP also showed a trend towards a reduction in the level of this neurotrophin in their parietal cortices. However, this reduction was not statistically significant. The limited number of samples per group and heterogenity in tauopathy disease could affect our results for PSP and CBD. None of the transgenic animals used for this study showed BDNF reductions. Again, the limited number of samples (3-4 animals/group for GSK-3β study or 3 animals older than 6 months in tau22 mice study) and also variety in age of samples could be reasons for lacking significant differences in this part of the research (see discussion for more detail).

Reduction in BDNF levels in CBD may support the idea that tau dysfunction could affect the level of this neurotrophin in AD brain. However, the results need to be confirmed and extended with more patient tissue before we can conclude with certainty that CBD parietal cortex has reduced BDNF mRNA levels.

# CHAPTER 4

# RESULTS:

## *PRONGF PROTEIN INCREASES IN PICK'S DISEASE BRAIN.*

NGF is produced as 32-34 and 25 kDa precursors (proNGF) (Darling et al., 1983) which undergo post translational modification to produce mature NGF (Greene et al., 1968, Seidah et al., 1996). ProNGF (NGF precursor) is expressed in many tissues and is the predominant form of NGF in the brain of human, mouse and rat (Fahnestock et al., 2001, Pedraza et al., 2005). Moreover, in AD brain, it is proNGF which increases two fold over normal levels in cortical tissue with almost no mature NGF detectable (Peng et al., 2004, Pedraza et al., 2005). Tau dysfunction has been shown to interrupt axonal transport which can lead to accumulation of unused proteins in the target tissues. It has been suggested that increased proNGF levels in AD is the result of impaired retrograde transport in specific neurons bearing tau dysfunction. Increase in proNGF levels in AD correlates with the level of dementia observed in this disease (Peng et al., 2004). As other factors such as amyloid-β and/or decreased TrkA can also be involved in transport impairment, studying proNGF levels in tauopathies other than AD and without amyloid-β interfering may shed light on the mechanism underlying increased proNGF levels in AD. To investigate whether proNGF levels change in tauopathies other than AD, we tested the levels of proNGF (32-34kDa proNGF) protein in human postmortem brain tissue (parietal cortex) of tauopathies (PiD, PSP, CBD) versus control using Western blotting.

### *4.1. Sensitivity of primary antibody for NGF:*

The primary antibody used for NGF detection (H-20) indicated 3 immunoreactive bands (34kDa, ~53kDa, ~80kDa) on Western blot for NGF with no mature NGF (13kDa) detectable (Figures 4.1A and 4.1C). In some cases there was an extra band of 24 kDa as well. All samples were within the range of the standard curve (Figures 4.2A, B and 3A, B, 34kDa proNGF and β-actin). To ensure all bands are specific for NGF, a blocking experiment was carried out

using NGF peptide obtained from Santa Cruz (the same company providing H-20 antibody). The 53kDa band did not disappear when primary antibody was blocked with 5-fold molar excess of NGF peptide for 2 hours (Figure 4.4A). All other bands (including strong 34kDa bands, 24kDa and 80kDa bands) disappeared during this blocking experiment. Therefore, 80kDa, 34kDa, 24kDa bands were considered as proNGF and used for statistical analysis. 80kDa band could be a dimer of glycosylated proNGF [glycosylated proNGF is 40kDa, (reviewed in Al-Shawi et al., 2007)] and 24kDa band could be the result of 34kDa cleavage. The blocking result showed that the 53 kDa immunoreactive band is a nonspecific band. Moreover, different lots of H-20 antibody (Santa Cruz), did not detect the 53 kDa immunoreactive band (Figure 4.4B), confirming that this band is nonspecific. Therefore, we excluded the 53 kDa band from statistical analysis.

The level of β-actin protein (as a housekeeping gene) was unchanged within the groups (Figure 4.5) ($p=0.6$, one-way ANOVA) and used to normalize proNGF levels.

***4.2. ProNGF levels increased in PiD parietal cortex:***

Equal amounts of total protein (30 μg) from each homogenate were run (in three independent experiments) on Western blots using an antibody against NGF. One outlier was removed from the control group (Figure 4.6). There was a significant effect of experimental groups ($p=0.007$, one-way ANOVA) in the level of 34kDa proNGF (normalized to β-actin). Differences among groups were further analyzed using post hoc Tukey's test. A significant increase was detected in the level of 34kDa proNGF normalized to β-actin in PiD compared to control ($p=0.036$) (Figure 4.7). This increase corresponds to what was described previously in AD brain (Fahnestock et al., 2001). The 24kDa band was detectable in some cases of PiD and control only. This band was probably produced through cleavage of 34kDa proNGF and was not used for separate statistical analysis. A combination of 34 kDa and 24 kDa bands did not show any significant increase in the ratio of proNGF to β-actin ($p=0.13$, non-parametric Mann-Whitney test) in

parietal cortex of PiD brain in comparison to control (Figure 4.8A). There was no change in the level of normalized 80 kDa NGF immunoreactive band in any of the tauopathy groups (p=0.6 one-way ANOVA) (Figure 4.8B).

The ratio of proNGF (34kDa) to β-actin did not show a significant difference (p=0.9, Tukey's test) in parietal cortex between PSP and normal brain (Figure 4.6). However, there was a significant decrease in the level of proNGF in PSP compared to PiD (p=0.006, Tukey's test) (Figure 4.6).

CBD subjects did not show any change in the level of normalized 34 kDa proNGF (p=0.79, Tukey's test) in comparison to normal brain (Figure 4.6).

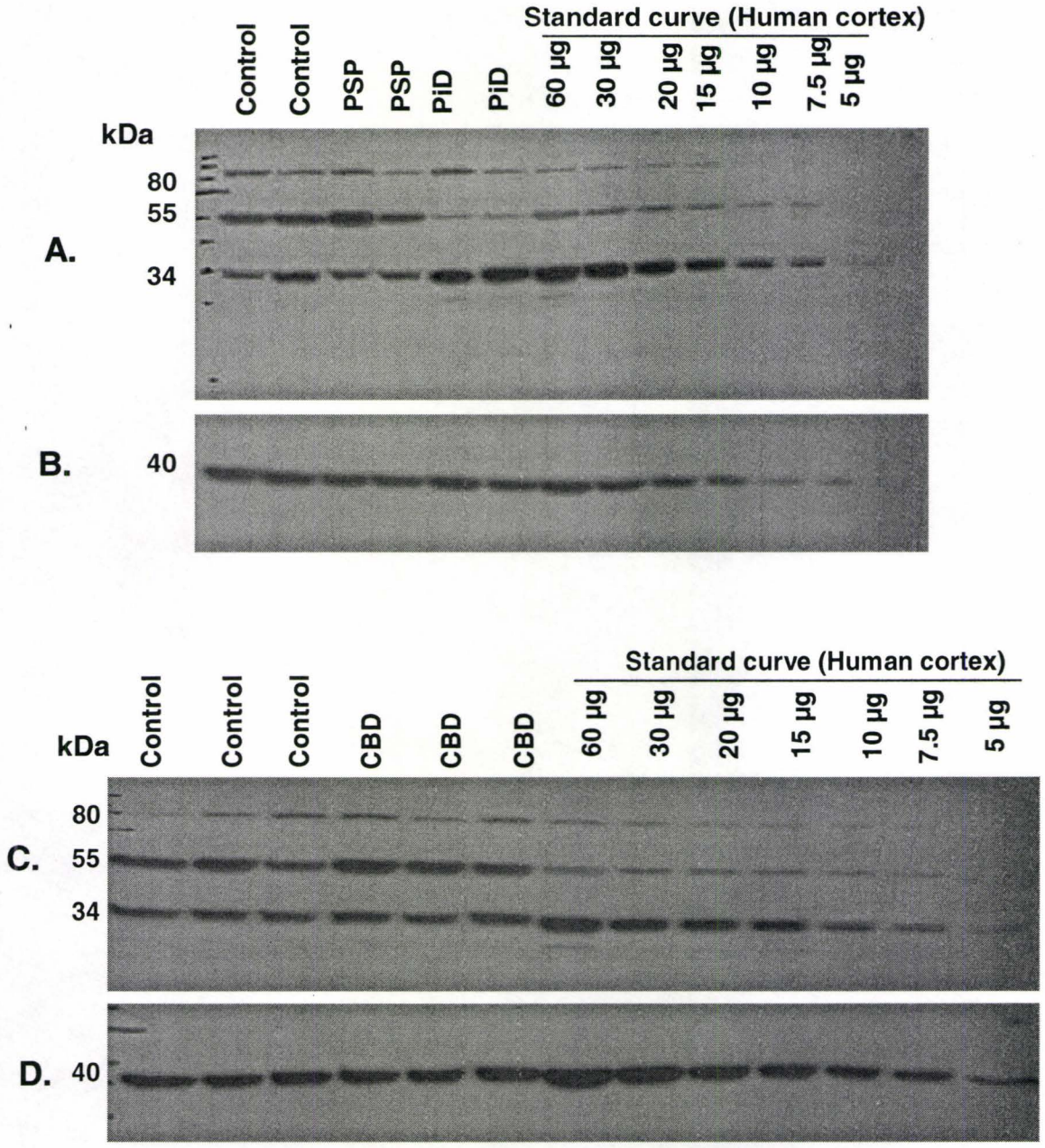
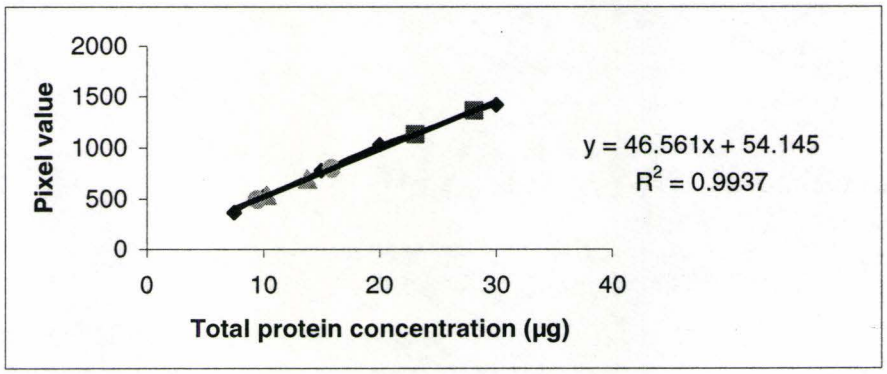



**Figure 4.1: Representative Western blot [NGF antibody (H-20) and β-actin antibody] carried out on tauopathy parietal cortex homogenates.** 30 µg total protein of each sample was loaded. Blots were probed with NGF antibody (H-20) (A and C) and reprobed with β-actin antibody (B and D). The gels are representative of 3 independent experiments.

**A.**




**B.**

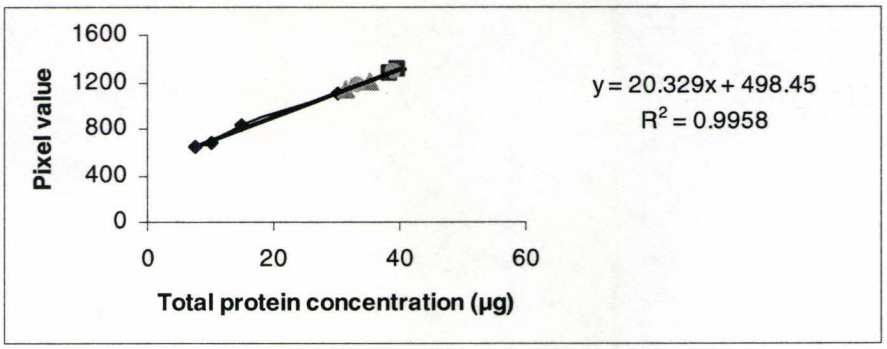
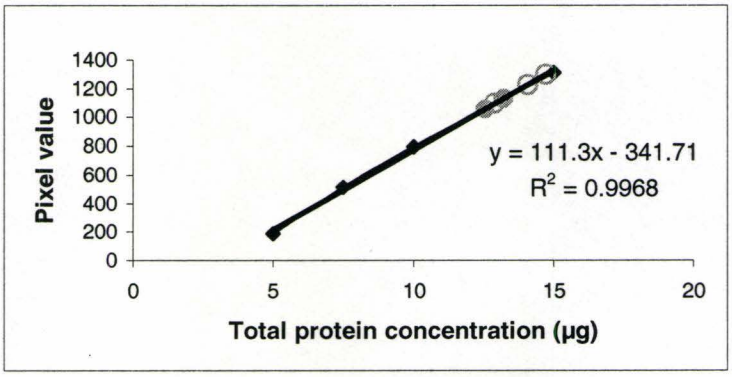



**Figure 4.2: All samples were within the range of the standard curve (small solid diamonds) in Western blotting using NGF and β-actin antibodies.** A. proNGF levels in PiD (square), PSP (triangle) and normal brain (circle). B. β-actin levels in PiD (square), PSP (triangle) and normal brain (circle). Pixel values were determined by densitometry.

A.




B.

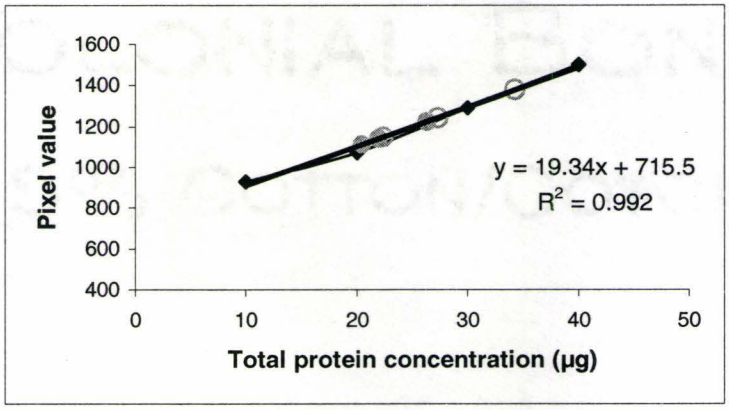



**Figure 4.3: All samples were within the range of the standard curve (small solid diamonds) in Western blotting using NGF and β-actin antibodies.** A. proNGF levels in CBD (empty circle) and normal brain (solid circle). B. β-actin levels in CBD (empty circle) and normal brain (solid circle). Pixel values were determined by densitometry.

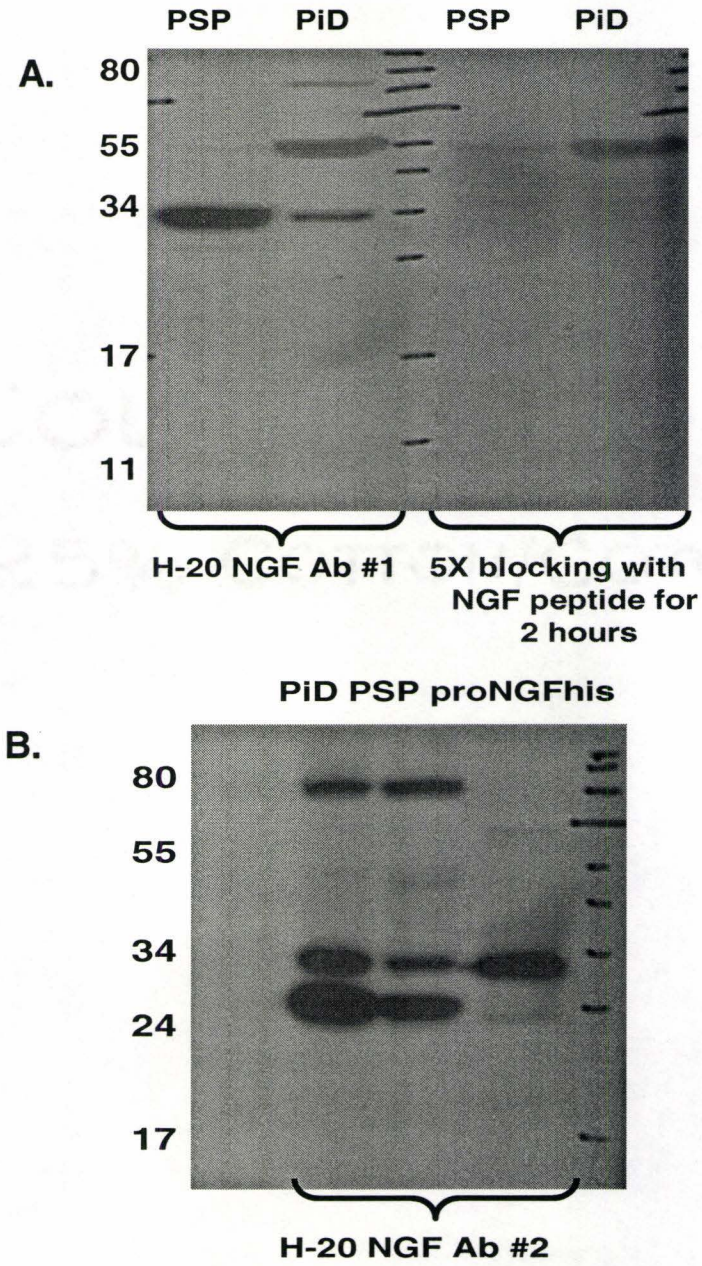



**Figure 4.4: Sensitivity of NGF antibody in Western blotting of tauopathy tissue.** A: The 53kDa NGF immunoreactive band did not disappear when primary antibody (H-20 NGF antibody) was blocked with 5-fold molar excess of NGF peptide for 2 hours. B: Another lot of H-20 NGF antibody (Ab#2) did not detect the 53 kDa

immunoreactive band which confirmed that this band was nonspecific for the first lot of H-20 NGF antibody (Ab#1).

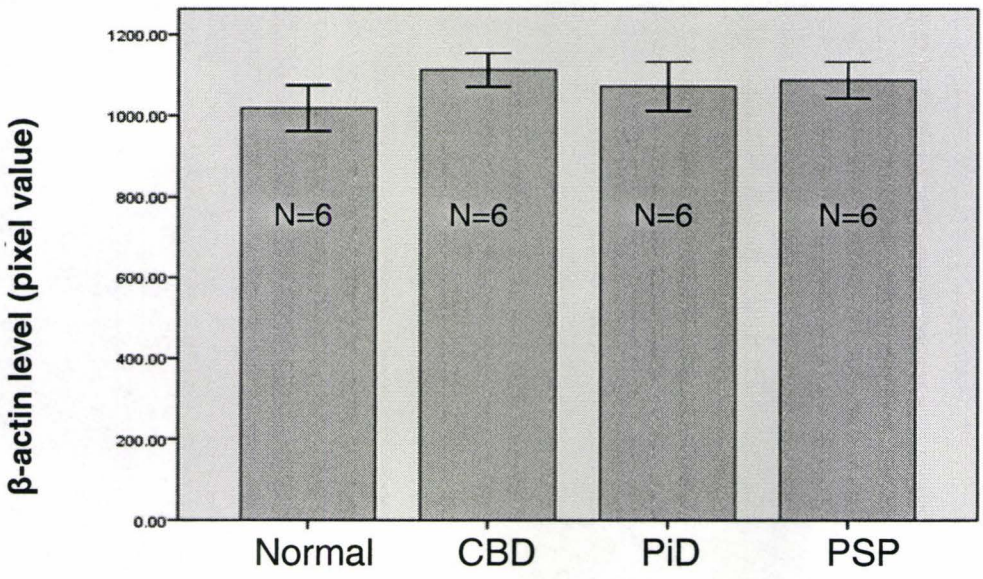



**Figure 4.5: No change in β-actin levels (protein) in tauopathies compared to control.** No significant differences were detected in the level of β-actin protein in any of the tauopathies compared to normal brain. All samples were measured in 3 separate experiments. Error bars represent standard error of the mean (S.E.M).

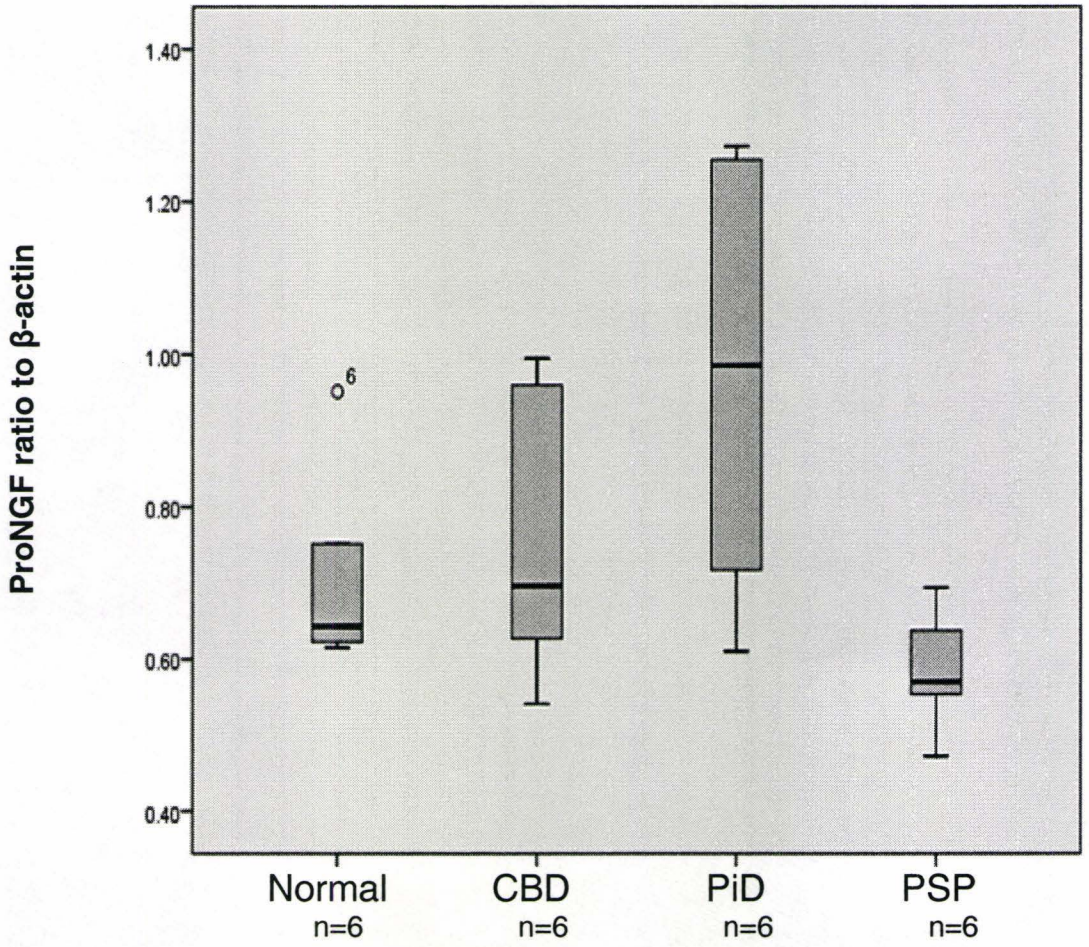



**Figure 4.6: Box plot of the ratio of proNGF (34kDa) to β-actin in tauopathies** (SPSS 16.0, SPSS Inc, Chicago, USA was used to determine the outliers). The first and last horizontal lines in each box (from the top) are the highest and lowest data values. The thick black line inside the box is the median. The top and bottom of the box are upper and lower quartiles. The upper quartile is the median of the first 50% of the data. The lower quartile is the median of the last 50% of the data. All samples were analyzed in triplicate.

°= Mild outlier: 1.5-3 IQR (interquartile range) above upper quartile.

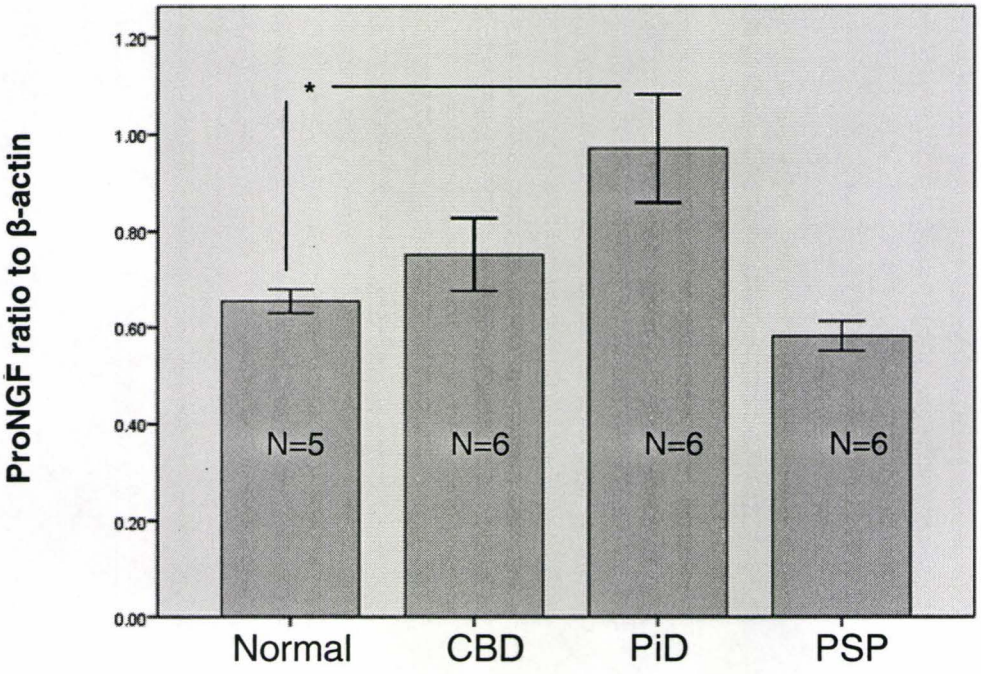



**Figure 4.7: Significant increase in the ratio of proNGF (34 kDa) to β-actin in PiD compared to normal brain.** All samples were measured in 3 separate experiments. Error bars represent standard error of the mean (S.E.M).

*= Significant increase (p=0.036, post hoc Tukey's test) was observed in the normalized level of proNGF (34kDa) compared to control.

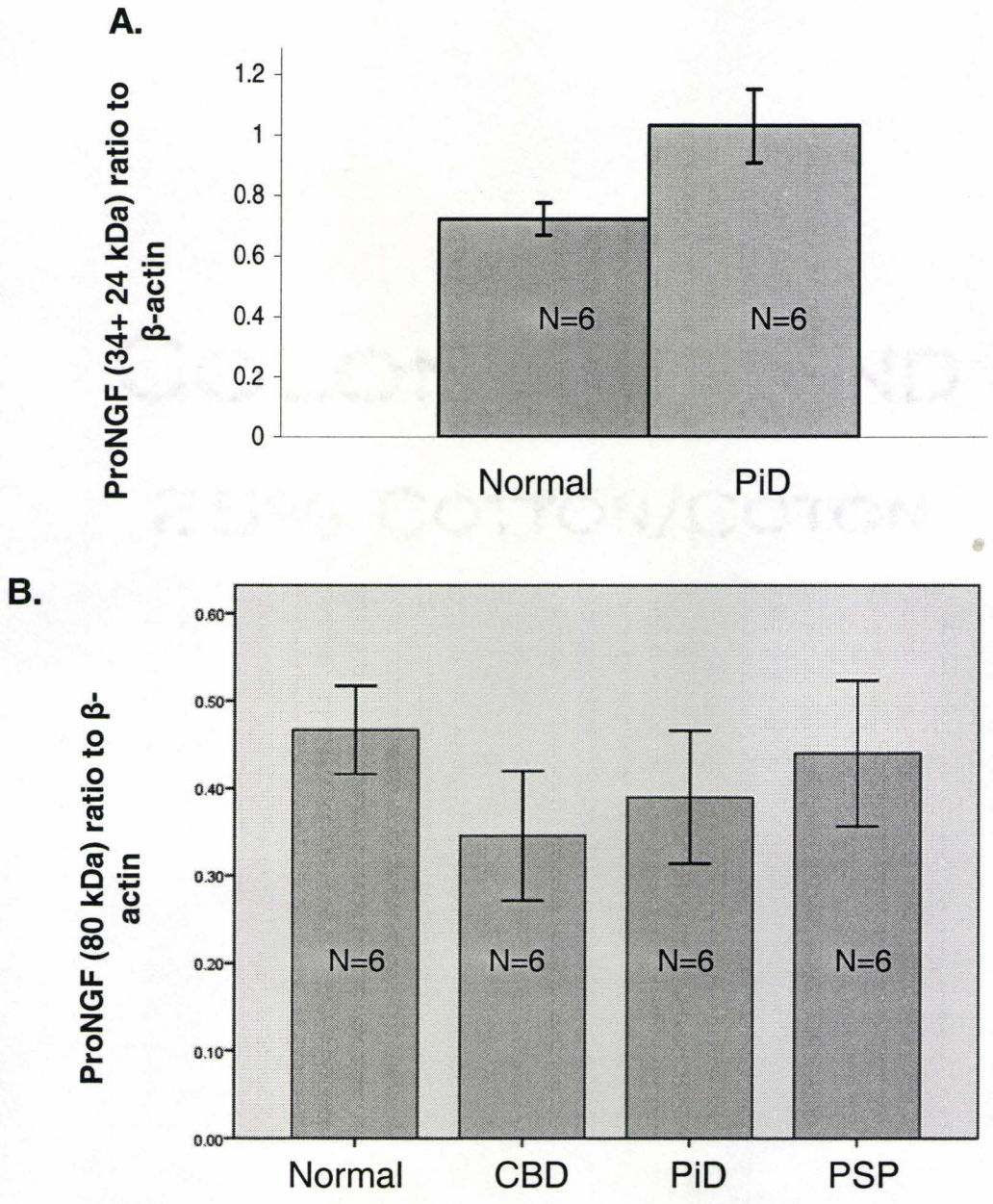



**Figure 4.8: No change in the level of 80kDa proNGF or combination of 24 and 34kDa proNGF was detected in tauopathy groups compared to control.** A. Normalized level of proNGF to β-actin (24kDa and 34kDa proNGF were combined) (24 kDa band was only detected in some of PiD and control subjects). B. Normalized level of 80kDa NGF immunoreactive band between tauopathy groups. Error bars represent standard error of the mean (S.E.M).

### *4.3. ProNGF levels did not show any significant increase in tau transgenic mice:*

As mentioned in section 2.2, GSK-3β overexpressing mice showed GSK-3β overexpression in both hippocampus and cortex while tau hyperphosphorylation was detected only in hippocampus (Lucas et al., 2001). According to our hypothesis, we expected to observe an increase in proNGF levels in hippocampus if tau hyperphosphorylation could interrupt proNGF retrograde transport. As no difference was detected in the level of β-actin in hippocampus (Figure 4.9B, C) (p=0.4, Student's *t*-test) or cortex (Figure 4.10B, C) (p=0.09, Student's *t*-test) of GSK-3β overexpressing mice compared to control, the level of proNGF was normalized to β-actin. Neither hippocampus (Figure 4.9A, D) (p=0.7, non-parametric Mann-Whitney test) nor cortex (Figure 4.10A, D) (p=0.7, non-parametric Mann-Whitney test) of transgenic mice overexpressing GSK-3β showed any changes in the level of proNGF normalized to β-actin compared to wild type littermates (see discussion for more details). All samples were within the range of the standard curve (Figures 4.9E, F and 4.10E, F).

JNPL transgenic mice are a model of tauopathy with motor impairment which show progression of tau abnormalities more in spinal cord compared to brain (reviewed in Lewis et al., 2000). β-actin did not show any significant differences (Figure 4.11B, C) (p=0.6, Student's *t*-test) in JNPL mice compared to their wild type littermates. Therefore, β-actin was used to normalize the level of proNGF in these mice. No change in the ratio of proNGF to β-actin (p=1, non-parametric Mann-Whitney test) was detected in the cortex of JNPL mice (Figures 4.11A, D) (see discussion for more details). All samples were within the range of the standard curve (Figures 4.11E, F).

PiD mice express tau containing the mutation for human Pick's disease but, unlike the human condition, show early motor impairment (reviewed in Janus, 2008). These transgenic mice did not show any difference in the level of

proNGF (p=0.2, Student's *t*-test) in comparison to wild type mice (Figures 4.12A, B) (see discussion for more details). All samples were within the range of the standard curve (Figure 4.12C).

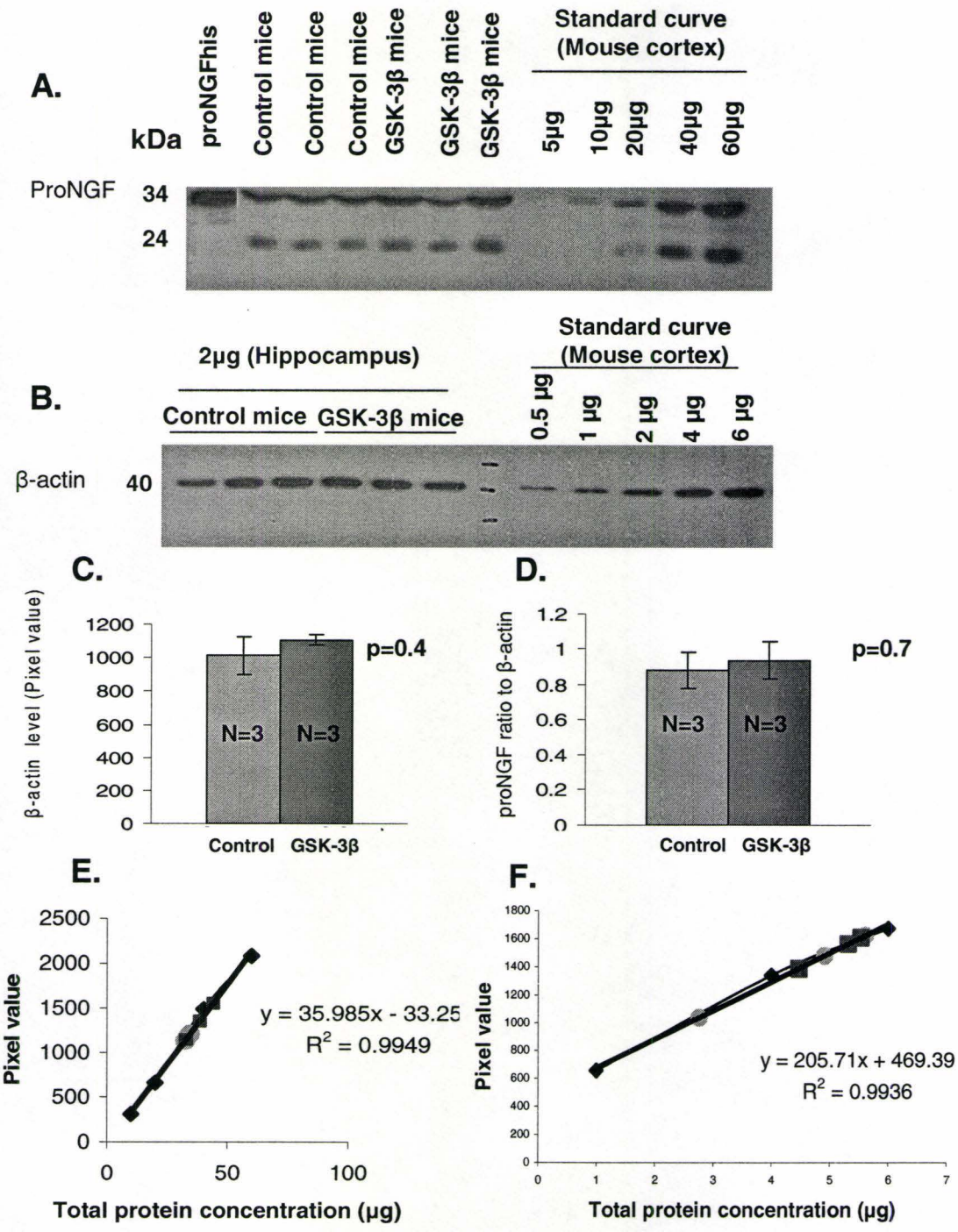



**Figure 4.9: No difference in the normalized level of proNGF (34kDa) in the hippocampus was detected between transgenic mice overexpressing GSK-3β and WT mice.** A. Representative Western blot using NGF antibody. B.

Representative Western blot using β-actin antibody. C. No significant difference in the level of β-actin between GSK-3β and WT mice. D. Ratio of proNGF to β-actin in hippocampus of GSK-3β overexpressing mice and control. Error bars represent standard error of the mean (S.E.M). All samples were within the range of the standard curve (solid diamonds) in Western blotting using NGF and β-actin antibody. E. ProNGF levels in GSK-3β animals (square) and WT animals (solid circle). F. β-actin levels in GSK-3β animals (square) and WT animals (solid circle). Samples were measured in 3 separate experiments for proNGF and one experiment for β-actin.

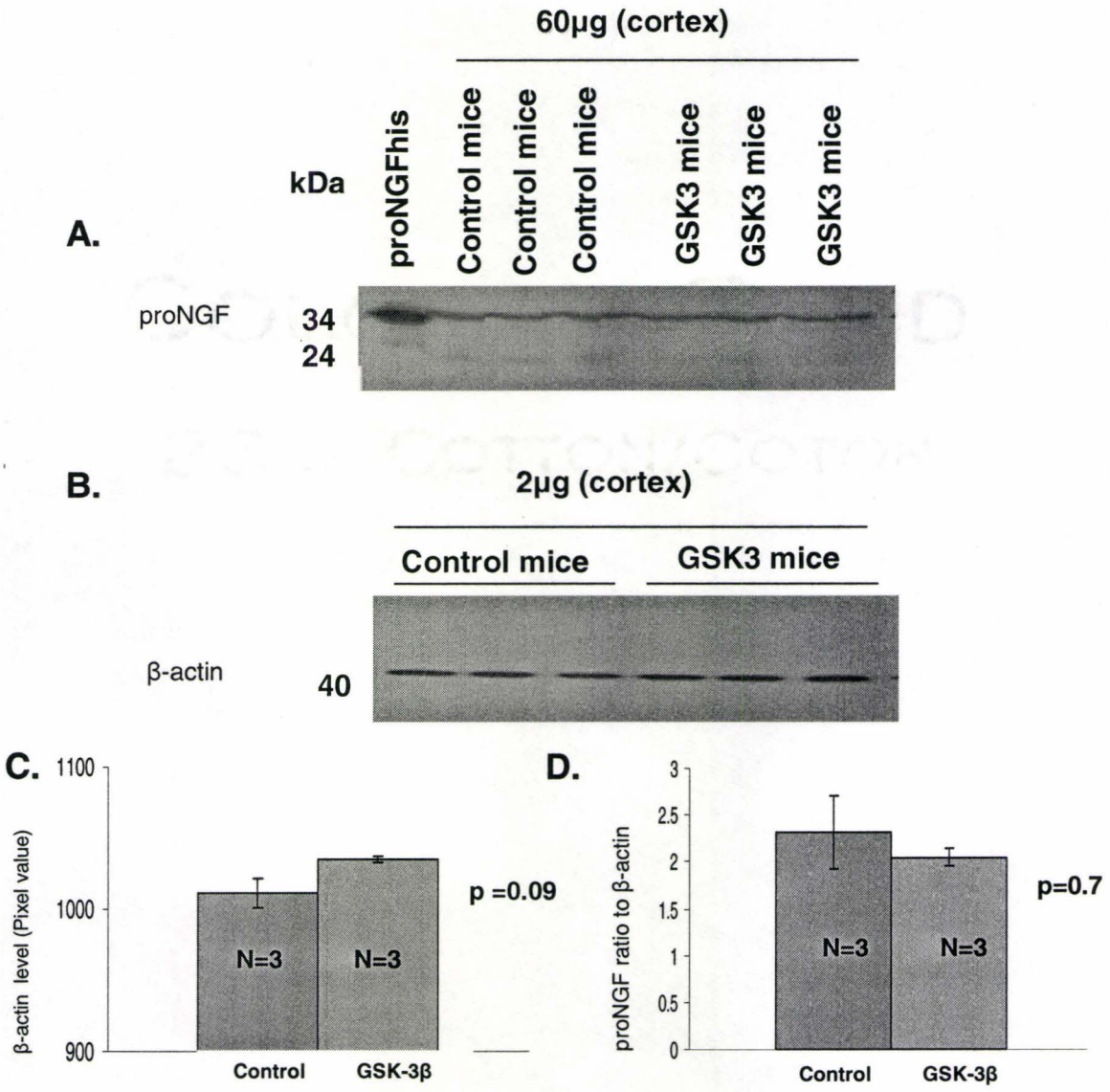



**Figure 4.10: No difference in the normalized level of proNGF (34kDa) was detected between transgenic mice overexpressing GSK-3β and WT mice cortex.** A. Representative Western blot using NGF antibody. B. Representative Western blot using β-actin antibody. C. No significant difference in the level of β-actin between GSK-3β and WT mice. D. Ratio of proNGF to β-actin in cortex of GSK-3β overexpressing mice and control. Error bars represent standard error of the mean (S.E.M). The experiment was carried out only once as there were complications with

Western blotting on the cortex of these Tg mice (bands did not get separated from each other which made it hard to determine the density of each band).

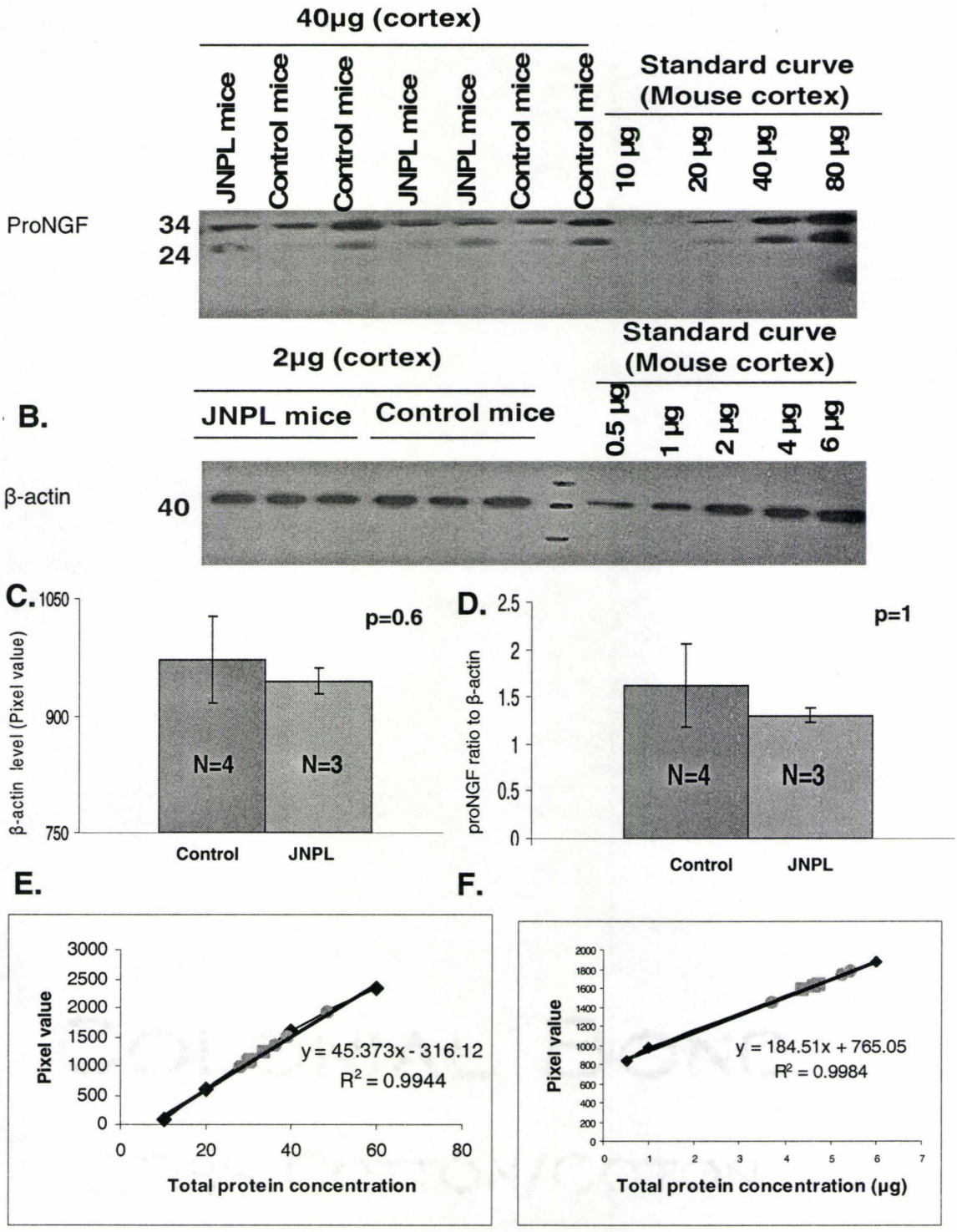
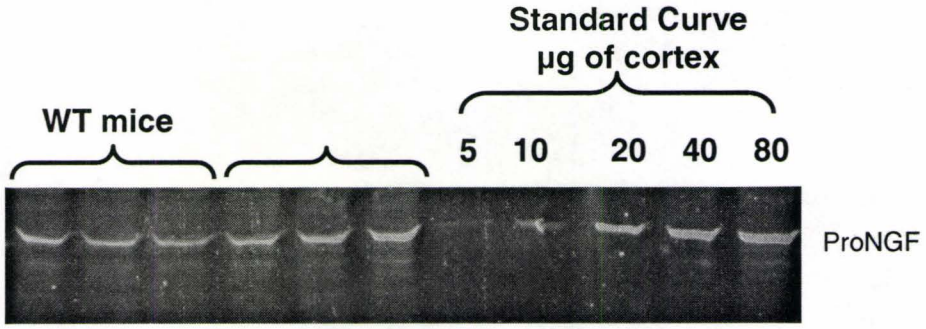


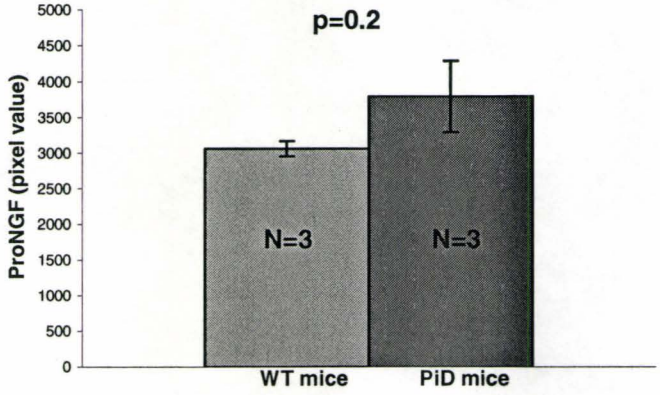

**Figure 4.11: No difference in the normalized level of proNGF (34kDa) in the cortex was detected between JNPL transgenic mice and WT mice.** A.

Representative of a Western blot using NGF antibody. B. Representative Western blot using β-actin antibody. C. No significant difference in the level of β-actin between JNPL and WT mice. D. Ratio of proNGF to β-actin in cortex of JNPL mice and control. Error bars represent standard error of the mean (S.E.M). All samples were within the range of the standard curve (solid diamonds) in Western blotting using NGF and β-actin antibody. E. ProNGF levels in JNPL (square) and WT animals (solid circle). F. β-actin levels in JNPL (square) and WT animals (solid circle). Samples were measured in 2 separate experiments.

A.




B.




C.

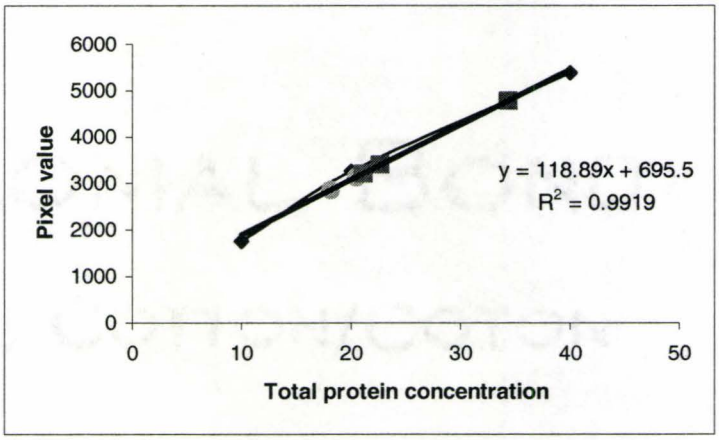



**Figure 4.12: No significant increase in the level of proNGF in PiD mice (cortex) compared to WT mice.** A. Representative Western blot using NGF antibody (secondary antibody was IRDye® 680 conjugated goat polyclonal anti-rabbit IgG). B. There was no significant increase in the level of proNGF in PiD mice compared to control. Error bars represent standard error of the mean (S.E.M). C. All samples were

within the range of the standard curve (solid diamonds) in Western blotting using NGF antibody. ProNGF levels in PiD mice (square) and WT animals (solid circle). Samples were run once. No Western blot was run for β-actin. Experiment is still in progress.

**SUMMARY** (CHAPTER 4)

In Chapter 4, we have shown that proNGF levels (34kDa) increase in human PiD parietal cortex compared to normal brain, while this protein did not change in PSP and CBD parietal cortices. PiD and AD share the same tau modification which may explain the increase observed in the level of proNGF in PiD brain but not in CBD and PSP. The increase in proNGF levels in PiD may support the idea that tau dysfunction could affect the level of this neurotrophin in AD brain.

None of the tau transgenic or GSK-3β overexpressing animals investigated in this study showed an increase in the level of proNGF. The limited number of samples per group is a factor which could affect the result. Moreover, in some tauopathies and transgenic mice, tau hyperphosphorylation occurs in areas other than hippocampus and cortex which could explain lack of change in proNGF levels in these mice (see Discussion for more details).

# CHAPTER 5

# RESULTS

## PRONERVE GROWTH FACTOR IS NEUROTROPHIC

### *5.1. ProNGF biological activity:*

As proNGF is the predominant form of NGF in the CNS and its level changes in neurodegenerative diseases such as AD and PiD, it may have biological activity in vivo other than its precursor activities. The role of this proform is controversial, as two different recombinant, cleavage-resistant types of proNGF reportedly show opposite activities (neurotrophic versus apoptotic) *in vitro*. Increases in the level of proNGF in AD may account for the neuronal degeneration, if proNGF is apoptotic. However, if proNGF is neurotrophic, neuronal degeneration may occur in the AD brain because of impaired axonal transport of this protein and the subsequent lack of trophic support.

The differences between these two cleavage-resistant proNGFs may affect their activity. There are four amino acid substitutions at -1 and -2 (R-R to A-A), 118 and 119 (R-R to A-A) in proNGF with apoptotic activity compared to one amino acid substitution (R-1G) in proNGF with neurotrophic activity. Some amino acid substitutions may change the protein structure interfering with its activity (Ibanez et al., 1992). The proNGF with more amino acid substitutions carries a C-terminal histidine tag, as well (this proNGF will be referred to as proNGFhis from this point onward). Addition of a histidine tag may also affect the activity of a protein as it can interfere with the protein structure and oligomerization (a histidine tag interferes with the oligomerization of a protein called TALL-1) (Ledent et al., 1997, Ramage et al., 2002, Zhukovsky et al., 2004, Hunt, 2005). ProNGFhis was purified over a nickel column which may cause oxidation leading to changes in the activity of the protein or protein aggregation (Ramage et al., 2002, Hunt, 2005, Al-Samarrai et al., 2007). Finally, different systems used to express these two cleavage resistant proNGFs may affect their activity; there are low amounts of endogenous neurotrophins in the mammalian

expression system used to express proNGFhis. Neurotrophin monomers can bind freely together to produce artifacts (Treanor et al., 1995). On the other hand, the baculovirus/insect cell system used to produce proNGF with neurotrophic activity may contain some chaperones that could cause the neurotrophic activity observed for (R-1G) proNGF (reviewed in Fahnestock et al., 2004b). Different bioassays were carried out to determine the activity of these two cleavage resistant proNGFs, as well. While (R-1G) proNGF showed neurotrophic activity in the neurite outgrowth/survival assay, proNGFhis showed apoptotic activity in the NGF withdrawal assay in which cell survival was already compromised. We hypothesized that differences in the number of amino acid substitutions, the presence/absence of a histidine tag, the protein expression system, purification methods or different methods used for bioassays may account for the opposite activity (apoptotic or neurotrophic) of these two types of cleavage-resistant proNGF (Table 1.2).

In order to address our hypothesis, cDNA from a proNGF with apoptotic activity (proNGFhis) was expressed in the same protein expression system used to produce proNGF (R-1G) with neurotrophic activity. The expressed proNGF was used in different bioassays, and then partially purified as reported for proNGF with apoptotic activity, and its activity was determined. The proNGF used in all the following sections of this chapter was proNGFhis expressed in a baculovirus/insect cell system unless stated otherwise.

### *5.1.1. ProNGFhis expressed in baculovirus/insect cells was intact:*

A proNGFhis construct was subcloned into pVL1393 and was expressed in an insect cell/baculovirus expression system. After protein expression, Western blotting was carried out in order to check the quality of expressed protein which demonstrated bands at expected molecular weights for both proNGFhis (34 kDa) and wild type NGF (WT NGF) (13 kDa) [made from cleavable proNGF described in Fahnestock et al., (2004a). This proNGF was expressed in a baculovirus/insect cell system and was largely cleaved to mature

NGF]. Western blotting using an antibody against NGF (Figure 5.1A), proNGF (Figure 5.1B), or the C-terminal histidine tag (Figure 5.1C) confirmed the right size band for proNGFhis. The latter Western blot verified that expressed protein in insect cells is proNGFhis carrying a histidine tag. Moreover, the anti-NGF Western blots showed that there was less than 2% (1.3% with NGF antibody) cleavage of proNGFhis. No band was detected for either wild type NGF (no his tag) or 2.5S NGF after using proNGF antibody (Dr Xin-Fu Zhou) or his tag antibody (Invitrogen) (Figure 5.1B, 5.1C) which confirmed the specifity of the antibodies for proNGFhis. No reactivity was shown for wild type baculovirus conditioned medium for any of the antibodies mentioned above (Figure 5.1A-C); therefore there is no proNGF production in baculovirus/insect cells and also the antibodies did not crossreact with any protein produced by the baculovirus/insect cell system alone.

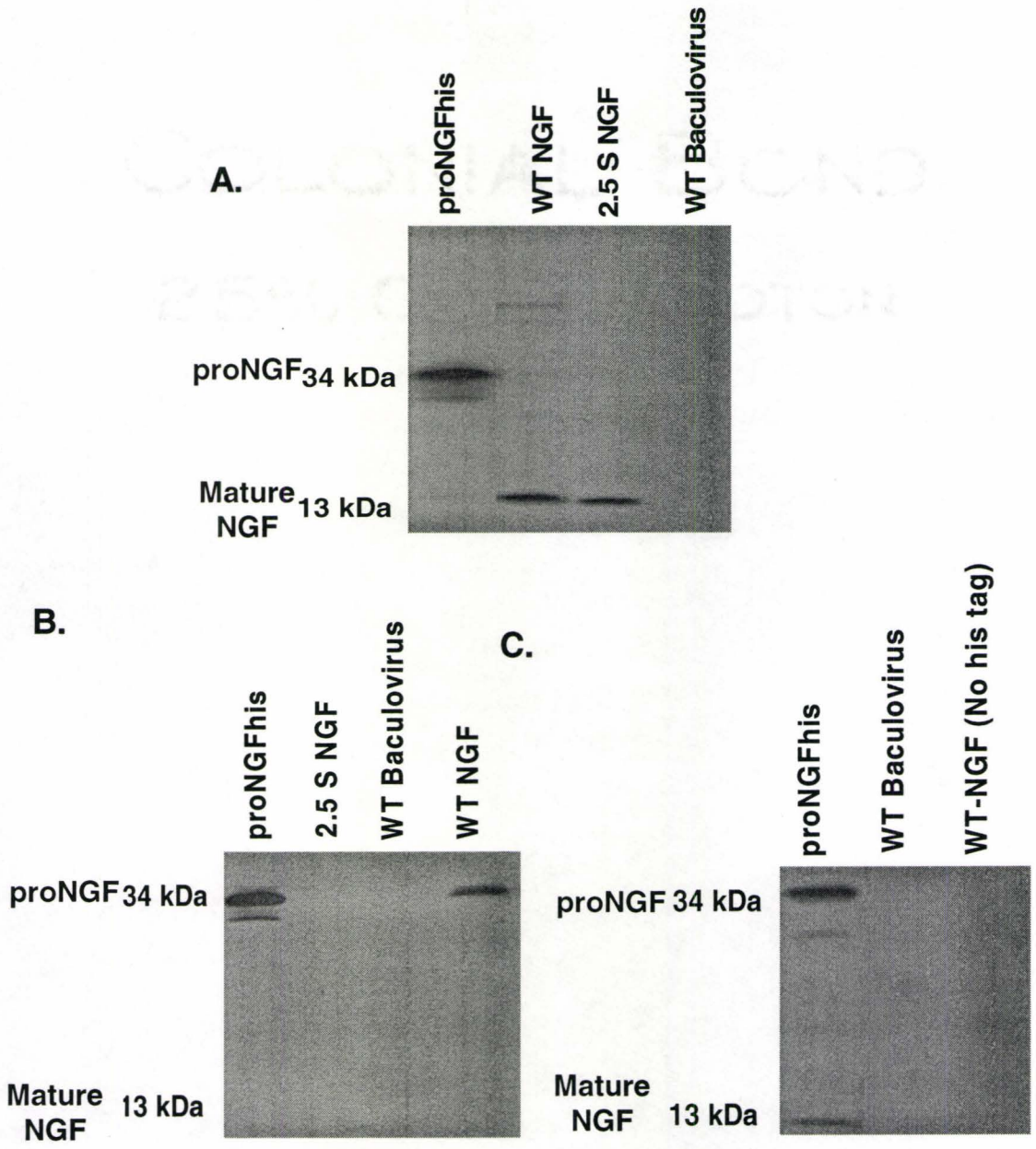



**Figure 5.1: Western blotting of expressed proteins from insect cell supernatant showed that proNGFhis was intact and at the right molecular size.** (A) Antibody against NGF (MC-51) (B) Antibody against proNGF (Dr Xin-Fu Zhou). (C) Antibody against histidine tag (Invitrogen).

### *5.1.2. ProNGFhis was intact after protein purification over a nickel column:*

Purified proNGFhis was run on Western blots to check the quality of the protein. There was less than 1% cleavage for this protein after purification (Figure 2A and 2B). Therefore, the purified material was of sufficient quality to be used in bioassays.

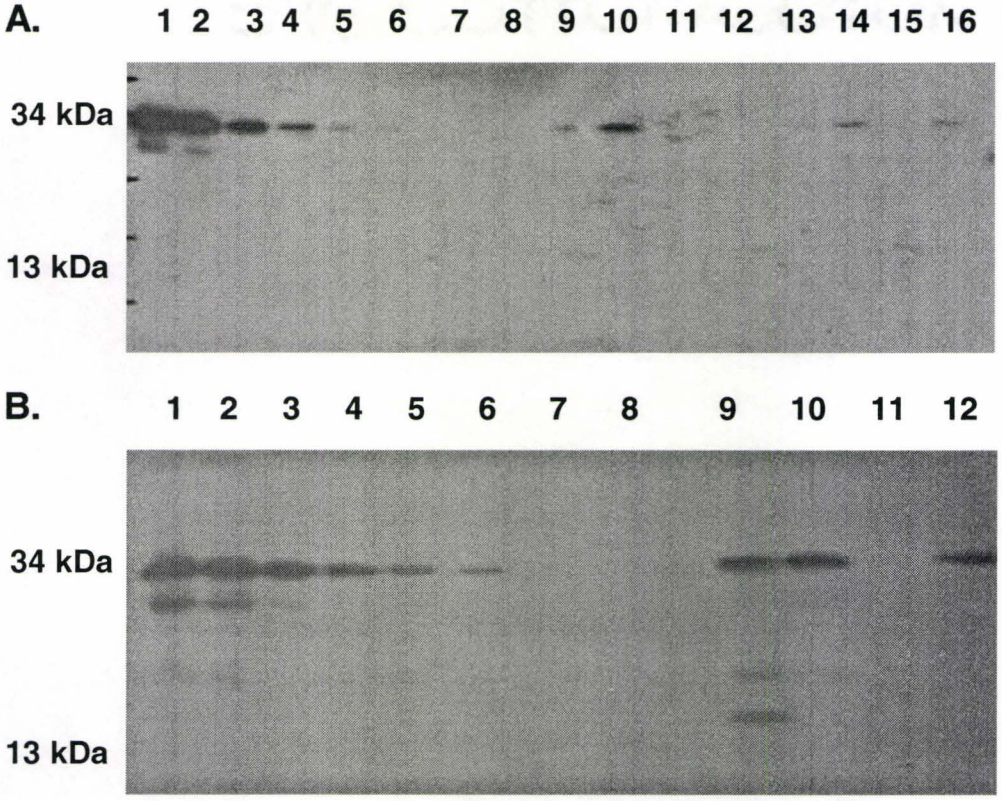



**Figure 5.2: ProNGFhis after purification over a nickel column was intact and at the right molecular size.** 1-8 in A and B: First eight fractions after elution step. 9 and 10 in A and B: starting material before and after dialysis, respectively, against buffer specific for the column. Pass through after binding to the column (11-13 in A and 11 in B). Washing (14-16 in A and 12 in B) (A): Antibody against histidine tag (Invitrogen) showing proNGFhis at the right molecular size (34kDa) and no cleavage to mature NGF at 13kDa after purification over a nickel column. (B): Antibody against NGF (MC-51) showing less than 1% cleavage for proNGF (34kDa) to mature NGF (13kDa) after purification over a nickel column.

***5.1.3. SCGs treated with proNGFhis in serum-free medium showed neurite outgrowth activity:***

ProNGFhis was prone to cleavage when serum-containing medium is used in bioassays (data not shown). Therefore, neurite outgrowth bioassays were carried out using serum-free medium. Both 2.5S NGF (Figure 5.3) and proNGFhis (Figure 5.4) were able to promote outgrowth from SCGs in serum-free medium. There was no outgrowth detected for baculovirus-infected insect cell medium (Figure 5.5). The EC50 for proNGFhis was 0.08 (logEC50=–1.1±0.15) compared to 0.03 (logEC50=–1.5±0.13) for 2.5S NGF (Figure 5.6A), meaning that proNGF is less active than mature NGF which was again consistent with the data published by Fahnestock et al., 2004a (Figure 5.6C). A Western blot on the material after SCG neurite outgrowth assay (with no serum) revealed that there was almost no cleavage (less than 2%) for proNGFhis after the assay (Figure 5.6B), which demonstrated that proNGF rather than mature NGF was the active species in promoting neurite outgrowth.

***5.1.4. There was no significant difference between 2.5S NGF and proNGFhis in inducing apoptosis:*** SCG neurons incubated with 0.2nM proNGFhis for 24 hours showed 32±8% dead cells in the TUNEL assay which was not significantly different from 2.5S NGF showing 27±6% dead cells at a concentration of 0.2nM ($p>0.05$, one-way ANOVA) (Table 5.6C).

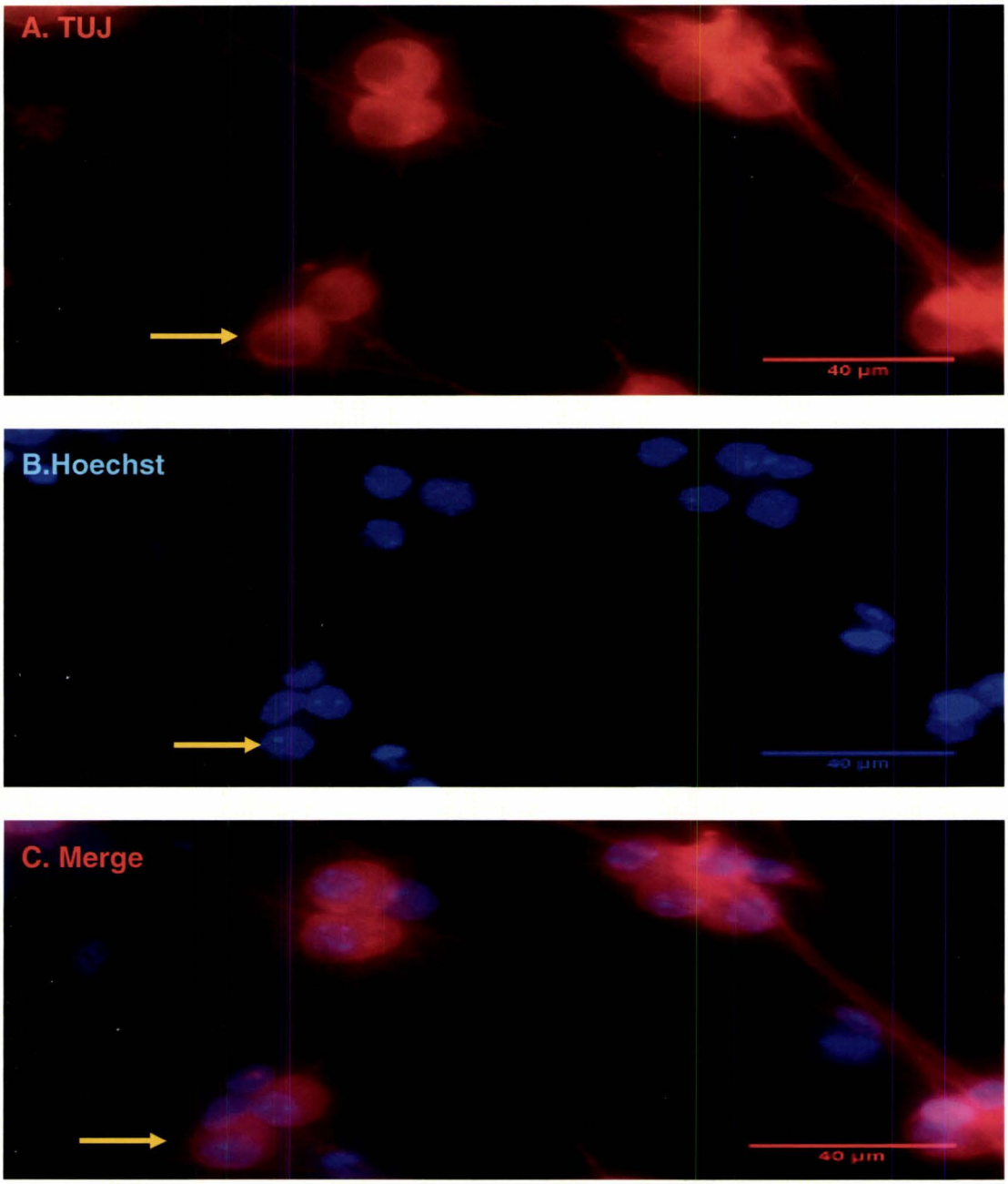



**Figure 5.3: Neurite outgrowth in SCG neurons treated with 2.5S NGF.** SCG neurons were cultured in serum-free medium [DMEM-F12, $10^{-5}$ M uridine, $10^{-5}$ M 5-fluoro-2-deoxyuridine, 0.5% penicillin/streptomycin (pen/strep) and 1% N2 supplement]

containing different dilutions of either NGF, proNGFhis or baculovirus conditioned medium for 48 hours. Fixed cells were stained with TUJ-1 antibody and Hoechst. TUJ staining (A), Hoechst staining (alive cells were transparent while dead cells looked condensed and in some cases fragmented) (B) and merged pictures (C) of cells treated with 0.2 nM 2.5S NGF. The scale bar is 40μm. The yellow arrows show a representative live cell and the white arrows indicate one example of a dead cell.

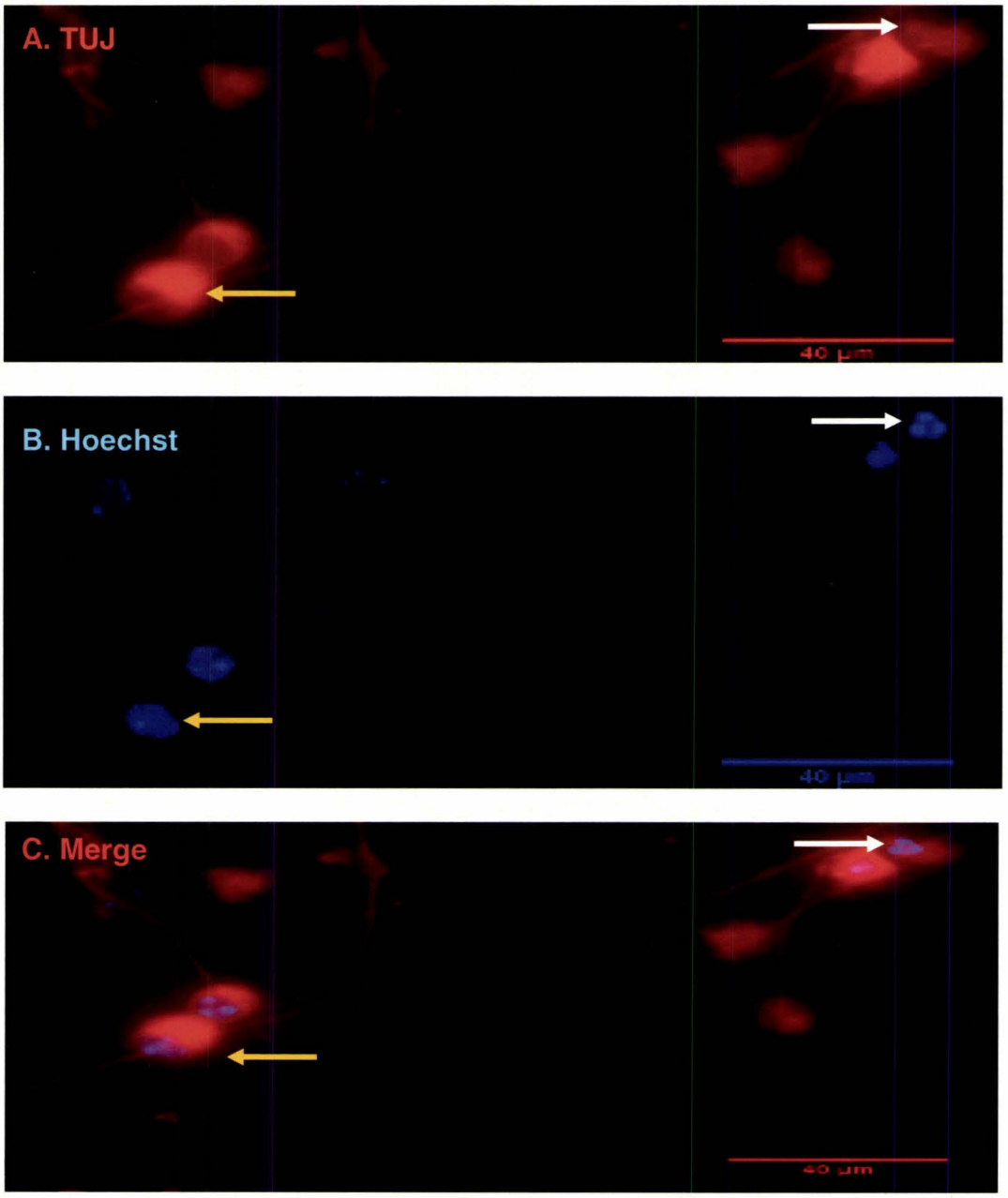



**Figure 5.4: ProNGFhis induced outgrowth of SCG neurons.** SCG neurons were cultured in serum-free medium [DMEM-F12, $10^{-5}$ M uridine, $10^{-5}$ M 5-fluoro-2-deoxyuridine, 0.5% penicillin/streptomycin (pen/strep) and 1% N2 supplement] containing different dilutions of either NGF, proNGFhis or baculovirus conditioned medium for 48 hours. Fixed cells were stained with TUJ-1 antibody and Hoechst. TUJ staining (A), Hoechst staining (alive cells were transparent while dead cells looked

condensed and in some cases fragmented) (B) and merged pictures (C) of cells treated with 0.2 nM proNGFhis. The scale bar is 40μm. The yellow arrows show a representative live cell and the white arrows indicate one example of a dead cell.

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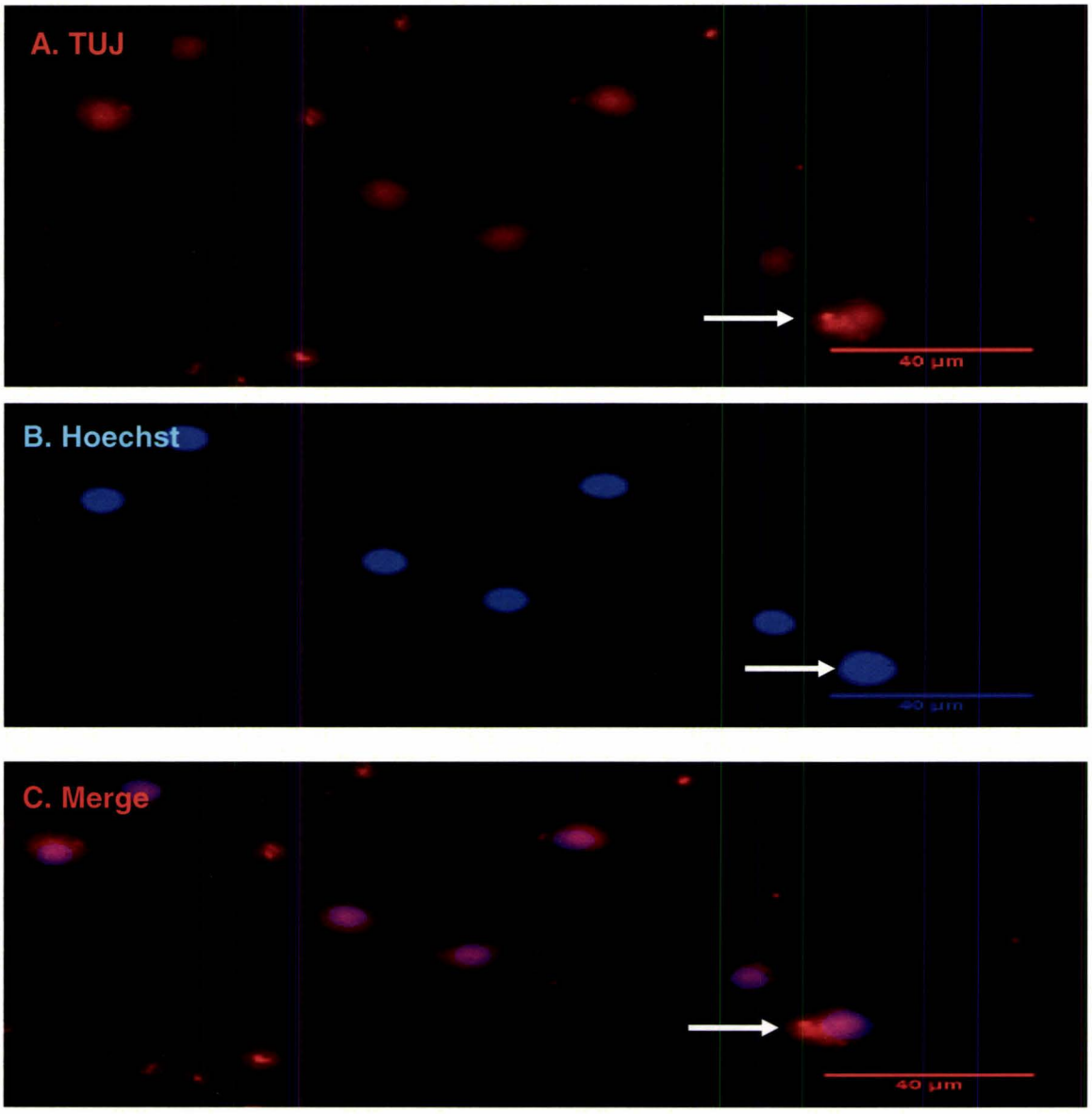
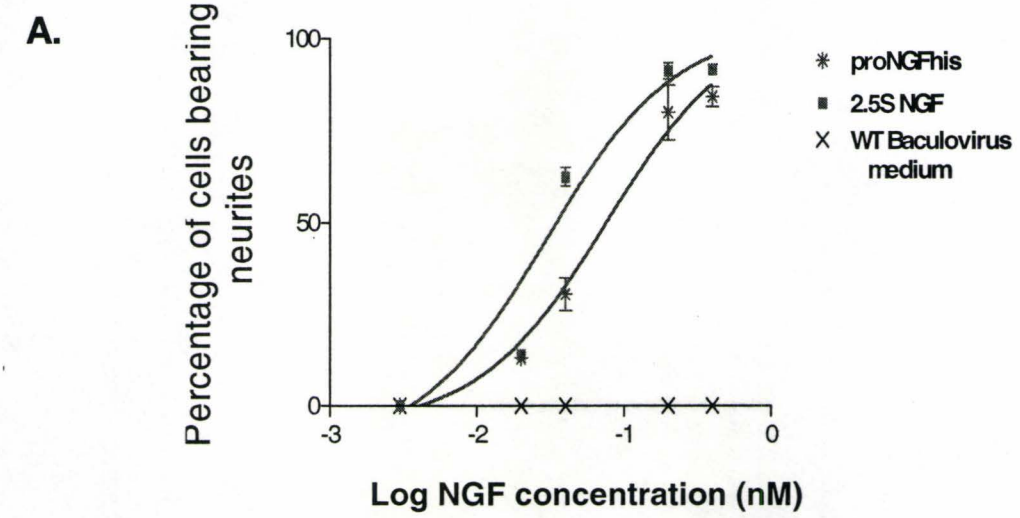
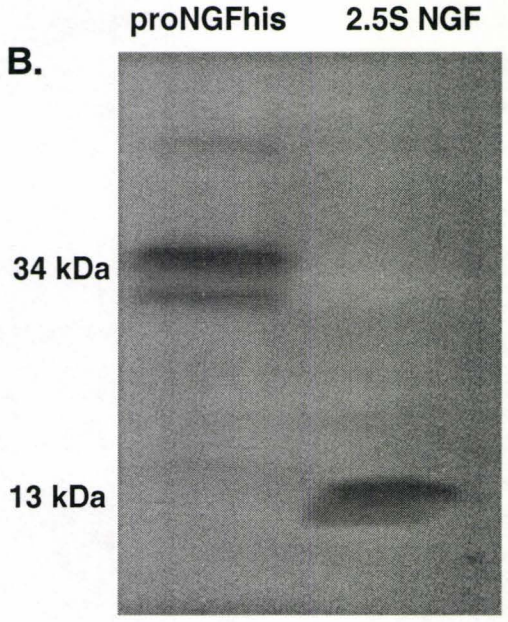



**Figure 5.5: No outgrowth was detected in SCG neurons treated with baculovirus-infected insect cell conditioned medium.** SCG neurons were cultured in serum-free medium [DMEM-F12, $10^{-5}$ M uridine, $10^{-5}$ M 5-fluoro-2-deoxyuridine, 0.5% penicillin/streptomycin (pen/strep) and 1% N2 supplement] containing different dilutions of either NGF, proNGF or baculovirus conditioned medium for 48 hours. Fixed cells were stained with TUJ-1 antibody and Hoechst. TUJ staining (A), Hoechst staining (alive cells were transparent while dead cells looked condensed and in some cases fragmented) (B) and merged pictures (C) of cells treated with

conditioned medium. The scale bar is 40µm. The white arrows indicate one example of a dead cell.




C.

| Sample | % Apoptotic cells | % neurons bearing neurites |
|---|---|---|
| 2.5S NGF (0.2nM) | 27 +/- 6 | 91 +/- 2 |
| proNGFhis (0.2nM) | 32+/-8 | 80+/-8 |
| WT Baculovirus medium | 70 +/- 6 | 0 |

**Figure 5.6: Both 2.5S NGF and proNGFhis promoted neurite outgrowth in SCG neurons, and there was no significant difference between 2.5S NGF and proNGFhis regarding the number of apoptotic cells.** SCG neurons were cultured in serum-free medium [DMEM-F12, $10^{-5}$ M uridine, $10^{-5}$ M 5-fluoro-2-deoxyuridine, 0.5% penicillin/streptomycin (pen/strep) and 1% N2 supplement] containing different dilutions of either NGF, proNGF or baculovirus conditioned medium

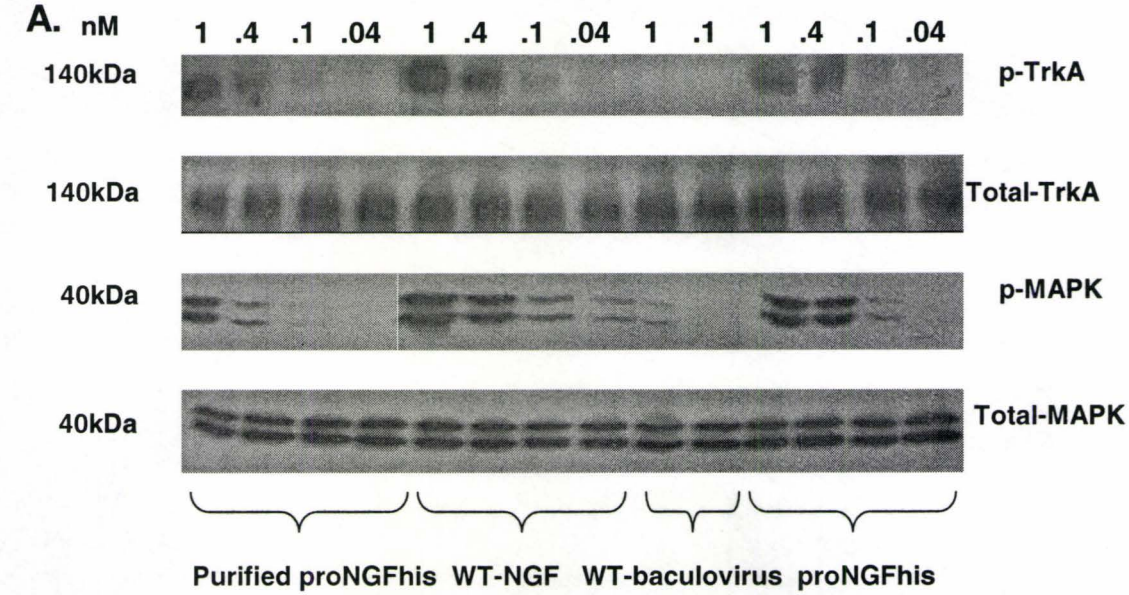
for either 48 hours for the outgrowth assay or 24 hours for the apoptosis assay. Fixed cells were stained with TUJ-1 antibody and Hoechst. A: Neurite outgrowth assay in serum-free medium (Graphpad software 3.03, Inc, San Diego, CA, USA). $R^2$ was 0.96 for both proNGFhis and 2.5S NGF. Error bars represent standard error of the mean (S.E.M). B: Medium used to treat the cells was tested on a 12% SDS-PAGE gel using NGF antibody (MC51). There was less than 2% cleavage of proNGFhis after the neurite outgrowth assay in serum-free medium. C: There was no significant difference between proNGFhis and 2.5S NGF in inducing apoptosis at 0.2nM concentration. Results were obtained from three independent experiments.

### *5.1.5. ProNGFhis activated the TrkA-MAP kinase pathway:*

NGF, as a neurotrophic factor, promotes TrkA and subsequent MAPK phosphorylation to signal cell survival and neurite outgrowth. To determine whether proNGFhis (unpurified and purified) is also able to activate the TrkA and MAPK pathway, a TrkA-MAPK activation assay was carried out on PC12 cells. WT proNGF expressed in baculovirus/insect cell system which was cleaved to mature NGF was used as a control (WT NGF). Results of Western blotting using antibodies against phospho-TrkA and phospho-MAPK showed that proNGFhis (unpurified and purified) promotes both TrkA and MAPK phosphorylation (Figure 5.7A). The EC50 for TrkA activation was 0.2nM (logEC50=–0.6±0.07), 0.6nM (logEC50=–0.2±0.2) and 0.7nM (logEC50=–0.16±0.2) for WT-NGF, purified proNGFhis, and unpurified proNGFhis, respectively (Figure 5.7B). For MAPK activation, the EC50 for WT-NGF was 0.08nM (logEC50=–1.1±0.1), while it was 0.4nM (logEC50=–0.4±0.2) for purified proNGFhis and 0.1nM (logEC50=–0.87±0.05) for unpurified proNGFhis (Figure 5.7C). As expected, for both TrkA and MAPK activation there was less activity for proNGFhis than WT NGF. There was no change in the level of total TrkA and total MAPK which showed that the concentration dependent phosphorylation of TrkA and MAPK is not due to changes in the expression of these receptors (Figure 5.7A). As can be seen in Figure 5.8A, no cleavage was detected for proNGFhis after the assay. This confirms the role of proNGF and not mature NGF in TrkA-MAPK activation. Moreover, blocking proNGFhis using an antibody specific for proNGF reduced the ability of this protein to activate MAPK phosphorylation, while there was no change in the ability of WT NGF to activate MAPK phosphorylation before and after treating with this blocking antibody (Figure 5.8B). This also verified that the MAPK activation observed for proNGFhis was specific to proNGF.

### *5.1.6. Furin inhibitor did not change the ability of proNGFhis to induce TrkA-MAPK activation*:

Furin processes proNGF intracellularly to mature NGF. It has been reported that proNGF requires intracellular proteolysis in order to activate TrkA (Boutilier et al., 2008). Here, we carried out a TrkA-MAPK activation assay in the presence of a cell permeable furin inhibitor. As can be seen from Figure 5.9A, proNGFhis activated TrkA-MAPK phosphorylation in the presence of furin inhibitor (Decanoyl-RVKR-CMK) (Figure 5.9A, B, C). This protease inhibitor was used to prevent proNGFhis cleavage inside the cells which did not affect the ability of proNGF to induce TrkA and MAPK phosphorylation. There was no difference in the ability of WT-NGF (13kDa) to activate the TrkA-MAPK signaling pathway with or without furin inhibitor treatment (Figure 5.9 A, B, C) (WT-NGF served as a control to ensure that furin inhibitor does not affect the ability of cells to activate the TrkA-MAPK signaling pathway). ProNGF quality was checked using Western blotting which showed presence of proNGFhis in PC12 cell lysates after the TrkA-MAPK activation assay (Figure 5.9D). Two NGF immunoreactive bands were detected at 34kDa and considered as endogenous and exogenous proNGF as both bands disappeared when NGF antibody used for Western blotting was blocked using NGF peptide. Exogenous proNGF ran slightly slower than endogenous NGF, as it carries a histidine tag. These data indicated the role of proNGF (not mature NGF from intracellular cleavage of proNGF) in activation of a neurotrophic pathway.



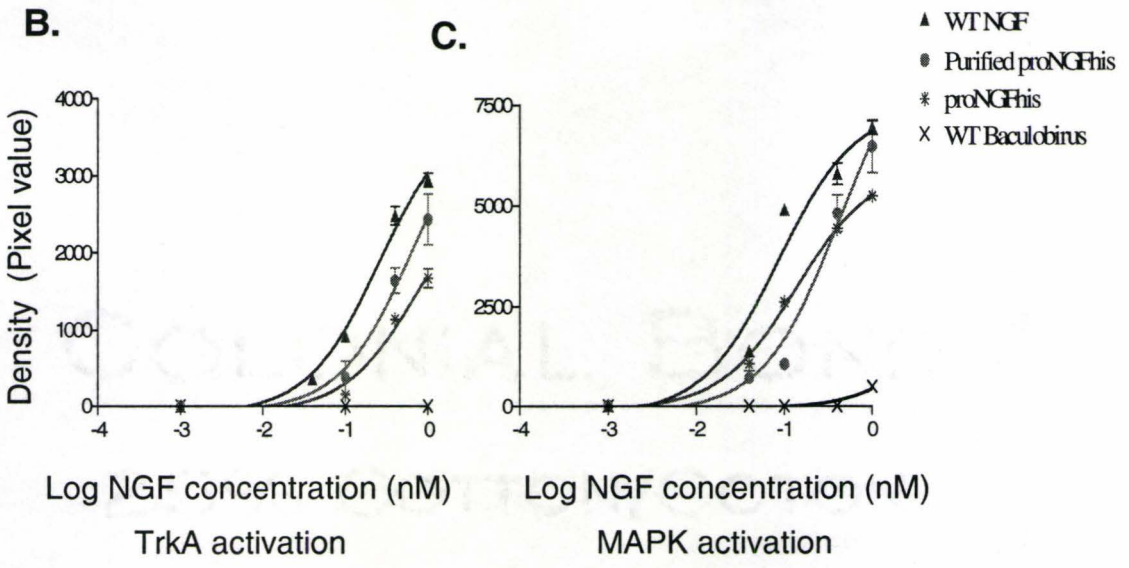




**Figure 5.7: Both unpurified and purified proNGFhis activated TrkA-MAPK phosphorylation in PC12 cells.** Cells were treated with serum-free medium one day before treating them with different dilutions of proNGFhis, WT NGF, or baculovirus conditioned medium. A: Representative blot showing proNGFhis (unpurified and purified) induced TrkA-MAPK phosphorylation while total TrkA and MAPK remained unchanged B, C: Results were obtained from 3 independent experiments. Error bars represent standard error of the mean (S.E.M) (Graphpad software 3.03, Inc, San Diego, CA,

USA). As there was not enough material to apply higher concentrations of proNGF, we could not achieve the characteristic plateau seen at higher concentrations. B: TrkA activation: $R^2$ was 0.98, 0.94, 0.96 for WT-NGF, purified proNGFhis, and unpurified proNGFhis, respectively. C: MAPK activation: $R^2$ was 0.96, 0.97, and 0.99 for WT-NGF, purified proNGF, and unpurified proNGF, respectively.

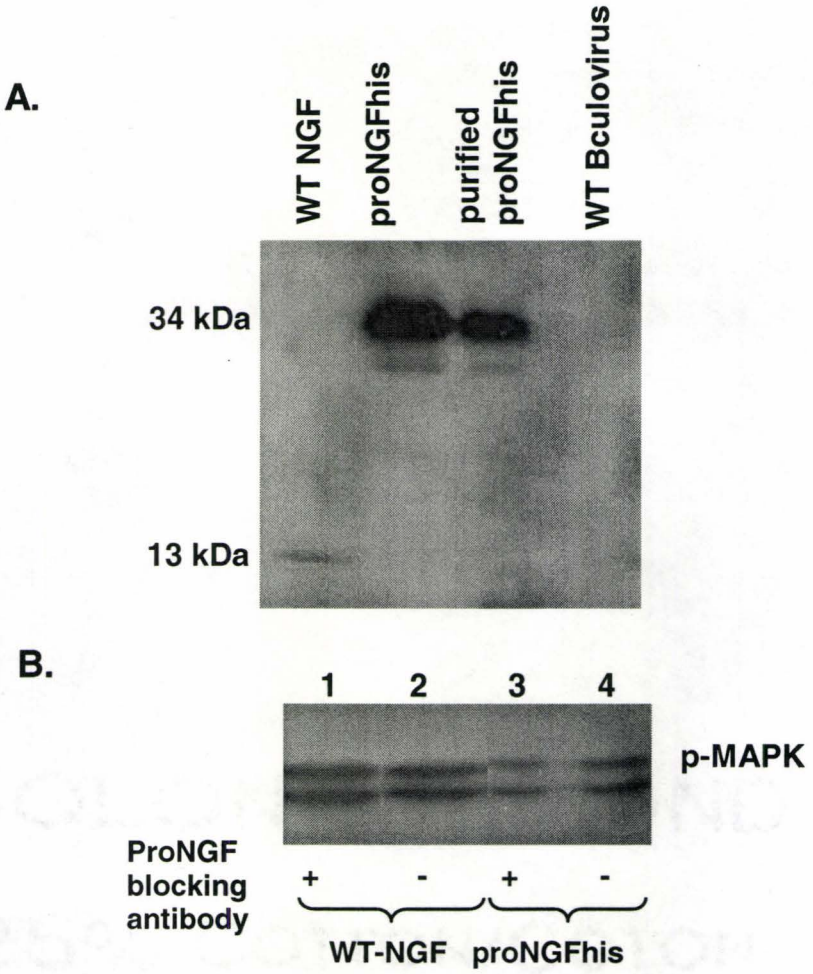



**Figure 5.8: TrkA-MAPK activation was specific for proNGFhis.** A. After the assay, conditioned medium (not concentrated) was tested on a 12% SDS-PAGE gel using NGF antibody (MC51). ProNGFhis remained intact after the TrkA-MAPK activation assay which confirmed that proNGF, not mature NGF, was responsible for the observed activation. B. ProNGFhis and WT NGF (cleavable WT proNGF expressed in baculovirus/insect cell system) were incubated with proNGF antibody (Dr Xin-Fu Zhou) for 30 min before adding them to PC12 cells. There was less MAPK activity in response to proNGFhis when it was blocked with an antibody specific for proNGF (3) compared to proNGFhis alone (4). There was no change in the ability of WT NGF to promote MAPK activation with (1) or without treating with proNGF antibody (2). Blocking assay was carried out twice with similar results.

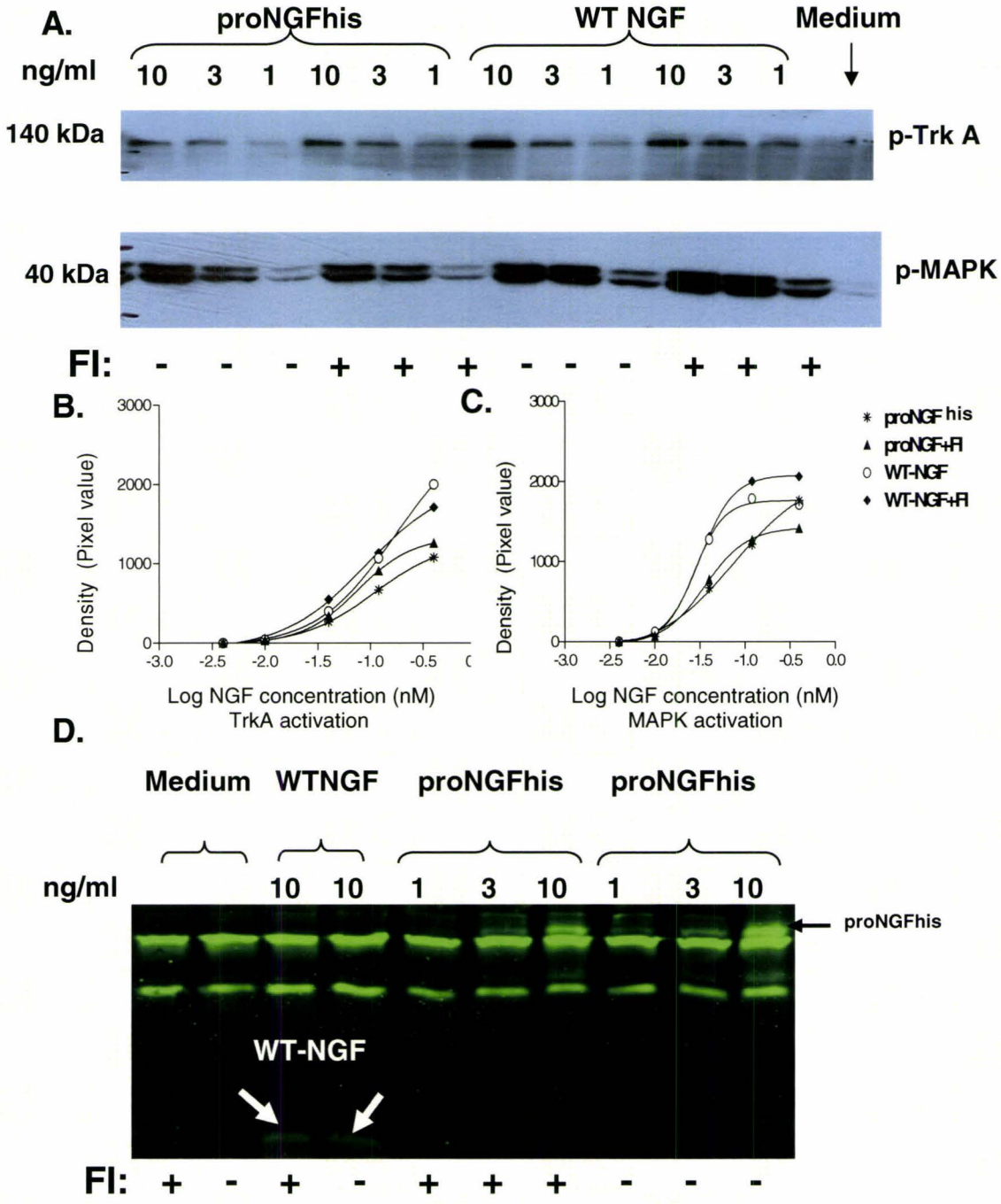



**Figure 5.9: ProNGFhis activated TrkA and MAPK phosphorylation even in the presence of furin inhibitor.** PC12 cells were treated with 30 μM furin inhibitor for 2 hours before the TrkA-MAPK activation assay. A. Western blotting using phospho-TrkA and phospho-MAPK antibodies for both WT-NGF and proNGFhis with (+FI) and

without (-FI) furin inhibitor. B. TrkA and C. MAPK phosphorylation activity of WT-NGF and proNGFhis with (+FI) and without (-FI) furin inhibitor (Graphpad software 3.03, Inc, San Diego, CA, USA). Result was obtained from a single experiment. $R^2$ was 0.99-1 for all experimental groups. D. Western blotting using NGF antibody (MC51) on the cell lysates of PC12 cells after the TrkA-MAPK activation assay: ProNGFhis (exogenous proNGF) was still intact in cell lysates with or without furin inhibitor.

### *5.1.7. ProNGF expressed in Escherichia coli was able to phosphorylate TrkA and MAPK:*

So far, we showed that neither structural differences (such as different amino acid substitutions or a histidine tag) nor purification method affect the activity of proNGFhis. As neurotrophic proNGF and apoptotic proNGF were expressed in different expression systems (insect cell/baculovirus versus mammalian expression system), we tested a proNGF expressed in another expression system other than the two systems mentioned above.

Interestingly, a human recombinant proNGF expressed in a bacterial expression system (E.coli) (proNGFE) showed the same activity in the TrkA-MAPK activation we have already shown using recombinant proNGF expressed in baculovirus/insect cells (Figure 5.10). ProNGFE carries two mutations at -1 and 1 (R-A and S-A) preventing proteolytic cleavage to mature NGF. ProNGFE is expressed in E.coli and purified from inclusion bodies. For TrkA activation, the EC50 was 7.4nM (logEC50=0.87±0.16) and 17.3nM (logEC50=1.2±0.13) for 2.5S NGF and proNGFE, respectively (Fig 5.11A). The EC50 for MAPK activation was 4.3nM (log EC50=0.6±0.2) for 2.5S NGF and 8.9nM (log EC50=0.95±0.13) for proNGFE (Fig 5.11B). In all TrkA-MAPK activation assays, Western blotting using total TrkA and total MAPK showed that there was no change in the level of total TrkA and total MAPK for different dilutions of proNGFE or 2.5S NGF, as expected (Figure 5.10). Coomassie blue staining for this proNGFE before assay confirmed its quality and purity (Figure 5.11C). A Western blot on the material after TrkA-MAPK assay showed no cleavage for proNGFE, which shows that proNGF, not mature NGF, activates the TrkA signaling pathway (Figure 5.11D).

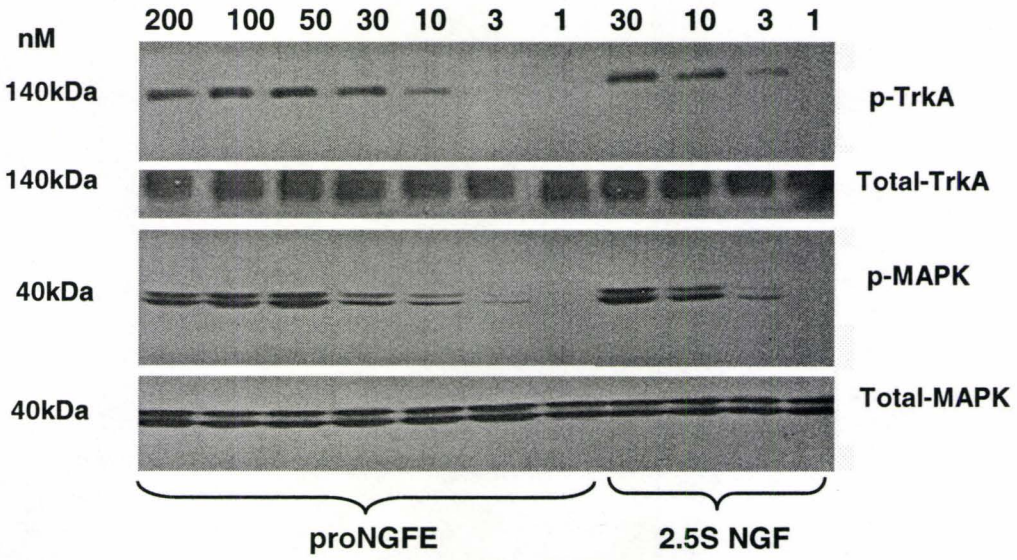



**Figure 5.10: ProNGFE expressed in E.coli promoted TrkA-MAPK phosphorylation.** Cells were treated with serum-free medium one day before treating them with different dilutions of proNGFE or 2.5S NGF. ProNGFE activated TrkA-MAPK signaling in a dose-dependent manner, while total TrkA and MAPK remained unchanged. Representative Western blot from 3 independent experiments using antibody against p-TrkA, p-MAPK, total TrkA, and total MAPK.

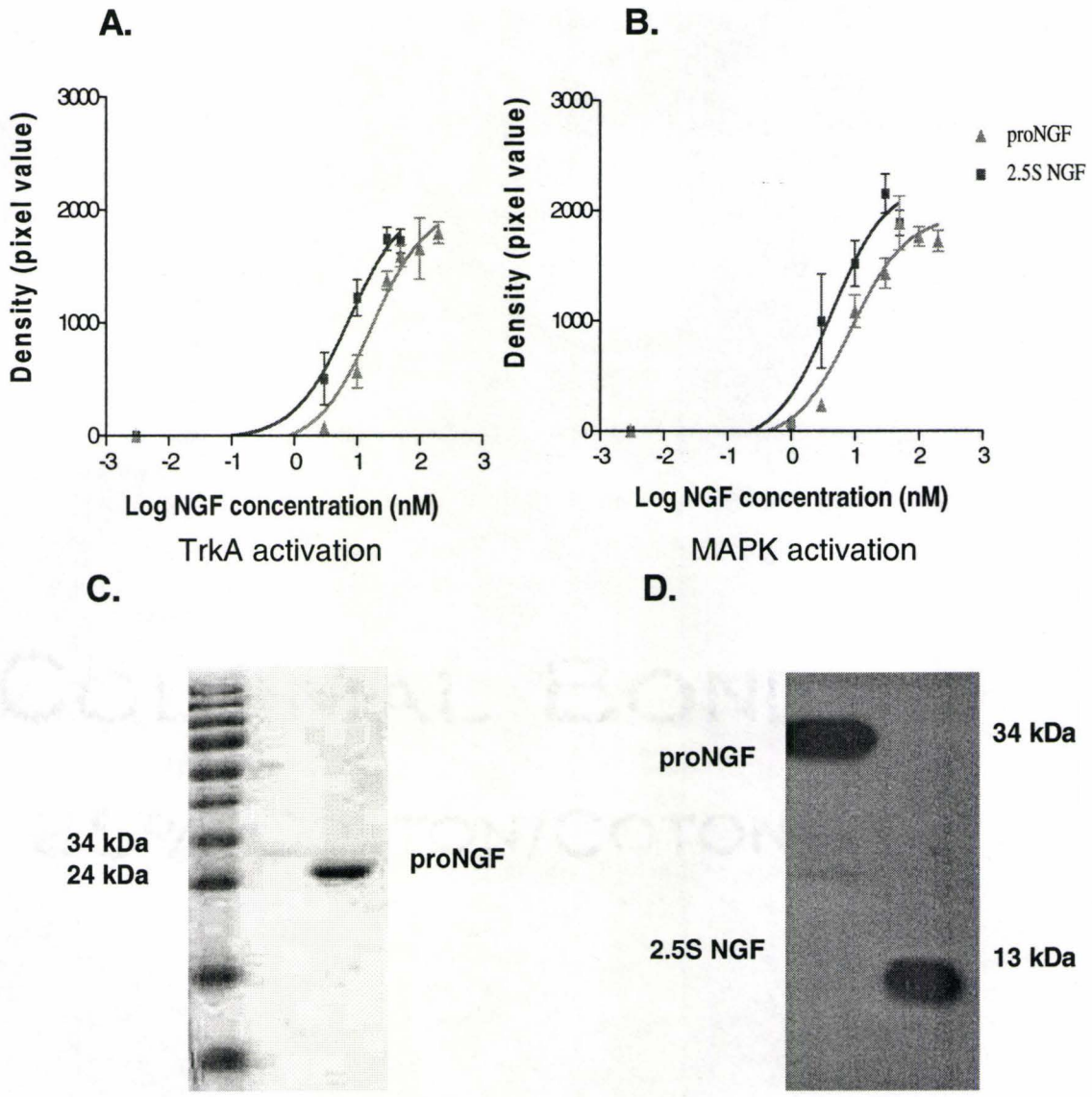



**Figure 5.11: ProNGFE expressed in a bacterial expression system promoted TrkA-MAPK signaling.** A, B: Results were obtained from 3 independent experiments. Error bars represent standard error of the mean (S.E.M) (Graphpad software 3.03, Inc, San Diego, CA, USA). A: TrkA phosphorylation: $R^2$ was 0.8 and 0.9 for proNGFE and 2.5S NGF, respectively. B: MAPK activation: $R^2$ was 0.9 for both proNGFE and 2.5S NGF. C: Coomassie blue staining for proNGFE expressed in E.coli showed an intact and pure proNGF. D: Western blotting using NGF antibody (MC51) on PC12 cell supernatant

after the TrkA-MAPK activation assay: ProNGFE expressed in E.coli was intact after the TrkA-MAPK activation assay.

### *5.1.8. ProNGFhis was neurotrophic even after NGF withdrawal:*

It has been shown that proNGFhis expressed in mammalian cells induces apoptosis (compared to medium alone) when NGF-primed SCG neurons are deprived of NGF (Nykjaer et al., 2004). As we have already shown repeatedly, proNGFhis has less activity than mature NGF. Therefore, after NGF withdrawal, when cells start to die, proNGFhis with lesser activity compared to mature NGF may not be able to rescue the cells. We repeated this NGF withdrawal experiment with proNGFhis expressed in baculovirus/insect cells. It was observed that in cells treated with medium alone (Figure 5.12C, TUJ), neurites started retraction very soon after NGF withdrawal (3-4 hours), while cells in proNGFhis (Figure 5.12B, TUJ) appear very similar to cells treated with 2.5S NGF (Figure 5.12A, TUJ) in terms of morphology. After NGF withdrawal, there was no significant difference between proNGFhis and 2.5S NGF in inducing apoptosis. Therefore, in contrast to medium alone, proNGFhis rescued cells from dying after NGF withdrawal (Figure 5.12D) and this result showed that proNGFhis is neurotrophic in the NGF withdrawal assay.

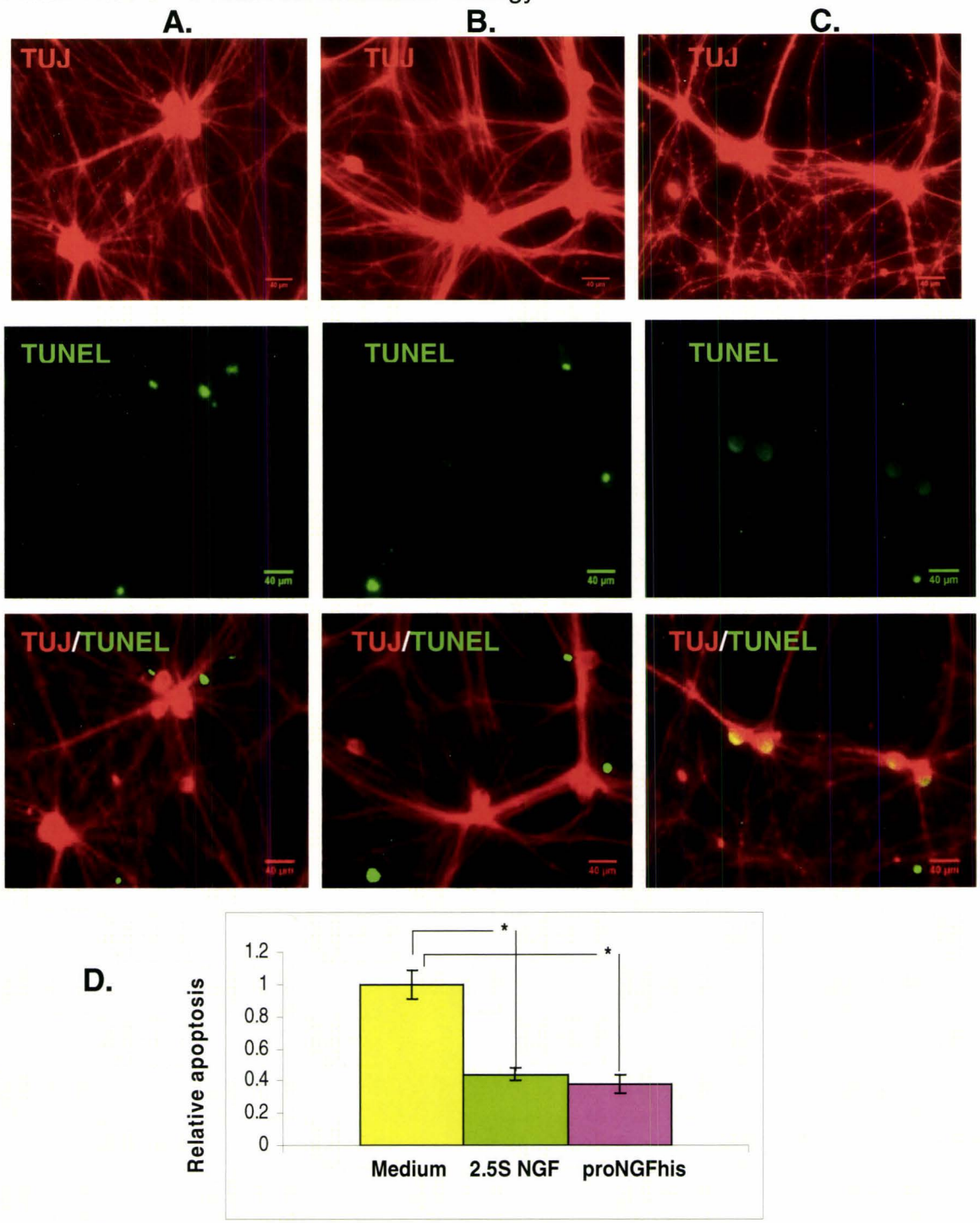



**Figure 5.12: ProNGFhis rescued SCG neurons after NGF withdrawal.** SCG cells were primed with 50 ng/ml (2 nM) of 2.5S NGF for 6-8 days and 10 ng/ml (0.4 nM) for 3 days. After NGF withdrawal for 2.5 hours, cells were treated with 10 ng/ml (0.4 nM) of 2.5S NGF (A), proNGFhis (B), or medium alone (C). After 32-36 hours, cells were fixed

and stained with TUJ-1 antibody and were subjected to TUNEL assay. Neurite degeneration can be clearly observed compared to proNGFhis or 2.5S NGF. There was no significant difference between proNGFhis and 2.5S NGF in rescuing the cells after NGF withdrawal. In contrast to medium alone, proNGFhis rescued cells from dying after NGF withdrawal. D: both 2.5S NGF and proNGFhis prevent apoptosis after NGF withdrawal. Results were obtained from three independent experiments.

* $p<0.05$, one way ANOVA followed by post hoc Tukey's test.

### *5.1.9. ProNGFhis showed apoptotic activity in the serum-deprivation assay in PC12 cells:*

Another type of deprivation assay was carried out using PC12 cells. These cells are dependent on serum for survival. Removal of serum leads them towards apoptosis. Interestingly, we found that proNGFhis induced more apoptosis (Figure 5.13B) in PC12 cells deprived of serum compared to WT-NGF (Figure 5.13A) and even compared to medium alone (Figure 5.13C). While WT-NGF (10ng/ml) showed about 13% dead cells after serum deprivation, there was 18% cell death for medium alone and 30% for proNGFhis (10ng/ml). Therefore, proNGF may function as an apoptotic factor when cell survival is already compromised.

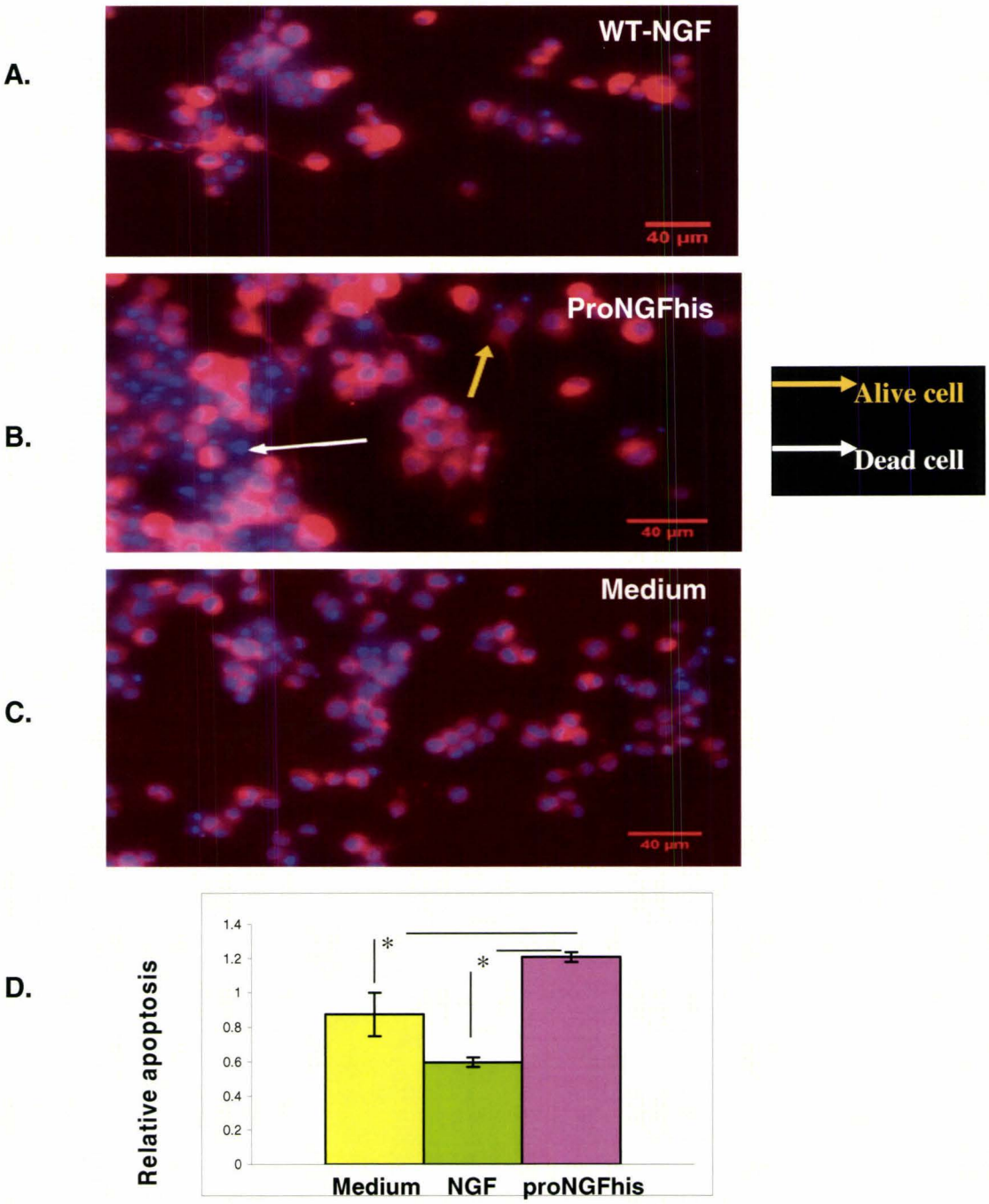



**Figure 5.13: ProNGFhis induced cell death in PC12 cells after serum removal.** PC12 cells (plated in 10% horse serum and 5% FBS) were treated with 10 ng/ml (0.4 nM) of either WT-NGF (A) or proNGFhis (B) in serum-free medium and were stained with TUJ-1 antibody (detects neuronal tubulin) and Hoechst after 48 hours

treatment. There are more dead cells in PC12 cells treated with proNGFhis (B) compared to WT-NGF (A) and even medium alone (C). There are more dead cells induced by proNGF compared to NGF and medium alone (D). However, cells treated with proNGFhis, similar to WT-NGF, show more neuronal characteristics (larger cell bodies and more staining with TUJ) compared to medium. Results were obtained from three independent experiments.

* $p<0.05$ one way ANOVA followed by post hoc Tukey's test.

**SUMMARY** (CHAPTER 5)

In Chapter 5, we showed that proNGFhis (carrying 4 amino acid substitutions and a his tag) is neurotrophic when it is expressed in a baculovirus/insect cell expression system. This protein promotes neurite outgrowth in SCG cells without inducing significant apoptosis compared to mature NGF, showing that structural differences do not change the activity of this protein.

ProNGFhis is neurotrophic (able to activate neuroprotective TrkA-MAPK phosphorylation pathway) even after purification through a nickel column. Therefore, purification was not responsible for different reported activities of proNGFs.

ProNGFE activates TrkA-MAPK phosphorylation when it is expressed in expression systems other than baculovirus (bacterial expression system). These data showed that proNGF is neurotrophic regardless of system used to express it.

ProNGFhis is neurotrophic even in the presence of a furin inhibitor, which ruled out the idea of intracellular cleavage of proNGFhis to induce TrkA-MAPK activation.

ProNGFhis rescues SCG cells from NGF withdrawal while it induces apoptosis in PC12 cells after serum deprivation. Different conditions such as priming the cells in the SCG assay may change the ratio of TrkA to p75 in such a way (increase the ratio) that proNGF is neurotrophic in the SCG NGF-withdrawal assay while it is apoptotic in the PC12 cell serum deprivation assay.

## DISCUSSION:

### *6.1. Trend in BDNF mRNA reduction in tauopathy subjects.*

BDNF plays a pivotal role in neuronal survival and differentiation (Yuan and Yanker, 2000), synaptic plasticity, learning and memory (reviewed in Lu et al., 2008). BDNF regulates neuronal survival upon binding to its receptor (TrkB) and activating the PI3Kinase signaling pathway Following activation of this pathway, apoptosis is blocked through prevention of a pro-apoptotic protein called BAD (Bcl-XL/Bcl-2-associated death promoter). Moreover, BDNF promotes survival through activation of a transcription factor (CREB) inducing the expression of genes responsible for survival (Yuan and Yankner. 2000).

Synaptic plasticity is the ability of the synapse to change its strength between two neurons. Long-lasting communication between neurons (long-term potentiation or LTP) is thought to be the mechanism underlying memory formation and learning (reviewed in Lu et al., 2008). Two types of LTP are early-phase LTP (E-LTP) and late-phase LTP (L-LTP). E-LTP is a short-lasting form (hours) of LTP which requires modification of existing proteins while L-LTP lasts longer (days) and requires new protein synthesis. BDNF is involved in E-LTP by enhancing synaptic responses likely through regulation of synaptic proteins. Moreover, BDNF has roles in maintenance of L-LTP (reviewed in Lu et al., 2008). Learning dysfunction has been reported in transgenic mice with reduced BDNF levels (Linnarsson et al., 1997). Moreover, BDNF is the only neurotrophin which shows down-regulation in AD. Levels of this protein and TrkB are reduced in cortical and hippocampal areas in AD (reviewed in Schindowski et al., 2008). Moreover, BDNF down-regulation which occurs early in AD correlates with the severity of dementia in this disease (Peng et al., 2005).

Two hallmarks of AD, Aβ deposition and tau aggregation, account for the major pathological insults in this disease. According to the amyloid cascade hypothesis, Aβ aggregation is the major factor in AD pathology (Selkoe, 1994), and other events such as tau hyperphosphorylation are downstream of Aβ

deposition. Recently, it has been shown that Aβ is able to reduce BDNF levels in vitro, probably through CREB down-regulation (Tong et al., 2004, Garzon and Fahnestock, 2007). However, it is not clear whether Aβ exerts its effect on BDNF directly or through tau hyperphosphorylation.

Treatment with lithium (a GSK-3 inhibitor), which decreases tau hyperphosphorylation, increases BDNF levels (Wada et al., 2005). This study suggests that tau hyperphosphorylation may be involved in BDNF down-regulation in AD. To investigate the role of tau dysfunction in BDNF regulation without the interference of other factors such as Aβ, we tested the level of BDNF mRNA in subjects with tau dysfunction (tauopathies). Levels of BDNF mRNA were quantified in tauopathy subjects with PiD, CBD, and PSP. These are types of dementia other than AD without any Aβ aggregation but with tau hyperphosphorylation.

### *6.1.1. BDNF mRNA levels significantly decrease in subjects with corticobasal degeneration:*

Although there is no significant decrease in BDNF mRNA levels in PSP and PiD parietal cortex compared to control, we have shown here that there is a significant reduction in BDNF mRNA in CBD when some outliers with higher BDNF levels are removed from the control and CBD groups. Interestingly, both outliers from the CBD group were of unknown race compared to all other subjects (white). No study has been carried out on the levels of BDNF in different races. However, it has been reported that there is a lower frequency of a specific BDNF allele in the Chinese population compared to other races (Bian et al., 2005). Therefore, according to our result, it would be interesting to check the levels of BDNF in different races, as well.

It is noteworthy that both PiD and PSP also showed a trend toward decreased BDNF mRNA levels compared to control. However, the trend was not statistically significant. Possible explanations for lack of significant BDNF reduction in PSP and PiD are outlined below:

1) The number of subjects per group was limited to 8-12 people which are small numbers statistically for studying human subjects. Normally, there is a huge variation between human subjects depending on the patients' clinical background. Therefore, in order to obtain significant results, a larger number of samples are required. In this study, we need more than 30 extra samples to obtain significant results for a reduction in BDNF levels in PiD and 80 additional samples for PSP, according to a power analysis with a power value of 80%.

As these disorders are rare, providing enough samples to study them is difficult. Many studies on these diseases are restricted to 1-3 patients per disease (Kasashima and Oda, 2003), which is too low statistically to draw any conclusions. As a result, there are inconsistent reports regarding neuronal loss in different areas of the brain in these neurodegenerative diseases (Kasashima and Oda, 2003).

2) Tauopathies, similar to AD, are age-related neurodegenerative diseases, and tau pathology increases with age. Moreover, it has been shown that the decline in synaptic plasticity is age-related in AD (reviewed in Horwitz, 1987). There is a wide range of ages among subjects (36-81) examined in this study (refer to Table 2.1 and 2.2). This variation in the age of samples could affect our results, as younger subjects may show less tau pathology, less cognitive decline, and less reduction in BDNF levels. However, there was no correlation ($p=0.87$, Pearson correlation) between age and BDNF levels in tauopathies investigated in this study.

3) Parietal cortex is not the most affected area of the brain in PiD or PSP compared to AD. Other areas of the brain such as the frontal and temporal cortex in PiD and midbrain in PSP may show significant changes in the levels of BDNF mRNA in those diseases. In CBD, parietofrontal cortex degeneration is characteristic of the disease, which is consistent with the decrease in BDNF levels we observed in parietal cortex of this group.

As a future direction, levels of BDNF mRNA can be determined in more affected areas such as frontal and temporal cortex in PiD and midbrain in PSP.

4) Other mechanisms (like the direct effects of Aβ) may also be involved in BDNF down-regulation in AD. It is still under investigation whether Aβ affects BDNF mRNA directly or through GSK-3β activation and tau hyperphosphorylation. If Aβ is the main culprit in BDNF mRNA reduction in AD, we expected to observe no change in BDNF mRNA levels in tauopathy brain, as there is no Aβ pathology in non-AD tauopathies. If this is so, the presence of a different mechanism underlying cognitive impairment in AD and other tauopathies is expected.

In AD, senile plaque distribution does not correlate with neuronal degeneration (Morris et al., 1991). Soluble oligomeric Aβ (soluble non-fibrillar assemblies of Aβ which is an intermediate state between Aβ monomers and non-soluble fibrils of Aβ) such as ADDLs (amyloid-beta derived diffusible ligands) has been suggested to play a major role in functional deficits in AD prior to neuronal loss (Catalano et al., 2006). Aβ aggregates block the activation of protein kinase A (PKA), which in turn, decreases CREB activation leading to memory dysfunction (reviewed in Vitolo et al., 2002). As CREB activation regulates BDNF gene expression, CREB inactivation could lead to BDNF down-regulation. Interestingly, it has been shown that soluble oligomeric Aβ causes BDNF down-regulation, most likely through CREB inactivation (Tong et al., 2004, Garzon and Fahnestock, 2007). The pathway mentioned in this paragraph (Aβ aggregates/PKA inhibition/CREB inactivation/BDNF down-regulation) could be involved in cognitive and memory deficits in AD without tau hyperphosphorylation involvement. Moreover, cognitive dysfunction observed in different kinds of transgenic animals with Aβ deposition and no tau hyperphosphorylation may support the idea that different mechanisms could be involved in cognitive dysfunction in AD and tauopathies.

5) Several transcripts are produced through alternative splicing of the human BDNF gene. Depending on the upstream promoters, different activation and tissue specific expression of transcripts occurs (reviewed in Garzon and Fahnestock, 2007). While in AD BDNF transcripts I, II, and IV are down-regulated (Garzon et al., 2002), Aβ decreases transcripts IV and V in cell culture (Garzon and Fahnestock, 2007). It is possible that tau dysfunction affects other transcripts of BDNF such as transcripts I and II which are also down-regulated in AD but are much less prevalent compared to transcript IV (Garzon et al., 2002). Decreases in those transcripts may not be enough to significantly reduce the total BDNF mRNA.

As a future direction, levels of different transcripts of BDNF can be determined in the tauopathies investigated in the present research.

### *6.2. BDNF mRNA levels in transgenic mice with tau pathology:*

#### *6.2.1. BDNF mRNA levels do not change in hippocampus of GSK-3β transgenic mice:*

GSK-3β is one of the enzymes responsible for tau phosphorylation in the brain and is involved in AD (reviewed in Hooper et al., 2007). This kinase is constitutively active and its activity is down-regulated through its phosphorylation at serine 9 by protein kinases A, B, and C (reviewed in Takashima. 2006). PI-3 kinase is another regulator of GSK-3β (reviewed in Hooper et al., 2007). All these kinases have inhibitory effects on GSK-3β activity. When cell survival is compromised and protein kinases or PI-3 kinase is inactive, increased GSK-3β activity leads to cell death. Aβ activates GSK-3β by inhibition of PI-3 kinase leading to tau hyperphosphorylation and axonal transport disruption, which in turn causes synaptic loss and dysfunction (reviewed in Takashima. 2006). Transgenic mice overexpressing GSK-3β (under control of the calcium/calmodulin kinase IIα promoter) showed a deficit in LTP which was improved after lithium treatment which inhibits GSK-3β, showing the involvement of GSK-3β in LTP dysfunction (Hooper et al., 2007). Tau hyperphosphorylation

has been detected in the hippocampus of these Tg animals (Lucas et al., 2001). Moreover, the role of BDNF in LTP has already been shown (Patterson et al., 1996). Therefore, we expected to observe a decrease in the level of BDNF in GSK-3β overexpressing mice compared to WT animals, if tau hyperphosphorylation is responsible for BDNF reduction in AD. However, we could not detect any reduction in BDNF levels in these transgenic mice compared to their WT littermates. Distribution of hyperphosphorylated tau varies among transgenic mice. As a future direction, level of hyperphosphorylated tau can be determined using Western blotting in these transgenic animals to make sure the tissues used in this experiment are actually affected by tau hyperphosphorylation.

***6.2.2. No reduction was detected in the level of BDNF mRNA in the cortex of tau transgenic mice (tau22 and tau30):***

These Tg mice express human 4R-tau under control of the Thy 1.2 promoter, which is specific for neurons and is activated postnatally. Tau30 mice exhibited motor dysfunction, as they had higher levels of human tau in the spinal cord than in the hippocampus and cortex (Schindowski et al., 2006). Therefore, we did not expect to see any changes in the level of BDNF mRNA in the cortex of those Tg mice. Our result confirmed that there is no difference between Tau30 mice and their littermates in terms of BDNF mRNA levels.

Tau22 mice, however, showed minor traces of human tau in the spinal cord and high levels of this protein in the whole brain homogenate. In these mice, abnormal tau was detected in the neocortex, hippocampus, and several other subcortical areas. The amount of abnormal tau in these areas increased with age. Abnormal conformations of tau (detected using a specific antibody) started from 3 months old, and PHF-1 immunoreactivity was detected from 6 months old in the hippocampus. Neuronal degeneration was observed at 12 months of age, and synaptic transmission showed a decrease in 14-15 month-old animals. However, they showed earlier learning impairment at age 2-3 months

(Schindowski et al., 2006). It was shown that BDNF mRNA was reduced in the entorhinal cortex, CA3 and dentate gyrus in hippocampus using in situ hybridization (Schindowski et al., 2007). Surprisingly, we could not detect any statistically significant changes in the level of BDNF mRNA between tau22 mice and WT mice in the cortex. However, there was a trend toward BDNF reduction in tau22 cortex. There are several factors which could affect our results for this strain of transgenic mice.

1) Schindowski et al. showed that BDNF decreases in the entorhinal cortex using in situ hybridization while we used whole cortex to detect the level of BDNF using q-PCR. Results of in situ hybridization can be more precise (Schindowski et al., 2007) as it determines the level of BDNF in specific areas. Lack of BDNF reduction in some areas of the cortex may obscure the reduction in the level of BDNF in other areas of the cortex when whole cortex is examined.

2) BDNF reduction was observed by 6 months in tau22 mice (Schindowski et al., 2007). Our samples ranged from ages 3-12 months, of which only 3 out of 13 were older than 6 months. This is a very small number for drawing any significant conclusions.

### *6.3. ProNGF levels in tauopathy subjects:*

NGF levels increase in AD and this increase correlates with the degree of cognitive impairment in the disease (Peng et al., 2004). There is no change in the level of NGF mRNA in AD brain (Jette et al., 1994, Fahnestock et al., 1996). Therefore, increase in the level of NGF is not due to NGF gene up-regulation in AD brain. Moreover, there is no correlation between NGF levels (in fact proNGF) and postmortem interval in human parietal cortex (Fahnestock et al., 2001). NGF is retrogradely transported from hippocampus and cortex to the basal forebrain, and its axonal trafficking is supported by MTs (Hendry et al., 1974). Tau promotes assembly and organization of the MT cytoskeleton (Matus 1994, Barlow et al., 1994, Mandelkow and Mandelkow, 1995). Tau hyperphosphorylation leads to tau detachment from MTs and causes MT depolymerization and instability which can interfere with the axonal transport of NGF (Goode et al., 1997, Utton et al.,1997, Buee et al., 2000, Sassin et al., 2000). Moreover, hyperphosphorylated tau forms large tangles of filaments (Alonso et al., 1996) which can block the axonal transport of NGF. Therefore, tau abnormalities could account for impaired retrograde transport of NGF. As proNGF, not mature NGF, is the predominant form of NGF in human brain (Fahnestock et al., 2001), it is suggested that, in AD, retrograde transport of proNGF is disrupted which leads to accumulation of this protein in target tissues such as hippocampus and cortex (Fahnestock et al., 2001, Peng et al, 2004). The NGF receptor, TrkA, is reduced in AD (for review, see Siegel and Chauhan, 2000 and Mufson et al., 2007), which may also reduce the transport of NGF. It is not clear whether tau dysfunction or TrkA reduction or both interferes with NGF (more correctly proNGF) transport in AD. Study of NGF levels in tauopathies other than AD may shed light on the role of tau in NGF transport impairment in AD.

### *6.3.1. Increased levels of proNGF in human PiD:*

In this study, we have shown that in some tauopathies such as PiD, proNGF levels (34kDa) are increased in the parietal cortex compared to normal brain. This change in the level of proNGF is similar to what happens in AD at early stages of cognitive impairment (Peng et al., 2004). However, we could not identify any significant changes in proNGF levels in the other tauopathies, PSP and CBD.

There is a consistent pattern of changes in tau during aging and AD from tau monomer to dysfunctional aggregated tau (Garcia-Sierra et al., 2003, Horowitz et al., 2004, Guillozet-Bongaarts et al., 2006). Phosphorylation at specific sites of tau is considered an early event in tau modification. It has been shown that this early change happens in tauopathies such as AD, PiD, PSP, and CBD (Figure 6.1 A) which is detected by Alz-50 antibody (Arai et al., 2003). However, there are some modifications such as tau truncation followed by phosphorylation at specific sites of tau which are not common among all tauopathies. A truncated form of tau has been detected only in AD and PiD using T-46 antibody (Figure 6.1 B) (Arai et al., 2003, Guillozet-Bongaarts et al., 2007). It has been shown that truncated forms of tau lead to tau hyperphosphorylation and tangle formation which are associated with behavioral deficits in transgenic animals expressing truncated tau (Hrnkova et al., 2007). As mentioned earlier, both tau hyperphosphorylation and tangle formation could block NGF transport. Moreover, immunoreactivity for PHF-1 antibody (which detects specific phosphorylation sites on tau which are found frequently in AD) is significantly higher in AD and PiD compared to PSP and CBD (Figure 6.1 C).

The differences in tau modifications may explain the normal level of proNGF in PSP and CBD, as they may have less modified tau which does not interfere with axonal transport of proNGF. These data may also support an increased level of proNGF only in PiD and AD. An increased level of proNGF in PiD shows that, similar to AD, retrograde transport of this protein is disrupted in

PiD, which supports an effect of tau dysfunction on retrograde transport of proNGF.

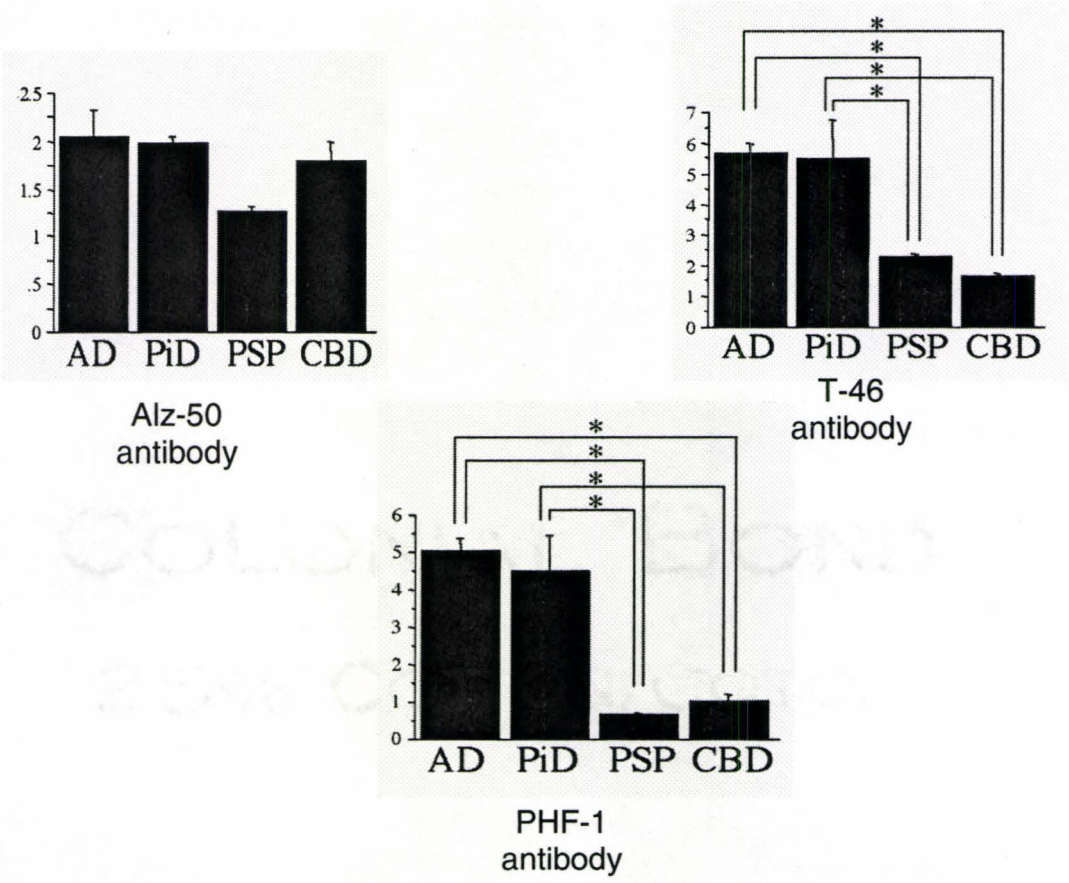



**Figure 6.1: There are differences in tau modification in AD, PiD, PSP, and CBD.** A: Alz-50 antibody recognizes early modifications that occur in tau protein. No significant difference is observed between tauopathies using Alz-50. B. T-46 detects truncated tau. Significant increase in the level of truncated tau is observed in AD and PiD compared to PSP and CBD using T-46 antibody. C. There is more PHF-1 immunoreactivity in AD and PiD than PSP and CBD. PHF-1 recognizes specific phosphorylation sites on tau which are found frequently in NFT in AD (Arai et al., 2003, *Acta Neuropathology).*

In addition, different areas of the brain are involved in each tauopathy and may explain the differences between their clinical symptoms. Both AD and PiD develop dementia and are considered primary cortical dementias, while PSP and CBD are motor-predominant syndromes (both develop dementia at later stages of the disease). This could also be a reason for observing increased proNGF levels in the cortex of PiD and AD, while not in PSP and CBD. However, in order to draw this type of conclusion we would need to access the clinical backgrounds of our experimental subjects. Unfortunately, clinical information for our tissues is unavailable, as they are from different clinical centers.

Finally, parietal cortex used in this study is not the most affected area in all groups that we examined. In PiD, frontotemporal cortex is affected the most. Midbrain, in PSP, is one of the areas of the brain with the most neuronal degeneration. Although parietofrontal cortical degeneration is characteristic of CBD brain, it is an asymmetric feature with more atrophy in one hemisphere than the other hemisphere (Kłodowska-Duda et al., 2006). This asymmetric cortical atrophy in CBD could affect our results, as we could study the wrong hemisphere with less atrophy.

### *6.4. ProNGF levels in transgenic mice with tau pathology:*

#### *6.4.1. No reduction was detected in the level of proNGF in the cortex of tau transgenic mice (JNPL):*

JNPL transgenic mice are a model of FTDP-17. These transgenic mice resemble tauopathies with motor impairment and not cognitive decline. They show progression of tau abnormalities more in spinal cord compared to the brain (reviewed in Lewis et al., 2000). Therefore, we did not expect to observe any difference in the level of proNGF in the cortex of these transgenic animals compared to WT littermates. No change was observed for proNGF levels in the cortex of this group of transgenic animals.

### *6.4.2. ProNGF levels do not change in either cortex or hippocampus of GSK-3β transgenic mice:*

Tau hyperphosphorylation has been detected in the hippocampus but not in the cortex of transgenic mice overexpressing GSK-3β (Lucas et al., 2001). If tau is responsible for impaired retrograde transport of proNGF, we expect to observe an increase in the level of proNGF in the hippocampus and not in the cortex of these transgenic mice. As expected, there was no difference in the level of proNGF between transgenic and WT animals in the cortex. Although we could observe an increase in proNGF levels in the hippocampus of some of these transgenic mice (2 out of 3 transgenic mice showed increased levels of proNGF), the increase was not statistically significant. In this experiment, the number of animals was limited to 3 per group. Increasing the number of animals per group could help us to draw a more reasonable conclusion.

### *6.4.3. No significant increase was detected in the level of proNGF in PiD mice:*

These transgenic mice overexpress a human tau mutation K369I under control of the Thy1.2 promoter. This mutation is implicated in PiD (Neumann et al., 2001). Substantia nigra, cortex, hippocampus, and amygdala showed tau overexpression and classical motor disturbances observed at age 4-6 weeks (reviewed in Janus, 2008). There is no information regarding tau hyperphosphorylation status and cognitive impairment in these animals. We could detect a trend toward increased proNGF levels in these transgenic animals, but the increase was not significant. This experiment is still under investigation and no specific conclusions can be drawn so far.

***6.5. ProNGF biological activity:***

NGF has been known for more than 50 years as a neurotrophic factor. Many papers are published on its structure, biochemical and biological properties and its role in neuronal survival, neurite outgrowth, and many other neurotrophic activities. There are also an extensive number of publications on NGF receptors, their structures, their interactions, and how NGF induces signals through these receptors. Moreover, it has been shown that NGF plays a role in learning and memory (Gutierrez et al., 1997, Woolf. et al., 2001).

Alteration in the normal level of NGF has been correlated with several neurodegenerative diseases and also with normal aging. A surprising discovery about NGF is that it can induce programmed cell death even though it is a neurotrophic factor. Apoptosis induced by NGF occurs through NGF binding to $p75^{NTR}$ with either low or absent Trk expression (Aloyz et al., 1998, Yoon et al., 1998).

Neurotrophin prodomains have roles in proper cleavage and folding of the mature domain (Edwards et al., 1988a, Suter et al., 1991, Rattenholl et al., 2001) and regulation of their secretion pathway (Farhadi et al., 2000). However, additional biological activity has been observed for proneurotrophins. In fact, in many tissues from the central and peripheral nervous system, proNGF is the predominant form of NGF (Fahnestock et al., 2001, Smith et al., 2002, Bierl et al., 2005, Pedraza et al., 2005, Bierl and Isaacson, 2007, Hayashi et al., 2007). Moreover, it is proNGF levels, not NGF, which increase in AD (Fahnestock et al., 2001) and correlate with the behavioral deficits observed in AD (Peng et al., 2004). Here (Chapter 4), we also showed changes in levels of proNGF in PiD, which is consistent with our previous observations in AD. What is the role of proNGF in AD and PiD pathology? Does an increase in proNGF levels in AD and PiD cause neuronal degeneration, or do neurons die due to the lack of neurotrophic support when retrograde transport of proNGF is disrupted? Does proNGF show the same neurotrophic activity as NGF or is it apoptotic? To

answer this question, many experiments have been carried out on the biological activity of proNGF in vitro. Nonetheless, the role of proNGF is still under debate and opposite activities (neurotrophic versus apoptotic) have been reported for this precursor.

### *6.5.1. Evidence supporting neurotrophic activity of proNGF:*

Many reports have shown neurotrophic activity for this precursor. 32 kDa proNGF isolated from mouse salivary gland promoted neurite outgrowth prior to processing (Saboori and Young, 1986). Lakshmanan et al., (1989) demonstrated that reduced forms of the 53 kDa NGF precursor promoted neurite outgrowth and survival activity of PC12 cells. A proNGF with a deletion within the processing site for conversion to mature NGF exhibited 50% of wild type neurite outgrowth activity on PC12 cells (Suter et al., 1991). Partially processed NGF precursors constructed and expressed by Ibanez et al., (1992) exhibited neurite outgrowth and survival activity. Full-length proNGF isolated from rat round spermatids exhibited neurite outgrowth activity on PC12 cells and survival activity on Sertoli cells (Chen et al., 1997). In 2004, Fahnestock et al. produced a cleavage-resistant proNGF which exhibited neurotrophic activity in promoting neurite outgrowth and TrkA-MAPK activation in cells expressing both p75 $^{NTR}$ and TrkA (Fahnestock et al., 2004a). A non-mutated human recombinant proNGF was able to activate MAPK in the presence of a furin inhibitor in pig oligodendrocytes carrying both p75$^{NTR}$ and TrkA (Althaus and Klöppner, 2006). ProNGF produced by oral mucosal keratinocytes induced neurite outgrowth in PC12 cells, and the application of a blocking antibody specific for NGF could not prevent this effect (Hayashi et al., 2007). Axonal growth with no apparent neuronal death was reported following ectopic expression of NGF in mouse cerebellum. Interestingly, proNGF not mature NGF was the form of NGF increased in the cerebellum of those transgenic mice (Buttigieg et al., 2007). In 2008, Al-Shawi et al. showed that proNGF promotes neurite outgrowth in young adult sympathetic neurons (Al-Shawi et al., 2008). Neurotrophic activity of a WT-proNGF in PC12 cells was just

recently shown by Boutilier et al (2008). This proNGF was able to phosphorylate Erk, Akt, and promote TrkA activation. The phosphorylation of Erk and Akt was blocked using K252a (TrkA inhibitor), confirming the role of TrkA in Erk and Akt activation by proNGF. However, they showed that this proNGF required intracellular cleavage to the mature form in order to exert its neurotrophic activity.

### *6.5.2. Evidence supporting apoptotic activity of proNGF:*

On the other hand, Lee et al., reported a cleavage-resistant proNGF with apoptotic activity (Lee et al., 2001b) on cells expressing only $p75^{NTR}$ or both $p75^{NTR}$ and TrkA. Beattie et al. (2002) showed that proNGF from the injured spinal cord was apoptotic for mouse oligodendrocytes expressing $p75^{NTR}$. It is noteworthy that rodent oligodendrocytes do not express TrkA (Althaus and Klöppner, 2006). Harrington et al. (2004) demonstrated that secreted proNGF after CNS injury induced apoptosis in rat oligodendrocytes and $p75^{NTR}$-expressing vascular smooth muscle cells. The induction of neuronal apoptosis on both SCG neurons and PC12 cells was shown using proNGF isolated from human AD brain (Pedraza et al., 2005). Recently, Peters et al. (2006) showed that proNGF is involved in hair follicle regression through binding to $p75^{NTR}$. A cleavage-resistant proNGF showed apoptotic activity and no ability to promote neurite outgrowth in PC12 cells (Pagadala et al., 2006). Apoptotic activity of proNGF has been reported in aged sympathetic neurons while this protein is neurotrophic in young adult sympathetic neurons (Al-Shawi et al., 2008).

Among these proNGFs, there are two different cleavage-resistant proNGFs which reportedly demonstrate opposite activity (neurotrophic versus apoptotic). These two proNGFs will be discussed in more detail:

### *6.5.3. Opposite activity for two cleavage-resistant proNGFs:*

ProNGF produced by Fahnestock et al showed neurotrophic activity (Fahnestock et al., 2004a, Althaus and Klöppner, 2006) while proNGF produced by Lee et al is apoptotic (Lee et al., 2001b, Nykjaer et al., 2004). The study of the differences in biological activity between these recombinant proNGFs (Chapter 5)

was undertaken to shed light on this controversy. Differences in the number of amino acid substitutions, addition of a histidine tag, purification methods, expression systems, and different methods of bioassays all were postulated to elicit the opposite biological activity (neurotrophic versus apoptotic) (Fahnestock et al., 2004b).

***6.6. Structural differences between two cleavage-resistant proNGFs were not responsible for the opposite activity reported for these two proNGFs:***

As mentioned earlier, cleavage-resistant proNGF with apoptotic activity carries four separate amino acid substitutions at -1 and -2 (R-R to A-A) designed to eliminate the prodomain cleavage site, and at 118 and 119 (R-R to A-A) designed to eliminate a carboxyl-terminal cleavage site that could release a polyhistidine tag. Cleavage-resistant proNGF with neurotrophic activity (R-1G proNGF) has one amino acid substitution at the -1 position (R to G) designed to eliminate the cleavage site for furin. More amino acid substitutions may change the protein folding more than a single amino acid substitution, which may lead to a less stable molecule. Some amino acid substitutions may disturb the ability of a molecule to bind to its receptor (Ibanez et al., 1992).

Moreover, there is a histidine tag at the carboxyl-terminus of the apoptotic proNGF (proNGFhis), but the neurotrophic proNGF does not carry any tags. Poly-His C-terminal extensions have been shown to modify the active site of an enzyme called β-lactamase (Ledent et al., 1997). In addition, poly histidine tags disturb protein structure (Ramage et al., 2002, Hunt, 2005) or alter fundamental biochemical properties of proteins such as dimerization/oligomerization. Recently, Zhukovsky et al. (2004) showed that the addition of a histidine tag causes higher order oligomers of protein to form as an artifact. Therefore, histidine tags may affect protein refolding and stability in such a way as to prevent binding to the TrkA receptor, while a protein without a tag may have a more stable structure.

In order to determine whether these differences can cause the opposite activity for those two cleavage-resistant proNGFs, we expressed the apoptotic proNGF construct, carrying four amino acid substitutions and a C-terminal histidine tag, in an insect cell/baculovirus expression system.

### *6.6.1. ProNGFhis promotes neurite outgrowth in SCG cells and not apoptosis:*

The activity of expressed protein was tested in a neurite outgrowth assay using SCG cells. ProNGFhis expressed in baculovirus was able to promote outgrowth in cultured SCG comparable to 2.5S NGF. There was no significant difference between proNGFhis and 2.5S NGF in inducing apoptosis in SCG cells. The ability of this proNGFhis to promote SCG outgrowth was completely different from its activity published in Lee et al. in 2001b. Our data were different from Lee's results in the apoptosis assay as well. They reported much lower outgrowth activity and much more apoptosis for proNGFhis than in our hands. Moreover, we demonstrated that the effective concentration showing 50% of maximum activity (EC50) for neurite outgrowth activity of proNGFhis expressed in baculovirus to be consistent with the EC50 for (R-1G) proNGF reported by Fahnestock et al (2004a).

### *6.6.2. ProNGFhis induces TrkA-MAPK phosphorylation in PC12 cells:*

ProNGFhis was also neurotrophic in other assays. It was able to promote both TrkA and MAPK phosphorylation in PC12 cells. This cell line expresses both TrkA and $p75^{NTR}$. Our result was consistent with the result published by Fahnestock et al (2004a) and in contrast with the results from Lee et al. (2001b) who reported no TrkA activation at all for proNGFhis expressed in mammalian cells.

To ensure that the MAPK activation was due to proNGF and not cleaved NGF, we incubated the proNGF with a proNGF antibody (obtained from Dr Xin-Fu Zhou, Human Physiology Department, Flinders University, Adelaide, Australia) before applying it in the MAPK activation assay. MAPK

phosphorylation was inhibited in a dose-dependent manner when proNGF was blocked. The activity of MAPK induced by WT-NGF was not inhibited by blocking which confirms that neurotrophic activity was dependent on proNGF.

Altogether, our observations showed that proNGFhis promoted both neurite outgrowth and TrkA-MAPK activation when it is expressed in baculovirus/insect cells. These results rule out the idea that structural differences (more amino acid substitutions or his tags) are responsible for the opposite activity of those recombinant proNGFs.

### *6.6.3. ProNGFhis promotes TrkA-MAPK phosphorylation even in the presence of furin inhibitor:*

We repeated our bioassays (mentioned above) in the presence of a furin inhibitor, and we have shown that the neurotrophic ability of proNGF remains unchanged when this protease inhibitor is used. This result is in contrast with the idea of intracellular cleavage of proNGF to NGF as a mechanism for its neurotrophic action (Boutilier et al., 2008). The same result was reported for a non-mutated recombinant human proNGF (Althaus and Klöppner, 2006). They detected Erk phosphorylation in pig oligodendrocytes even when they blocked proNGF processing using 25μM furin inhibitor. This result and our own data are in contrast with the observation published by Boutilier et al. (2008) demonstrating that the ability of a recombinant human proNGF to activate Erk and Akt is reduced by inhibiting furin. One explanation for this discrepancy could be the concentration of furin inhibitor used by our group and the Althaus group, 25-30 μM, compared to 50 μM applied by Boutilier et al. (2008). To investigate this possibility, we ran a Western blot using an NGF antibody on PC12 cell lysates exposed to proNGFhis in the presence of a furin inhibitor. Endogenous proNGF (expressed by PC12 cells) and exogenous proNGFhis ran at slightly different sizes due to the presence of the histidine tag in proNGFhis, so that we could detect both proNGFs [bands for both endogenous and exogenous proNGFs disappeared when NGF antibody was blocked using NGF peptide (data not

shown)]. In both conditions, with or without furin inhibitor, we could detect uncleaved proNGF in the cell lysates (there was less than 5% cleavage) which could activate TrkA-MAPK phosphorylation.

As a future direction, applying more furin inhibitor (50μM) on PC12 cells treated with proNGF could be appropriate.

### *6.7. Purification over a nickel column does not affect the activity of proNGFhis:*

ProNGFhis with apoptotic activity was purified over a nickel column while (R-1G) proNGF with neurotrophic activity was not. Unusual behavior of proteins purified through nickel column chromatography has been reported, as the column can cause oxidation and promote proteolysis (Ramage et al., 2002). Elution through a nickel column may change protein properties (Hunt, 2005) or cause protein aggregation (Al-Samarrai et al., 2007). We purified proNGFhis (expressed in a baculovirus system) through a nickel column and used this purified proNGF in a TrkA activation assay. This purified proNGFhis could induce both TrkA and MAPK phosphorylation in PC12 cells, about 3-5 times less than WT-NGF. This result is consistent with the data obtained by Fahnestock et al, (2004a). These data revealed that purification over a nickel column is not the reason for the observed opposite activity of the two cleavage-resistant proNGFs. Moreover, it argues against the existence of a neurotrophic agent in unpurified insect cell conditioned medium which could contribute to the neurotrophic activity observed for proNGFhis expressed in this system.

### *6.8. The expression system does not alter proNGFhis activity:*

#### *6.8.1: A mutant recombinant proNGF expressed in E.coli (proNGFE) activates TrkA-MAPK phosphorylation:*

There was also a difference in the expression system used to produce the two cleavage-resistant proNGFs with opposite activity. ProNGF with apoptotic activity was expressed in mammalian Human Embryonic Kidney (HEK) 293 cells,

while neurotrophic proNGF was produced in insect cells using a baculovirus expression system.

There are low but significant amounts of endogenous trophic factors in 293 cells. Neurotrophin subunits can freely combine with each other and produce artifacts (Treanor et al., 1995). However, in the baculovirus/insect cell expression system, there are no endogenous neurotrophic factors and protein is expressed in serum-free medium. The baculovirus–mediated insect cell expression system is one of the most common systems for recombinant protein production from a variety of organisms with similar but not identical processing and modifications to those in higher eukaryotes. Another advantage of using the baculovirus expression system is that it yields a higher level of protein compared to the mammalian cell systems (Hunt, 2005, Vermaa et al., 1998). On the other hand, it is believed that some chaperones in the baculovirus/insect cell expression system may be responsible for neurotrophic activity observed for proNGF (R-1G) (Fahnestock et al., 2004b).

Here, we have shown that proNGFhis is neurotrophic when it is expressed in insect cells using a baculovirus expression system, which is in contrast with its published role when it is expressed in mammalian cells. In all of the bioassays we carried out here, wild type baculovirus conditioned medium did not demonstrate any neurotrophic activity, arguing against the presence of any neurotrophic factor in this conditioned medium. Moreover, after purification over a nickel column, proNGFhis had more neurotrophic activity compared to unpurified proNGFhis, which is another piece of evidence showing that the neurotrophic activity of proNGFhis expressed in the baculovirus expression system is not due to some contaminating material in insect cell conditioned medium.

Therefore, our next aim was to determine whether proNGF is neurotrophic or apoptotic when it is expressed in an expression system other than the mammalian or baculovirus system. We used a mutant human recombinant

proNGFE expressed in E.coli and purified from inclusion bodies. This protein was also neurotrophic in terms of promoting TrkA-MAPK signaling activation.

ProNGFE expressed in E.coli activated the TrkA-MAPK signaling pathway with about 1/2 the activity of 2.5S NGF. This observation revealed that proNGF is able to trigger a neurotrophic pathway even when it is expressed in an expression system other than in baculovirus. It confirms, indirectly, that neurotrophic activity of proNGF expressed in the insect cell/baculovirus expression system is not due to presence of other neurotrophins or chaperones in this system.

***6.8.2: proNGFhis expressed in mammalian cells induces MAPK phosphorylation in PC12 cells:***

We detected MAPK phosphorylation activity for proNGFhis expressed in 293 cells at a concentration of 2.5ng/ml, which was approximately 2.5 times less active than WT-NGF at the same concentration (Figure 6.2). This result is consistent with the data we observed for proNGF expressed in both baculovirus and bacterial expression systems which is less active than mature NGF. Altogether, we demonstrated that proNGF is neurotrophic regardless of the expression system in which proNGF is produced.

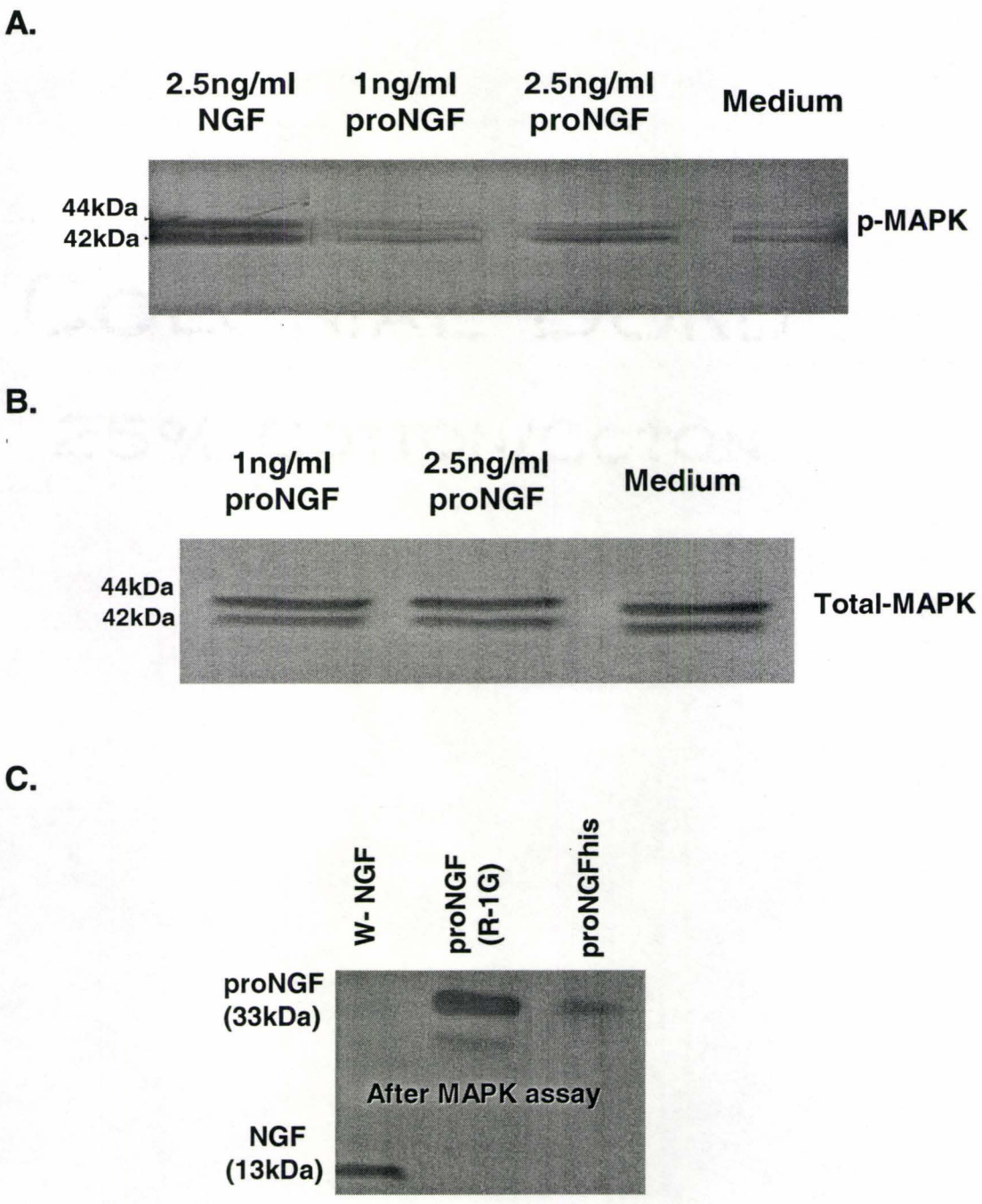



**Figure 6.2: MAPK assay carried out by Maria Ioannou (Graduate student in Dr Fahnestock's lab) for proNGFhis expressed in 293 cells.** A. proNGFhis induces MAPK phosphorylation. B. Total MAPK did not show any changes in different concentrations of proNGFhis. C: proNGFhis remains intact after MAPK assay.

### *6.9. ProNGFhis showed neurotrophic activity in an NGF-withdrawal assay in SCG cells:*

So far, this question still remained unanswered: why these two-cleavage resistant proNGFs have been reported to exhibit opposite activity. There are differences in bioassay methods used to test the proteins. ProNGF with neurotrophic activity was used in a neurite outgrowth assay in which the SCG cells were treated with this protein for 48 hours before analyzing them for neurite outgrowth (Fahnestock et al., 2004a). Although Lee et al. in (2001b), carried out the same assay as Fahnestock et al. in (2004a), they later switched to a different kind of experiment, a NGF-withdrawal assay, to show apoptotic activity for proNGF (Nykjaer et al., 2004). In this assay, SCG cells were primed with 2.5 S NGF. After 6-8 days, NGF was removed and cells were treated with mature NGF, proNGF, or medium alone (Nykjaer et al., 2004). NGF-primed cells undergo apoptosis if they are deprived of NGF (Xia et al., 1995). It is possible that proNGF cannot rescue the cells from NGF withdrawal, as proNGF shows less affinity for TrkA (Lee et al., 2001b). Surprisingly, when we repeated the withdrawal assay using proNGFhis, both NGF and proNGFhis rescued the SCG cells after NGF deprivation. There was no significant difference between 2.5S NGF and proNGFhis in rescuing cells after NGF withdrawal. Thus, this assay method used cannot account for the differences in biological activity for these two cleavage-resistant proNGFs, either.

### *6.10. ProNGFhis showed apoptotic activity in a serum withdrawal assay in PC12 cells:*

We conducted another bioassay in which undifferentiated PC12 cells were deprived of serum. These cells are a convenient and extensively used model to study NGF signaling pathways. PC12 cells require serum to survive and following serum removal, these cells undergo apoptosis. NGF can rescue the cells from apoptosis after removing serum (Greene, 1978). Interestingly, we found that proNGFhis rescued fewer PC12 cells deprived of serum compared to WT-NGF

and even compared to medium alone. While WT-NGF (10ng/ml) showed about 13% dead cells after serum deprivation, there was 18% cell death for medium alone and 30% for proNGFhis (10ng/ml). Therefore, proNGF induced more cell death than medium alone. We concluded that proNGF may be apoptotic when cell survival is already compromised. This result can be explained by the fact that proNGF shows higher affinity for $p75^{NTR}$ compared to WT-NGF (Lee et al., 2001b). Therefore, in situations such as serum deprivation in which cells are already moving towards death, NGF is able to rescue the cells while proNGF induces more death. Different conditions used to determine the activity of proNGF (such as differentiated versus undifferentiated cells) may change the level of NGF receptors in a way that proNGF shows different activities (more details in section 6.10.2).

***6.10.1. Discrepancy between the results from the SCG neurite outgrowth assay and the serum-deprivation assay in PC12 cells:***

We already mentioned that our SCG neurite outgrowth was also carried out in medium containing no serum. How is proNGF able to promote outgrowth and survival in this assay while in the serum removal assay for PC12 cells this protein was apoptotic?

One explanation could be the presence of the N2 supplement (1%) in the former experiment compared to a reduced amount of N2 supplement (0.5%) in the latter assay. Transferrin is one of the components in the N2 supplement which has been shown to be responsible for cell survival in serum-free medium (Ohnuma et al., 2006). Insulin is another protein found in N2 supplement which may also be involved in cell survival (Ohnuma et al., 2006). It may be possible that the presence of N2 supplement in the SCG neurite outgrowth assay prevents cells from progressing towards apoptosis and both NGF and proNGF are able to display their neurotrophic activity. In the serum removal assay for PC12 cells, a reduced amount of N2 supplement could be insufficient to maintain cell survival and as cells are already unhealthy, proNGF reveals another side of

its activity. As a future direction, application of different concentrations of N2 supplement in serum withdrawal assay can be tested to determine if this hypothesis is correct.

It is worthwhile to mention that in the serum deprivation assay, cells treated with proNGFhis showed neuronal features which were comparable to WT-NGF and completely different from cells treated with media. In cells treated with either proNGFhis or NGF, the ratio of cell body to nucleus was obviously larger than this ratio in cells treated with medium. An increase in the ratio of cell body to nucleus is one of the characteristics of PC12 cells when they start to differentiate. Due to this property, cells in proNGFhis or WT-NGF showed more intensive staining with the TUJ antibody. This antibody is specific for neuronal MTs which increase during PC12 cell differentiation in response to a growth-arrest specific protein (Lortie et al., 2005). Interestingly, this protein could induce neuronal properties in PC12 cells already treated with NGF, while it did not show any neuroprotective effects when cell survival was compromised. It can be concluded that different pathways are involved in survival and differentiation in PC12 cells which can be triggered separately and, while a protein may increase differentiation (proNGFhis in PC12 cells), it may not necessarily increase survival.

### *6.10.2. Discrepancy between the results of the NGF-withdrawal assay in SCG cells and the serum-deprivation assay in PC12 cells*

If we believe that proNGF is apoptotic when cells are already pushed towards death, how could we interpret the results of the NGF-withdrawal assay? In this assay, NGF treated SCGs were deprived of NGF for 2.5 hours. Cells were then treated with proNGF, NGF, or medium alone (serum-free conditions with no N2 supplement). Both NGF and proNGF rescued the SCG cells from death compared to medium alone.

First of all, we used two different types of cells (SCG versus PC12). Second of all, SCG cells were already primed with NGF. Priming has several

effects on the activity of cells. It has been shown that NGF is able to increase levels of TrkA both in vitro and in vivo (reviewed in Zhou et al., 1995). $P75^{NTR}$ either increases (reviewed in Zhou et al., 1995) or remains unchanged in response to NGF (reviewed in Althaus and Klöppner, 2006). Moreover, the ratio of TrkA to $p75^{NTR}$ is one of the factors that can determine the signaling pathway (death or survival) induced by NGF (Davies et al., 1993, Mahadeo et al., 1994, Barrett and Bartlett, 1994). Reduction in TrkA or increase in $p75^{NTR}$ can induce apoptosis in response to NGF. Therefore, priming with NGF may change the ratio of these receptors in such a way that both NGF and proNGFhis show neurotrophic activity in our NGF-withdrawal assay.

If we assume that $p75^{NTR}$ remains unchanged in our NGF-withdrawal assay after priming while TrkA was upregulated, as the ratio of TrkA to p75 increases, we expect to observe neurotrophic activity for proNGF in this assay. However, if we assume that both TrkA and p75 are upregulated in response to NGF, another explanation must be applied to interpret our NGF withdrawal results. NGF concentration is an important factor in the interaction between $p75^{NTR}$ and TrkA. In low levels of NGF (2.5ng/ml), both $p75^{NTR}$ and TrkA play a role in survival. At this concentration, reduction of each receptor alone does not affect cell survival; both receptors need to be reduced to induce cell death. However, in higher concentrations of NGF (10-50ng/ml), $p75^{NTR}$ and TrkA do not co-operate the way they do at lower concentrations of NGF regarding cell survival (Epa et al., 2004). Here, we used higher concentration of proNGF (10ng/ml). Therefore, an increase in TrkA is enough for proNGF to induce neurotrophic activity even though $p75^{NTR}$ is also upregulated.

The presence of 10-15% glia, although prevented from proliferating in our SCG assay compared to PC12 cells with no supporting cells also could account for the discrepancy observed in these two assays. These cells may support SCGs in a way that both NGF and proNGF are neurotrophic in the NGF-

withdrawal assay. All these assumptions open a wide range of opportunities to investigate more about the role of NGF, proNGF, and their receptors.

The ratio of TrkA and $p75^{NTR}$ varies during development. Interestingly, it has been shown that while reduction in $p75^{NTR}$ increases cell death in embryonic sensory neurons, it can decrease cell death in postnatal sensory neurons (Barrett and Bartlett, 1994). Most cell types used in the literature to show the apoptotic activity of proNGF do not carry TrkA (Beattie et al., 2002, Harrington et al., 2004). As a future direction, it can be proposed that proNGF is apoptotic when there is low level of TrkA compared to $p75^{NTR}$. This hypothesis can be examined by reducing TrkA expression using siRNA in cells expressing both TrkA and $p75^{NTR}$ and determining the activity of the proNGF under these circumstances. In cells treated with proNGF, less outgrowth and more apoptosis is expected when TrkA is reduced.

## *CONCLUSIONS:*

### *7.1: Tau dysfunction may be responsible for BDNF down-regulation in AD:*

AD shares tau dysfunction and some behavioral abnormalities with PiD, PSP, and CBD. There is a reduction in the level of BDNF mRNA which correlates with the level of cognitive impairment in AD. Here, we detected BDNF mRNA down-regulation in CBD and a trend to decrease in PSP and PiD. We concluded that tau dysfunction may contribute to BDNF dysregulation in AD.

### 7.2: Tau dysfunction can be involved in proNGF increase in AD:

ProNGF levels increase in AD, most likely due to tau dysfunction and transport impairment and/or TrkA reduction. In this report, we detected increased proNGF levels in human PiD but not in PSP and CBD. PiD and AD compared to PSP and CBD share common tau modifications. We concluded that tau modification may account for increased levels of proNGF in AD and PiD.

### *7.3: ProNGF is neurotrophic but can induce apoptosis when cell survival is already compromised.*

In this study, we have addressed several possibilities that might produce an apoptotic proNGF, and none of them resulted in apoptotic activity for this protein except the different bioassays used. Therefore, we can conclude that proNGF is neurotrophic regardless of expression system used to produce the protein or purification system used to purify the protein. However, proNGF can be apoptotic when cell survival is already compromised.

**Abstracts and Presentation:**

Raheleh Masoudi, Holly Buttigieg, Michael D Coughlin, and Margaret Fahnestock. (2005), Neurotrophic activity of two cleavage-resistant proNGFs in a baculovirus expression system. Southern Ontario Neuroscience Association (SONA), McMaster University, Hamilton, Canada.

Raheleh Masoudi, Holly Buttigieg, Michael D Coughlin, and Margaret Fahnestock. (2006), Neurotrophic activity of two cleavage-resistant proNGFs in a baculovirus expression system. Southern Ontario Neuroscience Association (SONA), University of Toronto, Toronto, Canada.

Raheleh Masoudi and Margaret Fahnestock, (2007), Effect of tau dysfunction on nerve growth factor retrograde transport. Canadian Association for Neuroscience (CAN), University of Toronto, Toronto, Canada.

Raheleh Masoudi, Michael Coughlin, Shelley Allen, David Dawbarn and Margaret Fahnestock, (2007), Neurotrophic activity of recombinant, cleavage-resistant proNGFs from different expression systems, Society for Neuroscience (SfN). San Diego, California, USA.

Raheleh Masoudi, Michael Coughlin, Shelley Allen, David Dawbarn and Margaret Fahnestock, (2008), Neurotrophic activity of a recombinant cleavage-resistant proNGF is unaffected by structural changes, purification and assay methods, or expression system. Southern Ontario Neuroscience Association (SONA), University of Western Ontario, London, Ontario, Canada.

Raheleh Masoudi, Maria Ioannou, Michael Coughlin, Shelley Allen, David Dawbarn and Margaret Fahnestock, (2008), Neurotrophic activity of a recombinant cleavage-resistant proNGF is unaffected by structural changes, purification and assay methods, or expression system. Canadian Association for Neuroscience (CAN), Montreal, Ontario, Canada.

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