UNIVERSIDAD DE LLEIDA

Facultad de Medicina

Departamento de Ciencias Médicas Básicas

# Implication of pro-NGF in the neurodegeneration: characterization of the underlying physiological mechanisms in Alzheimer´s Disease


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2008

Departamento de Ciencias Médicas Básicas
Universidad de Lleida

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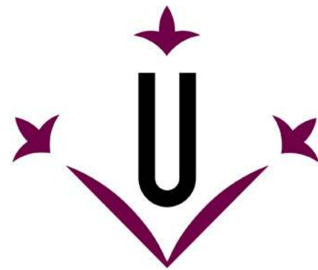
Anton Vladimirov Kichev

Lleida, 2008



Universitat de Lleida

Director of thesis

Carme Espinet Mestre

## Agradecimientos:

Quiero agradecer a toda la gente que me ha ayudado durante estos últimos cuatro años, tanto en el laboratorio como fuera de él. No quiero enumerarlos con nombres porque probablemente me olvidaría de alguien, ellos saben que les estoy muy agradecido y que los recordaré siempre.

## Благодарности

Искам да благодаря на всички хора които ми помагаха през последните 4 години, както в лабораторията така и извън нея. Няма да ги изброявам поименно защото вероятно ще пропусна някой. Тези които са ми помагали искам да знаят, че оценявам помоща им и няма да я забравя!
Освен това искам да добавя, че всъщност не искам да добавям нищо повече...

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# III. ABREVIATIONS

| | |
|---|---|
| 3T3-p75 | 3T3 cell line expressing p75NTR |
| ARA | arachidonic acid |
| Ach | acetylcholine |
| AD | Alzheimer´s disease |
| AGD | Argyrophilic grain disease |
| AGE/ALEs | advanced glycoxidation and lipoxidation end-products |
| AIF | Apoptosis Inducing Factor |
| Akt | PI3-kinase protein kinase B |
| ALS | Amyothrophic lateral sclerosis |
| AMPA | α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid |
| AP-3 | assembly protein-3 |
| APAF-1 | apoptotic protease-activating factor-1 |
| ApoE | Apolipoprotein E |
| APP | β-amyloid precursor protein |
| ARMS | Ankyrin rich membrane proteins |
| ATG | autophagy related genes |
| Aβ | amyloid β-peptide(s) |
| AβDE | Aβ-degrading enzymes |
| Bad | Bcl-2-associated death promoter |
| Bak | Bcl-2 agonist killer 1 |
| Bax | B-cell lymphoma 2-associated protein X |
| Bcl-xL | Bcl-2 associated x-protein |
| BDNF | Brain Derived Growth Factor |
| BFCN | Basal forebrain cholinergic neurons |
| BFCN | Basal forebrain cholinergic neurons |
| BID | BH3 interacting domain death agents |
| Bid | BH3 interacting domain death agonist |
| Bim | Bcl-2 interacting mediator of cell death |
| BMAA | β-methylamino-l-alanine |
| Bok | Bcl-2-related ovarian killer protein |
| BSA | Bovine serum albumin |
| CaMPK II | calmodulin-dependent protein kinase II |
| CARD | caspase recruitment domain |
| CBD | Cortico basalganglionic degeneration |
| CEL | Nε-(carboxyethyl)-lysine |
| CGNs | Cerebellar granule neurons |
| ChAT | Acetylcholine transferase |
| ChAT | Acetylcholine transferase |
| CJD | Creutzfeldt-Jacob disease |
| CML | Nε-(carboxymethyl)-lysine |
| CNS | Central nervous system |
| COX2 | Cyclooxigenase 2 |

| | |
|---|---|
| COX2 | cyclooxigenase-2 |
| CRDs | cysteine-rich domains |
| CSF | cerebrospinal liquid |
| CSN | Corticospinal neurons |
| CTF | C-terminal fragment |
| CYP | Cytochrome P450 |
| DAPT | N-[N-(3,5-difluorophenacetyl)-Lalanyl]-(S)-phenylglycine t-butyl ester |
| DED | death effector domain |
| DHA | Docosahexaenoic acid |
| DIABLO | direct IAP Binding protein with low pI |
| DISC | death inducing signalling complex |
| DJ1 | |
| DM | Myotonic dystrophy |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl Sulfoxide |
| EDTA | ethylenediaminetetraacetic acid |
| EGF | Epidermal Growth Factor |
| EMEA | European Medicines Agency |
| ER | Endoplasmatic reticulum |
| ERK | Extracellular signal regulated kinase |
| ERK | extracellular signal-regulated kinase |
| FAD | familial Alzheimer´s disease |
| FADD | Fas-associated death domain |
| FAIM | Fas apoptosis inhibitor molecule |
| FALS | Familial Amyothrophic lateral sclerosis |
| FBS | fetal bovine serum |
| FBS | fetal bovine serum |
| FDA | Food and Drug Administration |
| FGF | fibroblast growth factor |
| FGF-2 | fibroblast growth factor 2 |
| FTD | fronto-temporal dementia |
| FTDP-17 | frontotemporal dementia and parkinsonism linked to chromosome 17 |
| GC/MS | Gas chromatography coupled with mass spectrometry |
| GDNF | Glial-derived neurothrophic factor |
| GDNF | Glial-derived neurotrophic factor |
| GO | glyoxal |
| GRIK2 | glutamate receptor, ionotropic, kainate 2 |
| GSK3 | Glycogen synthase kinase 3 |
| hbi-pro-NGF | Human brain isolated pro-NGF |
| HD | Huntington´s disease |
| HNE | 4-hydroxynonenal |
| HNE-Lys | 4-hydroxynonenal-lysine |
| HS | horse serum |
| HS | horse serum |

| | |
|---|---|
| HSP | heat shock proteins |
| HTT | polyglutamine expansions in huntingtin |
| IAP | inhibitor of apoptosis protein |
| ICAD | Inhibitor of caspase-activated DNase |
| ICD | ntracellular domain |
| IGFR | Insulin like growth factor I receptor |
| IGFR | insulin like growth factor I receptor |
| IPTG | isopropyl thio-b-d-galactoside |
| JNK | c-Jun N-terminal kinase |
| JNKs | c-Jun N-terminal kinases |
| LAMP-2A | lysosomal receptor |
| LINGO | leucine rich repeat and Ig domain containing 1 |
| LRKK2 | leucine-rich repeat kinase 2 |
| LRRs | Leucine-rich repeats |
| LTD | long-term depression |
| LTP | long-term potentiation |
| MAG | Myelin associated glycoprotein |
| MAOS | membrane-associated oxidative stress |
| MAPK | mitogen activated protein kinase |
| MAPK | mitogen-activated protein kinases |
| MARK | microtubule affinity regulating kinase |
| MBGIs | Myelin-based growth inhibitors |
| MCI | mild cognitive impairment |
| MCL1 | myeloid cell leukaemia-1 |
| MDA-Lys | Nε-(malondialdehyde)-lysine |
| MEM | Minimum Essential Medium |
| MEM | Minimum Essential Medium |
| MGO | methylglyoxal |
| MMP | Matrix metaloproteases |
| mNGF | Mature form of NGF |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| MTT | 3-(4,5-dimethylthiazolys-2)-2,5-diphenyltetrazolium bromide |
| MTT | (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole |
| NAD | nicotinamide adenine dinucleotide |
| NADE | Neurothrophin associated cell death executor |
| NBM | Nucleus basalis of Meynert |
| NDD | neurodegenerative diseases |
| NFkB | nuclear factor kappaB |
| NFT | neurofibrillary tangles |
| NGF | Nerve Growth Factor |
| NICD | notch intracellular domain |
| NICD | Notch intracellular domain |
| NKs | Neuroketals |
| NMDA | N-methyl-D-aspartic acid |
| NMDA | N-methyl-d–aspartate |
| NPs | neuroprostanes |
| NRAGE | Neurothrophin receptor interacting MAGE homolog |
| NRH2 | neurotrophin receptor homolog 2 |
| NRIF | neurotrophin receptor interacting factor |
| NT | Neurothrophin(s) |

| | |
|---|---|
| NT-3 | Neurotrophin-3 |
| NT-4/5 | Neurotrophin-4/5 |
| OmgP | Olygodendrocytemyelin glycoprotein |
| p38 | p38 MAP kinase |
| p75NTR | p75 neurotrophin receptor |
| p75NTR-ICD | p75NTR intracellular domain |
| PAA | polyacryl amide |
| PAPS | 3'-phosphoadenosyl-5'-phosphosulphate |
| PARP | poly ADP-ribose polymerase |
| PCD | Programmed cell death |
| PCD | Programmed cell death |
| PD | Parkinson´s disease |
| PDPK | proline-directed protein kinases |
| PET | Positron emission tomography |
| PET | positron-emission tomography |
| PHFs | paired helical filaments |
| PI-3K | phosphoinositol-3-kinase |
| PiD | Pick's disease |
| PINK1 | phosphatase and tensin homologueinduced kinase 1 |
| PKA | cyclic-AMP-dependent kinase |
| PLC-γ | Phospholipase C-gamma |
| PNS | periferal nervous system |
| PRMTs | peptidylarginine methyltransferases |
| proBDNF | Proform of BDNF |
| pro-NGF | Proform of NGF |
| PrP | prion protein |
| PS | presenilins |
| PS-1 | Presenilin-1 |
| PS-2 | Presenilin-2 |
| PSP | progressive supranuclear palsy |
| PTMs | Post-translational modifications |
| PUFAs | polyunsaturated fatty acids |
| RCS | Reactive carbonyl species |
| RGC | retinal ganglion cells |
| Rho-GDIa | RhoGDPdissociation inhibitor |
| rh-pro-NGF | Recombinant human pro-NGF |
| RIP | Regulated intramembranal proteolisys |
| RNAi | RNA interference |
| ROS | reactive oxygen species |
| RT | Reminiscence therapy |
| RTK | Receptor tyrosine kinase |
| SAP kinases | Stress-activated protein kinases |
| SCA1 | type-1 spinocerebellar ataxia |
| SCG | Superior cervical ganglion |
| SDAT | senile dementia of the Alzheimer type |
| SDS | sodium dodecyl sulfate |
| SMAC | second mitochondrial activator of caspases |
| SOD1 | superoxide dismutase 1 |
| SPT | Simulated presence therapy |
| tBID | Truncated Bid |
| TGN | Trans Golgi network |

| | |
|---|---|
| TNF | tumor necrosis factor |
| TNFR | Tumor necrosis factor receptor |
| TNFR | tumor necrosis factor receptor |
| TOR | target of rypamicyn |
| TOR | target of rypamicyn |
| tPA | Tissue plasminogen activator |
| TPST | tyrosylprotein sulfotransferase |
| TRAFs | TNF receptor associated factors |
| TRAIL | TNF-related apoptosis-inducing ligand receptors |
| Trk A | Tyrosine receptor kinase A |
| Trk B | Tyrosine receptor kinase B |
| Trk C | Tyrosine receptor kinase C |
| TUNEL | Terminal Deoxynucleotidyl Transferase-Mediated dUTP nick end labeling |
| UCHL1 | ubiquitin carboxy-terminal hydrolase 1 |
| UPR | unfolded protein response |
| UPS | inhibition of the ubiquitin-proteasome system |

# *IV. SUMMARY*

# IV. SUMMARY

The aim of the present work is to investigate a possible relationship between the pro-form of the Neurotrophic Growth Factor (pro-NGF) and the cell death induced during pathogenesis of Alzheimer Disease (AD).

We studied the physiological effect of pro-NGF isolated from human brain (hbi-pro-NGF) in different cell models that express p75NTR. We show that hbi-pro-NGF is a potent pro-apoptotic agent. Interestingly, the hbi-pro-NGF isolated from control human brain was shown to be unable to induce cell death in the used models.

The specific receptor of pro-NGF was described to be p75NTR. We describe that higher amounts of its processing products – p75NTR ICD, are observed in brain tissue affected from AD.

In the cellular models that we used, the induction of apoptosis was preceded by processing of p75NTR with liberation of its ICD. p75NTR processing is γ-Secretase dependent. The induction of pro-apoptotic effect was shown to be entirely dependent of γ-Secretase activity. Released p75NTR ICD is shown to be translocated to nuclei. Hbi-pro-NGF isolated from control brain is not able to induce processing of p75NTR neither translocation of its ICD.

The levels of non enzymatic post-translational modifications of pro-NGF in the hippocampus and enthorinal cortex increase in AD affected brains. This can explain the greater stability and elevated physiological activity of pro-NGF isolated from human brains affected with AD.

The in vitro modified pro-NGF by glyoxal and methylglyoxal shows greater resistance against the furine cleavage and consequently greater ability to induce cell death trough p75NTR. Mice intracerebroventricularly injected with this modified rh-pro-NGF present significant learning difficulties compared with controls intracerebroventricularly injected with non modified pro-NGF and even greater differences compared with modified BSA.

The results that we describe suggest the possibility that highest levels of non enzymatic modifications of pro-NGF in human brain during the progress of AD could lead to neuron loss by a γ-Secretase dependent activation of p75NTR.

# IV. RESUMEN


El presente trabajo está centrado en la posible relación entre la form precursora del Factor de Crecimiento de Neuronas (pro-NGF) y la muerte celular inducida durante el patogénesis de la Enfermedad de Alzheimer (EA).

Hemos estudiado el efecto fisiológico de pro-NGF aislado de cerebro humano, utilizando diversos modelos celulares expresores de p75NTR. En presencia de pro-NGF aislado de cerebro afectado por EA (hbi-pro-NGF) se observa una fuerte inducción de apoptosis. El pro-NGF aislado de cerebros humanos control no es capaz de inducir muerte celular en los modelos estudiados.

P75 NTR esta descrito como receptor especifico para pro-NGF. Hemos observado niveles aumentados de su producto de procesamiento p75 ICD en tejido de cerebro afectado de EA.

En los modelos celulares estudiados, la inducción de apoptosis es posterior al procesamiento de p75NTR con liberación de su dominio intracelular (ICD). La liberación de p75NTR ICD depende de γ-Secretasa. La actividad de γ-Secretasa es imprescindible para la inducción de apoptosis. El fragmento ICD liberado se transloca hacia el núcleo. Hbi-pro-NGF aislado de cerebro control no es capaz de inducir procesamiento de p75NTR, ni translocación de su ICD.

Los niveles de modificaciones post-tranductionales no enzimáticos de pro-NGF en hippocampo y en cortex entorhinal, se muestran aumentados en los cerebros humanos afectados por EA.

Pro-NGF modificado in vitro por glyoxal y methylglyoxal demuestra una aumentada resistencia al procesamiento por furina y por consíguente una capacidad más elevada de inducir muerte celular por p75NTR. Ratones inyectados en ventrículo cerebral con este pro-NGF modificado presentan dificultades significativas en procesos de aprendizaje comparados con los ratones inyectados con pro-NGF no modificado y con ratones inyectados con BSA modificado de misma manera.

Los resultados descritos hasta ahora abren una posible vía de interpretación de los efectos de pro-NGF en cerebro humano durante desarrollo de EA pudiendo ser causantes de neurodegeneración a través de una via dependiente de γ-Secretasa y activación de p75NTR.

# V. INTRODUCTION

# V. INTRODUCTION

## 1. Neurodegenerative diseases

### 1.1. Overview

Neurodegeneration (Greek νέυρο-, néuro-, "nerval" and Latin dēgenerāre, "to decline" or "to worsen") is a general term that covers many neurlogical diseases including Alzheimer’s, Parkinson’s, Huntington’s diseases, Amyotrophic Lateral Sclerosis (ALS) and many others. Neurodegenerative disease (NDD) is a condition in which the brain and spinal cord neurons are markedly lost. Neurons die either because they are triggered to self-destruct or are damaged by toxic substances. A number of different factors may contribute to cell death, but the exact cause and the basic molecular mechanisms underlining behind the NDD are currently unknown. Although each disease has its own peculiar characteristics, many affect middle aged or older people, progress slowly over time, and result in premature death [Terry 1994]. Normally, neurodegeneration begins long before the patient experiences any symptoms. It can be months or years before any symptom is felt. Symptoms are noticed when many cells are already death or cease to function and a part of the brain begins to fail.

Aging greatly increases the risk of neurodegenerative disease. The percentage of the affected patients with neurodegenerative illness is closely correlated with their age. There is a dramatic increase in the probability of developing a neurodegenerative disorder during the sixth, seventh and eighth decades of life. There is a high probability that a person who lives to the age of 85 years will suffer from Alzheimer’s disease (AD); Parkinson’s disease (PD) is most common in those above the age of 70 years and the probability of developing Amyotrophic Lateral Sclerosis (ALS) rises sharply above the age of 40 years (figure 1i).

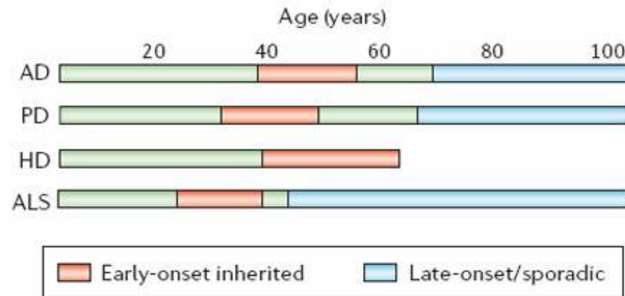



**Figure 1i.** *Ages of disease onset of early-onset inherited forms and late-onset sporadic forms of neurodegenerative disorders* [Mattson and Magnus, Nature Reviews / Neuroscience 7, April 2006, vol.7, 278-294]

A broad range of neurodegenerative disorders is characterized by neuronal damage that may be caused by toxic, insoluble aggregation-prone proteins that accumulate in and around the cells of the central nervous system (CNS) [Taylor 2002]. Neurodegenerative disorders as diverse as AD, PD, Prion diseases, Huntington's disease (HD), fronto-temporal dementia (FTD), and motor neuron disease (MND) all share a conspicuous common feature—aggregation and deposition of abnormal protein. Misfolded proteins are prone to aggregation. When all of a cell's protein quality controls fail, many proteins tend to accumulate in sufficient quantity in the affected cell.

Some of these aggregates, by adsorbing critical macromolecules to them, can severely damage cells and even cause cell death. The protein aggregates released from dead cells tend to accumulate in the extracellular matrix that surrounds the cells in a tissue, and in extreme cases they can also damage tissues. As the brain is composed of a highly organized collection of nerve cells, it is especially vulnerable. Many of the protein aggregates that cause problems form fibrils built from a series of polypeptide chains that are layered one over the other as a continuous stack, tends to be highly resistant to proteolysis. This resistance presumably may explain why this structure is observed in so many of the neurological disorders caused by protein aggregates [Molecular Biology of the Cell, $4^{th}$ edition]. Insoluble aggregates of disease-related proteins may be deposited in microscopically visible inclusions or plaques, the characteristics of which are often disease specific.

- Plaques: Amyloid precursor protein (APP) is an essential protein lodged in the surface of neurons. When unusually high levels of APP known as beta-amyloid (Aβ), are present, the Aβ fragments can clump together to form what are referred to as “plaques”. These plaques are found in Alzheimer’s disease and are thought to disrupt the signaling between cells and eventually result in massive cell death (figure 2i).
- Tangles: when microtubules, long thin fibers that transport nutrients throughout the cell, have been damaged, fibers twist together and form tangles that prevent nutrient transport. Tangles are also found in Alzheimer’s disease (figure 2i).
- Tau aggregates: Microtubule-associated tau is a protein with important functions in healthy neurons, but forms insoluble deposits in diseases now known collectively as tauopathies. Tauopathies encompass more than 20 clinicopathological entities, including AD, the most common tauopathy, progressive supranuclear palsy (PSP), Pick's disease (PiD), Argyrophilic Grain Disease (AGD), corticobasal degeneration and post-encephalitic parkinsonism.

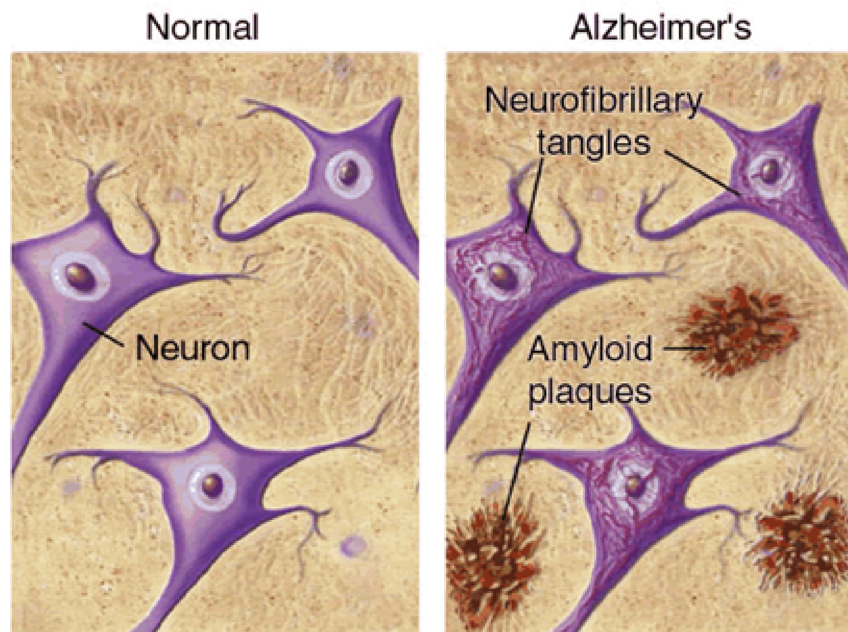


**Figure 2i.** *Intracellular neurofibrillary tangles and extracellulary amyloid plaques in Alzheimer's disease.*

Since the NDD are a vast group of disorders with different causes, locations, manifestation and mechanisms are quite difficult for classification. They are crudely divided into two groups according to phenotypic effects, conditions causing problems with movements, such as ataxia and conditions affecting memory and related to dementia. Many other diseases of the nervous system can manifest symptoms like neurodegeneration but with different pathology, like some tumors, ischemic changes, traumatic injuries, hormonal disorders, vitamin deficiencies (vitamin B, Folic acid), infections (Neurosyphilis). Rare cases of AD, PD and ALS are caused by mutations in specific genes and, in such cases, disease onset occurs at an early age (30s, 40s or 50s), which is up to 40 years earlier than the more common sporadic forms of these diseases. However, a purely genetically programmed neuronal fate seems unlikely given that late-onset neurodegenerative disorders are sporadic within families and that some individuals live for a decades or more with little or no evidence of neuronal degeneration [Hofer 2003; Mattson 2004; Cookson 2005; Moore 2005; Sieradzan 2001; Cleveland 2001].

## 1.2. Classification of neurodegenerative diseases

There are several different classifications of NDD based on different characteristics of the illnesses. The first classification based only on clinicopathological features, considerable overlap between the many disorders leading to difficulties in the diagnosis and distinction of the disorders. The complexities of the most common disorders and the overlap of the factors that contribute to the sickness, demande for a new classification of neurodegenerative disease, taking into account the age-correlation, zone of affection, symptoms, hereditary and environmental factors. However, up to now do not exist the ideal classification and they still partially overlap [Armstrong 2005]. So the NDD could be classified according to i) the type of the accumulated protein; ii) the area of the brain, the type of cells and the cellular structures that affects, and the manifested symptoms; iii) the genetic factor; and iv) environmental factors and infectious aetiologies of the NDD.

### 1.2.1. According to the type of the accumulated proteins-tauapathies and no tauopathies:

Advances in molecular neuropathology have allowed a classification system of NDD based on the protein accumulation as (i) tauopathies and (ii) no tauopathies [Armstrong 2005].

In each disease, one or more specific proteins have been identified to acumulate.

Neurodegenerative disorders as diverse as AD, PD, Lewy body disease, Prion diseases, HD, fronto-temporal dementia (FTD), and motor neuron disease all share a conspicuous common feature—aggregation and deposition of abnormal, misprocessed protein (Table 1). For that reason these diseases are known as a Proteopathies.

**Table 1.** Features of neurodegenerative disorders characterized by aggregation and deposition of abnormal protein.

| Disease | Protein deposits | Toxic protein | Disease genes | Risk factor |
|---|---|---|---|---|
| Alzheimer's disease | Extracellular plaques | Aβ | APP*<br>Presenilin 1†<br>Presenilin 2† | apoE4 allele |
| | Intracellular tangles | tau | | |
| Parkinson's disease | Lewy bodies | α-Synuclein | α-Synuclein*<br>Parkin†<br>UCHL1† | tau linkage |
| Prion disease | Prion plaque | $PrP^{Sc}$ | PRNP* | Homozygosity at prion codon 129 |
| Polyglutamine disease | Nuclear and cytoplasmic inclusions | Polyglutamine-containing proteins | 9 different genes with CAG repeat expansion* | |
| Tauopathy | Cytoplasmic tangles | tau | tau* | tau linkage |
| Familial amyotrophic lateral sclerosis | Bunina bodies | SOD1 | SOD1* | |

*Pathogenic mutations are associated with a toxic gain of function. †Pathogenic mutations are associated with a loss of function.

**Table 1.** *Aggregation and deposition of abnormal proteins is a common feature of diverse neurodegenerative disorders* [J. Paul Taylor, SCIENCE, VOL 296, 14 JUNE 2002]

Differences among neuronal populations in the production and/or clearance of abnormal proteins might be determinants of age-related neuronal

vulnerability in AD, PD and HD. The pathogenic proteins are amyloid β-peptide (Aβ and tau in AD), α-synuclein in PD and huntingtin (HTT) in HD. Genetic and age-related factors that can increase the amounts of pathogenic proteins (upstream events) include: in AD, increased levels of Aβ42 that are caused by mutations in APP or presenilins (for example, γ-Secretase), by reactive oxygen species (ROS) and by reductions in Aβ-degrading enzymes (AβDE), such as neprilysin and insulin-degrading enzyme, increases in tau concentrations that are effected by ROS, phosphorylation and calcium; in PD, increased levels of α-synuclein caused by triplication of its gene or mutations in gene for parkin enzyme, DJ1, ubiquitin carboxy-terminal hydrolase 1 (UCHL1), phosphatase and tensin homologueinduced kinase 1 (PINK1) or leucine-rich repeat kinase 2 (LRKK2), as well as proteasome impairment and ROS; in HD, polyglutamine expansions in huntingtin.

The protein aggregation process is enhanced by: increasing protein concentration; the action of transglutaminases; inhibition of the ubiquitin-proteasome system (UPS), a multicomponent system that identifies and degrades unwanted proteins; protein chaperone insufficiency; mutations in key protein genes; and post-translational modifications, such as glyco- and lipoidation; direct protein oxidation; and phosphorylation. Although the proteins involved can differ, there is considerable overlap in the mechanisms by which they damage and destroy neurons. Oligomers of Aβ, α-synuclein and HTT might act over neurons by inducing membrane-associated oxidative stress (MAOS), thereby impairing mitochondrial function and disrupting calcium homeostasis [Mattson 2006] (figure 3i).

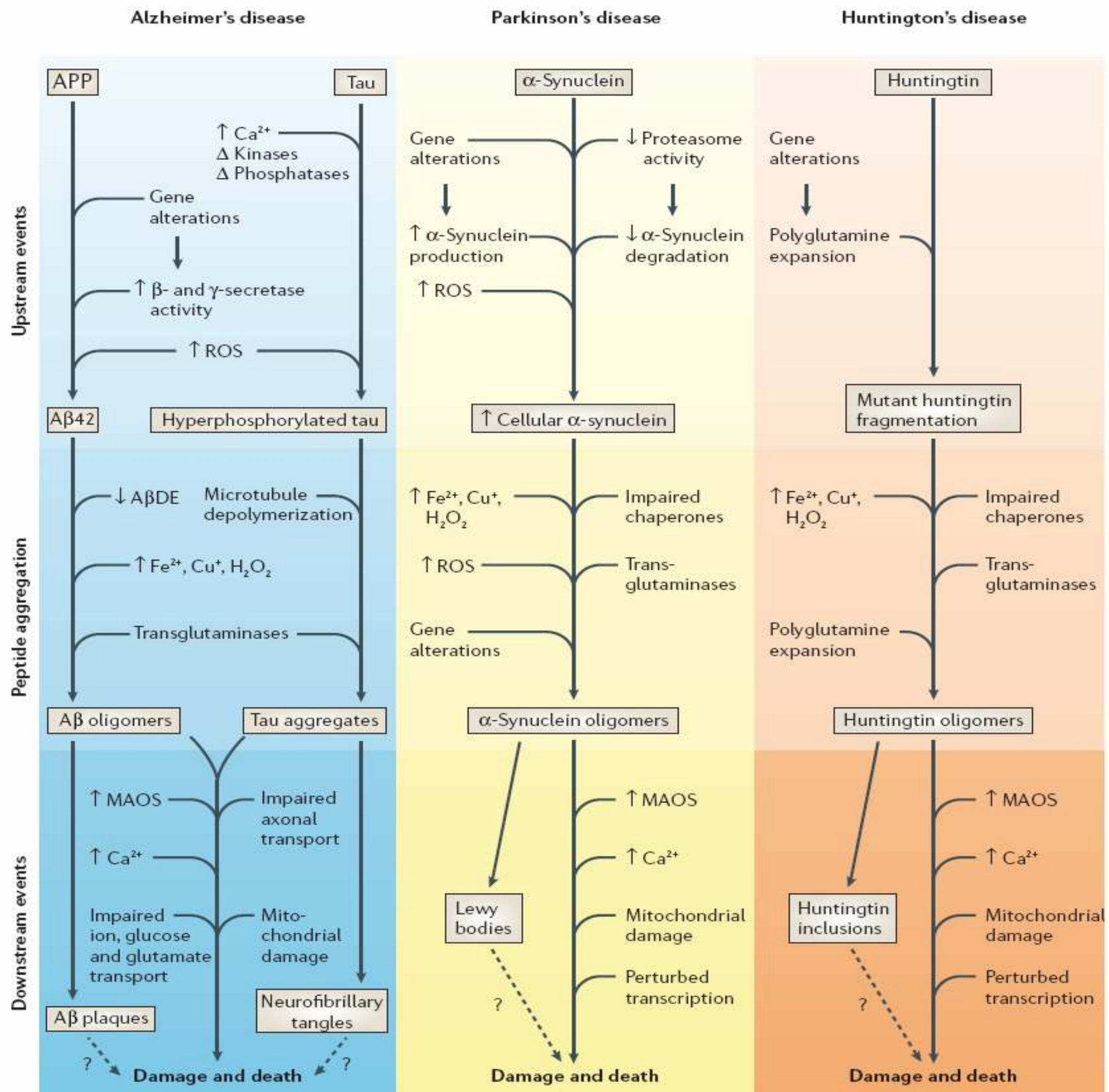



**Figure 3i.** *Mechanisms of abnormal protein accumulation in neurodegenerative disorders.* [Mattson and Magnus, Nature Reviews Neuroscience 7, April 2006, vol.7, 278-294]

## 1.2.1.1. Tauopathies: AD, PiD, AGD, FTD, PSP.

Taupathy is the commonest group of NDD, and as the most common tauopathy appears the Alzheimer disease [Tolnay 1999].

| Neurodegenerative tauopathies |
| --- |
| Alzheimer's disease (AD) |
| Amyotrophic lateral sclerosis/Parkinsonism-dementia complex of Guam* |
| Argyrophilic grain dementia* |
| Bliut disease |
| Corticobasal degeneration* |
| Dementia pugilistica* |
| Diffuse neurofibrillary tangles with calcification* |
| Down's syndrome |
| Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17)* |
| Gerstmann-Sträussler-Scheinker disease |
| Multiple system atrophy |
| Myotonic dystrophy |
| Neurodegeneration with brain iron accumulation type 1 (Hallevorden-Spatz disease) |
| Niemann-Pick disease, type C |
| Pick's disease* |
| Post-encephalitic parkinsonism |
| Prion diseases |
| Progressive subcortical gliosis* |
| Progressive supranuclear palsy* |
| Subacute sclerosing panencephalitis |
| Tangle only dementia* |

* Abnormal tau deposits are the most predominant neurodegenerative brain lesions of these diseases

**Table 2.** *The filamentous deposits of abnormally modified tau are also the hallmark brain lesion of several other neurodegenerative diseases collectively known as tauopathies* [C.-X. Gong et al., J Neural Transm (2005) 112: 813–838]

Five classes of tauopathies have been defined depending on the type of Tau aggregates that constitute the "Bar Code" for NDD:

- **Class 0** - frontal lobe degeneration:

Morphological changes comprise a neuronal cell loss, spongiosis and gliosis mainly in the superficial cortical layers of the frontal and temporal cortex. No tau aggregates are observed [Delacourte 1997; Zhukareva 2001, 2003].

- **Class I** - a major Tau triplet at 60, 64, 69 kDa:

Class I is characterized by a pathological tau triplet at 60, 64 and 69 kDa and a minor pathological tau at 72/74 kDa. This pathological tau triplet corresponds to the aggregation of the six tau isoforms [Sergeant 1997; Goedert 1992]. The pathological tau 60 is composed of shortest tau isoform. The tau 64

and 69 are a mix of tau isoforms with exon 10 or exon 2 alone, and exon 2+10 or exon 2+3, respectively.

- **Class II** - a major Tau doublet at 64 and 69 kDa and the concept of 4R tauopathies:

The class II profile is characterized essentially by the aggregation of 4R-Tau isoforms. This pathological tau profile is observed in PSP, corticobasal degeneration, AGD and FTDP- 17 [Sergeant 1999; Flament 1991; Tolnay 2002, 1997]. Dementia is a common feature at the end-stage of the disease [Litvan 1998, 2001].

- **Class III** - a major Tau doublet at 60 and 64 kDa and the concept of 3R tauopathies:

This class of tauopathy includes a single neurological disorder that is PiD. Pick's disease is a rare form of neurodegenerative disorder characterized by a progressive dementing process. Early in the clinical course, patients show signs of frontal disinhibition [Brion 1991]. Neuropathologically, PiD is characterized by prominent fronto-temporal lobar atrophy, gliosis, severe neuronal loss, ballooned neurons and the presence of neuronal inclusions called Pick bodies [Buee 1996; Delacourte 1996].

- **Class IV** - a major Tau 60:

Class IV is also represented by a single neurological disorder: Myotonic dystrophy of type I. Myotonic dystrophy (DM) is the commonest form of adult-onset muscular dystrophy. It is a multisystemic disease affecting many systems as the CNS (cognitive and neuropsychiatric impairments), the heart (cardiac conduction defects), the genital tract (testicular atrophy), the eyes (cataracts), Hypothyroidism, gastrointestinal tract (smooth muscle), endocrine system (insulin resistance), thus leading to a wide and variable complex panel of symptoms [Harper 1992; Meola 2000 a, b].

All these diseases have in common the presence of aberrant tau aggregates. Tau has long been suspected of playing a causative role in human NDD, a view supported by the observation that filamentous tau inclusions and/or microtubule defects are the predominant neuropathological feature of a broad range of sporadic disorders, known as the "tauopathies" [Lee 2001; Heutink 2000; McMurray 2000]. Tau was first implicated as a protein involved in

the pathogenesis of AD when it was discovered to be a major component of the neurofibrillary tangle [Lovestone 2002]. Subsequently, the occurrence of neurofibrillary tangles (NFT) in a wide range of conditions led to the suggestion that tau deposition may be an incidental nonspecific finding associated with cell death or cellular dysfunction. Later, the discovery that multiple mutations in the gene encoding tau are associated with frontotemporal dementia and parkinsonism (FTDP-17) provided strong evidence that abnormal forms of tau protein contribute to NDD and dementia [Spillantini 2000; Reed 2001; Martin 2001; Cole 1999; Sergeant 2005].

A substantial overlap of clinical features exists between taupathies, with many cellular lesions encountered in more than one disease. For example, neurofibrillary tangles can be seen in AD, FTDP-17, PSP and neuropil threads can be seen in AD, Cortico basalganglionic degeneration (CBD) and FTDP-17. Silver impregnation technique usually detects most of the tau inclusions (figure 4i).

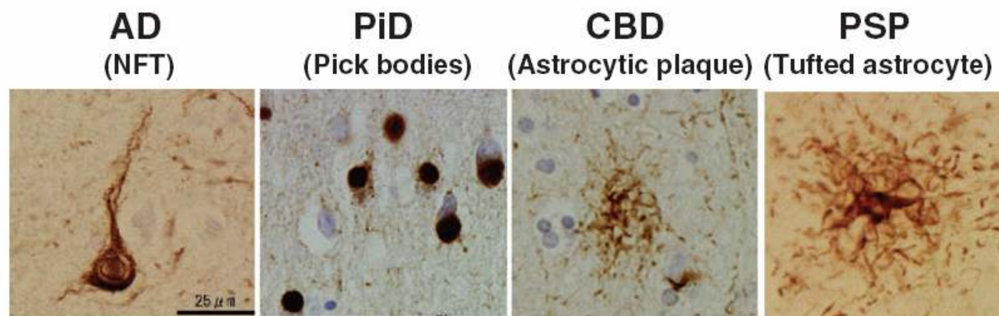



**Figure 4i.** *Characteristic tau pathologies in Alzheimer’s disease (AD), Pick’s disease (PiD), corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP)* [Masato Hasegawa, Neuropathology 2006; 26, 484–490]

The tau proteins are low molecular weight, normally cytosolic, microtubule-associated proteins that are abundant in the central and peripheral nervous system, where they are expressed predominantly in axons, but in the tauopathies they are redistributed to the cell body and dendrites [Binder 1985; Brandt 2005; Goedert 2004].

Tau protein promotes the polymerization of Tubulin into microtubules and stabilizes them [Hasegawa 2006]. Human tau proteins are encoded by a single gene on chromosome 17q21 that consists of 16 exons, and the CNS isoforms are generated by alternative splicing involving 11 of these exons (figure 5i) [Neve 1986; Goedert 1988].

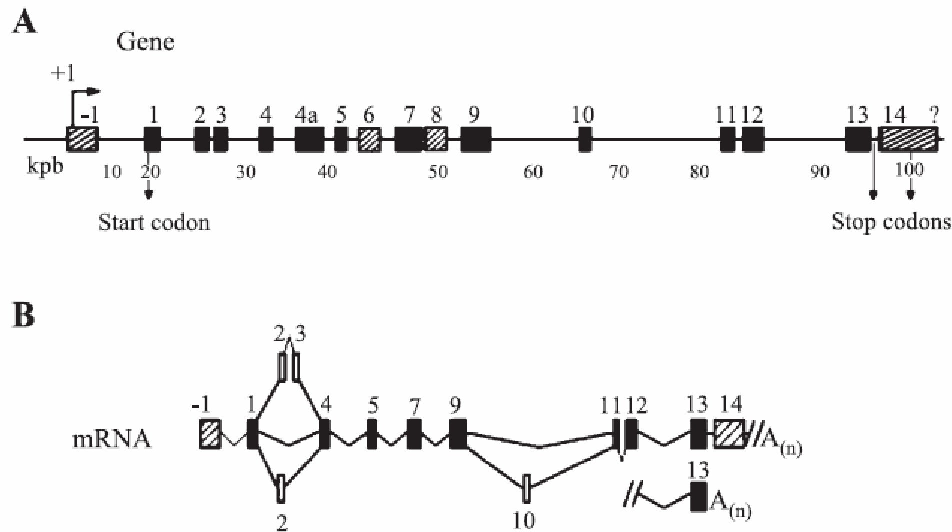



**Figure 5i.** *Tau gene structure and pre-mRNA alternative splicing in CNS. (A) The human tau gene (17q21), spans over 110 kb (B) Schematic representation of Tau primary transcript. In the CNS, exons 4A, 6 and 8 are constitutively skipped. In the CNS, the alternative splicing of exons 2, 3 and 10 generates six tau proteins. Inclusion of exon 3 is associated with that of exon 2, whereas exon 2 can be included alone* [Sergeant 2005]

In adult human brain, alternative mRNA splicing of exons 2, 3, and 10 generates six tau isoforms ranging from 352 to 441 amino acids in length. The adult tau isoforms are proteins of 441 amino acids (2 + 3 + 10 +), 410 amino acids (2 + 3+ 10-), 412 amino acids (2 + 3-10 +), 381 amino acids (2 + 3-10-) and 383 amino acids (2-3-10 +); the fetal tau isoform is a protein of 352 amino acids (2-3-10-) [Ferrer 2008]. The interaction between tau and microtubules are mediated by three or four C-terminal imperfect repeat domains (R1–R4, 31–32 amino acids each) encoded by exons 9–12 [Gustke 1994; Trinczek 1995]. Alternative splicing of exon 10 produces tau isoforms with either three (exon 10-) or four (exon 10+) repeat domains, known as 3R (PiD) and 4R (AGD, PSP)

tau, respectively (figure 6i). The isoforms differ in whether they contain three (tau-3L, tau-3S or tau-3: collectively 3R) or four (tau-4L, tau-4S or tau-4: collectively 4R) tubulin-binding domains/repeats (R) of 31 or 32 amino acids each at the C-terminal. They also differ on whether they have two (tau-3L, tau-4L), one (tau-3S, tau-4S) or no (tau-3, tau-4) repeats of 29 amino acids each in the N-terminal portion of the molecule [Robert 2007]. In the normal adult human brain, there are similar proportions of 3R-tau to 4R-tau isoforms, about 50% each one. The four repeat form of tau binds more strongly to microtubules and tends to aggregate more rapidly than the three-repeat form. NFT may result from polymerization of free tau subunits thus depleting the amount of tau available for binding [Dodart 2000].

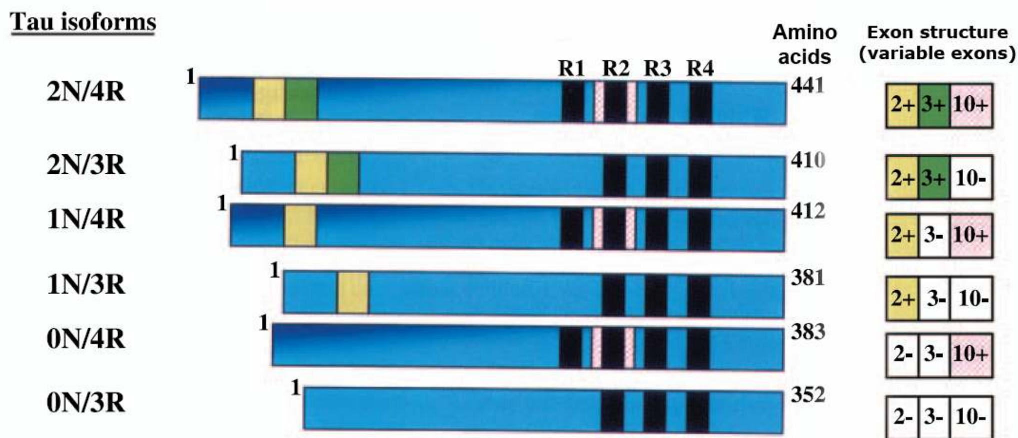



**Figure 6i.** *Tau alternative splicing: Schematic representation of the six human CNS tau isoforms. These isoforms differ by the absence or presence of one or two 29-amino acid inserts encoded by exons 2 and 3 in the amino-terminal part, in combination with either three (R1, R3 and R4) or four (R1–R4) repeat regions in the carboxy-terminal part* [R. Brandt, Biochimica et Biophysica Acta 1739 (2005) 331–354]

### 1.2.1.2. No tauopathies: PD, HD, Prion diseases, Polyglutamine disease and ALS.

- The pathological hallmark of PD is the deposition of cytoplasmic inclusions Lewy bodies, composed largely of α-synuclein, within dopaminergic

neurons. α -synuclein is a 14 kDa protein of unknown function that is located in the presynaptic terminal of the neuron [Wakabayashi 2000; McLean 2000].

Dopaminergic neurons may be more sensitive to the disease process than other neurons because they sustain more protein damage through oxidative stress induced by dopamine metabolism.

- The most common human prion disease is sporadic Creutzfeldt-Jacob disease (CJD). Prion diseases are distinct from other NDD because of their transmissibility. Although they share a common molecular aetiology, the prion diseases vary greatly in their clinical manifestations, which may include dementia, psychiatric disturbance, disordered movement, ataxia, and insomnia. The pathology of prion diseases shows varying degrees of spongioform vacuolation, gliosis, and neuronal loss. The one consistent pathological feature of the prion diseases is the accumulation of amyloid material that is immunopositive for prion protein (PrP), which is encoded by a single gene on the short arm of chromosome 20q13. Prions consist of an abnormal isoform of PrP [Colling 2001]. Structural analysis indicates that normal cellular PrP (designated $PrP_C$) is a soluble protein. In contrast, PrP extracted from the brains of affected individuals (designated $PrP_{Sc}$) is highly aggregated and detergent insoluble. The polypeptide chains for $PrP_C$ and $PrP_{Sc}$ are identical in amino acid composition, differing only in their three-dimensional conformation. Aggregation and deposition of $PrP_{Sc}$ may be a consequence of a rare, conformational change leading to sporadic cases.

- In animal models of the polyglutamine diseases and in the CNS of patients with these diseases expanded polyglutamine forms neuronal intranuclear inclusions [Ross 1997]. These inclusions consist of accumulations of insoluble aggregated polyglutamine-containing fragments, in association with other proteins. It has been proposed that proteins with long polyglutamine tracts misfold and aggregate [Perutz 1994].

- ALS is a progressive neurodegenerative disease of upper and lower motor neurons. Neuropathologically, ALS is characterized by degeneration and

loss of motor neurons. Intracellular inclusions are found in degenerating neurons and glia [Rowland 2001].

Three classifications of ALS have been described:

- Sporadic - the most common form of ALS - 90 to 95% of all cases [Cole 1999].
- Familial - occurring more than once in a family lineage (genetic dominant inheritance) accounts for a very small number of cases - 5 to 10% of all cases [Rosen 1993; Atkin 2008].
- Guamanian - an extremely high incidence of ALS was observed in Guam and the Trust Territories of the Pacific in the 1950's with both environmental and genetic components contributing to disease susceptibility [Simpson 2006].

The most common form of ALS is the "sporadic" ALS. There is also "Familial" ALS (FALS) in which the disease is inherited. Only about 5 to 10% of all ALS patients appear to have genetic or inherited form of ALS. In those families, there is a 50% chance for each offspring to inherit the gene mutation and to develop the disease.

Familial ALS in model animals is characterized neuropathologically by neuronal and astrocytic hyaline inclusions composed largely of mutant SOD1. Accumulated aggregates of mutant SOD1 might have a possible deleterious effect. The notion that aggregation is related to pathogenesis is supported by the observation that murine models of mutant SOD1-mediated disease feature prominent intracellular inclusions in motor neurons, and in some cases within the astrocytes surrounding them as well [Cleveland 2000]. Although a variety of inclusions have been described in sporadic cases of ALS, there is no evidence for deposition of SOD1 in these inclusions and no convincing evidence that aggregation contributes to the pathogenesis of sporadic ALS.

### 1.2.2. According to the area, the type of cells and the cellular structures that affects, and the manifested symptoms:

According to the affected area the disease could be separated in disorders that affect the brain, (AD, PD, PiD, AGD, prion disease, polyglutamine disease) and the illnesses that damage the spinal cord (ALS). Taking in to account the diverse symptoms that present, the NDD could be sorted out as dementias (AD, PiD, AGD, FTD, Lewy body dementia and Parkinson's dementia), motor neuron diseases (ALS, Proximal hereditary motor neuronopathy, Hereditary spastic paraplegia ).

= <u>Motor neuron disease:</u>

There are two types of motor neurons, the lower and the upper. Lower motor neuron cell bodies are located in the anterior horn of the spinal cord and in the brainstem. The upper motor neuron cell bodies are located in the brain cortex [Donaghy 1999].

Although some autors use the term 'motor neuron disease' to refer to amyotrophic lateral sclerosis (ALS), there are a wide variety of different motor neuron diseases, of varying severity, affecting all ages from infancy onwards (Table 3). The death of motor neurons is a form of NDD and results in paralysis of the limb and swallowing muscles. This degenerative process causes various disabilities, such as inability to walk, use the arms, swallow or speak, and it may lead to death when the breathing muscles become involved [Donaghy 2002].

**Combined upper and lower motor neuron involvement**

Amyotrophic lateral sclerosis

Sporadic

Familial adult onset

Familial juvenile onset

**Pure lower motor neuron involvement**

Proximal hereditary motor neuronopathy

Acute infantile form (Werdnig–Hoffmann)

Chronic childhood form (Kugelberg–Welander)

Adult onset forms

**Hereditary bulbar palsy**

With deafness (Brown–Violetta–Van Laere;

Without deafness (Fazio–Londe)

**X-linked bulbospinal neuronopathy**

**Hexosaminidase deficiency**

**Multifocal motor neuropathies**

**Postpolio syndrome**

**Postirradiation syndrome**

**Monomelic, focal or segmental spinal muscular atrophy**

**Pure upper motor neuron involvement**

Primary lateral sclerosis

Hereditary spastic paraplegia

Lathyrism

Konzo

**Table 3***. Classification of the motor neuron diseases* [Michael Donaghy, ENCYCLOPEDIA OF LIFE SCIENCES 2002]

### 1.2.3. Genetic Classification - According to the genetic factor: sporadic and genetic neurodegenerative disorders.

Familial aggregation had been recognized as a prominent characteristic of many NDD decades before the underlying molecular genetic or biochemical properties were known. Gene defects play a major role in the pathogenesis of degenerative disorders of the nervous system. The genetic studies have allowed the elucidation of the molecular mechanisms underlying the aetiology and pathogenesis of many NDD. Many NDD are also frequently described as “sporadic” or “idiopathic,” although there is a growing number of evidence suggesting that a large proportion of these cases are also significantly influenced by genetic factors. These risk genes are likely to be numerous, displaying intricate patterns of interaction with each other as well as with no genetic variable, and unlike classical Mendelian (“simplex”) disorders exhibit no simple or single mode of inheritance. Hence, the genetics of these diseases has been labeled “complex” [Bertram 2005]. Genetic analyses have laid the foundation for understanding a variety of disease mechanisms leading to neurodegeneration and associated symptoms. Majority of NDD have an early-onset familial cases that are associated with mutations in different genes transmitted in an autosomal dominant fashion. Usually these cases form a very small proportion of overall amount of cases compared with late-onset without obvious familial segregation cases (Table 4).

| Gene | Locus | Disease & Inheritance |
|---|---|---|
| APP | 21q21.2 | Alzheimer’s disease, Autosomal dominant |
| PSEN1 | 14q24.3 | Alzheimer’s disease, Autosomal dominant |
| PSEN2 | 1q31–q42 | Alzheimer’s disease, Autosomal dominant |
| SNCA(PARK1) | 4q21 | Parkinson’s disease, Autosomal dominant |
| Parkin(PARK2) | 6q25.2–q27 | Parkinsonism (juvenile), Autosomal recessive |
| SNCA(PARK4) | 4q21 (locus triplication) | Parkinson’s disease, Autosomal dominant |
| UCH-L1(PARK5) | 4p14 | Parkinson’s disease, Autosomal dominant |
| PINK-1(PARK6) | 1p36 | Parkinson’s disease, Autosomal recessive |
| DJ-1(PARK7) | 1p36 | Parkinsonism, Autosomal recessive |
| LRRK2(PARK8) | 12q12 | Parkinson’s disease, Autosomal dominant |

| | | |
|---|---|---|
| SOD1(ALS1) | 21q21 | ALS, Autosomal dominant |
| Alsin(ALS2) | 2q33 | ALS, Autosomal recessive |
| SETX(ALS4) | 9q34 | ALS (juvenile), Autosomal dominant |
| VAPB(ALS8) | 20q13.33 | ALS, Autosomal dominant |
| MAPT | 17q21 | ALS with FTD and PD, Autosomal dominant |
| IT15 | 4p16.3 | HD, Autosomal dominant |
| PRNP | 20pter-p12 | Prion diseases, Autosomal dominant |

**Table 4.** *The major causative genes in neurodegeneration* [Fabio Coppede, Biosci Rep (2006) 26:341–367]

-*Tau* mutations are either missense, deletion or silent mutations in the coding region, or intronic mutations located close to the splice-donor site of the intron following the alternatively spliced exon 10 [Goedert 2004]. Functionally, they fall into two largely non-overlapping categories—those whose primary effect is at the protein level and those that influence the alternative splicing of tau pre-mRNA. Most missense mutations reduce the ability of tau protein to interact with microtubules, as reflected by a reduction in the ability of mutant tau to promote microtubule assembly [Hasegawa 1998; Hong 1998]. A number of mutations in *Tau* may cause FTDP-17, at least in part, by promoting the aggregation of tau protein [Nacharaju 1999; Goedert 1999].

- In Alzheimer disease, rare, fully penetrant autosomal dominant mutations in 3 genes (*APP*, *PSEN1*, and *PSEN2*) have been shown to cause the disease while a common, incompletely penetrant susceptibility variant (ε4 in *APOE*) significantly increases the risk for AD.

- Parkinson’s disease at first has been considered a nongenetic disorder. However, recent data increasingly implicate genetic factors in its aetiology. Two genes are clearly associated with the disease: α-synuclein (PARK1) located on chromosome 4 [Polymeropoulos 1997] and parkin (PARK2) located on chromosome 6 [Kitada 1998]. Early onset of PD has been associated with two point mutations in α-synuclein that both increase the rate of fibril formation. There is evidence implicating a third, ubiquitin COOH-terminal hydrolase

(PARK5), [Leroy 1998; Maraganore 1999] and there are at least five other linkage loci (PARK 3, 4, 6, 7, and 8), indicating additional contributing genes [ Farrer 1999; Gasser 1998; Valente 2001; Van Duijn 2001; Hicks 2001; Funayama 2002]. When multiple genes influence a single disorder, those genes may define a pathogenic biochemical pathway. It is not yet clear what this pathway might be in PD. The notion that it could be a pathway involved in protein degradation has gained ground with the observations that parkin is an ubiquitin–protein ligase and that parkin and α-synuclein may interact [Shimura 2001].

- In Huntington's disease approximately 90% of HD cases are hereditary and transmitted in an autosomal dominant fashion. The HD gene was the first autosomal disease locus to be mapped by genetic linkage analysis (to chromosome 4q16), in 1983 [Gusella 1983]. It took 10 more years to identify the underlying gene defect, which proved to be a poly-CAG (encoding glutamina) repeat in exon 1 of a 350 kDa protein huntingtin. The mean repeat length in HD patients is 40–45, although variability is quite wide, [Gusella 1995] displaying an inverse correlation with onset age. Approximately 10% of all HD cases are considered "de novo," these cases originate from asymptomatic parents with normal repeat lengths that have expanded to symptomatic range. The precise function of huntingtin remains elusive, but cloning experiments show that it is highly conserved throughout evolution, which suggests an essential functional role of this protein in neuronal development.

In contrast to AD, PD, ALS and other multigene disorders HD is always attributable to a defect in a single gene, poly-Q expansion in *huntingtin*, although such defects only account for 50% of the cases. Between the several suggestive linkage regions the most promising is located on chromosome 6q25, close to the *glutamate receptor, ionotropic, kainate 2* (*GRIK2*), which has been associated with a younger HD onset age [Li 2003].

- Prion diseases include a rare and heterogenous spectrum of clinical and histopathological phenotypes, which are unique in the group of NDD, as they can be familial (familial Creutzfeld Jakob disease, fatal familial insomnia, Gerstmann-Sträussler-Scheinker syndrome), sporadic (Creutzfeld-Jakob

disease, sporadic fatal insomnia), or acquired (kuru, iatrogenic CJD, variant CJD).

Less common prion diseases are the hereditary forms, including familial CJD, Gerstmann-Straussler-Scheinker disease, and fatal familial insomnia [Prusiner 2001]. Hereditary prion disease is likely a consequence of a pathogenic mutation that predisposes $PrP_C$ to the $PrP_{Sc}$ structure as previously explained. The prion disease also possesses infectious aetiology.

- The Polyglutamine Diseases harbour at least nine inherited neurological disorders caused by trinucleotide (CAG) repeat expansion, including HD, Kennedy's disease, dentatorubro-pallidoluysian atrophy, and several forms of spinocerebellar ataxia [Zoghbi 2000; Nakamura 2001]. These are adult-onset diseases with progressive degeneration of the nervous system that is typically fatal. The genes responsible for these diseases appear to be functionally unrelated. The only known common feature is a CAG trinucleotide repeat in each gene's coding region, resulting in a polyglutamine tract in the disease protein.

In the normal population, the length of the polyglutamine tract is polymorphic, generally ranging from about 10 to 36 consecutive glutamine residues. In each of these diseases, expansion of the polyglutamine tract beyond the normal range results in adult-onset, slowly progressive neurodegeneration. Longer expansions correlate with earlier onset and more severe disease.

These diseases likely share a common molecular pathogenesis resulting from toxicity associated with the expanded polyglutamine tract. Expanded polyglutamine endows the disease proteins with a dominant gain of function that is toxic to neurons. Each of the polyglutamine diseases is characterized by a different pattern of neurodegeneration and thus different clinical manifestations. The selective vulnerability of different populations of neurons in these diseases likely is related to the expression pattern of each disease gene and the normal function and interactions of the disease gene product. Partial loss of function of individual disease genes, although not sufficient to cause disease, may contribute to selective neuronal vulnerability [Dragatsis 2000; Zuccato 2001].

- About 10% of ALS cases are inherited denominated familial ALS (FALS). Twenty to 40% of hereditary ALS cases are associated with mutations in the gene encoding Cu/Zn superoxide dismutase 1 (SOD1) on chromosome 21q, which catalyses conversion of toxic superoxide anion free radicals to hydrogen peroxide [Ogasawara, 1993]. More than 110 different pathogenic SOD1 mutations have been described. All of them are dominant except for the substitution of valine for alanine at position 90, which may be recessive or dominant. In FALS a deleterious gain of function by the mutant protein, might play a role consistent with autosomal dominant inheritance. In several studies was reported that Cu, Zn-SOD mutants retain high levels of dismutation activity [Gurney 1994; Borchelt 1994; Borchelt 1995], which suggests that the FALS mutations in SOD1 may act through a dominant cytotoxic gain-of-function [Ripps 1995; Pardo 1995; Rabizadeh 1995]. The cause of the other 80% of inheritated cases is practically unknown [Subramaniam 2002].

Another mutation related to ALS was identified in independent families with a rare, chronic juvenile onset form of amyotrophic lateral sclerosis (ALS2) and primary lateral sclerosis, a syndrome restricted to upper motor neuron degeneration [Yang 2001; Hadano 2001]. This mutation hit in a gene mapped to human chromosome 2q33 that codes for a protein named alsin. ALS2 is an autosomal recessive form and slow progression caused by loss of function of alsin. However the normal role of alsin is still obscure, but the protein product of the gene is found in all cell types, including motor neurons.

### 1.2.4. Accordin to the environmental factors and infectious aetiologies of the neurodegenerative disorders.

Despite several genetic mutations found in a large number of progressive and irreversible NDD [Haass 1994; Ancolio 1999; Munoz and Feldman 2000; Gatz 2005] more than 90% of the cases are sporadic [Bertram and Tanzi 2004]. Therefore, it is plausible that environmental exposure may be an etiologic factor in the pathogenesis of the NDDs.

-Parkinson’s disease - Environmental neurotoxins are related with this neurodegenerative disorder (table 5a). An incident in which several drug users

in California, USA, rapidly developed a PD-like syndrome led to the discovery of the dopaminergic neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [Langston 1983].

The widely used pesticide rotenone and the herbicide paraquat can induce PD-like pathology in rodents. The epidemiological data suggest that they might be involved also in some cases of PD [Monte 2003].

- Alzheimer's disease - The effects of the excitotoxic neurotoxin domoic acid was lighted in 1987 when more than 100 individuals who had recently eaten shellfish at restaurants in Canada became ill, with 25% of them suffering short-term memory loss that was clinically similar to that seen in patients with AD. Subsequent investigations established that the shellfish contained unusually high amounts of domoic acid, which was produced by the algal food source of the shellfish [Jeffery 2004].

Developmental exposure of rodents to the heavy metal lead (Pb) increases APP and Aβ production later in the aging brain [Basha 2005]. These findings indicate that early life experiences contribute to amyloidogenesis in old age perhaps through epigenetic pathways, as the underlying mechanism that mediates this early exposure-latent pathogenesis [Wu 2008].

- Huntington's disease - studies of several children in China who rapidly developed symptoms similar to those of HD led to the discovery of 3-nitropropionic acid, a potent inhibitor of mitochondrial complex II [Ludolph 1991].

- An amyotrophic lateral sclerosis-like syndrome was discovered in native populations in islands of Guam that, based on epidemiological and genetic studies, appears to have had an environmental cause. Evidence suggests that the cycad seed, a staple of the natives' diet, is a source of the putative toxin known as β-methylamino-l-alanine (BMAA) [Kisby 1992]. Exposure to toxins, or the influence of intense exertion, can interact with genes to cause or contribute to ALS. These are ideas researchers consider as possible reasons for the finding that some veterans and some athletes have increased incidence of ALS. As well as the Chamorro of Guam, U.S. veterans are another group of people who appear to develop ALS more often than the general population. A recent

study found that the relative risk of dying from ALS for veterans was 1.5 times that seen for the men who did not serve. The increased risk was apparent for veterans of World War II, the Korean War, and the War in Vietnam. A prior study had found that the rate of ALS in young Gulf War veterans was more than two times greater than expected for the general population.

In rodents and monkeys, MPTP, domoic acid, 3-nitropropionic acid and BMAA can destroy the specific populations of neurons that die in PD, AD, HD and ALS. Neurotoxin-based models have provided important evidence supporting the involvement of excitotoxicity in NDD. It is probable that multiple genetic and environmental factors determine whether exposure to a neurotoxin results in disease. Some findings suggesting that high calorie diets are also a factor associated with an increased risk of some NDD (PD, AD) [Mattson 1999; Duan 1999].

| Environmental factor | Increased risk for: |
|---|---|
| Aluminium, zinc, copper, iron, mercury | Alzheimer's disease |
| Severe traumatic brain injuries | Alzheimer's disease |
| Occupational exposure to pesticides | Alzheimer's disease |
| 1-methyl-4-phenyl-1,2-5,6-tetrahydropyridine (MPTP) | Parkinsonism |
| Pesticides, herbicides and fungicides | Parkinson's disease |
| Farming and living in rural areas | Parkinson's disease |
| Manganese and copper exposure | Parkinson's disease |
| Head injuries | Parkinson's disease |
| Respiratory trait infections | Parkinson's disease |
| Pesticide exposure | Amyotrophic lateral sclerosis |
| Lead and mercury exposure | Amyotrophic lateral sclerosis |
| Traumas to the spinal cord | Amyotrophic lateral sclerosis |
| Excessive physical activity | Amyotrophic lateral sclerosis |
| Professional football | Amyotrophic lateral sclerosis |
| Infection through contact with infected blood and/or animals | Prion diseases |

| Environmental factor | Protective role for: |
|---|---|
| Smoking and drinking coffee | Parkinson's disease |
| Unsaturated and omega-3 PUFA | Alzheimer's disease |
| Dietary manipulations | Huntington's disease |

**Table 5.** *Some examples of environmental factors linked to neurodegenerative diseases* [Fabio Coppede, Biosci Rep (2006) 26:341–367]

For several environmental factors linked to neurodegenerative pathologies a positive association has been observed only in some studies but not in others.

Docosahexaenoic acid (DHA) (22:6ω3) is an omega-3 polyunsaturated fatty acid (figure 7i) uniquely enriched in the brain grey matter phospholipids and retina, especially in synaptic neural membranes and in photoreceptor cells [Anderson; Bazan 1990; O'Brien 1965]. Astrocytes play an important role in the delivery of DHA to the blood-brain barrier endothelial cells and to neurons

[Bernoud 1998]. Although the physiologic basis for why DHA is enriched in the brain and retina remains unclear, reduced levels of DHA are associated with disturbances in visual acuity, behavior, and learning in young animals and humans [Jensen 1996; Makrides 1996; Werkman 1996]. DHA is a membrane polyunsaturated fatty acid that is especially vulnerable to free radical attack because hydrogen radicals easily remove its double bonds [Moore 1993; Salem 1986]. The brain may be especially susceptible to oxidative injury due to its high content of polyunsaturated fatty acids, its high oxygen consumption rate and its relative lack of antioxidant defenses. It was hypothesized that DHA can protect neural cells from apoptotic death [Kitajka 2004]. That is why changes in DHA levels might be attributed to a potential defensive response, since as DHA modulation is usually viewed as a protective mechanism for neuronal cells [Acbar 2002].

Other environmental factors also have been linked to neurodegeneration in small sample-size populations and require confirmation.

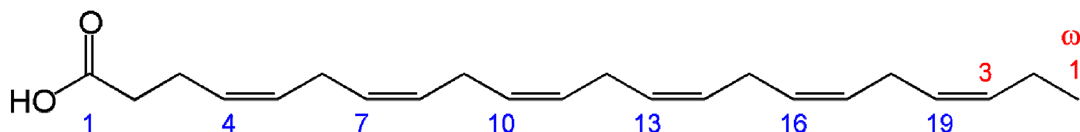



**Figure 7i.** *Structural formula and numbering of residues of Docosa hexaenoic acid* [Source www.wikipedia.org]

# 2. Alzheimer's disease

## 2.1. Short history of Alzheimer's disease

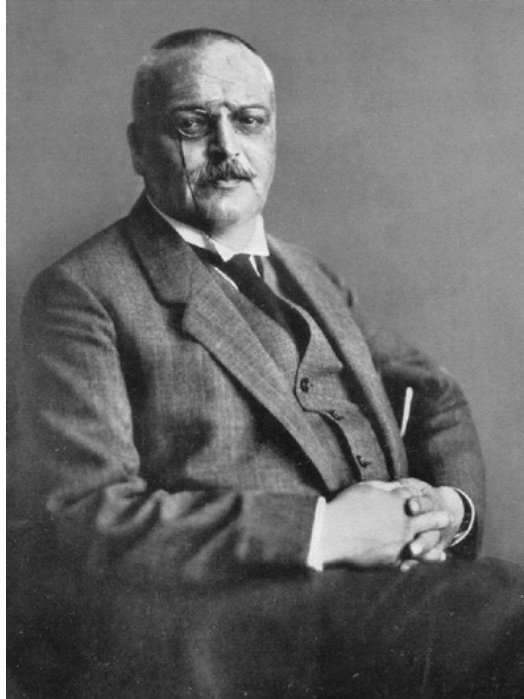


**Figure 8i.** *Alois Alzheimer (1864 - 1915)* (Photo: Alzheimer Society)

Aloysius "Alois" Alzheimer (born 14 June 1864, Marktbreit, Bavaria; died 19 December 1915, Breslau (now Wrocław, Poland), Silesia) was a German psychiatrist and neuropathologist. Alzheimer is credited with the first published case of "presenile dementia", which Kraepelin would later identify as Alzheimer's disease. In 1901, Alzheimer observed a patient at the Frankfurt Asylum named Mrs. Auguste Deter. The 51-year-old patient had strange behavioral symptoms, including a loss of short-term memory. This patient would become his obsession over the coming years. In April 1906, Mrs. D. died and Alzheimer had the patient records and the brain. Together with two Italian physicians, he would use the staining techniques to identify amyloid plaques and neurofibrillary tangles.

For most of the twentieth century, the diagnosis of Alzheimer's disease was reserved for individuals between the ages of 45 and 65 who developed

symptoms of dementia. The terminology changed after 1977 when a conference concluded that the clinical and pathological manifestations of presenile and senile dementia were almost identical, although the authors also added that this did not rule out the possibility of different etiologies [Katzman 1978]. This eventually led to the use of Alzheimer's disease independently of onset age of the disease [Boller 1998]. The term senile dementia of the Alzheimer type (SDAT) was used for a time to describe the condition in those over 65, with classical Alzheimer's disease being used for those younger. Eventually, the term Alzheimer's disease was formally adopted in medical nomenclature to describe individuals of all ages with a characteristic common symptom pattern, disease course, and neuropathology [Amaducci 1986].

## 2.2. Characteristics and stages

Alzheimer's disease is one of the most serious health problems in the industrialized world. It is an insidious and progressive NDD that accounts for the vast majority of age-related dementia [Tanzi 1999]. It is estimated that 26.6 million people worldwide were afflicted by AD in 2006, which could quadruple by 2050, although estimations vary greatly [Brookmeyer 2007]. Studies have shown that AD is more common among women than men by a ratio of 1.2 to 1.5 [Gao 1998]. The most common NDD is characterized by global cognitive decline and the accumulation of Aβ deposits and neurofibrillary tangles in the brain.

Each individual experiences the symptoms of AD in unique ways. The symptoms of AD are generally reported to a physician when memory loss becomes apparent. If AD is suspected as the cause, the physician or healthcare specialist confirm the diagnosis with behavioral assessments and cognitive tests, often followed by brain neuroimaging (CT scan or MRI). The duration of the disease is between 5 and 10 years. The disease can develop many years before it is eventually diagnosed. In its early stages, episodic memory loss, shown as a difficulty to remember recently learned facts, is the most common symptom, although it is often initially misdiagnosed as age-related memory-loss or stress [Waldemar 2007]. As the disease advances, symptoms include confusion, anger, mood swings, language breakdown, long-term memory loss,

and the general withdrawal of the sufferer as his or her senses decline [Waldemar 2007; Tabert 2005]. Gradually, minor and major bodily functions are lost, leading ultimately to death.

The cause and progression of AD are not well understood. Research indicates that the disease is associated with plaques and tangles in the brain. In general the progression of the desiese is correlated bether with the neurofibrilary tangles accumulation then with amyloid plaques deposition. No treatment has been found to stop or reverse the disease, and the current treatments used only slows a little the progression. Many preventive measures have been suggested for AD, but their value is unproven in reducing the course and severity of the disease. Mental stimulation, exercise and a balanced diet are often recommended, both as a possible prevention and as a sensible way of managing the disease. Also important is the control of the risk factors as arterial hipertension, diabetes mellitus and hipercolesterolemia.

The disease course is divided into four stages, with a progressive pattern of cognitive and functional impairment expressed during each stage.

### 2.2.1. Clinical classification

#### 2.2.1.1. Predementia

Detailed neuropsychological testing can reveal mild cognitive difficulties up to eight years before a person fulfills the clinical criteria of diagnosis. These early symptoms can have an effect on the most complex daily living activities. The most noticeable deficit is memory loss, shown as a difficulty to remember recently learned facts and an inability to acquire new information. In addition, subtle executive function problems (attention, planning, flexibility, abstraction, etc.) or impairments in semantic memory (memory of meanings and concept relationships) can also occur [Linn 1995; Perneczky 2006].

#### 2.2.1.2. Early dementia

In most people with the disease the increasing impairments in learning and memory will lead to diagnosis, while in a small proportion of them difficulties with language, executive functions, recognition of perceptions (agnosia) or

execution of movements (apraxia) will be more salient [Förstl 1999]. Nevertheless, memory problems do not affect all memory subcapacities equally. Older memories of the patient's life (episodic memory), facts learned (semantic memory), and implicit memory (the memory of the body on how to do things, such as using a fork to eat) are affected to a much lesser degree than the capacities needed to learn new facts or make new memories [Carlesimo 1992; Jelicic 1995]. The Alzheimer's patient is usually capable of adequately communicating basic ideas [Frank 1994; Becker 2002; Hodges 1995]. While performing fine motor tasks such as writing, drawing or dressing, certain visoconstructional difficulties, or apraxia, may be present. This may appear as clumsiness [Benke 1993]. As the disease progresses to the middle stage, patients might still be able to live and perform tasks independently for most of the time, but may need assistance or supervision with the most complicated activities.

#### 2.2.1.3. Moderate dementia

In the early stage, people with Alzheimer's can usually care for themselves. At the moderate stage, progressive deterioration seriously hinders the possibility of independence [Förstl 1999].

Speech difficulties become clearly noticeable: due to difficulties in finding words the person makes frequent incorrect substitutions (paraphasias) and content is poor. Reading and writing are also progressively forgotten. As time passes, complex motor sequences become less coordinated, costing the patient most of their daily-living abilities. Memory problems worsen, and the person may not recognize close relatives. Long-term memory, which was previously left intact, is now also impaired. At this stage, behavior changes, leading to crying or outbursts of unpremeditated aggression and physical violence, even in patients whose life-long behavior has been peaceful. Approximately 30% of the patients also develop illusionary misidentifications and other delusional symptoms [Galasko 2005; Sartori 2004].

#### 2.2.1.4. Advanced dementia

In the last stage of Alzheimer's disease the subject with the disease is fully dependent. Language is reduced to simple phrases or even single words before being lost altogether [Frank 1994]. Nevertheless many patients can receive and return emotional signals long after the loss of verbal language. Although aggressiveness can still be present, extreme apathy and exhaustion are much more common [Förstl 1999]. Patients will ultimately not be able to perform even the simplest tasks independently. Finally, deterioration of muscle and mobility will develop, leading the patient to become bedridden, and to lose the ability to feed oneself [Souren 1995].

### 2.2.2. Braak and Braak stage classification

Nowadays one of the most acsepted is the Braak stage method of staging the severity of Alzheimer's pathology. The staging method developed by Braak and Braak (figure 9i) postulates that the neurofibrillary pathology of Alzheimer's disease evolves in a relatively predictable sequence across the medial temporal lobe structures, subcortical nuclei and neocortical areas of the brain in six stages, with a seventh (Stage 0) representing the virtual absence of neurofibrillary changes.

0 = Braak stage 0
1 = Braak stage I
2 = Braak stage II
3 = Braak stage III
4 = Braak stage IV
5 = Braak stage V
6 = Braak stage VI

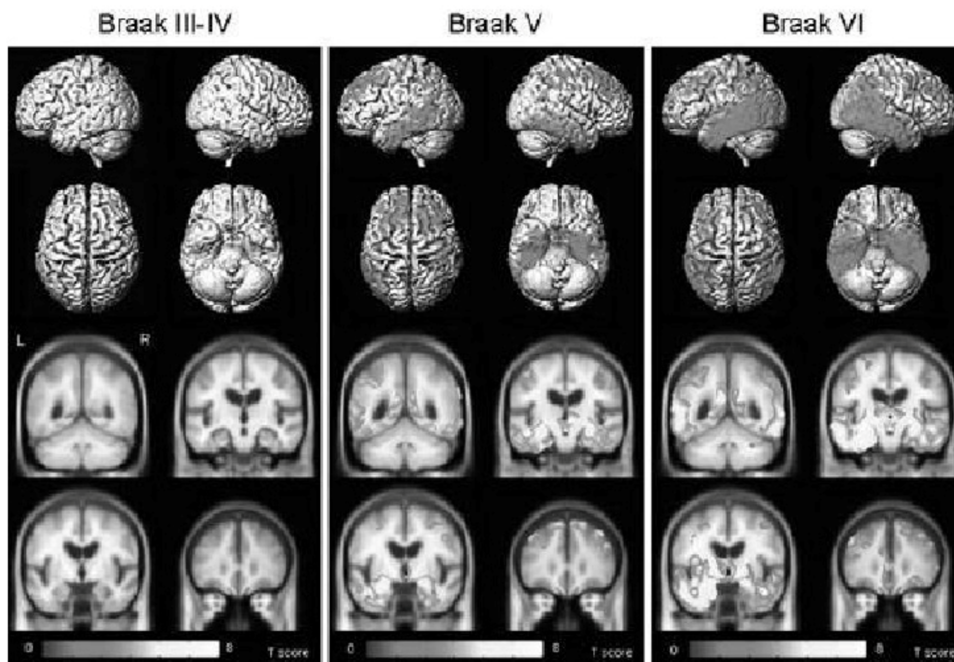



**Figure 9i.** *Voxel-based morphometry: Gray matter volume and three-dimensional surface rendering of human brains with different braak stages.*

The six stages can be collapsed into three stages. In the transentorhinal stage (Stages I and II), the neurofibrillary pathology is essentially confined to the transentorhinal and entorhinal cortex and mild involvement of the CA1/CA2 sections of the hippocampus. The limbic stage (Stages III and IV) involves severe involvement of the entorhinal areas, moderate tangles in the hippocampus, and spread to the amygdala, thalamus, hypothalamus and basal forebrain. Finally, the neocortical stage (Stages V and VI) involves abundant neurofibrillary pathology in the neocortex. The higher the Braak score the greater the spread of Alzheimer's pathology in the brain, and the more likely the person will display the symptoms of the disease.

0 = Braak stage 0
1 = Braak stage I or II
2 = Braak stage III or IV
3 = Braak stage V or VI

Braak´s neuropathologic diagnostic criteria for Alzheimer's disease is based only on topographic staining of NFT and do not evaluate SP.

### 2.2.3. CERAD stage classification

Another well accepted method of staging the severity of Alzheimer's pathology is accordinf to Consortium to Establish a Registry for Alzheimer's disease (CERAD) [Mirra 1991]. The stages are divided in 4: 0(Controls), A, B, and C stage. This method is based on semiquantitative assessment of neuritic SPs, SP frequency combined with patient age to generate an age-related plaque score. Age-related plaque score integrated with clinical information regarding the presence or absence of dementia to determine level of certainty of diagnosis. For this method the presence of NFTs are not required.

## 2.3. Pathophysiology of Alzheimer's disease

### 2.3.1. Neuropathology

At a macroscopic level, AD is characterised by loss of neurons and synapses in the cerebral cortex and certain subcortical regions (figure10i). This results in atrophy of the affected regions, including degeneration in the temporal lobe and parietal lobe, and parts of the frontal cortex and cingulate gyrus [Wenk 2003].

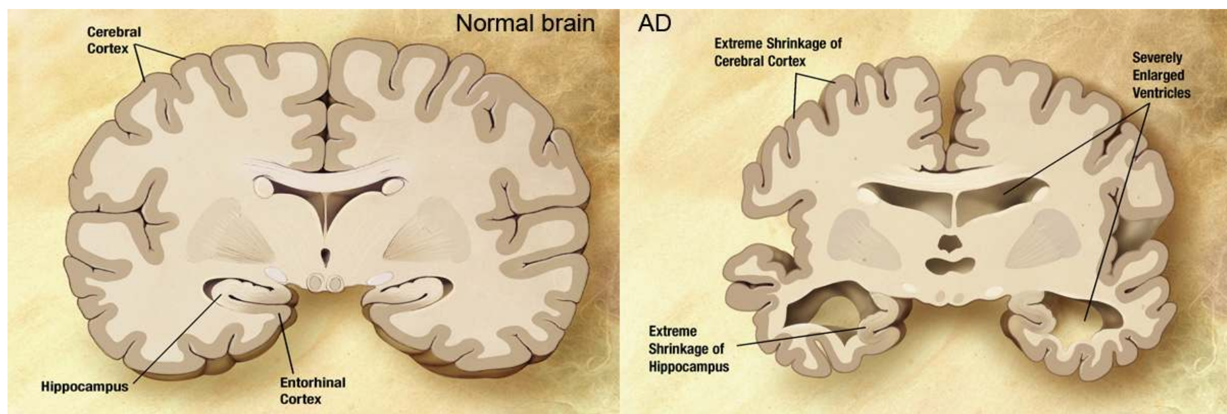



**Figure 10i.** *Cross-sections of a typical healthy brain (left) compared to an Alzheimer's-affected brain (right).* [Courtesy U.S. Nat'l Inst. on Aging]

Both amyloid plaques and neurofibrillary tangles are clearly visible by microscopy in AD brains [Tiraboschi 2004]. Plaques are dense, mostly insoluble deposits of amyloid-beta protein and cellular material outside and around

neurons. Tangles are insoluble twisted fibers that build up inside the nerve cell. Though many older people develop some plaques and tangles, the brains of AD patients have them to a much greater extent and in different brain locations [Bouras 1994].

### 2.3.2. Biochemical characteristic

Alzheimer's disease has been identified as a protein misfolding disease, or proteopathy, due to the accumulation of abnormally folded Amyloid-beta (Aβ), proteins in the brains of AD patients [Hashimoto 2003]. Aβ is a short peptide that is a proteolytic byproduct of the transmembrane protein APP, whose function is unclear but thought to be involved in neuronal development. The presenilins are components of a proteolytic complex involved in APP processing and degradation. Although Aβ monomers are soluble and harmless, they undergo a dramatic conformational change at sufficiently high concentration to form a beta sheet-rich tertiary structure that aggregates to form amyloid fibrils [Ohnishi2004] that deposit outside neurons in dense formations known as senile plaques or neuritic plaques, in less dense aggregates as diffuse plaques, and sometimes in the walls of small blood vessels in the brain in a process called amyloid angiopathy or congophilic angiopathy.

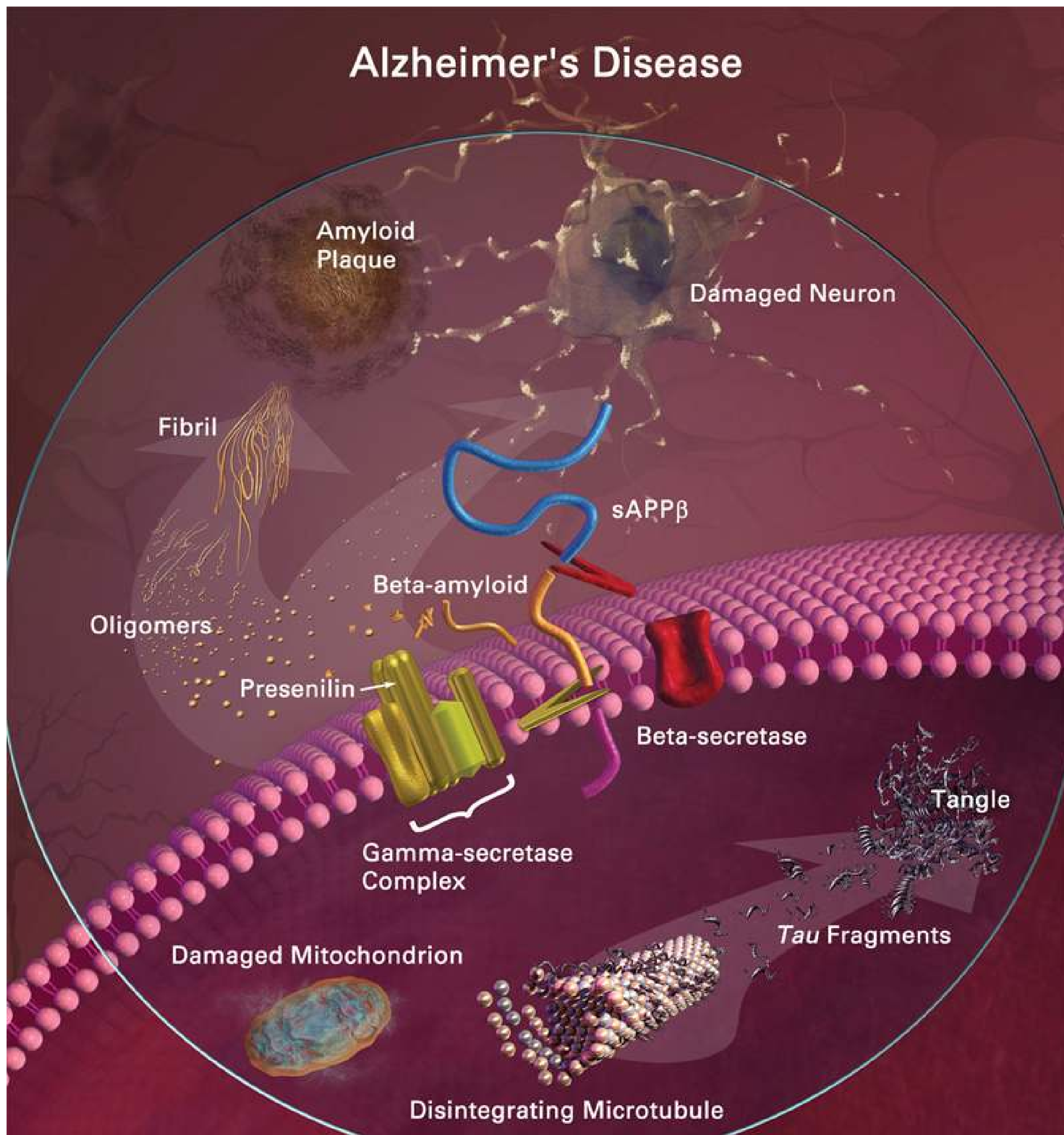



**Figure 11i.** *Characteristics of Alzheimer's disease* [Source National Institute on Aging www.nia.nih.gov]

AD is also considered a tauopathy due to abnormal aggregation of the tau protein, a microtubule-associated protein expressed in neurons that normally acts to stabilize microtubules in the cell cytoskeleton. Like most microtubule-associated proteins, tau is normally regulated by phosphorylation. However, in AD patients, hyperphosphorylated tau accumulates as paired helical filaments [Goedert 2006] that in turn aggregate into masses inside nerve

cell bodies known as neurofibrillary tangles and as dystrophic neurites associated with amyloid plaques.

Levels of the neurotransmitter acetylcholine are reduced. Levels of the neurotransmitters serotonin, norepinephrine, and somatostatin are also often reduced. Glutamate levels are usually elevated.

## 2.4. Genetic predisposition and risk factors

Genetic and environmental factors can determine the personal risk of AD and both playing an important role in the development of the disease.

The strongest risk factor for AD is aging. The incidence of AD is less than 1% in people aged 60-65 years. This possibility doubles every 4 years and becomes more than 6% in people older than 85 [Kawas 2001]. The discoveries of genetic aberrations that either cause or increase the risk of AD marked a rapid increase in the knowledge of the molecular and cellular alterations responsible for neuronal degeneration and cognitive dysfunction in AD [De Strooper 2000].

Late onset AD is influenced by the genetic risk-factor Apolipoprotein E (ApoE). [McMurray 2000] However, most of the early onset, familial forms of AD are caused by mutations associated with APP, and the presenilins (PS) [Hardy 1999; Dodart 2000]. Although it is predominantly a disease of late life, there are families in which AD is inherited as an autosomal dominant disorder of mid-life. Three genes have been described as implicated in the inherited form of the disease: the APP gene, [Citron 1992] which encodes the Aβ peptide; and the presenilin protein genes (PS1 and PS2), which encode transmembrane proteins Presenilins that participate in the cleavage of the APP protein within its Aβ region [Sherrington 1995; Levy-Lahad 1995].

After the determination of the amino acid sequence of APP and the gene was cloned was initiated a screening for possible mutations in this gene. Several causative mutations were discovered in families with dominantly inherited AD [King 2004]. Metabolism of APP generates a variety of Aβ species, predominantly a 40–amino acid peptide, Aβ1-40, with a smaller amount of a 42–amino acid peptide, Aβ1-42. This latter form of the peptide is more prone to forming amyloid deposits. Mutations in all three pathogenic genes alter the

processing of APP such that a more amyloidogenic species of Aβ is produced [Scheuner 1996]. Linkage analysis identified a region of chromosome 14 as a locus of a mutation(s) responsible for inherited AD in several different pedigrees. The Presenelin-1 (PS-1) gene was identified as the affected gene [Ho 2004]. Mutations in a gene of chromosome 1 with high homology to PS1, now called Presenellin-2 (PS-2), were then shown to cause a few cases of inherited AD [Kamal 2001]. Although the precise function of the presenilins is still the subject of debate, it is clear from gene ablation experiments that presenilins are intimately involved in the COOH-terminal cleavage of Aβ, [De Strooper 1998] and the simplest explanation of the effects of presenilin mutations on APP processing is that they lead to an incomplete loss of function of the complex that processes APP [Refolo 1999; Wolfe 1999].

Recently mutations in SORL1 gene encoding for Sortilin were proposed to be risky for late onset AD [Rogaeva 2007].

The vast majority of cases of AD are sporadic and they do not associate in families. Nevertheless, molecular genetic analyses suggest that there are some genes associated with increase of the susceptibility for developing AD. The first susceptibly gene identified was Apolipoproten E. There are three alleles that encode three different isoforms of ApoE-E2, E3 and E4. Individuals in which the E4 isoform is produced, the risk of developing AD are increased [Pietrzik 2004]. There are evidences that E4 enhances Aβ aggregation and reduce Aβ clearance. In addition data suggest that E4 might increase the risk of AD by enhancing amyloidogenic processing of APP, promoting cerebrovascular pathology, increasing oxidative stress and impairing neuronal plasticity. The inheritance of one or two E4 alleles of the ApoE gene lead to both a substantially higher likelihood and an earlier age of onset of the late-onset form of AD [Corder 1993], whereas inheritance of the E2 allele appears to do the opposite [Corder 1994]. Other phenotypic indication to the action of ApoE4 is the finding that AD patients harboring one or two E4 alleles have a significantly higher density of both plaque and vascular Aβ deposits in their brains than do AD subjects lacking this allele [Schmechel 1993; Rebeck 1993; Hyman 1995; Polvikoski 1995; Greenberg 1995]. This well confirmed observation has led to several *in vitro* studies in which purified ApoE4 (generally in a non-lipidated state and at supra- physiological concentrations) has produced enhanced

aggregation of synthetic Aβ peptides under cell-free conditions [Ma 1994; Evans 1995]. Although these studies suggest the role of the ApoE-E4 protein as a facilitator of Aβ aggregation, an alternate explanation for the phenotypic effect could be a decreased efficiency of clearance of the peptide from the extracellular space in the presence of ApoE-E4. Regarding Aβ production, evidence obtained in stably transfected cells co-secreting Aβ and each of the apoE isoforms at physiological levels suggests that ApoE-E4 does not lead to increased generation of Aβ [Biere 1995]. A second susceptibility locus for late-onset AD has been localized to chromosome 10, but the gene responsible has not yet been established [Kinoshita 2003].

As in other age-related diseases (cardiovascular disease, diabetes, cancers), is likely to have different behavioral, dietary and other environmental factors that may affect the risk of AD. However, this area of research has not yet matured to point where clear recommendations can be made. Epidemiological findings suggest that low educational level history of head trauma, consumption of high-calorie, high-fat diets and a sedentary lifestyle may each increase the risk of AD [Ninomiya 1993; Caille 2004]. When rodents are maintained in cognitively stimulating environment or on a dietary restriction regimen, the hippocampal neurons are more resistant to death and there is increased neurogenesis [Caille 2004; Small 2001]. Similarly, regular physical exercise enhances hippocampal synaptic plasticity and neurogenesis and is neuroprotective [Walsh 2002].

Specific dietary components may affect the risk of AD. Individuals with low dietary folate intakes are at increased risk of AD, as an apparent consequence of increased levels of homocysteine. Studies on mouse models of AD have demonstrated adverse effects of low dietary folate levels and high homocysteine levels on a disease process [Caille 2004]. Other dietary factors implicated as risk factors for AD include metals and lipids [Ninomiya 1993; Klein 2001; Coppede 2006].

Among environmental factors metals have been extensively studied for their posible contribution to neurodegeneration/AD; the brain is, among human organs, the most capable to concentrate metals, so that either human ingestion of metals present in drinking water or accumulated in animal meat, or environmental and occupational exposure to metals may result in their

accumulation in the brain. After accumulation metals can react with proteins, leading to protein damage or aggregation, ultimately leading to neurodegeneration. Moreover metals can contribute to an increased oxidative stress, thus predisposing to neurodegeneration [Bush 2000]. Several studies have been conducted to explore the role of heavy metals on brain development and dementia. Ingestion of aluminium in drinking water was associated with an increased risk of AD; however other studies failed to find such association [McLachlan 1996; Martyn 1997]. Conflicting results have been obtained concerning occupational or environmental exposures to aluminium [Gun 1997; Graves 1998] and the role of this metal in AD pathogenesis is still debated. The homeostasis of zinc, copper and iron are altered in the brain of AD individuals, and under mildly acidic conditions, such as those believed to occur in AD brain, iron and zinc ions have been observed to induce Aβ aggregation [Cherny 1999]. Elevated levels of iron, zinc and selenium in the brain have been associated with AD [Cornett et al. 1998]. The role of copper in Aβ aggregation is still controversial: APP has copper binding domains, and there is evidence that copper and iron interact with Aβ leading to the production of aggregates. However, APP is also supposed to regulate cellular concentrations of copper, whose role in the brain is not yet completely understood [Doraiswamy and Finefrock 2004]. Another risk factor for AD is inorganic mercury, often present in dental amalgam applications, and a role for ApoE as a mediator of the toxic effect of mercury has been largely suggested [Mutter et al. 2004]. Other environmental factors associated with AD are traumatic brain injury, inflammation and occupational exposure to pesticides [Jellinger 2004; Baldi 2003], whereas the consumption of unsaturated fatty acids and omega-3 polyunsaturated fatty acids, might reduce the risk of dementia [Morris 2003].

Lipids are structural components of cell membranes, serve as intra- and intercellular signaling molecules and can modify the functions of many different proteins. Individuals that consume diet high in cholesterol and those with increased cholesterol levels may be at increased risk of AD, whereas those who take cholesterol lowering drugs (statins) may be at reduced risk [Edbauer 2003; Kimberly 2003]. The well-known adverse effects of high-cholesterol diet on the vasculature could contribute to an increased risk of AD. Accumulating data suggest that cholesterol may feed more directly into the amyloid cascade by

promoting amyloidogenic processing of APP. Statin treatment decreases Aβ levels and plaque formation in APP mutant transgenic mice [Takasugi 2003]. And high levels of cholesterol and shifts in subcellular cholesterol metabolism can increase the production of Aβ in cultured cells and APP mutant transgenic mice [Edbauer 2003]. Alterations in cholesterol metabolism might also promote neuronal degeneration by perturbing membrane fluidity and signal transduction. A more directed explanation comes from the model of altered microdomain structure of lipid membranes. Microdomains of plasma membrane called lipid rafts, in which the outer leaflet of the lipid bilayer is enriched in cholesterol and sphingomyelin may be sites at which several molecular events implicated in AD pathogeny occurs, including synaptic signal transduction, APP processing and the initiation of apoptosis [Wong 2004]. Analysis of raft lipids in brain tissue samples from AD and controls subjects have demonstrated increased amount of cholesterol and ceramides in AD which are associated with increased levels of membrane-associated oxidative stress [Tournoy 2004]. Exposure of neurons to Aβ or oxidative insults results in increased accumulation of cholesterol and ceramides in the neurons and these alterations can be prevented by treating the neurons with Vitamin E [Tournoy 2004], suggesting that increased membrane oxidative stress may induce the production of cytotoxic ceramides in AD. Further studies in animal models and AD patients are required to clarify the roles of disturbed lipid metabolism in AD. Although the emerging data linking cholesterol and fatty acids to AD are encouraging, the potential of dietary modifications of fat intake to affect disease risk remains to be established.

## 2.5. Disease mechanism hypothesis

There are three major hypotheses about initialization and progression of the AD. The oldest hypothesis suggests that deficiency in cholinergic signaling initiates the progression of the disease. More recent hypotheses center on the effects of the misfolded and aggregated proteins, Aβ and tau. The two positions are lightheartedly described as "ba-ptist" and "tau-ist" viewpoints in scientific publications by Alzheimer's disease researchers. "Tau-ists" believe that the tau protein abnormalities initiate the disease cascade, while "ba-ptists" believe that beta amyloid deposits are the causative factor in the disease [Mudher 2002].

### 2.5.1. Cholinergic hypothesis

The oldest hypothesis is the "cholinergic hypothesis". It states that AD begins as a deficiency in the production of acetylcholine, a vital neurotransmitter. Much early therapeutic research was based on this hypothesis, including restoration of the "cholinergic nuclei". The possibility of cell-replacement therapy was investigated on the basis of this hypothesis. All of the first-generation anti-AD medications are based on this hypothesis and work to preserve acetylcholine by inhibiting acetylcholinesterases (enzymes that break down acetylcholine). These medications, though sometimes beneficial, have not led to a cure. In all cases, they have served to only treat symptoms of the disease and have neither halted nor reversed it. These results and other research have led to the conclusion that acetylcholine deficiencies may not be directly causal, but are a result of widespread brain tissue damage, that cell-replacement therapies are likely to be impractical. More recently, cholinergic effects have been proposed as a potential causative agent for the formation of plaques and tangles [Shen 2004] leading to generalized neuroinflammation [Wenk 2003].

### 2.5.2. Tau hypothesis

The hypothesis that tau is the primary causative factor has long been grounded in the observation that deposition of amyloid plaques does not correlate well with neuron loss [Schmitz 2004]. A mechanism for neurotoxicity has been proposed based on the loss of microtubule-stabilizing tau protein that leads to the degradation of the cytoskeleton [Gray 1987]. However, consensus has not been reached on whether tau hyperphosphorylation precedes or is caused by the formation of the abnormal helical filament aggregates. Support for the tau hypothesis also derives from the existence of other diseases (tauopathies) in which the same protein is identifiably misfolded [Williams 2006]. Moreover, all the six tau isoforms occur in Alzheimer’s disease [Goedert 2001; Hasegawa 2006] (figure 6i).

### 2.5.3. Amyloid cascade hypothesis

The amyloid hypothesis is initially compelling because the gene for the amyloid beta precursor APP is located on chromosome 21, and patients with trisomy 21 (Down syndrome) - who thus have an extra gene copy almost universally exhibit anatomopathological AD-like findings by 40 years of age [Nistor 2007; Lott 2005]. The traditional formulation of the amyloid hypothesis points to the cytotoxicity of mature aggregated amyloid fibrils, which are believed to be the toxic form of the protein responsible for disrupting the cell's calcium ion homeostasis and thus inducing apoptosis [Yankner 1990]. This hypothesis is supported by the observation that higher levels of a variant of the Aβ protein known to form fibrils faster in vitro correlate with earlier onset and greater cognitive impairment in mouse models [Iijima 2004] and with AD diagnosis in humans [Gregory 2005]. However, mechanisms for the induced calcium influx, or proposals for alternative cytotoxic mechanisms, by mature fibrils are not obvious.

A more recent and broadly supported variation of the amyloid hypothesis identifies the cytotoxic species as an intermediate misfolded form of Aβ, neither a soluble monomer nor a mature aggregated polymer but an oligomeric species, possibly toroidal or star-shaped with a central channel that may induce apoptosis by physically piercing the cell membrane [Blanchard 2000; Abramov 2004]. A related alternative suggests that a globular oligomer localized to dendritic processes and axons in neurons is the cytotoxic species [Barghorn 2005; Kokubo 2005].

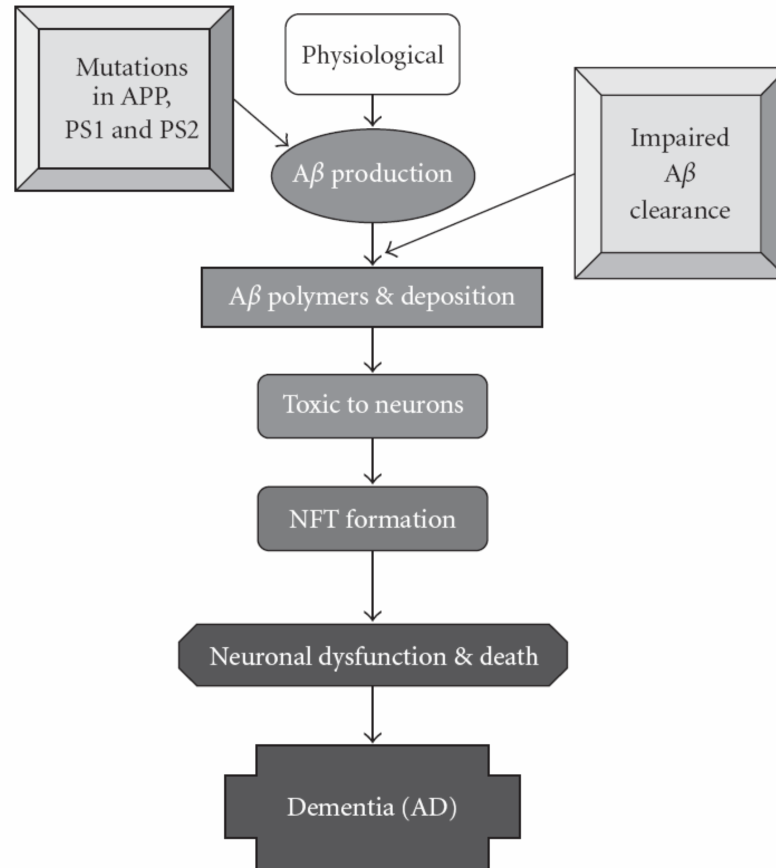



**Figure 12i.** *Amyloid cascade hypothesis. Aβ is a normal metabolite which, under physiological conditions, is constantly produced and quickly degraded. Accumulating Aβ will initially oligomerize, gradually form fibrils, and culminate in microscopically visible amyloid plaques. Soluble and fibrillar Aβ and associated plaque proteins are toxic to neurons, resulting in synaptic loss, the formation of neurofibrillary tangles, and eventual neuronal death and AD* [Deng-ShunWang, J. Biomedicine and Biotechnology Vol.2006, ID 58406,1–12]

## 2.6. Possible implications of the Neurothrophins in Alzheimer's disease

NGF plays a key role in stimulation, survival and phenotypic maintenance of adult basal forebrain neurons. It is synthesized in the cortex and the hippocampus and is retrogradely transported to BFCN (Basal forebrain

cholinergic neurons) cell bodies supporting normal cellular functions and morphology [Hefti 1989; Sofroniew 2001].

NGF has been shown to up-regulate several cholinergic markers, i.e. Acetylcholine transferase (ChAT) activity, gene expression and protein levels [Hefti 1985; Pongrac 1998], acetylcholine synthesis and release [Oosawa 1999; Auld 2001] and the expression of vesicular acetylcholine transporter [Takei 1997; Oosawa 1999].

In rats, radiolabeled NGF injected into the frontal and occipital lobes and the hippocampus has been shown to be retrogradely transported to BFCN cell bodies, the neuronal population particularly affected in AD [Seiler 1984; DiStefano 1992]. Lesions to the septohippocampal pathway result in increased levels of NGF in the hippocampus and cholinergic neuronal atrophy as also observed in AD. These effects can be prevented using intracerebroventricular infusion of NGF [Hagg 1988; Fischer 1991; Venero 1994]. A large number of studies indicate that BFCN degeneration is linked to a fail in NGF signaling.

The disruption of the trkA gene and by this means NGF signaling, leads to severe loss of ChAT staining in the basal forebrain neurons and their projections to the hippocampus and cortex [Smeyne 1994].

In support to failed retrograde transport of NGF in AD, it has been shown that increased amounts of NGF protein in the cortical areas were associated with decreased levels of this protein in the basal forebrain [Scott 1995].

The levels of Brain-derived neurotrophic factor (BDNF) in the hippocampus and parietal cortex of patients with AD are significantly reduced. In other parts of the brain as the levels of the other neurotrophin factors (NT-3, and NT-4/5) are no changed in AD [Hock 2000].

## 2.7. Models of Alzheimer's disease

The accumulated data of genetic base of AD permitted creation of genetic models bearing mutated human genes. The *knockout* of APP [Heber 2000], the main implicated in pathogenesis of AD gene do not show significant alterations. Similarly γ-Secretase knockouts are viable, without significant alterations in the development. Presenelin-1 knockout, in contrast exhibit neurodegeneration memory deficit and severe synapses alterations [Saura

2004]. Transgenic mice that express high levels of human mutant APP (with valine at residue 717 substituted by phenylalanine) progressively develop many of the pathological hallmarks of AD, including numerous extracellular Aβ deposits, neuritic plaques, synaptic loss, astrocytosis and microgliosis [Games 1995].

In several organisms, spontaneous formation of filaments or tangles has been reported similar to the sequence of events during AD in the human brain. This includes aged bears, sheep, goats, wolverines, and baboons [Braak 1994; Cork 1988; Nelson 1995; Roertgen 1996; Schultz 2000] and a closer examination of these organisms will be important to study correlations between hyperphosphorylation of tau, formation of filaments or tangles, synaptic dysfunctions, deficits in learning and memory, and neurodegeneration.

Unfortunately, standard laboratory animals, in particular mice and rat, do not develop tau filaments or tangles spontaneously but have to be specifically manipulated to study tau pathology. Injection of aluminium maltotate in white rabbits induces aggregation of hyperphosphorylated tau in neurons [Savory 1998] and apoptosis [Ghribi 2002]. However, it is unclear whether tau aggregation is causative for cell death in this experiment.

A triple-transgenic mouse model (expression of mutant APP, mutant presenilin 1 and mutant tau) develops plaques and tangles in AD-relevant brain regions in an age related manner [Oddo 2003]. However, in these animals, synaptic dysfunction as an early indicator of neuronal degeneration manifests before plaque and tangle pathology.

In human P301L tau mouse lines, co-expression of mutant human APP or the intracerebral injection of Aβ fibrils was reported to increase the number of tangle-bearing nerve cells [Lewis 2001; Götz 2001]. It thus appears that extracellular Aβ deposits can exacerbate the intraneuronal pathology caused by the expression of mutant human tau protein. In contrast to these findings, Aβ deposits failed to induce the formation of tau filaments in mice expressing wild-type human tau protein. Based on these findings, it would appear that Aβ can promote, but not induce, tau filament formation [Goedert2004].

Novel rat AD models was developed based on virally-mediated gene transfer used to selectively increase hippocampal levels of human Aβ42 and Aβ40 peptides in adult Wistar rats, allowing examination of the contribution of

each to the cognitive deficits and pathology seen in AD. AAV1 vectors encoding BRI-Aβ cDNAs, fusions between human Aβ peptides and the BRI protein envolved in amyloid deposition in British and Danish familial dementia, were used to achieve high-level hippocampal expression and secretion of the specific encoded Aβ peptide in the absence of APP overexpression. AAV-treated animals are tested for development of cognitive deficits, and brain tissue analyzed for Aβ levels and evidence of extra-cellular Aβ deposition. The results demonstrate that whilst virally-mediated overexpression of Aβ42 alone is sufficient to initiate plaque deposition, both Aβ40 and Aβ42 levels contribute to the development of cognitive deficits in this model [Lawlor 2007].

Animals injected with AAV1 vectors encoding BRI-Aβ40 or BRI-Aβ42, alone and in combination, developed behavioral deficits in a distinct pattern. AAV-BRI-Aβ42 animals had reduced exploration behavior during working memory evaluation but no significant deficits in passive avoidance or acquisition and retention of spatial information. Animals injected with AAV-BRI-Aβ40 alone were impaired in passive avoidance, but were not significantly impaired in the Morris water maze. However, animals co-injected with both BRI-Aβ vectors showed the most pronounced behavioral deficits with some impairment in all tests. Despite measurable impairments, only BRI-Aβ42 animals developed extracellular Aβ deposits. Taken together with the histology results, this behavioral data confirms observations from AD transgenic mouse models that measurable behavioral deficits are not dependent on the presence of Aβ plaques. Unexpectedly, the development of more pronounced cognitive deficits when Aβ42 and Aβ40 are co-expressed, suggests a role for Aβ40 (the role of Aβ40, the more prevalent Aβ peptide secreted by cells and a major component of deposits in the cerebral vasculature of AD brain is less cleary than that of Aβ42), along with Aβ42, in cognitive impairment [Lawlor 2007].

Both Aβ40 and Aβ42, not just Aβ42 alone, may contribute to the development of distinct cognitive deficits in rats as co-expression of Aβ40 and Aβ42 produced a more robust behavioral phenotype then expression of either Aβ peptide alone. The lack of correlation between severity of behavioral deficits and presence of Aβ deposits confirms previous studies of mouse transgenic models in which cognitive deficits precede visible Aβ deposition, but are associated with the accumulation of detergent insoluble Aβ.

## 2.8. Treatment of Alzheimer's disease

### 2.8.1. Pharmaceutical treatment

Four medications, to treat the cognitive manifestations of AD, are currently approved by regulatory agencies "Food and Drug Administration (FDA) and the European Medicines Agency (EMEA)". Three are acetylcholinesterase inhibitors and the other is memantine, an NMDA receptor antagonist. No drug has an indication for halting the progression of the disease.

Because reduction in the activity of the cholinergic neurons in the disease is well known, [Geula 1995] acetylcholinesterase inhibitors are employed to reduce the rate at which acetylcholine (ACh) is broken down and so to increase the concentration of ACh in the brain, thereby combatting the loss of ACh caused by the death of the cholinergin neurons [Stahl 2000]. Cholinesterase inhibitors currently approved include donepezil (brand name Aricept), galantamine (Razadyne/Reminyl), and rivastigmine (branded as Exelon) [US National Library of Medicine (Medline Plus)]. There is also evidence for the efficacy of these medications in mild to moderate AD, [Birks 2006] and some evidence for their use in the advanced stage. The use of these drugs in mild cognitive impairment has not shown any effect in a delay of the onset of AD. [Raschetti 2007] Most common side effects include nausea and vomiting, both of which are linked to cholinergic excess. These side effects arise in approximately ten to twenty percent of users and are mild to moderate in severity. Less common secondary effects include muscle cramps, decreased heart rate (bradycardia), decreased appetite and weight, and increased gastric acid.

Glutamate is a useful excitatory neurotransmitter of the nervous system, although excessive amounts in the brain can lead to cell death through a process called excitotoxicity which consists of the overstimulation of glutamate receptors. Excitotoxicity occurs not only in AD, but also in other neurological diseases such as PD and multiple sclerosis [Lipton 2006]. Memantine (brand names Akatinol, Axura, Ebixa/Abixa, Memox and Namenda) [US National Library of Medicine (Medline)], is a noncompetitive NMDA receptor antagonist first used as an anti-influenza agent. It acts on the glutamatergic system by

blocking NMDA glutamate receptors and inhibits their overstimulation by glutamate [Lipton 2006]. Memantine has been shown to be moderately efficacious in the treatment of moderate to severe AD. Its effects in the initial stages are unknown [Areosa 2004]. Reported adverse events with memantine are infrequent and mild, including hallucinations, confusion, dizziness, headache, seizures and fatigue. Memantine used in combination with acetilcolinesterasa inhibitors has been shown to be "of statistically significant but clinically marginal effectiveness" [Raina 2008].

Neuroleptic anti-psychotic drugs commonly given to AD patients with behavioural problems are modestly useful in reducing aggression and psychosis, but are associated with serious adverse effects, such as cerebrovascular events, movement difficulties or cognitive decline [Ballard 2006; Ballard2008; Sink 2005].

As production of neurotoxic forms of Aβ from APP appears to be a leading event in AD pathogenesis, there is intense interest in developing drugs that block the β- and γ-Secretase. Specific γ-Secretase inhibitors that decrease Aβ levels have been produced [Dewachter 2002], but their use in humans may be compromised by side effects resulting from blockade of γ-Secretase cleavage of Notch and other protein substrates. BACE inhibitors [John 2003] may reduce Aβ production without major side effects, as shown in BACE-deficient mice [Roberds 2001]. Another approach to reducing amyloid accumulation in the brain is the use of agents that chelate copper and iron [Bush 2003; Ritchie 2003]. Such chelators would also be expected to reduce oxidative stress in neurons.

One promising approach for preventing and treating AD is based upon stimulating the immune system to remove Aβ from the brain. The initial reports that immunization with human Aβ42 [Schenk 1999] or passive immunization with Aβ antibodies result in the clearance of Aβ plaques from the brains of APP mutant transgenic mice, were followed by the report that such immunization approaches can also diminish memory deficits in the mice [Morgan 2000]. The results of the initial clinical trial suggest that Aβ immunization may be an effective treatment for AD, although adverse reactions occurred in some patients and refinement of immunization methods will be required [Kotilinek 2002]. It should also be noted that there have been many failures of trials in

human patients of treatments that were shown effective in animal models of other NDD, including PD and stroke. Nevertheless, an effective vaccine would have a major impact on the disease, and is currently one of the most exiting areas of AD research.

Other therapeutic approaches being tested include: anti-inflammatory agents, such cyclooxigenase-2 (COX2) inhibitors and Aspirin [Hoozemans 2003], steroids that decline during normal aging such as estrogen and testosterone [Resnick 2002]. Drugs that target specific sites in neurodegenerative cascades, to date, only have been employed in cell culture and animal models of AD, and their potential use in the clinical practice remains unclear.

### 2.8.2. Psychosocial intervention

Psychosocial interventions are used as an adjunct to pharmaceutical treatment and can be classified within behavior, emotion, cognition or stimulation oriented approaches. Epidemiological findings in animal studies show that cognitively stimulating environments physical exercise and dietary restrictions regimens increase the resistance of neurons in the brain to degeneration enhance neurogenesis and improve learning and memory. They could posses beneficial effect through mechanisms involving increased production of BDNF [Mattson 2003; Young 1999; Lee 2002], although the importance of BDNF in AD remains to be determined. Vitamins supplementation may reduce disease risk, as suggested by a recent prospective study demonstrating reduced prevalence and incidence of AD in individuals taking vitamins C and E [Zandi 2004].

Emotion-oriented interventions include reminiscence therapy, validation therapy, supportive psychotherapy, sensory integration, and simulated presence therapy. Supportive psychotherapy has received little or no formal scientific study, but some clinicians find it useful in helping mildly impaired patients adjust to their illness. Reminiscence therapy (RT) involves the discussion of past experiences individually or in group, many times with the aid of photographs, household items, music and sound recordings, or other familiar items from the past. Although there are few quality studies on the effectiveness

of RT it may be beneficial for cognition and mood [Woods 2005]. Simulated presence therapy (SPT) is based on attachment theories and is normally carried out playing a recording with voices of the closest relatives of the patient. There is preliminary evidence indicating that SPT may reduce anxiety and challenging behaviors [Peak 2002; Camberg 1999]. Finally, validation therapy is based on acceptance of the reality and personal truth of another's experience, while sensory integration is based on exercises aimed to stimulate senses. There is little evidence to support the usefulness of these therapies [Neal 2003; Chung 2002].

The aim of cognition-oriented treatments, which include reality orientation and cognitive retraining, is the restoration of cognitive deficits. Reality orientation consists in the presentation of information about time, place or person in order to ease the understanding of the person about its surroundings and his place in them. On the other hand cognitive retraining tries to improve impaired capacities by exercitation of mental abilities. Both have shown some efficacy improving cognitive capacities, [Spector 2000; Spector 2003] although in some works these effects were transient and negative effects, such as frustration, have also been reported.

Stimulation-oriented treatments include art, music and pet therapies, exercise, and any other kind of recreational activities for patients. Stimulation has modest support for improving behavior, mood, and, to a lesser extent, function. Nevertheless, as important as these effects are, the main support for the use of stimulation therapies is the improvement in the patient daily life routine they suppose.

# 3. Neurotrophin theory

## 3.1. Overview

A hallmark of vertebrate evolution is the development of a complex nervous system. A common design principle in vertebrates is to sculpt the nervous system during embryonic development from an initial excess of neurons. Along with cell-intrinsic mechanisms, the final number is achieved by degeneration of those neurons that fail to establish functional contact with their targets and do not gain access to limited amounts of survival signals in the form

of secreted proteins. By this means, the number of innervating neurons is adjusted to the target tissue. This organizing principle, known as “neurotrophic theory”, allows the nervous system to flexibly adjust itself to changes. This theory is accepted as one of the most-important concepts in developmental neurobiology and has been further developed since its conception in the 1940s [Levi-Montalcini 1954].

## 3.2. The neurotrophin family

Neurotrophins are a small family of dimeric secretory proteins in vertebrates consisting of Nerve Growth Factor (NGF), Brain Derived Growth Factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4/5 (NT-4/5). They are essential for a broad spectrum of functions including: control of survival or death of neurons and other cell types, regulation of precursor cell numbers, modulation of neurite outgrowth and growth cone guidance, synaptic structure and plasticity, neurotransmitter release and long term potentiation [Mc Allister 1999; Poo 2001]. The neurotrophins are generated as pro-proteins with a functionality that is distinct from the proteolytically processed mature form.

The cellular responses to neurotrophins are mediated by three different types of receptor proteins, the Trk family of Tyrosine kinases receptors [Huang, 2003], the neurotrophin receptor p75NTR, which is a member of the tumor necrosis factor receptor (TNFR) superfamily [Roux 2002] and Sortilin, originally described as Neurotensin receptor.

## 3.3. Pro-neurotrophins

Neurotrophins are synthesized first as pre-pro-molecule and after removing the signal peptide, the precursors of about 270 amino acids called proneurotrophins are formed [Lessmann 2003].

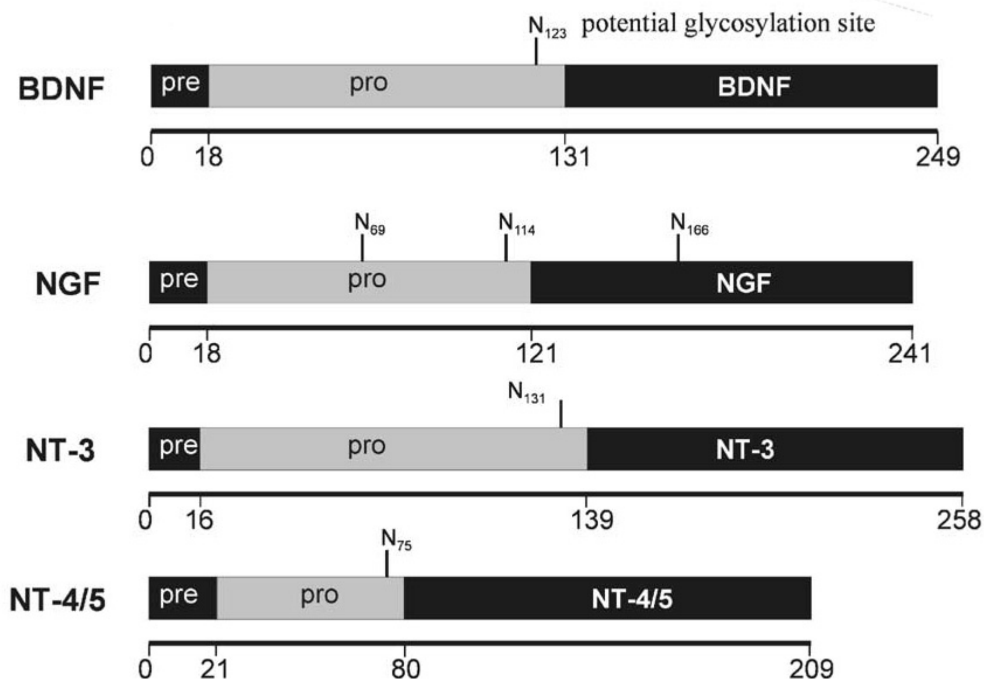



**Figure 13i.** *The structure of the rat pro-neurotrophins* [Lessmann Progress in Neurobiology 69 (2003) 341–374]

Pro-neurotrophins could be significant proportion (40%–60%) of total secreted neurotrophins, particularly in CNS [Farhadi 2000; Heymach 1996; Mowla 1999, 2001]. The N-terminal fragment, or the "pro-region," of around 120 amino acids is then proteolytically cleaved in either trans-Golgi by Furin or secretory granules by pro-protein convertases to form mature neurotrophins [Seidah 1996; Bresnahan 1990]. Proform could be also cleaved in the extracellular space depending on the availability of certain proteases. Specifically, matrix metalloproteases (MMP) as well as Plasmin have been shown to be responsible for extracellular cleavage [Lee 2001; Pang 2004]. Both processed and unprocessed neurotrophins form stable homodimers in solution [Kolbeck 1994]. The extent to which proteolytic processing occurs depend on the type of neurotrophin and, obviously, on the availability of active proteases. Importantly, NGF and BDNF also differ in their mode of secretion. While NGF is released through the constitutive secretory pathway, BDNF is predominantly secreted through regulated pathway [Mowla 1999]. Schwann cells (and presumably astrocytes) secrete predominantly pro-NGF and pro-BDNF,

suggesting that the intracellular cleavage system in glia is not very active [Mowla 1999].

In resume, neurotrophins are processed and secreted in distinctly regulated ways that suggest distinct biological functions. Biological activities of proneurotrophins pro-NGF and pro-BDNF, but not proNT-3 and proNT-4/5, have been documented in a variety of model systems. While both pro-NGF and pro-BDNF have been shown to provoke programmed cell death, [Teng 2005; Lee 2001; Beattie 2002; Harrington 2004; Srinivasan 2004] pro-NGF has further been implicated in cell migration [Shonukan 2003] and pro-BDNF in activity-dependent synaptic plasticity [Pang 2004;Woo 2005].

## 3.4. Neurotrophin's receptors

Neurotrophins and their precursors bind to three types of transmembrane receptor proteins: the members of the Trk family of receptor tyrosine kinases-TrkA, B and C, the structurally unrelated neurotrophin receptor p75NTR and Sortilin [Bibel 2000; Huang 2001]. In mammals, three Trk genes have been identified. They encode for TrkA, which displays highest affinity for NGF, TrkB, which preferably binds to BDNF and NT4/5, and TrkC, which interacts with NT3. These specificities are not absolute, and NT3 is also a weak ligand for TrkA and TrkB [Barbacid 1994]. p75NTR is a member of the tumor necrosis factor receptor (TNFR) superfamily and binds all neurotrophins with similar affinity.

Sortilin is the latest identified neurotrophin receptor. It is a member of the family of Vps10p-domain transmembrane receptors and was previously characterized as receptor for the neuropeptide neurotensin [Mazella 1998]. Sortilin has been shown to bind both pro-NGF [Nykjaer 2004] and pro-BDNF [Teng 2005]. When expressed alone both Trk receptors and p75NTR bind neurotrophins with an equilibrium binding constant of Kd $10^{-9}$M, so called low-affinity binding [Kaplan 1991; Hempstead 1991; Rodriguez-Tebar 1990, 1992]. However, when co-expressed, the equilibrium binding is increased to Kd $10^{-11}$M, creating high-affinity binding site [Hempstead 1991; Mahadeo 1994]. This result is consistent with the observation of steady state high-affinity binding sites for NGF in neurons with Kd $10^{-11}$M [Sutter 1979] suggesting that, in vivo, for creation of high-affinity binding sites for neurotrophins both receptors are

necessary. In fact, p75NTR and Trk receptors are frequently coexpressed and physically interact with each other [Bibel 1999]. Importantly, in Trk–p75NTR complexes, ligand specificity is determined by the Trk moiety and further refined by p75NTR. Thus, a TrkA–p75NTR complex is readily activated by NGF, but not by NT-3. Similarly, TrkB–p75NTR is responsive to BDNF, but responsiveness is markedly reduced for NT-3 and NT-4/5 [Roux 2002]. Based on these results, neurotrophins have been proposed to physically interact solely with the Trk constituent of a Trk–p75NTR receptor complex, with p75NTR altering the conformation of the Trk in a way that the affinity and specificity of Trk for the neurotrophin ligand is increased to form selective high-affinity binding sites [Esposito 2001; Zaccaro 2001]. This conformational model is supported by the finding that a p75NTR homologue, NRH2, can associate with and increase in the affinity of NGF for TrkA although it does not bind NGF on its own [Murray 2004]. Interestingly p75NTR has been shown to provide a high-affinity binding site for NT-3 in the absence of TrkC (Kd $10^{-11}$M) [Dechant 1997]. In addition to modulating the affinity and specificity for neurotrophins, Trk and p75NTR co-expression was demonstrated to result in downstream signaling cross-talk, both in a synergistic and antagonistic manner [Roux 2002; Nykjaer 2005]. The Trk receptors have close relationship to well-documented signaling pathways, such as of the epidermal growth factor (EGF) receptor, however characterization of p75NTR was more complicated. In fact, p75NTR was cloned before the Trk receptors (p75NTR [Johnson 1986; Radeke 1987] TrkA [Kaplan 1991; Klein 1991]) but a decade past before p75NTR was demonstrated to elicit signaling pathways on its own, independently of the Trks [Dobrowsky 1994; Carter 1996]. Thorough investigation of p75NTR was also attenuated by the early finding that the classic trophic properties of neurotrophins are basically mediated by either of the three Trk receptors [Barbacid 1994]. However, because of its influence on Trk receptors, as outlined above, p75NTR does participate in the trophic regulation of neuronal populations [Brennan 1999; Kuruvilla 2004]. To date, plenty of both transmembrane and intracellular partners for both Trk and p75NTR have been identified. While Trk receptors employ several signaling cascades which are typical for them [Huang 2003], p75NTR has been shown to recruit in a non-enzymatic manner a great variety

of, largely unique, intracellular binding partners that couple p75NTR to several distinct signaling pathways [Roux 2002].

Sortilin, finally, binds mature NGF with low affinity (Kd $10^{-8}$M), but has a significantly higher affinity for the unprocessed neurotrophins pro-NGF Kd $10^{-9}$M [Nykjaer 2004] and pro-BDNF (Kd $10^{-10}$M) [Teng 2005]. Sortilin associates with p75NTR if co-expressed and the affinity of this receptor complex to pro-NGF is further increased to Kd $10^{-10}$M [Nykjaer 2004]. p75NTR thus forms high-affinity binding sites with both Trk receptors and Sortilin for mature neurotrophins and pro-NGF, respectively. There is no data about the signaling pathways elicited by Sortilin. In analogy to p75NTR and Trk receptors, it is unclear whether Sortilin acts merely as co-receptor for p75NTR, facilitating binding of pro-NGF or whether it can trigger signaling cascades of its own. If so, they are assumed to be proapoptotic in character because of the likely involvement of Sortilin, together with p75NTR, in the induction of programmed cell death [Nykjaer 2004; Teng 2005].

## 3.5. Functions of neurotrophins

The positive effect of NGF on the growth of neurites of sensory neurons was early observed in vitro. Indeed, this response was used as a bioassay for the purification of NGF [Levi-Montalcini 1954]. NGF (and other neurotrophins) was demonstrated not only to promote but also to direct nerve growth [Campenot 1977]. They have been shown to modulate processes as diverse as cell migration, formation of neurites, synaptic transmission and differentiation in neuronal and non-neuronal cells.

Studies of neurotrophins knock-out mice models show impairment of adult brain functions and behavior. Homozygotes completely lacking neurotrophins die in first weeks of postnatal life. Heterozygous mice with reduced neurotrophin levels are viable, but show a complex deficits which are outlined in Table 6.

| | |
|---|---|
| **NGF+/- mice** | -Decreased cholinergic innervations of the hippocampus [Chen 1997]<br>-Deficiency in memory acquisition and retention [Chen 1997]<br>-Loss of neurons in the peripheral nervous system [Crowley 1994] |
| **BDNF+/- mice** | -Hyperplasia, obesity, increased aggressiveness [Lyons 1999]<br>-Impairment of long-term potentiation [Korte 1995;Patterson 1996;Bartoletti 2002]<br>-Elevated striatal dopamine levels [Dluzen 2001]<br>-Loss of mechanosensitivity [Carroll 1998]<br>-Loss of neurons of the peripheral nervous system [Ernfors 1994;Bianchi 1996]<br>Intracerebroventicular infusion of BDNF can reverse the hyperphagic phenotype [Kernie 2000]. The impaired serotonin function in forebrain, cortex, hypothalamus and hippocampus leading to aggressive behavior, hyperphagia and hyperlocomotor activity can be suppressed by administration of fluoxetine-a serotonine reuptake inhibitor [Lyons 1999]. A conditional BDNF knockout in postnatal mice also lead to similar behavior impairment also showing higher levels of anxiety [Rios 2001], which comes to show that this defect is not just a alteration during development. Consistently with the impaired LTP in hippocampus BDNF+/- mice show impaired spatial memory. |
| **NT3+/- mice** | -Deficient amygdale kindling activity [Elmer 1997]<br>-Cardiovascular defects [Donovan 1996]<br>-Reduced mechanoreceptors [Airaksinen 1996]<br>-Loss of neurons of the peripheral nervous system [Ernfors 1994] |

**Table 6.** *Complex deficits in heterozygous mice with reduced neurotrophin levels.*

A huge amount of accumulated data lead to general understanding that the neurotrophins (NT) are able to activate quite opposite effects depending on the context-the moment of development, cell type or their processing stage. We can consider them as quite polyvalent fine tool used to tune variety of processes.

### 3.5.1. Neuronal fate: survival or cell death

Neurotrophins were initially identified because of their survival-promoting activity for distinct neuronal populations, mostly within the peripheral nervous system [Bibel 2000; Huang 2001]. Considered as leading neurotrophic factor, NGF was unexpected to have the opposite function such as to elicit programmed cell death. The characterization of the three types of NGF

receptors and the pro- versus the mature form of NGF, has contributed to clarify these seemingly paradoxical findings.

#### 3.5.1.1. Survival signalling by matures neurotrophins through Trk receptors

Culturing embryonic peripheral nervous system (PNS) neurons requires the addition of the processed form of specific neurotrophins to the culture medium. This survival effect of mature neurotrophins is mediated by any of the three Trk receptors. Deleting Trk genes in the mouse abolishes the survival-promoting activity of neurotrophins and results in the loss of distinct neuronal populations [Klein 1993, 1994]. Neurotrophins acting by Trk receptors induce survival of many different PNS neurons, initially at prenatal stages of development. However, for reasons that are not fully understood, evidence for CNS neuron dependency on neurotrophins is limited. NGF–TrkA couple has been implicated in postnatal survival of cholinergic forebrain neurons [Chen 1997] and BDNF–TrkB has been shown to be vital for postnatal maintenance of specific subsets of cortical neurons [Xu B 2000]. A significant amount of information has been accumulated on the signaling mechanisms employed by Trk receptors. Typical for RTKs ligand binding induces receptor dimerization and triggers autophosphorylation of particular cytoplasmic tyrosine residues. This, in turn, leads to a conformational change and creates docking sites for specific adaptor proteins, “Shc” adapter proteins and phospholipase C (PLC-γ) being the most prominent, those that couple the receptor to intracellular signaling elements, including the Ras– ERK, the PI-3K–Akt, and the PLC-γ pathways. The PI-3K–Akt signaling module seems to be most important, supplemented by the Ras–ERK pathway. Ultimately, these signaling cascades result in the suppression of a default apoptosis program by neutralizing pro-apoptotic molecules, such as members of the Bcl-2 family, or by transcriptional repression of genes encoding apoptosis-promoting agents such as Fas ligand [Huang 2003]. p75NTR is frequently expressed along with Trk receptors. As noted above, it cross-talks with survival pathways elicited by Trk receptors in both a synergistic and antagonistic manner, depending on the cellular context.

#### 3.5.1.2. Cell death by pro-neurotrophin through p75NTR–Sortilin

Although p75NTR was the first cloned neurotrophin receptor [Johnson 1986; Radeke 1987], it was considered as an accessory coreceptor for Trks when they were identified as key mediators for the trophic function of neurotrophins. For this reason, it was a major surprise when a ligand-dependent proapoptotic function was described for p75NTR, independently of Trk receptors [Frade 1996; Casaccia-Bonnefil 1996]. Death-promoting activities of p75NTR have been observed in a number of model systems both *in vitro* and *in vivo* [Roux 2002; Nykjaer 2005]. Cell death by p75NTR in vitro can be triggered with both mature and pro-neurotrophins. However, a significantly higher concentration of the processed ligands is needed (2–4 nM) than of the uncleaved form (0.1–0.2 nM) in order to obtain an equivalent effect [Lee 2001].

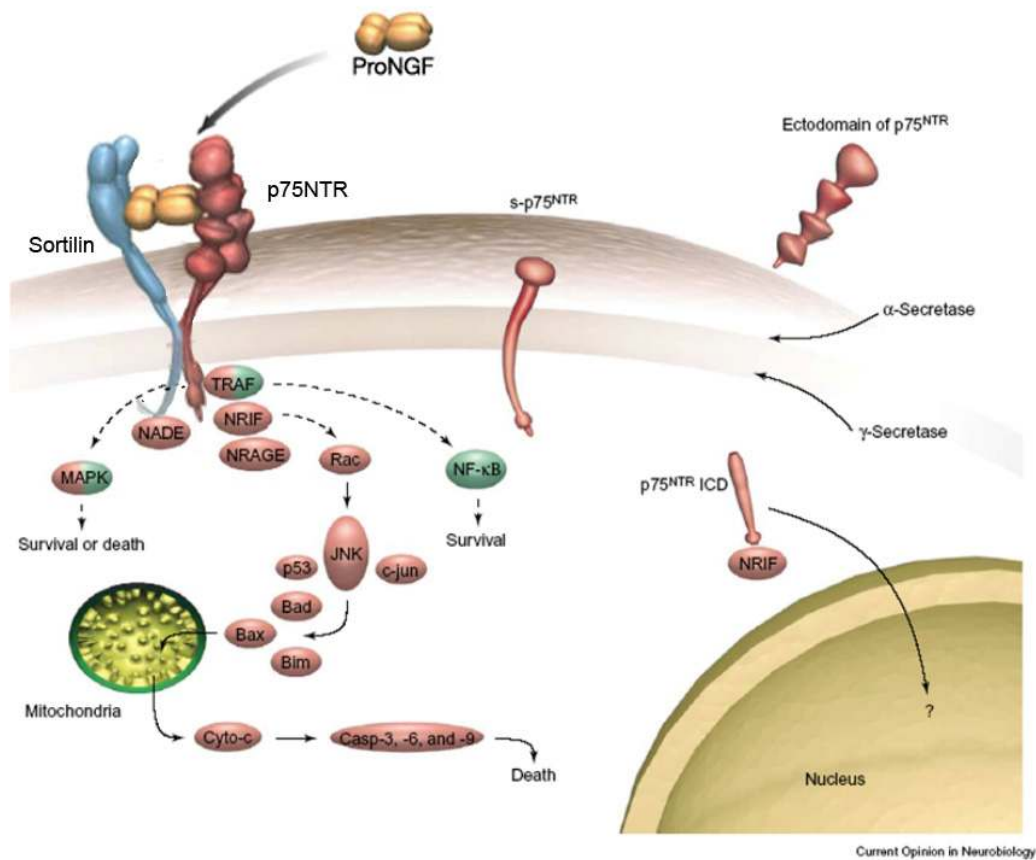



**Figure 14i.** *Cell death signalling induced by pro-neurotrophins through p75NTR* [Nykjaer 2005]

Given the presumably low concentration of neurotrophins in vivo, it is possible that pro-neurotrophins are the ones that physiologically account for p75NTR-mediated cell death. Since pro-neurotrophins bind very weakly to Trk receptors and activate them, there is no interference between p75NTR and Trks upon engagement of the pro-ligand in contrast to binding of the mature form. In cultured sympathetic neurons, which express both TrkA and p75NTR, pro-NGF elicits programmed cell death via p75NTR whereas mature NGF promotes cell survival via TrkA– p75NTR [Lee 2001]. Both pro-NGF and pro-BDNF have been demonstrated to exert pro-apoptotic activity through p75NTR [Teng 2005; Lee 2001; Beattie 2002; Harrington 2004; Srinivasan 2004]. Although major progress has been made in delineating the molecular mechanisms underlying p75NTR-mediated cell death, still there is not complete model. A hallmark of pro-neurotrophin-induced cell death seems to be the recruitment of Sortilin by

p75NTR. Blocking Sortilin with its specific ligand Neurotensin prevents induction of apoptosis by both pro-NGF and pro-BDNF in a variety of cell types [Nykjaer 2004; Teng 2005]. pro-NGF but not mature NGF, crosslinks with p75NTR and Sortilin binding its mature part to p75NTR and pro-domain to Sortilin [Nykjaer 2004]. The receptor complex p75NTR–Sortilin has a higher affinity for pro-NGF than each one of its constituents alone [Nykjaer 2004]. It is not yet known how the death signal is transduced from the p75NTR–Sortilin receptor complex. At present no Sortilin-specific pathways have been described. In contrast, p75NTR has been shown to bind a number of cytoplasmic molecules that have been implicated in propagating a neurotrophin-dependent death signal including NADE, NRAGE, NRIF or members of the TRAF family of proteins [Roux 2002].

### 3.5.2. Glial cell migration and differentiation

Myelination of axons is essential for the efficient and rapid propagation of action potentials. Schwann cells, the myelin-forming glia of the PNS, undergo distinct stages during development that include proliferation, migration along axons, ensheathment of individual axons, and formation of the mature myelin sheath [Lemke 2001]. Neurotrophins play a key role in the regulation of the glial–neuronal interactions. In vivo, as well as in vitro, neurotrophins have been found to regulate Schwann cells in an opposing manner dependent on the activation of either the Trk or p75NTR receptor. Migration of Schwann cells, isolated from sciatic nerve, is significantly enhanced by neurotrophin 3, but not by nerve growth factor or brain-derived neurotrophic factor that act through TrkC [Yamauchi 2003]. BDNF enhances myelination of Schwann cells through p75NTR and NT3 inhibits the process through TrkC [Yamauchi 2003].

Schwann cells derived from perinatal sciatic nerve were shown to be able to migrate significantly more efficient upon addition of NT-3, as compared to control cells [Yamauchi 2003, 2004]. TrkC is likely to be the NT-3 receptor for this activity since the effect was abolished by blocking Trk receptors, but not when deleting co-expressed p75NTR [Yamauchi 2003].

### 3.5.3. Activity-dependent synaptic plasticity

Among all the function that possess neurotrophins also have been implicated in activity-dependent synaptic plasticity. Synaptic plasticity accounts for long-term changes of synaptic efficacy, at least several hours, which are induced by specific patterns of electrical activity. Long-term changes of synaptic transmission are believed to form the basis for learning and memory. *In vivo*, they presumably arise from distinct patterns of electrical activity involving both the pre- and postsynaptic site [Paulsen 2000]. In vitro, they are studied in slices, usually from hippocampus, which are subjected to specific electrical stimulation. It is generally accepted that this in vitro model system reflects physiological processes in vivo [Bennett 2000]. Long-term changes of synaptic transmission are bidirectional, with long-term potentiation (LTP) enhancing synaptic efficacy, and long-term depression (LTD) reducing it.

The fact that BDNF is secreted in an activity- and $Ca^{2+}$-dependent manner is of particular interest. The activity-dependent secretion of BDNF may be critically involved in controlling synaptic transmission and long-term synaptic plasticity and may represent an important mechanism underlying local and synapse specific modulation by BDNF [Lu 2003]. Neurotrophins, in particular BDNF, have been shown to modulate both LTP and LTD via the mature form and the pro-form engaging either Trk or p75NTR receptor [Woo 2005;Korte 1995,1996; Patterson 1996; Minichiello 1999;Xu B 2000; Rosch 2005].

It has been reported that the levels of mRNA encoding NGF and BDNF are highly regulated by electrical stimulation and epileptic activity [Gall 1992], and BDNF is actively secreted during the periods of activity-dependent synaptic remodeling [Schoups 1995;Lein 2000;Kohara 2001;Balkowiec 2002].

## 3.5.3.1. LTP mediated by Trk receptor

Hippocampal slices derived from mice with a targeted disruption of the bdnf gene exhibit a significant impairment in LTP [Korte 1995]. Moreover, LTP deficits in bdnf or nt-4/5 knock-out mice can be rescued by acute treatment with either exogenous mature BDNF [Patterson 1996; Korte 1995; Xie 2000] or an adenoviral construct encoding the bdnf gene [Korte 1996]. The same LTP phenotype has been observed in trkB mutant mice lines indicating that the relevant BDNF receptor is TrkB [Minichiello 1999; Xu B 2000]. Currently, two

major mechanisms are discussed for BDNF–TrkB-mediated modulation of LTP. They both invoke $Ca^{2+}$ as a crucial regulatory factor for LTP induction [Sheng 2002]. First, mice that have the docking site for the adaptor protein PLC-γ, but not Shc, within TrkB mutated display substantial reduction of LTP [Minichiello 2002]. This finding thus implicates the PLC-γ pathway of TrkB in the regulation of LTP, presumably via $Ca^{2+}$ which is a downstream component of this signaling cascade [Huang 2003; Minichello 2002]. Alternatively, TrkB may instruct LTP by direct coupling to the Na channel Nav1.9 [Blum 2002]. Activating TrkB with BDNF has been shown to open Nav1.9. The resulting depolarization in turn gates voltage-dependent $Ca^{2+}$ channels thereby inducing $Ca^{2+}$ influx. These two models presumably describe two parallel mechanisms since they rely on completely different time scales. Activating the PLC-γ pathway takes seconds to minutes, whereas opening of Nav1.9 takes just milliseconds after addition of BDNF [Blum 2005].

### 3.5.3.2. LTD mediated by p75NTR

It also has been shown that p75NTR is important for development of the LTD [Woo 2005; Rosch 2005] and that pro-BDNF is a relevant ligand for p75NTR in this context [Woo 2005]. Specifically, LTD, but not LTP, was markedly impaired in two different lines of p75NTR knock-out mice. Addition of cleavage-resistant pro-BDNF to hippocampal slices derived from wild-type animals enhanced LTD. Two types of glutamate receptors have been proposed to provide the molecular basis for the effect of p75NTR on LTD. First, two subunits of the AMPA (α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid) type of glutamate receptor were shown to be significantly altered in their expression level in hippocampus of p75NTR knock-out mice [Rosch 2005]. Second, a specific subunit of the NMDA (N-methyl-D-aspartic acid) type of glutamate receptor, NR2B, which is considered a key molecule for LTD, but not LTP, [Massey 2004; Liu 2004] was found to have an altered expression level in hippocampus of p75NTR knock-out mice [Woo 2005]. Thus, p75NTR appears to contribute to the generation of LTD through different mechanisms in a parallel way. Conversion of pro-BDNF to mature BDNF by tPA (tissue plasminogen activator)-plasmin has been shown to be essential for occuring of LTP

[Khursigara 2001], a regulatory step that might otherwise provide unprocessed BDNF to p75NTR resulting in a diametrically opposite output.

It has been reported that p75NTR signaling might also influence some effects of behavior. Full-length p75NTR deficient mice show slight impairment in several learning tasks [Peterson 1999]. Additional deletions of short p75NTR isoforms lead to even more severe phenotype [Peterson 1999].

# 4. pro-NGF and p75NTR receptor

## 4.1. pro-NGF

### 4.1.1. Molecular structure of pro-NGF

The gen that encodes Human b-NGf is located in chromosiome 1 in locus 1p13.1. The coding sequence contains 727 nucleotides:

```
  1 atgtccatgt tgttctacac tctgatcaca gcttttctga tcggcataca ggcggaacca
 61 cactcagaga gcaatgtccc tgcaggacac accatccccc aagcccactg gactaaactt
121 cagcattccc ttgacactgc ccttcgcaga gcccgcagcg ccccggcagc ggcgatagct
181 gcacgcgtgg cggggcagac ccgcaacatt actgtggacc ccaggctgtt taaaaagcgg
241 cgactccgtt caccccgtgt gctgtttagc acccagcctc cccgtgaagc tgcagacact
301 caggatctgg acttcgaggt cggtggtgct gccccсttca acaggactca caggagcaag
361 cggtcatcat cccatcccat cttccacagg ggcgaattct cggtgtgtga cagtgtcagc
421 gtgtgggttg gggataagac caccgccaca gacatcaagg gcaaggaggt gatggtgttg
481 ggagaggtga acattaacaa cagtgtattc aaacagtact tttttgagac caagtgccgg
541 gacccaaatc ccgttgacag cgggtgccgg ggcattgact caaagcactg gaactcatat
601 tgtaccacga ctcacacctt tgtcaaggcg ctgaccatgg atggcaagca ggctgcctgg
661 cggtttatcc ggatagatac ggcctgtgtg tgtgtgctca gcaggaaggc tgtgagaaga
721 gcctga
```

The protein is originally translated as a pre-pro-NGF with 241 amino acid length. The signal peptide of 18 amino acids is cleaved off immediately after sequestration of the newly formed polypeptide chain into the endoplasmic reticulum (ER). The resulting protain is pro-NGF of 222 amino acid length.

PHSESNVPAGHTIPQVHWTKLQHSLDTALRRARSAPAAAIAARVAGQTRNITV
DPRLFKKRRLRSPRVLFSTQPPREAADTQDLDFEVGGAAPFNRTHRSKRSSS
HPIFHRGEFSVCDSVSVWVGDKTTATDIKGKEVMVLGEVNINNSVFKQYFFET
KCRDPNPVDSGCRGIDSKHWNSYCTTTHTFVKALTMDGKQAAWRFIRIDTAC
VCVLSRKAVRRA

According to some authors at this stage pro-NGF can spontaneously form non-covalently-linked homodimers directly in the ER after the synthesis. At this stage, can be generated also heterodimers of different NT monomers [Lessmann 2003].

pro-NGF can be possessed further in trans-Golgi Network (TGN) by convertase cleavage to mature NGF of 120 amino acids. In the protein sequence of pro-NGF has several suitable cleavage sites. The main member of the subtilisin/kexin-like convertase family is the furin, he cusually cleaves substrates at a consensus tipe I cleavage site Arg-Xaa-(Lys/Arg)-Arg1. Despite that in the pro-NGF molecule has several suitable sites for cleavage it was shown that convertase cleavage occurs only in 2 of the site Arg/Arg in position 115/116 and Lys/Arg in position 186/187 [Scott 1983]. Because of different cleavage sites in the molecule there are different pro-NGF molecules described in the literature. The cleavage in position 186/187 gives the mature form of NGF with protein sequence:

SSSHPIFHRGEFSVCDSVSVWVGDKTTATDIKGKEVMVLGEVNINNSVFKQYF
FETKCRDPNPVDSGCRGIDSKHWNSYCTTTHTFVKALTMDGKQAAWRFIRID
TACVCVLSRKAVRRA

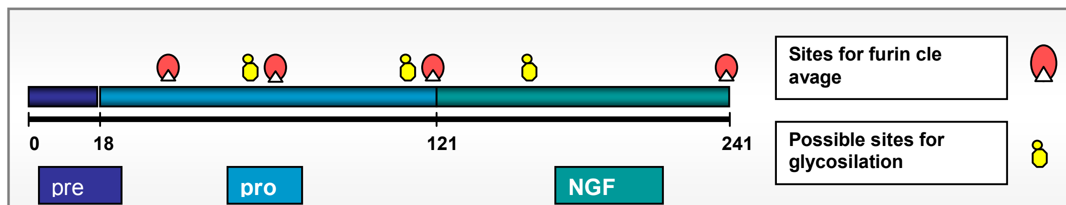



**Figure 15i.** *The structure of pre-pro-NGF with sites for glycosilation and furin cleavage*

These different sites for convertases proteolysis combined with different sites in which different modifications can take place result in several different pieces of pro-NGF that can usually be observed on the Western blot [Pedraza 2005]. These different forms of pro-NGF, combined with pro-NGF stable dimers can be observed on Western blots as bands with molecular weight between 14 and 53 kDa.

### 4.1.2. Neurotrophic and Neurotoxic Functions of pro-NGF

It has been found that pro-NGF, and not mature NGF, is the predominant isoform found in the human brain as well as in a variety of cell types. Thus, the case of the mouse salivary gland, which is the most abundant source of mature NGF, seems to be the exception and not the rule.

pro-NGF is the ligand with the highest affinity to p75NTR. Therefore, cleavage resistant pro-NGF (mutated at the cleavage site to insure that it retains the prodomain) induced apoptosis ten times more effectively than mature NGF in a vascular smooth muscle cell line expressing p75NTR but not Trk receptors [Lee 2001].

However, some authors claim that pro-NGF can also mediate survival. It is shown that pro-NGF was as effective as NGF in inducing survival of DRG neurons [Rattenholl 2001], and mutated pro-NGF exhibits neurite outgrowth on both PC12 cells and mouse sympathetic cervical ganglions [Fahnestock 2004]. It is reported also that in mature pig oligodendrocytes, which express p75NTR and Sortilin, pro-NGF induces survival [Althaus 2006].

Most accepted hypotesys so far is that pro-NGF can produce neuronal cell death interacting with p75NTR. The neurotoxic effects of pro-NGF are mediated through p75 in conjunction with Sortilin [Nykjaer 2004], which is identical to neurotensin receptor 3 [Petersen 1997; Mazella 1998]. The neurotoxicity of pro-NGF has been demonstrated most convincingly in embryonic early postnatal (P0–P1) neurons [Nykjaer 2004; Pedraza 2005; Volosin 2006]. Other evidences that support that hypotesis are the findings that in AD, brain levels of pro-NGF are increased in a stage-dependent manner. It is

expressed particularly in glial cells as well as in cortical and hippocampal neurons [Fahnestock 2001; Pedraza 2005; Peng 2004].

### 4.1.3. pro-NGF during development, injury and pathological conditions

Neurotrophins such as NGF, BDNF, NT-3 and NT-4/5, can support the survival and differentiation of neural cells during retinal development and regeneration [von Bartheld, 1998; Frade and Barde 1999; Harada 2005]. Accumulated data suggest that stratified retinal ganglion cells (RGCs) in chick retina, control their own number by secreting NGF, which kills the incoming migratory RGCs through p75NTR on their surface [Gonzalez-Hoyuela 2001; Frade 1996]. On the other hand, layered RGCs may survive the apoptotic effect of NGF by expressing the high-affinity TrkA receptor [Gonzalez-Hoyuela 2001].

NGF implication in programmed cell death in the developing chick retina [Srinivasan 2004] gives possibility for a similar role in retinal diseases. Activated retinal microglia expressed high molecular weight forms of neurotrophins, presumably not processed, which are able to induce cell death in retinoblastoma cell line. This effect can be abolished completely by NGF neutralizing antibody or by blocking p75NTR. Increased levels of pro-NGF mRNA and protein were observed in the rat model of retinal dystrophy.

It was reported also that pro-NGF can serve as mediator of secondary cell death after brain injury as it was induced and secreted in an active form capable of triggering apoptosis in culture [Harada 2000]. It was also demonstrated that pro-NGF binds p75NTR *in vivo* and that disruption of this binding results in complete rescue of injured adult corticospinal neurons [Srinivasan 2004]. These data together suggest that pro-NGF binding to p75NTR is responsible for the death of adult corticospinal neurons after lesion [Thanos 1992; Roque 1996], and they helps to establish pro-NGF as the pathophysiological ligand that activates the cell death program through p75NTR after brain injury. Interference in the binding of pro-NGF to p75NTR may provide a therapeutic approach for the treatment of disorders involving neuronal loss typically associated with trauma and degenerative or ischemic disorders of the nervous system.

## 4.2. p75NTR

### 4.2.1. Molecular structure and elements in p75NTR

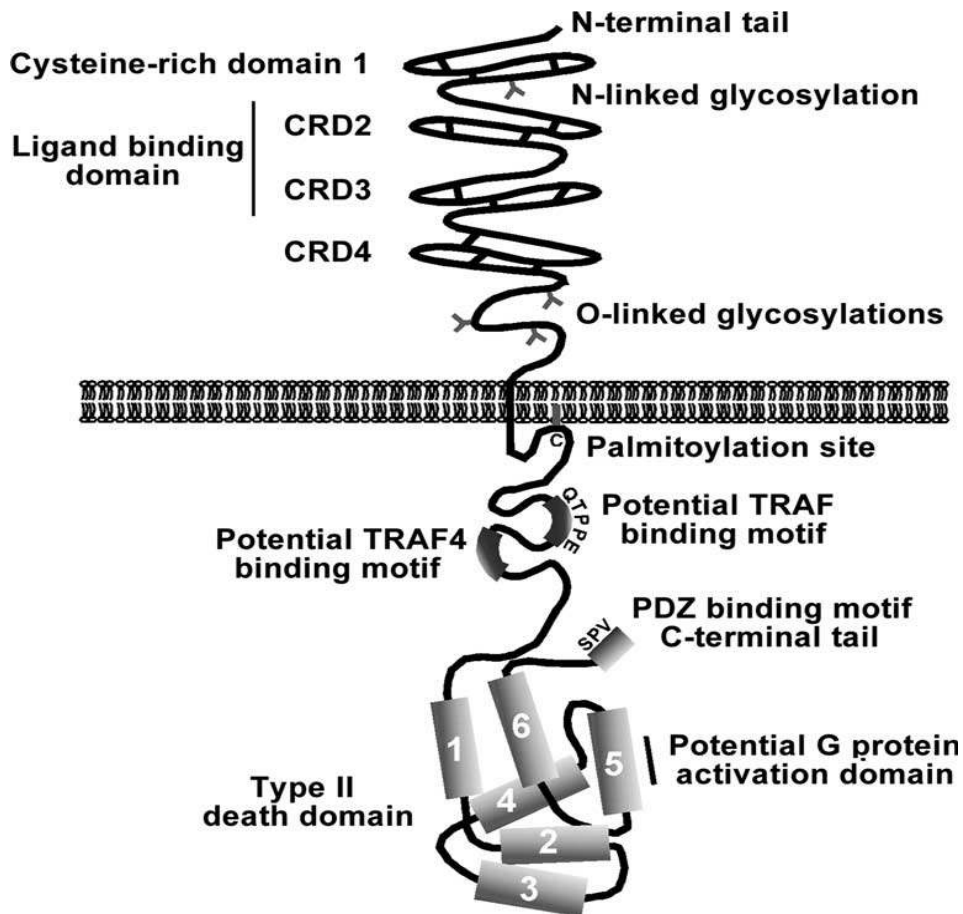



**Figure 16i.** *Main structural elements of p75NTR* [Roux and Barker, 2002]

The p75NTR was cloned for the first time in 1986[Chao 1986], it is a Type I transmembrane receptor with an extracellular domain that contains four cysteine-rich domains (CRDs). The CRDs in p75NTR are required for neurotrophin binding and were subsequently recognized as the defining characteristic of the TNFR superfamily that includes, apart of TNF receptor, Fas (CD95) antigen, CD30, and CD40 [Smith 1994]. They share also the single transmembrane sequence and noncatalytic cytoplasmic domain [Locksley

2001]. X-ray crystallographic analyses show high similarity between the cysteine-rich repeats of p75NTR and the TNFR [Banner 1993; He 2004].

TNFR superfamily members typically bind homotrimeric ligands that are produced as type II trans-membrane proteins, and most of them act as independent signaling units. In contrast, p75NTR binds soluble dimeric ligands and often requires (or acts as) a coreceptor to activate biological activity [Barker 2004]. Some TNFR family members, including p75NTR, contains approximately 80 amino acid death domain [Feinstein 1995]. Most common role of the death domain is protein binding module interacting with adaptor proteins that aggregate and activate Caspase-8 and thereby apoptosis. However, the death domain in p75NTR is structurally distinct from that in other TNFR superfamily members and signaling properties of p75NTR differs from its TNFR relatives. Indeed, p75NTR can be considered somehow particular in the TNFR family.

Expression of p75NTR is confined mainly to nervous system. In vitro p75NTR is expressed in similar amounts in the dendrites and axons of transfected hippocampal neurons [Jareb 1998]. In vivo immunohistochemistry in rat dentate gyrus and electron microscopy show more prominent p75NTR expression in axons than dendrites [Dougherty 1999]. p75NTR is up-regulated under different pathological or inflammatory conditions. For example, after seizures, there is prominent expression of p75NTR in cortical neurons [Roux 1999]. Induction of p75NTR has been observed in many cell types, including oligodendrocytes, Schwann cells, microglia, macrophages, and smooth muscle cells [Chao 2003; Barker 2004].

### 4.2.2. Functions of p75NTR

p75NTR can initiate a complex signaling pathway by itself, but also can be a component of three distinct receptor platforms that bind a broad spectrum of ligands and that, under different circumstances, facilitate cell survival [Huang 2001; Chao 2003], cell death [Frade 1996; Casaccia-Bonnefil 1996; Bredesen 1997; Bamji 1998] or growth and regeneration inhibition [Chao 2003; Wang 2002; Wong 2002].

#### 4.2.2.1. p75NTR as unassisted receptor

In the absence of Trk's receptors, p75NTR is capable of triggering independent signalling. The signalling pathways underlying the p75NTR-dependent apoptotic response are not completely understood but are believed to be involved in the activation of JNK. Further downstream events include phosphorylation of c-Jun, activation of both p53 and the 'BH3-domain only' family members Bad (Bcl-2-associated death promoter) and Bim (Bcl-2 interacting mediator of cell death), mitochondrial translocation of Bax and release of mitochondrial Cytochrome C and activation of Caspases 3, 6, and 9 [Gentry 2004 a; Bhakar 2003; Okuno 2004; Barker 2004; Becker 2004]. The proximal events that couple p75NTR to JNK are poorly understood but ligand binding to p75NTR induces activation of Rac, a GTP-binding protein and an established activator of JNK [Harrington 2002]. Although Rac does not itself directly associate with p75NTR, at some other cytosolic adaptor proteins do [Gentry 2004 a]. Whether some of them link the cytoplasmic tail of p75NTR to Rac remains to be determined. However, the p75NTR counterparts NT-receptor interacting factor (NRIF), NT-associated cell death executor (NADE), NT-receptor-interacting MAGE homolog (NRAGE) and TNF receptor-associated factors (TRAFs) have been shown to activate JNK and promote p75NTR-dependent apoptosis alone or in combination [Yeiser 2004]. For example, JNK activation requires coexpression of NRIF and TRAF6 in p75NTR-expressing cells, because either protein alone fails to reconstitute JNK signalling [Gentry 2004 b]. Intriguingly, coexpression of the two adaptors also induces nuclear translocation of NRIF and enhances TRAF-mediated activation of NF-kappaB, which paradoxically promotes survival rather than apoptosis [Gentry 2004 a, b]. The p75NTR signalling might be further extended because NT binding can also activate the MAP kinase signalling pathway and stimulate death in some cell types but survival in others. The multiple p75NTR-dependent signalling pathways might operate simultaneously and converge, divide or intercept, depending on the cellular context.

#### 4.2.2.2. p75NTR and Trk's

p75NTR is often named low-affinity NGF receptor, but its neurotrophin binding affinity is actually similar to that of monomeric TrkA, with a Kd of about

$1–10.10^{-9}$ [Chao 2003]. However, when the receptors are coexpressed, p75NTR enhances the ability of Trk receptors to bind and respond to neurotrophins [Chao 1995]. Peripheral tissues produce low concentrations of neurotrophins to maintain appropriate levels of neuronal survival and innervation, and p75NTR appears to act as a coreceptor and allows Trks to respond to limited NT levels. Structural analysis of p75NTR with NGF suggests the formation of an asymmetric complex of one dimeric NT bound to a single p75NTR.

Taking into account the conformation, the p75NTR–NT complex is not able to recruit more than one receptor molecule. Therefore, it is unable to form high-affinity two-chain sites that can 'compete' with Trk dimers for ligand binding [He 2004]. Trk mediated signaling is that p75NTR interacts with Trk through the cytosolic and transmembrane domains. p75NTR takes the role of a coreceptor that refines Trk affinity and specificity for NTs [Huang 2003]. The importance of this mechanism is revealed during sequential stages of neuronal development when Trk receptors are activated by different NTs in a p75NTR-dependent manner. For example, induction of p75NTR expression in maturing sympathetic neurons causes a shift in TrkA responsiveness that reduces NT-3 dependent axonal growth and promotes NGF-induced neuronal survival and differentiation [Kuruvilla 2004].

p75NTR also influences signaling pathways elicited by Trk activation. For example, p75NTR can bind Shc - an established Trk adaptor - stimulate its phosphorylation and thereby amplify Trk signalling [Epa 2004]. Thus, elevated levels of NGF induce increased p75NTR expression in sympathetic neurons, greater sequestration of NGF by p75NTR and reduced availability of NGF for binding to TrkA. Finally, Trk–p75NTR receptor complexes might possess signalling capacities that are inherently different from those of Trk dimers. Thus, activation of TrkA in the absence of p75NTR elicits the MAP kinase pathway but both receptors are required for a robust activation of the PI3-kinase–protein kinase B (Akt) cascade. The presence of Ankyrin rich membrane protein (ARMS), a binding partner for p75NTR and TrkA, is a possible explanation for the ability of NGF to activate distinct signalling pathways depending on the cellular context. ARMS can simultaneously associate with TrkA and p75NTR, but increased ARMS expression leads to a significant diminution of TrkA dimer-

p75NTR complex formation [Chang 2004]. Upon NGF binding, the 'isolated' TrkA dimer stimulates the phosphorylation of ARMS, which subsequently promotes prolonged MAP kinase signalling [Arevalo 2004]. This might explain why TrkA dimers are sufficient to sustain neurite outgrowth, whereas the heteromeric TrkA dimer-p75NTR complex is necessary for complete outgrowth and for long-term survival [Lad 2003 b]. Fas apoptosis inhibitor molecule (FAIM) was identified as another adaptor that can bind both TrkA and p75NTR and promote NGF dependent neurite outgrowth. The exact mechanism by which TrkA and p75NTR integrates FAIM is presently under study.

The morphology of the neuron, with axonal prolongation thousands of times larger than the cell body rises the question how after Trk-p75NTR complex is formed the signal is transported from axonal or dendritic sites of generation to the cell body. This question has been addressed primarily for Trk signal transduction, and the hypothesis raised include calcium/phosphorylation 'waves' progressing along the axon or retrograde transport of neurotrophin–Trk by signalling endosomes [Delcroix 2003; Ginty 2002]. Actually a direct interaction between Trk and the motor protein Dynein has been reported, which provides a possible mechanism for microtubule-associated transport of such endosomes [Yano 2001, 2004].

#### 4.2.2.3. p75NTR and Sortilin complex-the proneurotrophin receptor

Over the last decade, numerous studies have shown that the p75NTR can act as an apoptotic receptor during development and following injury, but the precise ligand requirements for these effects was controverted. Mature neurotrophins are not effective activators of p75NTR-induced apoptosis, and high non-physiological concentrations are often required to induce even modest levels of cell death. This led to speculation that there may be other ligands of p75NTR.

pro-NGF binds p75NTR with high affinity and is a potent inducer of p75NTR-dependent apoptosis in sympathetic neurons, oligodendrocytes, and in a vascular smooth muscle cell line [Nykjaer 2004]. It was also shown that pro-

NGF does not bind TrkA suggesting that pro-NGF is an apoptotic ligand that is specific for p75NTR.

The mechanism of how p75NTR mediates cell death is far from being completely understood but the identification of Sortilin as a p75NTR coreceptor that is necessary for pro-NGF-induced effect was a major breakthrough [Nykjaer 2004]. Sortilin is a type I transmembrane protein expressed in a wide variety of tissues but is most abundant in the central nervous system. Through crosslinking studies it was established that p75NTR and Sortilin form a receptor complex that binds pro-NGF at the cell surface [Nikjaer 2004]. Both receptors appear to be required to transduce the apoptotic effects of pro-NGF. Blocking the interaction of pro-NGF with Sortilin inhibits pro-NGF-mediated apoptosis, whereas expression of exogenous Sortilin in Schwann cells, which normally express only p75NTR, makes these cells sensitive to the apoptotic effect of pro-NGF [Nykjaer 2004]. Although p75NTR and Sortilin are required for pro-NGF-induced apoptosis, their coexpression does not invariably result in pro-NGF induced killing; for example, pro-NGF does not induce apoptosis of melanoma cells but instead enhances migratory activity [Shonukan 2003].

Sortilin is a member of the Vps10 family that is involved in transport from the trans-Golgi network (TGN) to the vacuole. In mammalian cells, Sortilin has been shown to play an important role in TGN-to-endosome and TGN-to-lysosome trafficking events, and greater than 90% of the Sortilin pool is retained in intracellular compartments [Nielsen 2001]. Pro-NGF directly binds Sortilin, even in the absence of p75NTR. Since Sortilin plays a role in TGN-to-endosome trafficking, it is possible that Sortilin functions not only as a cell surface pro-NT receptor but also directs intracellular movement of newly synthesized pro-NTs.

A further intriguing possibility is that proneurotrophin - Sortilin interactions could start intracellularly within the secretory pathway. In such a scenario, Sortilin binding to the pro-domain of the neurotrophin in Golgi apparatus could protect it from proteolytic cleavage, and the molecules could then travel to the cell surface in a preformed pro-apoptotic p75NTR activating complex. Regulating Sortilin levels might thereby provide the cell with an efficient mechanism of active suicide. Alternatively, cell-surface cleavage of Sortilin could release a soluble Sortilin–proneurotrophin complex into the extracellular

space. Such a complex might provide a circulating reservoir of proneurotrophin that is protected from being cleaved to the mature form and is available for interaction with receptors on adjacent or distant cells [Bronfman 2004].

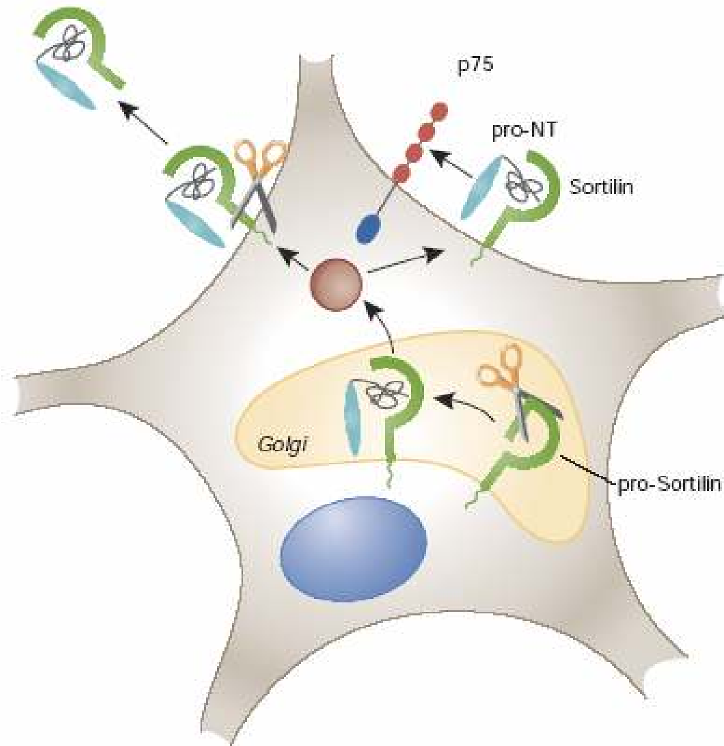



**Figure 17i**. *Different sites of interaction between pro-NGF and Sortilin. The possibility of intra-Golgi, plasma membrane and extracellular interaction is outlined.* [Brofman 2004]

#### 4.2.2.4. NogoR, LINGO-1 and p75NTR receptor complex

In addition to its roles as a Trk coreceptor and regulator of apoptosis, p75NTR has recently emerged as a key player in the regulation of neuronal growth. Definitive data linking p75NTR to this function came from studies of Barde and colleagues who demonstrated that unligated p75NTR was a potent activator of RhoA and showed that neurotrophins could suppress this effect [Yamashita 1999]. In adults, RhoA activation mediates the effects of CNS-

derived myelin-based growth inhibitors (MBGIs) that include Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgP). The discovery that RhoA could be regulated by p75NTR helped to determine p75NTR role in MBGI-induced growth inhibition. It was found that MAG-induced growth inhibition and RhoA activation were attenuated in sensory and cerebellar granule neurons (CGNs) derived from p75NTR null mice [Yamashita 2002].

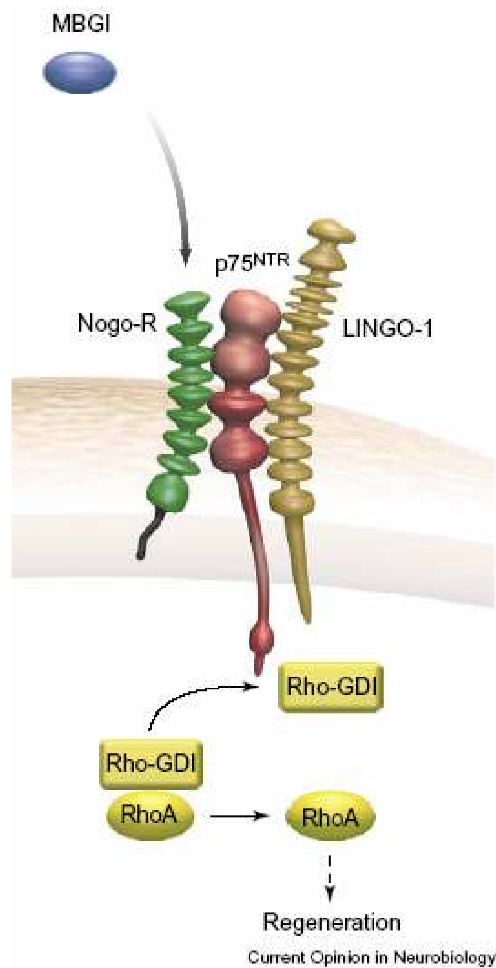



**Figure 18i.** *p75NTR, Nogo-R and LINGO-1 receptor complex* [Nykjaer 2005]

The MBGIs bind to NogoR, a GPIlinked protein with no intracellular signalling capability, and the discovery that p75NTR played a role in MAG signalling suggested that p75NTR may collaborate with the NogoR to mediate responses to MBGIs. Two groups have independently confirmed this prediction

and shown that a complex containing NogoR and p75NTR can be identified in cells coexpressing the two receptors [Wang 2002; Wong 2002]. The precise signalling mechanisms that are activated by the p75NTR-NogoR are still under investigation but some studies have shown that p75NTR regulates RhoA by directly binding Rho GDP dissociation inhibitor (Rho-GDIα). Binding of MBGIs to the p75NTR-NogoR complex appears to enhance the association of Rho-GDIα with p75NTR, whereas NGF binding abolishes the p75NTR-Rho-GDIα interaction [Yamashita 2003]. In a set of experiments, Yamashita and Tohyama took advantage of binding peptide that binds the p75NTR death domain. This peptide blocks the association of Rho-GDIα with p75NTR in vitro and was shown to antagonize Nogo- and MAG induced growth inhibition when delivered to primary sensory neurons. Thus, the association of MBGIs with the p75NTR-NogoR complex causes Rho-GDIα to bind the p75NTR death domain and causes RhoA-GDP to be released from Rho-GDIα. RhoA is then able to exchange GDP for GTP and thus being activated. LINGO-1 has been identified as an essential component of the p75NTR-NogoR receptor complex that is required for MBGI signalling. It was described that the extra cellular domain of LINGO-1 binds both NogoR and p75NTR and showed that full-length LINGO-1 can be immunoprecipitated with either NogoR or p75NTR [Mi 2004].

LINGO-1 is a member of a protein family (including also LINGO-2, -3, and -4), characterized by extracellular leucine-rich repeats (LRRs) and an IgC2 domain. The primary function of LRR and IgC2 domains is to mediate protein-protein interactions, but their role in LINGO-1 function is unknown. There are relatively few proteins that contain both LRR and Ig-like domains in their extracellular region, and it is noteworthy that TrkA, TrkB, and TrkC belong to this group. The NogoR does not contain IgC2 domains but does contain multiple LRRs, and it may be more than a coincidence that this structural motif is present within three classes of transmembrane proteins that form physical complexes with p75NTR.

Contradictory results have been published even using the same experimental model. For example, two studies of adult facial neuron regeneration in the p75NTR null mice have revealed increased regeneration and enhanced functional recovery [Boyd and Gordon 2001], but others have found that the rate of axonal elongation was not altered [Gschwendtner 2003].

In the injured spinal cord, the initial phase of RhoA activation is dependent upon p75NTR expression [Dubreuil 2003], yet p75NTR null mice do not show enhanced regeneration of lesioned corticospinal neurons or ascending sensory tracts after spinal cord damage [Song 2004].

#### 4.2.2.5. p75NTR processing as signalling mechanism

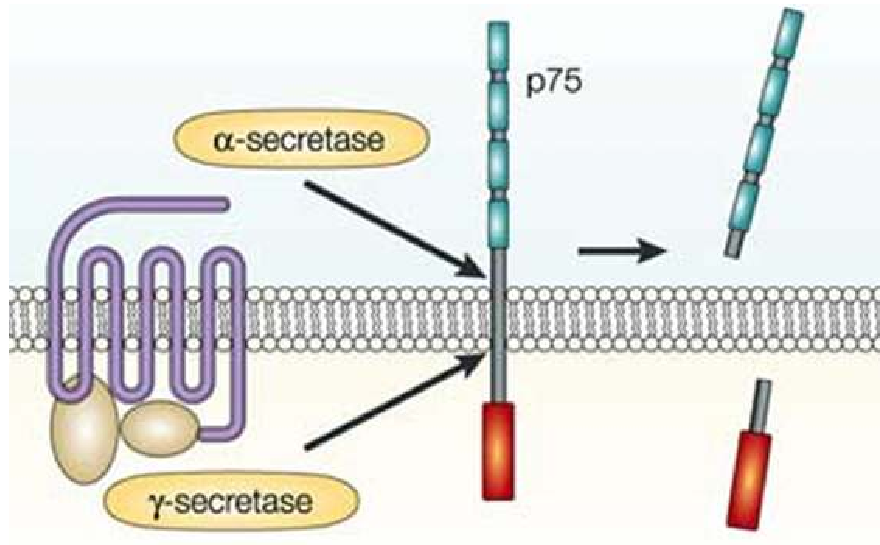



**Figure 19i.** *p75NTR processing with liberation of p75NTR ICD by α-and γ-Secretase* [Chao, 2003]

Intramembrane cleavage events have been described recently for p75NTR [Kanning 2003; Jung 2003]. Regulated intramembrane proteolysis (RIP) has emerged as a highly conserved mechanism in receptor signalling [Fortini 2002; Ebinu 2002; Wolfe 2004]. Presenilin-dependent γ-Secretase activity is responsible for the intramembrane proteolysis of an increasing number of membrane proteins, including Notch, ErbB4 tyrosine kinase receptors, CD44, low density lipoprotein, and Aβ precursor protein [Ebinu 2002]. γ-Secretase is a large protein complex with an unusual aspartyl protease activity that cleaves substrates within the transmembrane domain and requires Presenilin and other components, such as Nicastrin, Pen-2, and Aph-1

[Iwatsubo 2004]. The assembly of γ-Secretase complex is discussed in details below.

*γ*-Secretase cleavage occurs in the middle of the transmembrane domain of p75NTR [Jung 2003]. α-Secretase cleavage event is previous and indispensable for the γ-Secretase activity. Structural studies indicate that just a 15-aminoacid region of p75NTR, adjacent to the membrane spanning domain is necessary-for the activity of the α-Secretase [Zampieri 2005]. The NRH2, a p75NTR homolog that lacks the cysteine-rich domains, is also efficiently cleaved by α-Secretase activity [Kanning 2003]. The extracellular domain of NRH2 is short and does not have homology with p75NTR. NRH2 is a target of not only for γ-Secretase but also α-Secretase activities. It is striking that the action of γ-Secretase does not appear to depend strictly upon a specific amino acid motif in these molecules. Deletion mutants in the transmembrane region of p75NTR indicate that γ-Secretase effectively cleave the receptor even when large portions of the stalk domain are missing [Weskamp 2004]. Remarkably, it was found that the sizes of the C-terminal fragment (CTF) and the intracellular domain (ICD) produced by each deletion mutant are nearly identical, indicating that the γ-Secretase is recognizing a structural feature of the receptor for its proteolytic activity. Other related TNF receptor members, such as the Fas receptor, do not undergo cleavage like p75NTR, despite the overall similarity in structural features between the two receptors.

Cleavage of p75NTR by RIP is of particular importance for a number of reasons. First, each product of the cleavage may serve multiple functions. The release of the extracellular domain generates a binding protein for many potential ligands, including neurotrophins, pro-neurotrophin precursors, β-amyloid, and the rabies virus glycoprotein [Roux 2002; Dechant 2002]. Second, the ICD domain of p75NTR has the potential of binding many intracellular proteins, including TNF-associated factor 6, NADE, NRAGE, and RhoA [Hempstead 2002; Barker 2004].

The p75NTR cytoplasmic domain may bring these proteins to function in different cellular compartments. The cleavage of the CTF also gives rise to a small peptide the significance of which is unknown but that is analogous to the Aβ peptides generated from APP. The early induction and cleavage of p75NTR in selective neurons that are destined to apoptosis suggests that p75NTR

cleavage may be linked to other events occurring during neurodegeneration. The growing number of substrates undergoing intramembrane cleavage raises the issue of how particular target sequences are recognized by γ-Secretase.

##### 4.2.2.5.1. Structure and assembly of γ-Secretase complex

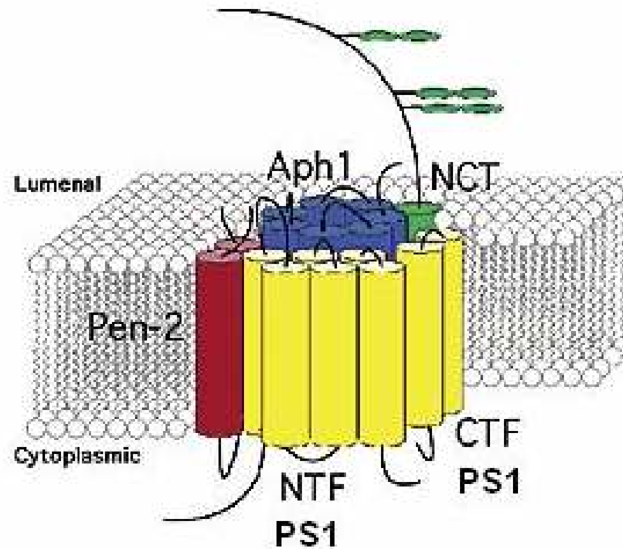



**Figure 20i.** *Structure of γ-Secretase complex* [www.crystaltenshi.com]

γ-Secretase is membrane-bound protease responsible for the intramembrane cleavage of membrane-spanning proteins as APP and Notch. The cleavage, which is expected to take place in the middle of the transmembrane segment of substrates, usually occurs after ectodomain shedding of the target protein, and does not depend entirely on their amino acid sequences [Struhl 2000]. The discovery of Presenilin (PS) 1 and 2 as the major pathogenic genes for early-onset FAD, in addition to APP, prompted the identification of γ-Secretase. PS, which is endogenously present in every type of mammalian cell, undergoes endoproteolysis, and the resulting amino-terminal and carboxy-terminal fragments remain closely associated [Thinakaran 1996]. The observed native size of γ-Secretase complex is 250–1000 kDa. These suggest the presence of binding partners (cofactors) of PS that stabilized and activated the γ-Secretase complex.

The first protein cofactor of PS to be identified in the γ-Secretase complex was Nicastrin, which is a type I membrane glycoprotein [Yu 2000]. RNA interference (RNAi) knockdown of aph-2 (a Nicastrin homolog) in C. elegans resulted in hypoplasia of the anterior pharynx. It was shown that the activation of Notch-1 in mammalian cells, through its cleavage in the transmembrane segment and liberation of the Notch intracellular domain (NICD), was also dependent on PS [De Strooper 1999].

Overexpression of Nicastrin alone in mammalian cells did not increase the levels of the functional γ-Secretase complex [Hu 2002], indicating the presence of additional protein cofactors was required for the formation and function of γ-Secretase. Recent studies have shown that disruption of the GXXXG motif within the 4th transmembrane domain of APH-1 caused by the mutation leads to failure in assembly and activation of the γ-Secretase complex [Lee 2004]. Two homologs of aph-1 genes (i.e. aph-1a and aph-1b) were identified in humans, both encoding possible multispan membrane proteins that were predicted to span the membrane seven times. Formation of the active γ-Secretase complex by overexpression of PS1, Nicastrin, Aph-1a and PEN-2 has also been demonstrated [Kimberly 2003; Kim 2003]. Edbauer and co-workers [Edbauer 2003] overexpressed human PS-1 together with Nicastrin, Aph-1a, and PEN-2 in yeast, an organism that lacks endogenous PS and cofactors, and observed γ-Secretase activity in vitro using a recombinant APP carboxy-terminal substrate, which strongly suggests that PS, Nicastrin, Aph-1 and PEN-2 are sufficient to reconstitute γ-Secretase activity.

One of the unresolved questions regarding the structure of the γ-Secretase complex is the stoihiometry of each component contained in the active enzyme. The sum of the molecular masses of the four components amounts to 150–200 kDa, which is smaller than the size of the active γ-Secretase complex predicted by gel filtration or glycerol velocity separation that might exceed 250–1000 kDa. By demonstrating the coimmunoprecipitation of differentially tagged PS molecules, Schroeter and co-workers [Schroeter 2003] suggest that PS could form a dimer at the core of the γ-Secretase complex. They propose that the substrate is cleaved at the interface of the two PS molecules.

#### 4.2.2.6. NRH2

Assessing the precise role of p75NTR in neuronal growth in vivo is interesting challenge but several factors complicate the progress in this area. Recently was identified neurotrophin receptor homolog 2 (NRH2). It is type I transmembrane protein that lacks the CRDs but otherwise is remarkably similar to p75NTR [Kanning 2003]. NRH2 has recently been shown to physically interact with TrkA receptors [Murray 2004], and possibly with Sortilin, NogoR, or LINGO-1 and participate in the effects of proNTs and MBGIs.

### 4.3.3. Processes in which p75NTR plays role

#### 4.3.3.1. Development of the nervous system

A well-established function of p75NTR is to promote cell death. This has been convincingly illustrated in transgenic animal models [Miller 2001]. Newborn p75NTR/exonIII null mice have a greater number of sympathetic neurons, suggesting that in early development p75NTR might control apoptosis rather than survival. Further support for this notion comes from studies of p75NTR/exonIII-/- and TrkA-/-double deficient mice. Whereas TrkA-/- sympathetic neurons die during the late neonatal and early postnatal periods because of lack of trophic stimulation, neurons from double knockout mice are substantially rescued from death by the parallel absence of p75NTR. However, adult p75NTR/exonIII knockout mice, in which exon III encoding the ligand-binding domain has been removed, exhibit massive loss of sympathetic and sensory neurons in agreement with a role of p75NTR in NGF-induced Trk activation. In summary, these data indicate a dual function for p75NTR—that is, refinement of target innervation by elimination of no preferred neurons during early development, and cooperation with Trk at later stages to facilitate survival and to refine neuronal connections [Gentry 2004a; Miller 2001].

#### 4.3.3.2. Pathological conditions and injury

| Induction of p75 receptor expression after injury | |
|---|---|
| **Cell type** | **Injury** |
| Motor neurons | Axotomy, regeneration[139–141] |
| Purkinje neurons | Traumatic injury[142] |
| Entorhinal neurons | Seizure[41] |
| Hippocampal neurons | Primary culture[143] |
| Striatal neurons | Ischaemia[144] |
| Cortical neurons | Zinc, ischaemia[145], Alzheimer's disease[146] |
| Schwann cells | Axotomy[147,148] |
| Oligodendrocytes | Spinal cord injury[42], multiple sclerosis lesions[40,149] |

p75 expression is also induced in experimental allergic encephalomyelitis[150,151] and Alzheimer's disease[152].

***Table 7.*** *Induction of p75 receptor expression after injury. Moses V. Chao, Nature Reviews Neuroscience 4, 299-309 (2003)*

p75NTR is abundantly expressed during development but is down regulated in the adult organism. Yet, p75NTR is re-expressed in conditions of increased neuronal cell death including mechanical damage, focal ischemia, axotomy, stroke and AD, suggesting a physiological role during neuronal degeneration [Dechant 2002]. For example, in a mouse model of ALS, p75NTR expression is up regulated in dying motor neurons, and disease onset is delayed on the p75NTR/exonIII knockout background and upon treatment with antisense p75NTR peptide nucleic acid constructs [Kust 2003; Turner 2003]. Reexpression of p75NTR has also been reported in the cerebral cortex of AD patients and might, along with the parallel down regulation of Trk receptors, account for an imbalance between survival and death signals [Mufson 2003]. However, the ligand responsible for triggering p75NTR-dependent cell death remains controversial because some studies report that Aβ induce neurotoxicity by binding to p75NTR and activation of JNK [Hashimoto 2004; Tsukamoto 2003], whereas others find that receptor expression protects neurons against Aβ-induced toxicity [Zhang 2003]. A more likely candidate is pro-NGF, which is up regulated during the initial and end-stages of AD. Some studies have provided evidence that pro-NGF constitutes an authentic death inducing ligand upon spinal cord injury. Thus, apoptosis of oligodendrocytes correlates with

increased pro-NGF synthesis [Beattie 2002], and axotomy induces the release of pro-NGF as well as death of corticospinal neurons in a p75NTR-dependent manner [Harrington 2004]. Interestingly, the disruption of the pro-NGF–p75NTR signalling cascade using p75NTR *knockout* mouse models or inhibitory antibodies completely rescues lesioned neurons from death [Harrington 2004].

The role of pro-NGF in p75NTR-dependent apoptosis *in vivo* has now been examined in two injury models. It was shown that oligodendrocyte apoptosis that occurs after spinal cord trauma correlates with the synthesis of bioactive pro-NGF making it possible inducer of cell death *in vivo* [Beattie 2002]. Corticospinal neurons (CSN) undergo p75NTR-dependent apoptosis following lesion, and Harrington used two loss-of-function approaches to establish a role for pro-NGF in this system. In the first, antibodies directed against either mature NGF or against the prodomain of NGF were shown to reduce CSN loss, and in the second, significant protection of CSNs was observed in mice containing only single NGF allele. Importantly, it was demonstrated that p75NTR immunoprecipitated from lesioned tissue is bound to pro-NGF and showed that infusion of antibodies directed against pro-NGF reduces this association.

## 5. Protein post-translational modifications

### 5.1. Overview

Post-translational modifications (PTMs) are covalent processing events that change the properties of a protein by proteolytic cleavage or by addition of a modifying group to one or more amino acids [Mann 2003]. This is one of the later steps in protein biosynthesis for many proteins. PTMs of a protein can determine its activity state, localization, turnover, and interactions with other proteins, so they were found conductive to various neurodegenerative disorders [Yokoyama 2008; Pamplona 2008]. Activity and molecular and signalling interactions of many proteins are determined by their extent of phosphorylation [Jacob 2008]. Common feature for all tauopathies is that tau protein is modified by phosphorylation at several sites for serine/threonine. Tau in NFT insoluble aggregates appear to occur in a hyperphosphorylated state [Anderton 2000; Andorfer 2000; Avila 2000; Bennecib 2000]. In signaling, kinase cascades are turned on and off by the reversible addition and removal of phosphate groups

[Cohen 2000]. In a number of NDD unfolded or aggregated proteins are marked by the ubiquitination and destined for proteolytic destruction [Grune 2004]. Recently was reported increase in the brain protein glyco- and lipoxidation for diverse NDD: AD, ALS, Prion disease, PiD [Pamplona 2005; Ilieva 2007; Pamplona 2008]. Post-translational modifications are key to the regulation of functional activities of proteins, so quantitative and qualitative information about PTM stages of proteins is crucial in the discovery of biomarkers of disease. Table 8 summarizes the features of a few important PTMs.

| PTM type | ΔMass[a] (Da) | Stability[b] | Function and notes |
|---|---|---|---|
| Phosphorylation | | | Reversible, activation/inactivation of enzyme activity, modulation of molecular interactions, signaling |
| pTyr | +80 | +++ | |
| pSer, pThr | +80 | +/++ | |
| Acetylation | +42 | +++ | Protein stability, protection of N terminus. Regulation of protein–DNA interactions (histones) |
| Methylation | +14 | +++ | Regulation of gene expression |
| Acylation, fatty acid modification | | | Cellular localization and targeting signals, membrane tethering, mediator of protein–protein interactions |
| Farnesyl | +204 | +++ | |
| Myristoyl | +210 | +++ | |
| Palmitoyl | +238 | +/++ | |
| etc. | | | |
| Glycosylation | | | |
| N-linked | >800 | +/++ | Excreted proteins, cell–cell recognition/signaling |
| O-linked | 203, >800 | +/++ | O-GlcNAc, reversible, regulatory functions |
| GPI anchor | >1,000 | ++ | Glycosylphosphatidylinositol (GPI) anchor. Membrane tethering of enzymes and receptors, mainly to outer leaflet of plasma membrane |
| Hydroxyproline | +16 | +++ | Protein stability and protein–ligand interactions |
| Sulfation (sTyr) | +80 | + | Modulator of protein–protein and receptor–ligand interactions |
| Disulfide bond formation | –2 | ++ | Intra- and intermolecular crosslink, protein stability |
| Deamidation | +1 | +++ | Possible regulator of protein–ligand and protein–protein interactions, also a common chemical artifact |
| Pyroglutamic acid | –17 | +++ | Protein stability, blocked N terminus |
| Ubiquitination | >1,000 | +/++ | Destruction signal. After tryptic digestion, ubiquitination site is modified with the Gly-Gly dipeptide |
| Nitration of tyrosine | +45 | +/++ | Oxidative damage during inflammation |

Stability: + labile in tandem mass spectrometry, ++ moderately stable; +++ stable.

**Table 8.** *Some common and important post-translational modifications* [Matthias Mann and Ole N. Jensen, Nature Biotechnology, 2003, vol. 21]

## 5.1.1. PTMs involving addition of functional groups

### 5.1.1.1. Protein oxidation

All sorts of macromolecules are prone to oxidation. Proteins are a major target for oxidants as a result of their abundance in biological systems, and their high rate constants for reaction. Proteins can be oxidized in different ways. Oxidation can occur at both the protein backbone and on the amino acid sidechains. Moreover, oxidized proteins undergo secondary changes in conformation, which may render them more susceptible to further (oxidative or nonoxidative) modifications [Gracy 2002].

The nonenzymatic protein modifications are spontaneous, random, and uncatalyzed chemical reactions. Oxidative protein damage arises from direct exposure to reactive oxygen, chlorine, or nitrogen species generating oxidative products such as glutamic and aminoadipic semialdehydes [Stadtman 1992; Davies 2005; Dean 1997; Stadtman 1998; Hawkins 2001]. These two carbonyl-containing compounds are the main carbonyl products of metal-catalyzed oxidation of proteins, accounting for 55–100% of the total carbonyl value [Requena 2001]. The protein modifications may also arise from reaction with low molecular weight reactive carbonyl compounds derived from amino acids, carbohydrates or polyunsaturated fatty acids such as glyoxal, glycolaldehyde, methylglyoxal, malondialdehyde and 4-hydroxynonenal. These carbonyl compounds could react primarily with lysine, arginine and cysteine residues, leading to formation of both adducts and cross-links (advanced glycation/lipoxidation endproducts) in protein. Because the generation of carbonyl derivatives occurs by many different mechanisms, the level of carbonyl groups in proteins is widely used as a marker of oxidative protein damage.

The formation of Maillard reaction products *in vivo*, commonly known as the "glycation" reaction, is one of many nonenzymatic modifications of proteins that may have an important regulatory role in physiological responses and pathological processes [Maillard 1912].

Reactive carbonyl species (RCS) formed on oxidation of carbohydrates, lipids and amino acids were identified as intermediates in the formation of irreversible, advanced glycoxidation and lipoxidation end-products (AGE/ALEs) on protein (figure 21i).

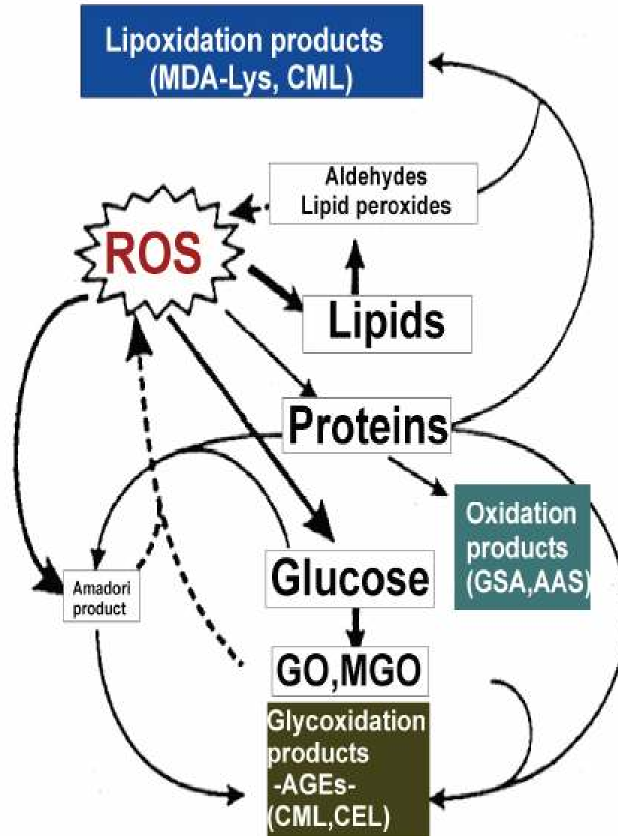



**Figure 21i.** *Molecular heterogeneity of protein oxidative modifications: All kind of macromolecules can be damaged by oxidation.* [Baynes et al. (2002), Diabetes]

The first stage in the classical Maillard reaction is the formation of a Schiff base and Amadori adducts between reducing sugars and free amino groups in protein [Baynes 1999]. The Schiff base and Amadori adduct undergo facile oxidation, especially in the presence of transition metal ions, and fragment to yield shorter chain sugars and reactive intermediates, such as glyoxal (GO) and methylglyoxal (MGO) [Nagai 2002]. A protein-bound dicarbonyl intermediate in protein crosslinking has also been described [Chen 1993] as intermediates formed during the second stage of the Maillard reaction, react with lysine and arginine (and other aminoacids) residues in protein to produce a wide range of protein-bound AGEs and crosslinks during the third and final stage of the classical scheme of the reaction. There are multiple pathways for formation of AGEs from reducing sugars: some proceed from the Amadori compound [Hodge 1953], while others proceed from the Schiff base [Hayashi

1986] or by direct autoxidation of carbohydrates (autoxidative glycosylation) [Wolff 1987]. Other AGEs, such as Nε-(carboxymethyl)-lysine (CML) and Nε-(carboxyethyl)-lysine (CEL), require oxidative fragmentation of the carbon skeleton of glucose, but may also be formed from other hexoses, pentoses, glycolytic intermediates or ascorbic acid.

The term, glycoxidation product [Monnier 2003], was originally introduced to characterize products formed by sequential glycation and oxidation reactions. However, some glucose-derived glycoxidation products may be derived from other precursors by non-oxidative routes, e.g. the formation of CEL from triose phosphates or MGO [Ahmed 1997]. CML may also be formed from a variety of non-carbohydrate sources, including lipid [Fu 1996] and amino acid [Anderson 1999] oxidation products. When CML is formed from lipids, it is described as an advanced lipoxidation end-product and, in those cases where its origin is uncertain, CML is best described as AGE-ALEs [Azzia 2004]. Thus, there is increasing evidence that lipids are as important as carbohydrates in the chemical modification of tissue proteins and development of pathology [Baynes 2000]. The chemical modification of proteins is, in both cases, primarily the result of carbonylamine chemistry or, in a broader sense, the reaction of nucleophilic groups on proteins (e.g. the side chains of lysine, arginine, histidine and cysteine) with electrophilic carbohydrates and lipids and their derivatives (e.g. hydroxyaldehydes, dicarbonyls, hydroxyalkenals and epoxides).

The formation of AGEs, like the metabolism of sugars, may be non-oxidative or oxidative, i.e. anaerobic or aerobic, while the formation of ALEs, like the metabolism of lipids, requires oxidative chemistry to form intermediates. Intermediates in carbohydrate metabolism, including products of glycolysis (triose phosphates) and the polyol pathway (fructose or fructose 3-phosphate), are potent modifiers of protein or precursors of reactive intermediates [Ahmed 2003; Hamada 1996]. In addition to the nonenzymatic formation of isoprostanes, cyclooxygenase and lipoxygenases are important sources of endo- and hydro peroxide precursors of ALEs in tissues. Thus, as direct oxidative damage as Maillard reaction products affect physico-chemical and biological processes.

Neuroketals (NKs) are isoprostanes, a class of prostaglandin-like compounds produced by free radical-induced peroxidation of docosahexaenoic

acid (DHA), which is highly enriched in the brain [Roberts 1998; Nourooz-Zadeh 1998]. NKs were found to be formed in abundance *in vitro* during oxidation of docosahexaenoic acid, and were shown to rapidly adduct to lysine, forming Schiff base adducts. Because DHA is highly concentrated in nervous system tissue these compounds were termed neuroprostanes (NPs). The fact that DHA is prone to free radical attack and free radicals have been implicated in a number of NDD makes neuroketals a unique and prominent marker of oxidative injury in the brain.

Unsaturated fatty acids are the cellular macromolecules most sensitive to ROS, making lipid peroxidation the major oxidative process in tissues. However, lipid peroxidation not only damages the lipids, because its final products can also alter tissue proteins and DNA. Oxidative decomposition of polyunsaturated fatty acids (PUFAs) initiates chain reactions that lead to the formation of specific ALEs such as Nε-(malondialdehyde)-lysine (MDA-Lys), 4-hydroxynonenal-lysine (HNE-Lys), and Nε-(hexanoyl)-lysine, among others. MDA-Lys is the major oxidative break down product of PUFA.

Most protein damage is non-repairable, and has deleterious consequences on protein structure and function. The major fate of oxidised proteins is catabolism by proteosomal and lysosomal pathways, but some materials appear to be poorly degraded and accumulate within cells. The accumulation of such damaged material may contribute to a range of human pathologies [Davies 2005].

#### 5.1.1.2. Acetylation

In living cells, acetylation occurs as a post-translational modification of proteins (histones, tubulins). Many proteins are modified at their N-termini following synthesis, and the modification is performed by N-alpha-acetyltransferases. In most cases the initiator methionine is hydrolyzed and an acetyl group is added to the amine group of the new N-terminal amino acid. Acetyl-CoA is the acetyl donor for these reactions. The removal of the acetyl group is deacetylation.

Some proteins have the 14 carbon myristoyl group added to their N-termini. The donor for this modification is myristoyl-CoA. This latter modification

allows association of the modified protein with membranes. Despite being such a conserved and widespread modification, little is known about the biological role of N-alpha-terminal acetylation.

### 5.1.1.3. Methylation and demethylation

Methylation is a term that denotes the attachment or substitution of a methyl group ($CH_3$) on various substrates. In biological systems, methylation is catalyzed by methyltransferase enzymes and occurs at lysine or arginine amino acid residues in the protein sequence [Walsh 2006]. Arginine can be methylated once (monomethylated arginine) or twice, with either both methyl groups on one terminal nitrogen (asymmetric dimethylated arginine) or one on both nitrogens (symmetric dimethylated arginine) by peptidylarginine methyltransferases (PRMTs). Lysine can be methylated once, twice or three times by lysine methyltransferases. Protein methylation has been most well studied in the histones. [Grewal 2004; Nakayama 2001] DNA methylation is a commonly occurring modification of human DNA and an important epigenetic event in disease progression.

Demethylation is another chemical process resulting in the removal a methyl group from a molecule. In biochemical systems, this process is also often catalyzed by an enzyme such as one of the Cytochrome P450 (CYP) family of liver enzymes.

### 5.1.1.4. Phosphorylation and dephosphorilation

Reversible protein phosphorylation is one of the most important and well-studied post-translational modifications in mammalian cells. The vast majority of phosphorylations occurs as a mechanism to regulate the biological activity of a protein, for instance activating or inactivating an enzyme, a phosphate (or more than one in many cases) is added and later removed [Soulie 1996]. The enzymes that phosphorylate proteins are termed kinases and those that remove phosphates are termed phosphatases.

In animal cells serine, threonine and tyrosine are the amino acids subject to phosphorylation. Serine/threonine kinases are the largest group of kinases that phsophorylate either serines or threonines. The ratio of phosphorylation of

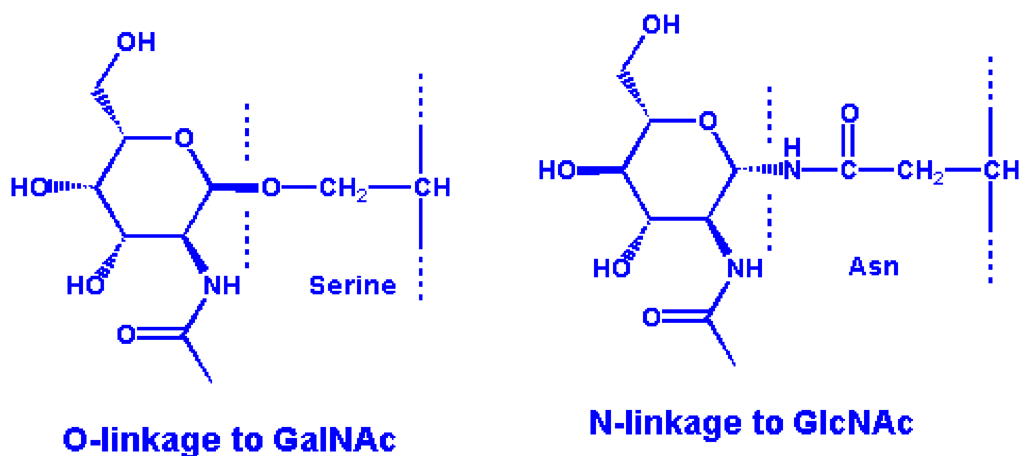
the three different amino acids is approximately 1000/100/1 for serine/threonine/tyrosine.

Although the level of tyrosine phosphorylation is minor, the importance of phosphorylation of this amino acid is profound. As an example, the activity of numerous growth factor receptors is controlled by tyrosine phosphorylation [Drewes 1992].

#### 5.1.1.5. Glycosylation

Glycosylation is the enzymatic process that links saccharides to produce glycans, either free or attached to proteins and lipids. This enzymatic process produces one of four fundamental components of all cells (along with nucleic acids, proteins, and lipids) and also provides a PTM mechanism that modulates the structure and function of membrane and secreted proteins. Glycoproteins consist of proteins covalently linked to carbohydrate. Protein glycosylation is acknowledged as one of the major PTMs with significant effects on protein folding, conformation distribution, stability and activity [Kanninen 2004]. The majority of proteins synthesized in the rough ER undergo glycosylation. It is an enzyme-directed site-specific process, as opposed to the non-enzymatic chemical reaction of glycation. The predominant sugars found in glycoproteins are glucose, galactose, mannose, and fucose. Carbohydrates in the form of aspargine-linked (N-linked) or serine/threonine (O-linked) oligosaccharides are major structural components of many cell surface and secreted proteins. Glycosylation is also present in the cytoplasm and nucleus as the O-GlcNAc modification.

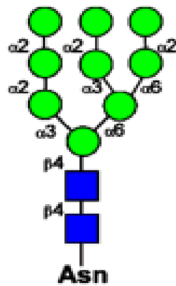
The carbohydrate modifications found in glycoproteins are rarely complex: carbohydrates are linked to the protein component through either O-glycosidic or N-glycosidic bonds. The N-glycosidic linkage is through the amide group of asparagine, and the O-glycosidic linkage is to the hydroxyl of serine, threonine or hydroxylysine (found only in the collagens). In ser- and thr-type O-linked glycoproteins, the carbohydrate directly attached to the protein is GalNAc, whereas in N-linked glycoproteins, it is GlcNAc.

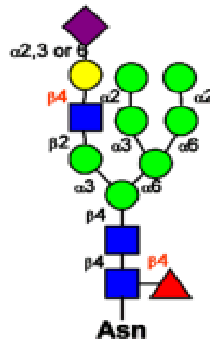


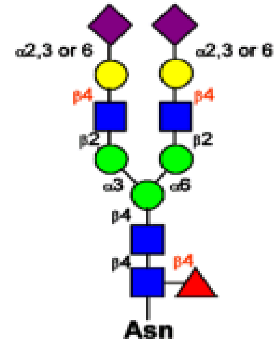

The predominant carbohydrate attachment in glycoproteins of mammalian cells is via N-glycosidic linkage. All N-linked glycoproteins contain a common core of carbohydrate attached to the polypeptide that consists of three mannose residues and two GlcNAc. A variety of other sugars is attached to this core and comprises three major N-linked families:

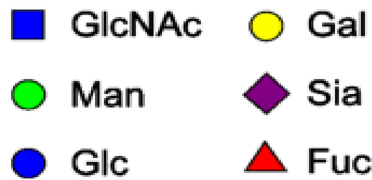
1. High-mannose type contains all mannose outside the core in varying amounts.
2. Hybrid type contains various sugars and amino sugars.
3. Complex type is similar to the hybrid type, but in addition, contains sialic acids to varying degrees.

High-Mannose Hybrid Complex



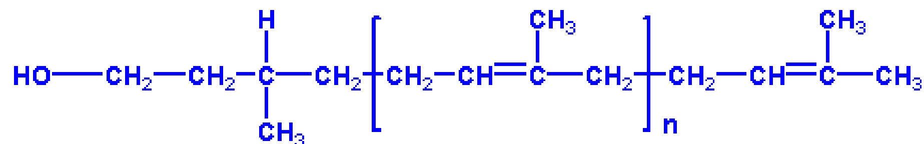




**O-linked sugars:** The synthesis of O-linked glycoproteins occurs via the stepwise addition of nucleotide-activated sugars directly onto the polypeptide. The nucleotide-activated sugars are coupled to either UDP or GDP (as with mannose). The attachment of sugars is catalyzed by specific glycoprotein glycosyltransferases. Evidence indicates that each specific type of carbohydrate linkage in O-linked glycoproteins is the result of a different glycosyltransferase.

**N-linked sugars:** In contrast to the step-wise addition of sugar groups to the O-linked class of glycoproteins, N-linked glycoprotein synthesis requires a lipid intermediate: dolichol phosphate. Dolichols are polyprenols (C80-C100) containing 17 to 21 isoprene units, in which the terminal unit is saturated.



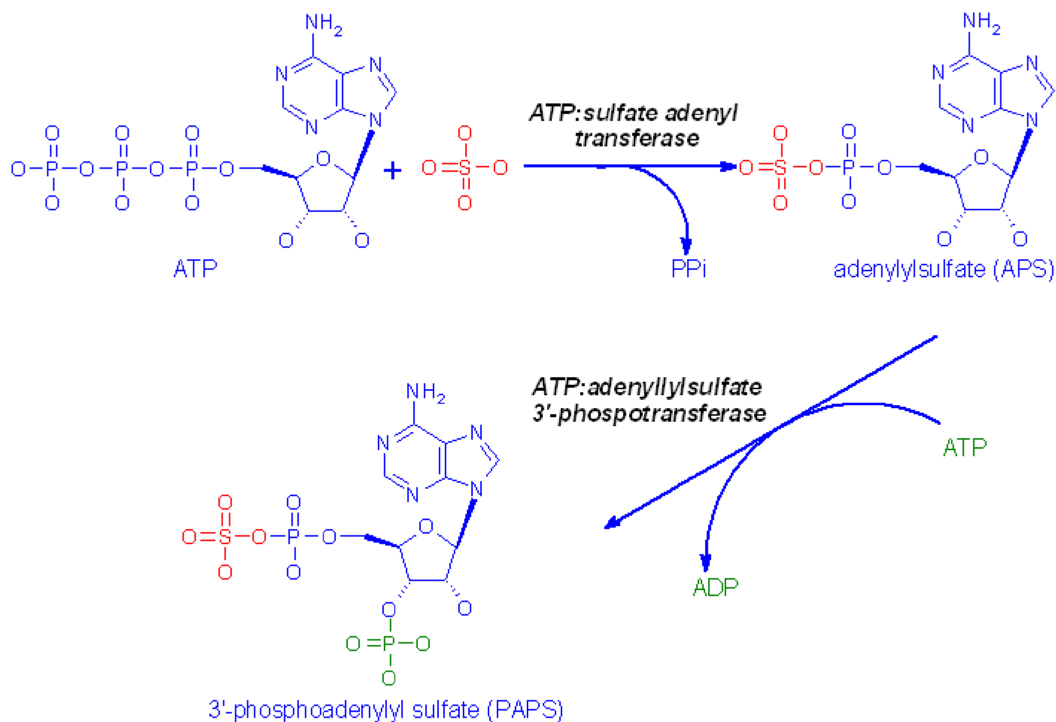
**DOLICHOL**

*The bracket denotes the reapiting isoprene unit. The phosphate in dolichol*

The formation of the GlcNAc-b-Asn linkage in proteins occurs in the endoplasmic reticulum (ER) through cotranslational addition of a preassembled carbohydrate core structure that is delivered via the carbohydrate-dolichol lipid intermediate.

## 5.1.1.6. Sulfation

Sulfate modification of proteins occurs at tyrosine residues, and is catalyzed by tyrosylprotein sulfotransferase (TPST) in Golgi apparatus. The universal sulfate donor is 3'-phosphoadenosyl-5'-phosphosulphate (PAPS). Secreted proteins and extracellular parts of membrane proteins that pass through the Golgi apparatus may be sulfated. The reaction catalyzed by TPST is a transfer of sulfate from the universal sulfate donor to the side-chain hydroxyl group of a tyrosine residue.




Sulfation plays role in strengthening protein-protein interactions. Since sulfate is added permanently it is necessary for the biological activity and is not used as a regulatory modification like that of tyrosine phosphorylation.

## 5.1.1.7. Lipidation

Proteins are covalently modified with a variety of lipids, including fatty acids, isoprenoids, and cholesterol through multiple mechanisms. Lipid modifications play important roles in the localization and function of proteins.

They can be broadly divided into two categories: those that occur in the cytoplasm or on the cytoplasmic face of membranes, and those that occur in the lumen of the secretory pathway. Three common lipid modifications that occur in the cytoplasm are: N-myristoylation, S-palmitoylation, and prenylation (figure 22i) [Nadolski 2007].

N-myristoylation is the covalent addition of the fatty acid myristate to an N-terminal glycine residue via an amide linkage [Resh 1999]. Prenylation is the addition of an isoprenoid, either a C15 farnesyl or a C20 geranylgeranyl group, to a C-terminal cysteine residue via a thioether linkage [Zhang 1996]. And S palmitoylation is the covalent addition of a long-chain fatty acid to a cysteine residue via a thioester linkage [Smotrys 2004; Resh 2006; Greaves 2007].

Three enzymes carry out lipidation in the cell: Farnesyltransferase, Geranylgeranyltransferase I and Rab geranylgeranyltransferase or Geranylgeranyl transferase II.

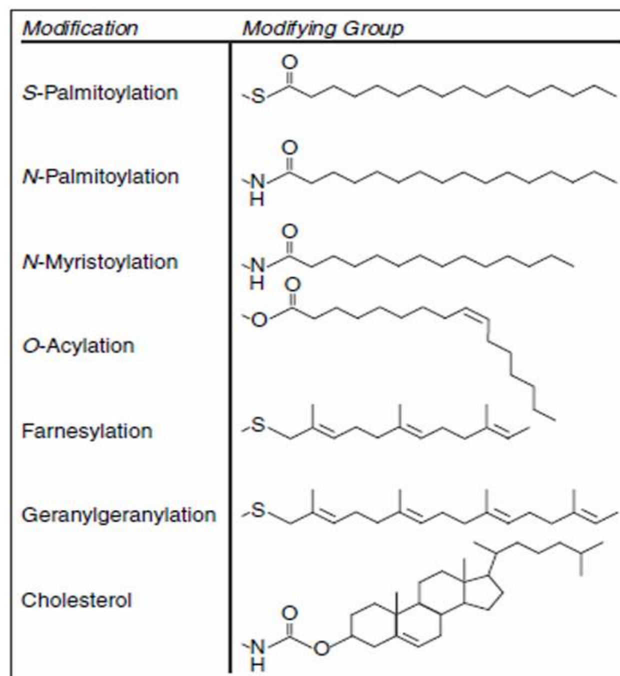



**Figure 22i.** *Structures of covalent lipid modifications.* [M. J. Nadolski and M. E. Linder, FEBS Journal 274 (2007) 5202–5210]

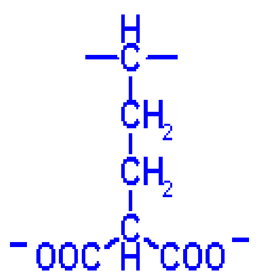
#### 5.1.1.8. Vitamin C-Dependent Modifications (amidation at C-terminus)

Modifications of proteins that depend upon vitamin C as a cofactor include proline and lysine hydroxylations and carboxy terminal amidation. The hydroxylating enzymes are identified as prolyl hydroxylase and lysyl hydroxylase. The donor of the amide for C-terminal amidation is glycine.

The most important hydroxylated proteins are the collagens. Several peptide hormones such as oxytocin and vasopressin have C-terminal amidation.

#### 5.1.1.9. Vitamin K-Dependent Modifications (gamma-carboxylation)

Vitamin K is a cofactor in the carboxylation of certain glutamic acid residues in proteins, to form gamma-carboxyglutamate residues. The result of this type of reaction is the formation of a γ-carboxyglutamate (gamma-carboxyglutamate), referred to as a gla residue.




*Structure of a gla residue*

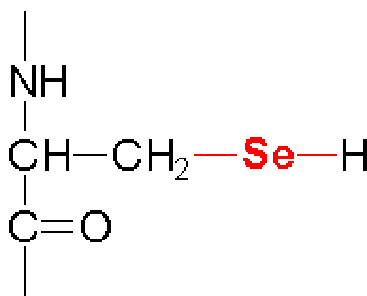
The modified residues are situated within specific protein domains called Gla domains. At this time 14 human proteins with Gla domains have been discovered, and they play key roles in the regulation of three physiological processes:

- Blood coagulation: prothrombin [Mann 1999]
- Bone metabolism: osteocalcin [Price 1988]
- Vascular biology [Berkner 2004]

The Gla-residues are essential for the biological activity of all known Gla-proteins [Furie 1999]. The formation of gla residues within several proteins of the blood clotting cascade is critical for their normal function. Gla-residues are also involved in binding calcium.The presence of gla residues allows the protein to chelate calcium ions and thereby render an altered conformation and biological activity to the protein.

#### 5.1.1.10. Selenoproteins

Selenium is a trace element and is found as a component of several prokaryotic and eukaryotic enzymes that are involved in redox reactions. The selenium in these selenoproteins is incorporated as a unique amino acid, selenocysteine, during translation. A particularly important eukaryotic selenoenzyme is glutathione peroxidase. This enzyme is required during the oxidation of glutathione by hydrogen peroxide ($H_2O_2$) and organic hydroperoxides.




*Structure of the selenocysteine residue*

### 5.1.2. PTMs involving addition of other proteins or peptides

#### 5.1.2.1. Ubiquitination

Ubiquitination is a cellular process by which short lived or damaged proteins are conjugated with multimers of ubiquitin, marking them for degradation in the proteosome [Voss 2007]. Ubiquitin is a small protein that occurs in all eukaryotic cells. Its main function is to mark other proteins for proteolysis. At least four ubiquitin molecules attach to a lysine residue on the condemned protein, in a process called polyubiquitination, and the protein then

moves to a proteasome. It is observed that at least four ubiquitins are required on a substrate protein in order for the proteasome to bind and therefore degrade the substrate, though there are examples of non-ubiquitinated proteins being targeted to the proteasome. Ubiquitin can also mark transmembrane proteins (receptors) for removal from membranes and fulfill several signaling roles within the cell.

The ubiquitination consists of a series of steps (figure 23i):

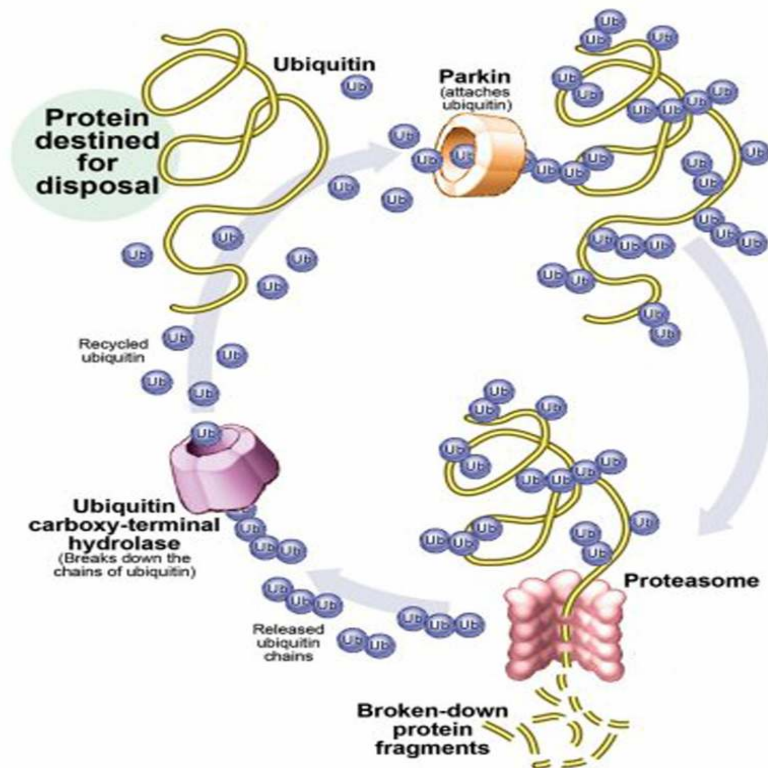



**Figure 23i.** *The Ubiquitin-proteasome system: polyubiquitination of the substrate and its degradation in the 20S catalitic core of the 26S proteasome.*

If the polyubiquitin chain is longer than 3 ubiquitin molecules, the tagged protein is rapidly degraded by the 26S-proteasome [Widmer 2006]. Ubiquitin moieties are cleaved off the protein by deubiquitinating enzymes and are recycled for further use [Jung 2007; Grune 2004].

## 5.1.3. PTMs involving structural changes

#### 5.1.3.1. Proteolytic Cleavage

Most proteins undergo proteolytic cleavage following translation. The simplest form of this is the removal of the initiation methionine. Many proteins are synthesized as inactive precursors (propeproteins) that are activated under proper physiological conditions by limited proteolysis [Perez 2001, Novak 1994].

Enzymes are synthesized as inactive precursors called zymogens, activated by proteolytic cleavage such as is the situation for several proteins of the blood clotting cascade.

#### 5.1.3.2. Disulfide bridges

A disulfide bond is a single covalent bond derived from the coupling between the thiol groups of cysteine residues. Disulfide bonds in proteins are formed by thiol-disulfide exchange reactions. The other sulfur-containing amino acid, methionine cannot form disulfide bonds. The linkage is also called an SS-bond or disulfide bridge. Disulfide bonds are usually formed from the oxidation of sulfhydryl (-SH) groups. Disulfide bonds play an important role in the folding and stability of some proteins, usually secreted to the extracellular medium. Since most cellular compartments are a reducing environment, disulfide bonds are generally unstable in the cytosol.

The disulfide bond stabilizes the folded form of a protein in several ways:1) It holds two portions of the protein together, 2) The disulfide bond may form the nucleus of a hydrophobic core of the folded protein, 3) Related to #1 and #2, a disulfide bond stabilizes secondary structure in its vicinity. For example, researchers have identified several pairs of peptides that are unstructured in isolation, but adopt stable secondary and tertiary structure upon forming a disulfide bond between them. In order to analyze the structure of proteins, it is necessary to break disulfide bonds. This reduction of disulfide bonds can be accomplished by treatment with 2-mercaptoethanol, dithiothreitol, or urea.

### 5.1.4. PTMs changing the chemical nature of amino acids

Citrullination or deimination - the conversion of arginine to citrulline, and deamidation - the conversion of glutamine to glutamic acid or asparagine to aspartic acid.

## 5.2. Protein modifications in Alzheimer's disease

Microtubule-associated protein tau undergoes several post-translational modifications and aggregates into paired helical filaments (PHFs) in AD. Tau in PHFs is different from normal tau. These modifications of tau include hyperphosphorylation, glycosylation, ubiquitination, glycation, polyamination, nitration, and proteolysis. Hyperphosphorylation and glycosylation are crucial to the molecular pathogenesis of neurofibrillary degeneration of AD. The others appear to represent failed mechanisms for neurons to remove damaged, misfolded, and aggregated proteins.

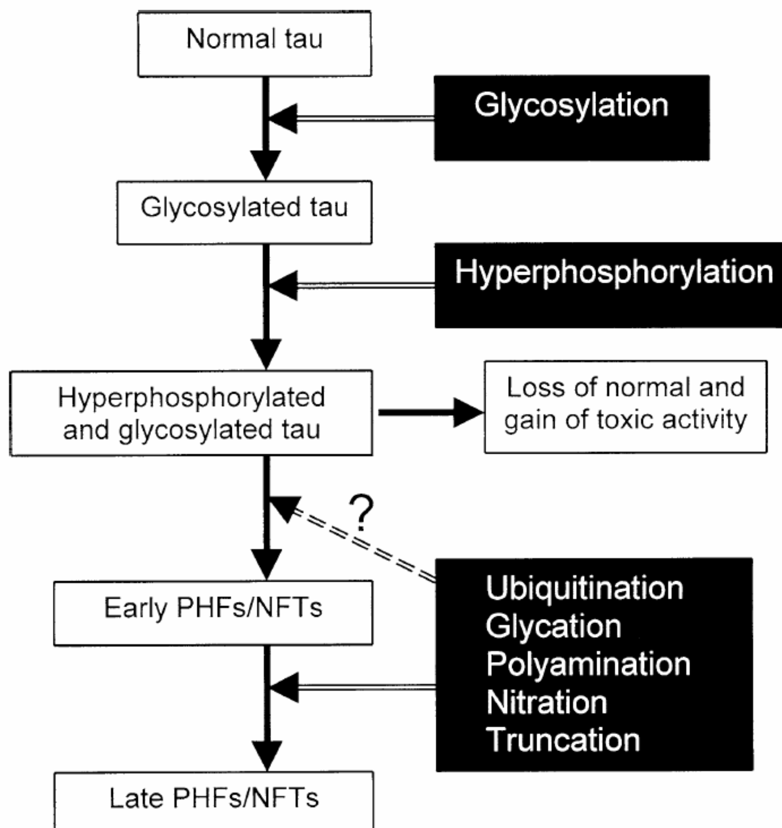


**Figure 24i.** *Post-translational modifications of tau in AD. Tau undergoes abnormal glycosylation and hyperphosphorylation at early stages of tau pathology. The hyperphosphorylated tau on one hand looses its normal activity to stimulate microtubule assembly and becomes toxic to the cell. On the other hand, hyperphosphorylation promotes tau's self-assembly into PHFs=NFTs. PHFtau is further modified by ubiquitination, glycation, polyamination, nitration, and proteolytic truncation. Some of these modifications might occur before or during self-assembly of tau into PHFs=NFTs* [C.-X. Gong et al., J Neural Transm (2005) 112: 813–838]

### 5.2.1. Protein oxidation

The pathological cascade of both familial and sporadic AD appears to involve protein oxidations. However, protein oxidations are non random and the susceptibility of proteins to oxidative damage is highly dependent on the nature of the protein [Gracy 1999; Gracy 2001]. This specificity is dependent on factors such as unique sequence motifs, exposure of labile groups to the surface environment, and the presence of oxidizing ligands.

For example, in the Aβ peptide of 40 residues Met35 is easily oxidized, but peptides with the same composition but different sequences are not oxidized.

Different studies have demonstrated that increased levels of protein oxidation [Keller 2005; Sayre 2001], lipid oxidation [Picklo 2002], and nucleic acid oxidation [Ding 2005] are all evident in AD. Recent studies have also found that increases in each of these forms of oxidative damage are present in MCI subjects [Markesbery 2005; Wang 2005].

Significant elevations of total oxidized proteins were observed in both the AD subjects and AD relatives [Conrad 2000]. As well levels of NKs are also increased in brain from patients with AD [Bernoud-Hubac 2001, 2002].

Montine have shown the implication of oxidation products of two fatty acids, arachidonic acid (ARA) and docosahexaenoic acid (DHA), in the pathogenesis of AD [Thomas 2005].

Analices of DNP-reactive carbonyls showed increased oxidative damage in AD simples in several bands around 40 to 65 kDa. AD samples exhibited increased glycoxidative (6D12 antibody) damage, and the targets of these

modifications showed diverse molecular masses, from 30 to 50 kDa, whereas staining with a polyclonal anti-AGE did not reveal any evident difference. Gas chromatography coupled with mass spectrometry (GC/MS) quantification of protein oxidation revealed that the more abundant products were those derived from metal-catalyzed oxidation, AASA and GSA (almost 95% of measured markers). GSA stood as the more frequent modification, with levels being 40-fold higher than of those of AASA. The mean concentrations of both GSA and AASA were significantly higher in brain samples from AD patients than in control, age-matched individuals. The mean concentrations of CEL and CML and MDAL, a lipoxidation product, were also significantly increased in brain proteins from AD patients than in control individuals.

The analyses of fatty acid revealed significant differences associated with AD in brain samples, both in individual fatty acids and in global indexes. AD samples showed significant decreases for monounsaturated fatty acids, with subsequent increases in the content of PUFA of the *n*-3 family.The highly peroxidizable docosahexaenoic acid (omega-3) showed a significant increase in its content in AD samples [Pamplona 2005].

### 5.2.2. Acetylation

There is evidence of DNA oxidation and altered DNA repair mechanisms in AD brain. Histones, which interact with DNA, conceivably provide a protective shield for DNA against oxidative stress. Because of their abundant lysine residues, histones are a target for 4-hydroxynonenal (HNE) modification. Electron paramagnetic resonance in conjunction with a proteinspecific spin label measurement disclosed that HNE binds to histones and this binding affects the conformation of the histone. The covalent modification to the histone by HNE affects the ability of the histone to bind DNA. But acetylated histones appeared to be more susceptible to HNE modifications than control histones. So, acetylated DNA-histone interactions subsequent to oxidative modification of histones by the lipid peroxidation product HNE, may contribute to the vulnerability of DNA to oxidation in AD brain [Drake 2004].

Exploration of the effect of epsilon-amino acetylation of the Lys28 residue on the magnitude of the unzipping force of Aβ 25-35 fibrils in AD, by atomic

force microscopy, revealed that although the gross structure of the Lys28-acetylated (Aβ 25-35_K28Ac) and wild-type Aβ25-35 (Aβ 25-35wt) fibrils were similar, the fundamental unzipping forces were significantly lower for Aβ 25-35_K28Ac (20 +/- 4 pN SD) than for Aβ 25-35wt (42 +/- 9 pN SD) [Karsai 2005].

### 5.2.3. Methylation

It was found that many neurodegenerative tauopathies include AD as a typical tauopathy, share the effect of decreased methylation that could lead to tau hyperphosphorylation. But hypomethylation and hyperphosphorylation may not necessarily be directly causally related [McCaddon 2007].

It was suspected that amyloid-beta overproduction and accumulation, which may be the cause of the disease, could be due to the loss of epigenetic control in the expression of the genes involved in Aβ protein precursor processing. In cell culture it has been shown, that two of the genes responsible for Aβ production are controlled by the methylation of their promoters. A further support to this thesis was the observation that in elderly DNA methylations are consistently lower than in young and mid aged people [Scarpa 2006].

### 5.2.4. Phosphorylation

A normal level of phosphorylation is required for tau's optimal function, whereas the hyperphosphorylated tau loses its biological activity [Gong 2000]. Phosphorylation, cleavage and conformational changes in tau protein all play pivotal roles during AD. Experiments in double transgenic mice model strongly support the notion that the conformational changes and truncation of tau occur after the phosphorylation of tau [Mondragón-Rodríguez 2008].

There are 80 putative Ser or Thr phosphorylation sites on the longest brain Tau isoform. Many of these phosphorylation sites are also phosphorylated in normal human brain at small extents, but they are rapidly dephosphorylated during postmortem delay [Matsuo 1994; Garver 1994]. However, the phosphate groups at these sites are not readily dephosphorylated during postmortem period of AD brain, probably because of the deficient protein phosphatase activities [Vogelsberg-Ragaglia 2001; Loring 2001; Sontag 2004] and aggregation of tau. Some of the phosphorylation sites seen in PHF-tau are not

phosphorylated at all in normal brain. The different states of Tau phosphorylation result from the activity of specific kinases and phosphatases towards these sites. Most of the kinases involved in Tau phosphorylation are part of the proline-directed protein kinases (PDPK), which include mitogen activated protein kinase (MAP) [Goedert 2005; Sergeant 1997; Reynolds 1997; Vulliet 1992], Tau-tubulin kinase [Takahashi 1995 a,b] and cyclin-dependent kinases including cdc2 and cdk5 [Baumann 1993; Liu 1995]. Stress-activated protein kinases (SAP kinases) have been recently involved in Tau phosphorylation [Jenkins 2000; Buee-Scherrer 2002]. Non Ser/Thr-Pro sites can be phosphorylated by many other protein kinases, including microtubule affinity regulating kinase (MARK) [Drewes 1997], Ca2+/ calmodulin-dependent protein kinase II (CaMPK II) [Baudier 1988; Steiner 1990], cyclic-AMP-dependent kinase (PKA) [Jicha 1999; Litersky 1992] and casein kinase I and II [Pierre 1983; Greenwood 1994]. Glycogen synthase kinase 3 (GSK3) is a Tau kinase able to phosphorylate both non-Ser/Thr-Pro sites and Ser/Thr- Pro sites.

Tau proteins from Alzheimer patient's brain tissue or neuroblastoma cells are rapidly dephosphorylated by endogenous phosphatases [Buee-Scherrer 1996; Sergeant 1995; Matsuo 1994]. Ser/Thr phosphatase proteins 1, 2A, 2B (calcineurin) and 2C are present in the brain [Cohen 1989] and are developmentally regulated [Dudek 1995]. Purified phosphatase proteins 1, 2A and 2B can dephosphorylate Tau proteins in vitro [Goedert 1995]. Like kinases, phosphatases have many direct or indirect physiological effects, and counterbalance the action of kinases. They are associated directly or indirectly with microtubules [Sontag 1999].

How tau becomes abnormally hyperphosphorylated in AD is not well understood. Theoretically, the abnormal hyperphosphorylation of tau could be the result of an upregulation of tau kinase(s) or a downregulation of tau phosphatase(s). Hence, several studies have focused on whether the activities and expression of these enzymes are altered in AD brain. Among protein kinases, cdk5 was reported to be upregulated in AD brain by one laboratory [Patrick 1999], but this result was not reproduced by others [Yoo and Lubec 2001; Taniguchi 2001]. On the other hand, both the activity and the expression of PP2A have been found to be reduced in selected areas of AD brain [Gong 1995; Vogelsberg-Ragaglia 2001; Loring 2001; Sontag 2004]. Consistent with

this finding, several other neuronal proteins such as neurofilaments, b-tubulin, and b-catenin are also hyperphosphorylated in AD brain [Vijayan 2001; Wang 2001]. It seems that downregulation of PP2A might underlie the abnormal hyperphosphorylation of tau in AD brain.

The phosphorylation level of tau isolated from autopsied AD brains is 3- to 4-fold higher than that from normal human brains [Ksiezak-Reding 1992; Köpke 1993; Kenessey and Yen 1993]. All six isoforms of tau are aggregated into PHFs in abnormally hyperphosphorylated forms in AD brain [Grundke-Iqbal 1986a, b; Goedert 1989, 1992].

The relationship between tau hyperphosphorylation and amyloid pathology is poorly understood. Fibrillar Aβ has been shown to induce phosphorylation of tau followed by progressive degeneration of neuronal processes [Buseiglio 1995] and to increase the numbers of NFTs in the brain of tauP301L-transgenic mice [Götz 2001]. Presenilin-1 may connect between tau pathology and amyloid pathology, because it regulates both tau phosphorylation and Aβ production [Baki 2004].

### 5.2.5. Glycosylation

Similar to phosphorylation O-GlcNAcylation is an abundant, dynamic, and inducible post-translational modification. The impairment of brain glucose uptake/metabolism is a known metabolic defect in Alzheimer's neurons, and several GlcNAcylated proteins are involved in it. A new linkage between Aβ and tau was recently proposed by which Aβ can promote tau hyperphosphorylation by impairing intraneuronal glucose metabolism and consequently decreasing O-GlcNAcylation of tau [Liu 2004]. AD-tau, which is active in stimulating microtubule assembly and not yet hyperphosphorylated, is also aberrantly glycosylated, whereas tau purified from normal control brain is not [Liu 2000, 2002a]. Hence, GlcNAcylation/phosphorylation may be important for AD disease pathology [Dias 2007]. When O-GlcNAc glycosylation of proteins from autopsied human brains with confirmed AD and non-AD age-matched controls was examined, the number of O-GlcNAc-containing proteins and the overall O-GlcNAc level do not appear to be different between AD and control brain tissues. The only observed significant change is a marked reduction of O-

GlcNAcylated clathrin assembly protein-3 (AP-3) in AD. The reduction was more evident in brain neocortical regions, and there appeared to be a negative correlation between O-glycosylated AP-3 and the density of neurofibrillary tangles. That suggests a possible association between the O-glycosylated AP-3 and AD pathology [Yao 1998].

When AD-tau was used as a substrate for in vitro phosphorylation with GSK- 3b, cdk5, and PKA, the deglycosylated AD-tau was phosphorylated at a lower rate and to a smaller extent than the control-treated AD-tau [Liu 2002c]. The effect of glycosylation on subsequent phosphorylation of tau is site-specific, and the influence at each phosphorylation site is different. When AD P-tau - abnormally hyperphosphorylated and glycosylated - was used as a substrate for PP2A and PP5 after enzymatic deglycosylation, deglycosylated ADP-tau was dephosphorylated, at a faster rate and to a greater extent, at some of the studied phosphorylation sites [Liu 2002b]. Together, these observations suggest that aberrant glycosylation facilitates hyperphosphorylation of tau both by promoting phosphorylation and by inhibiting dephosphorylation of tau at the substrate level.

Analysis of the glycans released from purified AD P-tau and PHF-tau revealed that galactose, mannose, N acetylglucosamine, and sialic acid are the major monosaccharides that modify tau [Liu 2002a].

By studies from cultured cells to animal model, was demonstrated that O GlcNAcylation negatively regulates tau phosphorylation and that the impaired brain glucose uptake and metabolism of AD brain may contribute to neurodegeneration via down regulation of tau O-GlcNAcylation and consequent hyperphosphorylation of tau [Liu 2004].

### 5.2.6. Lipidation

The production of Aβ by the sequential cleavage of APP by BACE [beta-site APP cleaving enzyme (β-Secretase)] and γ-Secretase and its aggregation into senile plaques is the basis of the amyloid hypothesis of AD [Haas 1993]. Recent research upon the protein lipid modification of BACE has demonstrated that lipidation influences the production of Aβ protein by promoting the dimerization of BACE [Parsons 2005]. Most of the interest in BACE cleavage of

APP is centred upon its regulation by cholesterol. Cholesterol influences the glycosylation of BACE without influencing its activity or APP cleavage site selectivity, which in turn influences its recruitment into cholesterol-rich rafts [Sidera 2004]. This is supported by studies, which show that patients on long-term statin treatment are 7-fold less likely to develop AD [Wolozin 2000], thus making BACE–lipid interactions attractive therapeutic targets.

### 5.2.7. Ubiquitination

Alzheimer's disease belongs to a growing family of pathologies related to protein accumulation such as HD, ALS and type-1 spinocerebellar ataxia (SCA1) [Bruijn 1998; Cummings 1998; Sisodia, 1998]. Neurofibrillary tangles (NFT), which major component is tau protein, are one of the pathologic hallmarks of AD. During the course of the disease such proteins aggregate into bulky NFT that get ubiquitinated [Arnaud 2006].

It is well known that PHF-tau, but not normal tau or AD P-tau, is modified with polyubiquitins [Bancher 1991; Iqbal 1991; Morishima 1994]. Ubiquitination normally labels misfolded or damaged proteins for ATP-dependent degradation through the ubiquitin-proteosome system. Although PHF-tau is highly ubiquitinated, it is apparently not degraded, but is deposited as NFTs in AD brain. It is not understood why ubiquitinated PHF-tau fails to be degraded and cleared from AD brain. A possible cause is a defective proteasome system in AD brain [Keller 2000; Lam 2000; Lopes Salon 2000]. PHF-tau has also been shown to be monoubiquitinated [Morishima-Kawashima 1993], that appears to modulate the activity and the location of proteins rather than to target protein for degradation [Hicke, 2001]. Therefore, the monoubiquitination of PHF-tau may play a regulatory role.

The accumulation of oxidized proteins in AD may involve dysfunctions in the ubiquitin/proteasome system. For example, 4-hydroxynonenal oxidation of Aβ generated a selective inhibitor of the proteasome [Shringarpure 2000]. Animal models of sporadic AD and cultured neuron models support the hypothesis that the ubiquitin/proteasome system is defective in AD and results in the accumulation of abnormal ubiquitin-protein conjugates [Mastiah 2000; Feany 2000; Checler 2000; Mah 2000; Favit 2000]. In addition to the oxidized protein substrates, abnormal ubiquitin or defective proteasomes could result in

the accumulation of proteins [Van Leeuwen 2000; 2000]. Data also support the possibility of defects in the proteasome including ubiquitin ligase (E3) and the deubiquitin step catalyzed by ubiquitin carboxy- terminal hydrolase [McNaught 2001; Lopez-Salon 2000]. Thus, the expression of Ub+1 or its conjugates with oxidized proteins could result in the inhibition of the proteasome system leading to the accumulation of these neurotoxic oxidized protein products.

### 5.2.8. Proteolytic Cleavage

APP plays a central role in the development of the AD, through the generation of a peptide called Aβ by proteolysis of the precursor protein. In addition to the well known changes in phosphorylation state, tau also undergoes multiple truncation and conformational changes that likely occur in an orderly pattern [Carmel 1996; Garcia-Sierra 2001, 2003; Ghoshal 2001; Gamblin 2003a,b; Binder 2005; Guillozet-Bongaarts A2005; Luna-Munoz 2005].

Although the mechanisms by which amyloid deposition promotes pathological tau filament assembly are poorly understood. Gambling at al. reported that tau is proteolyzed by multiple caspases (caspases-1, -3, -6, -7, and -8) at a highly conserved aspartate residue (Asp421) in its C terminus in vitro and in neurons treated with Aβ (1–42) peptide [Troy 2000]. Tau is rapidly cleaved at Asp421 in neurons treated with Aβ, and its proteolysis appears to precede the nuclear events of apoptosis. Also they demonstrated that caspase cleavage of tau generates a truncated protein that lacks its C-terminal 20 amino acids and assembles more rapidly and more extensively into tau filaments in vitro than wild-type tau [Gamblin 2003].

Aβ generation involves the combined action of beta- and gamma-Secretase. Cleavage within the Aβ domain by α-Secretase prevents Aβ generation. In some very rare cases of familial AD (FAD), mutations have been identified within the βAPP gene. These mutations are located close to or at the cleavage sites of the secretases and pathologically effect βAPP processing by increasing Aβ production, specifically its highly amyloidogenic 42 amino acid variant (Aβ42) [Steiner 1999].

AD involves alterations of the microtubule-associated protein tau. The truncation state of tau influences many of its pathologic characteristics, including its ability to assume AD-related conformations and to assemble into

filaments. Cleavage also appears to be an important marker in AD progression. Although C-terminal truncation of tau at D421 has recently been attributed to the apoptotic enzyme caspase-3, N-terminal processing of the protein remains mostly uncharacterized. It was found that later in the course of tangle evolution the extreme N-terminus of tau was lost, correlating temporally with the appearance of a C-terminal caspase-truncated epitope lacking residues 422-441. Mass spectrometry analysis confirmed that the in vitro caspase-6 truncation site is D13, a semicanonical and hitherto undescribed caspase cleavage site in tau, suggesting a role for caspase-6 and N-terminal truncation of tau during neurofibrillary tangle evolution and the progression of AD [Horowitz 2004].

### 5.2.9. Glycation

PHF-tau is also modified by glycation [Münch 2002], which refers non-enzymatic linkage of glucose or other reducing sugars to the amino side chain (such as that of lysine residues) of a polypeptide. Glycation is different from the enzymatic glycosylation by which polysaccharides or monosaccharides attach to asparagine and serine/threonine residues of a protein via N- or O-linkage, respectively. In contrast to abnormal hyperphosphorylation and glycosylation that are early events of tau pathology, glycation, like ubiquitination, is a late event. Glycation normally leads to subsequent oxidation, dehydration, condensation, and finally formation of heterogeneous advanced glycation end products. Glycation-induced cross-linking reactions may be related to the insolubility of PHFs. Glycated tau can also induce neuronal oxidative stress, resulting in cytokine gene expression and the release of Aβ peptide [Yan 1995].

### 5.2.10. Other post-translational modifications in Alzheimer's disease

It has been reported that tissue transglutaminase can incorporate polyamines into tau in vitro. Polyamination of tau does not affect its microtubule binding, but makes tau significantly less susceptible to degradation by the calcium-activated protease calpain [Tucholski 1999]. PHFs isolated from AD brain are immunoreactive to an antibody against tissue transglutaminase,

suggesting that this enzyme may play a role in PHF formation [Norlund 1999]. Tissue transglutaminase also catalyzes the linkage between glutamine residues and primary amines of lysine residues of proteins, leading to an insoluble and protease-resistant high molecular weight complex [Appelt 1997].

Also recently was shown that Tau in AD brain was nitrated [Horiguchi 2003]. A monoclonal antibody against nitrated tau and a-synuclein stains tau isolated from brains of AD patients [Horiguchi 2003]. Further studies are needed to elucidate whether nitrative injury is linked to the formation of NFTs.

## 6. The identity of Program Cell Death

### 6.1. Program Cell Death – overview

The occurrence of cell death during normal development of multicellular organisms has been observed and commented in the nineteenth century [Clarke 1996]. The first unequivocal description of developmental neuronal cell death was made at the beginning of the twentieth century by Collin, who observed cell death of motor neurons in the spinal cord and death of sensory neurons in the spinal ganglia of chick embryos [Collin 1906]. However, these early studies of neuronal cell death did not receive much attention in the scientific community. Two decades later, the studies of Ernst and Glücksmann described naturally occurring cell death in the neuroepithelium and recognized that cell death serves phylogenetic, morphogenetic, and histogenetic purposes [Glücksmann 1951]. Nevertheless, the question why so much neuronal death occurs normally during development remained. The systematic studies of cell death during chick development by Hamburger and Levi-Montalcini (1949) in the 1940s led to the general recognition that the amount of cell death during neuronal development is linked to the size of the neuronal target population. These observations laid the foundation for the concept of the neurotrophic hypothesis.

In 1964, Richard Lockshin and his colleagues, through their observations about insect embryogenesis, introduced the term of *"programmed cell death"* to describe the apparently predetermined pattern in metamorphosing insect muscle cells that undergo a sequence of controlled steps towards their own destruction [Lockshin 1964, 2001]. In 1966 it was shown that, at least in some

cases, this is a protein synthesis dependent process, thus indicating an active, regulated suicide program. The term apoptosis was introduced in 1972 by John Kerr, Wyllie and Currie [Kerr 1972] who suggested that active and precisely controlled cell death serves a homeostatic function in regulating the size of cell populations under both normal and pathophysiologic conditions. This inherently programmed form of cell death was termed apoptosis.

Kerr et al. (1972) were the first to recognize that apoptosis is a highly conserved cellular process in eukaryotes that consist of a stereotyped sequence of biochemical and morphological changes. Characteristic hallmarks of programmed cell death are shrinkage and condensation of the cell body and fragmentation of the nucleus, together with internucleosomal degradation of the DNA [Compton 1992; Kerr 1972]. Little damage occurs to the cell organelles in the initial stages of apoptosis. This stands in contrast to necrotic cell death, in which the dying cell and its contents swell and subsequently lyse, thereby provoking an inflammatory response [Farber 1994]. Te absence of cell lysis in apoptosis allows the affected cell to die without adversely affecting its neighbours. Indeed, characteristic final step in apoptosis is the fragmentation of the cell into apoptotic bodies, which are subsequently phagocytosed by surrounding cells [Kerr 1972].

In 1986, Robert Horvitz established the molecular basis of the apoptosis phenomenon. In 2002, Sydney Brenner, John Sulston and Robert Horvitz awarded the Nobel Prize in Physiology or Medicine for their studies using the nematode *C. elegans* as model organism to investigate genetic regulation of organ development and programmed cell death. Based on their work a number of genes involved in programmed cell death were described and their precise function was characterized [Ellis 1986]. During evolution the apoptotic machinery is highly conserved among species and human homologues of the *C. elegans* genes have been discovered and cloned. The identification of the four *C. elegans* cell death genes *ced-3*, *ced-4*, *ced-9*, and *egl-1* laid the foundation for the current understanding of the molecular mechanisms involved in cell death in general and in the nervous system in particular [Metzstein 1998].

## 6.2. Types of PCD

Cell deaths fall into several categories, the boundaries of which are not always distinct and there is some overlap between these groups. Deaths are controlled (physiological) or not. The controlled deaths frequently display substantial caspase-independent autophagy or they are predominantly apoptotic. Most apoptotic deaths are caspase-dependent, but there are claims of apoptotic morphology in situations in which caspase activity is equivocal. Caspase activation can occur by means of ligation of a membrane-bound receptor or by means of metabolic changes resulting in depolarization of mitochondria and release of cytochrome *c* and APAF-1. Necrosis is today the catch-all term for any deaths that do not fit in the other described categories. Necrosis has been defined as a type of cell death that lacks the features of apoptosis and autophagy, and is usually considered to be uncontrolled. Typically, cells entering necrosis lose control of their ionic balance, imbibe water and lyse.

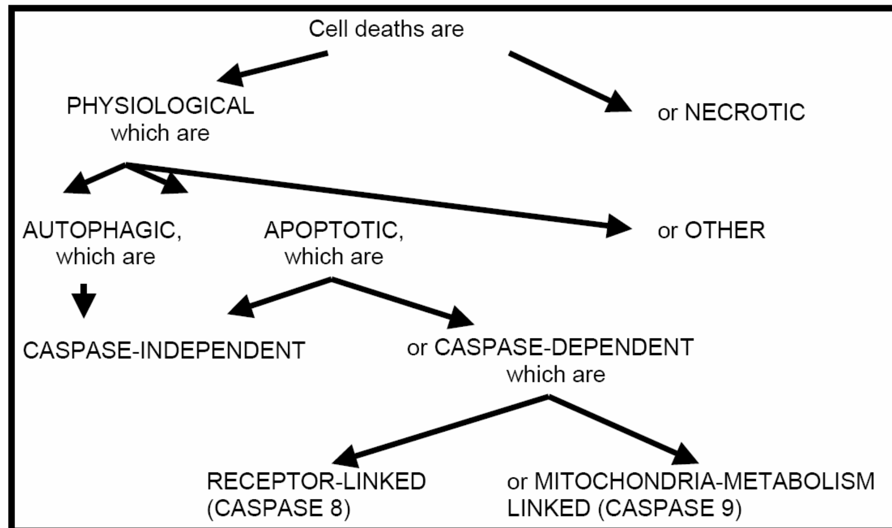



**Figure 25i.** *Schematic illustration of links between different types of cell death* [R.A. Lockshin & Z. Zakeri, The International Journal of Biochemistry & Cell Biology 36 (2004) 2405–2419]

Classical developmental studies support the view that at least three different types of PCD can be distinguished [Lockshin 2004]: apoptosis, autophagy and necrosis (figure 25i).

- Type I also known as nuclear or apoptotic is a characterized by chromatin condensation, followed by nuclear fragmentation and formation of apoptotic bodies.
- Type II also known as autophagic is characterized by formation of vacuoles enclosing and destroying cellular components.
- Type III is so called cytoplasmic or necrosis-like PCD [Clarke 1990; Cunningham 1982; Schweichel 1973; Oppenheim 1985], is a form of traumatic cell death that results from acute cellular injury.

However, features characteristic of both necrotic and apoptotic cell death can occur in the same tissue and even in the same cell simultaneously [Lemasters 1999]. The term "*Programmed cell death*" (Table 9) was from its inception an operational definition, referring to the fact that one could document, in cells that were doomed, a series of changes consistent with the impending failure but at a period at which one could experimentally prevent the death. The predictability of the death arose from examples in development, such as embryonic chick wings and the death of intersegmental muscles in moths [Haas 1995].

Definitions

| Term | Definition |
|---|---|
| Programmed cell death | Developmental<br>A sequence of (potentially interruptible) events that lead to the death of the cell<br>Now recognized to require the activity of specific genes (but often activation of pre-existing proteins, not necessarily transcription at time of death) |
| Necrosis | Apparently uncontrolled cell death<br>Loss of ATP or membrane pumps<br>Most commonly osmotic swelling of cell membrane and organelles, with extraction of contents and precipitation of proteins<br>Inflammatory response |
| Apoptosis | A specific morphology, cell shrinkage and blebbing; organelles (other than ER) do not swell; nucleus fragments; chromatin marginates; no inflammation<br>Degradation of DNA by 3′ cleavage to nucleosome-sized fragments<br>Exteriorization of phosphatidylserine<br>Activation of caspases such as caspase 3 |
| Lysosomal/Type II/autophagic cell death | Death characterized by formation of many large autophagic vacuoles<br>Caspase activation very late if at all<br>Primary proteases are cathepsins or proteasomal proteins<br>DNA fragmentation very late if at all<br>Exteriorization of phosphatidylserine<br>No inflammation |

**Table 9.** *Definition of the different types of cell death*

### 6.2.1. Type I: Apoptosis

Apoptosis, a term derived from the Greek words *apó* (from) and *ptosis* (fall), referring to the naturally occurring seasonal loss of leaves is the nuclear or type I PCD, the best characterized one. Apoptosis is considered a process of cell *suicide* that is regulated by complex molecular signalling systems that trigger orderly, energy-dependent enzymatic breakdown of DNA, lipids and other macromolecules. A characteristic feature of apoptosis is that it is an active ATP-dependent biochemical process. Apoptotic cell death is regulated through transcriptional as well as post-translational mechanisms.

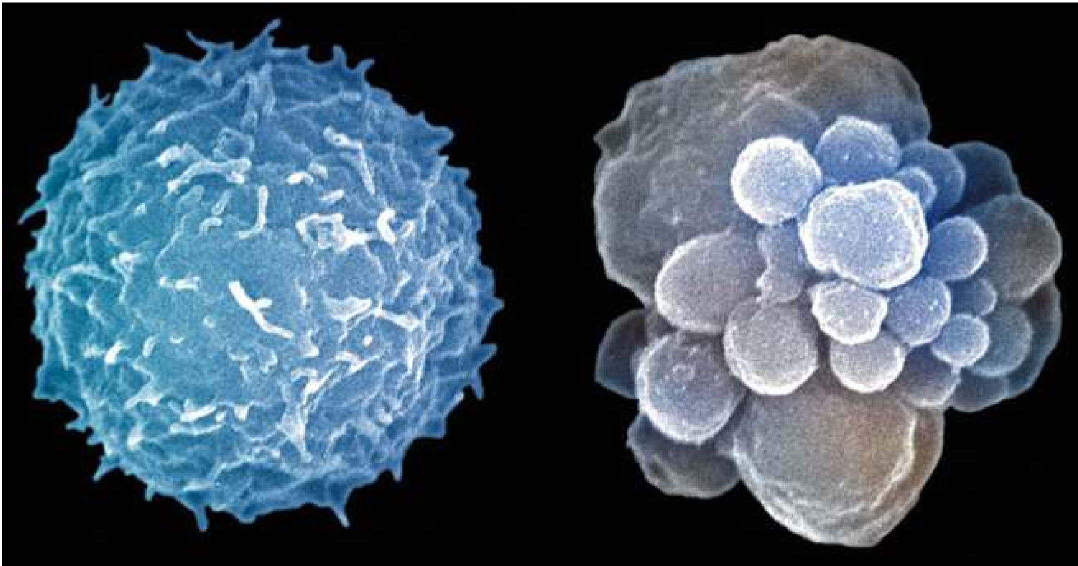


**Figure 26i.** *Characteristical morphological changes in lymphocyte culture observed during apoptotic process* [fig.cox.miami.edu]

Apoptosis is an important mechanism in embryonic development and organogenesis. In the CNS apoptotic cell death plays a key role in brain development and in neurodegenerative disease, and other types of CNS damage. Furthermore, apoptotic cell death can be the natural endpoint of a life span of a cell and is thereby involved in normal cell turnover of adult tissues. Apoptosis is used as a defense strategy to remove infected (by viruses, bacteria), mutated (tumor) or damaged cells. Thus, evading of apoptosis is one important of several steps in the transformation of a normal cell into a cancer

cell [Hanahan 2000]. Apoptosis can be triggered by cell surface death receptors, radiation and cytostatic drugs, which may target DNA, mitochondria or cytoplasmatic proteins. Furthermore, many environmental toxins and cellular stresses can also trigger apoptotic cell death (e.g., oxidative stress, endoplasmic reticulum stress, alcohol). Throughout this process the cell morphology suffers several changes: typical changes start with cells round up, the chromatin is condensed, the nucleolus is disintegrated and the nucleus weight is reduced. The cell undergoes swelling and grows denser, the cytoplasmatic organelles are condensed and the endoplasmatic reticulum is dilated, but the mitochondria do not change morphologically [Kanduc 2002]. The nucleus is fragmented and packaged into vesicles, called apoptotic bodies (figure 26i), which may be phagocytosed and reused. Phosphatidylserine which usually is restricted to inner layer of plasma membrane became randomly distributed [Fadok 1992]. These morphological and histochemical changes are largely the result of activation of set of cell-suicide cysteine proteases, the Caspases [Thornberry 1998; Yuan 1993].

### 6.2.1.1. The family of Caspases

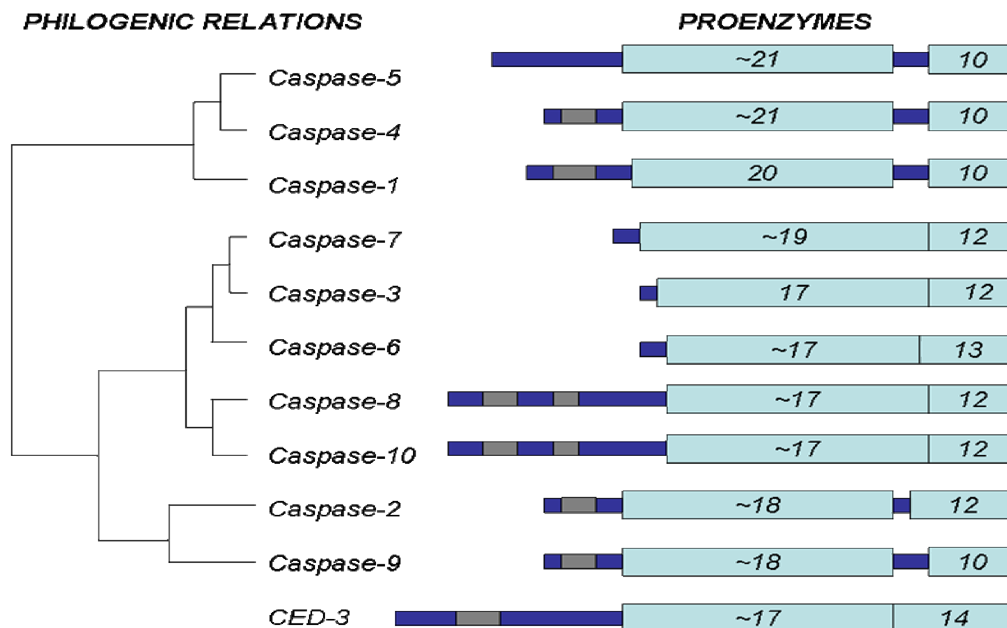


**Figure 27i.** *Phylogenic relations and outline of proenzme structure of the Caspase family* [modified from Shi 2006]

### 6.2.1.1.1. Caspase-dependent cell death

Caspases are cysteine-rich aspartic acid-specific proteases, which become specifically activated in most forms of cell death [Fadeel 2000]. In the cell, caspases are localized in the nucleus, cytoplasm, endoplasmatic reticulum, and mitochondrial intermembrane space, and they can be translocated to the plasma membrane [Hengartner 2000]. Many of these proteases have been implicated in neuronal apoptosis. The first evidence that caspase activity is required for neuronal apoptosis came from experiments using pharmacological inhibitors of caspases.

Currently, fourteen different mammalian homologues of CED-3 have been cloned, characterized, and named caspases from 1 to 14, eleven of which are present in humans. Although overexpression of all 14 caspases resulted in cell death, not all of them are involved in apoptosis. The known caspases are separated into several groups based on their function in the cell (Table 10), divided into two blocks:

- one include inflammatory caspases being primarily involved in procytokine activation (caspases-1, -4, -5, -11, , -13, and -14) [Earnshaw 1999], that have little or even unknown roles in the apoptotic pathways
- the other include caspases involved in apoptosis, both initiator (caspases -2, -8, -9, -10 and -12) and effector caspases (caspases-2, -3, -6, -7) that degrade vital cellular proteins [Budihardjo 1999; Cryns 1998]. After a cell receives an apoptotic signal, sequential activation of these caspases takes place; i.e. an upstream initiator caspase processes and activates downstream effector caspase(s). By this mechanism an apoptotic signal gets amplified rapidly, as proteolytic activation of preexisting molecules occurs instead of a much more slowly de novo-synthesis.

| Group | Caspases |
|---|---|
| Group I: **Inflammatory caspases** | Caspase-1<br>Caspase-4<br>Caspase-5<br>Caspase-11<br>Caspase-12<br>Caspase-13<br>Caspase-14 |
| Group II: **Apoptosis initiator caspases** | Caspase-2<br>Caspase-6<br>Caspase-8<br>Caspase-9<br>Caspase-10<br>Caspase-12 |
| Group III: **Apoptosis effector caspases** | Caspase-2<br>Caspase-3<br>Caspase-6<br>Caspase-7 |

**Table 10.** *The caspase family. Group I: inflammatory caspases; Group II: apoptosis initiator caspases; Group III: apoptosis effector caspases.*

The importance of Caspases in neuronal apoptosis has been demonstrated *in vivo* in various mouse models. Mice deficient in one of Caspase -1, -2, -3, -9, -11, or -12 show a neuronal phenotype [Yuan 2000]. Caspase-3 and -9 deficient mice show the most severe defects in neuronal development. In these mice, ectopic cell masses appear in the brain due to the absence of apoptosis [Hakem 1998; Kuida 1996, 1998]. In addition to developmentally regulated apoptosis, Caspases may play pivotal roles in a number of neurological disorders including stroke, amyotrophic lateral sclerosis and Huntington's disease [Friedlander 2003; Troy 2002; Yuan 2000]. For example, mice transgenic for a dominant negative mutant of Caspase-1 exhibit reduced sensitivity to ischemia as well as to trophic factor deprivation [Friedlander 1997]. Inhibition of Caspases is neuroprotective in a number of animal and human models of neurodegeneration [Friedlander 2003].

## 6.2.1.1.2. Procaspases and their activation

Caspases are present in cells as zymogens and they need to undergo proteolytic cleavage in order to achieve their enzymatic activity. Initiators caspases are capable of autocatalytic activation and generally have a long

prodomain. Downstream effector caspases need initiator caspases for their activation by transpro cessing figure 28i.

Two related motifs in the N-terminal region of initiator caspases have been identified: the death effector domain (DED), and the caspase recruitment domain (CARD). The DED is found in caspase-8 and -10, and CARD is found in caspase-1, -2, -4 and -9.

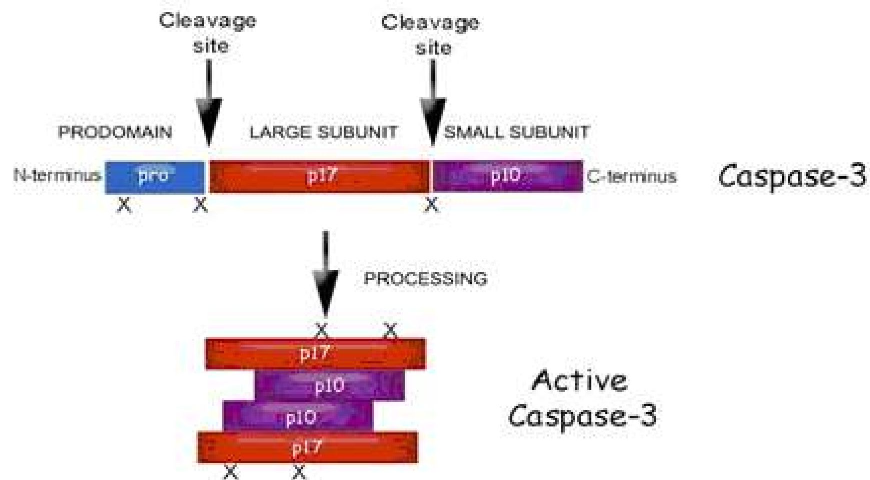



**Figure 28i.** *Caspase 3 activation*. [modified from Strasser, A., L, *Apoptosis signaling.* Annu Rev Biochem, 2000. 69: p. 217-45]

Thus, an active caspase molecule has two sites for substrate binding and cleavage. Initiator caspases are activated by oligomerization and conformational changes but do not require cleavage for activation, whereas executioner caspases require cleavage to the p10/p20 peptide fragments to assemble and form activated enzymes [Salvesen 1999].

Three different mechanisms of caspase activation are described so far [Hengartner 2000]:

1. Induced proximity: Several procaspase-8 zymogens are recruited to the DISC complex upon cell surface death receptor trimerization. In this membrane-bound signaling complex, a high concentration of the procaspase-8 molecules might be sufficient to process and activate each other.
2. Activation mediated by regulatory subunits: Caspase activation can be closely controlled by a regulatory subunit. Unlike other caspases,

proteolytic processing of procaspase-9 has only a minor effect in the enzyme's catalytical activity. The key requirement for caspase-9 activation is its association with the cytosolic protein Apaf-1 in the apoptosome complex.

3. Activation by upstream located caspases: An activated caspase can cleave and activate another caspase in a cascade of reactions.

### 6.2.1.1.3. Caspase cascades

Apoptotic stimuli can be functionally divided into extrinsic and intrinsic factors, which initiate the apoptotic process via interaction with extra- or intracellular targets. In response to different stimulus, two main signaling pathways of apoptosis have been well documented in the mammalian cells: the "extrinsic" or death receptor-initiated pathway and the "intrinsic" or mitochondrial pathway [Budihardjo 1999] (figure 29i). The extrinsic pathway engages the activation of death receptors on the cell surface. The intrinsic (metabolic) pathway involves mainly the triggering, regulation and amplification of apoptotic signals by mitochondria, but also by the endoplasmic reticulum (ER), lysosomes and the nucleus.

- *Extrinsic Pathway or Death receptors pathway:*

  Several extrinsic ligands can activate cell-surface "death receptors" of the tumor necrosis factor (TNF) family, such as apoptosis antigen-1 (APO1/FAS/CD95), TNF receptor member 1A (TNFR1) and the TNF-related apoptosis-inducing ligand receptors (TRAIL) DR4 and DR5 aggregate and form membrane bound signaling complexes [Sartorius 2001]. These complexes recruit the Fas-associated death domain (FADD) adaptor protein, which binds to procaspase-8, resulting in formation of the death inducing signalling complex (DISC) and promotes caspase-8 or caspase-10 activation [Rytomaa 1999; Scaffidi 1997]. Upon association of procaspase-8 to the DISC complex, it is activated and ready to activate downstream caspases [Sohn 2005; Li 2006; Velier 1999; Helfer 2006]. Additionally, caspase-8 can cleave Bid, a pro apoptotic member of the Bcl-2 family, to truncated Bid (tBid). Since Bid is

involved in the regulation of intrinsic mitochondrial pathway of apoptosis, its activation by cleavage provides a link between the extrinsic and intrinsic pathway of apoptosis.

- *Intrinsic Pathway or Mitochondrial pathway:*

  Mitochondria play an important role in the execution process of apoptosis by acting as a reservoir for a multitude of apoptogenic proteins [Kroemer 2007; Love 2003; Green 2004]. The intrinsic pathway involves the regulation of apoptosis via mitochondria and is characterized by the release of mitochondrial intermembrane space proteins including cytochrome *c*, Apoptosis Inducing Factor (AIF), second mitochondrial activator of caspases (SMAC)/ direct IAP Binding protein with low pI (DIABLO), Endonuclease G and Omi/HtrA2 into the cytosol [van Loo 2002]. This cell death pathway is controlled by Bcl-2 family proteins. Released cytochrome *c* together with the cytosolic Apaf-1 forms a complex in the presence of dATP/ATP, which is essential for proteolytic processing of procaspase-9 [Costantini 2002]. Apaf-1 is not only a caspase-9 activator, but it is an essential regulatory subunit of the caspase-9 holoenzyme. Apaf-1 bound together with mitochondrially released Cytochrome C, dATP/ATP, and procaspase-9, is often referred to as the apoptosome [Adams 1999]. The activated Caspase-9 subsequently cleaves and activates procaspase-3 [Susin 2000], resulting in DNA fragmentation and apoptosis [Li 1997; Zuo 1997].

Apaf-1 *knockout* mice die during late embryogenesis and show reduced apoptosis in the brain [Cecconi 1998; Yoshida 1998], thus suggesting that Apaf-1 is involved in the activation of the apoptotic machinery during neuronal development. However, impaired apoptosis in brains of apaf-1−/− mice may result from the absence of Apaf-1 in neural progenitor cells rather than post mitotic neurons [Honarpour 2001a, b]. Apaf-1 may play a role in apoptosis in post mitotic neurons since primary cortical neurons derived from apaf-1−/− mice are resistant to DNA damage. Apaf-1 has been shown to be a target of p53 in neuronal apoptosis [Fortin 2001].

Since caspase-3 is activated in the extrinsic as well as the intrinsic pathways of apoptosis, its activation provides a link between these different mechanisms. Thus, it is assumed that the extrinsic and intrinsic pathway lead to one common execution pathway, which explains that late apoptotic cells display uniform morphology and similar biochemical hallmarks, albeit the fact that apoptosis can be induced by variety of different stimuli.

A third, less characterized pathway originates from the endoplasmic reticulum and also results in the activation of Caspase-9 [Morishima 2002; Rao 2002; Yuan 1999]. Other organelles such as the nucleus and Golgi apparatus have damage sensors that link to apoptotic pathways [Green 2005].

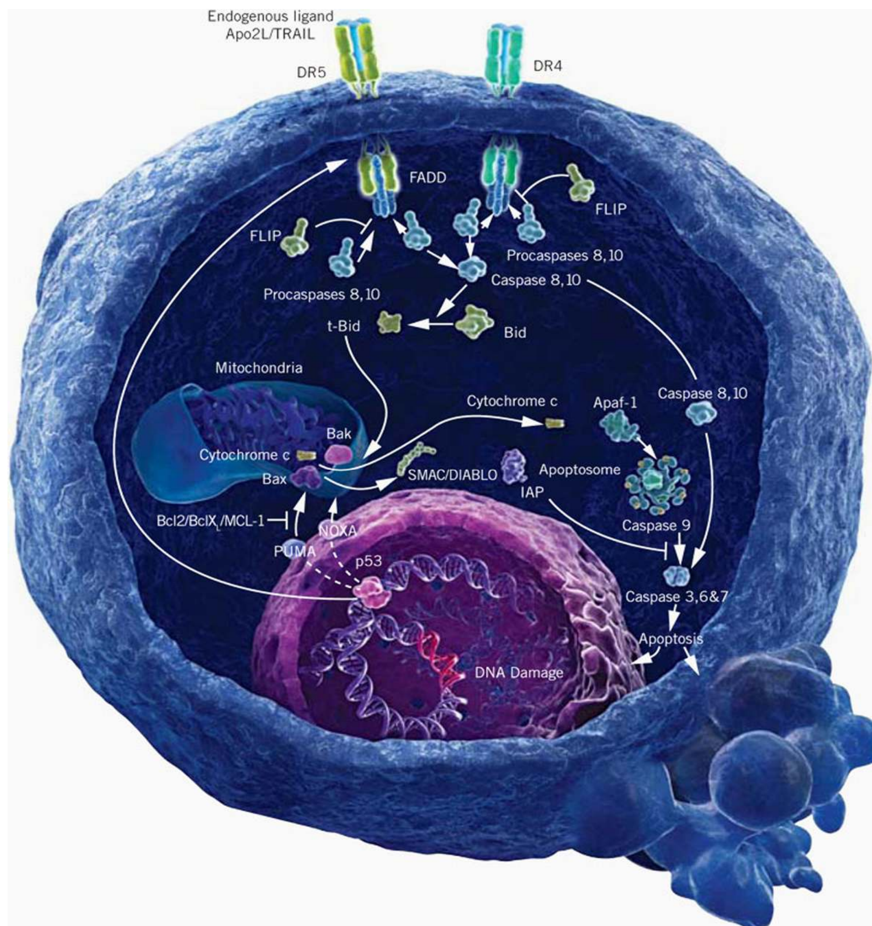



**Figure 29i.** *Two interconnected pathways - intrinsic and extrinsic - both lead to apoptotic process by activation of the Caspases.* [Jin Z. et al., Overview of cell death signaling pathways, Cancer Biol Ther. 2005; 4:139-163]

#### 6.2.1.1.4. The main executor: Caspase-3

Caspase-3 is a principal member of the cysteine protease family that is responsible for the execution of apoptotic cell death. It appears to be the most abundant of the known caspases in the brain, the most studied, and the convergence of all caspase-mediated pathways related to apoptosis. It is activated by both intrinsic and extrinsic cell signalling pathways [Persad 2004]. During normal brain development, when more than half of the neurons in some brain regions are removed through apoptosis, caspase-3 is highly upregulated, playing an essential role. In rats procaspase-3 is highly expressed from E17 (17th embryonic day) to P7 (7th postnatal day), and star to decrease after P14 (14th postnatal day) [Kurosu 2004; Roth 2001]. The importance of caspase-3 activation both in normal CNS development and after damage comes from studies using specific inhibitors or genetically modified animals. As a result, caspase-3 null mice display considerable neuronal expansion and unusually abnormal brain development, resulting in death by the second week of life [Le 2002; Kerr 2004]. In addition, specific inhibitors of this caspase have been shown to be neuroprotective in different neurodegenerative conditions [Han 2002; Joly 2004].

### 6.2.1.2. Balance between proapoptotic and antiapoptotic molecules

Apoptotic factors activated after an insult are usually accompanied by overexpression of molecules aimed to inhibit them: the antiapoptotic proteins. The balance between concentrations of both groups will finally determine if the cell will die or survive. Therefore, final effects of apoptotic caspase cascades cannot be studied independently form antiapoptotic mechanisms and caspase-independent proapoptotic pathways concomitant within the same cell.

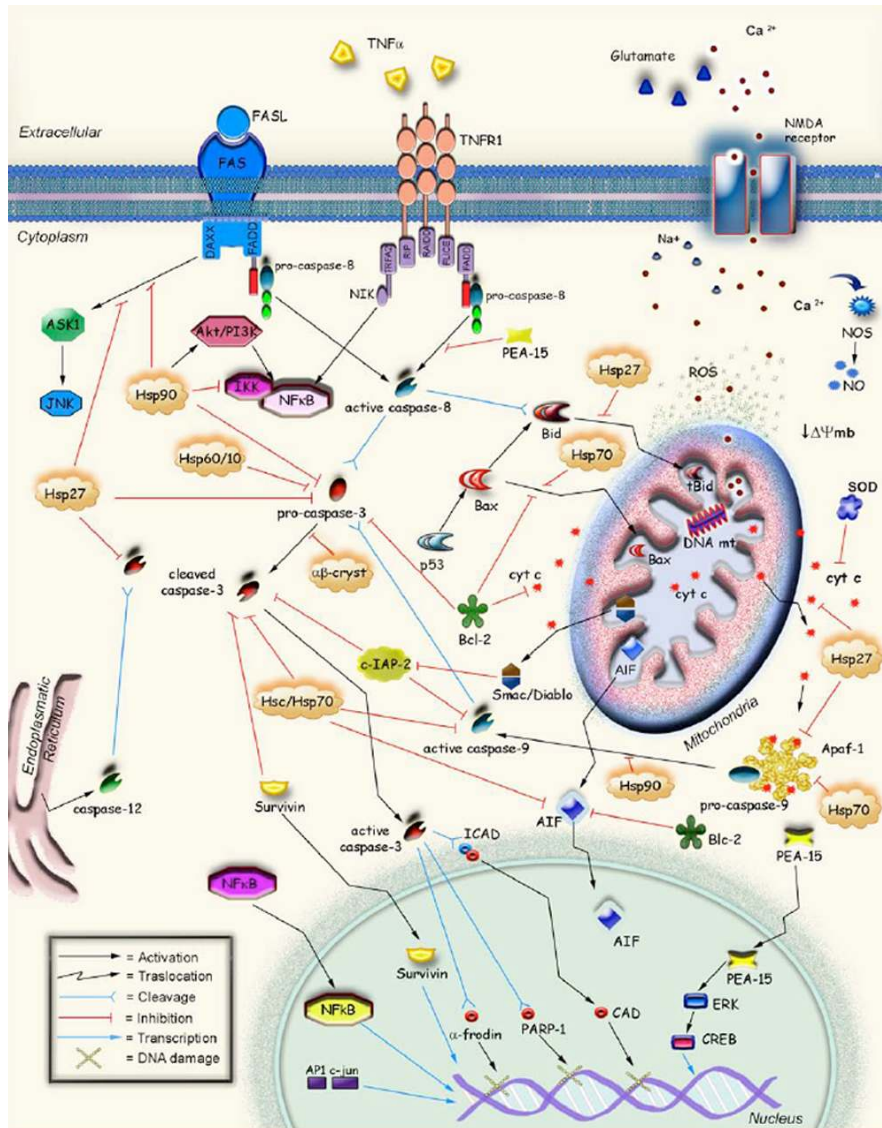



**Figure 30i.** *Proapoptotic and antiapoptotic signalling. Oxidative stress leads to the loss of mitochondrial membrane potential and the release of cytochrome C, which involves the formation of Apaf-1, leading to the activation of the intrinsic pathway via activation caspase-9 and effector caspase-3. In addition, ligand binding to FAS or TNFR1 stimulates the extrinsic pathway in a series of steps leading to the cleavage and activation of caspase-8, which activates effector caspase-3. Caspase-8 can also mediate BH3-interacting domain (Bid) cleavage, resulting in translocation of truncated bid (tBid) to mitochondria providing a link between intrinsic and extrinsic pathways. Key regulating proteins, such as AIF, Bcl-2 family proteins, the inhibitor of apoptosis*

*protein (IAP), and heat shock proteins (HSP) converge upon activation/inhibition of caspases and apoptotic cascades.*

**The family of proapoptotic and antiapoptotic Bcl-2 proteins** located on the surface of mitochondria appears to play a major role in the release of proapoptotic proteins and in the regulation of cell death [Adams 1999; Gross 1999; Strasser 2000].

The BCL-2 family has expanded significantly and now includes both pro- and antiapoptotic proteins, which form a complex network of checks and balances that regulate cell fate. Disrupting the balance imposed by the BCL-2 family can lead to a host of human conditions that are characterized by excessive cellular demise, such as in neurodegenerative disease or relentless cellular survival, such as in cancer (figure 31i) [Waldensky 2006].

The Bcl-2 family consists of several main subgroups, which are categorized according to their anti- or pro- death function, and the presence or absence of conserved structural motifs - the Bcl-2 homology (BH) domains.

The survival proteins such as BCL-2 and BCL-XL share three to four conserved BCL-2 homology (BH1-4) domains, and are thus termed ‘multidomain antiapoptotic’ members. The executioner proteins such as BAX and BAK share three conserved domains (BH1-3) and are known as ‘multidomain proapoptotic proteins. A subgroup of proapoptotic proteins only displays conservation in the third BH domain figure 32i [Waldensky 2006]. These ‘BH3-only’ members function as death sentinels that are situated throughout the cell, poised to transmit signals of cellular injury through multidomain members. A variety of physiological death signals, as well as pathological cellular insults, trigger the genetically programmed pathway of apoptosis [Cory 2002]. Depending upon the nature of apoptotic stimuli and the cellular context, a BH3-only protein’s death signal will either be neutralized by antiapoptotic proteins or delivered, directly or indirectly, to the mitochondrial executioners BAX and BAK. When activated, these proapoptotic multidomain members induce permeabilization of the outer mitochondrial membrane, enabling released mitochondrial factors to activate caspases, which irreversibly

execute the death program. The network of interactions among BCL-2 family members is complex and remains a focus of intensive investigation.

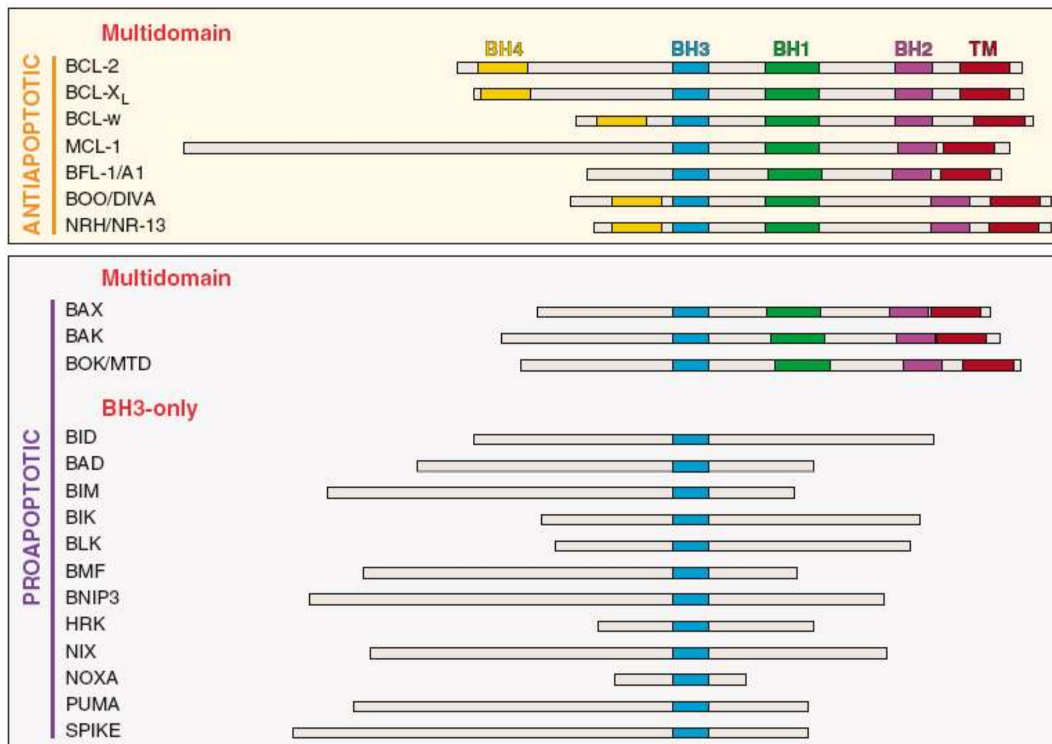



**Figure 31i.** *BCL-2 family proteins are structurally defined by their BCL-2 homology domains (BH domains) and functionally categorized by their ability to inhibit or activate cell death*. [Waldensky 2006]

**The tumor suppressor p53** acts in response to a variety of cellular stresses and ends up with cellular processes such as cell cycle arrest, DNA repair, senescence and differentiation [Culmsee 2005; Eizenberg 1996; Galderisi 2001]. p53 is also important in the control of apoptosis because it can engage both intrinsic and extrinsic apoptotic pathways, mainly through transcriptional regulation of an increasing number of target genes such as the proapoptotic Bcl-2 members Bax, PUMA, Apaf-1 and Noxa involved in the mitochondrial pathway, and the death receptors TRAIL-R1 and FAS in the extrinsic pathway (figure 30i).

**Apoptosis-inducing factor (AIF)** is a mitochondrial intermembrane flavoprotein with proapoptotic effects. It traslocates from mitochondria to nucleus in a caspase-independent pathway, causing DNA fragmentation [Susin 2000], usually earlier than the release of cytochrome C. SMAC/DIABLO is

released from the mitochondria along with cytochrome C [Munoz-Pinedo 2006] and it binds the inhibitors of apoptosis (IAPs) and disengages them from activated caspases, thus promoting caspase activity and enabling the execution of apoptosis. In fact, the balance between IAPs and SMAC/DIABLO establishes a threshold for caspase-3 activity [Siegelin 2005].

Another pathway involved in stress response and apoptosis is mediated by **mitogen-activated protein kinases (MAPK)**, a family of proteins that has three major members: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNKs) and p38 MAP kinase (p38), playing a significant role in neuronal survival or death. ERK is activated by growth factors or mitogens leading to proliferation, differentiation, and survival, for example activation of neurotrophins, such as the brain-derived neurotrophic factor (BDNF) [Han 2000]. On the other hand, JNK and p38 are preferentially activated by oxidative stress and cytokines resulting in inflammation and apoptosis through activation of mitochondrial related proapoptotic proteins [Kim 2005].

Finally, **nuclear factor kappaB (NFkB)** has been found to respond to a broad range of stimuli and conditions, including inflammatory cytokines, growth factors, adhesion molecules, cell surface receptors, extracellular stress and intracellular oxidative stress. NFkB is crucial for inflammatory reactions in the periphery and regulates expression of both apoptotic and antiapoptotic genes (figure 30i).

### 6.2.2. Type II Autophagic cell death

Another described form of cell death is autophagy (from Greek ‘self eating’) or lysosomal cell death, which can be recognized by formation of many large double membranebound autophagic vacuoles (autophagosomes) in the cytoplasm [Meijer & Codogno 2004]. The lysosomal compartment is expanded. Cell organelles are captured in the autophagosomes, which fuse with the lysosomes. Autophagy is a normal physiological process that does not necessarily lead to cell death. It is involved in routine turnover of cellular proteins and organelles. The autophagic pathway is used for bulk proteolysis, while the ubiquitin pathway is necessary for fine control of protein degradation. During starvation autophagy is used for recycling of materials. There is no clear

discrepancy between autophagy and apoptosis. Unused, starving, or hormone deprived cells can atrophy, removing the bulk of their cytoplasm by autophagy. It is conceivable that the autophagy is a cellular protective mechanism to reduce metabolic demand by the cell to stave off deaths. When autophagy has reached its limit, apoptotic cell death might occur. A connection between autophagy and apoptosis is further supported by the observation that lysosomal enzymes are capable to induce apoptosis in cells [Guicciardi 2004].

Autophagy occurs in diverse organisms and is subdivided into macroautophagy, microautophagy and chaperones-mediated autophagy (CMA) (figure 32i). It complements the proteosomal pathway of long-lived proteins, protein aggregates, and organelles which are degraded by this regulated lysosomal pathway of degradation. Once targeted for degradation, for example damaged mitochondria or aggregates of misfolded proteins are encircled by membrane in a process that is essentially an intracellular form of phagocytosis. The newly-membrane delineated structure (autophagosome), fusses with a lysosome resulting in the degradation of the content of the autophagosome. The molecular details of this process have been best characterized in yeast, in which a great number of genes have been identified, most of which have clear orthologues in higher eukaryotes. Because the degradation of molecules and organelles by autophagy results in the production of energy and amino acids for protein synthesis, it is a cellular protective pathway. Though constitutively active at a basal level, it can be markedly up regulated by nutrient starvation.

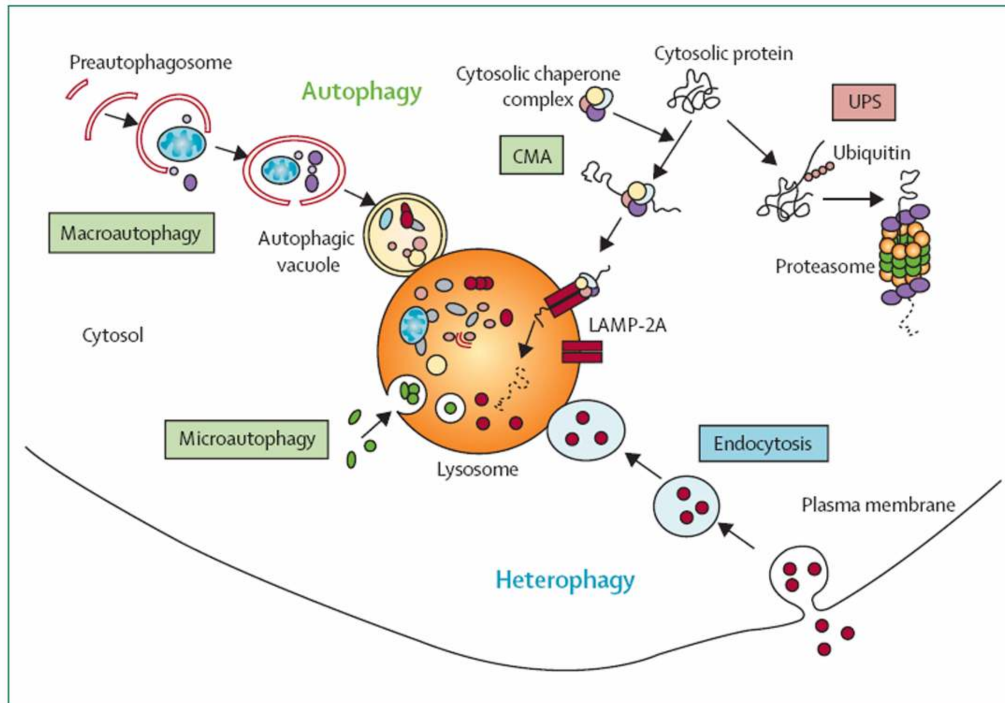



**Figure 32i.** Proteolytic systems in mammalian cells. *Substrate proteins are delivered to lysosomes from the extracellular media (heterophagy) or from inside the cell (autophagy). The best described heterophagic pathway is endocytosis. Three different types of autophagy have been described in mammalian cells: macroautophagy, microautophagy, and chaperone-mediated autophagy. In macroautophagy intracellular components are sequestered by a limiting membrane to form an autophagic vacuole that then fuses with lysosomes. In microautophagy, substrates are directly internalised through invaginations of the lysosomal membrane. In contrast to this "in-bulk" degradation, in CMA, selective substrate proteins are translocated into the lysosomes one by one after binding to a lysosomal receptor (LAMP-2A).* [M. Martinez-Vicente, A.M. Cuervo, Lancet Neurol 2007; 6: 352–61]

Nutrient starvation inactivates TOR (target of rypamicyn) activating ATG (autophagy related genes) complex [Levine 2005]. In a second step a vesicle nucleation occurs and then vesicle expansion followed finally by recycling of ATG proteins. The importance of this pathway in vivo has been illustrated by atg7 conditionally null mice [Komatsu 2005]. They show various cell abnormalities such as ubiquitin-positive aggregates and apparently damaged

mitochondria. Furthermore, mice deficient in autophagy due to knockout of beclin-1 are nonviable [Yue 2003]. Heterozygous beclin-1 animal show increased predisposition to tumor, indicating autophagy is tumor suppressive.

The roles of the autophagic process in protein and organelle degradation, and in cellular protection during nutrient starvation are well accepted [Shimizu 2004]. When mouse embryonic fibroblasts from Bax and Bak null mice are treated with staurosporine or etoposide they undergo a cell death which is associated with authophagosomes [Shimizu 2004]. Furthermore it was shown that general Caspase inhibitor zVAD-fmk in L929 cells results in autophagy-dependent PCD [Yu 2004], which has been proposed to be mediated by selective degradation of catalase [Yu 2006]. This may alert to the possibility that anti-apoptotic therapies carry the potential risk of inducing non-apoptotic PCD.

### 6.2.3. Type III and alternative cell-death programs

In comparison with apoptosis, little is known about autophagic PCD, and even less is known about other non-apoptotic forms of PCD. Other forms of PCD have not been generally accepted. However, as has been previously noted [Hengartner 2000], such forms of cell death are often observed. Their genetics and biochemical pathways are poorly understood. Type III PCD [Clarke 1990] or cytoplasmic cell death is a necrosis like form of PCD that includes swelling of the mitochondria and the endoplasmatic reticulum (ER), usually interpreted as overloading of the ER with misfolded proteins and lacks typical apoptosis features such as apoptotic bodies and nuclear fragmentation. It has recently been noted that the hyper activation of tyrosine-kinase receptor insulin like growth factor I receptor (IGFR) induces a non-apoptotic form of cell death known as paraptosis [Sperandio 2000]. This was shown to require transcription and translation and was found to be morphologically undistinguishable from type III PCD. Neither Bcl-2 nor caspase inhibitors block this form of PCD [Sperandio 2004].

Necrosis can be divided into primary and secondary necrosis. Primary necrosis is – in contrast to apoptosis - an apparently uncontrolled and mainly pathologic form of cell death. Loss of ATP and ion pump activity lead to swelling of organelles and the entire cell. Nuclear swelling leads to karyolysis. In cells

undergoing necrosis, the plasma membrane is destroyed early. Leakage of intracellular proteins into interstitial evokes an inflammatory response. Secondary necrosis occurs after apoptotic cell death, when apoptotic cells fail to be removed by heterophagy. Apart from the described differences between apoptosis and necrosis, there are a few arguments against the clear distinction between these two types of cell death. Aponecrosis is a term applied to a combination of apoptosis and necrosis [Formigli 2000]. Many cytotoxins induce PCD at low concentrations but at high concentrations induce primary necrotic cell death. In fact, this is the most common pattern seen with cellular toxins, from hydrogen peroxide and other oxidants to mitochondrial toxins such as antimycin A [Formigli 2000]. The “decision” whether a cell dies by apoptosis or necrosis mainly depends on the severity of the insult to the cell [Fadeel 1999a]. Many diseases labeled as necrotic involve also apoptosis (apoplexy, myocardial infarction, anoxia, ischemia-reperfusion damage). Mitochondrial is involved in both apoptosis and necrosis. It was shown that glutamate-induced cell death could proceed through apoptosis or necrosis, depending on mitochondrial membrane potential, permeability transition and cellular energy state [Ankarcrona 1995; Crompton 1999].

Furthermore, modulation of cellular ATP levels can shift apoptotic responses to necrosis (low ATP) and vice versa (high ATP) [Nicotera 1998]. Classical apoptosis inducers (serum withdrawal, etoposide, glucocorticoids) induce non apoptotic cell death in the presence of caspase inhibitors such as z-VAD-fmk.

Searching for patterns of cell death in a spectrum of models lead to description of forms of cell death, that do not fit the criteria for any of the three types of cell death described.

## 6.3. PCD during development

Programmed cell death (PCD) is naturally occurring or pathology induced cell response of cell removing without causing damage to the surrounding cells and it has a critical role in the development of the nervous system. Neuronal PCD during development was described for the first time more than 100 years ago [Studnicka 1905] and have passed more that 50 years since Levi-

Montalcini showed that NGF is able to suppress it [Levi-Montalcini 1966]. Deregulation of PCD is observed during development and neoplastic disorders of the nervous system as well as in neurodegenerative, infectious, traumatic, ischemic, metabolic, and demyelization disorders.

Apoptosis is a normal process needed for development in the womb, and to remove damaged, dangerous and surplus cells later in life. It is essential for the development and functioning of all multi-cellular animals including humans. If cells between our fingers didn't die during embryonic development, for instance, we would be born with webbed hands.

Programmed cell death or apoptosis of neurons is an integral process in the development of the nervous system. During development, the nervous system is initially generated with an excess number of neurons. This massive production of neurons is followed by apoptosis during a restricted developmental period, leading to the elimination of as much as half of the originally produced cells before the birth [Oppenheim 1991]. The main function of this developmental cell death appears to be the adjustment of the number of innervating neurons to the size of their target cell population. It is believed that only those neurons that receive the proper trophic input from their targets survive.

Neuronal apoptosis is a highly conserved cellular mechanism in the development of diverse organisms. Death of neurons has been observed in lower organisms including *C. elegans* and *D. melanogaster*, as well as in many neuronal structures of nearly every vertebrate species [Meier 2000]. The neuronal apoptosis in invertebrate organisms is critical to the normal development of their nervous system. However, in contrast to the vertebrates, neurons in these invertebrate species are thought to undergo cell death in a predetermined manner that is dependent on the genetic composition of the neuron rather than surrounding environmental cues [Meier 2000].

Two periods of cell death can be observed in the vertebrate's retina. The first period of cell death occurs during the main onset of neurogenesis, neuronal migration and initial axon outgrowth (e5–7 in the chick and e15–e17 in the mouse). This period of cell death is much more pronounced in chicks but can also be observed in the mouse retina [Frade 1996, 1999]. A second period, also called naturally occurring cell death or the physiological cell-death period,

coincides with the phase of tectal and thalamic innervation and synapse formation [Frade 1997]. In the rat, ~50% of the RGC population dies in the first postnatal week soon after reaching their target-regions, the superior colliculus and the lateral geniculate nucleus [Perry 1983]. In both periods cell death is apoptotic but the molecular mechanisms seem to be different. In the early period, the neurotrophin NGF induces cell death via stimulation of the p75 receptor and BDNF supports survival of chick RGCs [Frade 1996, 1997]. However, neither NGF nor BDNF have significant effects on RGC survival at the later period of cell death and *bdnf*-/- mice have normal RGC numbers [Cellerino 1997].

## 6.4. Triggering cell death in neurodegeneration

In maturity, apoptosis maintains the proper number of cells by balancing cell production. Apoptosis is also critical as a defense against disease. Cells dying prematurely can lead to neurodegenerative diseases; conversely, the failure of cells to die when they should can give rise to autoimmune disease and tumours. In addition to the extensive naturally occurring cell death in the developing nervous system, neuronal apoptosis is a prominent feature in a number of acute and chronic neurologic diseases [Friedlander 2003; Honig 2000; Mattson 2000; Vila 2003; Yuan 2000]. In some neurologic disorders, such as the hereditary motor neuron diseases, neuronal apoptosis is central to the pathogenesis of the disease [Cleveland and 2001; Julien 2001]. In other diseases including AD, a primary role for neuronal apoptosis in the pathogenesis of the disease is still debated. Neuronal cell death is increasingly recognized as a major contributor to the neuropathology underlying a variety of degenerative diseases of the brain. In general, neuronal apoptosis in these disorders might not only be the result of trophic factor withdrawal, but is thought to be associated with a number of toxic insults that may trigger PCD such as: abnormal protein aggregates, reactive oxygen and nitrogen species, mitochondrial-complex inhibition, calcium entry, excitotoxicity and death-receptors activation [Klein 2003; Taylor 2002].

The intracellular mechanisms regulating neuronal cell death are still not wery vell understood. Remarkably, the underlying molecular mechanisms in both developmentally regulated and disease-related neuronal apoptosis appear

to be similar. A growing number of signaling pathways have been proposed to link distinct death and survival signals with the cell-intrinsic death machinery. Aberrant re-entry into the cell cycle has emerged as a common mechanism leading to neuronal loss during normal brain development [Liu 2001a]. Similarly, re-expression and activation of cell cycle proteins have been observed in dying neurons in brains of patients afflicted by neurodegenerative disorders [Husseman 2000].

The pattern of neuronal loss in AD overlaps, but is not identical with that of normal neuronal loss during normal brain ageing [Lleo 2003]. Large numbers of neurofibrilary tangle-bearing neurons occur in AD, but neuronal loss exceeds the number of neurofibrilary tangles, which suggests that tangle-bearing neurons are removed or that some neurons die without forming tangles [Chen 2002].

The death of populations of neurons in the brain regions affected in AD apparently occurs over a prolonged time period of many years, which suggests that a relatively small number of neurons are dying simultaneously. Increasing evidence suggests that many neurons may die by apoptosis in AD, although it may be not the only form of cell death.

The role of the cell death machinery in NDD is controversial. Synaptic loss and electrophysiological abnormalities typically precede cell loss in these disease states. Recent studies suggest a critical role for cell-death mediators in neurodegenerative diseases, even before the reduction in neuronal number. For example, transgenic mouse model of AD that features amyloid plaques, synapse and memory loss, present little or any neuronal diminution. Moreover, mutation of caspase cleavage site at Asp 664 in APP surprisingly leads to complete synaptic lose, even though the plaques number and Aβ concentrations were unaffected [Galvan 2006]. In an analogous study with an HD-transgenic mouse model, mutation of Caspase-6 site (but not Caspase-3 site) in polyglutamine-expanded huntingtin prevented both the neurodegeneration and motor abnormalities characteristic of Huntington's disease [Graham 2006]. However, some neurodegenerative models and disease clearly demonstrate non-apoptotic forms of PCD [Dal Canto 1994].

Neuronal apoptosis is not restricted to the period of brain development but is believed to also contribute to loss of neurons occurring in acute and

chronic neurological diseases. In the adult CNS a distinct set of pathogenic stimuli rather than the lack of trophic support, predominate as inducers of apoptosis. One of the most widely studied extrinsic insults triggering neuronal cell death in the mature nervous system is the neurotransmitter glutamate [Choi 1994; Zipfel 2000]. Neurons undergo excitotoxic cell death following the excessive release of glutamate from presynaptic nerve terminals and astrocytes into the extracellular space. Olney and Sharpe [Olney 1969] initially observed that exposure of the brains of rhesus monkeys to an excess of glutamate causes toxicity and neuronal cell death. Subsequent studies established that excess glutamate results in over stimulation of glutamate receptors, including the *N*-methyl-d–aspartate (NMDA) receptors and the non-NMDA receptors [Ozawa 1998]. As a consequence of glutamate receptor activation, influx of calcium ions occurs, followed by the passive entry of chloride ions and water [Choi 1988]. The intracellular calcium concentration is very tightly regulated and even small variations result in the neuron's commitment to apoptosis [Orrenius 2003]. The increased influx of calcium ions via the NMDA channel in neurons is believed to play an essential role in the pathogenic mechanisms of excitotoxic cell death [Choi 1994].

AIF as an apoptosis inducer was first identified by Guido Kroemer's group as a mitochondrial protein that induces apoptosis in a caspase-independent manner [Azad 2003]. Stimuli that lead to apoptosis were found to promote the translocation of AIF from the mitochondria to the cytosol and nucleus [Susan 1999]. Once in the nucleus, AIF imparts a powerful apoptotic signal that leads to chromatin condensation and DNA fragmentation. The crystal structure of AIF revealed a surface with positive electrostatic potential that may mediate AIF's ability to bind to DNA in a sequence-independent manner leading to large-scale DNA cleavage [Ye 2002]. In genetic studies was confirmed that AIF is an important mediator of apoptosis: AIF-deficient embryonic stem cells are resistant to serum deprivation-induced apoptosis and the first wave of apoptosis during embryogenesis is defective in the AIF deficient mouse embryos [Joza 2001].

How apoptotic signals trigger AIF release remains to be elucidated. Apoptotic stimuli, including free radicals that induce DNA damage and activate poly ADP-ribose polymerase (PARP) in the nucleus have recently been

suggested to trigger the release of AIF from mitochondria as a secondary consequence of consumption of the metabolite nicotinamide adenine dinucleotide (NAD+) [Yu 2002]. However, how the alteration in NAD metabolism is involved in the translocation of AIF from the mitochondria to the nucleus remains unclear.

The intrinsic apoptotic pathway makes use of the release of Cyt C from the mitochondria to activate caspases [Wang 2001]. In vitro studies have shown that Cyt c together with apoptotic protease-activating factor-1 (APAF-1) generates an apoptosome structure that activates the initiator caspase (CASP)-9, which in turn activates downstream effectors CASP-3 and -7 [Wang 2001]. Organelle sites including endoplasmic reticulum, nucleus and mitochondria participate in apoptosis [Ferri & Kroemer 2001], and most organellespecific death responses ultimately trigger caspase activation. CASP-12 is activated specifically in response to ER stress [Nakagawa 2000], whereas CASP-2 is activated in response to stress induced by DNA-damaging agents [Lassus 2002]. However, it remains uncertain whether distinct organellespecific caspase activation pathways specifically mediate activation of initiator caspases including CASP-2 or CASP-12. Alternatively, CASP-2 or CASP-12 activation might represent amplification loops residing downstream of common sensors and/or effectors of cellular damage. For example, cells doubly deficient for BAX and BAK are resistant to both DNA-damage-induced and ER-stress-induced apoptosis [Danial & Korsmeyer 2004]. BAX and BAK seem to constitute an essential apoptotic gateway after DNA damage and can operate at the mitochondria and the ER (Danial & Korsmeyer, 2004) [Ruiz-Vela 2005].

Misfolded proteins are constantly being produced in the cell but when they accumulate in dangerous proportions in the lumen of ER and the cytoplasm the cell trigger a protective stress response, known as the unfolded protein response. These pathways are of particular interest in NDD studies because they share a common feature: accumulation and aggregation of misfolded proteins and are implicated in disorders such as AD, PD, HD, ALS and prion-protein related disease [Kopito 2000; Taylor 2002]. Moreover misfolded proteins also aggregates as oligomers and higher order multimers, both of which can interact with critical cellular targets as ER chaperones and transcription factors among others. When ER functions are severely impaired as

result of prolonged ER stress, the cell activates suicide pathways and is eliminated by apoptosis via transcriptional induction of CHOP/GADD153, the activation of CJUN NH2-terminal kinase, and/or the activation of caspase-12 [Araki 2003].

# *VI. OBJECTIVES*

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In the 2001, it was published the presence of NGF in brain tissue as a pro-form. It was also shown that using a recombinant mutated pro-NGF in *in vitro* models, not processed pro-NGF was a possible inductor of cell death, through its interaction with p75NTR.

The hypothesis based on these previous observations was established as follows: The alterations of pro-NGF in human brain affected by AD are possible inductors of neuronal cell death, activating apoptotic signalling through p75NTR receptor.

To evaluate this hypothesis we outlined the following objectives:

1. To extract pro-NGF from AD affected human brain tissue as well as from control brains and to compare their capacity to induce cell death in *in vitro* models.

2. To study possible alterations in p75NTR processing in AD affected tissue.

3. To obtain more data about the mechanism of p75NTR signalling upon binding of pro-NGF, particularly p75NTR processing and following events in cell cultures.

4. To study the effect of substances with possible protective function that may decrese the effects of pro-NGF.

5. To study the effect of pro-NGF in the development of motoneurons in chicken embryos.

6. To study the differences in post-translational modifications between pro-NGF from AD brain samples and brain samples from healthy individuals.

7. To introduce *in vitro* modifications of recombinant pro-NGF such as those observed *in vivo* in AD, and to study the differences in the physiological action of the modified and nonmodified pro-NGF.

# *VI. OBJETIVOS*

En 2001, se publicó que NGF estaba presente en su forma inmadura en cerebro. Utilizando pro-NGF mutado en los lugares diana de procesamiento por proteasas, en modelos in vitro, se demostró su capacidad de inducir apoptosis a través de p75NTR.

La hipótesis basada en estas observaciones previas, se formuló como sigue: Las alteraciones en el pro-NGF observadas en cerebro humano afectado por EA son posibles inductores de muerte neuronal, activando la señalización apoptotica a través de p75NTR.

Para evaluar la hipótesis propuesta se establecieron los siguientes objetivos de trabajo:

1. Aislar pro-NGF de tejido cerebral humano afectado por la enfermedad de Alzheimer y de tejido control y comparar sus capacidades de inducir muerte celular en modelos *in vitro.*

2. Estudiar la posible alteración en el procesamiento de p75NTR en tejido afectado por EA.

3. Obtener información detallada acerca del mecanismo de señalización por p75NTR respecto el procesamiento de p75NTR y los procesos que siquen.

4. Estudiar los efectos de substancias potencialmente protectoras, que puedan desminuir los efectos de pro-NGF.

5. Estudiar los efectos de pro-NGF en las motoneuronas de embriones de pollo en desarrollo.

6. Estudiar las diferencias en las modificaciones post-traducionales entre pro-NGF procedente de cerebros afectados por la enfermedad de Alzheimer y procedente de controles.

7. Introducir in vitro en el pro-NGF recombinante, las modificaciones observadas en la EA in vivo y estudiar las diferencias en los efectos fisiológicos entre pro-NGF modificado y no modificado.

# VII. MATERIALS AND METHODS

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## 1. Cell Cultures

### 1.1. Cell cultures used

#### 1.1.1. 3T3 and 3T3-p75 cell line

The 3T3 are originally obtained from Swiss mouse embryo tissue in 1962. Cells of the 3T3 cell line and the same line but stably transfected to express human p75NTR (3T3-p75st) were kindly provided by M.V.Chao, Skirball, New York University, NY).The line was maintained in Dulbecco's modified Eagle's medium (DMEM) from Gibco-BRL, Gaithersburg, MD, supplemented with 10% fetal bovine serum (FBS) and 20U/ml Penicilin 20ug/ml Streptomicyn. Subdivision was performed twice a week, detaching the cells with a brief Trypsin treatment (0,25% Trypsin-Gibco BRL), with usual dilution factor 1:6. For treatment, 15 000 cells/cm$^2$ were plated in 4-well, 24-well or 48-well plates in DMEM supplemented with 10% fetal bovine serum (FBS). Cell concentration was performed using Burker chamber. Before treatment, cells were washed twice with serum-free medium, and treatments were performed in 0.5% FBS medium.

#### 1.1.2. PC12 cell line

PC12 is a cancer cell line derived from a pheochromocytoma of the rat adrenal medulla. PC12 cells stop dividing and terminally differentiate when treated with nerve growth factor. PC12 cells were maintained in DMEM supplemented with 6% FBS, 6% horse serum, and 20U/ml Penicilin 20ug/ml Streptomicyn. Subdivision was performed twice a week with usual dilution factor 1:4. Cells were detached with repetitive washing with small volume of media and cell concentration determined with Burker chamber. For treatment cells were plated in 24-well or 48-well plates pre-coated with Polyornithine, at 15000 cells/cm2 in DMEM supplemented with 6%FBS 6%HS. Before treatment, cells were washed twice with serum-free medium, and treatments were performed in the absence of serum.

### 1.1.3. 293FT cell line

The 293FT Cell Line is a clonal isolate derived from transformed embryonic kidney cells. The 293FT strain is a fast-growing highly transfectable variant of the 293 cell line, which contains the large T antigen for high-level expression of the packaging proteins contributing to higher viral titers. 293FT cells were maintained in advanced MEM supplemented with 10% FBS, 20U/ml Penicilin 20ug/ml Streptomicyn, and 500µg/ml Geneticin. Subdivision was performed twice a week with usual dilution factor 1:4. Cells were detached with repetitive washing with small volume of media and cell concentration determined with Burker chamber.

### 1.1.4. NSC-34 cell line

NSC-34 cell line is a hybrid cell line obtained by somatic fusion of motor neuron-enriched embryonic mouse spinal cord cells (NSC) day 12–14 with mouse aminopterin-sensitive neuroblastoma N18TG2. The cell line maintained in DMEM from supplemented with 10% fetal bovine serum (FBS), 20U/ml Penicilin 20ug/ml Streptomicyn and 4mmol/l L-Glutamine.

### 1.1.5. Primary culture of superior cervical ganglion's neurons

Superior cervical ganglion (SCG) neuron cultures were prepared by isolating ganglia from 0 to 2 post-natal days mouse. After dissection, ganglia were incubated with collagenase type IV (20 U/ml) (Worthington, Lakewood, NJ) for 30 minutes at 37°C followed by incubation with trypsin (0.25%, Gibco, BRL, Paisley, Scotland) for 25 minutes at 37°C and washed with Minimum Essential Medium (MEM) (Gibco) supplemented with 10% horse serum (HS) and antibiotics (complete medium). Mechanical dissociation was performed by passing the ganglia through a 200-ul pipette tip. Cells were plated at 12,500 cells/cm2 in collagen pre-coated 24-well plates and maintained in complete medium supplemented with nerve growth factor (rh-mNGF, 100 ng/ml, Alomone Labs) and L-glutamine (2 mmol/L) (Gibco,BRL). To prevent the growth of fibroblasts, cells were cultured in the presence of 5-fluoro-2'-deoxyuridine

(50nmol/L) and uridine (50nmol/L) (Sigma). Treatments were performed after 5 days in culture. The cells were washed three times with serum-free medium and the reagents indicated were added in each case.

## 1.2. Pre-coating of cell culture plates

### 1.2.1. Poly-DL-ornithine

Cells were plated in 24-well or 48-well plates pre-coated for at least 1 hour with 20µg/ml Polyornithine diluted in 150mM Boric/Borate buffer. The pre-coation was followed by 3 washes with water.

### 1.2.2. Poly-Lysine/Collagen

Cells were plated in 24-well or 48-well plates pre-coated with Poly-lysine/ collagen. The plates were covered with Poly-lysine (Sigma cat# p7280) with final concentration 0.01mg/ml dissolved in MilliQ water for 30-60min at room temperature, followed by collagen (BD cat# 354236) covering with final concentration 0.1 mg/ml diluted in 0,1 N acetic acid and leaving them to evaporate over night.

## 1.3. Freezing and thawing of the cell lines

For freezing 100mm plates with confluent cell culture were used. The cells were detached from the plate end centrifuged for 5min at 130xg. The pellet was resuspended in 1ml freezing medium containing 90% Fetal Bovine serum and 10%DMSO and put in cryovials. The cryovials were freezed at -80$^{o}$ C for 24h and then moved to liquid nitrogen for long term maintainance.

For thawing cells were rapidly defrosted in water bath at 37$^{o}$ C, washed with basal medium, centrifuged at 130x g for 5min. and resuspended in appropriate medium.

## 1.4. Methods for cell transfection

### 1.4.1. Lipofectamine$^{TM}$ 2000

Lipofectamine$^{TM}$ 2000 (Invitrogen) is one of many cationic lipid-based transfection reagents available in the market. Lipofectamine is formulated to

give high transfection efficiency with little toxicity to wide variety of cells. For our experiments we followed the protocol listed by the manufacturers.

### 1.4.2. Calcium chloride ($CaCl_2$)

This method is based on coprecipitation of DNA with calcium chloride. We use this method of transfection for 293FT cell line. For this transfection we used 100mm plate with 90% confluent 293FT cell culture, changing the medium to fresh medium 1-2 hours before transfection. For precipitating DNA with $CaCl_2$ we use 20μg DNA adjusted to 250μl with mQ water buffered with 2.5mM HEPES pH 7,3 and mixed with 250μl 0,5M $CaCl_2$ and added slowly, dropwise on 500μl HeBS 2x buffer (0,28M NaCl; 0,05M HEPES; 1,5mM $Na_2HPO_4$; pH 7) while vortexing topspeed. The mixture is left for 30-40min on bench and added slowly, dropwise on the cell monolayer.

### 1.4.3. Electroporation

This method is based on opening of cell membrane pores by using electric field, and allowing the DNA to enter in the cell trough these pores. This method can be used for a broad variety of cells, but is not recommendable to be used for certain cell lines because of high mortality of the cells during electroporation.

For electroporation we used about $10^7$ cells, resuspend them in 800μl cold PBS and add 10μg of the plasmid DNA. The cells are leaved for 10min on ice and transferred to cooled electroporatin cuvette (Gene Pulser cuvette Bio Rad). The cuvette is placed in the Gene Pulser and the cells were exposed on the electric field 320V 975mF for PC12 cells and 250V 500mF for MEFs. Cells were immediately placed in 60 mm plate precoated with poliornithine with appropriate medium and left for 24h in the incubator. After 24h the cells can be plated for experiments.

### 1.4.4. Lentiviral transduction

For our experiments we used the second generation of lentiviruses designed and distributed from Laboratory of Virology and Genetics EPFL SV IBI LVG Lausanne, Switzerland http://tronolab.epfl.ch

For lentiviral production we used 100mm plate with 90% confluent 293FT end transfect them with 20µg pWPT-GFP or pWPI vector carrying the gene of interest; 15µg psPAX2 and 5µg pMD2G plasmids using the protocol for $CaCl_2$ transfection listed in section 1.4.2.

After approximately 12h the medium containing $CaCl_2$ precipitations was changed with fresh medium, from this moment we should use laminar hood appropriate for working with viruses. At this stage the 293FT cells start to produce lentiviruses and we can start harvesting 10ml media containing lentiviruses every 12h for 2-3 days and replacing it with fresh medium. The supernatant containing lentiviruses is spined down at 1000g for 10min and filter trough 0, 45µm filter. All the supernatants containing viruses were pooled together. Producing lentiviruses the 293FT cells start to fuse each other, the percentage of fusing cells is proportional of lentiviral production efficiency and can serve us for aprocsimal estimation of lentiviral titter. For precise calculation of lentiviral titter we plate one p24 plate with 20,000cells/well, made dilutions of lentiviruses, and count positive cells after 24h .The lentiviral pool can be concentrated using ultracentrifugation. We used 4ml of 20% sucrose in water in the bottom of ultracentrifuge tubes (Beckman) and fill up to the top with filtered supernatant. Spin down at 47000g for 90min and discard the supernatant, the pellet was resuspended with appropriate amount of medium to obtain desired concentration. The concentrated vector can be used directly or aliquoted and stored at -80°C for future use.

## 1.5. Neuritogenesis induction

PC12 cells were grown on poly-D-Ornithine precoted plates in the presence of 100ng/ml mNGF. The serum concentration was maintained at 1%. After 3-6 days the cells were examined under phase-contrast microscope using 20XOlimpus LCPlan objective. The stage of dendrite elongations was evaluated. As positively differentiated cells were considered those with dendrite length at least twice the diameter of the cell body.

## 1.6. Methods for detection of cell death and apoptosis

### 1.6.1. Hoechst 33258 staining

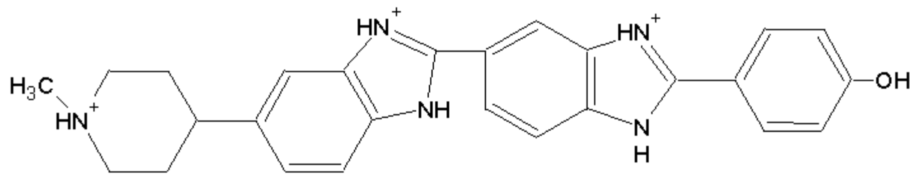



**Figure 1m.** *Hoecht 33258*

During the apoptotic process there are relevant changes in the nuclear morphology. A chromatin condensation usually occurs first, resulting in decreasing the overall size of the nuclei and the impossibility to distinguish between hetero- and euchromatin zones. Usually, a nuclear fragmentation takes place later, visualized as smaller highly condensed bodies of different shape occupying the area of disintegrated nuclei. The absence of phagocytosis (e.g. cell culture), as final stages the nonstructured chromatin is released in the media, is also characteristic. To follow and evaluate this characteristical changes a DNA binding dye Hoechst 33258 is used.

The Hoechst stain is part of a family of fluorescent stains for labeling DNA in fluorescence microscopy. Because these fluorescent stains label DNA, they are also commonly used to visualize nuclei and mitochondria. The Hoechst dye is excited by ultraviolet light at around 350 nm, and emits blue/cyan fluorescence light around an emission maximum at 461 nm. The Hoechst stain may be used on live or fixed cells. Because the Hoechst stains bind to DNA, they can disrupt DNA replication during cell division. Consequently they are potentially mutagenic and carcinogenic. Care should be taken in their handling and disposal.

We used 0,5 µg/ml Hoechst for 1h in both fixed and live cells.

The fluorescence was analyzed using an Olympus 20X LCPlan objective and documented with an Olympus DP70 camera. Olimpus U-MNAU2 setup was used with excitation at 360-370 and emission at 420-460.

The number of pyknotic nuclei was established, and the percentage of apoptosis was determined. At least 500 nuclei in random, no overlapping fields per condition were counted in each experiment.

## 1.6.2. MTT reduction

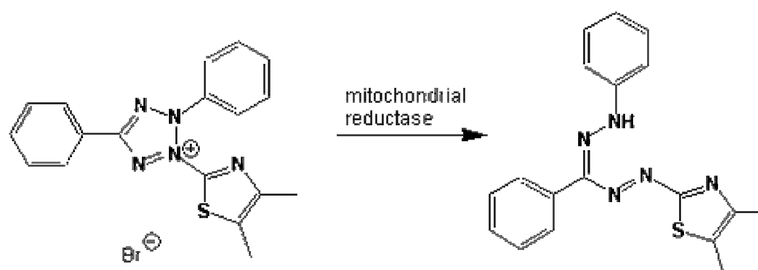



**Figure 2m.** *Reduction of MTT*

MTT assay is a laboratory test and a standard colorimetric assay for measuring cellular growth and for determine cytotoxicity of toxic agents. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve.

As disadvantage that we should mention, is that these assay do not distinguish the source of diminished viable cells (e.g. not apoptotic cell death) or different rates of proliferation. Changes observed by MTT measurement usually take place later than the nuclear morphology, and are associated with total disintegration of the affected cell.

We used final concentration of 400µg/ml MTT for our experiments. The MTT solution was added 48h after the treatment in both control and treated cells and incubated for one hour in incubator (37$^{o}$C / 5% $CO_2$). After that the medium was carefully aspirated and the cells were dissolved with DMSO (200µl for p24; 100µl for p48; 50µl for p96). The product gives a purple colored solution. The absorbance of this colored solution was quantified by measuring by a spectrophotometer at a 590 nm wavelength and a reference of 600 nm.

### 1.6.3. TUNEL

Terminal Deoxynucleotidyl Transferase-Mediated dUTP nick end labeling (TUNEL) is highly specific method for determination of apoptosis in cellular populations. It is based of labeling of free 5' ends generated after cleavage of cellular DNA associated with of activation of nucleases in the apoptotic process. TUNEL positive labeling can be observed in earlier stages of cell death even before the changes in nuclear morphology.

Apoptotic cells were detected by using TUNEL in situ cell death detection kit (Roche Applied Science, Mannheim, Germany), following the manufacturer's instructions. Briefly, after fixation and permeabilization with 1% of Triton-X100 cells were incubated with Terminal Deoxynucleotide Transferase in corresponding buffer. dUTP labeled with Alexa488 (Molecular probes) was used for fluorescent marker, and visualized with Olympus IX70 with 20X LCPlan objective and documented with an Olympus DP70 camera. TUNEL positive nuclei were counted against total number of nuclei visible after 24h after Hoecst staining.

## 1.7. γ-Secretase inhibition

For γ-Secretase inhibition, cells were pretreated with 1 umol/L N-[N-(3, 5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT; Calbiochem, San Diego, CA) for 1hour. The structure of DAPT is presented here:

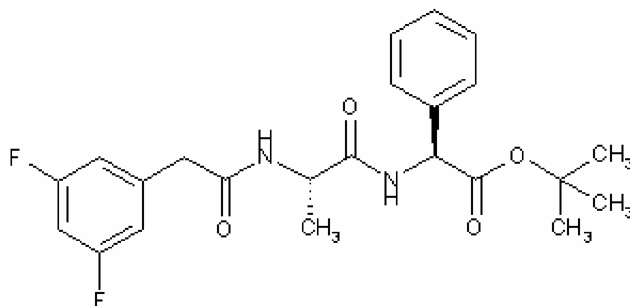



**Figure 3m.** *N-[N-(3, 5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT)*

### 1.8. p75NTR blocking

For blocking of p75NTR cells were pretreated with 200ng/ml REX antibody for 2hour. REX antibody recognizes the extracellular domain of p75 and prevents its activation from pro-NGF.

### 1.9. pro-NGF blocking

For blocking pro-NGF, cells were treated with 200ng/ml anty-pro-NGF antibody.

## 2. Immunocytochemistry and confocal imaging

The cells were grown and treated on precoated p-L-lysine coverslips. After the indicated times of treatment, the cells were fixed with 4% paraformaldehyde in phosphate buffered saline for 1 hour at room temperature and permeabilized with methanol for 10 minutes at room temperature. The incubation with corresponding primary antibody was done overnight at 4°C. Anti-human p75 (Promega) and anti-p75NTR (REX) antibody (kindly provided by L.F. Reichardt) were used to detect intra- and extracellular domains of p75NTR, respectively. The secondary antibody incubation with anti-rabbit Alexa Fluor 488 (Promega) was for 1 hour at room temperature. After washing with phosphate-buffered saline, the coverslips were mounted with Mowiol, and fluorescence images were acquired using a confocal microscopy setup (Model IX81; Olympus, Tokyo, Japan, employing 60X PlanApo objective Software control version Fluoview 4.3) or an inverted epifluorescence microscope (Olympus IX71, 20X LCPlanFl) equipped with Olumpus DP70 cooled sensor camera.

## 3. Proteomic methods

### 3.1. Preparation of cell and tissue lysates

It is very important for all types of analysis to have an efficient protein extraction with a minimum of protein degradation, in order to obtain reliable results.

### 3.1.1. Using denaturizing buffer for Western blot

For analysis in which the preserved conformation of the proteins is not necessary, as Western blot, cell lysates with denaturizing buffer (10mM TRIS; 1% SDS) was prepared. If cell cultures were used, the cell monolayer was washed 2 times with PBS and lysis buffer heated up to 90$^{o}$C was added. The cells were detached from the plate using plastic scraper and transferred to the eppendorf tube. The cells were sonicated for 1-2 min and centrifuged at 12000g for 10min. The supernatant was transferred in the new tube and protein concentration was estimated.

Protein extracts from the human brain samples were prepared from 0.3-1g tissue pieces. The pieces were sonicated for 1-2 min in denaturating buffer (10mM TRIS; 1% SDS) and centrifuged at 12000g for 10min. The supernatant was transferred to a new tube and protein concentration was estimated.

This protocol of protein extraction is very efficient as it breaks any protein interactions and denaturize all proteases making them inactive.

### 3.1.2. Using no denaturing buffer for immunoprecipitation

For immunoprecipitation the human samples were homogenized in RIPA buffer (150mM NaCl; 0.1% SDS; 50mM Tris pH 8.0; 1% NP-40; 0.5% deoxycholic acid) by sonication, and centrifuged at 12000g for 10min. The supernatant was transferred to a new tube and protein concentration was estimated.

### 3.1.3. Using no denaturing buffer for Ion-exchange chromatography

For Ion-exchange chromatography the human samples were homogenized in sterile water using a potter on ice.

## 3.2. Protein concentration determination

### 3.2.1. Modified Lowry method

The principle of Lowry method is based on the fact that under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue.

We used Lowry Kit (Bio-Rad cat N 500-0116). For our experiments we followed the protocol listed by the manufacturer. The resulting blue coloured samples were measured in the spectrophotometer at 600nm wave length. For analysis of the results a standard curve of absorbance versus micrograms protein (BSA) was prepared.

### 3.2.2. Bradford method

The assay is based on the observation that the maximum absorbance for an acidic solution of Coomassie Brilliant Blue G-250, when binding to protein, shifts from 465 nm to 595 nm. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

### 3.2.3. Measuring absorption at peptide bond at 230nm

The absorption at 230nm was measured using Nanodrop$^{TM}$ 1000 Spectrophotometer. After the measurement a calculation for correction of nuclear acid content was performed using the following formula:

Concentration of proteins (mg/ml) = (0,183*Abs230nm)-(0,0758* Abs260nm)

## 3.3. Protein separation using SDS-PAGE

30 µg of total protein was mixed with 5X Loading buffer (250mM TRIS/HCl pH 6,8; 10% SDS; 25% β-Mercaptoethanol; 50% Glycerol; 0,005% Bromphenol blue) and equilibrated to 30 µl total volume with sterile $H_2O$, boiled at 95$^{O}$C for 5min and centrifuged for 5min at 12000g. Proteins was separated using 5% Stacking gel (0,5ml 30%Acrylamide/bis 37.5%; 0,75ml 0,5M Tris-HCl

pH 6,8; 1,7ml $H_2O$; 30µl 10% SDS; 20µl 10% Ammonium Persulfate; 5µl TEMED) and 12% Resolving Gel(2ml 30%Acrylamide/bis 37.5%; 1,25ml 1,5M Tris-HCl pH 8,8; 1,65ml $H_2O$; 50µl 10% SDS; 40µl 10% Ammonium Persulfate; 5µl TEMED) for 1mm 6sm/9sm gels. The gels were runed on Mini-protean II (Bio-Rad) at 25v 300mA for 1h 15 min using running buffer (Tris base 30,3 g; Glycine 144 g; SDS 10 g for 1L 10x). To determine the molecular weight of proteins we used BenchMark$^{TM}$ (Invitrogen catN 10748-010) and MagickMark$^{TM}$ XP (Invitrogen catN LC5602).

After separation, the proteins were transferred to HYBOND-P PVDF membrane (Amersham cat N RPN303F) using Hoefer TE 77 (Amersham) for semi-dry method at 50mM for 1h 30 min with 20ml transfer buffer (29.3g Glycine; 58.1g TRIS; 3.75g SDS and 40% methanol for 1l 10x) or with tank transfer system using Mini Trans Blot Transfer Cell (BioRad) for 60 min at 15mA with 1l transfer buffer (21.6g Glycine; 4.5g Tris; 0.15g SDS and 300ml mathanol for 1,5l 1x).

## 3.4. Protein immunodetection (Western blot)

After the transfer the membranes were blocked for 1h at room temperature in TBS-T (50 mmol/L Tris, pH 8.0, 133mmol/L NaCl, 0.2% Tween 20) with 5% skimmed milk or blocking reagent (Tropix, MA, USA), depending on the antibodies used. For immunodetection the membranes were incubated overnight at 4$^{o}$C with agitation using the appropriate dilution for every antibody. After washing 3 times in TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000 in TBS-T) at room temperature for 1 hour. For detection, after washing 3 times in TBS-T, an ECL plus chemiluminescence system (Amersham Biosciences, Piscataway, NJ) was used in accordance with the manufacturer's instructions.

## 3.5. Protein staining in SDS-PAA gels and PVDF membranes

### 3.5.1. Coomassie blue staining

After the transfer the gels were stained in coomassie blue (0,1 % coomassie blue;25% Isopropanol; 10% Acetic acid) for 30min and washed 3 times for 30 min with coomassie destainer (10% Isopropanol; 10% Acetic acid) to determine the efficiency of the transfer.

### 3.5.2. Silver staining

To visualize smaller amounts of proteins more sensitive silver staining was used.

For gels we used Bio-Rad's Silver Stain Kit following the manufacturer instructions. For PVDF membranes we used Gallyas protein staining. This protocol was used to determine efficiency of the transfer and as a loading control. The PVDF membranes were washed 3 times for 5 minutes with 1% Sodium Acetate and 3 times for 5 minutes with 0,1% Sodium Thiosulfate. The Gallyas staining solutions (A and B), can be stored for months at room temperature protected from the light. Solution A contains 50g sodium carbonate and is dissolved in 1l of water, solution B contains 2g ammonium nitrate, 2g silver nitrate, 10g tungstosilicic acid, and 5ml 37% formaldehyde is dissolved in 1l water. The Gallyas stocks A and B were mixed 1:1 under stirring and the membranes developed in this solution for 10minutes. For improvement of the staining the development was repeated and stopped with 10% acetic acid for 5 minutes.

### 3.5.3. Reversible staining of PVDF membranes using Ponceau S solution

For reversible staining a Ponceau S solution (0.5 g Ponceau S dissolved in 1 ml glacial acetic acid. Bring to 100 ml with water) was performed for 30 min at room temperature.

## 3.6. Stripping PVDF membranes

When second rebloting with another antibody was needed the antibodies were removed from the membrane by washing 10 minutes with 0,1M Glycine

(pH 2,5), 10 min with 3M $MgCl_2$, 2 washes for 5 minutes with water, 10 min with 1% SDS and 3 times for 5 minutes with TBST.

## 3.7. Immunoprecipitation

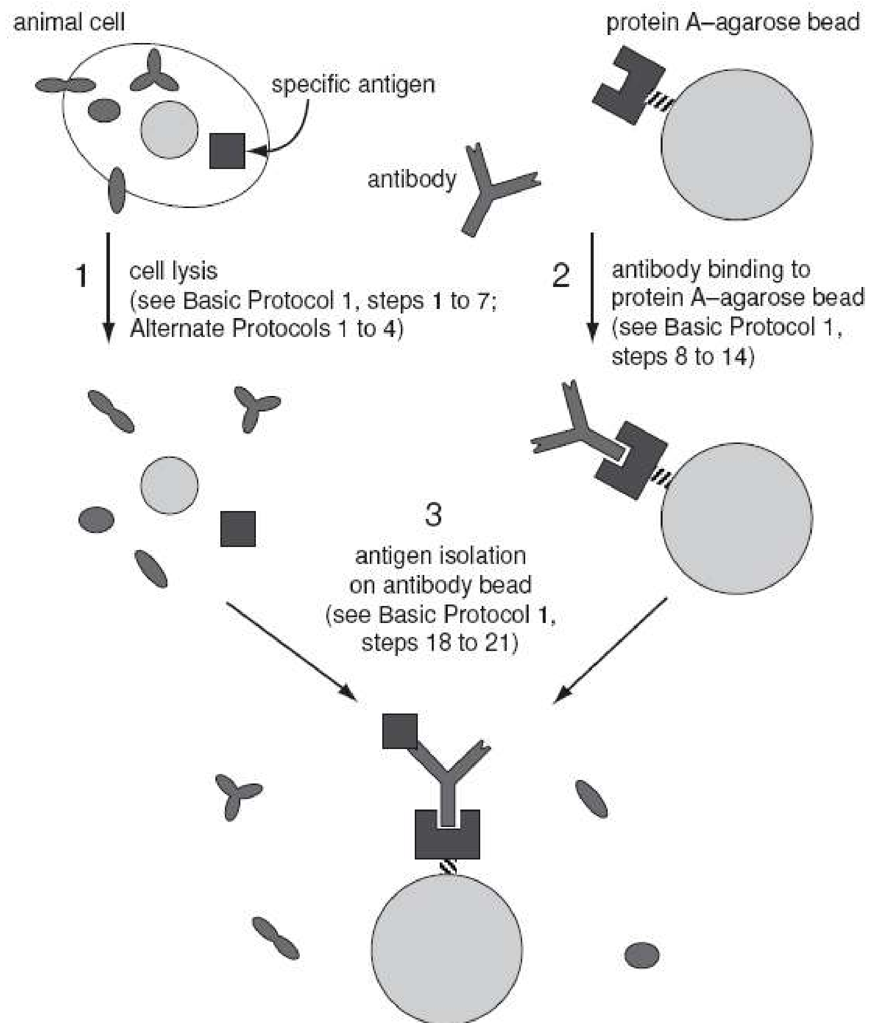



**Figure 4m.** *Schematic representation of the stages of the immunoprecipitation* [Current Protocols in Immunology]

Immunoprecipitation is a technique in which an antigen is isolated by binding to a specific antibody attached to a sedimentable matrix. Either polyclonal or monoclonal antibodies from various animal species can be used in immunoprecipitation protocols. Antibodies can be bound noncovalently to

immunoadsorbents such as protein A– or protein G–agarose, or can be coupled covalently to a solid-phase matrix.

For our experiments the antigen was solubilized by using ice-cold nondenaturing buffer as listed in section 3.1.2. The suspension was left on ice 15 to 30 min and centrifuged for 15 min at 16,000 × g (maximum speed), 4°C the supernatant was transferred to a fresh microcentrifuge tube.

The antibody-conjugated beads ware prepared by mixing 30µl of 50% protein A–Sepharose bead slurry, 0.5 ml ice-cold PBS, and 1µg of affinity-purified polyclonal antibody and mixed ≥1h at 4°C in a tube rotator. The mixture was centrifuged for 2 sec at 16,000 × g at 4°C, supernatant (containing unbound antibodies) was aspirated, and beads were washed with 1ml nondenaturing buffer 3 times.

The lysate was precleared by mixing 1ml of lysate with 30µl of 50% protein A–Sepharose bead slurry and tumble end over end 30 min at 4°C in a tube rotator and centrifuged for 5 min at 16,000 × g. The purpose of this step is to remove from the lysate proteins that bind to protein A–Sepharose, as well as pieces of insoluble material that may have been carried over from previous steps.

The Immunoprecipitate was performed by mixing 10 µl of 10% BSA to the tube containing specific antibody bound to protein A–Sepharose beads, and the entire volume of cleared lysate Incubated 1 to 2 hr at 4°C while mixing end over end in a tube rotator. The mixture was centrifuged for 5 sec at 16,000 × g, supernatant containing unbound proteins removed and beads were washed 3 times with 1 ml ice-cold washing buffer (0.1% (w/v) Triton X-100; 50 mM Tris□Cl, pH 7.4; 300 mM NaCl; 5 mM EDTA; 0.02% (w/v) sodium azide) and 1 time with PBS. Finally all the supernatant should be removed and remaining 15µl of sepharose beads containing bound antigen can be analyzed by electrophoresis and immunoblotting.

## 3.8. Isolation of pro-NGF from human brain

The methodology was based on the protocol for isolation of mature NGF from mouse submaxillar gland described by Longo et al. Briefly, frozen human brain tissue from AD-affected or control frontal cortex (8 to 10 g) was

homogenized in 20 ml of sterile water using a Potter on ice. After centrifugation of homogenates (2500 g for 1 hour at 4°C), supernatants were dialyzed overnight against 20 mmol/L Na2HPO4/NaH2PO4 (pH 6.8) using a 10 kDa molecular weight cut-off membrane (Sigma-Aldrich). The samples were loaded on a DEAE Sepharose FF column (Amersham Biosciences) pre-equilibrated in the same buffer. Eluted fractions having absorbance at 280nm more then 0.5 were equilibrated by a second dialysis against 20 mmol/L Na2HPO4/NaH2PO4 (pH 6.8) overnight. Salt concentration was adjusted to 0.4 mol/L NaCl in 50 mmol/L CH3COONa (pH 4.0). The sample was centrifuged at 2500 g for 30 minutes, and the supernatant was loaded on a DEAE-Sepharose FF column previously equilibrated with the same buffer. All of the procedures were performed at 4°C. Eluted fractions with absorbance at 280nm more than 0.1 were collected, concentrated, and analyzed by Western blot using antibodies against either mNGF (H20, Santa Cruz Biotechnology, Santa Cruz, CA) or pro-NGF antibody. mNGF protein was undetectable in all of the fractions obtained using H20 anti-NGF antibody. Absence of Aβ peptide or derived aggregates in the eluted fractions was excluded by Western blot using rabbit polyclonal anti-Aβ1–40 and Aβ1–42 antibodies (Dr. M. Sarasa) or anti-Aβ (Boehringer) used at a dilution of 1:50.

## 3.9. Gel filtration chromatography

Gel filtration chromatography was employed for separation of different molecular weight species of pro-NGF in the extract from human brain. A Superdex G75 (GE Healthcare) was used as packing material, which is able to separate proteins in the range of 15-120kD according to the manufacturer. Column used was 6mmX22cm. The column was packed and equilibrated with loading buffer of 50mM NaOAc pH 4.0, the same buffer was used for preparation of the samples and as mobile phase. After packing the column was checked and calibrated applying a mixture of proteins with known masses: Lyzosyme (14 kDa), Chimotrypsinogen (32 kDa) and Ovalbumine (56 kDa) 0, 25µg each. A buffer was applied at a speed of 0,1ml/min (~4mm/min) and separation monitored by absorbance at 280nm. The setup used was Amersham Pilot chromatography system. Three distinct picks were observed. The resolving

power was not enough for complete separation of the proteins, but at least separation of half-pick wide was achieved between neighbor picks. Without changing the set up brain, extract containing one mg of total protein was applied and separated under identical conditions. Fractions of 1ml were collected and labeled with mass ranges corresponding to the plotted calibration curved based on the separation of the controls.

## 3.10. Expression, purification and refolding of pro-NGF from Escheria coli

Part of the experimental work was realized thanks to collaboration with the group of Elisabeth Schwarz, University of Halle, Germany.

E. coli strain BL21 (DE3) for the expression of genes under the T7 promoter control was used. Human pro-NGF gene was subcloned from pMGL-SIG-pro-NGF (Roche) into pET11a (Novagene) expression vector. The resulting vector, pET11a-Pro-NGF, was co-transformed with plasmid pUBS520 which contains the dnaY gene, encoding the tRNA that recognizes the rare arginine-codons, in E. coli. pUBS520 contains the gene for kanamycin resistance and bears thep15A origin of replication which is compatible with ColE1-based pET vectors. BL21 (DE3) transformants were grown at 37$^{o}$C in 2 X YT medium (17 g tryptone, 10 g yeast extract and 5 g NaCl per L supplemented with the appropriate antibiotics) to a D600 of 0.5±0.08. Gene expression was induced by addition of 3 mM isopropyl thio-b-d-galactoside (IPTG) and subsequent cultivation for four hours. Cells were harvested by centrifugation 15 min 6000g. Inclusion bodies were isolated by resuspending the pellet in 5ml of buffer A (0.1M Tris-Cl pH 7; 1mM EDTA) for every 1g of bacterial pellet, 1mg Lysozyme was added, and kept on ice for 30 min. Then, $MgCl_2$ up to 3mM and DNAseI up to 10µg/ml were added and the suspension was sonicated well. After sonication the same amount of DNAseI was added and kept for 30 min at room temperature. After 30 min ½ volume of Buffer B (60mM EDTA 6% Triton X100 1,5M NaCl pH 7.0) was added and suspension was incubated for 30 min at room temperature with magnetic stirrer. The suspension was spinned down and resuspended in 5ml mixture 1:1 buffer B and buffer C (0.1M Tris-Cl; 20mM EDTA), then, spinned down and resuspended in 5 ml buffer C, and finally,

spinned down again( repeated 5 times). The pellet was dissolved with 1ml Solubilisation buffer (6M Guanidine; 0.1M Tris-Cl pH 8,0; 1mM EDTA; 100mM DTT added ex tempore) for every 0,2g pellet by shaking vigorously for more than 2h at 37°C. The solution was dialyzed against Dialysis buffer (4M Guanidine; 5mM EDTA pH 3-4) to remove completely DTT. The protein concentration was adjusted to 50mg/ml with Dialysis buffer and refolded by adding it stepwise (x100 µl) in 100 ml Refolding buffer (0,1M Tris-Cl; 1M L-Arginine; 5mM EDTA; 1mM Glutatione Ox; 5mM Glutatione Red; pH 9,5) with slow mixing at 4 °C. The proteins were concentrated after refolding using ultra filtration device (Millipore).

For purification of renatured rh-pro-NGF, the renaturation solution was dialyzed against 50 mm Na-phosphate pH 7.0, 1 mM EDTA and applied onto a Poros 20 HS column (4.6 X 100 mm). Refolded species eluted in a single peak at 980 mM NaCl after application of a linear gradient from 0 to 2 M NaCl. Purity was cheeked by SDS-PAGE and biological activity by application to cell cultures.

### 3.10.1. Modifications of rh pro-NGF

To test the effects of protein modifications in increasing the stability of recombinant pro-NGF we used several *in vitro* glycation using Glyoxal and Metylglioxal that react with Arginine in protein molecule producing different glycation products. The reactions for glycation were made by mixing 500µg recombinant protein with 250µl 50µM glyoxal or methylglyoxal and 250µl 100mM sodium phosphate and incubated at 37 °C for 24h. Effects of lipooxidation on recombinant pro-NGF were also tested. The oxidation was performed by mixing 500 µg recombinant pro-NGF with 70 µl 10mM Methyl linoleate; 70 µl 25mM Ascorbate; 70 µl 100 µM $FeCl_3$ ; 70 µl acid $H_2O$ (10µl 10% acetic acid in 990µ $H_2O$) and incubated for 6 h at 37 °C

## 3.11. Production of chicken antibodies

### 3.11.1. Immunization of hens

For producing antibodies we used Hy-Line White Leghorn hens immunized every 3 weeks with 100 µg antigen mixed with 100 µl of Complete Freeland's Adjuvant (CFA) by injecting beneath the loose skin of the chest on the hen (subcutaneously). The eggs were collected after 6 weeks for each hen daily and the yolks were separated from the whites and stored in 50 ml tubes at -20$^o$C.

### 3.11.2. Purification of antibodies from egg yolk

One egg yolk (approximately 15 ml) was mixed with 10 volumes of cold water and stirred at level 4 of intensity until the yolk is completely suspended in the water. The suspension was left overnight at 4$^o$C to let water-insoluble yolk particles to precipitated and centrifuged at 4$^o$C to remove the particles. To the supernatant containing water-soluble proteins slowly was added solid ammonium sulphate to 50 % saturation (313grams/liter) while stirring on ice or in a cold room, left for 30 min at 4$^o$C and centrifuged 30 min at 4$^o$C. The supernatant was discarded and the pallet dissolved in PBS and dialyzed against PBS. This solution contains all antibodies produced of the hen to obtain a single antibody of interest an affinity purification should be performed.

For affinity purification a CNBr-activated sepharose was used. 1ml sepharose was washed several times with 1 mM HCl for 15 min and 5-10mg antigen protein was dissolved in 5ml coupling buffer (0,1 M $NaHCO_3$ ; 0,5M NaCl ph 8,3). Sepharose and protein solution was mixed gently overnight at 4$^o$C the excess of loading buffer was washed with 5 gel volumes of coupling buffer remaining active groups ware blocked with 0,1M Tris-Cl pH 8,0 for 2h. The beds ware washed 3 times with alternating pHs: 0,1M NaAc; 0,5M NaCl pH 4 and 0,1M Tris 0,5M NaCl pH 8 and finaly washed with 10 ml PBS. 1ml of the solution containing antibodies and 4 ml PBS was mixed with the beds and let rolling overnight at 4$^o$C. The beds were washed with 10 ml PBS to remove no conjugated antibodies and at that stage the beds was packed into a column. The elution of antibodies was performed with 0,1M Glycine pH 2,4 in 1ml fractions in eppendorf tubes containing 50µl 1M Tris pH 9,5. The fractions containing protein ware pooled dialyzed against 3 changes of PBS and concentrated using Amicon Ultra Centrifugal filter devices (Millipore).

## 3.12. List of used antibodies

Anti-human ICDp75 was either from Promega (G3231; Madison, WI) or 9992 kindly provided by M.V. Chao.

Anti-p75NTR antibody (REX; directed against the extracellular domain) was kindly provided by L.F. Reichardt (Howard Hughes Medical Institute, University of California).

Anti-β-actin antibody (AC-15) was obtained from Sigma-Aldrich.

Anti-NRH2 antibody was kindly provided by P.A. Barker (Montreal Neurological Institute, McGill University, Canada).

Secondary antibodies (antimouse IgG-HRP and anti-rabbit IgG-HRP) were obtained from Amersham Biosciences.

Anti-pre-pro-NGF directed against the amino acid sequence 144 to 166 of pro-NGF was obtained from Pro-Hormone Science.

Antibodies against mNGF, H20, and anti-2.5S NGF were purchased from Santa Cruz and Cedarlane Laboratories, respectively.

Anti-GFAP antibody from Dako.

The antibody against pro-NGF pro-domain was raised as described previously by Beattie et al. In brief, GST fusion protein containing asp23-arg81 peptide from human pro-NGF was used to immunize New Zealand rabbits (Charles River Laboratories, Wilmington, MA). Specific anti-sera was purified by first incubating whole serum with GST to adsorb GST-specific immunoreactivity and then followed by adsorption to, and elution from, a glutathione column to which GST-pro-NGF was immobilized.

Anti-E2F1 (C20) scr-193 from Santa Cruz

Anti-Chicken IgY (IgG) Peroxidase conjugated from SIGMA

Anti-CML monoclonal antibody (clone No. SMS-10) Peroxidase conjugated from Cosmo Bio Co

Anti-CEL monoclonal antibody (clone No. KNH-30) Peroxidase conjugated from Cosmo Bio Co

Anti-AGEs monoclonal antibody (clone No. 6D12) Peroxidase conjugated from Cosmo Bio Co

Anti-MDA monoclonal antibody (clone 1F83) from JaICA

Goat anti-Neuroketals polyclonal antibody from Chemicon

# 4. Nucleic acids methods

## 4.1. Bacterial cell cultures

### 4.1.1. Growing bacterial cultures and preparing glycerol stocks for freezing

All bacterial cultures were grown in liquid medium LB Broth (Sharlab), containing 10g/l Casein peptone; 5g/l Yeast extract and solid medium LB Agar (Sharlab) containing 10g/l Casein peptone; 5g/l Yeast extract; 15 g/l Agar. Both medias ware sterilized by autoclave at 121$^{o}$C for 15 min. The solid medium was left to cool at room temperature until 50$^{o}$C and appropriate selective antibiotic (100µg/ml Ampicilin or 50µg/ml Kanamicin) was added and finally the medium was poured into 90mm plates by pouring 20 ml medium into each plate and leaving them to polymerize at room temperature. The polymerized plates were stored at 4$^{o}$C.

The bacterial colonies containing plasmid of interest were stored by mixing 500µl of saturated liquid culture with 500µl 50% Glycerol in criovials and can be stored at -80$^{o}$C for years.

### 4.1.2. Preparing of competent cells

The competent cells were prepared by growing bacterial culture in 500ml sterile LB without antibiotics on a shaker at 37$^{o}$C until culture reaches OD about 0,3-0,4 at 600nm for 1sm pathlenght. The bacterial culture was centrifuged at 5000 RPM for 10min at 4 $^{o}$C and the pellet was resuspended on ice in ¼ volume ice cold 100mM $MgCl_2$ and suspension was centrifuged at 4000 RPM for 10 min at 4 $^{o}$C. The pellet was resuspended on ice in 1/20 volume of ice cold 100mM $CaCl_2$ and additional 9/20 volume of ice cold 100mM $CaCl_2$ was added and suspension kept on ice for at least 20 min. The cell suspension was centrifuged at 4000 RPM for 10 min at 4 $^{o}$C and the pellet was resuspended on ice in 1/50 volume of ice cold 85 mM $CaCl_2$ in 15% glycerol w/v dispenced on 100 µl aliquots and freezed at -80 $^{o}$C. All solutions in this protocol must be steril!

### 4.1.3. Transforming competent cells with plasmid DNA and selection of positive colonies

The competent cells were removed from –80°C and thawed on ice for 5-10 min. The cells were gently mixed and 10-100 µg of plasmid (depending on plasmid size and quality), the volume of DNA solution should not exceed 5% of the volume of the competent cells or no more than 50 ng in a volume of 10 µL or less) was added and the tubes were gently mixed by finger flicking for a few seconds and incubated on ice for 30 minutes. The tubes were heat shocked by placing in 42°C water bath for ~30 seconds without shaking. Then, replaced on ice for 2-5 minutes and 900 µL of S.O.C. (2% Tryptone, 0.5% Yeast Extract, 0.4% glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, & MgSO4) or LB broth was added and the tubes were incubated at 37°C for 30-60 minutes with shaking. The 2 parts of every suspension (20 and 200 µl) was evenly spreaded on LB agar plates containing appropriate antibiotic using a bent glass rod spreader dipped in ethanol and flamed it before every spreading. The plates ware placed in the 37°C incubator with the agar side up and the lid side down and incubated.

### 4.1.4. Extraction of plasmid DNA from bacterial cell cultures

For extraction of plasmid DNA from bacterial cell cultures QIAprep Spin Miniprep Kit (50) and QIAGEN Plasmid Midi Kit (25) (QIAGEN) were used by following the protocols listed by the manufacturer.

## 4.2. Agarose gel DNA and RNA separation and analisys

For analysis of DNA fragments and plasmids and RNA, agarose gels were used containing between 0,8% and 2% of agarose depending of the size of the analyzed fragments. The gels were prepared by boiling agarose 2D (Conda) in TAE buffer (40 mM TRIS; 20mM Sodium Acetate; 1mM EDTA pH=8) in a microwave until complete dissolving of agarose particles and poured in gel rack with appropriate dimensions and was inserted a comb at one side of the

gel to form the slots in the polymerized gel. The gel was putted into a tank with TBE buffer and must be completely covered with TBE, with the slots at the end electrode that will have the negative current. After the gel has been prepared, with use of a micropipette about 2.5-5 µl of a DNA ladder (100bp or 1KB) or λHindIII was loaded. The DNA samples were mixed with 5X loading buffer (1mM EDTA; 5% glycerol; 0.04% Bromphenol blue) and loaded into the slots. The gels were typically run at 60V-100 V for 30 minutes-1h for 50 ml of gel. The colored dye in the DNA ladder and DNA samples acts as a "front wave" that runs faster than the DNA itself. When the "front wave" approaches the end of the gel, the current was stopped. The DNA is stained with ethidium bromide, or with sibergreen and is then visible under ultraviolet light.

## 4.3. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a technique widely used in molecular biology for amplification of a piece of DNA by in vitro enzymatic replication using Taq DNA polymerase. With PCR it is possible to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece.

For our experiments we used termocicler and for 50µl reaction we used the following reaction mix: 5µl 10x PCR buffer; 3,5µl 50 mM $MgCl_2$; 1µl 10µM Forward Primer; 1µl 10µM Reverse Primer; 1µl dNRPs; 0,3µl Taq DNA Polymerase; 33,2µl $H_2O$. (Biotools). We used a standart PCR program with variation of the numbers of cycles and the annealing temperature depending of primers used.

## 4.4. Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is a technique for amplifying a defined piece of a ribonucleic acid (RNA) molecule. The RNA strand is first reverse transcribed into its DNA complement or complementary DNA, followed by amplification of the resulting DNA using polymerase chain reaction. In the first step of RT-PCR, called the "first strand reaction," complementary DNA (cDNA) is made from a messenger RNA

template using dNTPs and an RNA-dependent DNA polymerase (reverse transcriptase) through the process of reverse transcription. Reverse transcriptase is incapable of de novo synthesis and requires a double-stranded primer. These primers will anneal to the 3' end of every mRNA in the solution, allowing 5' → 3' synthesis of cDNA by the reverse transcriptase enzyme. All the equipment and reagents must be kept RNase-free.

For our experiments we used Superscript II RT (Invitrogen) and Oligo (dT) $_{12\text{-}18}$ (Invitrogen) following the protocol listed by the manufacturer. After the cDNA creation the original RNA template was degraded by using Ribonuclease H (TaKaRa), leaving pure cDNA that facilitates the following PCR amplification.

## 4.5. Genes subcloning in prokaryotic and eukaryotic expression vectors

### 4.5.1. Gene fragment amplification

The desired gene of interest can be amplificated by PCR reaction or by bacterial amplification if it is inserted in a plasmid. When PCR amplification is used in the Forward and Reverse primers a restriction sites can be inserted that allows the more efficient blunt end ligation and facilitates the cloning.

### 4.5.2. Restriction enzymes digestion

For our experiments we used restriction enzymes from New England Biolabs and from TaKaRa following the protocols listed of the manufacturers and using recommended digestion buffers and temperatures.

### 4.5.3. Producing blunt end fragments

When blunt end products were needed we treat the nucleotide fragments with DNA polymerase I, Large (Klenow) Fragment (New England Biolabs) following the protocol listed byf the manufacturer.

### 4.5.4. Dephosphorilation

To prevent the plasmid backbone autoligation the ends of the cleaved plasmids were dephosphoryled using Antarctic Phosphatase (New England

Biolabs) that can be easily inactivated by heating at 65 $^oC$ for 5 min, following the protocol listed by the manufacturer.

### 4.5.5. DNA PCR purification and extraction from agarose gel

After PCR reaction the fragments were analyzed using agarose gel and if only one clear bad was observed the DNA fragments ware cleaned using MiniElute PCR purification Kit (Quagen). When cleaning of DNA fragments from termostable restrictases after restriction digestion or other DNA fragments was needed the DNA fragments were analyzed using agarose gel and bands of interest were cut and extracted from the gel using QIAquick Gel Extraction Kit (Quiagen) following the protocol listed of the manufacturer. This avoids the use of low melting agarose.

### 4.5.6. Ligation

Ligation of DNA fragments was performed by mixing 100ng open vector; insert 2:1 to 10:1 molar ratio of insert:vector; 1 µl 5 mM ATP; 1 µ 10x ligation Buffer (TaCaRa); 0,5 µl Ligase (TaCaRa); $H_2O$ up to 10 µl and incubating overnight at 16 $^oC$. For determine the quality of the vector, two controls were performed: control without insert and control without ligase. The next day competent bacterial cells were transformed and positive colonies (if any) were cultivated for checking the presence of plasmid with insert. If in the plates transformed with controls are observed same amount of colonies the procedure was repeated from the beginning. The colonies were checked by performing PCR reaction with plasmid extracted and forward primer complementary of T7 promoter and reverse complementary of the gene of interest.

## 4.6. Sequencing

To confirm not only the presence of desired insert but also the sequence the sequencing was performed using following sequencing primers:

T7 Forward /TAATACGACTCACTATAGGG/

CMV Forward /CGCAAATGGGCGGTAG/

BGH Reverse /TAGAAGGCACAGTCGAGG/

WPI Forward /AGACTGTATGTTTGGGAATGGG/
WPT Forward /TCTTTTTCGCAACGGGTTT/

The sequencing was performed by direct sequencing using ABI PRISM 3100 Genetic Analyzer (Applied biosystems). The reaction mixture contains 2 µl Terminator Ready Reaction Mix (Applied biosystems) 2 µl of template (should contains 100-1000ng DNA); 1ul 4 µM primer. The control reaction contains 2 µl Terminator Ready Reaction Mix (Applied biosystems); 1ul M13 primer; 0,5 µl pGEM plasmid; 1,5 µl $H_2O$. The reactions were run on the thermocicler with following program: 1min 96 $^oC$; (10 sec 96 $^oC$ ; 5 sec 50 $^oC$; 4min 60 $^oC$)x25; ∞ 4 $^oC$. After the reaction the reactions ware precipitated with 1 µl 125mM EDTA; 1 µl 3M AcNa pH 5,3; 50 µl 100% EtOH and leaved for 15 minutes at room temperature in a dark. The precipitates ware centrifuged at 12000 RPM for 20 minutes and the supernatant was aspirated with vacuum pump. The pellets were washed with 35 µl 70% EtOH and centrifuged again at 12000 RPM for 5 minutes. The supernatant was aspirated and the pellets let to dry in a dark. The dry pellets were rehydrated with 15 µl HiDi (high desionized) Formamid for 30 minutes at room temperature in a dark.

## 5. Densitometry and statistical Analysis

The density of the immunoreactive bands was determined by densitometry analysis using GS-800 Calibrated Densitometer (Bio-Rad). Pixel values for problem samples were compared to control values in at least four separate Western blots. Statistical significance between groups was calculated using Student's t-test.

## 6. Chicken embryos treatment

For our experiments chick embryos (*Hy-line white*) were used. Fertilized eggs were purchased from Avigan Terralta (Vinallop, Tarragona, Catalonia, Spain) and incubated at 37.5 ºC and 60% relative humidity. Embryos were staged *in ovo* or at the time of sampling according to the Hamburger and

Hamilton (1951) stage series. All experimental protocols used were approved by the Committee for Animal Care and Use of the University of Lleida.

For "in ovo" experiments the eggs were windowed on E5 and treated with 100μg pro-NGF (30μl of 3,3μg/μl ) and with 30μl saline solution for the controls, every day during next 3 to 5 days. The window in the shell was sealed with adhesive tape and the eggs returned to the incubator, where they remained until next day treatment. One part of the chicken embryos was killed at E8 and medulla spinal extracted and prepared for microscopical observation and counting of picnotic nucleus in the motoneuronal cells. Other part of the chicken embryos was killed at E8 and medulla spinal extracted and prepared for microscopical observation and counting the number of the motoneuronal cells.

# 7. Stereotaxis methods for in vivo mouse treatment

## 7.1. *In vivo* mouse treatment

For introducing pro-NGF in the mouse brain we used a Precision Stereotaxic Instrument. The mice were anaesthetizised with exposing them to inhalational anesthesia (Isoflurane) for 1-2 minutes and after that injected with mixture of 2% Rompun (Bayer) and 10% Ketamin (WDT) in 0,9% NaCl (10 μl for every 1g of mouse weight). Using the Hamilton syringle 4μl of pro-NGF or 0,9% NaCl solution for the controls were intracerebroventriculary injected in the right ventricle (A-0,0mm; L+0,8mm; V+2,2mm) with 1μl/min speed. The needle was left in place for 1minute before retraction and retracted slowly with 1mm/min speed. The Hamilton syringle used for intracerebroventriculary injections was repeatedly washed with distilled water, followed by flushing with 1 μg/ μl BSA solution, which reduces drastically protein binding to glass. This procedure was performed before every injection. The incisions were sewed up and the mousse left for 1 week to restore.

## 7.2. Behavioral tests

All of the behavioral procedures were conducted at the same time of the day in an isolated room every day during 5 consecutive days. The mice were trained to find a 50 $cm^2$ hidden platform 1cm under the water surface in a water

tank of 150 cm of diameter, with four different geometrical forms attached to the 4 sides of the water tank wall. Four trials per day with start positions close to the four geometrical signs were performed, and latency to reach the platform was recorded. Cutoff time to find the platform was 120 s, and mice failing to find the platform were placed on it and left there for 15 s. Each trail for single animal was 30min apart from the previous.

# VIII. RESULTS

# VIII. RESULTS

## 1. Increased levels of p75ICD from p75NTR processing were observed in vivo and in vitro

p75ICD is a 20 kDa product from γ-Secretase activity on the p75NTR transmembrane receptor. It has recently been suggested that it is involved in p75NTR signalling [Frade 2005; Zampieri 2005; Domeniconi 2005]. γ-Secretase is active in AD-affected human brain and has an important role in the etiopathogenesis of this disease [Selkoe 2003]. The increase of p75NTR in AD-affected human brain is not fully accepted in the literature. To investigate further the pathophysiological role of p75NTR, we first wanted to verify a possible increase in the 20 kDa p75ICD levels in AD. We used an antibody raised against the p75ICD, as described in the "Materials and Methods", to analyze human samples with AD pathology compared to control probes from frontal and entorhinal cortex at an advanced stage of the disease (stage C, following the staging of Aβ burden of Braak and Braak [Braak 1999]) (figure 1r A and B). The use of this antibody showed the presence of 25- and 20 kDa bands corresponding to p75CTF and p75ICD respectively, as sequential products of α- and γ-Secretase [Zampieri 2005]. Both fragments appeared to be increased in AD entorhinal cortex (figure 1r A). Densitometry analysis of the Western blots with anti-p75NTR revealed a significant rise of the 20 kDa band in entorhinal cortex, which was significantly increased in AD compared to control brains (figure 1r B). However, no differences between control cases and AD patients were observed with respect to the amount of 75 kDa band p75NTR in frontal and entorhinal cortex. Ultimately it was shown, from different groups as well as from our, an increase in pro-NGF levels in human AD cerebral cortex [Fahnestock 2001; Pedraza 2005; Peng 2004]. Our results showed not only a significant increase in the pro-NGF levels but also in the p75NTR processing to p75ICD in the same brain samples. The increase of pro-NGF levels in the entorhinal cortex in AD patients were lower than that observed in frontal cortex. But although it is evident that the levels of both p75NTR processing and pro-NGF increase in parallel in the entorhinal cortex. As it has been noted NGF is able to induce γ-Secretase-dependent p75ICD nuclear translocation [Frade

2005], we wanted to assess if this effect could also be observed following ADhbi-pro-NGF p75NTR stimulation. For this purpose, we used pro-NGF isolated from AD-affected human brain (ADhbi-pro-NGF), which was demonstrated earlier to induce apoptosis mediated by its interaction with p75NTR. As can be seen in Figure 2r, ADhbi-pro-NGF was able to induce higher p75ICD levels than NGF.

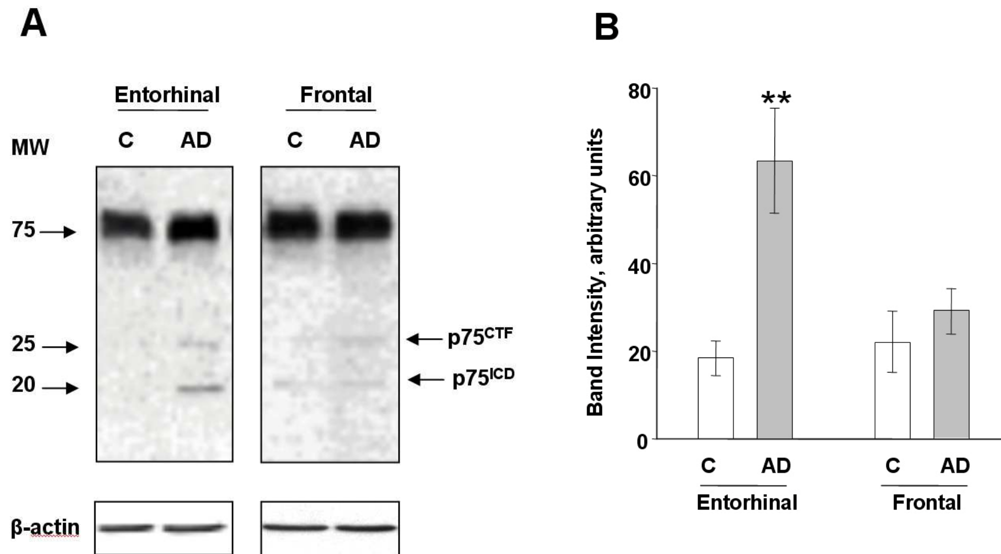



***Figure1r.*** *p75ICD is increased in entorhinal cortex in Alzheimer's disease. (**A**) The p75ICD and p75CTF are detected as 20- and 25 kDa immunoreactive bands, respectively, with an antibody directed against the intracellular domain of p75NTR. The content of p75ICD is clearly increased in stage C in AD entorhinal cortex (line AD) compared to controls (line C). β-Actin was used as a control of protein loading. (**B**) Densitometric analysis of anti-p75ICD immunodetected bands shows a significant increase of 20 kDa p75ICD in AD-affected entorhinal cortex. Bars represent the mean of five samples of stage C (AD) and five control samples (**C**). (**P < 0.01; Student's t-test)*

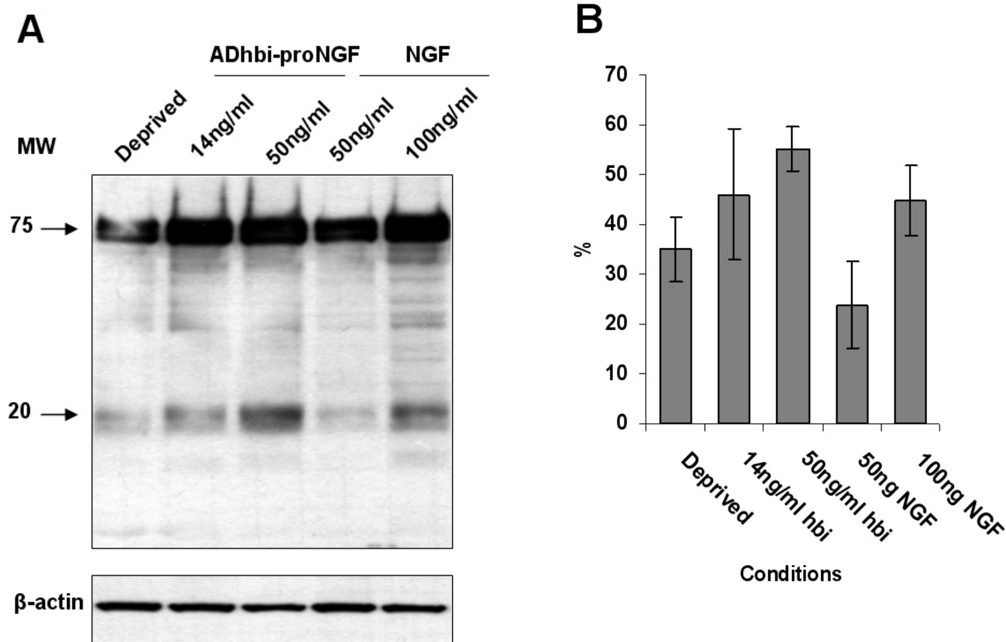



***Figure 2r.*** *p75NTR shedding yielding ICD is induced by ADhbi-pro-NGF and by mNGF treatment. 3T3-p75st cells were serum-deprived (0.5% FBS) and left untreated. The others were treated, with increasing concentrations of ADhbi-pro-NGF and NGF, for 12 hours. 30 µg of total protein lysate was used per lane. (**A**) Western blot using anti-p75ICD antibody (Promega) shows induction of the 20 kDa band. β-Actin was used as a control of protein loading. (**B**) The experiment was repeated three times with similar results. Bands were scanned, and the density of 20 kDa band is expressed as percentage of 75 kDa intensity. Bars represent the mean ±SEM of three independent experiments using Promega antibody (two experiments) and 9992 antibody (one experiment).*

## 2. pro-NGF activation of p75NTR induces nuclear translocation only of p75ICD but not of the full-length p75NTR

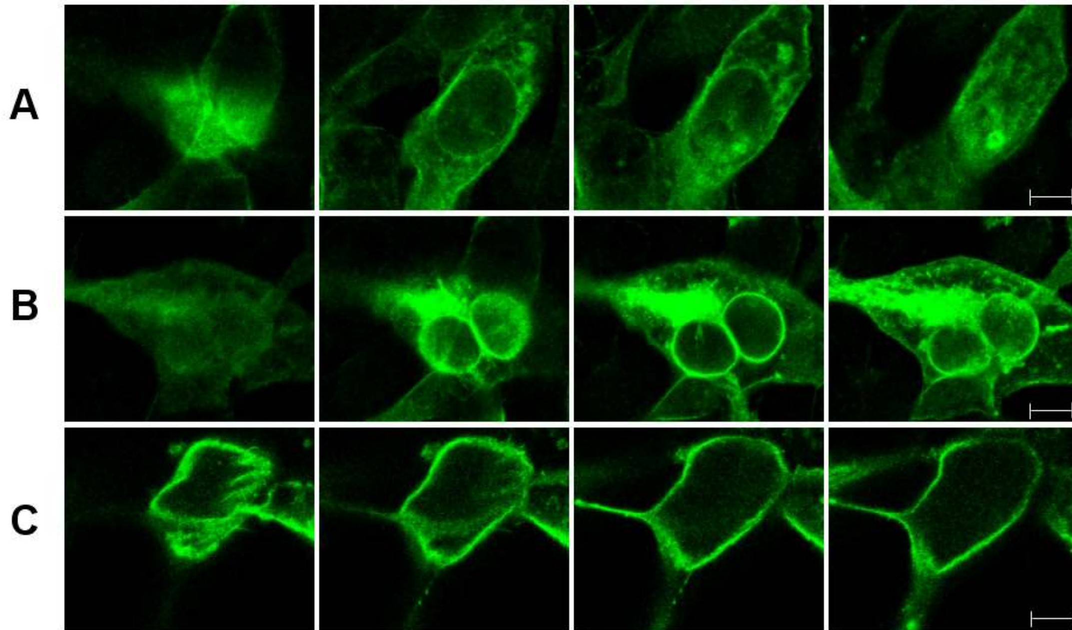



***Figure 3r.*** *ADhbi-pro-NGF induces internalization of p75ICD, but not of the entire p75NTR in 3T3-p75st cells. 3T3-p75st cells were serum-deprived (0.5% FBS) and left untreated (**A**) or treated with 25 ng/ml of ADhbi-pro-NGF (**B** and **C**) for 12 hours. Cells were washed, fixed, permeabilized, and immunostained using anti-p75ICD (**A** and **B**) or REX antibodies (**C**). Representative confocal sequential optical sections of 10 µm from the same cell are shown. Scale bars =40 µm.*

It has been reported that p75ICD translocates to the nucleus on neurotrophin stimulation in RN22-SC cell line and primary Schwann cells [Frade 2005]. Therefore, we wanted to assess whether ADhbi-pro-NGF was responsible for p75ICD yield and whether this was followed by nuclear translocation. For this purpose, we first used a cell model expressing high levels of p75NTR, such as 3T3-p75st, which has a good dose response to this pro-

neurotrophin in terms of a high degree of apoptosis [Pedraza 2005]. Confocal images of 3T3-p75st cells immunostained with an antibody raised against p75ICD showed increased perinuclear distribution in ADhbi-pro-NGF-treated cells (figure 3r A and B). To distinguish between the internalization of full-length p75NTR and the translocation of p75ICD, we used an antibody raised against the extracellular domain of p75NTR (REX). As shown in Figure 3r C, immunoreactive signal remained mainly in the cell membrane even in ADhbi-pro-NGF treated cells. Hoescht staining of the same cells showed the localization of the nuclei (figure 4r A). As shown in Figure 4r B, the percentage of apoptosis in ADhbi-pro-NGF-treated cells was low within the first 24 hours but increased significantly at 48 hours. This may explain the observation that positive cells for p75ICD perinuclear translocation show nonapoptotic nuclei.

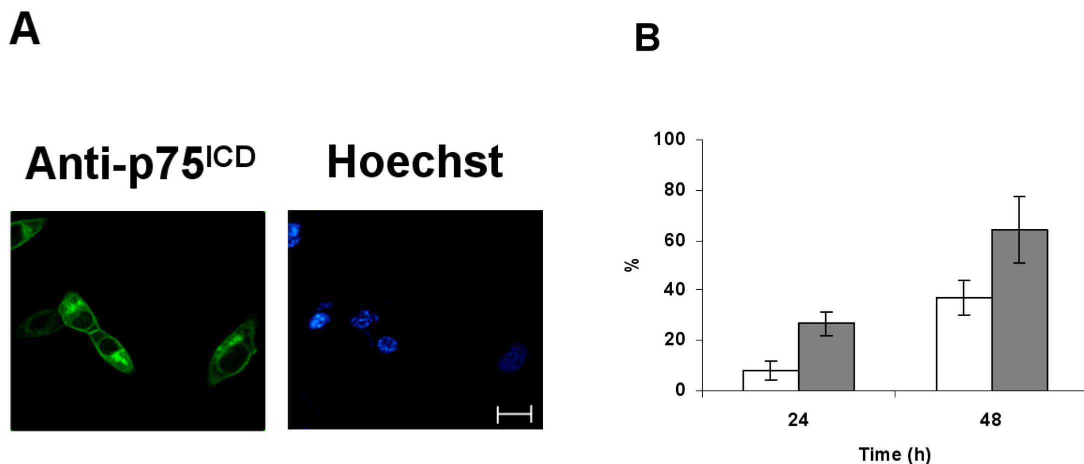



***Figure 4r.*** *Induction of translocation of p75ICD after ADhbi-pro-NGF treatment precedes the induction of apoptosis. (**A**) 3T3-p75st cells were serum-deprived (0.5% FBS), treated with 25 ng/ml ADhbi-pro-NGF for 24 hours, and immunostained for p75ICD. Scale bar* = 90 µm. *(**B**) At time points 24 and 48 hours, the nuclear translocation (**white bars**) and the induction of apoptosis (**gray bars**) were assayed. Percentages are corrected for serumdeprived cells (0.5% FBS). Bars represent the mean* ±SEM *of three independent experiments.*

Because the pattern of localization may depend on many factors, such as the level of expression of p75NTR, the presence or absence of other

membrane proteins or intracellular interacting proteins, we performed parallel experiments with PC12 cells. This model expresses more physiological levels of p75NTR than 3T3-p75st and undergoes apoptosis following ADhbi-pro-NGF treatment.

Figure 5r A shows that ADhbi-pro-NGF induced nuclear translocation of a proportion of p75ICD present in the cell, with immunofluorescence being more or less uniformly distributed throughout the nucleus (figure 5r A, panel c). It co-localized with Hoechst staining, even though there were remaining traces of the signal in the cytoplasm. We observed a lesser degree of nuclear p75ICD translocation with NGF (figure 5r A, panel b) and in deprived cells (figure 5r A, panel a). Following a similar approach, to make sure that this nuclear localization did not correspond to internalization of the full-length p75NTR, we also used REX antibody (figure 5r B). Unprocessed p75NTR remained mainly in the membrane in untreated and NGF-treated cells (figure 5r B, panels a and b) and showed a degree of internalization without nuclear translocation as a consequence of the addition of ADhbi-pro-NGF (figure 5r B, panel c).

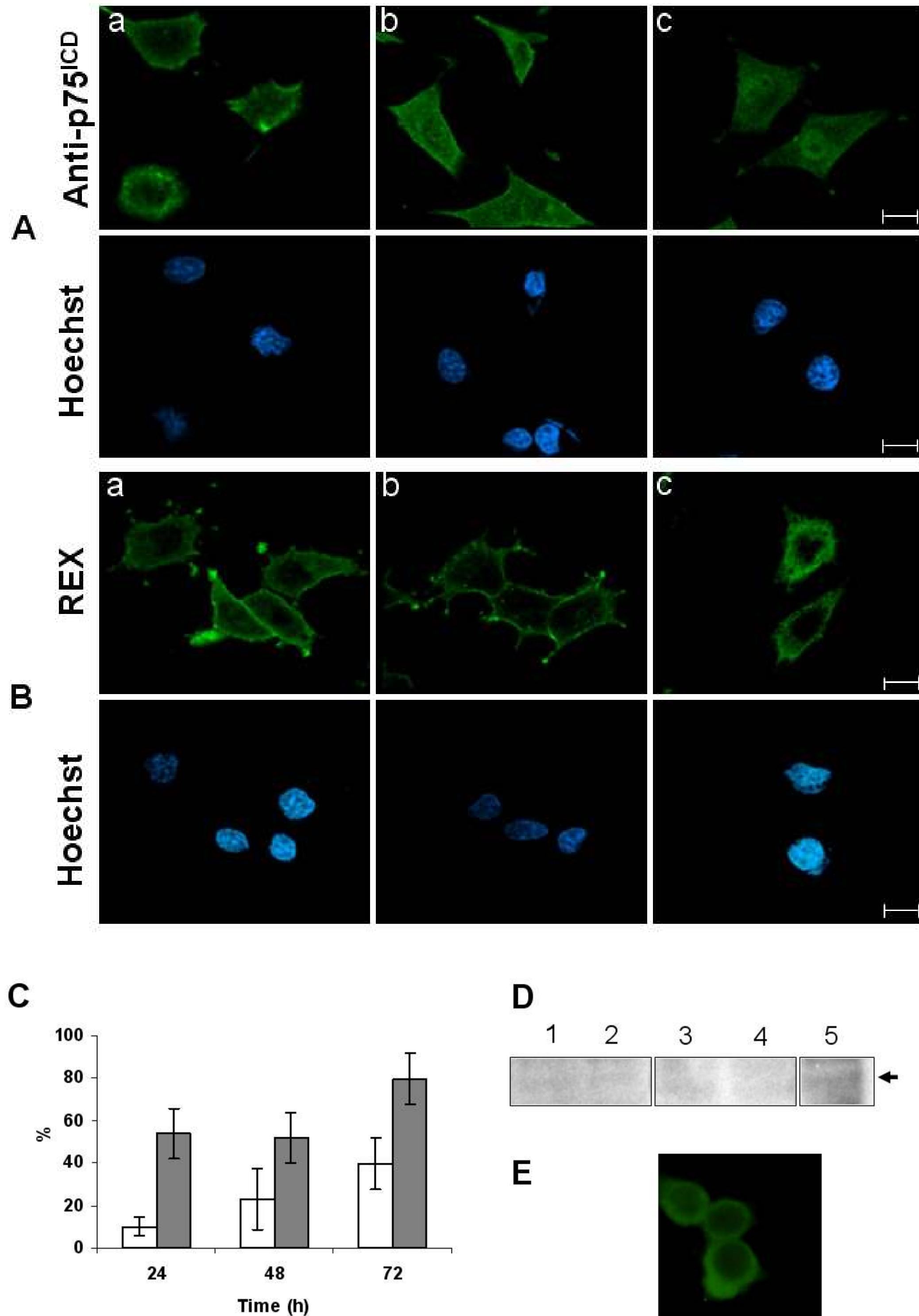



***Figure 5r.*** *ADhbi-pro-NGF induces nuclear translocation of p75ICD but not of the entire p75NTR in PC12 cells. PC12 cells were serum-deprived (**a**),*

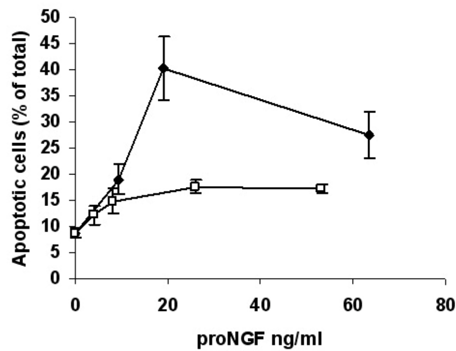
*serum-deprived and treated with 100 ng/ml NGF (**b**), or serum-deprived and treated with 25ng/ml ADhbi-pro-NGF (**c**). Cells were washed, fixed, permeabilized, immunostained with anti-p75ICD antibody (**A**) or with REX antibody (**B**) and visualized with confocal microscopy. Nuclei were stained with Hoechst. (**C**) Apoptosis (**white bars**) and nuclear translocation of p75ICD (**gray bars**) were quantified at 24, 48, and 72 hours. Bars represent the mean* ±SEM *of three independent experiments. (**D**) Western blot using anti-NRH2 antibody shows a band in PC12 total cell lysate (lane 5), which is barely detectable in total lysates from human entorhinal cortex (lanes 1 and 2) and frontal cortex (lanes 3 and 4). The **arrow** indicates apparent molecular weight of 24 kDa. (**E**) Epifluorescence NRH2 immunostaining image of PC12 cells treated with 25ng/ml ADhbi-pro-NGF for 24 hours shows the absence of NRH2 nuclear translocation. Scale bars =50 µm.*

The time course of ICD nuclear translocation and apoptosis of treated PC12 cells (figure 5r C) showed an initial effect of ICD translocation (at 24 hours) and a delayed increase in the number of apoptotic cells. At the time chosen to best observe nuclear translocation (24 hours), the percentage of apoptotic cells was still very low. Cells shown in the figures to be positive for translocation were still not positive for apoptosis. To exclude the possible cross-reaction between the anti-ICDp75NTR antibody and NRH2, we performed Western blotting with an anti-NRH2 antibody. As can be observed (figure 5r D), the 24 kDa positive band in PC12 lysates was not present in any of the control or AD-affected human brain samples. Furthermore, ICD nuclear translocation is clearly visible in an epifluorescence NRH2 immunostaining image of PC12 cells treated with ADhbi-pro-NGF in the same conditions. In addition, the patterns of NRH2 and ICD distribution were completely different. NRH2 labelling was not visible in the nucleus.

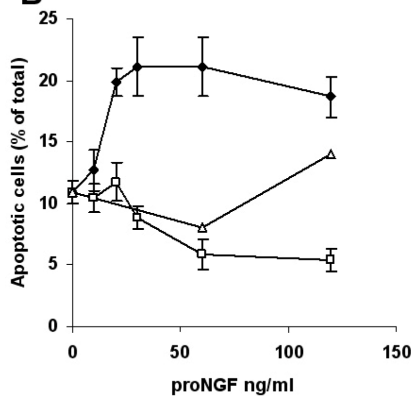
## 3. Control human cerebral cortex contains a pro-NGF that is not actively involved in inducing apoptosis or nuclear p75ICD translocation

An interesting question, related to the pathogenesis of AD, is whether there were any differences in the proapoptotic activity of pro-NGF obtained from AD compared with pro-NGF obtained from age matched controls. Western blot in cerebral cortex homogenates showed a similar molecular weight pattern in AD and controls, although the total amount of pro-NGF changed. [Pedraza 2005] Using the same purification procedure (see Materials and Methods), we obtained pro-NGF from both sources in similar yields. We performed four parallel purifications from each source and assayed induction of apoptosis in 3T3-p75st (figure 6r A) and in PC12 cells (figure 6r B). The percentage of apoptosis was higher in 3T3-75st cells than in PC12 cells.

A




B



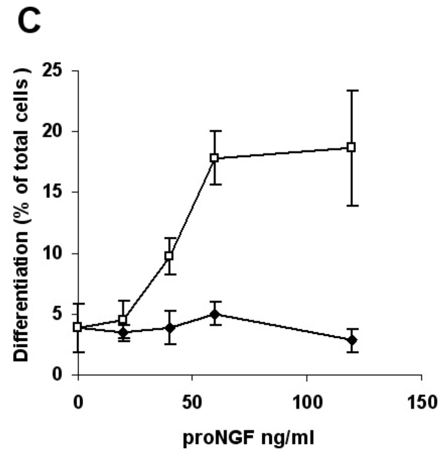
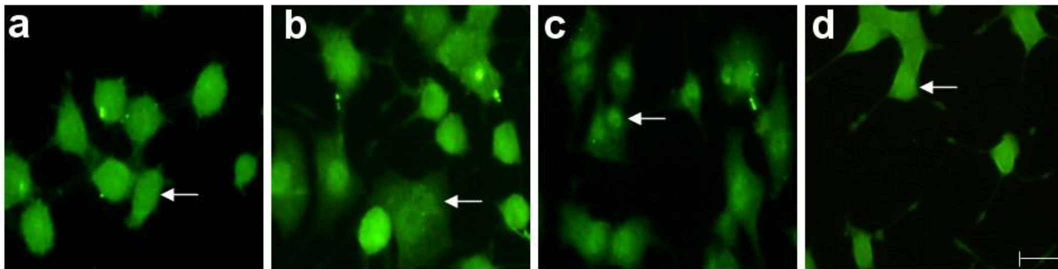



***Figure 6r.*** *ADhbi-pro-NGF but not Chbi-pro-NGF induces apoptosis in p75NTR-expressing cells. Cultured 3T3-p75st cells (**A**) and PC12 cells (**B** and **C**) were serum-deprived (0.5% FBS) and left untreated or treated for 24 hours with increasing concentrations of either ADhbi-pro-NGF (♦) or Chbi-pro- NGF (□) or with NGF (100 ng/ml) (∆)***.*** *Apoptosis was evidenced by nuclear morphology after Hoechst staining. Apoptosis increases in a dose-dependent manner with ADhbi-pro-NGF (**A** and **B**). In contrast, Chbi-pro-NGF treatment resulted in cell differentiation. Differentiated PC12 cells are those with neurite extensions longer than the cell body (**C**). Values represent the mean* ±SEM *of three independent experiments.*

Surprisingly, Chbi-pro-NGF was not able to induce apoptosis in 3T3-p75st or PC12 cells, even at high concentrations. Because 50ng/ml NGF is able to maintain survival and is very specific in inducing differentiation in PC12 cells, we used this cell line to further study whether protection from death following Chbi-pro-NGF treatment (figure 6r B) was due to pro-NGF processing to NGF. Moreover, at the incubation time used for the treatment (24 hours), even 100 ng/ml of mature NGF was not able to induce a significant increase of apoptosis in PC12 cells. Figure 6r C shows that Chbi-pro-NGF, but not ADhbi-pro-NGF, was able to differentiate PC12 cells to a similar degree that NGF. This is an indication that Chbi-pro-NGF is degraded in the cell culture to NGF. In contrast, ADhbi-pro-NGF was especially stable under these conditions.

To determine whether Chbi-pro-NGF had the capacity to induce p75ICD nuclear translocation, PC12 cells were deprived or treated with Chbi-pro-NGF, ADhbi-pro-NGF, or/and NGF. Nuclear or cytoplasmic localizations were counted with the help of an inverted epifluorescence microscope using anti-p75ICD antibody (figure 7r A). Arrows in Figure 7r A (panels a and d) represent the cytoplasmic distribution quantified in Figure 7r B. Arrows in Figure 7r A (panels b and c) represent the nuclear distribution quantified also in Figure 7r B. As can be observed in Figure7r, Chbi-pro-NGF did not induce nuclear p75ICD localization in PC12 cells treated with the same concentration of ADhbi-pro-NGF (25ng/ml). NGF was used at the concentration shown to be effective for inducing survival and differentiation in this cell line (100ng/ml). At this concentration, NGF was more active than Chbi-pro-NGF but less effective than ADhbi-pro-NGF.

A




B

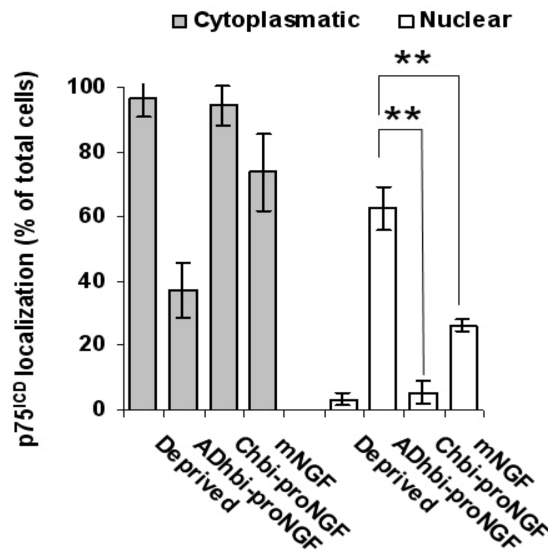
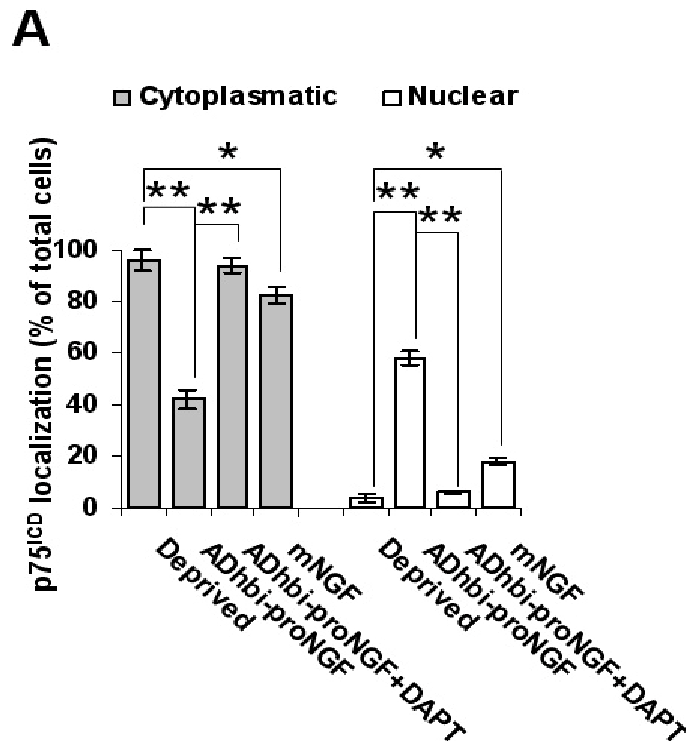


***Figure 7r. A:** ADhbi-pro-NGF but not Chbi-pro-NGF induces nuclear translocation of p75ICD in PC12 cells. PC12 cells were serum-deprived and left untreated (**a**) or treated for 24 hours with 100 ng/ml of NGF (**b**), 25ng/ml ADhbi-pro-NGF (**c**), or 25ng/ml Chbi-pro-NGF (**d**). Cells were washed, fixed, permeabilized, and immunostained with anti-p75ICD antibody. Arrows in **a** and **d** are representative of the cytoplasmic distribution quantified in **B**. Arrows in **b** and **c** are representative of the nuclear distribution quantified in **B**. Cytoplasmic and nuclear p75ICD was examined with the help of an inverted epifluorescence microscope. Bars represent the mean of percentages of at least 500 cells per condition (**B**). *P < 0.05; **P< 0.01; Student's t-test. Values result from three independent experiments. Scale bar 80 µm.*

## 4. γ-Secretase activity is necessary for p75NTR processing and induction of apoptosis as a consequence of hbi-pro-NGF binding



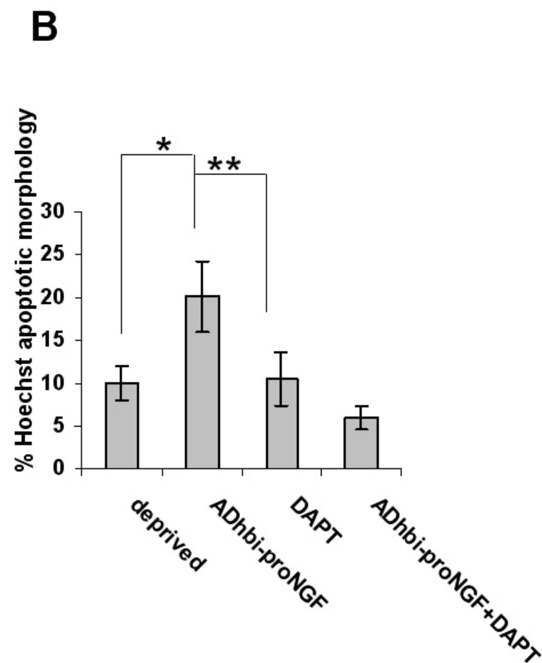



***Figure 8r.*** *Inhibition of γ-Secretase in PC12 cells involves loss of nuclear translocation and decreased apoptosis caused by ADhbi-pro-NGF. PC12 cells were serum-deprived and pre-treated with 1 μmol/L DAPT for 30 minutes, nontreated cells were used as controls. Cells were then treated, or not, with 25 ng/ml ADhbipro- NGF. After 12 hours, cells were washed, fixed, permeabilized, and immunostained with anti-p75ICD antibody. Localization was scored using an inverted epifluorescence microscope (**A**). Apoptotic morphology, as visualized with Hoechst staining, was quantified at 24 hours and expressed as a percentage of the total number of cells (**B**). Bars represent the mean of percentages of at least 500 cells per condition. *P < 0.05; **P < 0.01; Student's t-test. Values are the result of three independent experiments.*

To assess whether γ-Secretase processing of p75ICD could be involved in the induction of apoptosis, we used DAPT to specifically inhibit this enzyme. Nuclei were stained with Hoechst, and the percentage of pyknotic nuclei among the total number of nuclei was measured as described in Materials and Methods (figure 8r B). TUNEL yielded similar results as those obtained by morphological criteria.

When stimulated with pro-NGF, PC12 cells showed both apoptosis and p75ICD nuclear translocation. Pre-treatment of these cells with DAPT

completely blocked p75ICD nuclear translocation, as well as apoptosis induced by ADhbi-pro-NGF (figure 8r). The apoptosis level under these conditions was even lower than with serum deprivation, suggesting that p75NTR processing could also be, partially, involved in PC12 apoptosis under conditions of deprivation (figure 8r B). To confirm further that α- and γ-Secretase activities were involved in p75NTR processing under ADhbi-pro-NGF binding, we treated 3T3-p75st cells with ADhbipro-NGF in both the presence and absence of DAPT (figure 9r), under the same conditions shown to block p75ICD nuclear translocation in PC12 cells (figure 8r).

As seen in Figure 9r, the use of a γ-Secretase inhibitor completely blocked the yield of p75ICD, proportionally increasing the amount of p75CTF. As previously described in different cell models for p75NTR [Zampieri 2005] and also for different membrane-associated proteins such as Aβ precursor peptide and Notch, [Selkoe 2003] the cleavage of γ-Secretase is preceded by α-Secretase cleavage. We demonstrated (figure 9r) that DAPT blocked the yield of p75ICD producing an accumulation in p75CTF, which was also sensitive to ADhbi-pro-NGF p75NTR activation; p75CTF levels were also increased following pro-neurotrophin treatment.

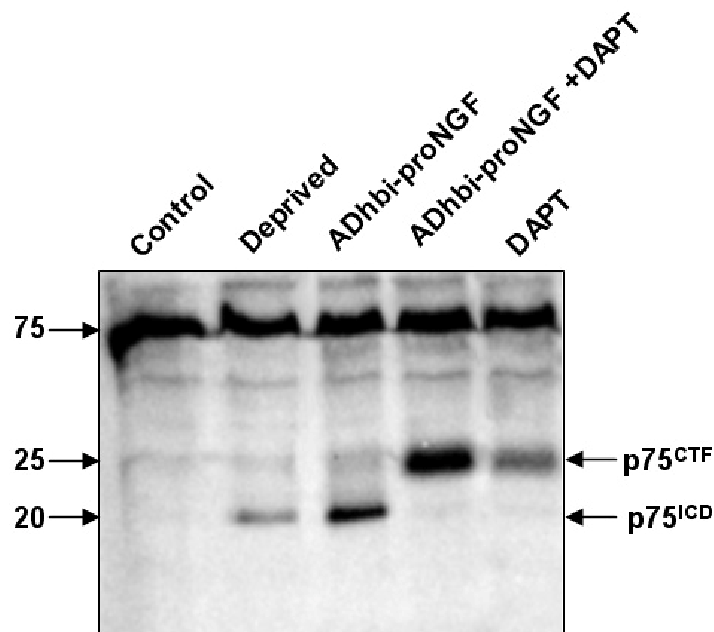


***Figure 9r.*** *Inhibition of γ-Secretase in 3T3-p75st cells results in loss of the 20 kDa p75ICD band together with accumulation of the 25 kDa p75CTF fragment. 3T3-p75st cells were serum-deprived (0.5% FBS) and pretreated for 30 minutes with DAPT. Subsequently, the cells were treated with 25ng/ml ADhbi-pro-NGF or with 100 ng/ml NGF for 12 hours in Dulbecco's modified Eagle's medium containing 0.5% serum, or they were maintained in media containing 10% serum as controls. 30 μg of total protein was used per lane. Western blots using 9992 anti-p75ICD antibody showed characteristic 20 kDa p75ICD and 25 kDa p75CTF bands. The experiment was repeated three times with similar results using 9992 (two experiments) and Promega (one experiment) anti-p75ICD antibodies.*

## 5. Study of Aβ induction of apoptosis mediated by p75NTR and nuclear translocation of p75ICD.

It is described that p75NTR binds Aβ and mediates Aβ toxicity in PC12, SK-NMC, NIH 3T3, and SK-N-BE cell lines, suggesting that p75NTR acts as a death receptor for Aβ toxicity [Rabizadeh et al., 1994; Yaar et al., 1997, 2002; Kuner et al., 1998; Perini et al., 2002]. According to our findings that hbi-pro-NGF is involved in inducing apoptosis and nuclear p75ICD translocation we decided to test whether p75NTR binding of Aβ would have the same effects and follow a similar mechanism. For that reason we treated the PC12 cells both cycling and differentiated with 10μM Aβ peptide (25-35) and as control with same concentration of Aβ peptide with reversed sequence. As described in methods, blocking of p75NTR was performed with pre-incubation for 2 hours before the Aβ treatment with an antibody (REX) raised against the extracellular domain of p75NTR. 72 hours after the Aβ treatment was performed the MTT assay. As it is shown in Figure 10r A and B we were not able to observe any significant difference between cells treated with REX antibody and not treated cells. The apoptotic effect of Aβ treatment itself was weak and no significant either in differentiated or in cycling PC12 cells.

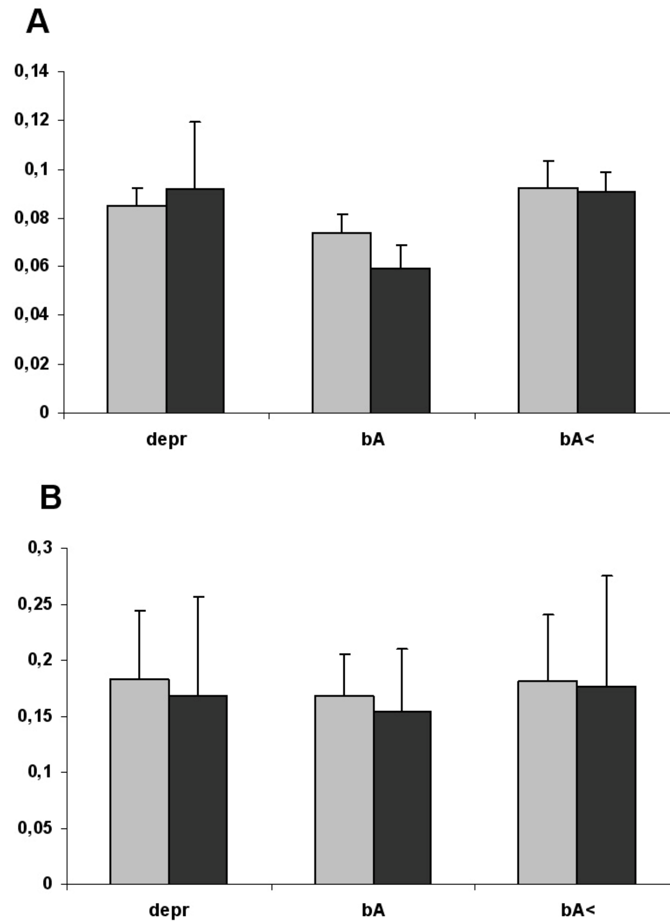



***Figure 10r.*** *Treatment of PC12 cells with REX antibody is not able to prevent cell death caused by Aβ peptide (25-35) treatment. PC 12 cells was differentiated with 50ng/ml NGF for 7 days (**A**) or let cycling (**B**), pre-treated with 50ng/ml REX antibody for 2 hours (**black bars**) or left without REX antibody (**grey bars**) and treated with 10μM Aβ peptide (25-35) and 10μM Aβ peptide (35-25).*

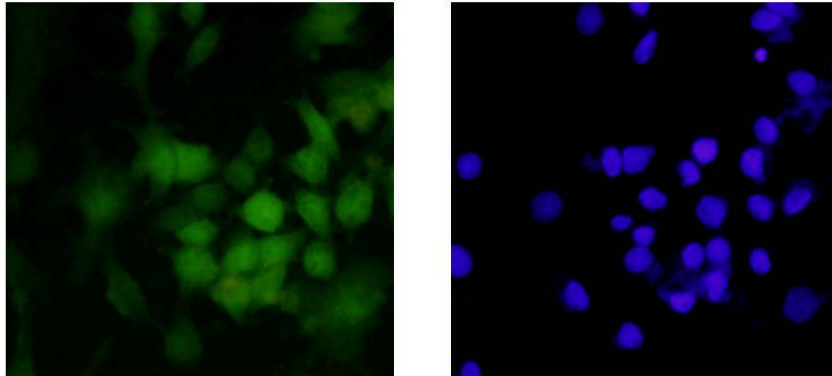
We also used Hoechst staining to observe pyknotic cell nuclei and to count the percentage of positive cells. We obtained similar results without significant differences between the treatments.

To examine the effects of Aβ in cell model that express higher endogenous levels of p75NTR we transfected 293FT cells using $CaCl_2$ with pcDNA3.1 plasmid containing p75NTR cDNA and performed the same experiments. The results did not show significant differences between conditions, neither using MTT assay nor Hoechst staining.

Immunostaining with antibody raised against the intracellular domain of p75NTR did not present differences in internalization and cellular localization of either p75NTR or of its ICD in any of the used cell lines.

## 6. Induction of cell death by over-expression of intracellular domain of p75NTR.

In order to study additionally the role of p75ICD nuclear translocation as a factor directly causing apoptosis under the activation of the receptor by pro-NGF, we studied the effect of the expression of ICD in different cell cultures. For that reason we subclonet the ICD fragment of rat p75NTR in pcDNA3.1 plasmid and transfected 293FT cells with it using $CaCl_2$. We also cloned the same construct into pWPTI lentiviral plasmid and packed it into lentivius vector which is able to transfect with high efficiency different cell lines. We used this lentiviral vectors to transfect PC12 cell line that express p75NTR and 3T3 cell line that do not express p75NTR. We were able to achieve very high cytoplasmatic concentrations of ICD in all cell types (figure 11r) but without any nuclear specific localization.



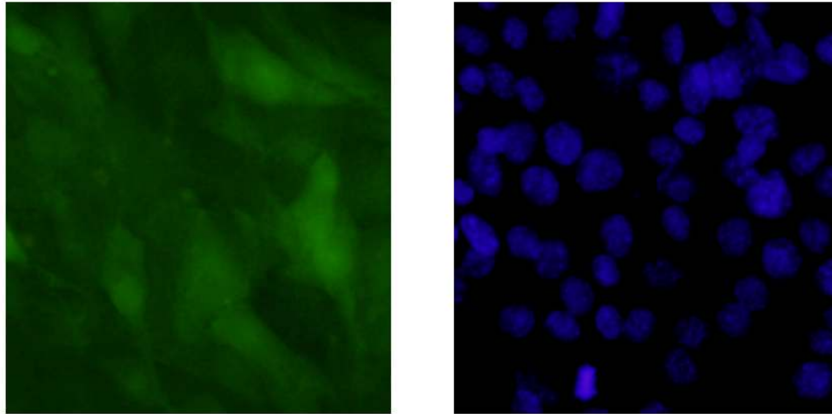
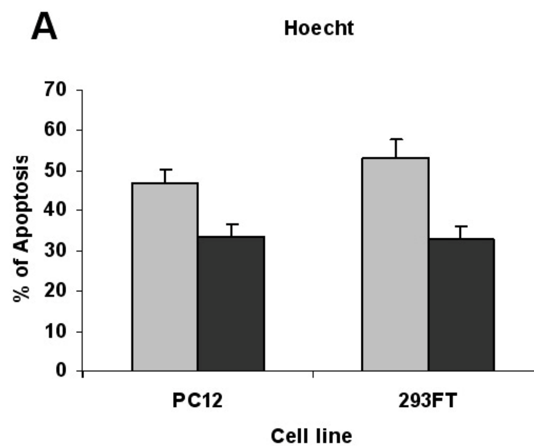



***Figure 11r.*** *PC12 cells transfected with lentivirus vector and expressing high concentration of p75NTR ICD and Hoechst staining of the same cells(**A**) and 3T3wt cells transfected using lentivirus vector and expressing high concentration of p75NTR ICD and Hoechst staining of the same cells (**B**).*

We were not able to detect an increase in apoptosis in the cells expressing high levels of ICD. In fact the cells transfected with ICD shows higher viability compared to the cells transfected with empty vector (figure 12r). In both MTT (figure 12r B) and Hoechst staining (figure 12r A) assays the results were very similar.



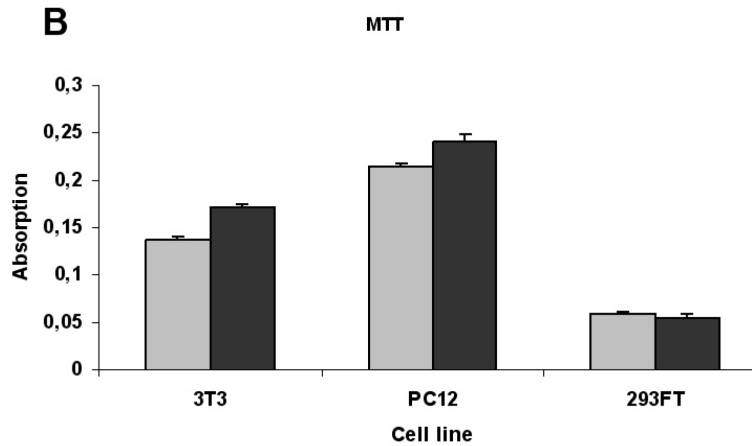



***Figure 12r.*** *3T3 and PC12 cells transfected with lentivirus vector and 293FT cells transfected with $CaCl_2$ are expressing high levels of p75NTR ICD. Viability of the cells was measured after 24 hours using Hoechst (**A**) and after 48 hours MTT (**B**) assays.*

## 7. Recombinant pro-NGF induces apoptosis and motoneuron loss in chicken embryo lumbar lateral motor column.

We wanted to study the role of pro-NGF and his interaction with p75NTR in neuronal development. As a model for our experiments we used chicken embryos. First we studied the p75NTR expression in different embryonal stages of chicken spinal cord. We extracted lumbar region of spinal cord and retina of chicken embryos in different days of embryonal development and prepared from them lysates for Western blot. The result from Western blot reveals an increase of p75NTR expression in the early days of embryonal development with highest levels in day 8 (figure 13).

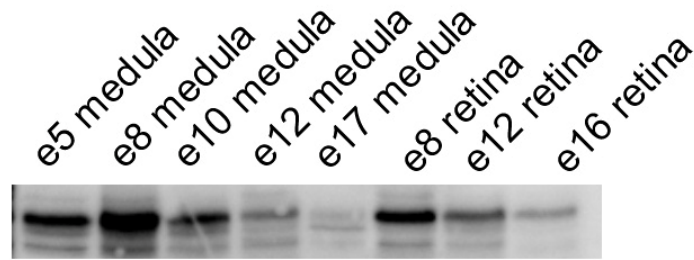



***Figure 13.*** *Western blot for p75NTR expression in chicken embryonal spinal cord and retina samples.*

We examined the role of pro-NGF in chicken embryonal development by introducing recombinant pro-NGF in the chicken embryos in days from e5 to e7 and preparing the spinal cords at day 8 for counting picnotic cell nucleus (figure 14A). Also we treated other group of chicken embryos with pro-NGF in days 5 to 9 and preparing the spinal cords at day 10 for counting total amount of motoneurons in the lumbar lateral motor column (figure 14B).

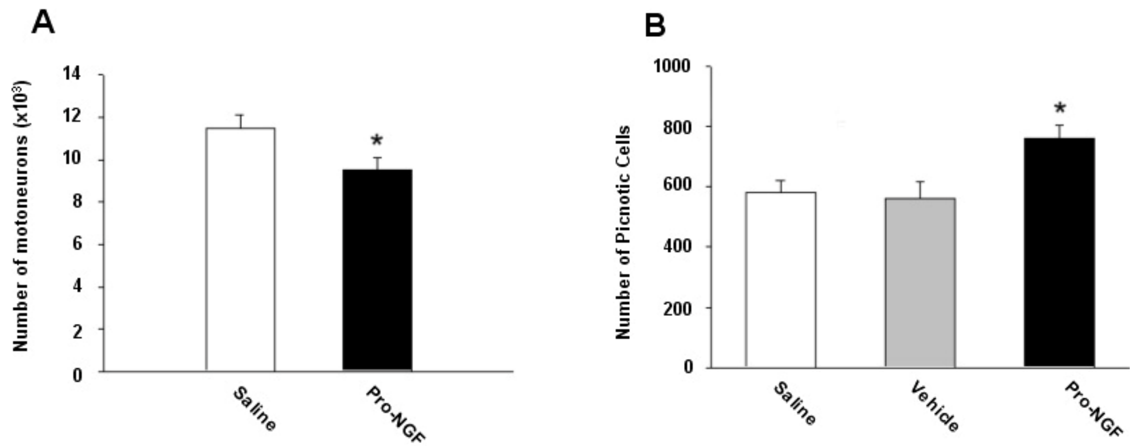



***Figure 14r.*** *Recombinant pro-NGF induces apoptosis and motoneuron loss in chicken embryo lumbar lateral motor column. (**A**) Number of picnotic cell sin the lumbar lateral motor column of e8 chick embryos after treatment with saline, pro-NGF vehicle and pro-NGF (100*μg*) from e5 to e7. (**B**) Number of motoneurons in the lumbar lateral motor column of e10 chick embryos after treatment with saline, pro-NGF vehicle and pro-NGF (100*μg*) from e5 to e9.*

We found a significant increase of picnotic cells and significant motoneuron loss in the chicken embryos treated with pro-NGF. This means that increased concentration of pro-NGF in early embryonal stages leads to significant motoneuron death.

## 8. The effect of DHA on apoptotic cell death induced by hbi-pro-NGF.

Referring to the described in the literature positive effects of the omega-3 fatty acids in particular Docosahexaenoic acid (DHA), we wanted to test weather the presence of DHA can prevent ADhbi-pro-NGF induced apoptosis. For cell treatment we prepared 1mM stock solutions of DHA in DMEM and in isopropanol and treat the cells with final concentration of 3µM DHA. We used DHA and DHA in combination with 6µM vitamin E as antioxidation agent. 24 hours after the DHA treatment we treated the cells with ADhbi-pro-NGF. Hoechst staining and MTT assay were performed 24 and 48 hours respectively after the treatment to estimate the cell death ratios in the different groups. We find that DHA dissolved in isopropanol has toxic effect over the cells due to the isopropanol presence. For that reason we continued the experiments using only DHA in DMEM. Our results show that DHA alone do not have ability to prevent ADhbi-pro-NGF induced apoptosis. To protect DHA from a possible oxidation during the period of cell treatment, we used the addition of vitamin E. When added in combination with vitamin E, DHA treatment reduced significantly the percentage of cell death caused by ADhbi-pro-NGF but this can be attributed mainly to the vitamin E action (figure 15). Although, this effect could be attributed to vitamin E, as the level of protection is similar with the use of both DHA and vitamin E together.

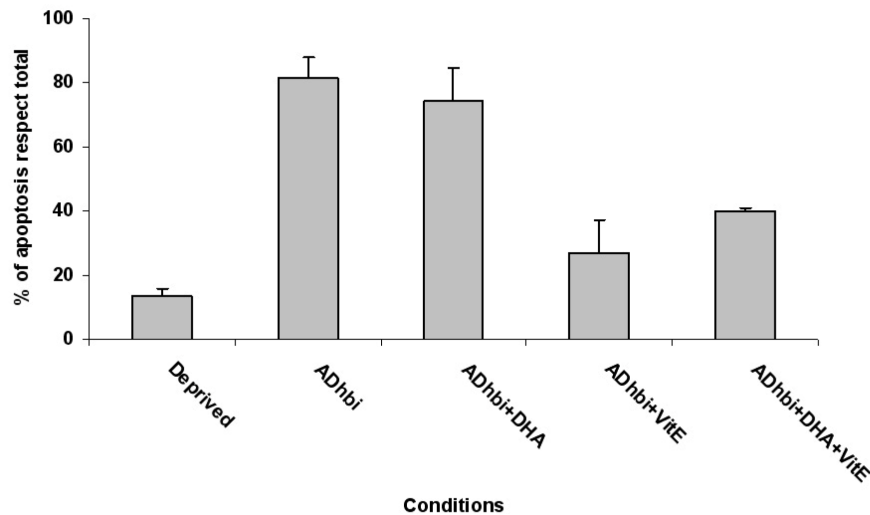



***Figure 15r.*** *PC12 cells were serum-deprived, then treated with* 3μM DHA*,* 6μM vitamin E, combination of 3μM DHA *and* 6μM vitamin E and untreated. 24 hours after the treatment 25 *ng/ml ADhbipro-NGF was added. Viability of the cells was measured after 24 hours using Hoechst.*

## 9. pro-NGF non-enzymatic modifications are increased in AD in a stage-dependent manner.

pro-NGF isolated from control brains differs functionally from pro-NGF isolated from AD-affected brains at comparable ages, which does not induce apoptosis but induces differentiation in PC12 cells. This fact gives strong indications that pro-NGF isolated from control brains is unstable and is processed to NGF when added to cell cultures. It is described that the level of the modifications of the proteins in AD-affected brain tissues is increased and AGE/ALEs are common non-enzymatic modifications of proteins in AD. The question whether differences in any kind of modifications in pro-NGF between control and AD-affected brains lead to the differences in resistance to protease degradation remain open.

In order to assess if pro-NGF is modified in vivo in human brains with AD pathology, we assayed different antibodies recognizing different AGE/ALEs products (figures 16r-20r). pro-NGF was immunoprecipitated from hippocampal and entorhinal cortex lysates of control and AD-affected human brain samples.

Western blots of immunoprecipitates with anti-N$^{\varepsilon}$ -(carboxyethyl)-lysine (CEL) antibody, which is known to generate from protein modification by methylglyoxal, show a general increase in the glycoxidation of pro-NGF in AD brains through the stages of the disease. The glycoxidation of pro-NGF increase gradually with the progression of the disease in the pools from hippocampus compared to the control pool. In enthorinal cortex also confronted to the control pool was observed a sudden increase in the first stage of the disease that is maintained during the development of the disorder (figure 16r).

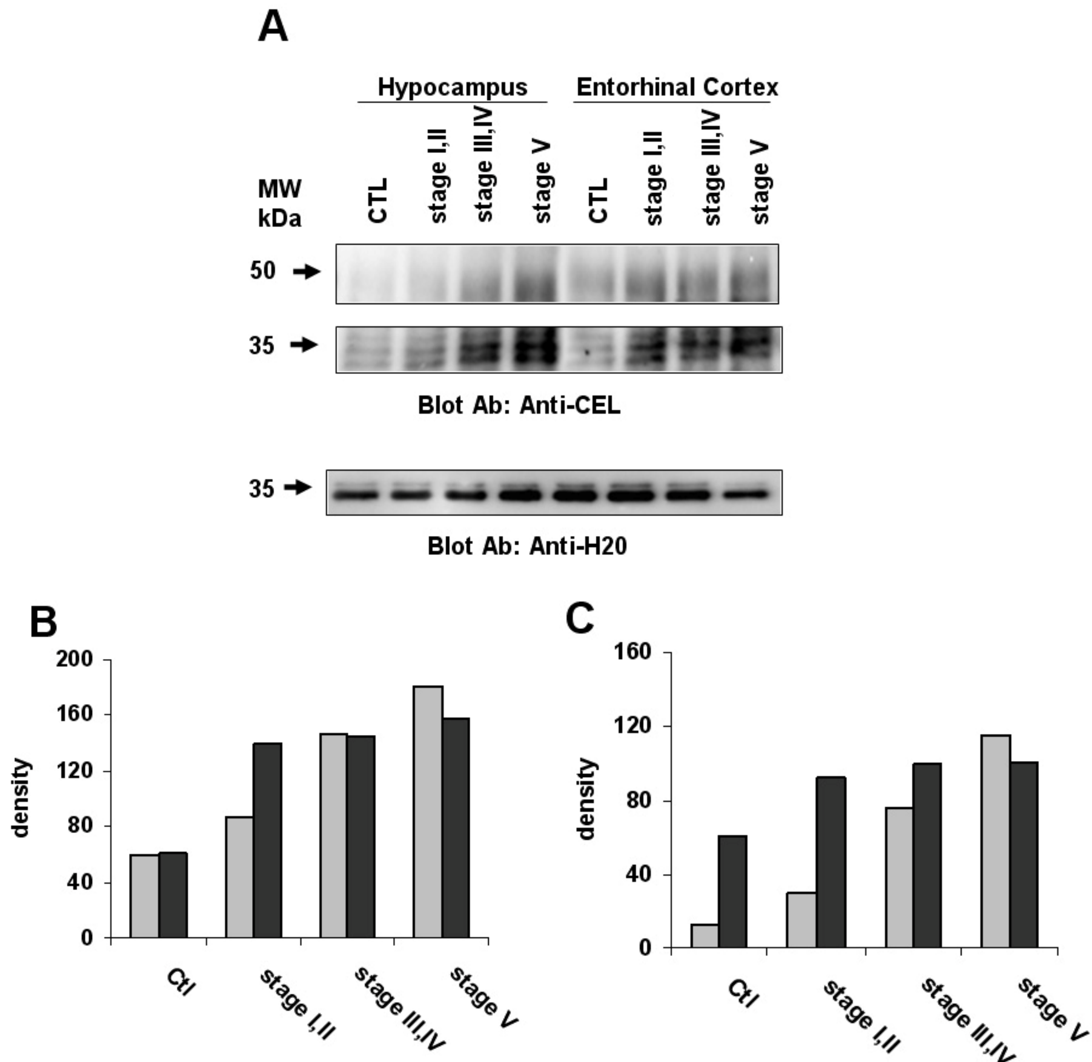
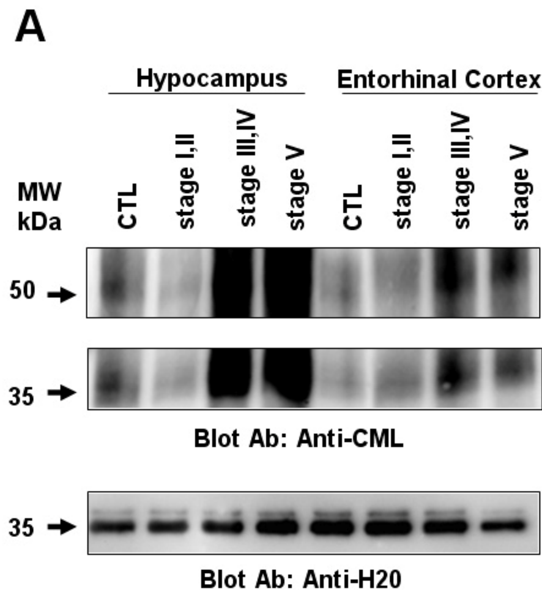



***Figure 16r.*** *pro-NGF from the affected hippocampus and enthorinal cortex in human AD is modified by N$^{\varepsilon}$ - (carboxyethyl)-lysine in an stage-dependent manner. Three samples of each, hippocampus and enthorinal cortex*

*were pooled and immunoprecipitated with anti-NGF antibody (H20), followed by immunostaining with the marker of non-enzimatic glycoxidation anti- $N^{\varepsilon}$-(carboxyethyl)-lysine (CEL) (**A**). Western blot analyses demonstrated a gradually increased glycoxidation of pro-NGF observed from stages I/II in enthorinal cortex (**black bars**), but not in hippocampus (**grey bars**). Increase in hippocampus is more evident from stages III,IV both in 35 and in 50 kDa molecular weight bands. Arrows indicate apparent molecular weight. The lower panel show the quantitation by densitometry, of these blots for 50 kDa (**B**) and 35 kDa (**C**) forms of pro-NGF, corrected for the total pro-NGF amount using as a loading control. Hippocampus (**grey bars**) and enthorinal cortex (**black bars**).*

$N^{\varepsilon}$- (carboxymethyl)-lysine (CML), a mixed marker of glycol- and lipoxidation is a major product of oxidative modification of glycated proteins that also may generate from protein modification by glyoxal. Western blot densitometric analysis of 50 and 35 kDa bands of the immunoprecipitated pro-NGF from AD samples, revealed a step by step increase in the degree of protein modification for carboxymethyl-lysine in both pools, from hippocampus and from enthorinal cortex (figure 17r).



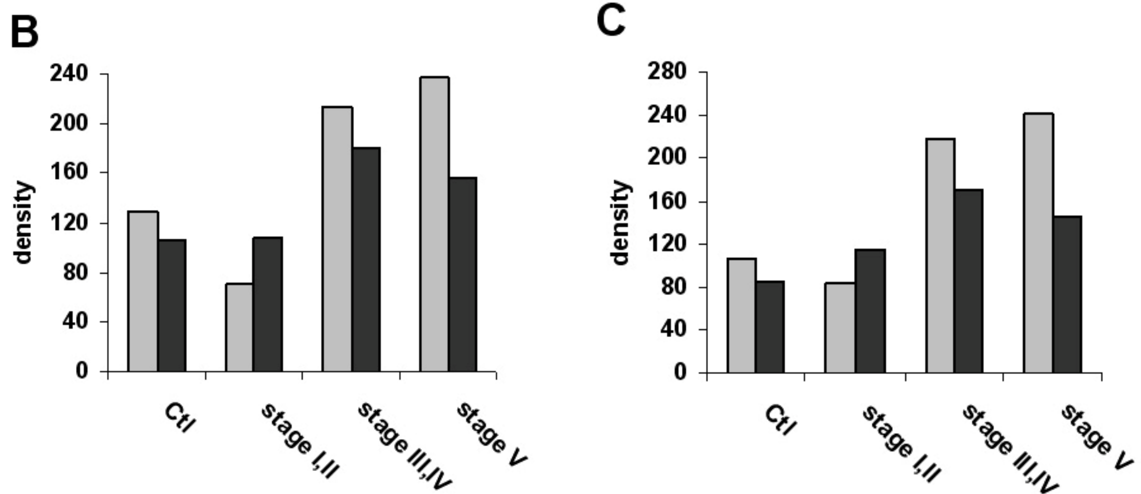



***Figure 17r.*** *$N^{\varepsilon}$ - (carboxymethyl)-lysine modification of human brain cortex pro-NGF in AD. (**A**) Western blot analyses of pro-NGF from AD samples, show a stage-dependent increase in the concentrations of $N^{\varepsilon}$ -(carboxymethyl)-lysine (CML) from stages I/II, in immunoprecipitates from hippocampus and enthorinal cortex with the progression of the disease. Arrows and numbers indicate apparent molecular weight. The panels represent the quantitation of 50 (**B**) and 35 kDa (**C**) forms of pro-NGF by densitometry, corrected for the total pro-NGF amount used as a loading control. Hippocampus (**grey bars**) and enthorinal cortex (**black bars**).*

The latter products of the Maillard reaction, termed advanced glycation end products (AGEs), adversely affect the functional properties of proteins, lipids and DNA. AGEs are produced from monosaccharides as glucose and fructose, but also from dicarbonyl compounds derived from the Maillard reaction, autoxidation of sugars and other metabolic pathways, e.g. glycolysis. Examples of AGE modified sites are carboxymethyllysine (CML), carboxyethyllysine (CEL) and Argopyrimidine which is the most common epitope. As a general marker for advanced glycation end products we used an Anti –AGE antibody (figure 18r).

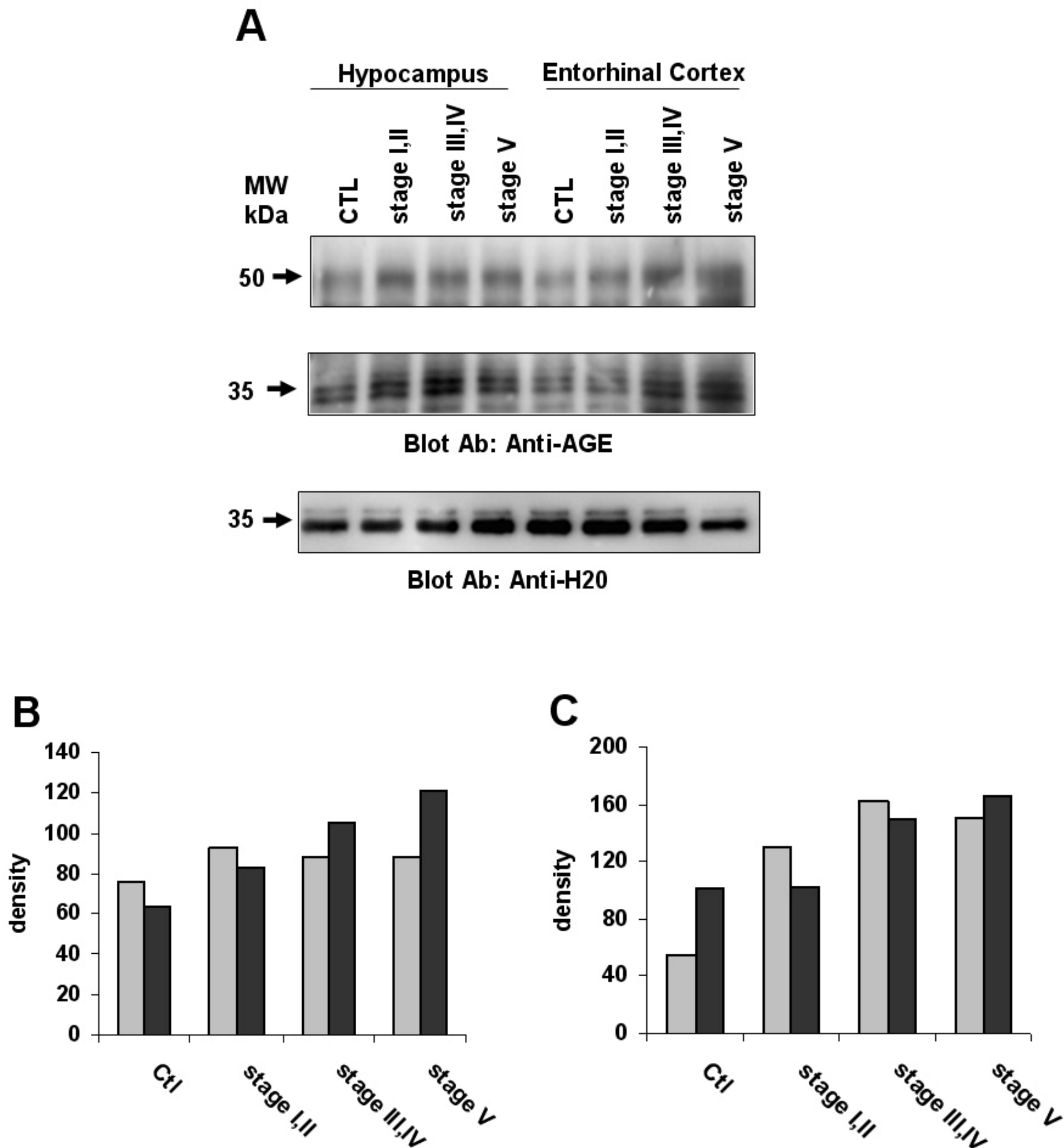



***Figure 18r.*** *Advanced Glication End products modifications of pro-NGF in the brain of patients with AD. (**A**) pro-NGF from enthorinal cortex compared to the control group in both modificated forms of the protein depict gradual augmentation in the amounts of advanced glycation end products (AGE) modification, arising from early products such as Schiff base and Amadori rearrangement products. The immunoprecipitates from hippocampus show rise in the AGE modifications only in the 35 kDa form with the progression of the disease, whereas there is no evident change compared to the control pools for the 50 kDa form of pro-NGF. Arrows and numbers indicate apparent molecular*

*weight. The panels represent the quantitation of 50 (**B**) and 35 kDa (**C**) forms of pro-NGF by densitometry, corrected for the total pro-NGF amount used as a loading control. Hippocampus (**grey bars**) and enthorinal cortex (**black bars**).*

It has been described an increase of lipoxidation in variety of neurodegenerative diseases. As this modification could also play a role in block pro-NGF processing, we wanted to test if this modification could be present specifically in this protein in AD. We used the same procedure described above to immunoprecipitate pro-NGF from control and different stages of AD affected hippocampal and entorhinal cortex human brain samples. Western blot of immunoprecipitates shows an increase in the signal lighted up using antibodies raised against MDAL (figure 19r) and Neuroketals (figure 20r). Densitometric analysis of the 35 kDa shows an evident increase of both kinds of modifications which correlates with the progression of the disease in both hypocampal and in entorhinal samples.

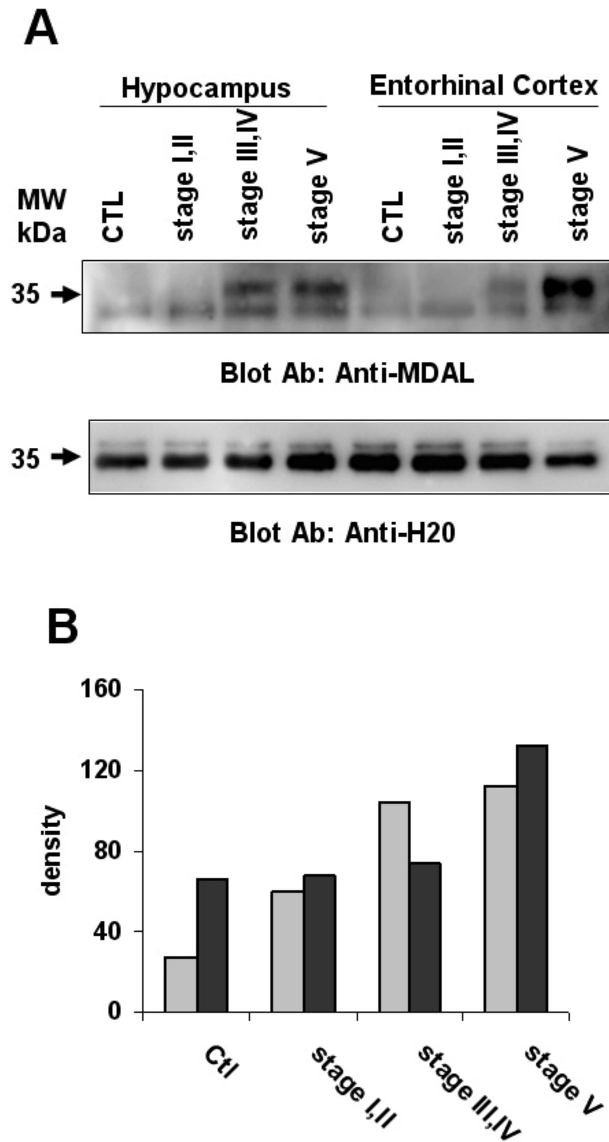



***Figure 19r.*** *pro-NGF from the hippocampus and enthorinal cortex in AD is glyco- and lipoxidated. (**A**). Western blot for lipoxidation of pro-NGF from AD samples, shows increases in lipid peroxidation-derived damage, by measurements of MDAL in 35 kDa form. MDAL is the major oxidative breakdown product of polyunsaturated fatty acids, those are abundant in the brain tissue. Arrows and numbers indicate apparent molecular weight. The panel (**B**) shows the quantification by densitometry, of the 35 kDa modified form of pro-NGF from hippocampus (**grey bars**) and enthorinal cortex (**black bars**), corrected for the total pro-NGF amount. Hippocampus (**grey bars**), enthorinal cortex (**black bars**).*

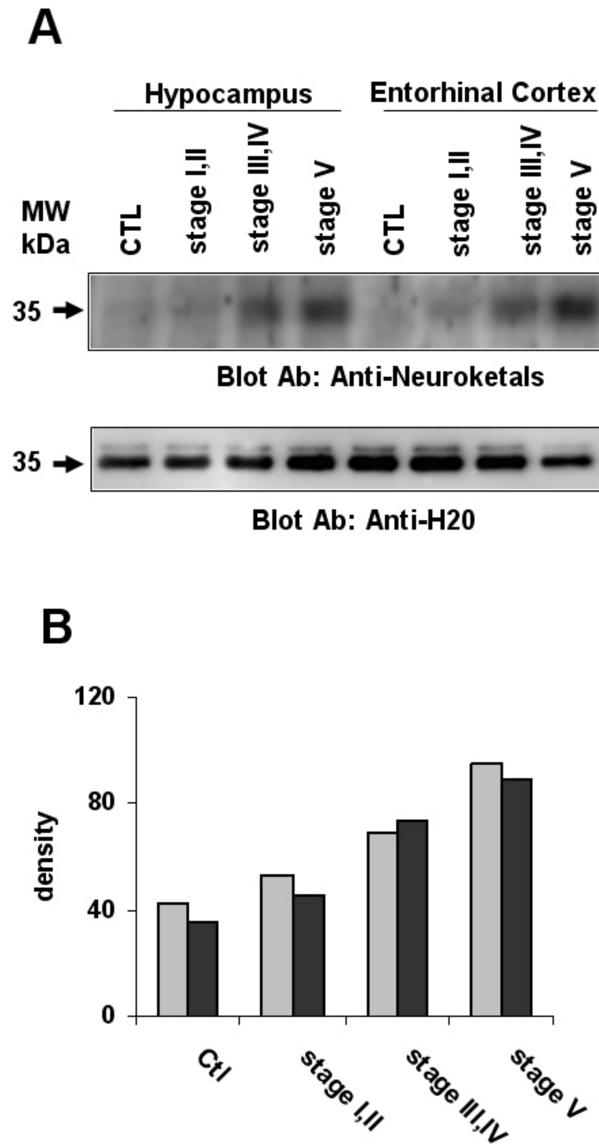



***Figure 20r.*** *Neuroketals, a unique marker of lipoxidative injury in the brain, also elevates in a stage-dependent manner in pro-NGF protein immunoprecipitated from AD patients (**A**). Western blot analyses with anti-Neuroketals demonstrated a gradual increased lipoxidation of pro-NGF, which is correlative to the progression of the disease, in both pools from hippocampus and enthorinal cortex, compared to the control pools. Arrows and numbers indicate apparent molecular weight. The panel (**B**) shows the quantitation by densitometry, of the blots for 35 kDa forms of pro-NGF, corrected for the total pro-NGF amount as a loading control. Hippocampus (**grey bars**), enthorinal cortex (**black bars**).*

## 10. pro-NGF modified by Glyoxal, Methylglyoxal and oxidized Methyl linoleate, increases its resistance to degradation and processing.

We wanted to test if GO-, MGO- and OML-modifications observed in pro-NGF from AD affected human brain, could be effective in block its processing by convertases activity, which rises lower Mw pro-NGF forms as well as NGF of 13.5 kDa. For that reason, we used human recombinant wild type pro-NGF (hr-pro-NGF) and modified it in vitro as described in methods. As we and others reported before [Podlesniy 2006; Rattenholl 2001], hr-pro-NGF, even when it is folded conveniently, is susceptible to be degraded.

Furin processing of pro-NGF accounts for the major part of intracellular physiological maturation of the protein raising the mature NGF form (13.5 kDa) [Seidah 1996; Bresnahan 1990]. At extracellular level, plasmin and MMP-7 are able to process pro-NGF in the cell media raising NGF of 13.5 kDa and fragments from 18 to 30 kDa. In order to assess if GO, MGO and oxidized Methyl linoleate protein modifications could block the degradation and processing of pro-NGF, we incubated the modified and the non modified pro-NGF in the presence of PC12 cells for 24 h. The media then was concentrated and the Western blot was performed. As can be observed in Figure 21r the modification by MGO, and at lesser extent GO and oxidized Methyl linoleate, block the hr-pro-NGF degradation. NGF cannot be detected in MGO-pro-NGF treated cells media, and only at a very low proportion in GO- and lipoxidized-pro-NGF when compared to the non-modified protein. Only faint bands of Mw from 17 to 30 kDa can be seen with H20 antibody in modified proteins, compared to the more evident corresponding bands in non-modified pro-NGF lines.

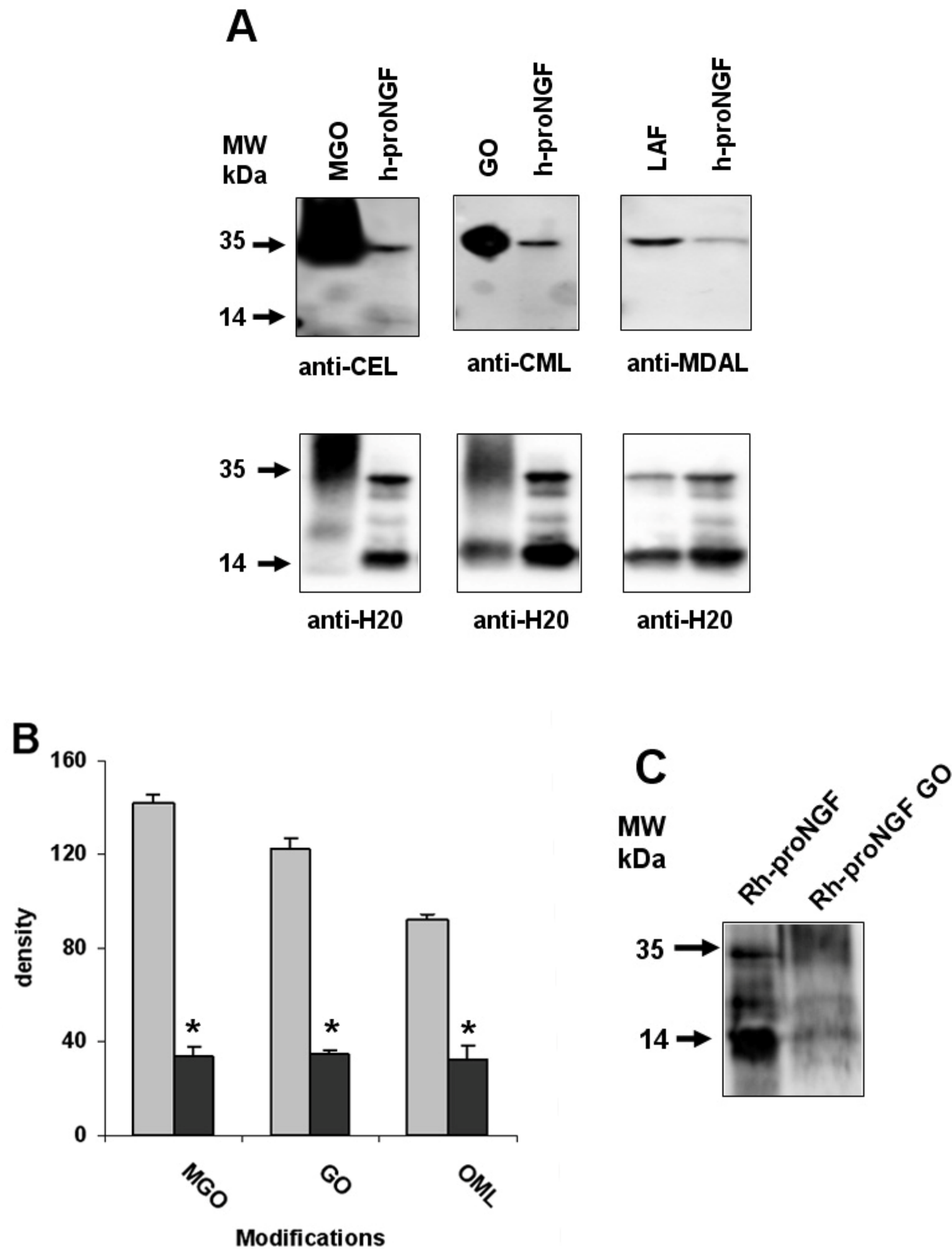



***Figure 21r.*** *Glycoxidized rh-pro-NGF and Lipoxidized rh-pro-NGF is more resitent to degradation. Western blots of rh-pro-NGF modified by glyoxal, metylglioxal and methyl linoleate methyl ester as well as the controls of no modificated rh-pro-NGF reveled with anti-CEL, anti-CML, anti-MDAL, and the same membranes rebloted with H20 (**A**). Densitometry of the membranes reveled with anti-CEL, anti-CML, anti-MDAL , grey bars represents the density of modificated rh-pro-NGF bands, black bars represents the density of no modificated rh-pro-NGF (**B**). GO-hr-pro-NGF and hr-pro-NGF control are*

*incubated for 30 min in the presence of recombinant furin. Western blot of the incubation mixture, using with H20 antibody, show that modified pro-NGF is not processed by the enzyme (**C**).*

In order to explore whether the reactive carbonyl compounds were able to block specific processing by Furin (which rise fragments of 14, 18 and 30 kDa), we incubated GO-pro-NGF and non-modified pro-NGF for 30 min in the presence of Furin following the manufacturer instructions (RD Systems). As can be seen in Figure 21r C, the band corresponding to NGF is visible only in non-modified pro-NGF. GO modification is able to block completely the processing by the enzyme.

## 11. pro-NGF modified by GO and MGO is physiologically active in inducing apoptosis through p75NTR.

A biological indicator of pro-NGF processing by convertases rising NGF is the ability of this product to differentiate PC12 cells in culture for 48 h. As can be seen in Figure 22r, 100 ng/ml of hr-pro-NGF is able to increase the differentiation of PC12. By contrast, neither glyco- nor lipoxidized -hr-pro-NGF are able to differentiate PC12 cells. These results indicate that the processing by convertases of modified pro-NGF is blocked throughout the 48h of incubation and as a consequence NGF is not raised. With the lack of NGF in the cell medium the PC12 cells does not differentiate and are able to respond to pro-NGF signal. This significantly increases the percentage of observed pyknotic cell nucleus revealing the apoptotic cell deaths that take place in these cells. To ensure that this apoptotic activity is not due to the residues of the glycoxidation modification reactions we used two controls, the first one containing non modified rh-pro-NGF and the second one-modification reaction buffer. These controls were managed in the same way as the modification reactions. As a control of lipoxidation reaction we used the reaction mixture without rh-pro-NGF and managed it in the same way. Also to ensure that the apoptotic effect is not due to the rests of GO and MGO that can be toxic in higher concentrations we treated the cells with the reaction mixtures for glycoxidation reactions without the addition rh-pro-NGF and they do not displey any effect on the cells.

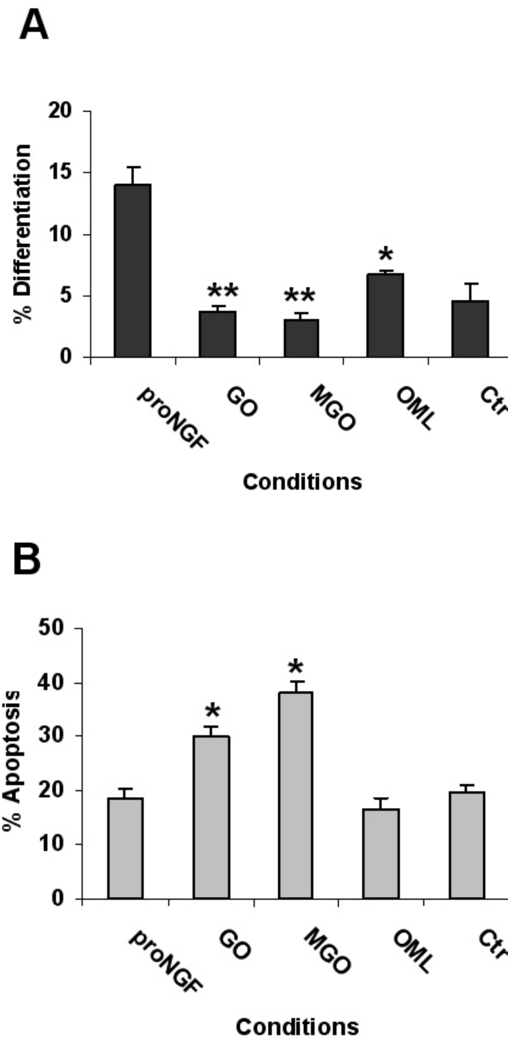



***Figure 22r.*** *GO and MGO modified rh-pro-NGF is physiologically active in inducing apoptosis through p75NTR in PC12 cells. PC12 cells were serum deprived and treated for 48 hours with 100 ng/ml of rh-pro-NGF, rh-pro-NGF modified by Glyoxal (GO), Methilglyoxal (MGO), and oxidized Methil linoleate (OML), and the same volume of the corresponding modification reaction buffers as controls. After 48h the cells were fixed and stained with Hoechst. Differentiation (**a**) and apoptosis (**b**) were quantified and expressed as percentage of the total number of cells. Differentiated PC12 cells are those with neurite extensions longer than the cell body. Values represent the mean ± SEM of three independent experiments. * p< 0.05; ** p< 0.01; Student's t-test.*

As can be observed in Figure 22r, GO and MGO are more effective than OML-hr- pro-NGF in inducing apoptosis and induce significantly higher degree of apoptosis than hr-pro-NGF.

To verify whether the effect of GO and MGO hr-pro-NGF was specifically mediated by its interaction through p75NTR, we blocked the receptor by a pre-incubation with an antibody (REX) rose against the extracellular domain of p75NTR. As it is shown in Figure 23r, the apoptosis induced by GO- and MGO hr-pro-NGF is reduced to the basal apoptosis caused by deprivation (indicated as 100%) which represent approximately the same percentage of blocking that is reported previously for pro-NGF isolated from human brain affected by AD (Pedraza 2005).

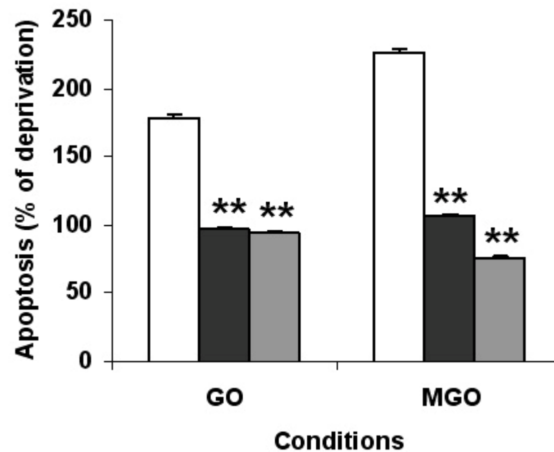



***Figure 23r.*** *PC12 cells were serum deprived and pre-treated for 2h with 50ng/ml REX (black bars), anti-pro-NGF ( grey bars) antibodies or left without blocking (white bars), then treated for 48 hours with 100 ng/ml of rh-pro-NGF modified by Glyoxal (GO) and Methylglyoxal (MGO). After 48h the cells were fixed and stained with Hoechst. Apoptosis was quantified and expressed as a percentage of the levels in non-treated control cells. Values represent the mean ± SEM of three independent experiments. ∗ p< 0.05; ∗∗ p< 0.01; Student's t-test.*

## 12. Modified pro-NGF *in vivo* administration induces cognitive impairment in mice.

Intracerebroventricular administration of Aβ peptide (25-35) in rat is described to produce memory and cognitive alterations in a pattern that is characteristic of AD-affected animal models [Ramirez 2005]. To examine whether either hr-pro-NGF or GO-hr-pro-NGF could be active in inducing a similar phenotype we administered 2 μg in a single injection as described in methods. In contrast to control mice (injected with GO-modified BSA, which was modified *in vitro* in parallel to hr-pro-NGF) that learned the spatial navigation tasks in about 2 days, mice injected with hr-pro-NGF had a significant impaired learning in the first 4 days (figure 24r), and presented afterwards learning and memory skills undistinguishable from control animals. By contrary, animals administered with GO-hr-pro-NGF, differed in learning test results and remain with a significant impaired behaviour until the day fifth (figure 24r). This difference is maintained for at least the following 2 days. This effect was not attributable to changes in the locomotor's activity of the animals and as it was expected, intra-ventricular administration of pro-NGF did not induce other adverse effects than cognitive impairment.

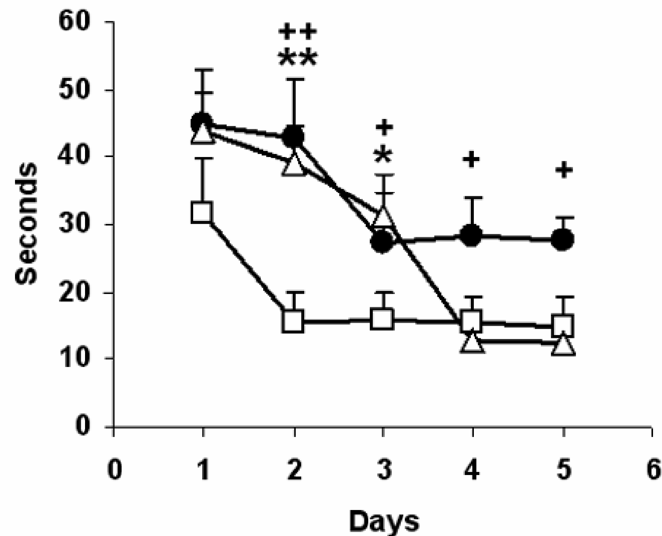


***Figure 24r.*** *Mice intracerebroventricularly injected with modified rh-pro-NGF present significant cognitive impariment compared with controls.*
*Mice injected with GO-rh-pro-NGF (●), non- modified rh-pro-NGF (∆) and the same volume and amount of GO-modified-BSA (□) present significant differences in learning progression, measured as seconds needed to find the submerged platform in the Morris water maze test. Values represent the mean ± SEM of five animals. + $p< 0.05$; ++ $p< 0.01$ Student's t-test for GO rh-NGF, ∗ $p< 0.05$; ∗∗ $p< 0.01$ Student's t-test for rh-NGF. Values result from three independent experiments.*

# *IX. DISCUSSION*

# IX. DISCUSSION

## 1. AD hbi-pro-NGF stimulation of p75NTR causes p75ICD nuclear translocation and apoptotic cell death.

p75NTR is known to be involved in signalling apoptosis in a number of cell models [Friedman 2000, Naumann 2002, Frade 1999]. However, some variations have been reported, depending on the plethora of intracellular adaptors and on the coreceptor pattern in a given cellular context [Chao 2002]. Later new insights were provided when it was shown that pro-NGF binds more strongly than NGF to p75NTR, inducing a higher degree of apoptosis [Lee 2001]. Furthermore, the death induced by ADhbi-pro-NGF through p75NTR is independent of the presence of TrkA, because the pro-neurotrophin is not a TrkA ligand. It has been reported that p75NTR is subjected to shedding in a sequential process of regulated intramembrane proteolysis. This process occurs by a mechanism involving α- and γ-Secretase processing on both sides near its trans-membrane domain, in a protein kinase C-dependent manner [Zampieri 2005]. The product of α-Secretase is p75CTF, comprising the transmembrane domain with an apparent molecular weight of 25 kDa [Zampieri 2005, Domeniconi 2005]. The product of γ-Secretase processing is p75ICD with an apparent molecular weight of 20 kDa [Zampieri 2005, Domeniconi 2005]. It has been described that α-Secretase activity is essential for the activity of γ-Secretase [Frade 2005]. p75ICD is difficult to be detected, because it is described as being very unstable [Kanning 2003]. The proportion of p75ICD:p75CTF observed can present variations between models or may be the consequence of different α/γ-Secretases activity and other processing mechanisms. The data that we present here show that the 20 kDa band is increased in AD-affected entorhinal cortex.

It has been shown that in a Schwannoma cell line [Frade 2005] the yield of p75ICD and its nuclear localization are dependent on neurotrophin binding to the receptor. Other authors have shown no p75NTR processing [Jung 2003] or only a weak p75ICD yield under NGF stimulation [Zampieri 2005]. In the present work, we have shown that in deprived 3T3-p75st cells both p75ICD and

p75CTF are only barely detected. Treatment with ADhbi-pro-NGF showed that the induction of p75ICD with a parallel p75ICD nuclear translocation was much stronger comparing with the induced by a corresponding concentration of mNGF.

Immunocytochemistry with an antibody raised against the extracellular domain of p75NTR (REX) does not show nuclear translocation, indicating that p75ICD follows a different trafficking pathway and localization than internalization of the whole p75NTR. NGF activation of p75NTR causes an internalization that is activated by TrkA phosphorylation. This occurs through sequential internalization of both receptors in Clathrin-coated vesicles and early endosome vesicles, recycling of p75NTR, and lysosomal degradation of TrkA. 3T3-p75st does not show internalization of the whole receptor in NGF- or in pro-NGF-stimulated cells. This can be explained by the lack of TrkA expression in these cells. Comparing localization of p75NTR using the REX antibody in pro-NGF-stimulated 3T3-p75st and PC12 cells, a degree of cytoplasmic internalization in PC12 not visible in 3T3-p75st can be observed, where immunolabeling is restricted to the plasmatic membrane. The fact that perinuclear or nuclear localization in 3T3-p75st and PC12, respectively, stimulated by pro-NGF, is only detected with anti-p75ICD and not with REX reinforces the idea that nuclear or perinuclear translocation only occurs with p75ICD from p75NTR processing by α- and γ-Secretases. Inhibitors of these Secretases completely abolished the process.

It is widely reported that p75NTR can interact with different sets of receptors, depending on their respective proportions and on the ligand availability. Sortilin receptor has been described to be essential in p75NTR induced apoptosis activated by pro-NGF [Nykjaer 2004]. The binding of the pro-domain to Sortilin and of the mature NGF domain to p75NTR starts a poorly understood cascade of events leading to apoptosis.

## 2. p75NTR ICD is increased in AD affected brain tissue.

Our results indicate that p75ICD levels significantly increase in AD in human entorhinal cortex in parallel with pro-NGF levels, as assayed by Western blot in samples from AD affected and control brains. As far as we know, up to

the moment no other data about activation of the processing of p75NTR in brain pathology is described.

## 3. pro-NGF from AD-affected cortex is not only present at higher levels but is also more active in inducing apoptosis through p75NTR processing.

It has been reported that pro-NGF is increased in human cortex brain in a manner that is dependent on the progress of the disease, reaching maximum levels in advanced stages [Pedraza 2005]. We have also shown that pro-NGF isolated from AD cerebral cortex can induce apoptosis in neuronal cell cultures through its interaction with the p75NTR receptor in a mechanism that is dependent on α-Secretase shedding of the receptor. As control brains also present some basal levels of pro-NGF [Pedraza 2005] it was interesting to explore whether there were any differences in the process of apoptosis induction by pro-NGF between AD-affected brains and controls. We first wanted to assess whether pro-NGF isolated from the two kinds of sources gave rise to similar physiological effects, or whether the only difference would be due to the increased amount of the pro-neurotrophin in AD. Surprisingly, pro-NGF isolated from different control samples was not able to induce apoptosis in any of the used cell models. Previous studies performed with the bacterially expressed wild-type pro-NGF showed the functionality of the protein by partially hydrolyzing the prodomain with Trypsin, demonstrating that the NGF resulting from the subsequent hydrolysis could induce the survival of dorsal root ganglia neurons at a similar rate as seen with the same concentration of NGF [Rathenhol 2001]. NGF is the only neurotrophin reported as inducing neuronal differentiation in PC12 cells through its interaction with TrkA. This interaction also activates strong survival signals that are able to block apoptosis acting on a number of molecular targets. This would make it difficult to detect apoptosis caused by stimulation of p75NTR under conditions of phosphatidylinositol 3-kinase/protein kinase A activation. ADhbi-pro-NGF could not sustain the survival and/or the differentiation of PC12 cells. However, when ADhbi-pro-NGF was partially digested by Trypsin, PC12 survival and differentiation were observed. Moreover, partially digested ADhbi-pro-NGF protected PC12 cells

from death caused by deprivation, to the same extent as NGF, thus indicating that NGF is obtained as a product of Trypsin digestion. Based on these findings, we wanted to assess whether Chbi-pro-NGF was either inducing apoptosis, as does ADhbi-pro-NGF, or survival and differentiation, as does NGF. The assay on PC12 cells shows that Chbi-pro-NGF was processed to NGF when it was added to the culture, inducing neuritogenesis. As we described, the 53 kDa pro-NGF isoform is at least partially deglycosylated by *N*-glycanase treatment [Pedraza 2005].

## 4. Aβ peptide 25-35 is not able to induce p75NTR nuclear translocation in PC12 cells.

We wanted to assess whether the processing of p75NTR rising ICD and its nuclear translocation caused by the Aβ interaction with p75NTR was similar to the processing caused by the stimulation with pro-NGF.

It is described that p75NTR binds Aβ and mediates Aβ toxicity in PC12, SK-NMC, NIH 3T3, and SK-N-BE cell lines, suggesting that p75NTR acts as a death receptor for Aβ toxicity [Rabizadeh 1994; Yaar 1997, 2002; Kuner 1998; Perini 2002]. The authors described Aβ (25-35) peptide as able to induce cell death trough his interaction with p75NTR. Our experiments in PC12 cell culture showed Aβ (25-35) as an agent with moderate toxicity. We were not able to see any significant differences between cells treated with Aβ (25-35), treated with Aβ (35-25) and nontreated deprived cells. In 3T3 cell line that not express p75NTR there is any difference between the conditions at all. Also the blocking of p75NTR with REX antibody does not show any significant differences in PC12 cells treated with Aβ. The moderate toxicity of Aβ is compatible with the observed lack of perinuclear translocation of p75NTR.

These results should not be considered as controversy of published earlier articles because of relatively low expression levels of p75NTR in the PC12 cells. Usually the authors that relate the Aβ (25-35) toxicity with its interaction with p75NTR used for their experiments cell lines with higher p75NTR expression levels. Usually they use neuroblastoma SK-N-BE cell line transfected with expression plasmids and in this way 3 to 5 times higher p75NTR expression can be achieved [Bunone 1997]. For that reason we

decided to produce a cell line with higher p75NTR expression levels. We transfected 293FT cell line with p75NTR cloned in pcDNA 3.1 plasmid using $CaCl_2$. We obtained very high p75NTR expression but despite that no significant cell death after Aβ (25-35) treatment were observed. Probably this is because 293FT cell line does not have neuronal origin and do not express either p75NTR or other important proteins needed for p75NTR induced apoptosis.

## 5. The overerexpression of p75 ICD is not sufficient for inducing apoptosis in different cell lines.

According to our results with AD hbi-pro-NGF the cell death is accompanied with cleavage of p75 ICD and his subsequent nuclear translocation. We wanted to study whether the increase of ICD concentration within the cell is sufficient for activating the cell death cascade. We also wanted to see whether expressed alone the ICD of p75 is able to “connect” with the intracellular structures and translocate to the nucleus. In our experiments we used two cell lines that normally do not express p75: 3T3 and 293FT, and PC12 cells that normally express p75NTR. For that reason we developed viral expression system encoding ICD and used it to transfect 3T3 and 293FT cells.

We found that despite of higher ICD concentration obtained in all cell types no other physiological effects were manifested. The homogenic distribution of ICD in all cells and the absence of increased cell death suggest that probably only the expression of ICD part is no able to carry out properly its physiological functions. Probably the whole p75NTR molecule is needed for proper ICD positioning and connection with other cell structures responsible for targeted protein translocation.

## 6. Recombinant pro-NGF induces apoptosis and motoneuron loss in chicken embryo lumbar lateral motor column.

It is broadly described that pro-NGF is high affinity ligand for p75NTR and induces cell death by activating it. Our results show an increase of p75NTR

expression in chicken embryos spinal cord in days from e5 to e12 with the highest point in day e8. This means that p75NTR has an important role in chicken neuronal embryogenesis. This early stages of neurogenesis are related with motoneuronal loss, the new formed (by division of the stem cells) neurons are much more than the motoneurons in the adult animal. At this stage of embryogenesis the decision which motoneurons will live should be taken. The neurons start to express high levels of p75NTR and increase its sensitivity to all ligands that can activate this receptor. Increasing of concentration of pro-NGF at these stages logically leads to an increase of motor neuron loss. And that is exactly what we have seen counting picnotic cell nucleus or total number of motoneurons.

## 7. DHA administration in cell cultures is not able to prevent cell death caused by AD hbi-pro-NGF.

Lipid peroxidation (i.e. the process of structural alteration of polyunsaturated fatty acids induced by free radicals), is involved in neurodegeneration pathogenesis, as assessed by the increased levels of lipid peroxidation derived markers found in experimental models and in human tissues.

Because of broadly described beneficial qualities of DHA over different human diseases, including neurodegeneration, and because of the high content of DHA in human brain we wanted to test whether the additional DHA administration in the cell cultures can reduce the cell death caused by AD hbi-pro-NGF. We found that 3μM DHA is not able to rescue the cells from cell death caused by AD hbi-pro-NGF. The combination of 3μM DHA and 6μM vitamin E shows some positive effects but those effects are mainly caused by vitamin E function. Since, DHA is polyunsaturated fatty acid with 22 carbon chain it is insoluble in water solutions such as DMEM medium. DHA with water solutions make an emulsion but does not dissolves. In the beginning we used isopropanol to dissolve DHA and to treat the cells with this solution, but the concentration of isopropanol result to be high enough to kill substantial percentage of the cell. The treatment of PC12 cells with DHA in DMEM did not show any positive effect for the cell survivel, but more studies are needed to conclude whether that is

why DHA has no positive effect over AD hbi-pro-NGF induced cell death or just because water insoluble DHA can not reach the cells and to incorporate in the membrane.

## 8. The levels of nonenzimatic modifications of pro-NGF are elevated in stage dependent manner in some regiones of AD affected brains.

The presence of 53, 37 and 32 kDa bands in control and AD-affected human frontal and entorhinal cortex, was described using different antibodies raised against the pro-domain of pro-NGF [Pedraza 2005]. Among them, the 32 and the 53 kDa forms showed a significant increase in AD samples which correlate to the progression of the disease. It has been described that the 53 kDa form is glycosylated [Pedraza 2005, Seidah 1996] as it varies its apparent molecular weight when it is deglycosilated *in vitro*.

So far, no other post-translational enzymatic and/or nonenzymatic protein modifications for pro-NGF had been described in vivo. The extent of ALEs in a given protein is indicative for oxidative stress and constitutes another post-translational modification of pathogenic interest, as an increase in glycoxidation and lipoxidation has been described in variety of neurodegenerative diseases. Given that, these modifications could also play a role in the blocking of pro-NGF processing. As we wanted to test whether this modification could be present specifically in one protein (pro-NGF) in AD, we used immunoprecipitation to isolate pro-NGF from control and AD-affected from different stages hippocampal and entorhinal cortex human brain samples.

Our data indicate an increased degree of modifications in pro-NGF, which correlate positively with the progression of the disease and are barely detectable in control individuals. The entorhinal cortex is the earlier cortical region affected by AD (stages I/II) followed by the hippocampus (stages III/IV) [Mowla 1999]. Increased CEL adducts are present in the entorhinal cortex but not in the hippocampus at stages I/II. However, evident increase of CML, AGE, MDAL and NKTLs modifications occur in the hippocampus and entorhinal cortex between stages II and stage IV. The anatomic specificity of the extent of

modification through the progression of the disease supports its pathogenic relevance that warrants further study.

## 9. *In vitro* modified recombinant pro-NGF is more stable than nonmodified and can induce cell death trough p75NTR and an AD-similar behaviour in mice.

Glycoxidation and lipoxidation are widely described non-enzymatic modifications present in relevant proteins such as ATP synthase and GFAP [Pamplona 2005] in AD. Pro-NGF contains Lys, Arg and Cys residues susceptible to non-enzymatic modification by glycoxidation and lipoxidation reactions. As well, these modifications could block pro-NGF processing by directly modifying targets of Furin or of other convertases. Several processing targets in the pro-NGF primary sequence contain putative AGE/ALEs formation (R and K) sites [Ullrich 1983]. The 13.5 kDa NGF is obtained by Furin processing in arg-ser-lys-arg103/ser-ser-thr site [Seidah 1996].

The described modifications block rh-pro-NGF degradation and processing, thus potentiating its pro-apoptotic effect through its interaction with p75NTR. The lack of maturation and as a consequence the lack of NGF production is evidenced by the fact that once modified, it does not induce differentiation of PC12 cells in culture. As was previously reported [Pedraza2005; Podlesniy 2006], ADhbi-pro-NGF could not sustain the survival and/or the differentiation of PC12 cells. However, when ADhbi-pro-NGF was partially digested by trypsin, PC12 survival and differentiation were observed. Moreover, partially digested ADhbi-pro-NGF protected PC12 cells from death caused by serum deprivation, to the same extent as NGF, thus indicating that NGF is obtained as a product of trypsin digestion. This also furnishes information about the biological activity of rh-pro-NGF expressed and folded *in vitro*.

Furthermore, modifications in different residues in the tertiary and/or quaternary structure, and/or possible formation of inter- and intra-molecular crosslinks, could also make the pro-NGF refractory to the access of convertase action. It was suggested [Pedraza2005; Podlesniy 2006] the possibility that inter-molecular crosslinks could be responsible for dimerization or stabilization

of higher molecular weight complexes resistent to disaggregation even in strong dissolving conditions such as standard SDS denaturing PAGE. In fact, in the present study we show (figure 3a) that Mw rh-pro-NGF forms bigger than 35 kDa are specifically visible using the H20 antibody, when the pro-neurotrophin is modified by MGO or GO, and that these forms remain stable after cell culture incubation. As both pro-NGF and NGF have been described as functionally working as dimmers [Hempstead 2002], the formation in vivo of these complexes could be compatible with their physiological action. Furthermore, p75NTR has been described as forming functionally active dimers or even trimers which are able to be stimulated and induce apoptosis [Chao 1994; Yaar 2002]. The possibility that in AD MGO, GO and OML protein modifications are increased could also be relevant to the formation of p75NTR complexes with the receptor itself, or even with the co-receptor Sortilin, thus making the whole complex pro-NGF-p75NTR-sortilin structurally more stable and functionally more efficient in inducing neuronal apoptosis.

Previous data have demonstrated that several growth factor receptors such as EGFR, PDGFR and insulin receptor are modified by MGO [Cantero 2007; Portero-Otín 2002], but this is the first evidence of a non-enzymatic modification in vivo of a soluble signalling protein, both by GO and MGO. During the aging procces and under conditions of oxidative stress, in AD the levels of reactive carbonyl compounds continuously increase. Increased number of protein damages by glyco- and lipoxidation has been implicated in neuronal cell death leading to AD [Ahmed 2005]. Accumulating carbonyl levels might be caused by an impaired enzymatic detoxification system. The major dicarbonyl detoxifying system is glyoxalase I, which removes Methylglyoxal in order to minimize cellular impairment. This enzyme plays a critical role in the detoxification of dicarbonyl compounds and thereby reduces the formation of advanced glycation end products. In mice AD models, an increased expression of glyoxalase I has been described in neurons, and populations with gloxylase I polymorphisms present a higher susceptibility to developing AD [Chen 2004].

It has been revealed that cross-linking modifications of Tau protein are likely to contribute to the characteristic features of paired helical filaments, including their insolubility and resistance to proteolytic degradation in the context of AD [Kuhla 2007]. MGO, GO and MDA are some of the most reactive

compounds in terms of formation of Tau dimers and higher molecular weight oligomers. These modifications have been suggested as accelerating tangle formation in vivo, and as a consequence interference with the formation or the reaction of these reactive carbonyl compounds could be a promising way of inhibiting tangle formation and neuronal dysfunction in Alzheimer's disease [Kuhla 2007]. Our results reinforce this idea and give these modifications a new pathophysiological role in terms of increasing the induction of neuronal apoptosis.

We used two different approaches to investigate the pathological relevance of the modified pro-NGF species. First, we demonstrate that this non-enzimatically modified pro-NGF was unable to differentiate or sustain the survival of PC12 cells in culture. In addition these proteins markedly increase p75NTR mediated apoptosis. More importantly we show that pro-NGF delays the learning of a spatial navigation task when injected *in vivo* to mice, and that this cognitive impairment is exacerbated when the protein is GO modified.

# X. CONCLUSIONS

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The results from our work can be summarized as follows:

First: It is possible to isolate pro-NGF from brain tissue, which shows stability and activity *in vitro*.

Second: The pro-NGF isolated from AD affected human brain induces apoptosis in cell cultures expressing p75NTR.

Third: The pro-NGF isolated from the control brains is not able to induce cell death, in opposite to those from AD affected tissue.

Fourth: The pro-NGF isolated from AD affected tissue show higher resistance to proteolytic degradation compared to the one isolated from control brains as assayed in *in vitro* models.

Fifth: The activation of p75NTR with pro-NGF from AD affected tissue *in vitro* results in processing of p75NTR and nuclear translocation of its ICD.

Sixth: p75NTR processing, that lead to accumulation of p75NTR ICD, is activated in human brain affected by AD.

Seventh: The pro-NGF isolated from control brains is significatively less effective in inducing the processing and nuclear translocation of p75NTR ICD, than the isolated from AD affect brain.

Eight: γ-Secretase activity is necessary for p75NTR activation by pro-NGF and for induction of cell death.

Ninth: Recombinant pro-NGF induces apoptosis and motoneuron loss in chicken embryo lumbar lateral motor column.

Tenth: The non-enzymatic modifications of pro-NGF are increased in AD in a stage-dependent manner.

Eleventh: The recombinant pro-NGF modified by Glyoxal, Methylglyoxal and oxidized Methyl linoleate, increases its resistance to degradation and processing.

Twelfth: pro-NGF glycoxidated by GO and MGO is physiologically active in inducing apoptosis through p75NTR.

Thirteenth: The modified pro-NGF intracerebroventriculary injected *in vivo* in mice induces an AD-related behaviour.

# X. CONCLUSIONES

Los resultados de nuestro trabajo se pueden resumir en las siguientes conclusiones:

Primera: Es posible aislar pro-NGF funcional de tejido cerebral, indicando que es estable y activo *in vitro*.

Segunda: El Pro-NGF aislado de cerebro humano induce apoptosis en cultivos primarios y líneas celulares expresoras de p75NTR.

Tercera: Pro-NGF aislado de muestras procedentes de individuos control no es capaz de inducir muerte celular, al contrario del pro-NGF procedente de tejido afectado por EA.

Cuarta: Pro-NGF aislado de tejido afectado por EA, demuestra una resistencia aumentada a la degradación proteolítica, comparada con el procedente de cerebros control.

Quinta: El procesamiento de p75NTR, demostrado por acumulación del dominio intracelular de p75NTR (ICD), esta activado en tejido afectado por EA.

Sexta: La activación *in vitro* de p75NTR por pro-NGF aislado de muestras de cerebro humano afectado por la enfermedad, es seguida de su procesamiento y translocación nuclear del fragmento ICD.

Séptima: El pro-NGF aislado de muestras control tiene una capacidad de activar p75NTR e inducir su procesamiento, significativamente inferior al aislado de muestras procedentes de individuos afectados.

Octava: La actividad de γ-Secretasa es necesaria para la activación de p75NTR y la inducción de muerte celular.

Novena: El pro-NGF recombinante induce apoptosis y pérdida de motoneuronas en la columna vertebral de embriones de pollo.

Décima: Las modificaciones no enzimáticas de pro-NGF en EA estan aumentadas de manera dependiente de estadio.

Undécima: El pro-NGF recombinante modificado por Glyoxal, Methylglyoxal y Methyl linoleato oxididado, aumenta su resistencia contra degradación y procesamiento.

Duodécima: El pro-NGF recombinante modificado por Glyoxal, Methylglyoxal es fisiológicamente activo e induce apoptosis a través de p75NTR.

Decimotercera: El pro-NGF recombinante modificado por Glyoxal, inyectado en cerebro de ratones in vivo induce comportamiento asimilable a EA.

# XI. REFERENCES

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# XII. PUBLICATIONS AND PRESENTATIONS

## 1. Publications

Podlesniy Petar, Kichev Anton, Pedraza Carlos, Saurat Jordi, Ferrer Isidre and Espinet Carme. “Pro-NGF from Alzheimer s Disease and normal human brain display distinctive abilities to induce processing and nuclear translocation of p75NTR with secondary apoptosis”. American Journal of PatholOlgy, (2006) 169: 119-131

Antón Kichev, Ekaterina V.Ilieva, Gerard Piñol-Ripoll, Petar Podlesniy, Isidro Ferrer, Manuel Portero-Otín, Reinald Pamplona and Carme Espinet “Cell death caused by *in vitro* modified pro-NGF can be related to its increased modifications in Alzheimer’s disease.” - In revision

# 2. Presentations

31st FEBS Congress 24-29 Jun, 2006, Istanbul Turkey
A. Kichev, P. Podlesniy, C. Pedraza, J. Saurat, M. Encinas, I. Ferrer, C. Espinet
Pro-NGF from Alzheimers disease and normal human brain displays distinctive abilities to induce processing and nuclear translocation of intracellular domain of p75NTR and apoptosis
Poster

31 st FEBS Congress, 24-29 June 2006, Istanbul, Turkey
E. Ilieva, V. Ayala, A.G. Estevez, K. Ricart4, C. Espinet, A. Kichev, P. Podlesniy, A. Naudi, I. Ferrer, R. Pamplona, M. Portero-Otin:
Molecular heterogeneity of protein oxidative stress in cellular models of familial amyotrophic lateral sclerosis (ALS).
Poster

1st Brain research Conference “Advances in Alzheimer´s Disease” 29-30 de September, 2006, Barcelona Institute of Neuroscience, UAB

XXIX Congreso de SEBBM -10 Septiembre 2006, Elche, España
E. Ilieva, V. Ayala, A. Naudi, A. Estevez, I. Ferrer, E. Dalfo, MJ. Bellmunt, C. Espinet, A. Kichev, R. Pamplona, M. Portero-Otin:
Heterogeneidad molecular de la lesion oxidative proteica en modelos celulares y pacientes de enfermedad motoneuronal
Poster

XXX Congreso de SEBBM 12-15 September 2007, Malaga, España
P. Podlesniy, A. Kichev, I. Ferrer, C. Espinet
Pro-NGF aislado de cerebro afectado por la enfermedad de Alzheimer, a diferencia del aislado de cerebro control, induce precesamiento y translocacion nuclear de p75NTR y apoptosis

SFRR Europe 2008 Meeting, 05-09 July 2008, Berlin, Germany
Ekaterina V.Ilieva, Alba Naudí, Antón Kichev, Ester Dalfó, J.Boada, Isidre Ferrer, Reinald Pamplona and Manuel Portero-Otín
Oxidative stress in Pick’s Disease is associated with mitochondrial and ER dysfunctions