PTEN AND AKT SIGNALLING IN ALZHEIMER´S DISEASE

Annika Rickle

Stockholm 2005

Printed by Larserics Digital Print AB
Bromma, Sweden

ISBN-number 91-7140-514-3

I am still confused but on a higher level ☺

CONTENTS

ABSTRACT

Akt / protein kinase B is an important cell survival kinase that modulates the activity of a range of pro-apoptotic proteins. A major downstream target of Akt is glycogen synthase kinase-3β (GSK3β) that phosphorylates the microtubule-associated protein tau. Previous studies have shown that GSK3β activity is increased in Alzheimer's disease (AD) brain in parallel to the accumulation of neurofibrillary changes. This study aimed to determine whether signal transduction pathways upstream of GSK3β are also deregulated in AD brain. The study focussed on Akt and PTEN. The latter is a dual lipid and protein phosphatase the functions of which include inhibition of Akt. Akt and PTEN activity states and levels were determined in AD brain as compared to material from other diseases and controls. Also studied was the basic biology of PTEN and the role of this enzyme as a modulator of rat primary cortical neuron cell death.

Paper I studied the levels and activity of Akt in post-mortem brain material from sporadic AD, controls and other neurodegenerative disorders. Using enzyme immunoprecipitations and phosphorylation of exogenous substrate, as well as immunoblotting with phospho-specific antibodies, we showed that Akt enzyme activities were significantly increased in soluble fraction of mid-temporal cortex from AD cases as compared to both control groups. This increase correlated with Braak staging for neurofibrillary changes. Immunohistochemistry revealed that this increase also occurred partly due to gliosis.

Paper II determined whether altered levels or distribution of PTEN are also found in AD brain. No significant changes of PTEN protein levels were seen in the nuclear, membrane or homogenates of medial AD temporal cortex as compared to control cases. A small but significant decrease in the levels of Ser^{380}PTEN was found in AD medial temporal cortex as shown by dot-blot analysis. Immunohistochemistry of layer III temporal cortex showed the majority of pyramidal neurons in this region were PTEN immunopositive (93-100%). The total numbers of pyramidal neurons and PTEN immunopositive neurons were significantly lower in AD as compared to control cases.

Paper III is a methodological study designed to optimise conditions for studying toxic effects of Aβ, HNE and TNFα on rat primary cortical neurons using propidium iodide/annexin V staining and flow cytometry analyses. These stressors were chosen as being implicated in AD and as having reported effects on Akt signalling. We showed time and dose-responses for all stressors and chose optimal conditions (24h 10μM Aβ, 24h 1μM or 5μM HNE, 24h 500 ng/ml TNFα) that demonstrated effects on numbers of viable, apoptotic and late apoptotic cells. Flow cytometry generated data were confirmed by immunocytochemistry of treated cells. Our results also provided important information on the culture conditions of primary neuron cultures.

Paper IV assessed the regulation of Akt and PTEN during neuronal death induced by the stress factors of TNFα and HNE. We used optimal conditions from study III to determine TNFα and HNE effects on the regulation of PTEN, Akt and GSK3β levels and activity. The inhibitors bpV(Pic) and LY294002 were used to determine PTEN and PI3K involvement, respectively, in TNFα and HNE modulation of neuronal cell death. Immunoblotting with phospho-epitope specific antibodies was used to determine effects on PTEN/PI3K/Akt/GSK3β signalling. In parallel, flow cytometry was used to determine effects on populations of viable and early apoptotic cells. Immunoblotting showed that neither PTEN nor PI3K inhibition alone had an effect on Ser^{473}Akt, or Ser^{9}GSK3β phosphorylation. Instead, effects of these inhibitors were apparent when used together with TNFα and to a lesser extent HNE. TNFα together with PTEN inhibition increased phosphorylation on Ser^{473}Akt and Ser^{9}GSK3β that was paralleled by an increased number of viable and decreased number of early apoptotic neurons. These effects were all reversed by PI3K inhibition. Thus, inhibition of PTEN results in a mild neuroprotection against TNFα induced toxicity. HNE did not show this protective effect. These studies provide evidence for a deregulated Akt and PTEN signalling in AD brain.

LIST OF ORIGINAL PUBLICATIONS

I **Rickle, A**, Bogdanovic, N, Volkmann, I, Winblad, B, Ravid, R and Cowburn, R F. Akt activity in Alzheimer's disease and other neurodegenerative disorders. Neuroreport (2004) 15 (6) 955-9

II **Rickle, A**, Bogdanovic, N, Volkmann, I, Zhou X W, Pei J J, Winblad, B, and Cowburn, R F. PTEN levels in Alzheimer´s disease medial temporal cortex. Neurochemistry International (2005). In press (published on line)

III Behbahani H*, **Rickle A***, Concha H, Ankarcrona M, Winblad B and Cowburn R F. Flow cytometry as a method for studying effects of stressors on primary rat neurons. *Authors contributed equally to the study. Journal of Neuroscience Research (2005) 82 (3) 432-441

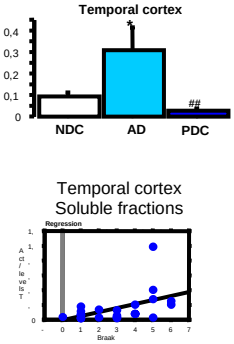
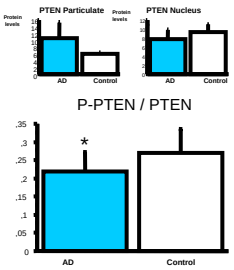
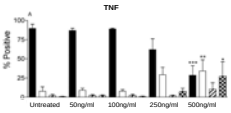
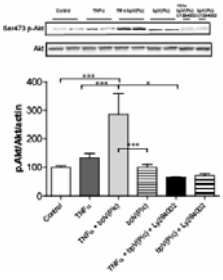
IV **Rickle A**, Behbahani H, Ankarcrona M, Winblad B and Cowburn R F. Involvement of PTEN and the PI3K signalling pathway in rat primary cortical neuronal toxicity mediated by the Alzheimer´s disease associated stresses of TNFα and HNE. Manuscript

* These authors contributed equally to this work

ABBREVIATIONS

Aβ	Amyloid β
AD	Alzheimer´s Disease
Akt	Protein kinase B also known as Akt
APP	Amyloid precursor protein
BDNF	Brain derived neurotrophic factor
CA	Cornu ammonis
CERAD	Consortium to establish a registry of AD
ChAT	Choline acetyltransferase
CREB	cAMP-response-element-bindning protein
DSM IV	Diagnostic and statistical manual of mental disorders 4th edition
FAD	Familial Alzheimer´s disease
FTDP-17	Frontal temporal dementia and Parkinsonism linked to Chromosome 17
GFAP	Glial fibrillary acid protein
GSK3	Glycogen synthase kinase 3
HNE	4-Hydroxynonenal
IGFI	Insulin-like growth factor-I
IL	Interleukin
mTOR	The mammalian target of rapamycin, also known as FRAP1
NDC	Non-diseased control
NFκB	Nuclear factor κB
NFT	Neurofibrillary tangles
NGF	Nerve growth factor
NINCDS-ADRDA	The National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer´s Disease and Related Disorders Association
PD	Parkinson's disease
PDC	Positive-disease control
PDKI	Phosphoinositide-dependent kinase I
PDKII	Phosphoinositide-dependent kinase II
PHF	Paired helical filaments
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol-3,4-biphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PS1	Presenilin 1
PS2	Presenilin 2
PTEN	Phosphatase and tensin homologue
ROS	Reactive oxygen species
TNF	Tumour necrosis factor
TUJ-1	β-tubulin III

THESIS AT A GLANCE

Question		Material & methods		Result	Conclusion
I	Is Akt activity involved in the pathology of AD?	Sub cellular fractionation of post-mortem brain from AD, positive-disease (PDC) and non-disease control (NDC) temporal and frontal cortex. Western blot and immunohisto-chemistry (IHC).		↑ total Akt activity in AD brain as compared to both PDC and NDC and is correlated to Braak staging for NFTs. Ser^{473}Akt positive astrocytes in AD.	↑ Akt activity could be involved in a defence mechanism in AD. Akt could also have a role in gliosis ↓ Akt activity in PDC.
II	Is PTEN, the upstream regulator of Akt involved in AD pathology?	Sub cellular fraction of post-mortem brain from AD and age- matched control (C) temporal cortex. Western blot, dot-blot and IHC.		Unaltered PTEN levels but in both nuclear, particulate and homogenates and a small ↓ in Ser^{380}PTEN in homogenates of AD as compared to C brain.	↓ in Ser^{380}PTEN could be involved in a defence mechanism in AD. PTEN seen in neurons, oligo-dendrocytes, astrocytes and capillaries in AD.
III	What are the optimal conditions for studying toxicity of Aβ, TNFα and HNE stressors in rat cortical neurons?	Flow cytometry and confocal microscopy for studying viable and early apoptotic cells after stress induced in rat primary cortical neurons.		Aβ, TNFα and HNE showed dose response toxicities in rat cortical neurons.	The optimal concentrations for studying toxicities are 24h 10μM Aβ, 500 ng/ml TNFα and 1 or 5μM HNE without B27 supplement.
IV	Is PI3K signalling involved in TNFα and HNE induced toxicity?	Immunoblotting with phospho-specific antibodies for Ser^{473}Akt, Ser^{380}PTEN and Ser^{9}GSK3β and PI3K, PTEN (bpVPic) inhibitors and flow cytometry used for studying toxicity.		TNFα together with bpVPic ↑ Ser^{473}Akt and Ser^{9}GSK3β as compared to TNFα.	PTEN inhibition exerts a slight neuroprotection against TNFα induced toxicity. HNE did not show this effect.

The initial idea "Thesis at a glance" was made by Kajsa Ericson

INTRODUCTION

ALZHEIMER´S DISEASE

General introduction

Alzheimer´s disease (AD) is the most common form of dementia in the aging population (Small *et al.*, 2001). The prevalence of AD is around 1% at age 65 and 20% after 85 years (Lobo *et al.*, 2000). Known risk factors for the development of AD include genetic factors and increased age. Other possible factors may include gender, low education, hypertension, head trauma and chemical exposure. AD is complex both clinically and neuropathologically.
A proportion (~5%) of AD cases can be described as familial, the remainder being classified as sporadic of unknown aetiology. Much of the understanding of the pathogenic mechanisms in AD is based on the study of involved mutations. So far, three genes causative for early-onset (before 65 years of age) familial AD (FAD) have been identified. These are the amyloid precursor protein (APP) gene on chromosome 21 and the presenilin 1 (PS1) and 2 (PS2) genes on respectively chromosomes 14 and 1. FAD is genetically heterogeneous and is inherited in an autosomal dominant fashion. In addition, inheritance of the ε4-allele of the apolipoprotein E (ApoE) gene has been recognised as a strong genetic risk factor for late-onset (after 65 years of age) disease (Selkoe, 2001).

Symptoms

AD is clinically diagnosed by memory deficits and progressive loss of cognitive abilities. This is followed by deterioration of language, motor and spatial abilities. Symptoms such as aphasia, disorientation and short-term memory impairment are common early in the disease. Later in the disease spatial and motor abilities become more affected.

Clinical and neuropathological diagnosis

Several different clinical tests are commonly used to diagnose AD. The assessments are based on the medical history combined with the exclusion of other dementia diseases. The National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer´s Disease and Related Disorders Association (NINCDS-ADRDA) criteria divide AD into probable, possible or definite (McKhann *et al.*, 1984), although the latter must be diagnosed in combination with a neuropathological determination. Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM IV). These criteria classify subtypes of AD into early and late onset and according to the major features of the clinical presentation such as delirium, delusions and depression (Association, 1994). The Global Deterioration Scale is a seven point score instrument for clinical assessment of the overall magnitude of severity of age associated memory impairment, primary degenerative dementia (PDD), especially PDD and also the progression of PDD (Reisberg *et al.*, 1988). The Consortium to Establish a Registry of Alzheimer´s Disease (CERAD) is a neuropathological diagnosis that is based on the presence of senile plaques and/or neurofibrillary tangles in the affected brain. CERAD is not sufficient for diagnosing a definitive AD. Thus a clinical diagnosis must also be considered in order to exclude other types of dementia (Mirra *et al.*, 1991).

Neuropathological hallmarks

AD is characterised by specific pathologies in the brain, in particular the neocortex, entorhinal area, amygdala hippocampus and specific sets of subcortical nuclei. The neuropathological hallmarks of AD include amyloidosis in the cerebrovasculature and senile plaques, as well as neurofibrillary tangles, synapse and neuron loss (Selkoe, 2001).

At the macroscopic level, the neuropathology of AD is characterised by cortical atrophy, a 20% reduction in brain weight or more, that affects mainly the temporal and then frontal lobes (Selkoe, 2002). The microscopic neuropathological hallmarks of AD include amyloidosis in the cerebrovasculature and as senile plaques, as well as neurofibrillary tangles (NFTs) (Figure 1) and loss of synapses and neurones. There are two morphologically distinguishable forms of plaques, namely senile neuritic and "diffuse" plaques. Neuritic plaques are extracellular deposits consisting mainly of a dense core of fibrillous amyloid β peptides ($A\beta_{1-40}$ or $A\beta_{1-42}$), surrounded by microglia near the core and by dystrophic neurites and astrocytes that encircle the outside of the plaque. Aβ is a product of APP that is proteolytically processed by α, β and γ secretases. The diffuse ("preamyloid") plaques are more light and amorphous and occur in a granular pattern without a clear fibrillar compact centre. NFTs are intracellular neuronal inclusions composed of paired helical filaments made up of hyperphosphorylated forms of the tau protein (Selkoe, 2001). Tau is a microtubule-associated neuronal protein predominantly located in axons (Binder *et al.*, 1985), the function of which is to promote tubulin polymerisation and stabilise microtubules (Drechsel *et al.*, 1992). Hyperphosphorylation impairs the microtubule binding function of tau (Lovestone and Reynolds, 1997). It has been suggested that reduced binding of tau to microtubules and lower levels of normal tau result in a destabilisation of the cytoskeleton and a perturbation of axonal transport which in turn leads to degeneration of affected neurons (Hong and Lee, 1997).

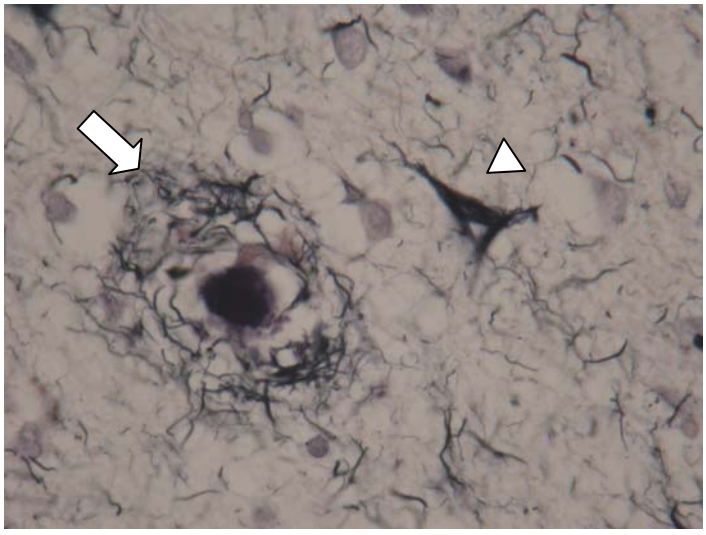


Figure 1. The main neuropathological hallmarks of AD. Extracellular neuritic plaques (arrows) and intracellular NFT (arrowhead). Bielschowsky silver staining. Photo by Nenad Bogdanovic.

Synaptic loss, plaques and NFTs correlated to clinical symptoms

Early studies have shown that synaptic loss seems to be best correlated to cognitive impairment in AD patients (DeKosky and Scheff, 1990; Terry *et al.*, 1991). This was recently confirmed using a PS1/APP/tau triple transgenic AD model, showing that synaptic loss appeared before plaques and tangles (Oddo *et al.*, 2003). It is still uncertain how the neuropathological hallmarks of AD, tangles and plaques, are correlated to cognitive impairment. Studies have shown that neither tangles nor plaques were correlated to the severity of dementia seen in AD (Morris *et al.*, 1991). Other groups have shown that the number of senile plaques (Roth *et al.*, 1966), Aβ load in plaques (Cummings and Cotman, 1995) or levels of total $A\beta_{x-40}$ and $A\beta_{x-42}$ (Näslund *et al.*, 2000) are correlated to cognitive impairment in AD patients. In contrast, others have shown that cognitive dysfunction is correlated to NFTs (Arriagada *et al.*, 1992; McKee *et al.*, 1991), but not senile plaques (Arriagada *et al.*, 1992). The relationship between neurofibrillary changes and the progression of AD neuropathology has been described in Braak´s staging protocol, a model that describes the topography of NFTs and neuropil threads in the progression of the disease. The model permits a differentiation of six stages based on the distribution of neurofibrillary changes. The first two stages demonstrate mild or severe alterations in a single layer of the transentorhinal region (transentorhinal I-II). The next stages (limbic III-IV) are characterised by severe involvement of the entorhinal and transentorhinal layers. The last two stages (isocortical V-VI) involve severe destructions of the isocortex (Braak and Braak, 1991). The amyloid deposition does not follow any specific distribution pattern during the progression of disease. These deposits are also found in the cerebral cortex of non-diseased eldery persons (Braak and Braak, 1991; Braak *et al.*, 1996).

Amyloid cascade hypothesis

Biochemical investigations have revealed that FAD gene mutations increase total Aβ or specifically the more amyloidogenic $A\beta_{1-42}$ form of this peptide. This has provided arguably the strongest support for the amyloid cascade hypothesis, which is currently the predominant model for the molecular pathogenesis of AD (Figure 2). According to the amyloid cascade hypothesis, accumulation of Aβ in the brain is the primary event that triggers other secondary pathological events in the disease such as inflammation, synaptic injury, oxidative injury and altered protein kinase/phosphatases activities, leading to tangle formation and cell death (Selkoe, 2002). The strongest support for the hypothesis is that the majority of mutations in the APP and PS genes increase Aβ production, especially $A\beta_{1-42}$. An increased $A\beta_{1-42}$ aggregates into fibrils that then deposits in plaques and the cerebrovasculature. Even though increased production or decreased clearance of Aβ appears to be a common mechanism for FAD gene mutations, the precise links between the Aβ peptide and neuronal dysfunction are not well understood. It has long been argued as to whether increased Aβ and senile plaque formation precedes NFT formation. Three groups have independently shown that Aβ influences the formation of tau tangles in transgenic mice (Gotz *et al.*, 2001; Lewis *et al.*, 2001; Oddo *et al.*, 2003). Lewis *et al* demonstrated an increased level of NFTs in the limbic system and the olfactory cortex of bigenic mice, expressing both mutant human tau (P301L) and mutant APP (APPsw) as compared to transgenic mice expressing only mutant tau (P301L). This study suggested that APP or Aβ might influence NFT formation (Lewis *et al.*, 2001). In comparison, Götz *et al* directly injected fibrillar $A\beta_{1-42}$ into the hippocampus of tau mutant (P301L) mice and showed an enhanced level of NFTs in the amygdala, an area affected in AD patients (Gotz *et al.*, 2001). Also using PS1/APP/tau triple transgenic mice, Oddo *et al* showed that amyloid deposition precedes tangle formation (Oddo *et al.*, 2003). The same group also showed using PS1/APP/tau triple transgenic mice that intraneuronal Aβ is strongly implicated in the early onset of cognitive impairment (Billings *et al.*,

2005). Together, this data suggest that changes in Aβ metabolism occur before NFTs, in accordance with the amyloid cascade hypothesis. However, NFT pathology is also a characteristic of neurodegenerative diseases other than AD, in the absence of β-amyloidosis, including that of FTDP-17 (frontal temporal dementia and Parkinsonism linked to chromosome 17) associated with point mutations in the tau gene (Spillantini and Goedert, 1998), Pick's disease, progressive supranuclear palsy, corticobasal degeneration and argyrophilic grain disease (Trojanowski and Lee, 2002). The finding that mutations in the tau protein gene cause frontal lobe dementia, strongly argues that tau pathology is the driving force in developing neurodegeneration independently of Aβ.

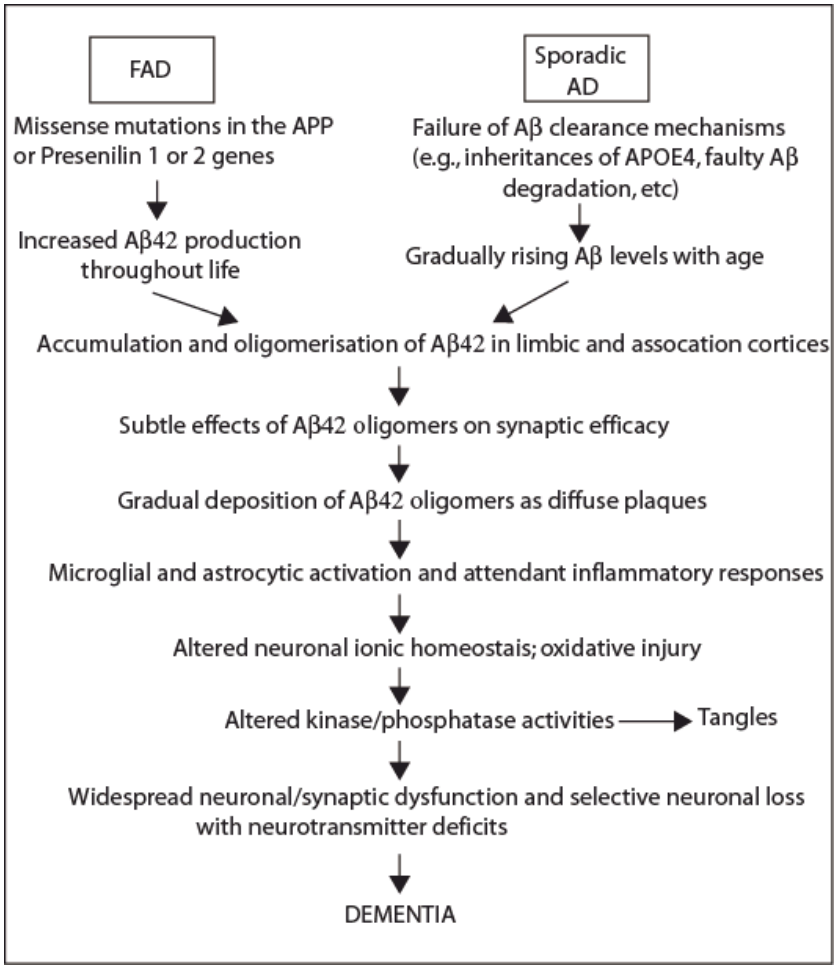


Figure 2. A hypothetical sequence of the AD pathogenic steps as suggested by Selkoe, 2002.

NERVE CELL DEATH IN ALZHEIMER´S DISEASE

The exact relationship between nerve cell loss and the pathogenesis of AD is still unclear. Although, the neurodegenerative process starts probably several years before any symptoms of cognitive impairment are apparent in the patient. The following chapter summaries neuronal cell death in specific areas of AD brain and also the possible mechanisms behind neurodegeneration.

Neuronal loss

A wide range of evidences demonstrate that nerve cell loss occurs in different subcortical and cerebral cortical areas in AD brain. Neurons from the basal forebrain degenerate in AD. The majority of cholinergic axons derive from the basal forebrain in the nucleus of Meynert that innervates the neocortex (Whitehouse *et al.*, 1981). Other subcortical areas affected by nerve cell loss and NFT accumulation in AD include the noradrenergic nuclei of the locus coeruleus, where the changes occur mainly in neurons that send projections to the temporal cortex and hippocampus, areas that are heavily damaged in the AD brain. Also affected are the serotonergic nuclei of the Raphé nucleus and dopaminergic neurons in the ventral tegmental area (Pearson, 1996). Brun *et al* demonstrated degeneration in basal medial temporal limbic areas, posterior cingulate gyrus and in the superior parietal lobe (Brun and Englund, 1981). Terry *et al* showed a specific neuronal loss in AD temporal and frontal cortex (Terry *et al.*, 1981). In these areas nerve cell death and NFT accumulation occurs predominantly in pyramidal cells in laminae III and V (Pearson, 1996). The hippocampus CA1 (cornu ammonis) region has tended to show most severe neuronal loss in AD as compared to in normal aging controls. There are evidences suggesting that neuronal loss in the CA1 region of the hippocampus is AD specific and not related to aging (West *et al.*, 2000) and it seems that degeneration is more profound in later stages of the disease than in pre-clincal stages (West *et al.*, 2004). A relationship between loss of neurons and NFTs has also been shown in hippocampal cortex in late onset sporadic AD (Fukutani *et al.*, 2000). Simic *et al* showed a significant loss in the number of neurons in the CA1 region of normal aged hippocampus, although no changes could be detected between AD and control cases. Instead, a significant decrease in the volume of the hippocampal formation was observed in AD cases suggesting that it is the atrophy in the CA1 region and not the loss of neurons that distinguishes AD brains from controls (Simic *et al.*, 1997). Interestingly, of the neuronal loss seen in the CA1 region (~60%), only 2.2-17.2% could be accounted for by NFTs, suggesting that there is more than one mechanism behind neuronal death in AD brain (Kril *et al.*, 2002).

Synapse loss

The synaptic pathology seen in AD includes not only the loss of the synapses but also an impaired ability to communicate with unaffected synapses. Neurons that lose synapses stay alive in the tissue as "ghost cells" before they are phagocyted by astrocytes or microglia. Synaptic loss seems to be the first morphological sign in the neuronal dysfunction in AD, preceding cell death. A recent study revealed that both synapse loss and disrupted long term potentiation (LTP) appeared before plaques and tangles in a PS1/APP/tau triple transgenic AD model (Oddo *et al.*, 2003). Early neurochemical analyses of post-mortem AD brain tissues showed a massive loss of choline acetyltransferase (ChAT), a marker for cholinergic synapses (Bowen *et al.*, 1976; Davies and Maloney, 1976). Although deficits in several neurotransmitters such as somatostatin, GABA and serotonin accumulate as the disease progresses (Selkoe, 2002), early symptoms appear to be linked to dysfunction of cholinergic (Bowen *et al.*, 1976; Davies and Maloney, 1976; Whitehouse *et al.*, 1981) and glutamergic synapses (Selkoe, 2002). Synaptic loss occurs in the hippocampus and temporal cortex (Honer *et al.*, 1992). Synaptic loss has clearly been shown to correlate to cognitive dysfunction in frontal areas (DeKosky and Scheff, 1990; Terry *et al.*, 1991) and inferior parietal lobe (DeKosky and Scheff, 1990) of the diseased brain. The loss of synapses is most

apparent in outer parts of the molecular layer of the dendate gyrus (Hamos *et al.*, 1989), most likely as a consequence of less input from the enthorinal cortex.

Cell death

Cell death can be divided into apoptosis and necrosis on the level of specific biochemical and morphological features, both of which have been implicated in AD. Apoptosis is a non-inflammatory process and is less harmful to neighbouring neurons than necrosis that can trigger neuroinflammation, resulting in a secondary damage to healthy neighbouring neurons. Apoptosis is an active process regulated by such stimuli as withdrawal of trophic factors, DNA damage, oxidative stress, drugs, toxins, viruses and exposure to hormones. Apoptosis is characterised by cell shrinkage, chromatin condensation and budding phenomena. It is under genetic control and the morphology of mitochondria and most organelles remains mostly intact. The apoptotic process avoids inflammatory responses by forming apoptotic bodies that are directly phagocytosed by neighbouring cells and macrophages (Behl, 2000). Studying post-mortem tissues provides some difficulties when considering the type of cell death that occurs in a disease. For example, as there is only one time point/incident for each case it is complicated to understand the ongoing neurodegenerative process in the brain. The time span between the apoptotic processes lasts for ~24h in tissue, whereas the disease progresses for ~20 years in a patient. Nevertheless, a great deal of recent evidence indicates that the nerve cell loss seen in AD can occur by apoptosis or programmed cell death. Several groups have demonstrated characteristic signs of apoptosis in AD brain, such as cell shrinkage, DNA fragmentation and caspase activation. Histochemical studies have shown DNA fragmentation in AD brain (Anderson *et al.*, 2000; Dragunow *et al.*, 1995; Lassmann *et al.*, 1995; Lucassen *et al.*, 1997; Overmyer *et al.*, 2000; Smale *et al.*, 1995; Stadelmann *et al.*, 1998; Troncoso *et al.*, 1996), although the majority of neurons with DNA fragmentation do not demonstrate the typical morphological features of apoptosis such as chromatin condensation and nuclear fragmentation (Lassmann *et al.*, 1995; Lucassen *et al.*, 1997; Overmyer *et al.*, 2000; Stadelmann *et al.*, 1998). Other evidence suggesting that apoptosis occurs in AD brain has come from studies of the cysteine protease caspases that play an essential role in apoptosis by cleaving a number of key proteins. Some reports have demonstrated an up-regulation of caspase-3 protein in AD brain (Marx, 2001; Shimohama, 2000). There is also evidence for apoptosis in synapses (Gylys *et al.*, 2004).

In contrast to apoptosis, necrosis is a passive pathological event that occurs from spontaneous insults or trauma to the cells. It is characterised by cellular and mitochondrial swelling, and plasma membrane disintegration. Necrosis leads to a massive inflammatory response and phagocytosis by macrophages (Behl, 2000). Aβ can mediate oxidative stress and thereby induce necrotic processes causing the generation of reactive oxygen species (ROS). Such Aβ-induced damage is thought to induce rapid necrotic events (Behl, 1999; Behl *et al.*, 1994). In addition, Aβ exposure in primary cultures has been shown to induce neither nuclear condensation nor DNA laddering, but rather a rapid breakdown of both plasma membranes and neurite filaments (Behl *et al.*, 1994). This may suggest that Aβ induces a rapid necrotic response in neurons and in the long term apoptosis. It feasible that necrosis occurs in parallel with apoptosis in the progression of AD.

Aβ

Aβ is known to be neurotoxic, but the link between Aβ accumulation in brain and cognitive decline is still uncertain. Several lines of evidence indicate that Aβ induces apoptosis and that accumulation of Aβ peptides and disruption of tau function are also important in cell death. A study of cultured mouse hippocampal neurons, revealed also the importance of tau in the mechanisms leading to Aβ-induced neurodegeneration (Rapoport *et al.*, 2002). The mechanisms behind Aβ neurotoxicity are complex and have been shown to involve oxidative stress, disruption of calcium homeostasis and the likely involvement of many other biochemical pathways such as those involving, JNK (c-jun N-terminal kinase), tumour necrosis factor (TNF), ER (endoplasmatic reticulum) stress via caspase-12, NO (nitric oxide) and cyclin-dependent kinase 4/ 6 (cdk 4/6) (see review for more details (Small *et al.*, 2001)).

Inflammation

Aβ can upregulate and activate astroglia and microglia which may act as inflammatory cells for the production of pro-inflammatory cytokines including Interleukin-1 α and β (IL1α, IL-1β), Interleukin-6 (IL-6) and TNFα and the anti-inflammatory cytokine, Transforming Growth Factor β (TGFβ) (Ho *et al.*, 2005). The key players implicated in the inflammatory process are thought to be microglia and astrocytes and to a lesser extent neurons. Activated microglia and astrocytes have been demonstrated to localise with neuritic plaques. Inflammatory responses have been suggested in the pathogenesis of AD and several neuropathological and neurochemical inflammatory markers have been identified in AD brain. TNFα is an important pro-inflammatory cytokine that is increased in AD. Genetic evidence suggests a role for TNFα in late onset AD. TNFα activates nuclear factor κB (NF-κB) that in turn triggers a positive feedback of TNFα, whereby more cytokines are released (Hemmer *et al.*, 2001). Also, polymorphisms for IL1α, IL-1β, IL-6 and TNFα have been shown to either accelerate the progression of AD or increase the risk of developing AD (Cacquevel *et al.*, 2004). These proinflammatory cytokines have all been shown to be upregulated in AD (Akiyama *et al.*, 2000). Aβ can also activate astrocytes and microglia followed by increased generation of nitric oxide (NO) and ROS products which lead to cellular damages(Tuppo and Arias, 2005).

Oxidative stress

Oxidative stress leads to apoptotic injury as well as necrosis and seems to be involved in aging and age-related disorders. Oxidative stress results in free carbonyls derived from lipids, proteins, carbohydrates and nucleic acids (Smith *et al.*, 1996), and chronic oxidative stress leads to extensive damage to neurons. Reactive oxygen species (ROS) are the primary products in oxidative stress and are generated by several metabolic pathways. Mitochondria have been linked to oxidative stress, acting as a notorious source of superoxide radicals and other ROS. The mitochondrial derived superoxide anion radical, gives rise to the non-radical hydrogen peroxide, which is converted further to the extremely aggressive hydroxyl radical (•OH), which induces lipid peroxidation. Other ROS are the singlet oxygen, nitric oxide and the derived peroxynitrite radical (Eckert *et al.*, 2003). Oxidative stress and AD is discussed below and in more details in the discussion section. Accumulating evidence indicates that oxidative stress is an early event in the pathogenesis of AD (Perry, 1998). Lipid peroxidation (Butterfield *et al.*, 2002) and protein oxidation (Choi *et al.*, 2003) have been demonstrated to occur in AD. Lipid peroxidation has also been linked to synapse pathology (Chakraborty *et al.*, 2001). In AD brains, toxic carbonyls accumulate with NFTs and also in the neuronal cytoplasm, nuclei and glia (Smith *et al.*, 1996). Furthermore, activated microglia that surround senile plaques, produce cytotoxic and neurotoxic

free oxygen radicals (NO and O_{2^-}) that can damage neurons by producing peroxynitrite (Mattson, 2002).

Tau and NFTs

Several lines of evidence indicate that disruption of tau function is also important in cell death. When tau is hyperphosphorylated it forms the abnormal paired helical filaments (PHFs) in the NFTs seen in AD brain (Lee *et al.*, 2001). Tau function seems to be regulated by phosphorylation due to different kinases and is counterbalanced by phosphatases. A number of kinases have been implicated in AD and can be grouped into two families: Proline-directed protein kinases (PDPK) and non-proline-directed kinases (non-PDPK). The PDPKs that have been involved in tau phosphorylation include glycogen synthase kinase-3 (GSK3), mitogen-activated protein kinase (MAPK), cyclin-dependent kinases cdk2 and cdk5, stress activated protein kinases (SAP kinases) (Buee *et al.*, 2000). GSK3β, and maybe also GSK3α, have been strongly implicated in AD pathogenesis, in particular tau hyperphosphorylation, as well as the production and mechanism of action of Aβ. GSK3β is also heavily implicated in modulating apoptotic cell death (Grimes and Jope, 2001). The non-PDPKs include Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II), microtubule-affinity regulating kinases (MARK), protein kinase (PKA) (for review see (Buee *et al.*, 2000; Lovestone and Reynolds, 1997). A number of phosphatases counterbalance the effects of kinases by dephosphorylating tau. The protein phosphatases 2A and 2B (PP2A, PP2B) are present in brain and dephosphorylate tau at different sites in neurons (Buee *et al.*, 2000). Evidences have shown that different apoptotic markers are associated with tau phosphorylation which may give rise to the PHF-1 epitope seen in AD. Studies have shown that caspase-cleaved tau is present in AD but not control brain (Gamblin *et al.*, 2003; Rohn *et al.*, 2002). An antibody recognising cleavage products of fodrin after digestion by caspase-3, has been reported to show increased immunostaining in most PHF-1 tau-positive neurons in AD brain (Rohn *et al.*, 2001). Caspase-3 tends also to be located around and in senile plaques and in NFTs (Marx, 2001), indicating that the enzyme may have a link to disease pathology. Another study showed that caspase-3 induces cleavage of PHF-1 tau in staurosporine treated mouse cortical neurons. These authors also showed that active caspase-3 was co-localised with PHF-1 immunoreactivity in AD temporal cortex (Kang *et al.*, 2005). Guo and colleagues showed that active caspase-6 is two-three fold increased in frontal and temporal cortex of AD brain and is associated with neuropil threads, NFTs and neuritic plaques (Guo *et al.*, 2004). Tau and NFTs have also been linked to oxidative stress. 4-hydroxynonenal (HNE) is the most cytotoxic form of lipid peroxidation (Esterbauer *et al.*, 1991). HNE has been demonstrated to co-localise with NFTs (Sayre *et al.*, 1997) and has also been shown to inhibit tau dephosphorylation in hippocampal neurons (Mattson *et al.*, 1997).

CELL SIGNALLING IN ALZHEIMER´S DISEASE

As summarised above, the cell death mechanisms occurring in AD include accumulation of ROS, disturbances of inflammatory compounds, altered protein kinases and protein phosphatases resulting in tau hyperphosphorylation, Aβ toxicity and apoptosis. All of these mechanisms can be linked to altered cell signalling pathways. This thesis focuses on the role of phosphatidylinositol-3-kinase (PI3K) and Akt signalling in AD. As discussed below, this pathway is strongly implicated in neuronal survival, tau phosphorylation and oxidative stress.

PI3K regulation

PI3K is a heterodimer composed of a regulatory p85 subunit and a catalytic p110 subunit. When a survival factor/growth factor binds to its receptor, PI3K becomes activated by the binding to the regulatory subunit either directly or via adaptors to activated receptor tyrosine kinase (RTK). Activation of RTK results in recruitment of the p110 subunit to the plasma membrane where it interacts and phosphorylates membrane phosphoinositides (Sofroniew *et al.*, 2001). PI3K generates phosphatidylinositol-3,4,5-triphosphate (PIP3) that is crucial for the activation of Akt, a critical mediator of cell survival by the activation of downstream substrates such as GSK3β (Brazil and Hemmings, 2001). An upstream negative regulator of the PI3K pathway is PTEN (phosphatase and tensin homologue deleted from chromosome 10, also named MMAC1 or TEP-1) (Maehama *et al.*, 2001) (Figure 3). PI3K is activated by various stimuli such as growth factors, insulin, insulin-like growth factor-I (IGFI), cytokines and cellular stresses (Brazil and Hemmings, 2001; Lawlor and Alessi, 2001).

Akt

Akt, the homologue of the retroviral oncogene v-Akt, is also known as PKB (Vivanco and Sawyers, 2002) and belongs to the AGC (cAMP-dependent kinase/protein kinase G/protein kinase C) superfamily. The Akt family of proteins exist as at least three isoforms in mammalian cells, encoded by separate gene loci: Akt1, Akt2 and Akt3 (Brazil and Hemmings, 2001). Akt is ubiquitously expressed in mammals, and Akt1 is the most expressed isoform whereas Akt2 is expressed at lower levels in skeletal muscle, heart, kidney, liver and adipose tissue (Altomare *et al.*, 1995). Akt3 expression seems to be limited to brain and testis, with lower expression in kidney, pancreas, heart and adipose tissue (Nakatani *et al.*, 1999). All isoforms have an amino-terminal pleckstrin homology (PH) domain, a catalytic domain and a C-terminal regulatory domain, that modulates interactions with phospholipids and binds to lipids in the membrane when activated. Akt is downstream of PI3K and is translocated to the membrane when activated by phosphorylation at the Threonine 308 (Thr^{308}) and Serine 473 (Ser^{473}) residues by phosphoinositide-dependent kinase I (PDK1) and (PDKII) (Brazil and Hemmings, 2001). Phosphorylation of both residues is necessary for full activity of the enzyme. Substrates of Akt include a number of apoptosis regulators and transcription factors such as GSK3β, Bad (a pro-apoptotic member of the Bcl-2 family) (Yuan and Yankner, 2000), mTOR (the mammalian target of rapamycin, also known as FRAP1) (Vivanco and Sawyers, 2002), transcription factors NF-κB, Forkhead and cAMP-response-element-binding protein (CREB), that stimulate cell survival signals (Yuan and Yankner, 2000). Caspase-9 is also thought to be an Akt substrate (Cardone *et al.*, 1998), although regulation of this is unclear (Rodriguez *et al.*, 2000). Akt has recently been shown to also phosphorylate the microtubule-associated protein tau at Ser^{214} and Thr^{212} (Ksiezak-Reding *et al.*, 2003).

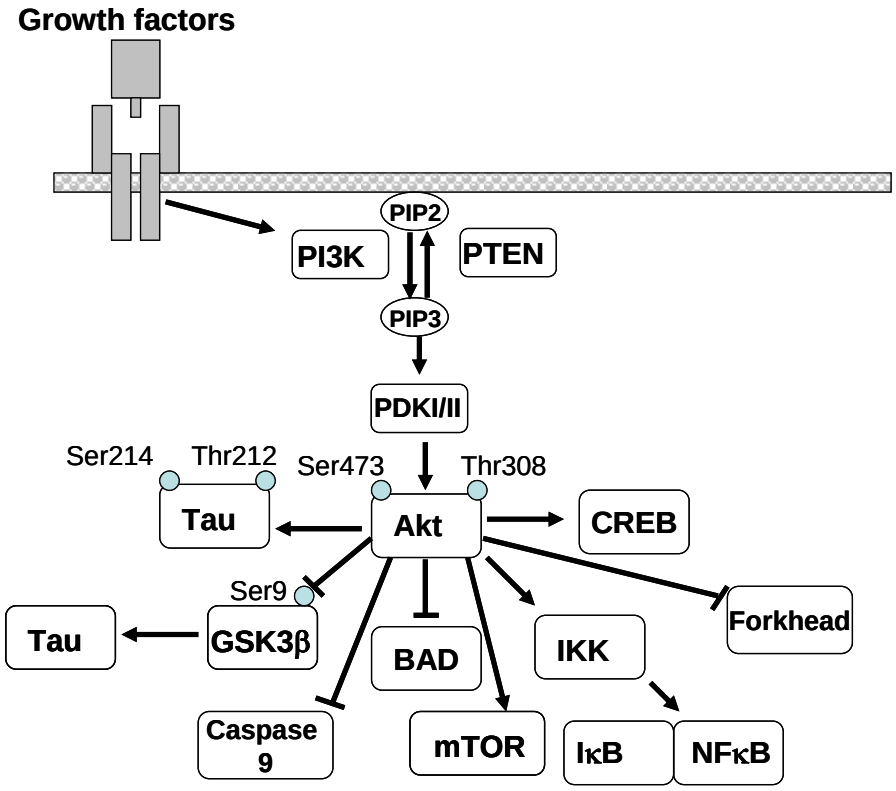


Figure 3. Simplified PI3K signalling pathway. Details of Akt regulation and substrates are given in the text. Akt also regulates Ask1 (apoptosis signal-regulating kinase 1) (Kim *et al.*, 2001), the cell cycle protein cyclin D and the tumour suppressor gene p53 (Vivanco and Sawyers, 2002) - not shown here.

GSK3β

GSK3 was originally discovered as the rate-limiting enzyme of glycogen synthesis. GSK3 phosphorylates and thereby inactivates glycogen synthase (Cohen, 1979; Embi *et al.*, 1980). Two closely related isoforms, GSK3α and GSK3β, are ubiquitously expressed in mammalian tissues (Woodgett, 1990; Woodgett, 1991). GSK3β is expressed in adult rat brain. Highest brain levels are seen during development (Leroy and Brion, 1999). The high expression of GSK3β in brain suggests a role of the enzyme in neuronal intracellular signalling pathways. GSK3β has been shown to affect many functions such as cell cycle, gene transcription and cytoskeletal integrity, due to its ability to phosphorylate proteins crucial for these processes (Frame and Cohen, 2001). Phosphorylation of GSK3β can be accomplished at Ser^{9} and Tyr^{216} and for GSK3α at Ser^{21} and Tyr^{279} in its kinase domain. Akt phosphorylates both isoforms at Ser^{21} in GSK3α and Ser^{9} in GSK3β which inactivates the enzymes (Kaytor and Orr, 2002). Inhibition of GSK3 activity occurs also due to serine phosphorylation by cyclic AMP-dependent protein kinase (protein kinase A), p70 S6 kinase, p90Rsk (also called MAPKAP kinase-1), and protein kinase C, cyclic AMP-dependent protein kinase (PKA) (Grimes and Jope, 2001). GSK3 can also be activated by phosphorylation of Tyr^{216} in GSK3β and of Tyr^{279} in GSK3α. This regulation of GSK3 could be due to autophosphorylation or by other kinases but the exact regulation behind the tyrosine phosphorylation remains to be elucidated (Jope and Johnson, 2004).

PTEN

PTEN negatively regulates the PI3K/Akt signalling pathway (Stambolic *et al.*, 1998). PTEN antagonises PI3K/Akt signalling by dephosphorylating the D3 position of the inositol ring of PIP3, to generate phosphatidylinositol-3,4-biphosphate (PIP2). The PTEN gene encodes a 403 amino acid protein and contains a protein tyrosine phosphatase (PTP) domain with features resembling those of "dual-specificity" phosphatases (DSPs). PTEN functions as both a protein tyrosine phosphatase and as a lipid phosphatase. As a lipid phosphatase, PTEN antagonises PI3K/Akt signalling. The structural features of PTEN are a 179 residue N-terminal phosphatase domain, containing the PTP signature motif and a 166 residue C-terminal domain. In addition, the extreme C-terminal segment of PTEN contains two PEST sequences and a consensus PDZ-binding site that can facilitate protein-protein interactions (Maehama *et al.*, 2001). The C-terminus of PTEN is phosphorylated by casein kinase II (ck2) a cluster of Ser/Thr residues. The ck2 sites have been shown to be important in PTEN protein stability and have been identified as residues Ser^{370}, Ser^{380}, Thr^{382}, Thr^{383} and Ser^{385} (Torres and Pulido, 2001). In contrast, a very recent report, could not show that ck2 phosphorylates Ser^{380}, but demonstrate phosphorylation of Ser^{370} and Ser^{385} sites. The same group, showed also that GSK3β could regulate PTEN at two phosphorylation sites, Ser^{362} and Thr^{366}, suggesting a possible PTEN regulation in a negative feedback loop (Al-Khouri *et al.*, 2005).

PTEN was originally discovered as a candidate tumour suppressor gene located on chromosome 10q23, a region deleted in several sporadic human cancers such as glioblastomas, prostatic and breast cancers. Germ-line mutations of PTEN result in Bannayan-Zonana syndrome, Cowden´s disease and Lhermitte-Duclos disease. Some of the patients with these hereditary disorders demonstrate defects in neural development such as mental retardation, macrocephaly, cerebellar hypertrophy, ataxia and seizure (Ross *et al.*, 2001). Homozygous deletion of PTEN is embryonic lethal in mice (Di Cristofano *et al.*, 1998; Suzuki *et al.*, 1998), while heterozygous PTEN animals are viable through adulthood. However, heterozygous mice develop a broad range of tumours, including breast cancers, endometrium, prostate and T cell lymphomas that resemble the etiology of human PTEN mutations (Di Cristofano *et al.*, 1998). PTEN expression in brain was first shown by using human brain biopsy samples (Sano *et al.*, 1999). PTEN has also been shown to be expressed in mouse (Lachyankar *et al.*, 2000) and rat brain (Omori *et al.*, 2002)], in particular in cerebellar granule cells (Kyrylenko *et al.*, 1999) and in hippocampal neurons (Gary and Mattson, 2002)

PI3K, Akt and GSK3 in neurodegeneration and in Alzheimer´s disease

PI3K and Akt have been implicated in neuronal survival by several different neurotrophic factors. Nerve growth factor (NGF) is part of the neurotrophin family and shares strong structural homology with brain derived neurotrophic factor (BDNF) (Sofroniew *et al.*, 2001). Both neurotrophic factors can be involved in cell survival by activating the signalling cascades mediated by PI3K and Akt, thereby inhibiting apoptosis. IGFI is another neurotrophic factor, that acts via the PI3K/Akt pathway (Dudek *et al.*, 1997). After nerve growth factor withdrawal, IGFI rescued dorsal root ganglion neurons from apoptosis via the PI3K pathway (Russell *et al.*, 1998).

Several lines of evidence suggest that a number of neurotrophic factors are differentially changed in AD brain. NGF has been implicated in AD and it has been suggested that decreased supply of NGF at the level of basal forebrain cholinergic cell bodies leads to degeneration (See review (Salehi *et al.*, 2004)). Decreased BDNF protein levels have also been reported in the hippocampus and temporal cortex (Murer *et al.*, 2001). A very recent study has shown that BDNF decreased tau phosphorylation at AT8, Tau1 and AT180 sites through the PI3K/GSK3β pathway in

differentiated p19 mouse embryonic carcinoma cells (Elliott *et al.*, 2005). IGFI regulates tau phosphorylation and binding to microtubules via an Akt mediated inhibition of GSK3β (Hong and Lee, 1997). IGFI has also been shown to prevent tau hyperphosphorylation on two sites in a gene target mouse model (IGFI-/-). Brain homogenates from (IGFI-/-) KO mice showed that tau sites phosphorylated by GSK3β, increased 7 fold (serine 396) and 10 fold (serine 202) as compared to WT mice (Cheng *et al.*, 2005) Recently, IGFI was shown to be able to ameliorate the AD like disturbances in APP/PS2 transgenic mice that demonstrate severe brain amyloidosis and cognitive impairment (Carro *et al.*, 2005).

Several lines of evidences have shown that increased Akt activity can promote cell survival during a number of injurious insults such as oxidative stress (Chong *et al.*, 2005), Aβ exposure (Martin *et al.*, 2001), neuronal axotomy (Namikawa *et al.*, 2000) and DNA damage (Henry *et al.*, 2001). Akt can also foster cell survival in primary hippocampal neurons and neuronal cell lines after free radical exposure (Chong *et al.*, 2005).

As mentioned previously, perhaps the most important downstream targets of Akt are GSK3β and maybe also α. Akt negatively regulates GSK3β activity and thereby regulates neuronal survival. Inactivation of GSK3β has been suggested as an important modulator of apoptosis downstream of PI3K/Akt signalling in cerebellar granule neurons (Cross *et al.*, 2001), in PC12 cells and in Rat-1 fibroblasts (Pap and Cooper, 1998). Bijur *et al* demonstrated that adequate inactivation of GSK3β is critical for cell survival. In their study they showed that overexpression of GSK3β in SH-SY5Y neuroblastoma cells, increased the sensitivity of the cells to staurosporine-induced apoptosis and also to heat shock-induced caspase-3 activation (Bijur *et al.*, 2000). A role for decreased inactivation of GSK3β in neuronal death has also been shown in cultured cortical neurons, subjected to serum deprivation or treatment with PI3K inhibitors (Hetman *et al.*, 2000). In contrast, phosphorylation at Tyr^{216} in GSK3β results in increased activity that occurs during neuronal degeneration (Bhat *et al.*, 2000). Taken together this data strongly suggest that the inhibitory control of GSK3β, by anti-apoptotic mechanisms via the PI3K pathway, is important in promoting cell survival.

With respect to AD, there are many studies suggesting that the PI3K/Akt/GSK3β signalling pathway may contribute to a number of aspects of pathology. *In vitro* studies showed that PI3K and Akt protect neurons against Aβ-induced toxicity (Martin *et al.*, 2001; Wei *et al.*, 2002). Takashima *et al* first reported that $Aβ_{25-35}$ and $Aβ_{1-40}$ inhibit PI3K and activate GSK3β in rat hippocampal neurons (Takashima, 1996). Another report provided evidence that Aβ stimulates the Akt pathway in SH-SY5Y neuroblastoma cells (Cedazo-Minguez *et al.*, 2003). GSK3α also facilitates APP processing thereby regulating the production of $Aβ_{1-40}$ and $Aβ_{1-42}$ (Phiel *et al.*, 2003). GSK-3β phosphorylates tau, of which hyperphosphorylated forms comprise the major component of AD NFTs (Lovestone and Reynolds, 1997). Liu *et al* demonstrated that inhibition of PI3K leads to tau hyperphosphorylation and impairment of spatial memory (Liu *et al.*, 2003a). GSK3β is strongly implicated in tau phosphorylation at various sites including the PHF-1, Tau1, AT8 and AT270 epitopes (Lee *et al.*, 2003; Munoz-Montano *et al.*, 1997). Yoon *et al* investigated okadaic acid-induced tau phosphorylation in rat primary cortical neurons and found that increased Ser^{473}Akt and increased Ser^{9}GSK3β phosphorylations occurred in proportion to the progression of okadaic acid-induced tau phosphorylation (Yoon *et al.*, 2005). Increased intracellular calcium levels have also been shown to increase GSK3β tyrosine phosphorylation associated with increased tau phosphorylation (Hartigan and Johnson, 1999)

Pei *et al* investigated the role of GSK3β in PHF formation and compared the distribution of the active Tyr^{216} and the inactive Ser^{9} forms of the enzyme in the entorhinal cortex, hippocampal formation and temporal cortical regions of a series of brains staged for AD neurofibrillary changes. The data from Pei *et al* indicated that the active form of GSK3β phosphorylated at Tyr^{216} is co-distributed with the progressive accumulation of abnormal hyperphosphorylated tau proteins in AD brains. The inactive form of GSK3β phosphorylated at Ser^{9} showed no accumulation in pretangle or tangle bearing neurons (Pei *et al.*, 1999). In contrast, the group of Ferrer *et al*, demonstrated strong immunoreactivity of Ser^{9}GSK3β in neurons with neurofibrillary tangles, in dystrophic neurites of senile plaques in AD and in other tauopathies such as Pick´s disease, progressive supranuclear palsy and corticobasal degeneration, suggesting a possible role of GSK3β in protecting abnormal tau-containing cells from dying (Ferrer *et al.*, 2002). Akt has been shown to phosphorylate the microtubule-associated protein tau at Ser^{214} and Thr^{212} (Ksiezak-Reding *et al.*, 2003). These two phosphorylation sites form the phospho-epitope AT100 (Matsuo *et al.*, 1994) that is specific to PHFs seen in AD and other age-related dementias with tauopathy (Goedert *et al.*, 1998). Recently, Kyoung et al provided evidences that Akt exerts its anti-apoptotic role by phosphorylation of tau at Ser^{214}, rather than at Thr^{212} (Kyoung Pyo *et al.*, 2004).

Akt signalling has also been implicated in the mechanism action of FAD mutations. A deregulation of Akt activity has proposed as one of the mechanisms by which mutant forms of the PS1 protein cause neurons to die by apoptosis in FAD. Studies with PC12 cells transiently transfected with PS1 A246E and PS1 C410Y mutations showed that mutant PS1 down regulates Akt resulting in increased GSK3β activity and thereby inducing apoptosis (Weihl *et al.*, 1999). In contrast, the mutated PS1 ΔE9 and L250S proteins do not cause altered Akt activity in SH-SY5Y neuroblastoma cells (Vestling *et al.*, 2001). De Sarno *et al* also reported no mutation differences in Akt activity induced by heat shock in PS1 wt and PS1 L286V mutant transfected SH-SY5Y cells (De Sarno *et al.*, 2001). Baki *et al* demonstrated using mouse fibroblasts that PS1 knockout gives a decreased phosphorylation of Akt and GSK3 and increased tau phosphorylation, indicating a PS1 regulation of tau phosphorylation through Akt/PI3K/GSK3 signalling (Baki *et al.*, 2004). Ryder and colleagues also reported using human embryonic kidney cells (HEK293) expressing the Swedish APP mutation and in AD presenilin1 (Canadian cell line) and presenilin2 mutation (German cell line) lymphoblast cells, a downregulation of both Ser^{473}Akt and inactive Ser^{9} phosphorylated GSK3β, also suggestive of increased GSK3 activity due to these mutations (Ryder *et al.*, 2004). Although, the underlying mechanisms of PI3K/Akt/GSK3β regulation in AD are still unclear they are currently being intensively investigated.

AIMS

The general aim of this study was to determine the role of Akt and PTEN in AD neurodegeneration.

The specific aims were to:

I Map levels and activity of Akt in post-mortem brain material from AD and control cases.

II Determine levels and distribution of PTEN in temporal cortex of AD and control brains.

III Optimise conditions for studying toxic effects of TNFα, HNE and Aβ on rat primary cortical neurons using flow cytometry analyses.

IV Assess the function of PTEN in neuronal death induced by different AD associated stress factors

RESULTS AND DISCUSSION

Pei *et al* demonstrated that Thr^{308}Akt was increased in the soluble fraction of sporadic AD frontal cortex (Pei *et al.*, 2003). These authors also demonstrated that GSK3β phosphorylated at Tyr^{216} is co-distributed with the progressive accumulation of abnormal hyperphosphorylated tau proteins in AD brains, whereas the inactive form of Ser^{9}GSK3β showed no accumulation in pretangle or tangle bearing neurons (Pei *et al.*, 1999). In contrast, Ferrer *et al* demonstrated strong immunoreactivity of Ser^{9}GSK3β in neurons with neurofibrillary tangles, in dystrophic neurites of senile plaques and in tau containing astrocytes in AD brain (Ferrer *et al.*, 2002).

The study in **Paper I** aimed to determine whether signal transduction pathways upstream of GSK3β are also deregulated in AD brain. We compared the enzyme activities of Akt in post-mortem mid-temporal and mid-frontal cortices (TC and FC) from groups of AD patients, positive disease controls (PDC) with other neurodegenerative disorders, and non-disease controls (NDC) that were closely matched for age, gender, post-mortem delay and tissue pH at autopsy. Since Akt activation involves phosphoinositide dependent recruitment of the enzyme to the plasma membrane followed by PDK1 and 2 mediated phosphorylation (Brazil and Hemmings, 2001), we chose to determine potential differences in Akt activity by measuring activities in soluble and particulate fractions generated by sub fractionation of homogenised tissue. We found that Akt activities were not significantly different in mid-frontal cortex soluble fractions from AD and NDC cases, whereas AD mid-temporal cortex soluble fractions showed a markedly increased enzyme activity as compared to fractions from both NDC and PDC groups. The Akt enzyme activities in temporal cortex soluble fractions revealed a positive correlation with Braak staging for neurofibrillary changes. This finding is in accordance with that of Pei *et al* who provided evidence that increased immunostaining for Thr^{308}Akt occurred in parallel to the progressive accumulation of Braak staged neurofibrillary changes in the entorhinal cortex, hippocampal CA1 region and temporal cortex (Pei *et al.*, 2003). In contrast to the results pattern seen in AD, the PDC cases with other neurodegenerative disorders showed reduced Akt activities in both frontal and temporal cortices, as demonstrated by enzyme measures (this study) and by immunoblotting for Thr^{308} phosphorylated Akt (Pei *et al.*, 2003). In our study we have to be aware of the interpretation of enzyme activity in the PDC cases since this group was heterogeneous and included cases (Vascular dementia, Pick's disease and Parkinson's disease (PD)) that do not share the same pathology. Recently Ser^{473}Akt was shown to be downregulated by caspase-3 cleavage in a rat model of Huntington's disease as well as in patients with the disease (Colin *et al.*, 2005). Pei *et al* also showed decreased Ser^{308}Akt in Huntington's disease as compared to control cases (Pei *et al.*, 2003).

To gain insight into the morphological structures showing increased Akt activity in AD temporal cortex we performed immunohistochemistry with an antibody against Akt phosphorylated at Ser^{473}. We found that AD cases showed a population of pyramidal neurones in temporal cortical layers II and III that were moderately immunopositive for Ser^{473}Akt, as well as neurons that were strongly immunopositive. In contrast, both NDC and PDC cases showed only occasional pyramidal neurones that were moderately Ser^{473}Akt immunopositive. In addition to neuronal staining we found that AD cases showed reactive astrocytes that were intensely immunopositive for Ser^{473}Akt, suggesting that a component of increased Akt activity in AD brain may be due to gliosis around neuritic plaque pathology. Akt immunopositive reactive astrocytes have not been described previously and were not seen using a Thr^{308} Akt antibody. As earlier described, GSK3β is downstream of Akt and the group of Ferrer *et al* showed strong immunoreactivity of Ser^{9}GSK3β in astrocytes in AD (Ferrer *et al.*, 2002). The current data together with that of Pei *et al* and the recent data of Griffin *et al* provide complementary evidence that Akt signalling is up

regulated in AD concomitant with the severity of neurofibrillary pathology (Griffin *et al.*, 2005; Pei *et al.*, 2003). Although, Griffin *et al* showed decreased Ser^{473}Akt in AD soluble fractions, whereas increased Ser^{473}Akt was seen in particulate fractions. Since our study investigated the total Akt enzyme activity we would expect increased Akt in the cytosol rather than in the particulate fraction, as phosphorylated Akt is translocated from the plasma membrane to the cytosol and nucleus and returned to the inactive state by protein phosphatase 2A (PP2A) (Brazil *et al.*, 2002). Griffin *et al* revealed that increased Ser^{473}Akt corresponded to increased Ser^{9}GSK3β and Ser^{214}tau in AD temporal cortex (Griffin *et al.*, 2005).

In conclusion, we showed that the total Akt enzyme activities were significantly increased in soluble fractions of mid-temporal cortex from AD cases as compared to matched positive disease and non-disease controls. This increase correlated with Braak staging for neurofibrillary changes and may also have occurred due to gliosis. In contrast, Akt activities in soluble fractions of post-mortem mid-frontal cortex were significantly reduced in cases with other neurodegenerative disorders. To elucidate if increased Akt was due to altered upstream regulators, we then investigated PTEN in AD temporal cortex.

In **Paper II** we determined whether altered levels of PTEN also occurs in AD post-mortem brain. The function of PTEN in the nervous system is still in the early stage of investigation. Targeted conditional deletion of PTEN was shown to give brain enlargement (Backman *et al.*, 2001; Groszer *et al.*, 2001; Kwon *et al.*, 2001) due to increased cell proliferation, decreased cell death and enlarged cell size (Groszer *et al.*, 2001). Griffin *et al* provided the first evidences of a deregulated PTEN in AD brain. These authors investigated the hippocampus using immuhistochemistry and revealed decreased PTEN immunoreactivity in the nuclei of AD CA1 and entorhinal cortical pyramidal neurons, whereas increased PTEN immunoreactivity was seen in the apical dendrites of CA1 region pyramidal neurons (Griffin *et al.*, 2005). In our study, we compared nuclear and membrane subcellular fractions from AD (n=6) and control brain (n=6) temporal cortex. Using western blot analyses, no significant changes in PTEN protein levels were found in either the nucleus or in the particulate fraction of AD as compared to control cases. The discrepancy between our study and that of Griffin *et al*, could be due to different methodological approaches. However, one should emphasise the difficulties of interpretation of the nuclear fractions as one may lose proteins when striving towards a pure nuclear fraction during the purification process. PTEN is regulated by the balance of phosphorylation and dephosphorylation at three residues (Ser^{380}, Thr^{382} and Thr^{383}) at the C-terminal domain of the protein, which seems to be important for PTEN stability and function (Torres and Pulido, 2001; Vazquez *et al.*, 2000). Phosphorylated PTEN is less active, whereas dephosphorylated PTEN is in a more active form (Vazquez *et al.*, 2000). As we used a small number of human brain samples for the nuclear fraction experiment, we increased the number of AD (n=21) and control (n=10) brains, and further investigated the total amounts of PTEN protein levels in homogenates by dot-blot analyses. Griffin *et al* showed by western blot analyses, that the PTEN protein level was down regulated in AD temporal cortex brains as compared to age matched controls. We could not detect any differences in the amount of PTEN protein between AD as compared to control brain samples. Nevertheless, we showed a small but significant decreased inactivation of PTEN, as shown by the reduction of Ser^{380}PTEN levels in AD as compared to control brains. This suggests that PTEN may be more activated in AD brain. This finding was somewhat unexpected. However, Choi *et al* reported in rats that 17 β-estradiol prevented focal cerebral ischemic damages via the activation of Akt and CREB associated with reduced PTEN phosphorylation. These authors reported an increased Ser^{380}PTEN/PTEN ratio and a decreased Ser^{473}Akt/Akt ratio as well as decreased p-CREB positive cells after focal cerebral ischemic damages, that was reversed by estradiol treatment after 24 hours. This data indicates a possible protection pathway

whereby reduced PTEN phosphorylation could be accompanied by increased p-Akt and p-CREB (Choi *et al.*, 2004).

The PTEN distribution pattern in nerve cells has been shown to be both nuclear and cytoplasmatic (Lachyankar *et al.*, 2000; Sano *et al.*, 1999). In our study, immunohistochemistry demonstrated PTEN in the nuclear membrane, cytoplasm and apical dendrites of control brain pyramidal cells. This staining pattern was also seen in AD temporal cortex. Lachyankar *et al* reported PTEN immunostaining in the cortex and cerebellum of mouse brain. PTEN expression was detected in a wide range of neurons, including pyramidal neurons in the cortex, the Purkinje neurons in the cerebellum, olfactory mitral neurons, magnocellular neurons in the basal forebrain, hippocampal and amygdalar neurons and dentate granule neurons (Lachyankar *et al.*, 2000). We also performed counting of pyramidal cells and found that the majority of neurons (between 93-100%) were immunopositive for PTEN. There was a significant lower number of pyramidal neurons (12% loss) in the AD as compared to control cases. Also, the PTEN immunopositive pyramidal neurons showed a 15% loss in AD as compared to control cases.

Our immunohistochemistry study revealed a complex PTEN immunostaining of structures other than neurons both in AD and control brains. In AD cases, PTEN staining was detected in oligodendrocyte-like structures, and in occasional capillaries. Weak PTEN immunostaining was also seen in the nucleus of astrocyte-like structures. In control brains, PTEN immunoreactivity was found in oligodendrocyte-like structures, in capillaries (although these appeared occasionally), and also very pale immunoreactivity in astrocyte-like structures. Lachyankar *et al* showed no PTEN expression in glial cells, although a weak transient expression of PTEN was observed in immature astrocytes as well as in differentiating CNS stem cells (Lachyankar *et al.*, 2000). The PTEN stained capillaries may be indicative of micro-ischemic changes in the brain. In this respect, Ohmori *et al* reported an involvement of PTEN and p-PTEN in rat brain ischemia following transient middle cerebral artery occlusion (MCAO), suggesting an important role of PTEN in cell survival (Omori *et al.*, 2002). In addition, PTEN immunostained AD neurons appeared both with and without neurofibrillary pathology as shown by co-immunostaining for phosphorylated tau using the AT-8 antibody. Although, we did not find any correlation to Braak staging for neurofibrillary tangles. The complex PTEN staining pattern seen in both AD and control brain makes it difficult to interpretate. It remains to be determined whether PTEN in these other different structures has a role in the disease.

In conclusion, using both immunoblotting and dot-blot analyses we could not show any significant differences in the levels of PTEN protein in nuclear and particulate fractions and in homogenates of AD as compared to control brain. However, a significant reduction of Ser^{380}PTEN levels was seen in AD as compared to control cases. Quantification of total and PTEN immunopositive neurons showed that PTEN containing neurons did not appear to be more or less vulnerable in AD brain. Our immunohistochemistry data also revealed structures other than neurons including oligodendrocytes, capillaries, astrocytes, that were PTEN immunoreactive. Whether these different structures have a role in AD needs to be further investigated, although for the moment we chose to continue investigating PTEN in a neuronal culture to limit other potential interacting cells in the process. As we did not show any differences in PTEN protein levels between AD as compared to controls cases, even though a deregulated Ser^{380}PTEN was seen in AD, we could speculate that decreased Ser^{380}PTEN levels might be involved in a defence mechanism via its downstream targets such as p-Akt. To test this hypothesis we chose to investigate PTEN/Akt/GSK3 signalling in a rat primary neuron culture system in response to neurotoxic stressors. There are few studies investigating the role PTEN and Akt signalling in neuronal death, therefore we chose to perform our study with rat primary cortical neuron cultures.

Paper III is a methodological study designed to optimise conditions for assessing neuronal cell death (analysed by flow cytometry) in primary rat cortical neurons in response to the neurodegeneration associated stressors of Aβ exposure, oxidative stress and proinflammatory cytokines. Flow cytometry analysis has several advantages as it is rapid and quantitative and different subsets of cells can be studied simultaneously. Flow cytometry has been widely used to study cell death, in particular for the identification and characterization of apoptotic cells (Ormerod *et al.*, 1992; Sun *et al.*, 1992). However, there are few reports investigating primary cultures and neuronal death using flow cytometry. Therefore, we thought it important to design and optimise conditions for studying toxic effects of $A\beta_{1-42}$, TNFα and HNE. These stressors were chosen as being implicated in AD and as having a reported effect on Akt signalling (studied in paper IV). Both HNE and TNF have been reported in other neurodegenerative disease. HNE has been reported in amyotrophic lateral sclerosis (ALS), lewy body related disease and in PD (Zarkovic, 2003). TNF has been implicated in PD (Hirsch *et al.*, 2005) and in vascular dementia (VD) (Roman, 2004). For the cell death analyses we chose an apoptosis assay kit, that contains annexin V conjugated to fluorescin (FITC-annexin V), as well as red-fluorescent propodium iodide (PI) nucleic acid binding dye. Apoptosis is associated with exposure of phosphatidylserine (PS) to the cell surface of the cell. Annexins, including annexin V, bind to PSs. Living cell and apoptotic cells are impermeable to PI, while necrotic cell are permeable to PI and allow nuclei acidic binding. Viable cells show little or no staining. Dead cells show both green and red fluorescence and apoptotic cells show green fluorescence. In addition to flow cytometry analysis, we performed confocal microscopy following PI and annexin V labelling. To distinguish different cell populations in our primary cell cultures, we performed double staining with TUJ-1 and GFAP by flow cytometry and also confocal microscopy. The neuronal cell cultures were incubated with B27 supplement that contains anti-oxidants. In the early stage of the study, cells were exposed both with or without B27 supplement. Only very small toxic effects were observed when using B27 supplement together with the stressors. Therefore, we continued the experiments without B27. Brewer *et al*, earlier reported that the B27 supplement used in primary cultures reduces growth of other cell types such as glial cells in primary culture to less than 0.5% (Brewer *et al.*, 1993). Our primary neuron cultures subsequently labelled with a specific neuronal marker, TUJ-1, revealed 82% pure neuronal populations, whereas approximately 7% of cells were astrocytic as shown by GFAP staining and by flow cytometry.

Aβ has been shown to be neurotoxic. In cultured primary neurons, Aβ is toxic after microinjections, or when applied extracellulary (Zhang *et al.*, 2002). In cortical cultures, Loo and colleagues demonstrated detectable DNA laddering after Aβ treatment (25μM) for 48 hours (Loo *et al.*, 1993). In our primary cultures Aβ-induced toxicity at concentrations of 10 μM and 60μM. Cells exposed to 10 μM $A\beta_{1-42}$ demonstrated statistically significantly increased numbers of late apoptotic cells, whereas 60μM $A\beta_{1-42}$ increased the numbers of both early and late apoptotic cells.

TNFα-treated human fetal CNS cultures showed increased apoptosis as seen by characteristic nuclear morphology and positive TUNEL labelling (Downen *et al.*, 1999). In our study, we showed that TNFα induced significant toxicity at 500ng/ml, as seen by increased numbers of early apoptotic and late apoptotic and reduced numbers of viable cells.

Kruman *et al* presented evidence that HNE induces neuronal apoptosis by oxidative stress in PC12 cells and in rat hippocampal neurons (Kruman *et al.*, 1997). Another study Lovell *et al* revealed that HNE was neurotoxic in a time- and concentration-dependent manner in hippocampal neuron cultures (Lovell *et al.*, 2001). Consistent with our analysis, we demonstrated

a dose-response as well as a significantly increased numbers of early apoptotic and reduced numbers of viable cells after 1 or 5μM HNE exposure.

In conclusion, we performed stress treatments for 24 hours without B27 supplement as it exerts antioxidant effects, thereby reducing the toxic effects of all stressors investigated. We also checked the purity of our neuronal cultures both with immunocytochemistry and flow cytometry, and found that 82% of cells were neurons and 7% were astrocytes. The appearance of glial cells was first seen after seven days in our primary cultures. We showed dose-responses for all stressors after 24 hours and chose optimal conditions for 10μM $A\beta_{1-42}$, 1μM or 5μM HNE, 500 ng/ml TNFα that revealed a mix between healthy, early apoptotic and late apoptotic cells. Flow cytometry generated data were confirmed by immunocytochemistry of treated cells.

Paper IV. As earlier discussed in the introduction, PI3K/Akt signalling plays a crucial role in the regulation of cell death. In view of these studies and the fact that little is known about PTEN involvement in regulating the PI3K/Akt pathway with respect to neuronal cell death we determined whether stresses associated with AD could affect this signalling pathway. For this we decided to use TNFα and HNE since both have been implicated in AD pathology (Lovell *et al.*, 1997; Montine *et al.*, 1997a; Montine *et al.*, 1997b; Sayre *et al.*, 1997) and have been shown in non-neuronal cells as being able to modulate Akt signalling (Dozza *et al.*, 2004; Gustin *et al.*, 2001; Liu *et al.*, 2003b; Ozes *et al.*, 1999). We used rat primary cortical neurons that were treated with TNFα or HNE in the absence and presence of the potent PTEN phosphatase inhibitor bpV(Pic) both alone and together with the PI3K inhibitor LY294002. To evaluate the effects of TNFα and HNE induced toxicity on these primary cultures, we assayed toxicity by performing flow cytometry. Enzyme activities were determined by western blot using antibodies that recognise phosphorylation of Ser^{473}Akt, Ser^{9}GSK3β and Ser^{380}PTEN. The Ser^{473}Akt antibody was chosen because it is primarily dependent on PI3K activity (Franke *et al.*, 1997).

In our study, TNFα alone demonstrated toxic effects to primary cortical neurons as shown by flow cytometry, although no differences could be detected in the phosphorylation state of either PTEN, Akt or GSK3β. TNFα in combination with PTEN inhibition gave increased Ser^{473} Akt and Ser^{9} GSK3β phosphorylation that was paralleled by a partial neuroprotection as shown by an increased number of viable cells, as compared to TNFα alone treated cultures. These effects were all reversed by PI3K inhibition. This data indicate that PTEN inhibition exerts a slight neuroprotection against TNFα-induced toxicity that is mediated via Akt/GSK3β signalling in rat cortical neurons.

TNFα can generate both a degenerative and a neuroprotective pathway mediated by two distinct receptors, TNFR1 and TNFR2. The TNFR1 receptor is associated with a death domain and induces neuronal degeneration and apoptosis, whereas TNFR2 does not contain a death domain and instead activates the anti-apoptotic NFκB pathway. In AD post-mortem brain TNFR1 immunoreactivity is reportedly increased whereas TNFR2 is decreased (Zhao *et al.*, 2003).

We also showed by flow cytometry that HNE induced toxicity of rat primary cortical neurons. HNE gave no changes in the activity states of either PTEN, Akt or GSK3β. Nevertheless, HNE in combination with PTEN inhibition gave an increased Akt signalling via the PI3K signalling pathway. However, these effects were not as large as those seen with TNFα, and were not accompanied by an increased Ser^{9} phosphorylation of GSK3β in rat primary neurons. Also, in contrast to the effects seen with TNFα, HNE did not give an apparent neuroprotection as determined by flow cytometry. It has previously been shown in neuroblastoma cells that HNE

can increase PI3K signalling to give elevated Ser^{473} Akt and Ser^{9} GSK3β phosphorylations (Dozza *et al.*, 2004). Such effects were seen after 30 min treatment with HNE and remained elevated after 6 h exposure. In our studies we used a single time point of 24 h (in order to be able to perform toxicity studies). It may be the case that HNE induced stimulation of PI3K signalling is more apparent short term or is more pronounced in neuroblastoma as compared to primary cultures. Nevertheless, our data indicate that PTEN inhibition is not sufficient to give neuroprotection after HNE toxicity in primary cortical neurons, nor does HNE toxicity seem to involve changes in Ser^{9} GSK3β phosphorylation.

In conclusion, both TNFα and HNE induced toxicity in primary neurons, as shown by flow cytometry. Using both immunoblotting and flow cytometry, we provide new data showing that PTEN inhibition results in a mild neuroprotective effect against TNFα-induced toxicity mediated by the Akt/GSK3β signalling pathway in rat primary neurons. In contrast, HNE did not show this protective effect.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

As pointed out in the introduction, AD is not a homogeneous disorder. Understanding the biochemical and pathophysiological mechanisms behind the disease is currently being investigated by many groups all over the world. Importantly, the main research tasks are to achieve early reliable diagnostic markers for AD and to also develop therapeutic drugs that could slow down the progression of disease. Currently, many investigators are focusing on trying to suppress the formation of Aβ aggregates in the brain as a strategy for AD prevention and treatment. It is also important to elucidate the intracellular signal transduction pathways during cell death in AD that may link the pathologies of NFTs and Aβ accumulation. The inability to link a single pathogenic mechanism has during the years led to different theories that each investigate only one specific pathway in AD brain.

In this thesis, we focused on the role of Akt and PTEN signalling in AD brain, as well as studying the biology of PTEN and Akt activity after stress using rat primary cortical neurons. The different AD post-mortem studies of PTEN and Akt are (summarised in Figure 4). Separate studies have shown decreased PTEN levels (Griffin *et al.*, 2005), unchanged PTEN levels (Rickle *et al.*, 2005), and a slight decreased phosphorylation of Ser^{380}PTEN in AD brain. There is more of a consensus for increased Akt activity (Griffin *et al.*, 2005; Pei *et al.*, 2003; Rickle *et al.*, 2004) that was correlated to Braak staging for neurofibrillary changes. Increased Ser^{9}GSK3β (Ferrer *et al*, Griffin *et al*) as well as increased Ser^{214}tau (Griffin *et al.*, 2005) and also increased Tyr^{216}GSK3β (Pei *et al.*, 1999) have all been shown in AD brain. However, there is no study that has looked at PTEN/Akt/GSK3β signalling in its entirety in AD brain. It would be of interest to continue investigating this process in an *in vivo* model. A good model for this could be the triple transgenic PS1/APP/tau mouse that gives AD pathology. However, the tau mutation used in this model is a human four repeat tau harbouring the P301L mutation which makes it unfortunately not a pure AD model. We could also speculate that if both the active (Tyr^{216}) and the inactive (Ser^{9}) form of GSK3β are increased in AD brain (Figure 4), then the overall effect would be no increase in total GSK3β activity (assuming that this happens in the same cells). It could also be of interest to further investigate neurons for co-localisation of tau and for both the active and inactive forms of GSK3β, as well as a co-localisation of both forms of GSK3β with an apoptotic marker. Since Tyr^{216}GSK3β is the active form, a therapeutic approach against this enzyme would be favourable, so that the inactive form of Ser^{9}GSK3β could exert its beneficial features thereby leading to neuroprotection.

It has been speculated that increased Akt activity could have a role in the defence mechanism in AD. Increased Ser^{214}tau has been shown in AD brain and this site is involved in inhibiting NFTs (Figure 4). Kyoung *et al* provided evidence that Akt exerts its anti-apoptotic role by phosphorylation at Ser^{214} rather than Thr^{212} (Kyoung Pyo *et al.*, 2004). The upstream regulators of Akt are many and include neurotrophic factors, oxidative stress, insulin and inflammation. Since oxidative stress occurs in AD and that Akt is known to be increased and protect cells from insults after oxidative stress, one could speculate that this defence mechanism could be triggered in AD brain. Another possible explanation could be that IGFI signalling also has a role in this protection mechanism, as IGFI receptor levels have shown to be increased in cortical areas of AD brain as compared to age-matched controls (Crews *et al.*, 1992). It has previously been reported and that IGFI immunoreactivity is induced in a population of GFAP-positive astroglia in AD temporal cortex. The same study showed strong immunoreactivity for IGFI in reactive astrocytes associated with Aβ-positive plaques (Connor *et al.*, 1997). These evidences argue for an increased IGFI signalling in AD. In addition, we found in AD cases increased Ser^{473}Akt

immunostaining of astrocytes like structures around plaques. To further investigate the role of Akt in astrocytes, it would be of interest to test both Ser^{473} and Thr^{308} antibodies, in primary human astrocytes. Interestingly, a study has shown increased Ser^{473}Akt and Ser^{214}tau activity in human astrocyte cultures after okadaic treatment (Kyoung Pyo *et al.*, 2004). In contrast, recently Steen and co-workers reported decreased levels of insulin, IGFI and IGFII receptors in AD brains and also decreased Thr^{308}Akt levels in hippocampal samples (Steen *et al.*, 2005). Even though, IGFI receptors are increased, it might be the case that impaired receptor responsiveness is involved in AD, similar to the insulin resistance seen in diabetes mellitus type II (de la Monte and Wands, 2005) However, due to a lack of consistent studies that link AD to diabetes mellitus such a hypothesis remains controversial. Instead, IGFI could have a role in another pathway as it has been implicated in Aβ clearance from the brain mediated by the transport proteins albumin and transthyretin (Carro *et al.*, 2002).

Hypothetically, if an impaired responsiveness of insulin and IGFI receptor is involved in AD then it could argue for an involvement of oxidative stress as Akt activity is increased in AD. Therefore, it would be of interest to investigate oxidative and inflammatory stresses that are known to induce this signalling pathway, especially oxidative stress. Although, we could not show (Paper IV) any differences in the PI3K signalling pathway after either TNFα or HNE treatment in our rat primary cortical neurons. As we used a pure neuronal culture, one could speculate that neurons have to interact with glial cells to give an effect after these stressors. As astrocytes are involved in the inflammatory response, it would be of interest to investigate the role of astrocytes alone or in the interaction with neurons in the regulation of a specific function. To continue investigating the involvement of the PI3K pathway in cell death after TNFα and HNE stressors, a mixed culture or a pure glial cell line could be used. It could be of interest to investigate if the mild protection, seen after PTEN inhibition together with TNFα, is still apparent in a mixed culture or in a glial cell line.

These studies provide evidence for a deregulated Akt and PTEN signalling in Alzheimer's disease brain. It is therefore of interest to also investigate the effects of PTEN on tau phosphorylation. We are currently investigating this issue in Chinese Hamster Ovary (CHO) cells together with the group of Simone Lovestone in London. Recently, mutations in PTEN-induced kinase 1 (PINK1) were identified as causing hereditary early-onset PD thus establishing the importance of PTEN signalling for other human neurodegenerative disease (Valente *et al.*, 2004). The neuronal function of PTEN in cell death is still poorly understood. To further investigate the role of PTEN in neurodegeneration is very topical given the recent report that deregulated PTEN signalling, may contribute to the neurodegeneration associated with PD. Very recently, Yang and colleagues demonstrated that DJ-1A, a Drosophila homologue of the familial PD-associated gene DJ-1, downregulates Akt activity and thereby impairment of PI3K/Akt signalling (Yang *et al.*, 2005). Further studies of PTEN and PI3K regulated signalling relevant to neurodegeneration will give novel insights into disease pathology and may also reveal new targets for potential therapeutic intervention also in AD and other neurodegenerative diseases.

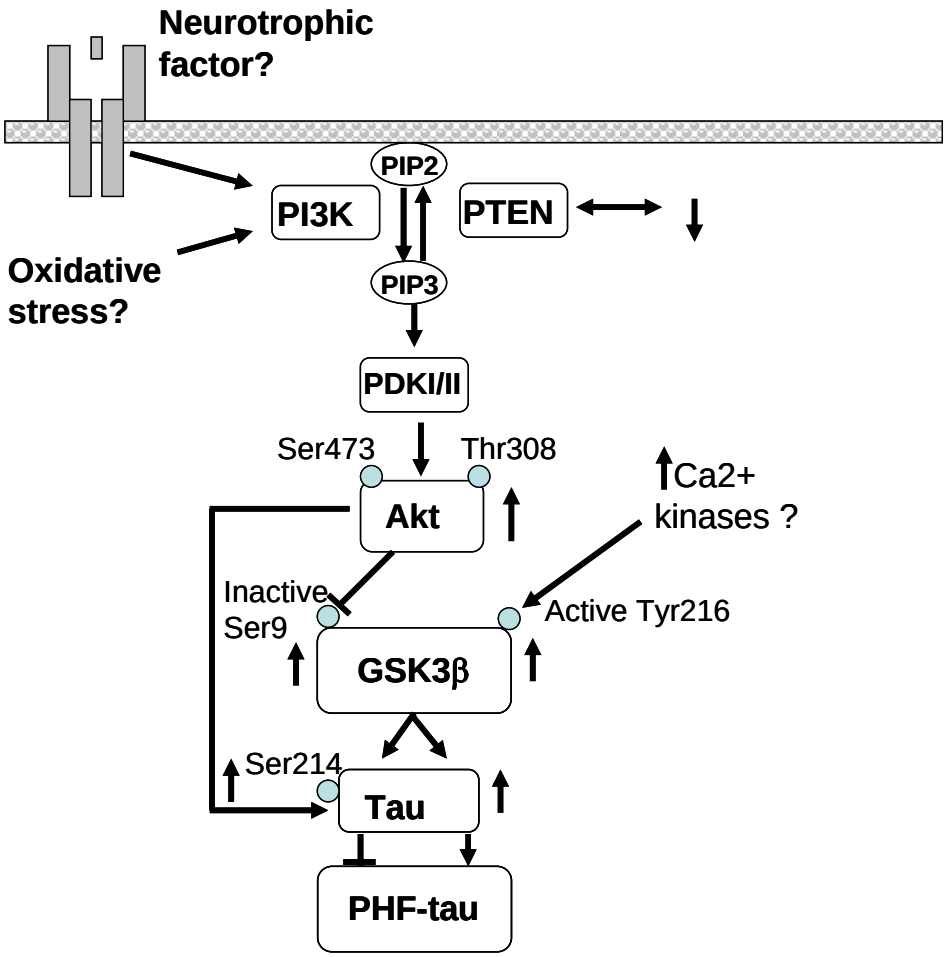


Figure 4. Studies showing a deregulation of PI3K signalling in AD post-mortem brain.

MATERIAL AND METHODS

The methods used in this thesis are described in more detail in each study. Following is a short summary of all methods used that are described in brief. Ethical approval was received from the local Ethical Committee at Karolinska Institutet Huddinge Universitetssjukhus (for human brain samples Study I & II Dnr 231/02, study II 157/02 and animal samples S88/044).

Human brain samples

Post-mortem brain material used in Paper I included the medial-temporal cortex and medial-frontal cortex from a series of sporadic AD (n=9), positive-disease control (PDC) (n=8) and age matched non-disease control (NDC) (n=9) cases that were collected via the rapid autopsy system of the Netherlands brain bank. The patients were diagnosed according to NINCDS-ADRDA criteria and the severity of dementia estimated according to the Global Deterioration Scale. All cases were pathologically Braak staged for neurofibrillary changes. For Paper II, post-mortem brain material was obtained from two sources. Twelve medial-temporal cortex brain samples were obtained from the Huddinge Brain Bank, Sweden. We selected six definite sporadic AD and six controls. The controls did not have any history of dementia or of other neurological disease. All AD patients fulfilled the clinical diagnosis of probable AD (DSM-IV criteria) as well as definite AD on CERAD criteria. Additionally, tissue of the medial temporal cortex was from 21 AD and 10 controls and was obtained from the Kuopio Brain bank (Dr Irina Alafuzoff, Dept of Neuroscience and Neurology, Kuopio University Hospital, Kuopio University, Kuopio, Finland). All demented cases fulfilled the diagnosis of Alzheimer's disease according to CERAD neuropathological criteria. All cases were pathologically Braak staged for neurofibrillary changes.

Cell culture

Human neuroblastoma SH-SY5Y cells (Paper I) were obtained from the European Collection of Cell Cultures (Center for Applied Microbiology and Research, Salisbury, Wiltshire, UK) and cultured in minimum essential medium with Earle's salts (ENEM), supplemented with 2 mM L-Glutamine, 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2/air.

Rat primary cortical neurons

The neuron cultures (Paper III & IV) were established from cortices dissected from E17 fetal Sprague-Dawley rats. Animals were purchased from B&K Universal (Sollentuna, Sweden). Ethical approval was obtained from the Animal Ethics Committee of South Stockholm, Sweden. Primary cells were dissociated by mechanical triturating and seeded on poly-D-lysine coated dishes at a density of 700 000 cells/ml and 24 well plates at 400 000/ml, in neurobasal medium supplemented with B27 (2 ml/100 ml medium) and L-glutamine (1 ml/100 ml). Cells were incubated at 37°C in 5% CO_2 air.

Subfractionation of brain tissue

In Paper I, we measured total Akt activity in post-mortem brain. Since Akt activation involves recruitment of the enzyme to the plasma membrane followed translocation to the cytosol when fully activated, we chose to investigate potential differences in Akt activity by performing subfractionation of homogenised tissue into soluble and particulate fractions. In brief, brain tissue was homogenised with a glass-teflon homogeniser in cold (4°C) buffer (5 mM Tris-HCl, 25 mM NaF, 5 mM MgCl2, 1 mM EGTA, 0.1 mM Sodium Orthovanadate, 1 protease inhibitor tablet / 10 ml, pH 7.4). Homogenates were centrifuged at 100 000 g for 60 min at 4°C. The

supernatant (soluble fraction) was collected and stored at -70°C. The pellet (particulate fraction) was resuspended and homogenised in 1 ml of the homogenisation buffer and stored at -70 °C. We also performed subfractionation in study II, although here we investigated nucleus, soluble and particulate fractions. Brain tissue was homogenised using a glass-teflon homogeniser in cold (4°C) homogenisation buffer [5 mM Tris-HCl, 25 mM NaF, 5 mM MgCl2, 1 protease inhibitor tablet / 10 ml, pH 7.4]. Unbroken tissue fragments were removed by filtration through a nylon net filter. The filtrated homogenates were centrifuged at 600 g for 10 min at 4°C. Pellets were resuspended in 9 vol. 2.2 M sucrose, 1 mM MgCl2, 10 mM Tris-HCl, 1 mM PMSF, pH 7.4 buffer and homogenised at 1000 rpm. This fraction was then centrifuged at 70 000 - 80 000 g for 80 min and the resultant pellets (nuclei fraction) resuspended in cold (4°C) homogenisation buffer. For non-nuclear fractions, the supernatants were further centrifuged at 100 000 g for 60 min after the 600 g centrifugation step and pellets (particulate fraction) resuspended in homogenisation buffer. The resultant supernatants were kept as soluble fractions. All fractions were then frozen in liquid nitrogen and stored at -70°C.

Immunohistochemistry

For immunohistochemistry (Papers I & II) medial temporal cortex tissue blocks were fixed in buffered 4% formaldehyde and embedded in paraffin. Cryostat 7μm-thick sections were mounted onto alum-gelatine coated slides. The mounted sections were baked at 58°C for 30 min, deparafinised and hydrated. The sections were pre-treated with 3% H_2O_2 in TBS (TRIS-buffered-saline, pH 7.6) for 10 min, followed by blocking of non-specific sites with Dako-protein block for 30 min. The same procedure was used for all primary antibodies. The primary antibodies were incubated at 4°C overnight. After this, sections were incubated with biotinylated horse anti-rabbit antibody in TBS, for 30 min, followed by incubation in ABC-Elite HRP (Vector laboratories Burlingame, UK) for 1 h. Reactions were visualized by developing the sections in DAB + (DAKO Cytomation, Denmark). To optimise the immunohistochemistry and to determine the morphological features stained by the antibodies several systems besides ABC-Elite HRP were also used to enhance the immunostaining. One set of sections was stained with DAKO-Envison. Finally, the sections were dehydrated and mounted in Depex (BDH, Broom Rd, Poole U.K.). All sections were treated simultaneously under the same conditions. The protocol for antibodies was repeated to assure reproducibility of results. For control staining the primary serum was omitted. Sections were counterstained with haematoxylin.

Characterisation of rat primary neuron cultures using flow cytometry analyses

In (Paper III), primary cultures were treated with 6.84 mM EDTA and 0.05% Trypsin for 5-7 min. The cells were washed twice by centrifugation with cold PBS-2% FCS at 1500 rpm for 5 minutes with cold PBS, following fixation in 2% formaldehyde for 10-20 min at RT. After blocking with PBS containing 0.1% AURION BSA-c, cells were incubated for one hour with primary antibodies; β-Tubulin III, as a neuronal marker (dilution 1:600, TUJ-1), glial fibrillary acidic protein (GFAP), an astrocytic marker (dilution 1:500 clone G-A-5). Thereafter, the cells were washed by centrifugation at 1500 rpm for 5 min with cold PBS and incubated for an additional 1h with secondary mouse IgG Alexa Fluor® 488 antibody (1:500, emission 530 nm) diluted in PBS-0.5% saponin at 4°C. Finally, the cells were washed with cold PBS prior to flow cytometry analyses. The flow cytometry analysis was performed using a FACSCalibur (Becton & Dickinson, San Jose, CA, USA) equipped with a 488 nm argon laser and a 635 nm red diode laser. Data from the experiments were analyzed using CellQUEST software (Becton & Dickinson). The collected events per sample were 10 000. Flow cytometry data were plotted as cell size (FSC) as a function of fluorescence intensity (FL-1). Triplicate analyses were performed.

The secondary mouse IgG Alexa Fluor® 488 antibody was used alone to control for non-specific staining reactions.

Confocal microscopy

We also performed identification of TUJ-1 and GFAP positivity in cultures grown on poly-D-lysine coated cover slips (Paper II). For double staining, the cells were first incubated with GFAP antibody followed by incubation with secondary antibody (Alexa Fluor® 488, emission 530 nm). After the first staining procedure, to minimize the background, cells were blocked with mouse normal serum (DAKO) before addition of TUJ-1 antibody, followed by a Cy3-conjugated goat anti-mouse antibody (emission 565 nm). The presence of TUJ-1 and GFAP positive cells was analyzed using confocal fluorescent microscopy (BioRad, Radiance plus confocal scanning system).

Flow cytometry analyses of cell death

In (Paper II & IV) apoptosis analyses were performed using the Vybrant™ Apoptosis Assay Kit (Molecular Probes, Inc) with annexin V-FITC and Propidium iodide (PI) labelling following the manufacturer's instructions. Primary cultures were treated with 6.84 mM EDTA and 0.05% Trypsin, as described above. Harvested cells were then twice washed by centrifugation with cold PBS at 1500 rpm at 4°C. The cells were diluted in 100µl of 1× annexin-binding buffer per assay. The primary cultures were incubated with annexin V-FITC and PI for 15 min at RT in the dark. Later, 400µl of 1× annexin-binding buffer was added to the cells and mixed gently. The stained cells were analyzed immediately by flow cytometry. As a positive control, the primary cultures were exposed to 0.5 nM colchicine. Flow cytometry data were plotted as a function of fluorescence intensity FL-1 (green) versus FL-3 (red) fluorescence. We used annexin V-FITC (emission 518 nm) versus PI (emission 617 nm) to identify populations of viable cells (annexin V-PI-), early apoptotic cells (annexin V+PI-), necrotic cells (annexin V-PI+) and late apoptotic cells (annexin V+PI+). The flow cytometry analysis was performed using a FACSCalibur (Becton & Dickinson, San Jose, CA, USA) equipped with a 488 nm argon laser and a 635 nm red diode laser. Data from the experiments were analyzed using CellQUEST software (Becton & Dickinson). The collected events per sample were 10 000. For flow cytometry analysis, in order to have a complete picture of the total neurons, we pooled floating cells with trypsinized cells and measured both together. Furthermore, since we wanted to investigate the effect of stress factors on the total cell populations, we included all events during analyses.

Western blot analyses

All immunoblotting in (Papers I, II & IV) were performed using standard protocols. The detailed procedures are given in each study. A standard with neuroblastoma SH-SY5Y cells was made for the immunoblotting to make it possible to better quantify data (Papers I & II).

Akt kinase assay

In Paper I, Akt enzyme activities in soluble and particulate fractions were determined using a commercially available non-radioactive Akt kinase assay kit from Cell Signalling Technology (Beverly, MA).

Quantification of blots

For quantification, (Paper I & II) samples were run with a series of standards prepared from human SH-SY5Y neuroblastoma cells. CCD camera images (Fluor-S^{TM} Multi Imager and Discovery series Quantity One 1-D Analysis software [Bio-Rad]) were taken every minute up to 30 min. Those showing good linearity for the standard curves and no saturation of signal were quantified. Optical densities were converted to cell standard equivalent amounts by reading off from the standard curves. In Paper IV, to minimize intrablot variability, samples were loaded on gels in duplicate (two different samples). Images were taken by CCD camera every minute up to 30 min and only images where saturation of signal had not occurred were quantified. The levels of phosphorylated PTEN, Akt and GSK3β were calculated as a ratio of total respective protein (p-PTEN/PTEN, p-Akt/Akt and p-GSK3β/GSK3β) following normalisation for amount of actin for each sample.

Counting of neurons

In (Paper II), a stereological approach was used to estimate the number of immunopositive neurons on the uniformly random systematic sampling and the unbiased optical fractionator method. The method involves counting objects (neurons) with an optical dissector in a uniform systematic sample that constitutes a known fraction of the area of the region being analyzed (for details see (West *et al.*, 1991). The counting equipment consisted of the CAST-GRID v 1.2 PC program (Olympus, Denmark) and a Zeiss microscope with a high-numerical-aperture (NA=1.4) x100 oil-immersion objective, a motorized stage, and an electronic microcator with digital readout for measuring movements in the Z-direction with 0.5μM precision. Briefly, the area of interest was delineated at low magnification (x2.5) using the cursor. For stepping through the delineated area, a meander sampling function of the GRID v2.0 (Olympus Danmark) program was used. Then, a 100x oil-immersion objective was moved into place and the appropriate counting frame superimposed on the screen. Only neuronal profiles falling in the counting frame or crossing two green lines of the frame are counted. To differentiate neuronal cells from other cellular elements morphological criteria were used as described by Berry et al and Dawson et al (Berry M, 2002; Dawson TP, 2003).

Statistics

All statistics were performed in Statview statistical software. For all experiments analyses of between group differences were tested for statistical significance by analysis of variance ANOVA followed by Fisher´s post-hoc analysis.

ACKNOWLEDGEMENTS

I have looked forward to writing this part of the thesis for many reasons. The most important is that it give me the chance to express my sincere gratitude and appreciation to all the kind people that have helped me along the way. I would never had completed this thesis within five years without you. Big thank to you all, I would especially thank the following people

First of all, **Richard Cowburn**, my supervisor and English teacher☺, who accepted me as a PhD student, for trusting me, for your support and encouragement. For your positive way of thinking, your devotion and excellent expertise in signal transduction. For your enthusiasm about new data, for giving me the chance to work independently and for giving me great guidance. Thanks Richard!

Bengt Winblad for his support and giving me the opportunity to work at Karolinska Institutet.

All my co-authors: **Maria Ankarcrona**, **Homira Behbahani**, **Hèrnan Concha**, **Nenad Bogdanovic**, **Inga Volkmann**, **XinWen Zhou** and **Jin-Jing Pei** for excellent collaboration and contribution, for sharing their knowledge and fruitful discussions.

Maria Ankarcrona, for good scientific discussions and for teaching me primary cultures. It was easy when I had such a good teacher. For all help and for all good times in and outside the lab.

Homira Behbahani for teaching me flow cytometry, for being such a nice person, supportive with all kinds of things, I admire your enthusiasms in science, for always taking the time including help with the front page to the thesis, for great layouts, pictures. We are a great team together, things gets done!

Nenad Bogdanovic for your excellent skill in neuropathology, for all neuroscience discussion about brain and for fruitful collaboration.

Inga Volkmann for your help, kindness and brilliant expertise in immunohistochemistry.

I would like to thank **Simon Lovestone** in London and **Fiona Kerr** for a great collaboration and their kind hospitality when I was in London.

I especially thank the members of the committee of my half time-seminar; **Marianne Schultzberg**, **Ratan Bhat** and **Elisabeth Englund** for all questions and great inputs.

The group of **Eric Sundström**, for sharing their knowledge, for always being friendly and helping me whenever needed **Eva-Britt**, **Hullan, Elisabeth, Jenny, Jing-Hua, Mia** and **Maria**.

Takeshi Nisimura, for nice inspiring collaboration, for all help and fruitful scientific discussions.

Eirikur Benedikz and **Ronnie Folkesson**, for all help with the gene arrays that Takeshi and I worked with.

Agneta L, Kristina L, Monika W, Siw L and **Maria R** for all help with different administration tasks.

All people at KASPAC both former and present, for creating a nice atmosphere, **Anna, Camilla, Caroline, Hedvig, Hiro, Homira, Janne, Ji-Yuen, Karin A, Karin D, Lars, Lena, Lotta, Mark, Mikio** and **Susanne.** A specially thank to **Gunilla** for all lunches together, for being so kind and supportive, for all fruits and chocolates. **Karin D** and **Susanne F** for sharing their knowledge and helping me at the lab.

All the other present and past colleagues: **Alexandra, Amelia, Angel, Angela, Anna B, Anna J, Atique, Atsu, Benosh, Camilla H, Camilla N, Catarina L, Cecilia, Dorota R, Daniel, Erik H, Ewa, Ezra, Hal, Hanna, Helena, Katarzyna, Lisa, Jenny C, Jenny F, Mircea, Naggat, Nahid, Nodi, Stefan, Susanne A, Sara J, Tatjana, Wen-Lin, Ola, Zhiguo** and **Åsa** for creating a good atmosphere and social events. Especially a thank to **Angel** who introduced me in playing tennis, for all help in the beginning in the lab at KFC and all Spanish lunches. **Sara** for all help I got in the final with the thesis and also for all times in and outside the lab. **Anna N** that has become a close friend, thanks for all support, dinners, parties, skiing, ice-skating and tennis etc.

Eva, Annika, Lisa, Kerstin, Irene, Eva-Britt and **Hullan** at KFC for enjoyable fika in the lunchroom and for running together at KI-loppen.

My former colleagues **Monika, Susanne** and **Beata**. For being nice friends, for having creative scientific discussions, for great trips together, especially NYC and having nice dinners.

Bitti for having such a nice attitude, being a true friend, all your support and help in and outside the lab has meant a lot to me, for always having fun together, bada det är livet det, Åh så skönt! Big hug!

Min underbara familj och vänner (ingen nämnd ingen glömd, ni vet vilka ni är). Tack för att ni alltid finns där!

The work was supported by Sumitomo Pharmaceuticals Co, Osaka

REFERENCES

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G.M., Cooper, N.R., Eikelenboom, P., Emmerling, M., Fiebich, B.L., Finch, C.E., Frautschy, S., Griffin, W.S., Hampel, H., Hull, M., Landreth, G., Lue, L., Mrak, R., Mackenzie, I.R., McGeer, P.L., O'Banion, M.K., Pachter, J., Pasinetti, G., Plata-Salaman, C., Rogers, J., Rydel, R., Shen, Y., Streit, W., Strohmeyer, R., Tooyoma, I., Van Muiswinkel, F.L., Veerhuis, R., Walker, D., Webster, S., Wegrzyniak, B., Wenk, G. and Wyss-Coray, T. (2000) Inflammation and Alzheimer's disease. *Neurobiol Aging*, **21**, 383-421.

Al-Khouri, A.M., Ma, Y., Togo, S.H., Williams, S. and Mustelin, T. (2005) Cooperative Phosphorylation of the Tumor Suppressor Phosphatase and Tensin Homologue (PTEN) by Casein Kinases and Glycogen Synthase Kinase 3{beta}. *J Biol Chem*, **280**, 35195-35202. Epub 32005 Aug 35117.

Altomare, D.A., Guo, K., Cheng, J.Q., Sonoda, G., Walsh, K. and Testa, J.R. (1995) Cloning, chromosomal localization and expression analysis of the mouse Akt2 oncogene. *Oncogene*, **11**, 1055-1060.

Anderson, A.J., Stoltzner, S., Lai, F., Su, J. and Nixon, R.A. (2000) Morphological and biochemical assessment of DNA damage and apoptosis in Down syndrome and Alzheimer disease, and effect of postmortem tissue archival on TUNEL. *Neurobiol Aging*, **21**, 511-524.

Arriagada, P.V., Growdon, J.H., Hedley-Whyte, E.T. and Hyman, B.T. (1992) Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology*, **42**, 631-639.

Association, A.P. (1994) Diagnostic and statistical manual of mental disorders. *American Psychiatric Association,*, **4th ed. Washington, DC**.

Backman, S.A., Stambolic, V., Suzuki, A., Haight, J., Elia, A., Pretorius, J., Tsao, M.S., Shannon, P., Bolon, B., Ivy, G.O. and Mak, T.W. (2001) Deletion of Pten in mouse brain causes seizures, ataxia and defects in soma size resembling Lhermitte-Duclos disease. *Nature Genetics.*, **29**, 396-403.

Baki, L., Shioi, J., Wen, P., Shao, Z., Schwarzman, A., Gama-Sosa, M., Neve, R. and Robakis, N.K. (2004) PS1 activates PI3K thus inhibiting GSK-3 activity and tau overphosphorylation: effects of FAD mutations. *Embo J*, **23**, 2586-2596. Epub 2004 Jun 2510.

Behl, C. (1999) Alzheimer's disease and oxidative stress: implications for novel therapeutic approaches. *Prog Neurobiol*, **57**, 301-323.

Behl, C. (2000) Apoptosis and Alzheimer's disease. *J Neural Transm*, **107**, 1325-1344.

Behl, C., Davis, J.B., Klier, F.G. and Schubert, D. (1994) Amyloid beta peptide induces necrosis rather than apoptosis. *Brain Res*, **645**, 253-264.

Berry M, B.A., Wilken G, Perry VH. (2002) Structure and function of neurons and glia in the central nervous system. *Greenfield´s Neuropathology, 7th edition*, 75-121.

Bhat, R.V., Shanley, J., Correll, M.P., Fieles, W.E., Keith, R.A., Scott, C.W. and Lee, C.M. (2000) Regulation and localization of tyrosine216 phosphorylation of glycogen synthase kinase-3beta in cellular and animal models of neuronal degeneration. *Proc Natl Acad Sci U S A*, **97**, 11074-11079.

Bijur, G.N., De Sarno, P. and Jope, R.S. (2000) Glycogen synthase kinase-3beta facilitates staurosporine- and heat shock-induced apoptosis. Protection by lithium. *J Biol Chem*, **275**, 7583-7590.

Billings, L.M., Oddo, S., Green, K.N., McGaugh, J.L. and Laferla, F.M. (2005) Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron*, **45**, 675-688.

Binder, L.I., Frankfurter, A. and Rebhun, L.I. (1985) The distribution of tau in the mammalian central nervous system. *J Cell Biol*, **101**, 1371-1378.

Bowen, D.M., Smith, C.B., White, P. and Davison, A.N. (1976) Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies. *Brain*, **99**, 459-496.

Braak, H. and Braak, E. (1991) Demonstration of amyloid deposits and neurofibrillary changes in whole brain sections. *Brain Pathol*, **1**, 213-216.

Braak, H., Braak, E., Bohl, J. and Reintjes, R. (1996) Age, neurofibrillary changes, A beta-amyloid and the onset of Alzheimer's disease. *Neurosci Lett*, **210**, 87-90.

Brazil, D.P. and Hemmings, A. (2001) Ten years of protein kinase B signalling: a hard Akt to follow. *Trends Biochem Sci*, **26**, 657-664.

Brazil, D.P., Park, J. and Hemmings, B.A. (2002) PKB binding proteins. Getting in on the Akt. *Cell*, **111**, 293-303.

Brewer, G.J., Torricelli, J.R., Evege, E.K. and Price, P.J. (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res*, **35**, 567-576.

Brun, A. and Englund, E. (1981) Regional pattern of degeneration in Alzheimer's disease: neuronal loss and histopathological grading. *Histopathology*, **5**, 549-564.

Buee, L., Bussiere, T., Buee-Scherrer, V., Delacourte, A. and Hof, P.R. (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res Brain Res Rev*, **33**, 95-130.

Butterfield, D.A., Castegna, A., Lauderback, C.M. and Drake, J. (2002) Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiol Aging*, **23**, 655-664.

Cacquevel, M., Lebeurrier, N., Cheenne, S. and Vivien, D. (2004) Cytokines in neuroinflammation and Alzheimer's disease. *Curr Drug Targets*, **5**, 529-534.

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S. and Reed, J.C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science*, **282**, 1318-1321.

Carro, E., Trejo, J.L., Gerber, A., Loetscher, H., Torrado, J., Metzger, F. and Torres-Aleman, I. (2005) Therapeutic actions of insulin-like growth factor I on APP/PS2 mice with severe brain amyloidosis. *Neurobiol Aging*, **22**, 22.

Carro, E., Trejo, J.L., Gomez-Isla, T., LeRoith, D. and Torres-Aleman, I. (2002) Serum insulin-like growth factor I regulates brain amyloid-beta levels. *Nature Medicine*, **8**, 1390-1397.

Cedazo-Minguez, A., Popescu, B.O., Blanco-Millan, J.M., Akterin, S., Pei, J.J., Winblad, B. and Cowburn, R.F. (2003) Apolipoprotein E and beta-amyloid (1-42) regulation of glycogen synthase kinase-3beta. *Journal of Neurochemistry*, **87**, 1152-1164.

Chakraborty, H., Ray, S.N. and Chakrabarti, S. (2001) Lipid peroxidation associated protein damage in rat brain crude synaptosomal fraction mediated by iron and ascorbate. *Neurochem Int*, **39**, 311-317.

Cheng, C.M., Tseng, V., Wang, J., Wang, D., Matyakhina, L. and Bondy, C.A. (2005) Tau is hyper-phosphorylated in the IGF1 null brain. *Endocrinology*, **25**, 25.

Choi, J., Malakowsky, C.A., Talent, J.M., Conrad, C.C., Carroll, C.A., Weintraub, S.T. and Gracy, R.W. (2003) Anti-apoptotic proteins are oxidized by Abeta25-35 in Alzheimer's fibroblasts. *Biochim Biophys Acta*, **1637**, 135-141.

Choi, Y.C., Lee, J.H., Hong, K.W. and Lee, K.S. (2004) 17 Beta-estradiol prevents focal cerebral ischemic damages via activation of Akt and CREB in association with reduced PTEN phosphorylation in rats. *Fundam Clin Pharmacol*, **18**, 547-557.

Chong, Z.Z., Li, F. and Maiese, K. (2005) Activating Akt and the brain's resources to drive cellular survival and prevent inflammatory injury. *Histol Histopathol*, **20**, 299-315.

Cohen, P. (1979) The hormonal control of glycogen metabolism in mammalian muscle by multivalent phosphorylation. *Biochem Soc Trans*, **7**, 459-480.

Colin, E., Regulier, E., Perrin, V., Durr, A., Brice, A., Aebischer, P., Deglon, N., Humbert, S. and Saudou, F. (2005) Akt is altered in an animal model of Huntington's disease and in patients. *Eur J Neurosci*, **21**, 1478-1488.

Connor, B., Beilharz, E.J., Williams, C., Synek, B., Gluckman, P.D., Faull, R.L. and Dragunow, M. (1997) Insulin-like growth factor-I (IGF-I) immunoreactivity in the Alzheimer's disease temporal cortex and hippocampus. *Brain Res Mol Brain Res*, **49**, 283-290.

Crews, F.T., McElhaney, R., Freund, G., Ballinger, W.E., Jr. and Raizada, M.K. (1992) Insulin-like growth factor I receptor binding in brains of Alzheimer's and alcoholic patients. *J Neurochem*, **58**, 1205-1210.

Cross, D.A., Culbert, A.A., Chalmers, K.A., Facci, L., Skaper, S.D. and Reith, A.D. (2001) Selective small-molecule inhibitors of glycogen synthase kinase-3 activity protect primary neurones from death. *J Neurochem*, **77**, 94-102.

Cummings, B.J. and Cotman, C.W. (1995) Image analysis of beta-amyloid load in Alzheimer's disease and relation to dementia severity. *Lancet*, **346**, 1524-1528.

Davies, P. and Maloney, A.J. (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet*, **2**, 1403.

Dawson TP, N.J., Llewellyn L, Thomas C. (2003) Neuropathology techniques Oxford University press. 162-179.

de la Monte, S.M. and Wands, J.R. (2005) Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. *J Alzheimers Dis*, **7**, 45-61.

De Sarno, P., Lesort, M., Bijur, G.N., Johnson, G.V. and Jope, R.S. (2001) Cholinergic- and stress-induced signaling activities in cells overexpressing wild-type and mutant presenilin-1. *Brain Res*, **903**, 226-230.

DeKosky, S.T. and Scheff, S.W. (1990) Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. *Ann Neurol*, **27**, 457-464.

Di Cristofano, A., Pesce, B., Cordon-Cardo, C. and Pandolfi, P.P. (1998) Pten is essential for embryonic development and tumour suppression. *Nat Genet*, **19**, 348-355.

Downen, M., Amaral, T.D., Hua, L.L., Zhao, M.L. and Lee, S.C. (1999) Neuronal death in cytokine-activated primary human brain cell culture: role of tumor necrosis factor-alpha. *Glia*, **28**, 114-127.

Dozza, B., Smith, M.A., Perry, G., Tabaton, M. and Strocchi, P. (2004) Regulation of glycogen synthase kinase-3beta by products of lipid peroxidation in human neuroblastoma cells. *J Neurochem*, **89**, 1224-1232.

Dragunow, M., Faull, R.L., Lawlor, P., Beilharz, E.J., Singleton, K., Walker, E.B. and Mee, E. (1995) In situ evidence for DNA fragmentation in Huntington's disease striatum and Alzheimer's disease temporal lobes. *Neuroreport*, **6**, 1053-1057.

Drechsel, D.N., Hyman, A.A., Cobb, M.H. and Kirschner, M.W. (1992) Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. *Mol Biol Cell*, **3**, 1141-1154.

Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R. and Greenberg, M.E. (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science*, **275**, 661-665.

Eckert, A., Keil, U., Marques, C.A., Bonert, A., Frey, C., Schussel, K. and Muller, W.E. (2003) Mitochondrial dysfunction, apoptotic cell death, and Alzheimer's disease. *Biochemical Pharmacology*, **66**, 1627-1634.

Elliott, E., Atlas, R., Lange, A. and Ginzburg, I. (2005) Brain-derived neurotrophic factor induces a rapid dephosphorylation of tau protein through a PI-3 Kinase signalling mechanism. *Eur J Neurosci*, **22**, 1081-1089.

Embi, N., Rylatt, D.B. and Cohen, P. (1980) Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur J Biochem*, **107**, 519-527.

Esterbauer, H., Schaur, R.J. and Zollner, H. (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology & Medicine*, **11**, 81-128.

Ferrer, I., Barrachina, M. and Puig, B. (2002) Glycogen synthase kinase-3 is associated with neuronal and glial hyperphosphorylated tau deposits in Alzheimer's disease, Pick's disease, progressive supranuclear palsy and corticobasal degeneration. *Acta Neuropathol (Berl)*, **104**, 583-591. Epub 2002 Jul 2013.

Frame, S. and Cohen, P. (2001) GSK3 takes centre stage more than 20 years after its discovery. *Biochem J*, **359**, 1-16.

Franke, T.F., Kaplan, D.R. and Cantley, L.C. (1997) PI3K: downstream AKTion blocks apoptosis. *Cell*, **88**, 435-437.

Fukutani, Y., Cairns, N.J., Shiozawa, M., Sasaki, K., Sudo, S., Isaki, K. and Lantos, P.L. (2000) Neuronal loss and neurofibrillary degeneration in the hippocampal cortex in late-onset sporadic Alzheimer's disease. *Psychiatry Clin Neurosci*, **54**, 523-529.

Gamblin, T.C., Chen, F., Zambrano, A., Abraha, A., Lagalwar, S., Guillozet, A.L., Lu, M., Fu, Y., Garcia-Sierra, F., LaPointe, N., Miller, R., Berry, R.W., Binder, L.I. and Cryns, V.L. (2003) Caspase cleavage of tau: linking amyloid and neurofibrillary tangles in Alzheimer's disease. *Proc Natl Acad Sci U S A*, **100**, 10032-10037. Epub 12003 Jul 10029.

Gary, D.S. and Mattson, M.P. (2002) PTEN regulates Akt kinase activity in hippocampal neurons and increases their sensitivity to glutamate and apoptosis. *Neuromolecular Medicine.*, **2**, 261-269.

Goedert, M., Spillantini, M.G. and Davies, S.W. (1998) Filamentous nerve cell inclusions in neurodegenerative diseases. *Curr Opin Neurobiol*, **8**, 619-632.

Gotz, J., Chen, F., van Dorpe, J. and Nitsch, R.M. (2001) Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Abeta 42 fibrils. *Science*, **293**, 1491-1495.

Griffin, R.J., Moloney, A., Kelliher, M., Johnston, J.A., Ravid, R., Dockery, P., O'Connor, R. and O'Neill, C. (2005) Activation of Akt/PKB, increased phosphorylation of Akt substrates and loss and altered distribution of Akt and PTEN are features of Alzheimer's disease pathology. *J Neurochem*, **93**, 105-117.

Grimes, C.A. and Jope, R.S. (2001) The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. *Prog Neurobiol*, **65**, 391-426.

Groszer, M., Erickson, R., Scripture-Adams, D.D., Lesche, R., Trumpp, A., Zack, J.A., Kornblum, H.I., Liu, X. and Wu, H. (2001) Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. *Science.*, **294**, 2186-2189.

Guo, H., Albrecht, S., Bourdeau, M., Petzke, T., Bergeron, C. and LeBlanc, A.C. (2004) Active caspase-6 and caspase-6-cleaved tau in neuropil threads, neuritic plaques, and neurofibrillary tangles of Alzheimer's disease. *Am J Pathol*, **165**, 523-531.

Gustin, J.A., Maehama, T., Dixon, J.E. and Donner, D.B. (2001) The PTEN tumor suppressor protein inhibits tumor necrosis factor-induced nuclear factor kappa B activity. *Journal of Biological Chemistry*, **276**, 27740-27744.

Gylys, K.H., Fein, J.A., Wiley, D.J. and Cole, G.M. (2004) Rapid annexin-V labeling in synaptosomes. *Neurochem Int*, **44**, 125-131.

Hamos, J.E., DeGennaro, L.J. and Drachman, D.A. (1989) Synaptic loss in Alzheimer's disease and other dementias. *Neurology*, **39**, 355-361.

Hartigan, J.A. and Johnson, G.V. (1999) Transient increases in intracellular calcium result in prolonged site-selective increases in Tau phosphorylation through a glycogen synthase kinase 3beta-dependent pathway. *J Biol Chem*, **274**, 21395-21401.

Hemmer, K., Fransen, L., Vanderstichele, H., Vanmechelen, E. and Heuschling, P. (2001) An in vitro model for the study of microglia-induced neurodegeneration: involvement of nitric oxide and tumor necrosis factor-alpha. *Neurochemistry International*, **38**, 557-565.

Henry, M.K., Lynch, J.T., Eapen, A.K. and Quelle, F.W. (2001) DNA damage-induced cell-cycle arrest of hematopoietic cells is overridden by activation of the PI-3 kinase/Akt signaling pathway. *Blood*, **98**, 834-841.

Hetman, M., Cavanaugh, J.E., Kimelman, D. and Xia, Z. (2000) Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal. *J Neurosci*, **20**, 2567-2574.

Hirsch, E.C., Hunot, S. and Hartmann, A. (2005) Neuroinflammatory processes in Parkinson's disease. *Parkinsonism Relat Disord*, **11**, S9-S15.

Ho, G.J., Drego, R., Hakimian, E. and Masliah, E. (2005) Mechanisms of cell signaling and inflammation in Alzheimer's disease. *Curr Drug Targets Inflamm Allergy*, **4**, 247-256.

Honer, W.G., Dickson, D.W., Gleeson, J. and Davies, P. (1992) Regional synaptic pathology in Alzheimer's disease. *Neurobiol Aging*, **13**, 375-382.

Hong, M. and Lee, V.M. (1997) Insulin and Insulin-like Growth Factor-1 Regulate Tau Phosphorylation in Cultured Human Neurons. *The journal of Biological Chemistry*, **272**, 19547-19553.

Jope, R.S. and Johnson, G.V. (2004) The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci*, **29**, 95-102.

Kang, H.J., Yoon, W.J., Moon, G.J., Kim, D.Y., Sohn, S., Kwon, H.J. and Gwag, B.J. (2005) Caspase-3-mediated cleavage of PHF-1 tau during apoptosis irrespective of excitotoxicity and oxidative stress: an implication to Alzheimer's disease. *Neurobiol Dis*, **18**, 450-458.

Kaytor, M.D. and Orr, H.T. (2002) The GSK3 beta signaling cascade and neurodegenerative disease. *Curr Opin Neurobiol*, **12**, 275-278.

Kim, A.H., Khursigara, G., Sun, X., Franke, T.F. and Chao, M.V. (2001) Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Mol Cell Biol*, **21**, 893-901.

Kril, J.J., Patel, S., Harding, A.J. and Halliday, G.M. (2002) Neuron loss from the hippocampus of Alzheimer's disease exceeds extracellular neurofibrillary tangle formation. *Acta Neuropathol (Berl)*, **103**, 370-376. Epub 2001 Dec 2012.

Kruman, I., Bruce-Keller, A.J., Bredesen, D., Waeg, G. and Mattson, M.P. (1997) Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J Neurosci*, **17**, 5089-5100.

Ksiezak-Reding, H., Pyo, H.K., Feinstein, B. and Pasinetti, G.M. (2003) Akt/PKB kinase phosphorylates separately Thr212 and Ser214 of tau protein in vitro. *Biochim Biophys Acta*, **1639**, 159-168.

Kwon, C.H., Zhu, X., Zhang, J., Knoop, L.L., Tharp, R., Smeyne, R.J., Eberhart, C.G., Burger, P.C. and Baker, S.J. (2001) Pten regulates neuronal soma size: a mouse model of Lhermitte-Duclos disease. *Nature Genetics.*, **29**, 404-411.

Kyoung Pyo, H., Lovati, E., Pasinetti, G.M. and Ksiezak-Reding, H. (2004) Phosphorylation of tau at THR212 and SER214 in human neuronal and glial cultures: the role of AKT. *Neuroscience*, **127**, 649-658.

Kyrylenko, S., Roschier, M., Korhonen, P. and Salminen, A. (1999) Regulation of PTEN expression in neuronal apoptosis. *Brain Research. Molecular Brain Research*, **73**, 198-202.

Lachyankar, M.B., Sultana, N., Schonhoff, C.M., Mitra, P., Poluha, W., Lambert, S., Quesenberry, P.J., Litofsky, N.S., Recht, L.D., Nabi, R., Miller, S.J., Ohta, S., Neel, B.G. and Ross, A.H. (2000) A role for nuclear PTEN in neuronal differentiation. *Journal of Neuroscience.*, **20**, 1404-1413.

Lassmann, H., Bancher, C., Breitschopf, H., Wegiel, J., Bobinski, M., Jellinger, K. and Wisniewski, H.M. (1995) Cell death in Alzheimer's disease evaluated by DNA fragmentation in situ. *Acta Neuropathol*, **89**, 35-41.

Lawlor, M.A. and Alessi, D.R. (2001) PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci*, **114**, 2903-2910.

Lee, C.W., Lau, K.F., Miller, C.C. and Shaw, P.C. (2003) Glycogen synthase kinase-3 beta-mediated tau phosphorylation in cultured cell lines. *Neuroreport*, **14**, 257-260.

Lee, V.M., Goedert, M. and Trojanowski, J.Q. (2001) Neurodegenerative tauopathies. *Annu Rev Neurosci*, **24**, 1121-1159.

Leroy, K. and Brion, J.P. (1999) Developmental expression and localization of glycogen synthase kinase-3beta in rat brain. *J Chem Neuroanat*, **16**, 279-293.

Lewis, J., Dickson, D.W., Lin, W.L., Chisholm, L., Corral, A., Jones, G., Yen, S.H., Sahara, N., Skipper, L., Yager, D., Eckman, C., Hardy, J., Hutton, M. and McGowan, E. (2001) Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science*, **293**, 1487-1491.

Liu, S.J., Zhang, A.H., Li, H.L., Wang, Q., Deng, H.M., Netzer, W.J., Xu, H. and Wang, J.Z. (2003a) Overactivation of glycogen synthase kinase-3 by inhibition of phosphoinositol-3 kinase and protein kinase C leads to hyperphosphorylation of tau and impairment of spatial memory. *J Neurochem*, **87**, 1333-1344.

Liu, W., Akhand, A.A., Takeda, K., Kawamoto, Y., Itoigawa, M., Kato, M., Suzuki, H., Ishikawa, N. and Nakashima, I. (2003b) Protein phosphatase 2A-linked and -unlinked caspase-dependent pathways for downregulation of Akt kinase triggered by 4-hydroxynonenal. *Cell Death Differ*, **10**, 772-781.

Lobo, A., Launer, L.J., Fratiglioni, L., Andersen, K., Di Carlo, A., Breteler, M.M., Copeland, J.R., Dartigues, J.F., Jagger, C., Martinez-Lage, J., Soininen, H. and Hofman, A. (2000) Prevalence of dementia and major subtypes in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. *Neurology*, **54**, S4-9.

Loo, D.T., Copani, A., Pike, C.J., Whittemore, E.R., Walencewicz, A.J. and Cotman, C.W. (1993) Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. *Proc Natl Acad Sci U S A*, **90**, 7951-7955.

Lovell, M.A., Ehmann, W.D., Mattson, M.P. and Markesbery, W.R. (1997) Elevated 4-hydroxynonenal in ventricular fluid in Alzheimer's disease. *Neurobiology of Aging*, **18**, 457-461.

Lovell, M.A., Xie, C. and Markesbery, W.R. (2001) Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures. *Neurobiol Aging*, **22**, 187-194.

Lovestone, S. and Reynolds, C.H. (1997) The phosphorylation of tau: a critical stage in neurodevelopment and neurodegenerative processes. *Neuroscience*, **78**, 309-324.

Lucassen, P.J., Chung, W.C., Kamphorst, W. and Swaab, D.F. (1997) DNA damage distribution in the human brain as shown by in situ end labeling; area-specific differences in aging and Alzheimer disease in the absence of apoptotic morphology. *J Neuropathol Exp Neurol*, **56**, 887-900.

Maehama, T., Taylor, G.S. and Dixon, J.E. (2001) PTEN and myotubularin: novel phosphoinositide phosphatases. *Annual Review of Biochemistry*, **70**, 247-279.

Martin, D., Salinas, M., Lopez-Valdaliso, R., Serrano, E., Recuero, M. and Cuadrado, A. (2001) Effect of the Alzheimer amyloid fragment Abeta(25-35) on Akt/PKB kinase and survival of PC12 cells. *J Neurochem*, **78**, 1000-1008.

Marx, J. (2001) New Leads on the "How" of Alzheimer´s. *Science*, **293**, 2192-2194.

Matsuo, E.S., Shin, R.W., Billingsley, M.L., Van deVoorde, A., O'Connor, M., Trojanowski, J.Q. and Lee, V.M. (1994) Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau. *Neuron*, **13**, 989-1002.

Mattson, M.P. (2002) Oxidative stress, perturbed calcium homeostasis, and immune dysfunction in Alzheimer's disease. *Journal of Neurovirology*, **8**, 539-550.

Mattson, M.P., Fu, W., Waeg, G. and Uchida, K. (1997) 4-Hydroxynonenal, a product of lipid peroxidation, inhibits dephosphorylation of the microtubule-associated protein tau. *Neuroreport*, **8**, 2275-2281.

McKee, A.C., Kosik, K.S. and Kowall, N.W. (1991) Neuritic pathology and dementia in Alzheimer's disease. *Ann Neurol*, **30**, 156-165.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D. and Stadlan, E.M. (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology*, **34**, 939-944.

Mirra, S.S., Heyman, A., McKeel, D., Sumi, S.M., Crain, B.J., Brownlee, L.M., Vogel, F.S., Hughes, J.P., van Belle, G. and Berg, L. (1991) The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology*, **41**, 479-486.

Montine, K.S., Kim, P.J., Olson, S.J., Markesbery, W.R. and Montine, T.J. (1997a) 4-hydroxy-2-nonenal pyrrole adducts in human neurodegenerative disease. *Journal of Neuropathology & Experimental Neurology*, **56**, 866-871.

Montine, K.S., Olson, S.J., Amarnath, V., Whetsell, W.O., Jr., Graham, D.G. and Montine, T.J. (1997b) Immunohistochemical detection of 4-hydroxy-2-nonenal adducts in Alzheimer's disease is associated with inheritance of APOE4. *American Journal of Pathology*, **150**, 437-443.

Morris, J.C., McKeel, D.W., Jr., Storandt, M., Rubin, E.H., Price, J.L., Grant, E.A., Ball, M.J. and Berg, L. (1991) Very mild Alzheimer's disease: informant-based clinical, psychometric, and pathologic distinction from normal aging. *Neurology*, **41**, 469-478.

Munoz-Montano, J.R., Moreno, F.J., Avila, J. and Diaz-Nido, J. (1997) Lithium inhibits Alzheimer's disease-like tau protein phosphorylation in neurons. *FEBS Lett*, **411**, 183-188.

Murer, M.G., Yan, Q. and Raisman-Vozari, R. (2001) Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. *Prog Neurobiol*, **63**, 71-124.

Nakatani, K., Sakaue, H., Thompson, D.A., Weigel, R.J. and Roth, R.A. (1999) Identification of a human Akt3 (protein kinase B gamma) which contains the regulatory serine phosphorylation site. *Biochem Biophys Res Commun*, **257**, 906-910.

Namikawa, K., Honma, M., Abe, K., Takeda, M., Mansur, K., Obata, T., Miwa, A., Okado, H. and Kiyama, H. (2000) Akt/protein kinase B prevents injury-induced motoneuron death and accelerates axonal regeneration. *J Neurosci*, **20**, 2875-2886.

Näslund, J., Haroutunian, V., Mohs, R., Davis, K.L., Davies, P., Greengard, P. and Buxbaum, J.D. (2000) Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. *Jama*, **283**, 1571-1577.

Oddo, S., Caccamo, A., Kitazawa, M., Tseng, B.P. and LaFerla, F.M. (2003) Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiol Aging*, **24**, 1063-1070.

Omori, N., Jin, G., Li, F., Zhang, W.R., Wang, S.J., Hamakawa, Y., Nagano, I., Manabe, Y., Shoji, M. and Abe, K. (2002) Enhanced phosphorylation of PTEN in rat brain after transient middle cerebral artery occlusion. *Brain Research.*, **954**, 317-322.

Ormerod, M.G., Collins, M.K., Rodriguez-Tarduchy, G. and Robertson, D. (1992) Apoptosis in interleukin-3-dependent haemopoietic cells. Quantification by two flow cytometric methods. *J Immunol Methods*, **153**, 57-65.

Overmyer, M., Kraszpulski, M., Helisalmi, S., Soininen, H. and Alafuzoff, I. (2000) DNA fragmentation, gliosis and histological hallmarks of Alzheimer's disease. *Acta Neuropathol (Berl)*, **100**, 681-687.

Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M. and Donner, D.B. (1999) NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase.[see comment]. *Nature*, **401**, 82-85.

Pap, M. and Cooper, G.M. (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *J Biol Chem*, **273**, 19929-19932.

Pearson, R.C. (1996) Cortical connections and the pathology of Alzheimer's disease. *Neurodegeneration*, **5**, 429-434.

Pei, J.J., Braak, E., Braak, H., Grundke-Iqbal, I., Iqbal, K., Winblad, B. and Cowburn, R.F. (1999) Distribution of active glycogen synthase kinase 3beta (GSK-3beta) in brains staged for Alzheimer disease neurofibrillary changes. *J Neuropathol Exp Neurol*, **58**, 1010-1019.

Pei, J.J., Khatoon, S., An, W.L., Nordlinder, M., Tanaka, T., Braak, H., Tsujio, I., Takeda, M., Alafuzoff, I., Winblad, B., Cowburn, R.F., Grundke-Iqbal, I. and Iqbal, K. (2003) Role of protein kinase B in Alzheimer's neurofibrillary pathology. *Acta Neuropathologica*, **105**, 381-392.

Perry, G., Castellani, R.J., Hirai, K., Smith, M. (1998) Reactive oxygen species mediate cellular damage in Alzheimer disease. *Journal of Alzeimer´s Disease*, **1**, 45-55.

Phiel, C.J., Wilson, C.A., Lee, V.M. and Klein, P.S. (2003) GSK-3alpha regulates production of Alzheimer's disease amyloid-beta peptides. *Nature*, **423**, 435-439.

Rapoport, M., Dawson, H.N., Binder, L.I., Vitek, M.P. and Ferreira, A. (2002) Tau is essential to beta -amyloid-induced neurotoxicity. *Proc Natl Acad Sci U S A*, **99**, 6364-6369.

Reisberg, B., Ferris, S.H., de Leon, M.J. and Crook, T. (1988) Global Deterioration Scale (GDS). *Psychopharmacol Bull*, **24**, 661-663.

Rickle, A., Bogdanovic, N., Volkman, I., Winblad, B., Ravid, R. and Cowburn, R.F. (2004) Akt activity in Alzheimer´s disease and other neurodegenerative disease. *Neuroreport*, **15**, 955-959.

Rickle, A., Bogdanovic, N., Volkmann, I., Zhou, X., Pei, J.J., Winblad, B. and Cowburn, R.F. (2005) PTEN levels in Alzheimer's disease medial temporal cortex. *Neurochem Int*, **17**, 17.

Rodriguez, J., Chen, H.H., Lin, S.C. and Lazebnik, Y. (2000) Caspase phosphoryaltion, cell death, and species variability. *Science*, **287**, 1363a.

Rohn, T.T., Head, E., Su, J.H., Anderson, A.J., Bahr, B.A., Cotman, C.W. and Cribbs, D.H. (2001) Correlation between caspase activation and neurofibrillary tangle formation in Alzheimer's disease. *Am J Pathol*, **158**, 189-198.

Rohn, T.T., Rissman, R.A., Davis, M.C., Kim, Y.E., Cotman, C.W. and Head, E. (2002) Caspase-9 activation and caspase cleavage of tau in the Alzheimer's disease brain. *Neurobiol Dis*, **11**, 341-354.

Roman, G.C. (2004) Vascular dementia. Advances in nosology, diagnosis, treatment and prevention. *Panminerva Med*, **46**, 207-215.

Ross, A.H., Lachyankar, M.B. and Recht, L.D. (2001) PTEN: a newly identified regulator of neuronal differentiation. *Neuroscientist*, **7**, 278-281.

Roth, M., Tomlinson, B.E. and Blessed, G. (1966) Correlation between scores for dementia and counts of 'senile plaques' in cerebral grey matter of elderly subjects. *Nature*, **209**, 109-110.

Russell, J.W., Windebank, A.J., Schenone, A. and Feldman, E.L. (1998) Insulin-like growth factor-I prevents apoptosis in neurons after nerve growth factor withdrawal. *J Neurobiol*, **36**, 455-467.

Ryder, J., Su, Y. and Ni, B. (2004) Akt/GSK3beta serine/threonine kinases: evidence for a signalling pathway mediated by familial Alzheimer's disease mutations. *Cell Signal*, **16**, 187-200.

Salehi, A., Delcroix, J.D. and Swaab, D.F. (2004) Alzheimer's disease and NGF signaling. *J Neural Transm*, **111**, 323-345.

Sano, T., Lin, H., Chen, X., Langford, L.A., Koul, D., Bondy, M.L., Hess, K.R., Myers, J.N., Hong, Y.K., Yung, W.K. and Steck, P.A. (1999) Differential expression of MMAC/PTEN in glioblastoma multiforme: relationship to localization and prognosis. *Cancer Research*, **59**, 1820-1824.

Sayre, L.M., Zelasko, D.A., Harris, P.L., Perry, G., Salomon, R.G. and Smith, M.A. (1997) 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. *Journal of Neurochemistry*, **68**, 2092-2097.

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

Selkoe, D.J. (2002) Alzheimer's disease is a synaptic failure. *Science*, **298**, 789-791.

Shimohama, S. (2000) Apoptosis in Alzheimer's disease--an update. *Apoptosis*, **5**, 9-16.

Simic, G., Kostovic, I., Winblad, B. and Bogdanovic, N. (1997) Volume and number of neurons of the human hippocampal formation in normal aging and Alzheimer's disease. *J Comp Neurol*, **379**, 482-494.

Smale, G., Nichols, N.R., Brady, D.R., Finch, C.E. and Horton, W.E., Jr. (1995) Evidence for apoptotic cell death in Alzheimer's disease. *Exp Neurol*, **133**, 225-230.

Small, D.H., Mok, S.S. and Bornstein, J.C. (2001) Alzheimer's disease and Abeta toxicity: from top to bottom. *Nature Reviews Neuroscience*, **2**, 595-598.

Smith, M.A., Perry, G., Richey, P.L., Sayre, L.M., Anderson, V.E., Beal, M.F. and Kowall, N. (1996) Oxidative damage in Alzheimer's. *Nature*, **382**, 120-121.

Sofroniew, M.V., Howe, C.L. and Mobley, W.C. (2001) Nerve growth factor signaling, neuroprotection, and neural repair. *Annu Rev Neurosci*, **24**, 1217-1281.

Spillantini, M.G. and Goedert, M. (1998) Tau protein pathology in neurodegenerative diseases. *Trends Neurosci*, **21**, 428-433.

Stadelmann, C., Bruck, W., Bancher, C., Jellinger, K. and Lassmann, H. (1998) Alzheimer disease: DNA fragmentation indicates increased neuronal vulnerability, but not apoptosis. *J Neuropathol Exp Neurol*, **57**, 456-464.

Stambolic, V., Suzuki, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P. and Mak, T.W. (1998) Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell.*, **95**, 29-39.

Steen, E., Terry, B.M., Rivera, E.J., Cannon, J.L., Neely, T.R., Tavares, R., Xu, X.J., Wands, J.R. and de la Monte, S.M. (2005) Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? *J Alzheimers Dis*, **7**, 63-80.

Sun, X.M., Snowden, R.T., Skilleter, D.N., Dinsdale, D., Ormerod, M.G. and Cohen, G.M. (1992) A flow-cytometric method for the separation and quantitation of normal and apoptotic thymocytes. *Anal Biochem*, **204**, 351-356.

Suzuki, A., de la Pompa, J.L., Stambolic, V., Elia, A.J., Sasaki, T., del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., Fukumoto, M. and Mak, T.W. (1998) High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Curr Biol*, **8**, 1169-1178.

Takashima, A., Murayama, M., et al. (1996) Exposure of rat hippocampal neurons to amyloid beta peptide (25-35) induces activation of phosphatidyl inositol-3 kinase and the

activation of tau protein kinase I/glycogen syntahse kinase -3 beta. *Neurosci Lett*, **203**, 33-36.

Terry, R.D., Masliah, E., Salmon, D.P., Butters, N., DeTeresa, R., Hill, R., Hansen, L.A. and Katzman, R. (1991) Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol*, **30**, 572-580.

Terry, R.D., Peck, A., DeTeresa, R., Schechter, R. and Horoupian, D.S. (1981) Some morphometric aspects of the brain in senile dementia of the Alzheimer type. *Ann Neurol*, **10**, 184-192.

Torres, J. and Pulido, R. (2001) The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. *J Biol Chem*, **276**, 993-998.

Trojanowski, J.Q. and Lee, V.M. (2002) The role of tau in Alzheimer's disease. *Med Clin North Am*, **86**, 615-627.

Troncoso, J.C., Sukhov, R.R., Kawas, C.H. and Koliatsos, V.E. (1996) In situ labeling of dying cortical neurons in normal aging and in Alzheimer's disease: correlations with senile plaques and disease progression. *J Neuropathol Exp Neurol*, **55**, 1134-1142.

Tuppo, E.E. and Arias, H.R. (2005) The role of inflammation in Alzheimer's disease. *Int J Biochem Cell Biol*, **37**, 289-305.

Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M.K., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., Albanese, A., Nussbaum, R., Gonsález-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W.P., Latchman, D.S., Harvey, R.J., Dallapiccola, B., Auburger, G. and Wood, N.W. (2004) Hereditary Early-Onset Parkinson´s Disease Caused by Mutations in PINK1. *Science*, **304**, 1158-1160.

Vazquez, F., Ramaswamy, S., Nakamura, N. and Sellers, W.R. (2000) Phosphorylation of the PTEN tail regulates protein stability and function. *Mol Cell Biol*, **20**, 5010-5018.

Wei, W., Wang, X. and Kusiak, J.W. (2002) Signaling events in amyloid beta-peptide-induced neuronal death and insulin-like growth factor I protection. *J Biol Chem*, **277**, 17649-17656. Epub 12002 Mar 17646.

Weihl, C.C., Ghadge, G.D., Kennedy, S.G., Hay, N., Miller, R.J. and Roos, R.P. (1999) Mutant presenilin-1 induces apoptosis and downregulates Akt/PKB. *J Neurosci*, **19**, 5360-5369.

West, M.J., Kawas, C.H., Martin, L.J. and Troncoso, J.C. (2000) The CA1 region of the human hippocampus is a hot spot in Alzheimer's disease. *Ann N Y Acad Sci*, **908**, 255-259.

West, M.J., Kawas, C.H., Stewart, W.F., Rudow, G.L. and Troncoso, J.C. (2004) Hippocampal neurons in pre-clinical Alzheimer's disease. *Neurobiol Aging*, **25**, 1205-1212.

Vestling, M., Wiehager, B., Tanii, H. and Cowburn, R.F. (2001) Akt activity in presenilin 1 wild-type and mutation transfected human SH-SY5Y neuroblastoma cells after serum deprivation and high glucose stress. *J Neurosci Res*, **66**, 448-456.

Whitehouse, P.J., Price, D.L., Clark, A.W., Coyle, J.T. and DeLong, M.R. (1981) Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. *Ann Neurol*, **10**, 122-126.

Vivanco, I. and Sawyers, C.L. (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*, **2**, 489-501.

Woodgett, J.R. (1990) Molecular cloning and expression of glycogen synthase kinase-3/factor A. *Embo J*, **9**, 2431-2438.

Woodgett, J.R. (1991) cDNA cloning and properties of glycogen synthase kinase-3. *Methods Enzymol*, **200**, 564-577.

Yang, Y., Gehrke, S., Haque, M.E., Imai, Y., Kosek, J., Yang, L., Beal, M.F., Nishimura, I., Wakamatsu, K., Ito, S., Takahashi, R. and Lu, B. (2005) Inactivation of Drosophila

DJ-1 leads to impairments of oxidative stress response and phosphatidylinositol 3-kinase/Akt signaling. *Proc Natl Acad Sci U S A*, **102**, 13670-13675. Epub 12005 Sep 13679.

Yoon, S.Y., Choi, J.E., Huh, J.W., Hwang, O., Nam Hong, H. and Kim, D. (2005) Inactivation of GSK-3beta in okadaic acid-induced neurodegeneration: relevance to Alzheimer's disease. *Neuroreport*, **16**, 223-227.

Yuan, J. and Yankner, B.A. (2000) Apoptosis in the nervous system. *Nature*, **407**, 802-809.

Zarkovic, K. (2003) 4-hydroxynonenal and neurodegenerative diseases. *Mol Aspects Med*, **24**, 293-303.

Zhang, Y., McLaughlin, R., Goodyer, C. and LeBlanc, A. (2002) Selective cytotoxicity of intracellular amyloid beta peptide1-42 through p53 and Bax in cultured primary human neurons. *J Cell Biol*, **156**, 519-529. Epub 2002 Jan 2028.

Zhao, M., Cribbs, D.H., Anderson, A.J., Cummings, B.J., Su, J.H., Wasserman, A.J. and Cotman, C.W. (2003) The induction of the TNFalpha death domain signaling pathway in Alzheimer's disease brain. *Neurochemical Research*, **28**, 307-318.