PTEN AND AKT SIGNALLING IN ALZHEIMER´S DISEASE

Annika Rickle

Stockholm 2005
I am still confused but on a higher level 😊
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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^{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^{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


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
<table>
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<tr>
<td>Aβ</td>
<td>Amyloid β</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
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<tr>
<td>Akt</td>
<td>Protein kinase B also known as Akt</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
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<tr>
<td>CA</td>
<td>Cornu ammonis</td>
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<td>CERAD</td>
<td>Consortium to establish a registry of AD</td>
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<td>ChAT</td>
<td>Choline acetyltransferase</td>
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<tr>
<td>CREB</td>
<td>cAMP-response-element-bindning protein</td>
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<tr>
<td>DSM IV</td>
<td>Diagnostic and statistical manual of mental disorders 4th edition</td>
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<td>FAD</td>
<td>Familial Alzheimer’s disease</td>
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<tr>
<td>FTDP-17</td>
<td>Frontal temporal dementia and Parkinsonism linked to Chromosome 17</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acid protein</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
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<tr>
<td>HNE</td>
<td>4-Hydroxynonenal</td>
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<tr>
<td>IGFI</td>
<td>Insulin-like growth factor-I</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>mTOR</td>
<td>The mammalian target of rapamycin, also known as FRAP1</td>
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<tr>
<td>NDC</td>
<td>Non-diseased control</td>
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<td>NFkB</td>
<td>Nuclear factor κB</td>
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<td>NFT</td>
<td>Neurofibrillary tangles</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NINCDS-ADRDA</td>
<td>The National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer’s Disease and Related Disorders Association</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
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<tr>
<td>PDC</td>
<td>Positive-disease control</td>
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<tr>
<td>PDKI</td>
<td>Phosphoinositide-dependent kinase I</td>
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<tr>
<td>PDKII</td>
<td>Phosphoinositide-dependent kinase II</td>
</tr>
<tr>
<td>PHF</td>
<td>Paired helical filaments</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
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<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-3,4-biphosphate</td>
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<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
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<tr>
<td>PS1</td>
<td>Presenilin 1</td>
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<tr>
<td>PS2</td>
<td>Presenilin 2</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>β-tubulin III</td>
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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 (Aβ_{1-40} or Aβ_{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 Aβ$_{40}$ and Aβ$_{42}$ (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 elderly 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 Aβ$_{42}$ 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 Aβ$_{42}$. An increased Aβ$_{42}$ 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 Aβ$_{42}$ 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 phagocytosed 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 dentate gyrus (Hamos et al., 1989), most likely as a consequence of less input from the entorhinal 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 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, 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 (Thr308) and Serine 473 (Ser473) 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 Ser214 and Thr212 (Ksiezak-Reding et al., 2003).
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 Ser⁹ and Tyr²¹⁶ and for GSK3α at Ser²¹ and Tyr²⁷⁹ in its kinase domain. Akt phosphorylates both isoforms at Ser²¹ in GSK3α and Ser⁹ 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 Tyr²¹⁶ in GSK3β and of Tyr²⁷⁹ 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 Ser370, Ser380, Thr382, Thr383 and Ser385 (Torres and Pulido, 2001). In contrast, a very recent report, could not show that ck2 phosphorylates Ser370, but demonstrate phosphorylation of Ser370 and Ser385 sites. The same group, showed also that GSK3β could regulate PTEN at two phosphorylation sites, Ser362 and Thr366, suggesting a possible PTEN regulation in a negative feedback loop (Al-Khoury 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. IGF1 is another neurotrophic factor, that acts via the PI3K/Akt pathway (Dudek et al., 1997). After nerve growth factor withdrawal, IGF1 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 Tyr216 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 Aβ25-35 and Aβ1-40 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 Aβ1-40 and Aβ1-42 (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 Ser393Akt and increased Ser9GSK3β 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\textsuperscript{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\textsuperscript{216} is co-distributed with the progressive accumulation of abnormal hyperphosphorylated tau proteins in AD brains, whereas the inactive form of Ser\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{473}Akt immunopositive. In addition to neuronal staining we found that AD cases showed reactive astrocytes that were intensely immunopositive for Ser\textsuperscript{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\textsuperscript{308} Akt antibody. As earlier described, GSK3β is downstream of Akt and the group of Ferrer et al showed strong immunoreactivity of Ser\textsuperscript{9} GSK3β in astrocytes in AD (Ferrrer 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\textsuperscript{473}Akt in AD soluble fractions, whereas increased Ser\textsuperscript{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\textsuperscript{473}Akt corresponded to increased Ser\textsuperscript{\beta}GSK3 and Ser\textsuperscript{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 immunochemistry 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\textsuperscript{380}, Thr\textsuperscript{382} and Thr\textsuperscript{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\textsuperscript{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\textsuperscript{380}PTEN/PTEN ratio and a decreased Ser\textsuperscript{\textsuperscript{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 Aβ$_{1-42}$, 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 nucleic acid 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 extracellularly (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 Aβ$_{1-42}$ demonstrated statistically significantly increased numbers of late apoptotic cells, whereas 60µM Aβ$_{1-42}$ 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.

Kru man et al presented evidence that HNE induces neuronal apoptosis by oxidative stress in PC12 cells and in rat hippocampal neurons (Kru man 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 Aβ1-42, 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 Ser473 Akt, 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\textsuperscript{473} Akt and Ser\textsuperscript{\beta} GSK3\beta phosphorylations (Dozza \textit{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\textsuperscript{\beta} GSK3\beta phosphorylation.

In conclusion, both TNF\alpha 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 TNF\alpha-induced toxicity mediated by the Akt/GSK3\beta signalling pathway in rat primary neurons. In contrast, HNE did not show this protective effect.
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 Ser380PTEN 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 Ser3GSK3β (Ferrer et al., Griffin et al.) as well as increased Ser214tau (Griffin et al., 2005) and also increased Tyr9GSK3β (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 (Tyr9) and the inactive (Ser9) 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 Tyr9GSK3β is the active form, a therapeutic approach against this enzyme would be favourable, so that the inactive form of Ser3GSK3β 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 Ser214tau 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 Ser214 rather than Thr212 (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 Ser473Akt
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$^{308}$ and Thr$^{308}$ antibodies, in primary human astrocytes. Interestingly, a study has shown increased Ser$^{308}$Akt and Ser$^{312}$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 IGFI receptors in AD brains and also decreased Thr$^{306}$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% CO₂/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% CO₂ 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-terflon 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, deparaffinised and hydrated. The sections were pre-treated with 3% H2O2 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 trypsinated 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™ 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.
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