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Pathogenesis of Alzheimer's Disease

Focus on amyloid β-peptide, homocysteine and metals

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To my family

Abstract

Alzheimer's disease (AD) is a complex dementia disorder. It is characterized by the neuronal and synaptic loss, presence of neurofibrillary tangles and senile plaques, composed of amyloid β-peptide (Aβ) in the brain. Biochemical and genetic studies implicate a central role for Aβ in the pathogenesis of AD, however how amyloid leads to neurodegeneration is still unknown. The present work focused on investigating the role of Aβ in AD and other relevant neurological and psychiatric disorders. The study was based on the analysis of Aβ in cell culture media and post-mortem brain tissue.

In *paper I*, we measured Aβ in cell culture media from cells transfected with APP mutations causing familial AD. We could see that mutations in familial AD are primarily pathogenic through their effect on APP processing and not through altered cell signaling.

In *paper II*, we compared the levels of Aβ in the brain of elderly schizophrenics with and without dementia versus controls. We demonstrated that in the brains from people with schizophrenia and dementia there is no increase of Aβ. Thus the pathogenic pathway of dementia in elderly schizophrenics is different from that seen in AD. Additionally, in schizophrenia cases with AD neuropathology, levels of brain Aβ were decreased as compared to 'pure' AD cases. This may be explained by high smoking prevalence among schizophrenics, the use of neuroleptic drugs or could be a result of the disease state per se.

In *paper III*, we further investigated the hypothesis that stimulation of nicotinic receptors may diminish amyloidosis in the brain. We could prove that deposition of Aβ is attenuated in the cortex of normal elderly people that used to smoke tobacco. However, the mechanism of this attenuation is unknown.

Metals have been implicated in AD pathogenesis and some metal chelators have shown therapeutic promise in animal and human studies. In *paper IV*, we studied the interaction of human brain Aβ with biometals, such as zinc, copper, aluminium, iron and manganese. We extracted and measured cortical Aβ in AD patients and control groups. The levels of the metals were assessed in a parallel set of samples. We found that zinc is strongly elevated in AD brains and is correlated with Aβ and dementia severity.

In *paper V*, we focused on other important factors in AD pathogenesis, such as homocysteine and vitamin B status. We compared plasma homocysteine levels in controls, AD patients and in patients with mild cognitive impairment. We observed hyperhomocysteinemia in AD. We also confirmed that ApoE 4 allele is a risk factor in the development of sporadic AD. We did not find any evidence that polymorphism of the enzyme involved in homocysteine biogenesis, methylenetetrahydrofolate reductase (MTHFR), has a clinical significance in these groups.

In summary, these studies suggest a multifactoral pathogenesis of AD, where Aβ, zinc and homocysteine are important factors. They also give insight into targets to develop therapeutic strategies for treatment of dementia. Those include: substances stimulating nicotinic receptors, metals chelators, anti-hyperhomocysteinemia therapies, and anti-Aβ strategies.

List of original publications

The thesis is based on the following publications, which are referred to in the text by their roman numerals.

I. Bergman A., **Religa D.**, Karlström H., Laudon H., Winblad B., Lannfelt L., Lundqvist J., and Näslund J.
APP intracellular domain formation and unaltered signaling in the presence of familial Alzheimer's disease mutations.
Experimental Cell Research, 287:1-9 (2003)

II. **Religa D.**, Laudon H., Styczynska M., Winblad B., Näslund J., and Haroutunian V.
Amyloid β pathology in Alzheimer's disease and schizophrenia.
American Journal of Psychiatry, 160:867-872 (2003)

III. Court J.A., Johnson M., **Religa D.**, Keverne J., Kalaria R., Jaros E., McKeith I.G., Perry R., Näslund J., and Perry E.K.
Attenuation of Aβ deposition in the entorhinal cortex of normal elderly individuals associated with tobacco smoking.
Neuropathology and Applied Neurobiology, 31:522-535 (2005)

IV. **Religa D.**,* Strozyk D.,* Cherny R., Volitakis I., Haroutunian V., Winblad B., Näslund J., and Bush A.I.
Elevated cortical zinc in Alzheimer disease.
Neurology, 67:69-75 (2006)

V. **Religa D.**, Styczynska M., Pepłonska B., Gabryelewicz T., Pfeffer A., Chodakowska M., Łuczywek E., Wasiak B., Stepien K., Gołebiowski M., Winblad B., and Barcikowska M.
Homocysteine, apolipoprotein E and methylenetetrahydrofolate reductase in Alzheimer's disease and mild cognitive impairment.
Dementia and Geriatric Cognitive Disorders, 16:64-70 (2003)

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List of abbreviations

AD	Alzheimer's disease
Aβ	Amyloid β-peptide
APP	Amyloid Precursor Protein
ApoE	Apolipoprotein E
BBB	Blood Brain Barrier
CDR	Clinical Dementia Rating Scale
CSF	Cerebrospinal Fluid
DSM	Diagnostic and Statistical Manual of Mental Disorders
FAD	Familial Alzheimer's Disease
FTD	Fronto-Temporal Dementia
GVP	Gal4 VP16
ICD	International Classification of Disorders
LOAD	Late Onset Alzheimer's Disease
LTP	Long-Term Potentiation
MCI	Mild Cognitive Impairment
MMSE	Mini Mental State Examination
MTHFR	Methylenetetrahydrofolate Reductase
NFT	Neurofibrillary Tangles
PS	Presenilin
SP	Senile Plaques
PCR	Polymerase Chain Reaction

Introduction

Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia. AD leads to cognitive impairment, decreased quality of life both for the affected people and theirs caregivers (Almberg et al., 1997; Grafström and Winblad, 1995; Jansson et al., 1997) and premature death (Tschanz et al., 2004). Dementia affects 8–12% of people above 65 years of age and nearly 50% of those over 85 (Evans et al., 1989; Fratiglioni et al., 1991). The name for AD was given after Alois Alzheimer (1864–1915) by Emil Kraepelin. Alzheimer described a case study of his 51-year-old patient, Auguste D (Alzheimer A, 1907). The patient was suffering from cognitive and language deficits, auditory hallucinations, delusions, paranoia and aggressive behaviour. Auguste D died in 1906 and her brain underwent a post mortem examination. The gross pathology included cortical atrophy in the form of gyral shrinkage and widening of the sulci, especially in the temporal and frontal lobe. Microscopically, Alzheimer noted the occurrence of numerous neurofibrillary tangles and many amyloid plaques in specific brain regions, especially in the upper cortical layers of the brain (Graeber et al., 1998).

The term dementia ("loss of mind" in Latin) includes a number of different subtypes, where AD is the most frequent. Clinical diagnosis of dementia can be made according to several established diagnostic classification systems such as the Diagnostic and Statistical Manual of Mental Disorders (DSM-IIIR, DSM-IV) and the International Classification of Disorders (ICD-10). DSM-IV criteria define dementia as the development of multiple cognitive deficits manifested by memory impairment (an impaired ability to learn new information or recall previous knowledge) together with one or more cognitive disturbances. The latter cognitive dysfunctions include aphasia (language disturbances), apraxia (impaired ability to carry out motor activities despite intact motor function), agnosia (failure to recognize or identify object despite the intact sensory function), and disturbance in executive functions such as planning, organizing, abstract thinking. In the ICD-10 criteria, dementia is defined as a decline in memory and in two other cognitive domains, and impairment in basic activities of daily-living that lead to dependency on the caregivers. A decline must be present for at least 6 months according to the ICD-10 criteria.

Clinical diagnosis of probable and possible AD relies on the DSM-IV criteria for dementia and NINDS-ADRDA criteria for AD (McKhann et al., 1984), however a definitive diagnosis can only be obtained by postmortem examination of the patient's brain. The most prominent and common symptoms in AD are short-term memory impairment, aphasia and cognitive decline. These symptoms can reflect brain regions affected by the disease (Haroutunian et al., 1998). Other clinical signs of AD, although not present in every patient, are neuropsychiatric symptoms, such as insomnia, aggressiveness, agitation, delusions and hallucinations. As the disease progresses late symptoms appear. These include spatial and motor disabilities and autonomic dysfunction. Finally, AD leads to premature death due to other causes, such as: cardiac infarct, hip fracture, pneumonia or other infections.

Epidemiology and risk factors

It is estimated that 25 million people worldwide suffered from AD in 2000. Almost half of the demented people, 46%, lived in Asia, 30% in Europe, and 12% in North America (Wimo and Winblad, 2003). The worldwide direct costs for dementia in 2003 are estimated to be in the range of 156 billion USD based on a worldwide prevalence of 27.7 million demented persons (Wimo et al., 2006). The direct cost for the Swedish society for caring and treatment of AD is 40 billion SEK (ca 5,5 billion USD or 4,3 billion EUR) per year. Indirect costs are harder to estimate. In a future perspective, given a growing aged population, the number of worldwide demented elderly is expected to increase to 63 million in 2030 and to 114 million in 2050 (Wimo and Winblad, 2003).

The prevalence of AD doubles every five years after 65 up to 90, and then remains stable. The most important risk factor for the development of AD is increasing age (Qui et al., 2005). Other well established risk factors include family history of dementia, hyper- and hypotension, high cholesterol, head trauma, low physical activity, obesity, low education and the presence of the E4 allele of apolipoprotein E (Kivipelto et al., 2001; Huang et al., 2004; Kivipelto et al., 2005). The more recently proposed risk factors are heart failure (Qui et al., 2006) and anaemia (Atti et al., 2006).

Genetics

The majority of AD patients has no obvious family history of disease and is therefore classified as so-called sporadic AD cases. There are however rare genetic forms of AD known (Tanzi et al., 1996). These genetic forms of AD, collectively referred to as familial Alzheimer's disease (FAD), are associated with specific mutations that are inherited in an autosomal dominant manner. The discovery of these mutations has been instrumental in supplying tools for studying the molecular pathology of AD. The FAD-linked mutations are located on 3 different genes: the amyloid precursor protein (APP) gene on chromosome 21; the presenilin (PS) 1 gene on chromosome 14; and the PS-2 gene on chromosome 1. An updated list of mutations on all three genes can be found at: http://molgen-www.uia.ac.be/ADMutations. One of the most studied FAD mutations is the so-called Swedish mutation APP 670/671 (Citron et al., 1992; Lannfelt et al., 1993; Mullan et al., 1992). Another mutation discovered in a large family in the Northern part of Sweden is the so-called Arctic mutation (Nilsberth et al., 2001; Stenh et al., 2002). The discovery of such mutations leads to development of different transgenic animals which can aid in the study of AD pathogenesis (McGowan et al., 2006).

Another identified genetic risk factor for AD is the E4 allele of the apolipoprotein E (ApoE) gene (Corder et al., 1993). The gene for ApoE is located on chromosome 19 and the variant ApoE4 constitutes the major genetic risk factor associated with both sporadic (Saunders et al., 1993) and familial (Strittmatter et al., 1993) late onset AD (LOAD). It appears that the presence of the ApoE4 allele decreases the age of onset of AD in a dose dependent way (Corder et al., 1993). As the number of ApoE4 alleles increases from 0 to 2, the risk of developing familial LOAD increases from 20% to 90%, and the mean age at onset decreases from 84 to 68 years [reviewed in (Huang, 2006)]. The 299-residue ApoE protein plays a major role in transport and metabolism of cholesterol and triglycerids in humans, and is produced mainly by the liver and the brain. In the central nervous system (CNS), ApoE is synthesized by astrocytes, microglia and to a lesser extent by neurons (Cedazo-Minguez and Cowburn, 2001). The role of ApoE in AD pathogenesis is not fully resolved, but it has, among other things been suggested that ApoE is important in trafficking of the amyloid β-peptide (Aβ) (Holtzman, 2001).

In addition, the presence of ApoE leads to increased Aβ fibril formation in vitro (Wisniewski et al., 1994). ApoE4 has also been shown to facilitate deposition of Aβ in the brain (Bogdanovic et al., 2002; Holtzman et al., 2000a; Holtzman et al., 2000b; Schmechel et al., 1993) and contribute to faster brain atrophy (Wahlund et al., 1999). ApoE4 can together with Aβ42 contribute to apoptosis and tau hyperphosphorylation (Cedazo-Minguez et al., 2003). ApoE4 carriers may potentially respond differently to AD treatment (Roses et al., 2006) and other drugs (Cornelius et al., 2004). ApoE4 may also be one of the predictors of which patients with mild cognitive impairment (MCI) that will convert to AD (Modrego, 2006).

Neuropathology

The definitive diagnosis of AD requires the presence of three types of changes in the brain: neuronal loss, neocortical amyloid deposits, so-called senile plaques (SP), and neurofibrillary tangles, within specific regions of the cerebral cortex. The macroscopic findings also include diffuse, most often symmetrical, brain athrophy which leads to shrinkage and widening of sulci. Brain weight is frequently reduced. Changes in brain vasculature are also observed (Buee et al., 1997). Although diagnosis of definitive AD is based on counting plaques in several brain areas [Consortium to Establish a Registry for Alzheimer's disease CERAD criteria, (Mirra et al., 1991) and Khachaturian criteria (Khachaturian, 1985)], there is no direct correlation between number of plaques and cognitive status of patients. However, Näslund et al. have shown that there is a correlation between extractable amyloid from the brain and ante mortem cognitive status of the patients (Näslund et al., 2000). The numbers of tangles in specific brain regions, assigned as Braak stages, correlate better than plaques with cognitive deficiency (Braak and Braak, 1991). The consensus description of post mortem AD criteria from the National Institute on Aging and Reagan Institute Working Group requires both tangles and plaques in defined brain regions in order to allow definitive diagnose of AD (Hyman and Trojanowski, 1997). SPs consist of a central amyloid core, which can be stained with the β-sheet-specific histological dye Congo red. The main protein component of the extracellular plaques is the Aβ peptide whereas the intracellular tangles are composed of the microtubule-associated protein tau.

Amyloid β-peptide

In a landmark paper, Glenner and Wong described the initial purification and characterization of Aβ from cerebrovascular amyloid and SPs (Glenner and Wong, 1984). It was subsequently shown that Aβ is an integral part of a larger protein, the amyloid precursor protein (APP) (Kang et al., 1987). There are two major forms of Aβ, the 40-residue Aβ40 form and the 42-residue Aβ42 form, where the latter is more amyloidogenic. The term amyloidogenic pertains to the ability of the peptide or protein to form amyloid fibrils (Makin et al., 2005). It is well established that the amyloid protein is predominantly present in a β-sheet structure (Makin et al., 2005). Aβ belongs to a larger group of proteins that can form amyloid structures in humans. Examples of other amyloidogenic proteins are lung surfactant protein C (Johansson, 2001), the prion protein (Tanaka et al., 2006) and transthyretin (Westermark et al., 1990).

Aβ in the brain is produced mainly by neurons. However, it has been difficult to directly test the role of other types of cells in the brain such as astrocytes, other type of glia cells, pericytes and endothelial cells in the production of Aβ. The shorter Aβ40 form is the major species secreted by cells and is mainly deposited in cerebral vessels resulting in congophilic amyloid angiopathy (CAA) (Gravina et al., 1995; Iwatsubo et al., 1994). Aβ40 is also the most common Aβ form in cerebrospinal fluid (CSF) and plasma (Scheuner et al. 1996). In contrast, Aβ42 is predominantly found in SPs and appears to be the initially deposited species (Iwatsubo 1994, 1996). This is in agreement with results showing that Aβ42 polymerizes more rapidly than Aβ40 into oligomers (Bitan et al., 2003) and fibrils (Jarrett et al., 1993) in vitro. There is growing evidence suggesting that Aβ42 is the initiating species in AD pathogenesis (McGowan et al., 2005). This allowed to formulate the leading working hypothesis in AD field, so-called "amyloid cascade hypothesis" (Hardy, 2006; Hardy and Selkoe, 2002). Nevertheless, it is still not fully known whether aggregation of Aβ is sufficient to induce the other neurophatological hallmarks of AD, such as, neurofibrillary tangles and neuronal and synaptic loss.

The Aβ peptide has been shown to be involved in several toxic processes, such as apoptosis and disrupted calcium homeostasis

(Small et al., 2001), however the exact and definitive mechanism by which Aβ causes dementia is still unknown. It has also been shown that Aβ is toxic in vivo when injected into the brain of animals and that oligomers of Aβ have a potential to inhibit hippocampal long-term potentiation (LTP) (Townsend et al., 2006; Walsh et al., 2002). LTP is a core event in memory processing in humans and other mammals (Maren, 2005). Aβ also has the potential to affect dendritic spine density and can, through this mechanism, possibly contribute to altered neural system function and behavioral impairments (Spires et al., 2005).

The polymerization of the Aβ into protease-resistant fibrils is an important step in AD pathogenesis. Models of Aβ fibril formation suggest that the process occur via more or less stable intermediates (e.g. dimers, oligomers, and protofibrils). Recently, a soluble toxic form of oligomeric Aβ, named "Aβ*56" was identified (Lesne et al., 2006). Aβ*56 causes memory deficits in transgenic mice independent of plaque formation or neuronal loss. Oligomeric Aβ may lead to the cognitive deficits associated with AD by contributing to synaptic dysfunction. It has also been hypothesized that Aβ oligomers assemble into pore-like structure inside membranes. The Aβ-induced membrane permabilization may cause intracellular calcium dyshomeostasis, production of reactive oxygen species, altered signaling pathways, and mitochondrial dysfunction (Glabe and Kayed, 2006). Recent studies in transgenic mice have also indicated that intracellular Aβ oligomerization may play a role in the induction of tau pathology (Oddo et al., 2006).

Amyloid Precursor Protein

Aβ peptides are proteolytic fragments of the precursor protein APP, a Class I transmembrane glycoprotein expressed throughout most tissues in the body. The APP gene maps to chromosome 21q21.3-21. At present, the biological function of the APP protein is unknown. However, it has been suggested that APP regulates trophic functions, cell adhesion, neurite outgrowth, neuronal migration, synaptic function, and the induction of apoptosis (Russo et al., 2005). All these functions occur via interaction with multiple components of the nervous system. Several proteins interact with the intracellular domain of APP including kinases and adaptor proteins such as Fe65, mDAP, JIP18 and X11 [reviewed in (Reinhard et al., 2005)]. Proteolytic processing of APP in vivo is a normal physiological process. There are two major APP processing pathways: the amyloidogenic and the non-

amyloidogenic one. In the non-amyloidgenic pathway, the key processing events are carried out by the so-called α- and γ-secretases, respectively (Figure 1).

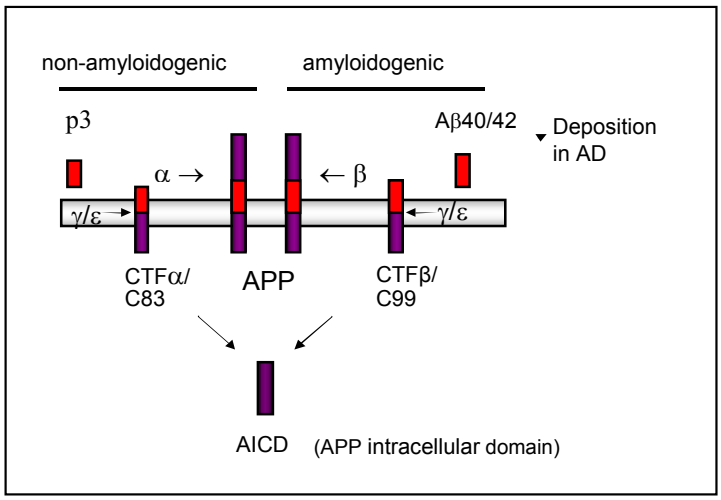

Figure 1. Two pathways of APP processing: the non-amyloidogenic and the amyloidogenic. In the non-amyloidogenic process (left panel) APP is cleaved by α-secretase (α) and γ-secretase (γ). During these cleavages the p3 and sAPPα/C83 fragments are formed. Aβ40 and Aβ42 are produced in the amyloidogenic pathway after cleavage by β and γ secretases. The intermediate C99 fragment is the direct precursor to γ-secretase cleavage. Processing at the ε-site generates the AICD fragment in both the amyloidogenic and non-amyloidogenic pathways. CTF (C-terminal fragment), AICD (APP intracellular domain), α (α-secretase), β (β-secretase), γ (γ-secretase).

The α-secretase cleavage is performed by the enzyme TACE (Buxbaum et al., 1998), a member of the ADAM metalloprotease family. Another ADAM family member, ADAM-10 has also been shown to act as an α-secretase (Lammich et al., 1999). The γ-secretase cleavage is executed by a set of proteins including PSs, nicastrin, Pen-2 and Aph-1 (Edbauer et al., 2003; Francis et al., 2002; Takasugi et al., 2003). The site of γ-secretase site of action is unusual in that it occurs within the hydrophobic milieu of the lipid bilayer. The end-product of non-amyloidogenic processing of APP is the p3 peptide, a truncated variant of Aβ that cannot form plaques. In contrast, the amyloidogenic pathway generates intact, plaque-competent Aβ. Here, the N terminus of Aβ is generated by the action of β-secretase. a transmembrane aspartyl protease, with the alternative name BACE (β-site APP cleaving enzyme) (Vassar et al., 1999). Recently, an additional cleavage site in the APP transmembrane

domain was discovered. This cleavage has been named the ε-cleavage and occurs mainly after position 49 in C99 (Yu et al., 2001). The C-terminal fragment formed by ε-cleavage is called the APP intracellular domain (AICD) (Figure 1). The temporal relationship between cleavages at the γ and ε sites is presently being studied.

The amyloid cascade hypothesis

At present the amyloid cascade hypothesis is the most prevailing theory to explain the pathogenesis of AD. It states that the deposition of Aβ is a key factor in AD and that other pathological events (NFT, neuronal and synaptic loss, vascular and cell dysfunction in CNS with attendant neurotransmitters changes) are secondary as a result of Aβ deposition. The following arguments are commonly used to support this statement: modified from (Selkoe, 2001) (i) patients with Down's syndrome, that have an extra copy of chromosome 21 (and thus an extra copy of APP) produce more Aβ throughout life and develop AD-like neuropathological lesions in their third or fourth decade of life; (ii) all known APP and PS mutations that are considered causative for AD increase the Aβ42 over Aβ40 ratio and (iii) transgenic mice that overexpress mutant APP alone or together with mutant PS recapitulate many of the pathological features seen in human AD. Adding to these arguments, it was very recently shown that APP gene duplication causes early-onset AD with abundant parenchymal and vascular deposition of Aβ (Rovelet-Lecrux et al., 2006).

One of the clinical AD treatment approaches based on the amyloid cascade hypothesis is vaccination against Aβ. This protocol has shown promise in mice, where active immunization with Aβ reduce plaque burden and increase performance in memory tests (Schenk et al., 1999; Janus et al., 2000; Morgan et al., 2000). Naturally occurring antibodies directed against Aβ are present in the CSF and plasma in humans. It was shown that CSF anti-Aβ antibody levels are significantly lower in patients with AD compared to controls (Du et al., 2001). Active immunization has also been tested in humans, however the first trial had to be terminated after reports of meningoencephalitis among the patients (Orgogozo et al., 2003). It appears that Aβ vaccination leads to increased clearance of plaques in humans too (Nicoll et al., 2003), and that patients that produced higher titers of own antibodies against Aβ scored better in the cognitive tests (Hock et al., 2003). Current protocols are investigating

the use of shorter Aβ immunogens, and the feasibility of passive instead of active immunization (Weiner and Frenkel, 2006).

Tau

Neurofibrillary tangles are composed of filaments of hyperphosphorylated tau (Delacourte and Defossez, 1986; Grundke-Iqbal et al., 1986), a microtubule-associated protein. The tau gene is located on chromosome 17 (17q21). In human brain, alternative splicing of the primary tau transcript can result in six different tau isoforms [rewieved in (Delacourte et al., 2003)]. Tau proteins belong to the microtubule-associated proteins (MAP) family and are found mostly in neurons. Tau is required for initiation and stabilization of neuronal microtubules through binding to tubulin (Delacourte and Buee, 1997). The balance between tau phosphorylation and dephosphorylation modulates the stability of the neuronal cytoskeleton and the morphology of axons. There is more than 20 potential phosporylation sites described (Hanger et al., 1998). In AD brains, hyperphosporylated tau forms paired helical filaments that in turn form neurofibrillary tangles.

Mutations in the tau gene have been described in patients with an autosomal dominant form of fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Hutton et al., 1998). This disease is characterized by behavioral disturbances, parkinsonism, dementia and amyothrophy. The characterization of these mutations, of which 35 are known today, demonstrated clearly that tau genetic dysfunction is an etiological factor in some cases of FTDP-17. However, the role of tau in sporadic forms of frontotemporal dementia is less obvious. In addition to AD and FTDP-17, tangles are detected in the brain of people diagnosed with other diseases where a massive neuronal death is evident. Among these are: Down syndrome, Pick's disease, VD, stroke, CJD, progressive supranuclear palsy, corticobasal degeneration, myotonic dystrophy, subacute sclerosing panencephalitis, post-encephalic parkinsonism, dementia pugilistica, Niemann-Pick disease type C, and Hallenvorden-Spatz disease [(reviewed in (Delacourte, 2005)]. There is no good explanation as to why plaques and tangles simultaneously accumulate in the brain of patients with AD. It may occur via direct interaction between the two

molecules, as has been recently suggested (Guo et al., 2006). However, there is a need for future studies explaining the possible crosstalk between tau and Aβ in the pathogenesis of AD.

Metals in the brain

Abnormal levels of metals (zinc, copper and iron) have been implicated in AD pathogenesis, where both excess and deficiency can be harmful to the brain (Bush, 2003; Frederickson et al., 2005). It has been shown that there is an interaction between Aβ and the metals (Maynard et al., 2005). The toxicity of Aβ in cell culture depends upon the peptide binding to copper, generating active radicals (Aβ42 more so than Aβ40) (Huang et al., 1999; Opazo et al., 2002). It has also been shown that zinc can induce rapid Aβ amyloid formation in vitro (Bush et al., 1994).

In AD post-mortem studies, elevated zinc levels have been demonstrated in several brain regions, such as: hippocampus (Cornett et al., 1998; Danscher et al., 1997; Deibel et al., 1996) amygdala (Cornett et al., 1998; Danscher et al., 1997; Deibel et al., 1996; Samudralwar et al., 1995; Thompson et al., 1988), basal nucleus of Meynert (Lovell et al., 1998; Thompson et al., 1988), the olfactory region (Cornett et al., 1998; Samudralwar et al., 1995) and frontal, temporal, and parietal (inferior) cortices (Cornett et al., 1998; Deibel et al., 1996).

It has been reported that treatment with a copper-zinc chelator inhibits Aβ accumulation in AD transgenic mice (Cherny et al., 2001). Metal chelators have also been tested as AD drugs in clinical trials with a promising effect (Ritchie et al., 2003). Still, further studies exploring the connection between Aβ and metals in AD are required.

Nicotine and AD

The relationship between nicotine use and AD is not fully established. Case control and follow up epidemiological studies have indicated that smoking is associated with a reduced risk of developing AD and that smoking delays onset of familial AD (Lee, 1994), even if these findings are controversial. A more recent population based study demonstrated a significantly increased risk of developing AD in smokers (Ott et al., 1998), although this was only evident in individuals not carrying the

ApoE4 allele. In ApoE4 carriers, tobacco smoking tended to reduce the risk of AD, consistent with a previous case control study (van Duijn et al., 1995). In a prospective community-based study (Merchant et al., 1999), smoking was shown to be associated with an increased risk of AD though there may have been a slight risk reduction in ex-smokers. Thus, the data from the epidemiological studies are somewhat conflicting and more studies are needed to determine the effect of smoking on the development of AD.

Many in vivo and in vitro studies have indicated that nicotine, acting through neuronal nicotinic receptors, can enhance neuronal survival in response to a range of neurototoxic insults including exposition to Aβ peptides, neonatal ischaemia, and excitotoxins (Akaike et al., 1994; Chen et al., 1995; Kaneko et al., 1997; Kihara et al., 1997; Kihara et al., 1998; Marin et al., 1994; Nanri et al., 1997; Nanri et al., 1998; Shimohama et al., 1996; Zamani et al., 1997; Zanardi et al., 2002). It has also been demonstrated that nicotine directly inhibits the aggregation of Aβ in vitro (Salomon et al., 1996) and that this effect is a result of nicotine stabilizing the α-helical conformation of Aβ (Zeng et al., 2001). Nicotine can also induce the production of nerve growth factor (French et al., 1999), fibroblast growth factor-2 (Belluardo et al., 2000) and brain derived neurotrophic factor (Kenny et al., 2000), which in turn have been shown to modulate Aβ toxicity and accumulation.

Homocysteine and MTHFR

Epidemiological and case-controls studies have shown elevated levels of homocysteine in AD (Seshadri et al., 2002), stroke (Bos et al., 2005; Brattström and Lindgren, 1992; Kelly et al., 2002; Pniewski et al., 2003; Sachdev et al., 2006), coronary heart disease (Chambers et al., 2000), silent white matter infarcts (Vermeer et al., 2002), brain atrophy (Sachdev et al., 2002), Parkinson's disease (Brattström, 2001; Kuhn et al., 1998; Religa et al., 2006) and many other disorders. The supplementation of folic acid, B12 and B6 is used in the treatment of hyperhomocysteinemia (Bolander-Gouaille, 2002).

Homocysteine is a sulphur-containing amino acid that is formed during the metabolism of methionine in the so-called methionine cycle. Metabolism of homocysteine occurs through two pathways: re-methylation (B12 dependent) or trans-sulphuration (vitamin B6

dependent) (reviewed in (Selhub, 1999). Hyperhomocysteinemia can be caused by genetic mutations or environmental factors, such as low levels of folic acid or vitamin B12. The other important determinants of plasma homocysteine concentration are age, gender, serum creatinine and multivitamin usage (Brattström et al., 1994). The most prevalent mutation leading to elevated plasma homocysteine levels is the methylenetetrahydrofolate reductase (MTHFR) C677T mutation (chromosome 1p36.3), which makes the MTHFR enzyme thermolabile. The MTHRF enzyme influences re-methylation of homocysteine and can through this mechanism cause hyperhomocysteinemia.

Diagnostic tools and biomarkers

Depression, delirium, B12 deficiency, and hypothyroidism and other causes of cognitive disturbances should be ruled out and treated in patients with dementia. A detailed clinical interview and examination should be followed by neuropsychological testing. One of the most frequent instruments used for testing cognition is the Mini Mental State Examination (MMSE) (Folstein et al., 1975). It has been translated to several languages and is used worldwide both by primary care doctors and by doctors working in the university clinics. The weakest feature of MMSE is that the scoring may depend on age, education and social class; therefore newer tests are being developed and validated. In advanced AD centers, a battery of detailed neuropsychological tests and questionnaires is commonly used to diagnose dementia (Almkvist et al., 1993; van Crevel et al., 1999).

Diagnosis of dementia requires one radiological examination of the brain to rule out other causes of cognitive impairment (e.g. brain tumor or hydrocephalus). It could also guide in the diagnosis by determining the presence of hippocampal and/or cortical atrophy and vascular changes. As a research tool or to specify other dementia forms, more advanced neuroimaging techniques can be used, such as CT with contrast, SPECT, fMRI, PET. The EEG examination is also a useful diagnostic help in differential diagnosis and contribute to classification accuracy (Jelic et al., 1998; Lindau et al., 2003).

In the dementia diagnostic procedure, laboratory methods are also used. These consist of blood and lumbar cerebrospinal fluid (CSF) tests (Andreasen and Blennow, 2002). Studies on Aβ in CSF have shown that Aβ42 levels in CSF decreases in AD compared to controls (Andreasen et al., 2001; Andreasen et al., 1999; Vanmechelen et al., 2001). This test has good sensitivity, especially if combined with measuring the NFT-forming protein tau, both total tau and phospho-tau in CSF (Blennow et al., 1995). Despite that the test has lower specificity when differentiating between AD and other dementias (Wallin et al., 2006). However, it was recently suggested that subgroups of AD could be identified based on CSF markers (Iqbal et al., 2005). These subgroups might benefit differently from different therapeutic drugs. The CSF biomarkers can also contribute to better characterization of MCI and prediction for the conversion to dementia (de Leon et al., 2006; Wahlund and Blennow, 2003).

At present, guidelines do not require CSF analysis to diagnose AD, although it is a very important and a cheap diagnostic tool. In Sweden, CSF is taken routinely at most centers for dementia diagnosis where the levels of total tau, phospho-tau and Aβ42 are analyzed. In many other countries CSF analysis will be a standard very soon. Measurements of Aβ42, tau and phospho-tau may also be used in the testing and monitoring of new treatments of AD (Thal et al., 2006; Tucker et al., 2005).

There is a need for developing blood-based biomarkers for AD. In most studies there was no difference in blood Aβ levels between sporadic AD cases and controls (Fukumoto et al., 2003; Tamaoka et al., 1996; Teunissen et al., 2002). One study showed lower levels of Aβ42 in AD as compared with controls and MCI (Pesaresi et al., 2006). Another group reported higher levels of Aβ42 in plasma in AD patients associated with polymorphisms in the urokinase-type plasminogen activator (Ertekin-Taner et al., 2005). Recent study suggests that high plasma Aβ40, especially when combined with low concentrations of Aβ42, indicate an increased risk of dementia (van Oijen et al., 2006). Another recent work has shown that plasma Aβ40 is correlated with white matter changes in AD, MCI and CAA (Gurol et al., 2006). If confirmed in longitudinal studies, these data would implicate circulating Aβ40 as a potential biomarker for microvascular damage in AD.

Mild cognitive impairment and other forms of dementia

It is a diagnostic challenge to distinguish between normal populations, mild cognitive impairment (MCI) and mild/moderate AD. MCI frequently precedes dementia, and the MCI group is very heterogeneous. The prevalence of MCI in population-based epidemiological studies ranges from 3% to 19% in adults older than 65 years (Gauthier et al., 2006). Individuals with the MCI diagnosis progress at different rates to dementia. Some individuals remain stable over the years whereas some experience amelioration. People with MCI have problems at work and when confronted with a complex task, but they do not experience any problems with daily life activities.

The term MCI was used for the first time in GDS 3 (Global Deterioration Scale) (Reisberg et al., 1988; Reisberg et al., 1986). A similar description was proposed by the Mayo Clinic Group (Petersen et al., 1997; Smith et al., 1996). The inclusion criteria for this group consists of memory complains from patients and/or others, good daily life activities, a Clinical Dementia Rating (CDR) score of 0,5, no diagnosis of dementia, and no cognitive impairment 1,5-2 SD less than expected when considering the age of the individuals (Jelic et al., 2006; Jelic and Winblad, 2003).

Several subgroups are included in the term MCI, such as amnestic MCI (aMCI) and MCI with global dysfunction, but there is no clear consensus on the different definitions. The aMCI group has a high risk of progression to AD (Gauthier et al., 2006). The neuropathologic features of aMCI match the clinical features and neurofibrillary changes intermediate between normal aging and very early AD (Petersen et al., 2006). MCI patients may also progress to other forms of dementia (Wahlund et al., 2003), and these include vascular dementia (VD), frontotemporal dementia (FTD), dementia with Lewy bodies, and Parkinson's disease dementia (Jicha et al., 2006). In clinical practice, a major challenge is to differentiate between the dementia seen in VD and AD (Roman and Royall, 2004). In the differential diagnosis of AD, one also have to consider less common or rare disorders, such as normotensive hydrocephalus, dementia with Huntington disease, Fahr's disease and many others.

Schizophrenia

Schizophrenia is a multiform psychiatric disorder with unknown etiology. The term schizophrenia literally means split mind, but nowadays it includes disorders with a cluster of symptoms such as delusions, hallucinations, disordered thinking and emotional unresponsiveness. The diagnosis encompasses a pattern of so called positive and negative symptoms, in conjunction with impaired occupational or social functioning, as defined by DSM-IV criteria. Schizophrenia affects 1% of the population worldwide and cognitive impairment is present in the clinical picture. Indeed, Kraepelin described schizophrenia as dementia praecox. Nowadays, detailed neuropsychological tests are used to define the cognitive deficits in schizophrenia (Goldberg TE, 2004). Patients with schizophrenia underperform in several tests such as Continuous Performance Test (CPT), Wechsler Memory Scale-Revised (WMS-R), and Wisconsin Card Sorting Test (WCST). There are deficits in attention, abstract thinking, solving new problems and many others, but they are uncharacteristic.

Memory deficits are also common in schizophrenia. However, most schizophrenic patients display only subtle memory deficits on clinical examination, unlike patients with dementia (Budson and Price, 2005). It is more likely that the memory deficits seen in schizophrenia are not widespread but limited to specific domains. Some recent studies have shown that relational memory, which is associated with impaired function of the parietal cortex and hippocampus, is particularly impaired in schizophrenia (Ongur et al., 2006).

Aims

The general aim of this PhD project was to increase knowledge concerning Aβ deposition in human brain. The relationship between brain Aβ and biometals and nicotine in humans was investigated. In addition, we have also studied hyperhomocysteinemia in AD and MCI patients.

The specific aims include:

1. To develop specific and sensitive ELISA assays to enable measurement of total Aβ, Aβ40 and Aβ42 in cell culture, brain, plasma and CSF *(paper I)*

2. To determine the levels of Aβ in brains from healthy humans, AD patients and demented patients with schizophrenia *(paper II)*

3. To determine the effect of smoking on brain Aβ levels *(paper III)*

4. To assess the association between biometals and Aβ in the brain *(paper IV)*

5. To compare plasma homocysteine levels, B12 and folic acid in AD, MCI and controls and determine ApoE and MTHFR status in these groups *(paper V)*

Materials & methods

Cell culture experiments

Cells (CHOPro5 and HEK 293) were transfected with C99-GVP wild type and mutant constructs. The C99 constructs included a Gal4-VP16 domain in order to achieve sensitive and quantitative detection of intramembrane proteolysis via a luciferase reporter gene. Levels of expression of transfected C99-GVP in cell lysates were determined by immunoblotting, while the levels of Aβ40 and Aβ42 in cell media were monitored by sandwich ELISAs. The ELISA assays were performed using monoclonal 4G8 as capture antibody and end-specific polyclonal rabbit antibodies against Aβ40 and Aβ42, respectively, as detection antibodies. The specificity and cross-reactivity of the end-specific antibodies were determined by spot-blotting, immunoblotting and peptide ELISAs as presented below in Figure 2. The correct reactivity of the antibodies was also corroborated in neurosphere cultures incubated with Aβ1-40, Aβ40-1, Aβ1-42 and Aβ42-1 as shown below in Figure 3.

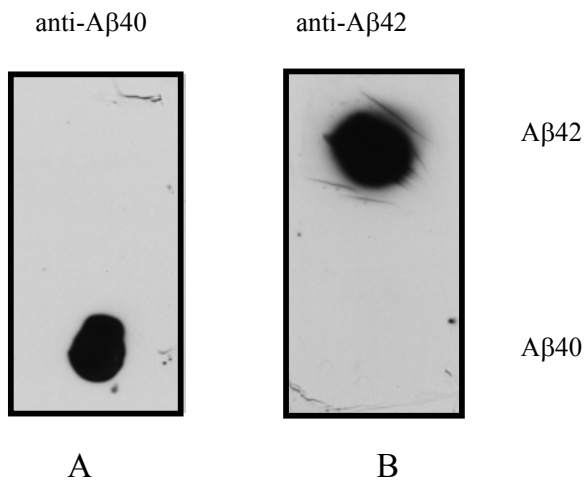

Figure 2. Characterization of Aβ40– and Aβ42–specific antibodies (A and B, respectively). Spot blotting: Amyloid β-peptides Aβ1-40 and Aβ1-42 were spotted onto nitrocellulose membranes and probed with the indicated affinity-purified antibodies.

Sandwich ELISA assays for brain Aβ

Sandwich ELISAs were used to measure extracted brain Aβ40 and Aβ42 using 6E10 as a capture antibody and secondary end-specific polyclonal antibodies. Total Aβ was measured using 6E10 as capture antibody and biotinylated 4G8 as detection antibody. The antibody 6E10 recognizes an epitope between residues 1-16 of Aβ, while the antibody 4G8 recognizes an epitope between residues 17-24. Plates were developed using alkaline phosphatase-labeled antibodies (anti-rabbit or anti-biotin) and phosphatase substrates yielding a colorimetric product.

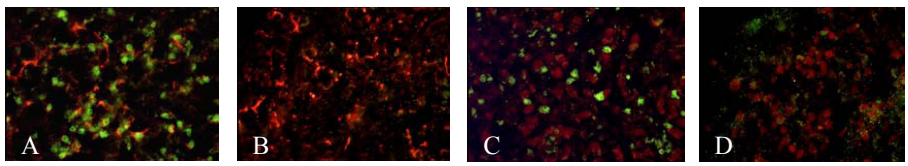

Figure 3. Further characterization of the specificity of the anti-Aβ40- and anti-Aβ42 antibodies.

A &B: Neurospheres were incubated with human Aβ1-40 peptide (A) and Aβ40-1 (B). Sections were stained with Aβ40 polyclonal antibody (green) and a neuronal marker NeuN (red). As, seen Aβ40 staining is only obtained from spheres incubated with Aβ1-40. C & D: The staining procedure as decribed aboved, but here neurospheres were incubated with human Aβ1-42 peptide (C) and Aβ42-1 (D) and stained with Aβ42 polyclonal antibody (green) and NeuN (red). Aβ42 staining is only obtained after incubation the neurospheres with Aβ1-42. [Courtesy of Johan Aarum, CMM, KI]

Human brain tissue

In studies II and IV we have used brain material from well-characterized brain bank in the USA (Mount Sinai School of Medicine Department of Psychiatry Brain Bank). Every subject underwent evaluation for the extent of neuropathological lesions using the CERAD neuropathological battery (Mirra et al., 1991). Aβ was extracted from dorsolateral prefrontal cortex, Brodmann area (BA) 46 from control subjects and subjects with AD and schizophrenia. Some schizophrenia cases also had AD pathology. Metals were measured in the same hemisphere from the superior temporal gyrus (BA 22).

In paper III we have used brain samples from the Newcastle Brain Tissue Resource. In this study Aβ was extracted from the entorhinal cortex (BA 28). The smokers were defined as people that have smoked for the main part of their lives or within 15 years before death. Non-smokers were defined as people that never smoked or ceased smoking at least 25 years before death. Pack year (PY) means the consumption of 20 cigarettes daily and was calculated on the basis of smoking since adulthood.

Aβ extraction from brain

The cortex was sub-dissected from coronal sections and frozen tissue stored at -80 °C. Tissue homogenates (42-56 mg) were thawed and homogenized by sonication in 600 µl of phosphate-buffered saline solution (PBS) containing 0.05% sodium dodecyl sulfatate and a protease inhibitor cocktail (PIC Complete, Boehringer-Mannheim), supplemented with 1 µM pepstatin. The PBS homogenate was centrifuged at 100,000 x g for 60 min at 4 °C and the supernatant flash frozen (PBS-soluble Aβ). The pellet was delipidated using a protocol modified from Wessel et al. (Wessel and Flugge, 1984). In brief, the pellet was suspended in 400 µl methanol by sonication before addition of 300 µl $CHCl_3$ and 500 µl H_2O. The emulsion was centrifuged at 10,000 x g for 2 min and the aqueous phase aspirated and discarded. The protein interphase was pelleted by addition of 400 µl MeOH followed by centrifugation at 10,000 x g for 5 min. The organic phase was aspirated and discarded. The remaining pellet was rehomogenized by sonication in 150 µl 70% formic acid, containing 1 µM pepstatin and Aβ was extracted by vortexing the homogenate for 30 min at room temperature. The extract was centrifuged at 100,000 x g for 60 min at 4 °C in a Beckman TLA110 rotor. The extracted formic acid-soluble Aβ was collected from the clear supernatant and the remaining pellet was flash frozen. The formic acid extract was neutralized by adding 18 vol of 1 M Tris-NaOH, pH 10.6, 100 mM betaine (850 µl neutralizing buffer to 50 µl formic acid extract). The visible precipitate was sonicated until the solution appeared clear. Before sandwich ELISA analysis, the neutralized extract was further diluted 50 times in ELISA buffer (PBS, 0.1% BSA, 0.05% Tween-20, 0.05% ELISA blocking reagent, 0.005% Thimerosal (Sigma-Aldrich Chemie GmbH, Germany).

Metal measurements

In paper III, Aβ was obtained from prefrontal cortex (BA 46), and metals were obtained from ipsilateral superior temporal gyrus (BA 22). Neuritic plaque density was averaged across five neocortical areas including middle frontal gyrus to give the plaque score number used in analyses. Severity of cognitive impairment was assessed with the Clinical Dementia Rating Scale.

The metal (zinc, copper, iron, aluminium and manganese) measurements were performed according to the following protocol. To each lyophilized, pre-weighed tissue sample, 0.1 ml of concentrated HNO3 (Aristar, BDH) was added and allowed to digest overnight at 4° C. Samples were further digested by heating them for 20 minutes at 90°C. One hundred µl of hydrogen peroxide (Aristar, BDH) was added immediately to each sample for 30 minutes, before heating again for a further 15 min at 70 °C. Samples were diluted with a 1% HNO_3 diluent in acid-washed 5 ml polypropylene tubes and measured in triplicate. As an internal control for the digestion procedure, triplicate preparations of NIST Bovine Liver SRM 1557B were also included. Measurements were made using a Varian UltraMass ICPMS instrument under operating conditions suitable for routine multi-element analysis. The instrument was calibrated using 0, 10, 50 and 100 ppb of a certified multi-element ICPMS standard solution (ICP-MS- CAl2-1, Accustandard). A certified internal standard solution containing 100 ppb of Yttrium (Y 89) was used as an internal control (ICP-MS- IS-MIX1-1, Accustandard).

Patients and controls characteristics

In study V, AD and MCI patients were diagnosed in a Day Clinic of the Department of Neurodegenerative Disorders of Medical Research Centre at the Polish Academy of Sciences in Warsaw. The detailed characteristics of study groups are given in Table 3 in Results and Discussion section.

ApoE genotyping

ApoE genotype determination was done using the modified method of restriction genotyping (Chapman et al., 1996). Briefly, after DNA extraction from peripheral blood, polymerase chain reaction (PCR)

amplification with specific primers and digestion with restriction enzymes were performed.

MTHFR genotyping

The MTHFR genotype was determined using PCR performed in a Biometra UNO II Thermocycler with previously described primers (Frosst et al., 1995). The primers generated a 198-bp fragment. The MTHFR polymorphism, C>T, creates a HinfI restriction site. When a C-to-T substitution is present, HinfI digests the 198-bp fragment into a 175-bp and a 23-bp fragment. These fragments were detected by agarose gel electrophoresis.

Homocysteine, folic acid and cobalamine measurements

Total fasting homocysteine (tHcy) concentrations in plasma were measured with a fluorescence polarization assay (Abbott IMx Homocysteine Assay). Serum folate levels were determined using Abbott Laboratories AxSYM Folate Reagent assay, and plasma vitamin B12 was quantified by immunoassay. Normal ranges for these analytes are from 4 to 12 μmol/L for homocysteine in plasma, 5.3-14.4 ng/ml for folate in serum and 157-1059 pg/ml for plasma cobalamine.

Ethical considerations

For study I there was no need for a separate ethical application, since only cell-based approaches were used. All studies when human material was analyzed complied with the Declaration of Helsinki. They were approved by the Ethics Committee of the Karolinska Institutet (KI372/02, studies II and IV, KI440/02, study III, KI441/02, study V). Studies II and IV were also approved by Ethical Committee from Mount Sinai School of Medicine, NY, USA (NA083). Study III was approved by Newcastle and North Tyneside Local Research Ethics Committee (2002/295). The protocol for study V was approved by the Ethical Committee in Hospital MSWiA Warsaw, Poland (20/2000). The patients and controls for study V were recruited with their and/or theirs caregivers written informed consent.

Results & discussion

Paper I

APP intracellular domain formation and unaltered signaling in the presence of familial Alzheimer's disease mutations

Aβ is a normal product of cell metabolism (Haass et al., 1992; Seubert et al., 1992). In FAD, there is a specific increase in the production of the Aβ42 variant in the body (Scheuner et al., 1996). Aβ is released from APP through the consecutive proteolytic actions of β- and γ-secretase. AICD is generated close to the cytoplasmic face of the membrane leaflet, via cleavage at the ε site (see Figure 1 in Introduction). AICD has been proposed to regulate gene transcription, and it can be hypothesized that altered AICD production and nuclear signaling is linked to a part of the phenotype seen for FAD mutations.

A sensitive ELISA assay distinguishing between Aβ40 and Aβ42 was developed and validated (as described in the Methods section). We measured Aβ and AICD generation in intact cells from a truncated APP construct, C99. This C99 construct had been derivatized to include a Gal4-VP16 domain to allow sensitive and quantitative detection of intramembrane proteolysis events via a luciferase reporter gene (Figure 4). We focused the study on determining the intracellular site of AICD formation and the effect of FAD-associated mutations on Aβ and AICD production. AICD generation was PS-dependent and sensitive to specific γ-secretase inhibitors indicating that authentic AICD formation and signaling was measured in the transfected CHOPro5 cells. The results obtained in the luciferase reporter assay could be verified by immunoblotting for AICD-GVP, since it was discovered that the introduction of the GVP domain into AICD significantly decreased degradation of this otherwise extremely labile molecule.

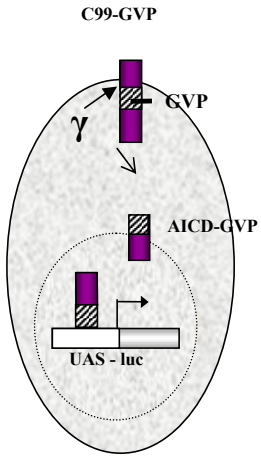

Figure 4. The luciferase reporter assay. The C99 reporter molecule with GVP domain inserted after transmembrane domain is presented. The intracellular domain (AICD-GVP) is released upon transmembrane cleavage and actives transcription of luciferase via the upstream activating sequence (UAS-luc).

Similar to Aβ, AICD was found to be formed in compartments downstream of the endoplasmic reticulum (ER), a fact that was highlighted by the introduction of a double-lysine ER retention signal tag in the C99 molecule. AICD formation and signaling was completely abolished from this construct. Moreover, it was shown that introduction of FAD-associated mutations did not alter AICD signaling compared to the wild type molecule. To ascertain that the introduction of the GVP domain did not change the properties of C99, Aβ40 and Aβ42 secreted into the medium was measured using sandwich ELISA assays. The FAD-associated mutants tested displayed the expected increase in Aβ42. Interestingly, the increase in Aβ42 production was followed by a simultaneous decrease in production of the more abundant Aβ40 variant.

In conclusion, both Aβ and AICD generation are critically dependent on the presence of the PS1 protein and both processes mainly occur in compartments downstream of the ER. No change in AICD formation could be seen for substrates containing FAD-associated mutations that increase Aβ42 production, suggesting that these mutations are primarily pathogenic through their effect on APP processing and not through altered AICD signaling.

Our data are consistent with other papers showing that mutations in APP do not lead to changes in AICD formation (Chen et al., 2002; Hecimovic et al., 2004). In contrast, presenilin FAD mutations have been demonstrated to increase Aβ42 and modulate AICD levels (Moehlmann et al., 2002). Our data contribute to the growing line of evidence suggesting differences between APP and PS mutants in terms of AICD generation. We speculate that the APP mutant-induced increase in Aβ42 is followed by a simultaneous decrease in Aβ40, thus leaving the formation of AICD unchanged. However, we cannot rule out that APP mutants also lead to a cleavage shift in AICD, as has recently been shown for PS1 mutants using mass spectrometry (Kakuda et al., 2006).

Paper II

Aβ pathology in AD and schizophrenia

Severe cognitive decline is a common phenomenon in elderly patients with schizophrenia (Purohit et al., 1998). AD patients often have psychiatric manifestations of the disease, such as psychosis and disruptive behavior, especially in the later stages of the disease. It has been shown that psychosis and aggression are associated with more rapid rates of cognitive and functional decline in AD (Stern et al., 1997). Although schizophrenia and AD may not share common neuropathologic lesions, such as neuritic plaques, the commonality of some cognitive and psychiatric symptoms in the two diseases suggests the potential existence of overlapping molecular pathogenetic pathways. We wanted to examine the possible involvement of Aβ in the cognitive impairment seen in elderly patients with schizophrenia by measuring total brain Aβ, Aβ40 and Aβ42.

Total Aβ, Aβ40, and Aβ42 were extracted from the brain using an established protocol and quantified by sandwich ELISAs as described in the Methods section. The brain region investigated in this study was the dorsolateral prefrontal cortex (DLPC), Brodmann Area (BA) 46. The levels of Aβ42 were similar in demented schizophrenics without AD and in controls, but they were significantly higher in schizophrenics with AD pathology relative to controls or to schizophrenics without AD pathology (vs. controls, $p=0.003$; vs. schizophrenics without AD, $p=0.006$). The levels of Aβ42 in schizophrenics with AD neuropathology was significantly lower than the Aβ42 levels in the AD cohort ($p=0.0001$). The results are shown in Figure 5.

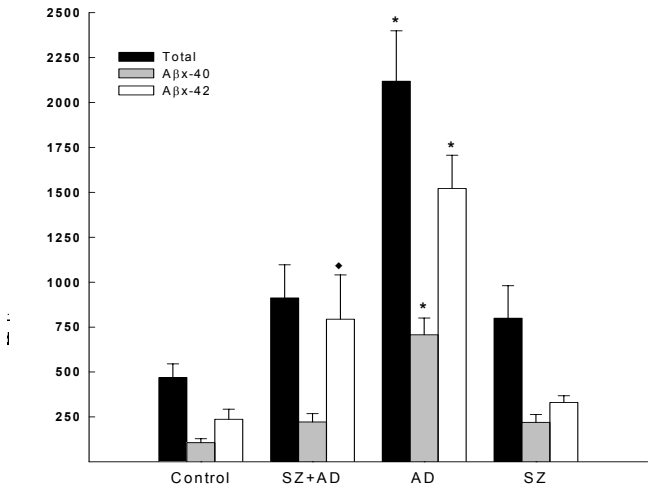

*Figure 5. Levels of total Aβ, Aβx-40 and Aβx-42 in the DLPFC of control, Alzheimer's disease (AD), schizophrenic (SZ) and schizophrenic with Alzheimer's neuropathology (SZ+AD) subjects. Values in pmol/g tissue represent means + SEM. * vs. all other groups, p<0.001. ♦vs. control and vs. AD, p<0.004.*

Some suggestions can be brought forward to explain why the schizophrenic patients with AD neuropathology had decreased levels of Aβ compared to AD patients: specific drug treatment, heavy smoking or the disease state per se. It has been shown that treatment with neuroleptics, such as droperidol and haloperidol, can lead to decrease of Aβ formation in vitro (Higaki et al., 1997). It was not possible to prove that the decrease of Aβ is connected with a specific drug in our cohort, since we did not have reliable information about drug treatment for all patients and the group was relatively small for creating further subgroups. Prevalence of smoking among patients with schizophrenia is high (Dalack et al., 1998). Nicotine has previously been shown to decrease Aβ levels both in vitro (Salomon et al., 1996; Ono et al., 2002) and in vivo (Hellström-Lindahl et al., 2004b; Lahiri et al., 2002; Utsuki et al., 2002; Zamani and Allen, 2001). The lack of detailed information for most of the patients regarding the smoking status throughout life precluded us from drawing any conclusions between smoking and brain Aβ levels. However, we have further explored the connection between smoking and Aβ production in study III.

In contrast to elderly schizophrenia patients having AD pathology, those without AD pathology had Aβ levels that were not significantly different from those of normal subjects, hence the Aβ does not account for the cognitive deficits in this group. These results suggest that the causes of cognitive impairment in 'pure' schizophrenia are different from those in AD. It also implicates that anti- Aβ strategies, tested for AD would not be effective in dementia in schizophrenics. More studies are needed in order to explain the cause of cognitive impairment in this large group of patients.

Paper III

Aβ deposition attenuated in smokers

In contradictory epidemiological studies, smoking appears to be protective or a risk factor for developing AD (Winblad et al., 1999). It has been demonstrated that nicotine directly inhibits the aggregation of Aβ in vitro (Salomon et al., 1996, Ono et al., 2002) and it has also been suggested that nicotine may stabilize the β-helical structure of Aβ (Zeng et al., 2001).

Based on the above mentioned data and on our previous study, we hypothesized that nicotine may decrease Aβ production and accumulation in human brain. We designed a study where we focused on ante mortem smoking habits. Postmortem tissue samples from the entorhinal cortex were obtained from dementia free controls. According to their smoking status the individuals were subdivided into two groups, smoker and non-smokers. Alzheimer-type pathology, such as Aβ and abnormally phosphorylated tau, and vascular changes in the brain were evaluated immunohistochemically and quantified by image analysis. The characteristics of the groups and Aβ peptides levels are shown in the Table 1.

The total Aβ and diffuse Aβ immunoreactivity, together with formic acid-extractable Aβ42 but not Aβ40, was reduced in smokers compared with non-smokers ($P<0.05$). There was also a reduced percentage of cortical and leptomeningeal vessels associated with Aβ immunoreactivity in smokers compared with non-smokers. The formic acid-extractable Aβ42 and pack years ($n=34$, $r=-0.389$, $P=0.025$) were inversely correlated. There was a similar trend between total Aβ immunoreactivity and pack years which did not reach statistical significance ($n=30$, $r=-0.323$,

P=0.082). In contrast, there were no significant group dif-ferences for vascular markers, phospho-tau markers or phosphate-buffered saline (PBS) soluble Aβ40, Aβ42 or total Aβ.

Table 1. Extracted insoluble and soluble Aβ40 and Aβ42 from BA28 in smokers and non-smokers. Aβ levels in pmol/g tissue. PD (post mortem delay), PY (pack years)

	Smokers		Non-smokers	
	Male (10)	Female (6)	Male (6)	Female (12)
Age (years)	78,9 (1,8)	78,8 (2)	80,4 (9,9)	79,4 (1,9)
PD	35 (6)	17 (4)	24 (4)	41 (8)
PY	48 (5)	38 (7)	16 (7)	5 (4)
Insoluble Aβ40	2440 (566)	1497 (331)	1541 (497)	3277 (782)
Insoluble Aβ42	3334 (674)	2892 (679)	6753 (3876)	12909 (4236)
Soluble Aβ40	8 (1,2)	7,1 (0,5)	7 (0,3)	7,2 (0,7)
Soluble Aβ42	21,1 (3,3)	26,6 (6,5)	29,2 (4,9)	27,6 (4,2)

Studies in transgenic mice have shown a similar pattern where both extracellular and PBS-soluble Aβ was decreased when animals were exposed to nicotine (Hellström-Lindahl et al., 2004a). The same group has also demonstrated that both soluble and insoluble Aβ42 and Aβ40 in frontal cortex and Aβ40 in temporal cortex and hippocampus were significantly decreased in smoking AD patients compared to nonsmoking AD patients (Hellström-Lindahl et al., 2004b). In our study we observed a more than 60% reduction of formic-acid extracted Aβ42, but no change in Aβ40 levels. Different roles of Aβ40 and Aβ42 in relation to vascular and parenchymal pathology have been suggested on the basis of experiments performed in double transgenic APPdutch and presenilin 1 mutant mice (Herzig et al., 2004). Recently it was shown that Aβ42, but not Aβ40 is essential for parenchymal and vascular Aβ deposition in mice. When mice expressing Aβ42, were crossed with APPswe mice, there was a massive increase in Aβ deposition both in parenchyma, and in vessels (McGowan et al., 2005). This pattern was not seen in mice expressing Aβ40.

The observation of correlation of tobacco smoking with decreased levels of Aβ42 leads to the obvious question about mechanism of action of nicotine on the deposition of Aβ. Nicotine may directly prevent the formation of amyloid fibrils (Ono et al., 2002; Salomon et al., 1996). This concept is consistent with our finding that we detected a reduction of insoluble Aβ42, but not of soluble forms of Aβ42 or Aβ40. Similar

results were also reported using APPswe mice treated with nicotine (Hellström-Lindahl et al., 2004a; Hellström-Lindahl et al., 2004b). However, the concentration of nicotine in the in vitro studies where Aβ fibril formation was modulated much exceeded those present in blood of smokers.

It may also be possible that nicotine indirectly affects Aβ production, for example via nicotinic receptors. Stimulation of nicotinic receptors may alter APP processing and thus Aβ production (D'Andrea and Nagele, 2006). Alternative mechanisms of nicotine action could involve changes of neurotrophic factors production, such as nerve growth factor (Eriksdotter Jönhagen et al., 1998; French et al., 2006; Rattray, 2001), fibroblast growth factor (Belluardo et al., 2004) or other neurotrophic factors. These factors may in turn modulate Aβ metabolism. Recently, it was shown that nicotine attenuated Aβ-induced neurotoxicity by regulating metal homeostasis, especially by decreasing the intracellular copper concentration (Zhang et al., 2006).

In summary, our data together with an emerging body of evidence from the literature encourage future trials with analogs of nicotine for Aβ -targeted anti-dementia treatment.

Paper IV

Association of cortical zinc with Aβ burden and clinical severity in AD

In several studies brain metals levels in AD post-mortem tissue have been measured and a consistent elevation of zinc (Danscher et al., 1997) and decrease of copper has been identified (Loeffler et al., 1996). It can be hypothesized that a biochemical interaction between Aβ and biometals play a role in the pathogenesis of AD. However, to date no study has explored the biochemical relationship of metals with Aβ burden in humans.

In order to determine whether changes in biometals in the brain are associated with Aβ burden we examined post mortem brain tissue from AD, controls and patients with schizophrenia with or without mild AD pathology. All the subjects had been assigned CDR score between 0 (normal cognition) and 5 (severe dementia). The characteristics of the study group for paper II and IV are presented in the Table 2.

Table 2. Descriptive statistics for the study groups.

	Normal controls	Schizophrenia	Schizophrenia plus AD	AD	ANOVA
	n=14	n=26	n=8	n=10	p-value
DEMOGRAPHICS					
Age	82.8 (11.2)	70.3 (13.1)	76.0 (14.6)	81.6 (11.0)	**0.02**
Female/Male (n)	12/2	8/18	5/3	7/3	0,01
PMI	479 (370)	686 (377)	735 (360)	532 (608)	0.36
CDR	0.25 (0.38)	1.85 (1.14)	2.25 (1.39)	4.1 (1.2)	**<0.0001**
METALS (nmol/g wet wt)					
Zinc	11.3 (1.9)	10.7 (1.8)	10.5 (1.8)	26.0 (13.8)	**<0.0001**
Copper	2.85 (0.9)	2.89 (0.9)	2.53 (0.5)	2.32 (1.0)	0.33
Iron	34.7 (5.0)	33.3 (6.2)	36.8 (7.6)	38.5 (13.1)	0.23
Manganese	0.17 (0.05)	0.14 (0.02)	0.15 (0.03)	0.15 (0.05)	0.09
Aluminum	0.67 (1.1)	1.01 (2.5)	0.79 (1.1)	0.31 (0.1)	0.69

The Aβ levels were assessed from the dorsolateral prefrontal cortex (BA 46) and metals levels from ipsilateral superior temporal gyrus (BA 22). There was a significant, more than twofold, increase of tissue zinc in patients with AD as compared to the other groups. After adjusting for age, this metal was positively associated with total Aβ (p= 0.03), Aβ40 (p=0.02) and Aβ42 (p=0.02). Zinc levels were significantly (p< 0.001) elevated in the most severely demented cases (CDR 4-5) (Figure 6) and in cases that had an amyloid burden greater than 8 plaques/mm^2. Copper showed a trend to decline with age. Levels of other metals did not differ between groups.

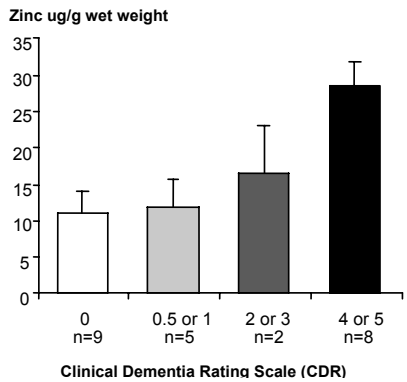

Figure 6. Zinc levels in different CDR groups. CDR 0 = non dementia, CDR 4 or 5 = severe dementia

These findings are in concordance with previous reports of elevated zinc levels in several brain regions in AD including hippocampus (Cornett et al., 1998), amygdala (Cornett et al., 1998; Danscher et al., 1997), basal nucleus of Meynert (Thompson et al., 1988), the olfactory region (Cornett et al., 1998), and frontal, temporal, and parietal (inferior) cortices (Cornett et al., 1998; Deibel et al., 1996). Studies that have not found zinc elevations in AD brain have either used small sample sizes or used formalin-fixed tissue which artifactually lowers zinc levels because of denatured zinc binding sites (Andrasi et al., 1995; Ward et al., 1987).

To our knowledge, this is the first human study to report an association between zinc levels in cortex and Aβ burden or dementia severity (CDR). Previously, zinc has been shown to accumulate within Aβ plaques in humans (Lee et al., 1999; Lovell et al., 1998; Suh et al., 2000). Zinc ions are released by neurotransmission from a subset of glutamatergic neocortical fibers achieving concentrations of approximately 300 μM. Zinc is concentrated into synaptic vesicles in these fibers through the activity of ZnT3, found only in the synaptic vesicular membrane. This pool of zinc represents 15-30% of total brain zinc, and genetic ablation of ZnT3 abolishes amyloid plaque deposition and cerebral congophilic angiopathy in the APPswe mice (Lee et al., 2002).

The elevation of zinc observed in our study is unlikely to be explained solely by the accumulation of zinc within Aβ deposits: while the levels of Aβ in the AD cases were 1.45 nmoles/g wet weight greater than levels in control tissue, the associated elevation in Zn was about 150-fold greater (222 nmoles/g wet weight). Thus the elevated zinc in AD could reflect accumulation within cells rather than merely indicating zinc sequestered into solid Aβ deposits or diffuse forms of Aβ deposits. Supporting this possibility, chemically exchangeable Zn^{2+} has been found to accumulate within the bodies of neurons in AD-affected neocortex (Suh et al., 2000). As we measured total zinc in the brain, we cannot speculate if intra- or extracellular zinc plays a role in AD pathogenesis. Since the elevation in zinc correlates with Aβ levels, it is still possible that Aβ and Zn accumulations are causally associated through a mechanism that is yet unclear. There is a need to determine whether altered zinc levels precede or follow deposition of Aβ.

The limitation of our study is that the analysis of metals and Aβ were done in the same hemisphere, but in different cortical regions. With some confidence we may assume that the chosen regions are to a similar extent affected by Aβ pathology and have similar zinc levels. The choice of the distinct brain regions may have reduced the strength of biochemical association between Aβ and zinc (increase the probability of a type 2 error). Further studies on identical areas are therefore warranted to confirm our findings. In contrast, the zinc comparison between AD and controls was performed using the same regions, thus the previous concern does not affect our finding of increased zinc levels in AD patients compared to controls.

Our results showing a correlation between zinc and dementia severity may suggest that zinc accumulation causes neuronal dysfunction. Excess of zinc is neurotoxic by several mechanisms. Abnormal elevated zinc inhibits mitochondrial reparation (Brown et al., 2000), proteosome activity (Kim et al., 2004) and induces abnormal microtubule assembly (Kamimura and Mandelkow, 1992). It has also been demonstrated that zinc induces memory impairments in rats (Flinn et al., 2005).

It is also possible that zinc indirectly or directly affect APP proceeding. It has been reported that zinc supplementation in combination with low-copper diet significantly decreased APP expression in platelet (Davis et al., 2000). In the same study it was confirmed that zinc tablets intake increase extracellular superoxide dismutase activity. There is a large group of patients that takes zinc supplementations daily. Elderly patients with inadequate dietary habits tend also to be at risk for mild to moderate zinc deficiency with symptoms including slow wound healing, increased risk of infection, and a loss of acuity in taste and smell (Keenan and Morris, 1993). In animal studies, chronic zinc treatment increases the pool of synaptic zinc in the hippocampus, a region that is affected early in the AD neurodegenerative process (Szewczyk et al., 2006).

The levels of other metals, including copper, which we measured in this study, remained unchanged between AD and control. Thus, our data do not support the hypothesis that imbalance in copper metabolism is

associated with AD. However, a subtle difference in copper levels could have escaped detection since the number of the patients included in the study was relatively small.

In summary, these data indicate that brain zinc accumulation is a prominent feature of advanced AD, and suggest that brain zinc accumulation is biochemically associated with brain Aβ levels and dementia severity in AD. These results support the hypothesis that zinc accumulation in the brain might be causally related to Aβ accumulation. The implementation of our finding in the clinics should be followed by trials with metal chelating agents for AD treatment. One such drug, clioquinol, has shown promising results in Phase II clinical trials (Ritchie et al., 2003). It has been documented that clioquinol had the potential to decrease plasma Aβ42 levels and slow the progress of AD.

Paper V

Hyperhomocysteinemia, APOE4 and MTHRF polymorphism in AD

There is a need to find genetic and environmental risk factors for AD. Increased levels of homocysteine in the blood are associated with various neurological, cardiological and psychiatrics disorders (Seshadri and Wolf, 2003). Recent longitudinal data show an association between hyperhomocysteinemia and a higher risk of AD (Seshadri et al., 2002). However, this finding has not been confirmed in some other studies (Miller et al., 2002) and more research into the possible relationship between homocysteine and MCI and AD is needed.

The aim of this study was to analyze the levels of homocysteine, vitamin B12 and folic acid in the blood from patients with AD, MCI and a control group. Importantly, we also determined the ApoE and MTHFR polymorphisms in these groups. A total of 100 patients with AD, 99 with MCI and 100 controls were included in this cross-sectional study. Homocysteine, B12 and folic acid were analyzed in the blood, and in parallel, APOE and MTHFR genotyping were performed. The descriptive statistics is shown in Table 3.

*Table 3. Descriptive statistics for study groups. Results are expressed in mean (SD). The laboratory reference values: homocysteine = 4-12 μmol/l, folate = 5,3-14,4 ng/ml, B12 = 157-1059 pg/ml; ApoE4 and MTHFR T in numbers (percent). ApoE (apolipoprotein E), MTHFR (methylenetetrahydrofolate reductase). *p<0.05,**p<0.0001.*

	Controls	AD	MCI
	100	99	98
Homocysteine	14,43 (4,5)	18,03 (9,9)**	14,15 (4,1)
Folate	7,56 (5,4)	8,51 (3,4)	10,87 (3,9)
B12	413,5 (241)	316,7 (140)*	386,3 (159)
ApoE4 +	21 (21%)	56 (56.6%)	27(27.6%)
ApoE4-	79 (79%)	43 (43.4%)	71 (72.4%)
MTHFR allele T fequency	0.260	0.273	0.291

Plasma total homocysteine was increased in AD patients, compared to controls (18,0 μmol/l and 14,4 μmol/l, respectively). Hyperhomocysteinemia was associated with decreased blood levels of folic acid and B12. In contrast, patients diagnosed with MCI did not have hyperhomocysteinemia. The increased frequency of the ApoE4 allele among AD patients was confirmed in our study group. The ApoE4 effect seen in our study was independent of homocysteine, folic acid and vitamin B12 levels and MTHFR status. The distribution of the MTHRF C677T polymorphism in the study group did not differ between AD, MCI and controls.

The association between total homocysteine and AD, which was found in our study, was consistent with results reported in the majority of retrospective and prospective studies (Anello et al., 2004; Guidi et al., 2006; Luchsinger et al., 2004; Ravaglia et al., 2005; Seshadri et al., 2002). The design of the study was to examine homocysteine once in patients with different cognitive levels. We have studied patients with moderate to severe AD and patients with MCI, the latter which have a high risk of progressing to AD dementia. In our study, the MCI group did not have hyperhomocysteinemia, so we cannot confirm that hyperhomocysteinemia does occur early in AD. In addition, we cannot deduce that hyperhomocysteinemia is a risk factor for progressing from

MCI towards AD, mostly because of cross-sectional design of the study. The lack of association between homocysteine and the MCI group could also, at least in part, be due to the relatively small numbers of patients included and the fact that the MCI study group is very heterogeneous. The hypothesis that hyperhomocysteinemia may contribute as a risk factor for converting from MCI to AD should be addressed by longitudinal prospective epidemiological studies. This has been explored by some authors and it was shown that severe to moderate hyperhomocysteinemia facilitated the conversion from MCI to AD dementia during the 3 year observation period (Annerbo et al., 2005). Previously, it was observed that homocysteinemia is an independent risk factor for development of AD, however the MCI group was not included in this study (Seshadri et al., 2002).

Homocysteine is elevated in the blood, brain and CSF also in many different neurological disorders (Seshadri and Wolf, 2003) and homocysteinemia cannot be a good biomarker for MCI or AD. Elevation of homocysteine in the CSF parallels that in serum; however, serum concentrations are 20–100-fold higher than levels in the CSF (Obeid and Herrmann, 2006). The mechanism of toxicity of homocysteine has been studied in animals, cell culture and humans, however, it still remains poorly understood (Jakubowski, 2006; Zieminska et al., 2006). Homocysteine itself or folate and vitamin B12 deficiency can cause disturbed methylation and/or redox potentials, thus promoting calcium influx, Aβ and tau protein accumulation, apoptosis, and neuronal death (Obeid and Herrmann, 2006). The homocysteine effect may also be mediated by activation of the N-methyl-D-aspartate receptor (Luchowska et al., 2005). Homocysteine potentiates copper- and Aβ-mediated toxicity in primary neuronal cultures (White et al., 2001). Aside from the direct neurotoxic effect, hyperhomocysteinemia in AD may lead also to cerebrovascular damage. Hyperhomocysteinemia is an established risk factor for vascular disorders (Castro et al., 2006; McCaddon et al., 2001; Nilsson et al., 2006) and white matter changes in the brain (Hogervorst et al., 2002). It has been suggested that anti-hyperhomocysteinemia therapy (vitamin B12-B6-folate combination) has a potential to improve the function of the blood-brain barrier in patients with MCI (Lehmann et al., 2003).

There is a need to evaluate the effectiveness of anti-hyperhomocysteinemia interventions in the clinic. So far there is no evidence suggesting that supplementation with folic acid, B12 and B6 may repair the cognitive loss seen in AD (Lonn et al., 2006; Malouf et al., 2003). It has been reported that vitamin supplementation did not improve cognitive function in elderly patients with vascular lesions which may indicate that the neuronal and synaptic loss is difficult to restore (Stott et al., 2005). These data also suggest that anti-hyperhomocysteinemia treatment should be started early in the disease process and be considered as a preventive method and not as a curative one.

In summary, we have showed that hyperhomocysteinemia is present in AD patients but not in the MCI group. There is a need for clinical trials using middle-aged subjects to asses the effectiveness of anti-hyperhomocysteinemia treatment (vitamin supplementation) as a preventive method for AD.

Conclusions & future perspectives

Although the finer molecular details of dementia and AD still remain largely unknown, the results presented in this thesis, together with a body of genetic and biochemical evidence gathered throughout the years, points to a pivotal role for Aβ in the pathogenesis of AD. During the process which leads to the neuropathology seen in AD, there appears to be an interplay between Aβ and other elements, such as tau, homocysteine and metals.

Based on the results from paper I we conclude that the pathogenic effect of APP mutations associated with familial AD occur through a subtle shift in the production of Aβ (i.e. a changed Aβ42/Aβ40 ratio), rather than through changes in intracellular signaling, since the AICD production was not affected by the mutations. Paper II demonstrates that Aβ is not causative agent for dementia observed in patients with schizophrenia. Additionally, extracted Aβ is decreased in the brains of schizophrenics with AD neuropathology compared to patients with AD. The decrease of brain Aβ in the schizophrenia patients may be due to treatment with neuroleptics, smoking or the pathological process underlying schizophrenia. Based on the results from paper III we conclude that smoking lead to decreased levels of Aβ in human brain, however the mechanism for this reduction remains to be determined. One possibility is that it can occur by stimulation of nicotinic receptors, and thus drugs that can mimic nicotine action in the brain are worth pursuing as potential therapeutic anti-amyloid agents. We also speculate that the effect of nicotine on reducing Aβ deposition can be partly mediated by metal homeostasis regulation.

In paper IV we show that zinc levels are elevated in AD brain and are correlated with brain Aβ and severity of dementia. This is an important mechanistic finding in light of the positive results seen with a metal chelator in clinical trials. Further studies will be required to establish the temporal relationship between Aβ and zinc deposition in AD brain and the molecular mechanism whereby zinc affects plaque genesis or turnover. Moreover, the potential use of zinc together with Aβ in CSF

and/or plasma as biomarkers for AD should be explored. It might also prove worthwhile to investigate the metal-Aβ relationship in CSF and plasma and its possible impact on Aβ turnover in humans. Finally, we performed a cross-sectional study where we analyzed homocysteine and vitamin B status and genetic polymorphisms of the MTHFR and ApoE genes. In paper V, we confirm that homocysteine and ApoE4 contribute to AD pathogenesis. The Aβ ELISA described in this thesis can also be used to measure Aβ1-40 and Aβ1-42, in plasma thus one apparent future study would be to explore the relationship between plasma Aβ and homocysteine.

In summary, the results presented in this thesis provide information that can be useful in research aiming at diagnosing and treating AD. Potential AD therapies highlighted by the results could be the use of nicotinic receptor stimulators, metal chelators and anti-hyperhomocysteinemia agents. In future studies it will be important to explore the mechanistic relationship between the factors, described here and Aβ in brain.

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