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A MOLECULAR GENETIC STUDY AND EXPRESSION-BASED ANALYSIS OF RISK FACTORS OF ALZHEIMER'S DISEASE

Emphasis on Aβ-degrading enzymes, IDE and NEP

Doctoral dissertation

To be presented with assent of the Medical Faculty of the University of Kuopio for public examination in Auditorium ML3, Medistudia building, University of Kuopio, on 23rd November 2007, at 12 noon

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ABSTRACT
Alzheimer`s disease (AD) is the most common cause of dementia in the elderly, affecting up to 20% of population aged over 90 years. With the increasing longevity of our population, AD is already approaching epidemic proportions with no cure or preventative therapy yet available. AD is a progressive, fatal neurodegenerative disorder leading to loss of independence and ability to cope with the tasks in everyday life which usually requires hospitalization. Neuropathologically, AD is characterized by the accumulation of amyloid β-protein (Aβ) and the formation of intracellular neurofibrillary tangles in the brain. AD is multifactorial disease to which both genetic and environmental factors contribute. Age is the major risk factor for AD, with a doubling of risk every five years after the age of 65. In addition to age, a positive family history of dementia is considered to be a definite risk factor for AD. Mutations in APP, PSEN1, and PSEN2 genes are known to cause the autosomal dominant form of early onset AD. Additionally, APOE ε4 allele was the first recognized and still only clear genetic risk factor in both early and late onset AD. Since the vast majority of AD occurs after the age of 65, putative risk genes affecting to pathogenesis of late onset AD have particular interest. The APOE ε4 allele accounts for about 50% of the genetic background of the late onset AD suggesting that additional susceptibility genes exist**.** Identifying new genes involved in the pathogenesis of AD could open new avenues for the development of rational treatment and prevention for AD.

In the present series of studies, the objective was to identify new risk genes for AD and to study their expressional changes in AD models using biological approaches. Six gene regions were studied and particularly polymorphisms in three genes, i.e. insulin degrading enzyme (IDE), neprilysin (NEP) and somatostatin (SST), showed a significant risk effect among Finnish AD patients. Aβ accumulation is thought to derive from an imbalance between Aβ production and degradation and recently, IDE and NEP have been identified as key enzymes responsible for the degradation and clearance of Aβ. In this thesis, we show that genetic variants of IDE and NEP increase the risk of AD among un-stratified Finnish AD cases. Additionally, the first genetic association study between somatostatin (SST) and AD indicates that genetic variations in the SST gene may increase the risk of AD among the APOE ε4 carriers. Interestingly, SST is known to modulate NEP expression and in that way it can influence Aβ degradation. Our data also suggest that accumulation of the pathological Aβ peptide may modify its own degradation. In particular, expression levels of IDE may be regulated through APP or its derivatives and there may be cross-regulation between IDE and NEP. We also demonstrated that analyzed polymorphisms of HHEX, BDNF and its receptor TrkB genes are not contributing significantly to risk in Finnish AD patients. In conclusion, the factors which affect Aβ degradation show particular relevance in AD. More studies are warranted to investigate further these associations and their functional implications as well as to evaluate the potential of using genes associated with AD as therapeutic targets in the search for prevention and treatment of this devastating disease.

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Medical Subject Headings: Alzheimer Disease/genetics; Amyloid beta-Protein; Amyloid beta-Protein Precursor/metabolism; Apolipoproteins E; Disease Models, Animal; Finland/epidemiology; Gene Expression; Insulysin; Mice, Transgenic; Neprilysin; Polymorphism, Single Nucleotide; Polymorphism, Genetic; Receptor; trkB/genetics; Risk Factors; Somatostatin

To Jouni, Jenna, Tanja and Lauri

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Saila Vepsäläinen

ABBREVIATIONS

Aβ	amyloid β-protein
AD	Alzheimer`s disease
ADAM	a disintegrin and metalloprotease family
AICD/CTFγ	APP Intra Cellular Domain
APOE	apolipoprotein E
APH	anterior pharynx-defective
APLP	amyloid precursor-like protein
APP	amyloid precursor protein
BACE1	β-secretase 1
BDNF	brain-derived neurotrophic factor
CALLA	common acute lymphoplastic leukemia antigen
cDNA	complementary DNA
CERAD	The Consortium to Establish a Registry for Alzheimer`s Disease
CI	confidence interval
CSF	cerebrospinal fluid
CT	computed tomography
Ct	threshold cycle
CO	control
ΔE9	exon 9 deletion
D`	a measure of linkage disequilibrium
DM2	type 2 diabetes mellitus
DNA	deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
ELISA	enzyme linked immunosorbent assay
EOAD	early onset Alzheimer`s disease
ER	endoplasmic reticulum
FAD	familial Alzheimer`s disease
fMRI	functional magnetic resonance imaging
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HHEX	hematopoetically expressed homeobox gene
HNE	hydroxynonenal
IDE	insulin degrading enzyme
kDa	kilodalton
LD	linkage disequilibrium
LOAD	late onset Alzheimer`s disease
MCI	mild cognitive impairment
MRI	magnetic resonance imaging
mRNA	messenger RNA
NCSTN	nicastrin
NEP	neprilysin
NFT	neurofibrillar tangle
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer`s Disease and Related Disorders Association
NT	non-transgenic

OR	odds ratio
PCR	polymerase chain reaction
PEN-2	presenilin enhancer II
PET	positron emission tomography
PHF	paired helical filament
PSEN	presenilin
RNA	ribonucleic acid
RT	reverse transcription
SAP	shrimp alkaline phosphatase
SD	standard deviation
SE	standard error
SNP	single nucleotide polymorphisms
SPSS	statistical package for social sciences
SST	somatostatin
Tg	transgenic
TrkB	tyrosine kinase receptor B

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numerals I-V

I Vepsäläinen S, Castren E, Helisalmi S, Iivonen S, Mannermaa A, Lehtovirta M, Hänninen T, Soininen H, Hiltunen M. Genetic analysis of BDNF and TrkB gene polymorphisms in Alzheimer`s disease. Journal of Neurology 2005; 252:423-428.

II Helisalmi S, Hiltunen M, Vepsäläinen S, Iivonen S, Mannermaa a, Lehtovirta M, Koivisto A M, Alafuzoff I, Soininen H. Polymorphisms in neprilysin gene affect the risk of Alzheimer`s disease in Finnish patients. Journal of Neurology, Neurosurgery and Psychiatry 2004; 75:1746-1748, cited in Journal of Medical Genetics 2005; 42:158

III Vepsäläinen S, Parkinson M, Helisalmi S, Mannermaa A, Soininen H, Tanzi R, Bertram L, Hiltunen M. Insulin degrading enzyme is genetically associated with Alzheimer`s disease in the Finnish population. Journal of Medical Genetics 2007; 44(9):606-608.

IV Vepsäläinen S, Helisalmi S, Koivisto A M, Tapaninen T, Hiltunen M, Soininen H. Somatostatin genetic variants modify the risk for Alzheimer`s disease among Finnish patients. Journal of Neurology (in press). Authors Vepsäläinen and Helisalmi contributed equally to the article.

V Vepsäläinen S, Hiltunen M, Helisalmi S, Wang J, van Groen T, Tanila H, Soininen H. Increased expression of Aβ degrading enzyme IDE in the cortex of transgenic mice with Alzheimer`s disease-like neuropathology. Submitted for publication.

CONTENTS

1. INTRODUCTION

The most common form of dementia is Alzheimer's disease (AD). This insidious and devastating neuronal degeneration robs its victims of their most human qualities such as memory, reasoning, abstraction, and language, and it is believed to afflict more than 30 million people worldwide (Townsend et al., 2006). AD frequency increases strongly with age, from less than 1% in people aged 65 to 69 years to over 20% in those who are 90 years or older. As the population ages, AD is now recognized as a major public health problem and financial burden on society. Unless effective prevention or treatment is found for AD, the magnitude of the problem will continue to increase.

Our knowledge of pathogenesis of AD has grown enormously in the past decade but the causes and mechanisms of the disease still remain unclear. In addition to age, both genetic and environmental factors contribute to the pathogenesis of AD. Compared to monogenic disorders, in which defects in a single gene result in a characteristic disease phenotype it is extremely challenging and laborious to recognize the risk factors and genes for such a multifactorial, complex disease. Mutations in amyloid precursor protein (APP) (Goate et al., 1991), presenilin 1 (PSEN-1)(Sherrington et al., 1995), and presenilin 2 (PSEN-2) (Levy-Lahad et al., 1995, Rogaev et al., 1995) genes are known to cause the autosomal dominant form of early onset AD (EOAD) (Corder et al., 1993b, Saunders et al., 1993b, Chartier-Harlin et al., 1994, van Duijn et al., 1994) while the apolipoprotein E (APOE) ε4 allele has been associated with an increased risk of developing both EOAD and late onset AD (LOAD) (Roses, 1997). APOE ε4 allele accounts for about 50% of the genetic background of the LOAD, suggesting that additional susceptibility genes exist (Saunders et al., 1993a, Combarros et al., 2002, Bertram and Tanzi, 2004a, Raber et al., 2004).

The objective of the present series of studies was to study genes which might affect the risk of AD and to assess the function of putative risk genes. Candidate genes in close proximity to the AD associated loci and also genes which are biologically relevant for AD provided the primary targets for genetic and functional studies. The patients and controls used in the study derived from a geographically restricted area in eastern Finland. Genetic isolates with a history of a small founder population, long-lasting isolation and population bottlenecks can modify gene pools significantly, rendering some populations more suitable than others for

gene mapping strategies (Peltonen, 2000). In line with these ideas, the strength of the present study originates from the homogeneity of patients and control subjects used. Additionally, considering the possibility to find true differences between the compared groups, there was an adequate number of subjects in the whole study populations. The diagnostic procedure was also carried out by experienced personnel. The diagnosis of AD was based on the globally used DSM-IIIR criteria for dementia (APA, 1987) and NINCDS-ADRDA criteria for AD (McKhann et al., 1984). Additionally, the function of putative risk genes of AD was studied by using well characterized transgenic APP/PSEN1 mice lines with AD-like amyloid pathology (Borchelt et al., 1997, Jankowsky et al., 2004).

It is well known that AD is characterized by the progressive and severe accumulation of amyloid ß-protein (Aß) and neurofibrillary tangles in the brain. Prevention of Aß production and accumulation is currently being evaluated as a potential therapeutic intervention for AD. Thus a considerable amount of research activity has been expended on clarifying the cellular mechanisms of Aß formation but relatively less effort has been aimed at understanding Aß metabolism and degradation, although these factors are also noteworthy when trying to clarify the mechanisms of AD pathogenesis. In this thesis, we show that genetic variants of two Aβ degrading genes, IDE and NEP increase the risk of AD. Additionally, genetic variants of the SST gene which is known to modulate NEP expression (Saito et al., 2005) increase the risk of AD in the APOE ε4 carriers. Our data also suggest that age- and region-specific changes in the proteolytic degradation of Aβ may contribute to pathogenic mechanisms in AD. Moreover, accumulation of the pathological Aβ peptide may modify its own degradation. In particular, expression levels of IDE may be regulated through APP or its derivatives and there may be cross-regulation between IDE and NEP. In conclusion, the factors which affect Aβ degradation show particular relevance in AD and further studies are warranted. One important challenge for the future is moving from the genes to proteins, to elucidate the biological relevance resulting from altered genetic information. This kind of progress is also likely to be the key to finding a curative treatment.

2. REVIEW OF THE LITERATURE

2.1. Alzheimer's Disease (AD)

AD is the most common cause of dementia in the elderly. AD is a progressive, fatal neurodegenerative disorder characterized by global cognitive decline involving memory, orientation, judgment, and reasoning. The expected survival time after the disease onset is estimated to be 5 to 12 years and the patients usually die of infectious or other incidental illnesses. The disease is named after Alois Alzheimer, a Bavarian psychiatrist with expertise in neuropathology (Figure 1a). Alzheimer presented the case of his first patient Auguste D in 1906 (Figure 1b) (Maurer et al., 1997)). Fifty one year old Auguste was diagnosed with what we would now refer to as "early onset dementia". However, although there are rare examples of early-onset dementia due to genetic mutations, the vast majority of AD occurs after the age of 65; prevalence is 0.6% for individuals aged 65 to 69 years and 22% for those aged over 90 years (Lobo et al., 2000). In Finland it is estimated that in 2005 there were 120 000 patients with dementia and 65-70 % of the dementia cases were attributable to AD (Erkinjuntti et al. 2006). AD is categorized according to the age of onset into early and late onset forms by using 65 years as the cut-off point (Terry and Katzman, 1983). With the increasing longevity of our population, AD is already approaching epidemic proportions with no cure or preventative therapy in sight.

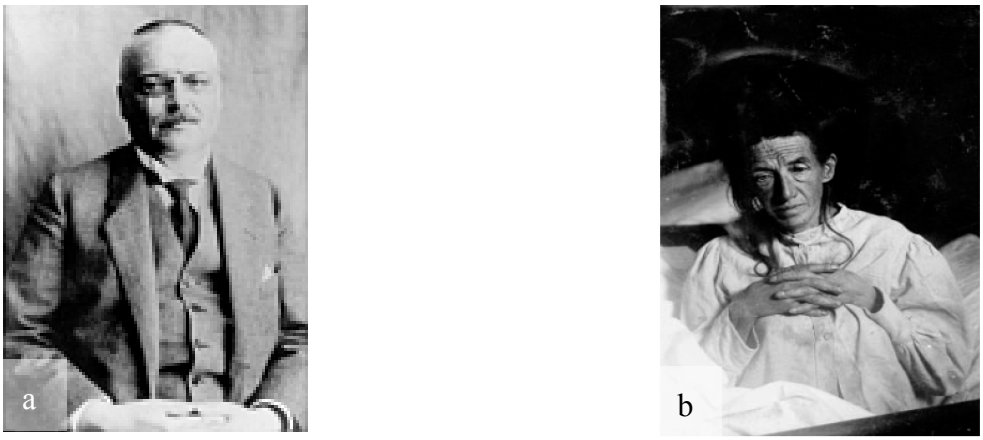


Figure 1. a) De Alois Alzheimer (1864-1915) and b) Auguste D., the first AD patient in 1906 (Graeber et al., 1998, Graeber and Mehraein, 1999).

2.1.1. Clinical diagnosis of AD

The diagnosis of AD is of importance since it permits the initiation of the currently available symptomatic treatment as early as possible. One widely used system for the clinical diagnosis of AD is based on the criteria defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R and DSM-IV, American Psychiatric Association 1987 and 1995) and on the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association Work Group (NINCDS-ADRDA) (McKhann et al., 1984). The NINCDS-ADRDA criteria divide AD into three categories i.e. probable, possible and definite. Although a clinical diagnosis of AD can be made with considerable certainty during life, a diagnosis of definite AD still requires postmortem observation of the classical lesions in microscopic sections of hippocampus, amygdala, and the association cortices of the frontal, temporal, and parietal lobes. The criteria defined by the Neuropathology Task Force of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) are usually the basis for the neuropathological diagnosis of AD (Mirra et al., 1991).

The development of different imaging techniques such as computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and functional MRI (fMRI) has provided tools for diagnostic purposes. Functional imaging techniques represent non-invasive methods of mapping human brain functions in vivo, while structural imaging allows the detection of atrophy and ischemic changes. Cerebrospinal fluid (CSF) biological markers may also be of valuable help in the diagnosis of AD, with total tau, phosphorylated tau (P-tau) and Aβ42 being the most promising candidates (Blasko et al., 2005, Herukka et al., 2005). CSF Aβ42 has been consistently shown to be decreased and total tau and P-tau increased in AD when compared to healthy subjects (Vandermeeren et al., 1993, Jensen et al., 1995, Motter et al., 1995, Vigo-Pelfrey et al., 1995). It has been proposed that the most accurate assay for AD among the patients with mild cognitive impairment (MCI) would be the combination of Aβ42 and P-tau. MCI is considered a transition state between normal aging and dementia. Subjects with MCI exhibit cognitive decline but do not fulfill the criteria for dementia.

2.1.2. Characteristic features of AD brain

AD is characterized by the accumulation of abnormally folded proteins, amyloid β–protein (Aβ) to neuritic plaques and tau to neurofibrillar tangles, in the brain leading to neuronal degeneration and synaptic loss. Neuritic plaques, which are also found to some extent in the brains of normal aged individuals, are mainly composed of Aβ in its insoluble, fibrillar form (Figure 2a) (Masters et al., 1985). Other essential constituents of neuritic plaques are dystrophic neurites, reactive astrocytes, and activated microglia cells (Terry and Katzman, 1983). Additionally, neuritic plaques have been shown to contain many other compounds such as specific proteoglycans, apolipoprotein E (APOE), α_1-antichymotrypsin and complement factors (Abraham et al., 1988, Eikelenboom et al., 1989, Snow and Wight, 1989, Namba et al., 1991). In addition to neuritic plaques, non-fibrillar plaques (diffuse) primarily composed of the Aβ component can be observed in AD. It has been suggested that diffuse and neuritic plaques actually exist in the cortex in a morphological continuum, rather than as two distinct types of lesions (Dickson et al., 1995). The formation of abnormally phosphorylated tau protein which is also detected in other degenerative brain diseases, leads to production of the neurofibrillary tangles, which are intraneuronal inclusions made up of the paired helical filaments (PHF) of the abnormally phosphorylated tau (Figure 2b) (Terry and Katzman, 1983).

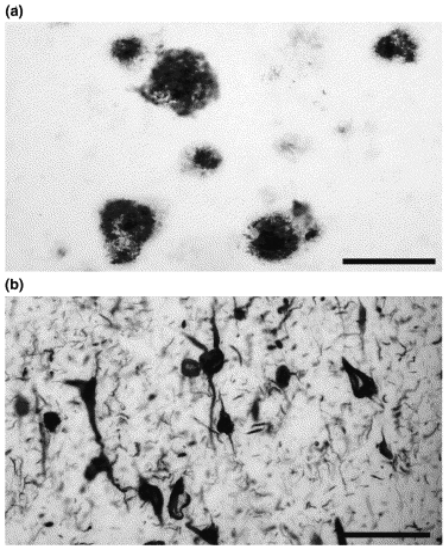


Figure 2. Plaques and tangles in the AD brain. (a) A representative micro-photograph of amyloid plaques in the AD brain. Amyloid plaques were visualized by immunostaining with an anti-Ab42 specific antibody. Scale bar: 125 mm. (b) A representative microphotograph of neurofibrillary tangles. Tangles were visualized by immunostaining with an anti-PHF1 specific antibody. Scale bar: 62.5 mm (LaFerla and Oddo, 2005).

2.2. Amyloid precursor protein (APP) and amyloid β-protein (Aβ)

During most of the 20th century, very little progress was made in understanding the etiology of AD and the majority of AD cases display no clear mode of inheritance. Finally, in 1981 Heston et al. reported that relatives of 125 subjects who had autopsy-confirmed AD exhibited a significant excess of dementing illness consistent with genetic transmission (Heston et al., 1981). In the same study, when compared to controls, the relatives of affected individuals derived from families with a significantly greater incidence of Down`s syndrome (DS; chromosome 21 trisomy). AD-type neuropathology was also observed in the brains of middle-aged patients with DS. These observations pointed to a possible genetic link between AD and an abnormal gene or structural defect on chromosome 21. In 1984, Glenner and Wong published the Aβ sequence (Glenner and Wong, 1984) and two years later four different groups isolated the gene encoding the β-amyloid precursor protein (APP) which Glenner had predicted mapped to chromosome 21 (21q21.3) (Goldgaber et al., 1987, Kang et al., 1987, Robakis et al., 1987, Tanzi et al., 1987).

The biological function of a widely expressed cell surface protein APP itself is unclear. Cell biological studies are strongly suggestive for a regulatory role of APP in cell adhesion and perhaps in this way it contributes to neurite outgrowth and synaptogenesis (Reinhard et al., 2005). However, APP and its gene family members do not share strong structural and functional similarities with other cell adhesion molecules, and it remains unclear how APP can influence to cell-cell or cell-matrix interactions. For instance, APP-induced neurite outgrowth depends on the intracellular domain but not on the ectodomain (Leyssen et al., 2005) evidence that intracellular interactions with the cytoskeleton and signaling contribute importantly to this adhesion function. Several proteins interact with the intracellular domain of APP including kinases and adaptor proteins (King and Scott Turner, 2004). Cu^{2+} homeostasis is one aspect of the ectodomain`s function, but how relevant this is for the organism remains to be elucidated (Barnham et al., 2003, Kong et al., 2007). The possible role of APP as a growth factor remains also to be clarified.

2.2.1. APP metabolism

APP has a long extracellular or luminal N-terminal domain and a short intracellular C-terminal domain (Figure 3) (Hardy, 1997). APP is ubiquitously expressed in different human tissues where it is alternatively spliced, leading to the production of APP isoforms of different sizes (695,751 and 770; 110-130 kD) (Neill et al., 1994). All of these encode multidomain proteins with a single membrane-spanning region. They differ from each other in that APP751 and APP770 contain exon 7, which encodes a serine protease inhibitor domain. The isoform of 695 amino acids is the predominant form in neuronal tissue, whereas APP751 is the predominant variant elsewhere. In neurons, APP is trafficked from the ER, where some Aβ may be generated, to the Golgi apparatus, where a significant amount of Aβ is produced in the trans-Golgi network and then to the plasma membrane, where additional Aβ appears to be generated (Gouras et al., 2005). Although APP localizes especially to the trans-Golgi network, both APP and Aβ appear to be present in vesicles within neuronal processes. An important site of Aβ generation is in the endocytic pathway after APP internalization from the plasma membrane. In AD, Aβ42 accumulates within multi vesicular compartments of vulnerable neurons, especially within distal neuronal processes and the pre- and post-synaptic compartments.

There are two proteolytic processing pathways for APP and its metabolic derivatives are presented in Figure 3. N-terminus of Aβ is generated from APP as a result of proteolytic processing through the β-secretase pathway (Esler and Wolfe, 2001). Beta secretase is an aspartyl protease known as BACE1 or Asp2 (Vassar et al., 1999). BACE1 is a transmembrane protein with a luminal active side. The transmembrane domain enables the enzyme to properly access the APP ectodomain at the β-cleavage site. BACE activity is highest in the acidic subcellular compartments of the secretory pathway, including Golgi apparatus and endosomes (Koo and Squazzo, 1994). During BACE overexpression, BACE also cleaves APP between Tyr10 and Glu11 of Aβ (Liu et al., 2002). The generation of the C-terminus of Aβ involves an unusual intramembranous cleavage by a multiprotein γ-secretase complex. Gamma-secretase also catalyzes the proteolysis of Notch and other type I transmembrane receptors. Gamma-secretase is a complex of at least four proteins such are presenilin (PSEN), nicastrin (NCSTN), anterior pharynx-defective-1 (APH-1) and presenilin enhancer-2 (PEN-2) (Vassar et al., 1999, Kimberly et al., 2003, Vetrivel et al., 2006). PSEN proteins are transmembrane proteins with

nine transmembrane domains which are encoded by 12 exons in chromosome 14q24 (Spasic et al., 2006). Gamma-secretase complex has a PSEN heterodimer in its catalytic site and a highly glycosylated NCSTN associated with the enzyme (Hu and Fortini, 2003). NCSTN and APH-1 associate in an early subcomplex, leading to stabilization of APH-1. The subcomplex regulates stabilization of PSEN holoprotein and its incorporation into a complex with NCSTN. Maturation of NCSTN in the Golgi/trans-Golgi network and entry of Pen-2 into the maturing complex promote PSEN endoproteolysis and formation of the active γ-secretase (Periz and Fortini, 2004). Endoproteolysis of the PSEN holoprotein cytoplasmic loop leads to formation of C- and N- terminal fragments (CTF and NTF). Only a small portion of PSEN is found as a holoprotein, suggesting that the heterodimers formed by CTF and NTF are the active forms of the protein. Gamma-secretase cleavage does not seem to be site restricted since it generates Aβ fragments of 37-43 amino acids in length. While the β-secretase pathway is favored in neurons, processing of APP by the α-secretase pathway is predominant in all other cell types. Alpha-secretase cleaves APP within the Aβ sequence (between amino acids 16 and 17) and this is followed by γ-secretase cleavage generating an N-terminally truncated non-amyloidogenic version of the Aβ peptide called p3.

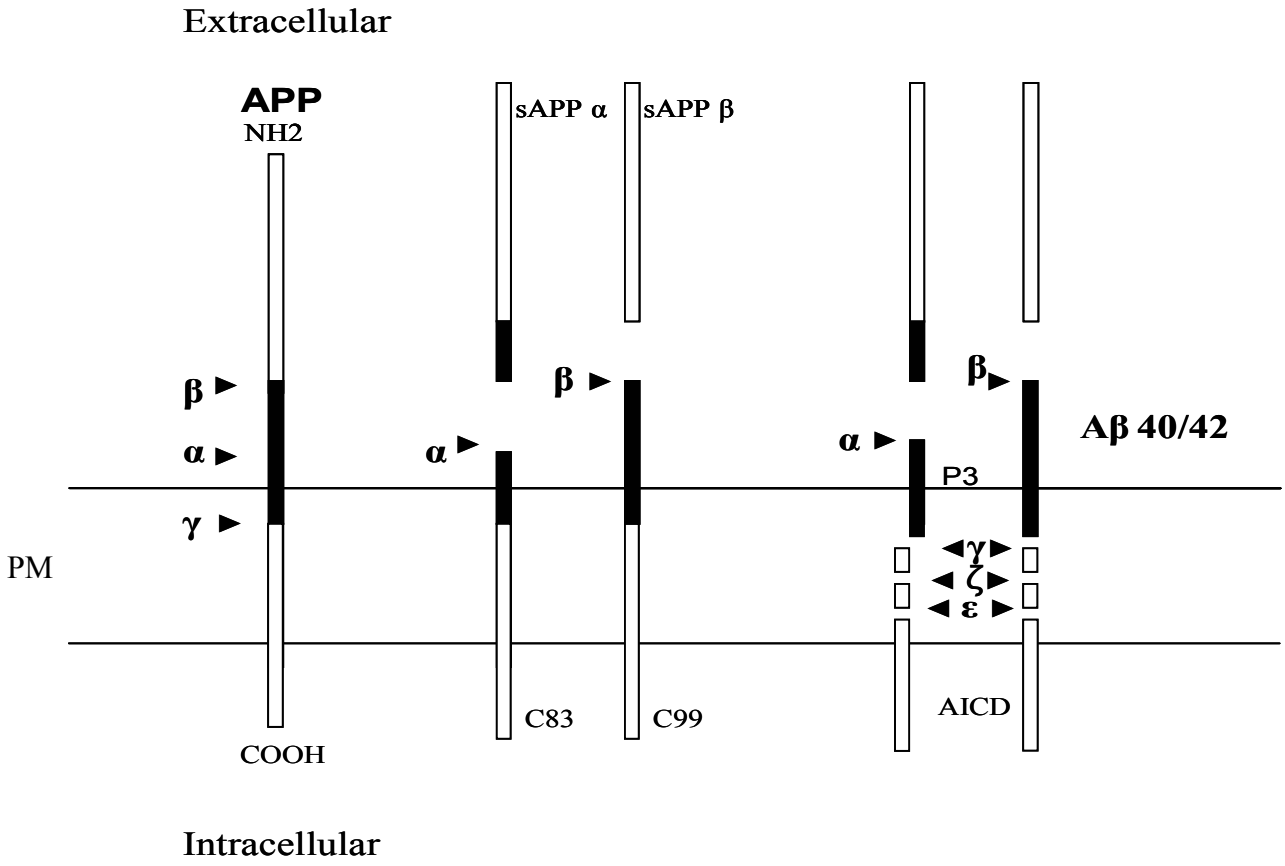


Figure 3. Metabolism of APP. Alpha-secretase cleaves APP within the Aβ sequence which leads to generation of a non-amyloidogenic version of the Aβ peptide called p3. Cleavage of APP by β-secretases produces a large soluble N-terminal fragment, soluble APPβ (sAPPβ), and C99 membrane-bound C-terminal fragments (in the middle), respectively, which can be further cleaved by γ-secretase, leading to the release and secretion of Aβ (on the right). AICD is produced after ε-cleavage. The novel ζ-cleavage site locates between γ- and ε-cleavage sites. PM = plasma membrane.

Recent studies have identified a PSEN-dependent C-terminal fragment of APP, AICD/CTFγ (APP Intra Cellular Domain/ C-terminal fragment γ), that may mediate APP signaling in a manner analogous to the NICD fragment of Notch. AICD is not a direct product of cleavage at the γ40/42 site(s) but is generated by cleavage in a site close to the cytoplasmic border (ε-cleavage site occurring between Aβ residues 49-50) of the APP transmembrane domain (Sastre et al., 2001). The exact role of AICD remains unclear. It has been shown that AICD enters the nucleus and acts as a transcription factor (Cao and Sudhof, 2001, Gao and Pimplikar, 2001, Baek et al., 2002, Pardossi-Piquard et al., 2005). Additionally, in vitro studies indicated that AICD can alter cell signaling and induce apoptosis (Passer et al., 2000,

Kinoshita et al., 2002, Leissring et al., 2002). However, Hèbert et al. (2006) claimed that AICD is a poor gene transcription stimulator (Hebert et al., 2006).

There has been speculation on whether AICD and Aβ are generated simultaneously or sequentially. Tesco et al. (2005) reported that APPV715F substitution, which has been previously shown to dramatically decrease Aβ40 and Aβ42 while increasing Aβ38 levels, did not affect *in vitro* generation of AICD (Tesco et al., 2005).They also revealed that the APPL720P substitution, which has been previously shown to prevent *in vitro* generation of AICD, could completely prevent Aβ generation. Additionally, a fragment of 50- and 49-residue resulting from cleavage of APP at Val50 (Aβ numbering) and also the longer Aβ species (1-45, 1-46, 1-48) has been identified from cells and brain tissue (Sastre et al., 2001, Zhao et al., 2004, Qi-Takahara et al., 2005). These observations support the possibility that γ-secretase generates Aβ only after the ε-cleavage. However, since some PSEN1 familial forms of AD (FAD)-linked mutations dramatically decrease the levels of AICD while still causing an increase in Aβ42 levels, it seems that ε-cleavage of APP may not always be a requirement for the generation of Aβ (Moehlmann et al., 2002).

Additional cleavage site between γ- and ε-cleavage sites has been reported (Zhao et al., 2004). The novel ζ-cleavage site at Aβ46 is also the APP717 mutation site. It has been shown that ζ-cleavage occurs after ε-cleavage and before γ-cleavage (Zhao et al., 2005). The data also suggest that γ-cleavage not only occurs last but also depends on ζ-cleavage, indicating that ζ-cleavage may be crucial for the formation of secreted Aβ.

2.2.2. Pathogenic role of Aβ

Although Aβ is highly heterogenous both at its amino and carboxyl ends, the minor isoform ending at Ala42 is believed to play a major pathogenic role in AD. This hypothesis is supported by several pieces of evidence: 1) Aβ42 is the major component of senile plaques (Masters et al., 1985), 2) Aβ42 has a lower critical concentration for amyloid formation in vitro as compared to the more abundant soluble form, Aβ40 (Jarrett and Lansbury, 1993), 3) many mutations in the APP and presenilin genes associated with autosomal dominant forms of AD increase the steady state ratio of Aβ42/Aβ40 in cultured cells and brains of transgenic mice carrying such mutants (Scheuner et al., 1996) and 4) Aβ42 is highly toxic for neurons in

culture either in fibrillar or non-fibrillar oligomeric forms (Lambert et al., 1998). However, Aβ is not likely to be the only cause of neuronal loss in AD; several other factors have been proposed as mediators of AD pathogenesis, including oxidative damage, impaired energy metabolisms, inflammation, mitochondrial dysfunction and APOE ε4 allele.

The amyloid cascade hypothesis of AD is that generation and deposition of Aβ leads to neuronal and synaptic degeneration and loss. Extracellular deposits of Aβ may reduce significantly the space and have harmful effects on these regions. Interestingly, naturally secreted Aβ oligomers have been shown to be potent and selective inhibitors of certain forms of hippocampal long-term potentiation (Townsend et al., 2006). It has also been shown that Aβ causes a disruption of synaptic vesicle endocytosis in cultured hippocampal neurons (Kelly and Ferreira, 2007). The chronic failure of certain synapses to function normally in the ongoing presence of natural Aβ oligomers is likely to contribute to the downstream neuropathology of AD. One study also showed that accelerating Aβ fibrillation could reduce oligomer levels and evoke a functional deficit in AD mouse model (Cheng et al., 2007). Additionally, Zhao et al. (2007) showed that signal transduction by neuronal insulin receptors is strikingly sensitive to disruption by soluble Aβ oligomers (Zhao et al., 2007). These studies suggest that specifically Aβ oligomers have detrimental effects on the cellular processes. Moreover, intraneuronal Aβ accumulation has been reported to be critical in synaptic dysfunction, neurodegeneration, cognitive dysfunction and the formation of plaques in AD (LaFerla et al., 1995, Takahashi et al., 2002, Billings et al., 2005, Heinitz et al., 2006, Oddo et al., 2006). One study investigated the effect of different Aβ42 aggregates by using the cholinergic cell line (Heinitz et al., 2006). Interestingly, many of the genes affected by oligomeric Aβ42 shown to present in the endoplasmic reticulum (ER), Golgi apparatus and/or otherwise involved in protein modification and degradation, indicating a possible role for ER-mediated stress in Aβ-mediated toxicity. One hypothesis states that the neurotoxic action of Aβ involves generation of reactive oxygen species and disruption of cellular calcium homeostasis (Mattson, 2004). When Aβ aggregation occurs at the cell membrane, membrane-associated oxidative stress results in lipid peroxidation and consequent generation of 4-hydroxynonenal (HNE), a neurotoxic aldehyde that covalently modifies proteins. Oxidative modification of tau by 4HNE and other reactive oxygen species can promote tau aggregation and may thereby induce the formation of neurofibrillary tangles. Aβ can also cause

mitochondrial oxidative stress which eventually leads to generation of the hydroxyl radical, a highly reactive oxyradical and potent inducer of membrane-associated oxidative stress that contributes to the dysfunction of the endoplasmic reticulum. By disturbing cellular ion homeostasis and energy metabolism, relatively low levels of membrane-associated oxidative stress can render neurons vulnerable to excitotoxicity and apoptosis.

2.3. Genetic risk factors for AD

AD is a heterogeneous disorder to which both genetic and environmental factors contribute. In addition to age, a positive family history of dementia is considered to be a definite risk factor for AD. Approximately 10% of AD patients show clear autosomal dominant transmission of disease and 30% of AD cases have a positive family history of AD, but insufficient evidence of autosomal dominant transmission. Mutations in APP (Goate et al., 1991), PSEN1 (Sherrington et al., 1995), and PSEN2 genes (Levy-Lahad et al., 1995, Rogaev et al., 1995) are known to cause the autosomal dominant form of EOAD (Corder et al., 1993a, Saunders et al., 1993b, Chartier-Harlin et al., 1994, van Duijn et al., 1994) while the APOE ε4 allele has been associated with an increased risk of developing both EOAD and LOAD (Roses, 1997). The APOE ε4 allele accounts for about 50% of the genetic background of the LOAD suggesting that additional susceptibility genes exist (Saunders et al., 1993a, Combarros et al., 2002, Bertram and Tanzi, 2004a, Raber et al., 2004)**.** Since the vast majority of AD occurs after the age of 65, putative risk genes affecting to pathogenesis of LOAD is of particular interest. Identifying new genes involved in the pathogenesis of AD could open new avenues for the development of rational treatment and prevention for AD. On the basis of full genome screens (used either linkage or association method; discussed in pages 44-46) there are a number of loci that have been linked with AD by at least two independent groups (Table 1) (Bertram and Tanzi, 2004b).

Table 1. Linkage and association regions observed in full-genome screens, and potential candidate genes locating at the regions (revised from Bertram and Tanzi, 2004b). The samples in Pericak-Vance et al., Myers at al., Li et al. and Blacker et al. partly overlap. Chr = chromosomal

Chr location	**Study method**	**Study**	**Candidate gene**	**Relevance to AD pathogenesis**
1p36	linkage linkage association	(Kehoe et al., 1999) (Myers et al., 2002) (Hiltunen et al., 2001b)	MTHFR	Homocysteine metabolism
3q28	linkage linkage association	(Hahs et al., 2006) (Lee et al., 2006) (Hiltunen et al., 2001b)	SST	Effects on Aβ degradation
4q35	linkage linkage	(Li et al., 2002) (Blacker et al., 2003)	SNCA	Tau fibrillization, component of Aβ plaques
5p13-15	linkage linkage linkage linkage association	(Kehoe et al., 1999) (Pericak-Vance et al., 2000) (Myers et al., 2002) (Blacker et al., 2003) (Hiltunen et al., 2001a)	-	
6p21	linkage linkage linkage association	(Kehoe et al., 1999) (Myers et al., 2002) (Blacker et al., 2003) (Hiltunen et al., 2001a)	TNFA HLA-A2	Inflammation and apoptosis Inflammation
6q15	linkage linkage linkage	(Pericak-Vance et al., 1997) (Kehoe et al., 1999) (Myers et al., 2002)	ESRA	Estrogen mediated action
9p21	linkage linkage linkage	(Kehoe et al., 1999) (Pericak-Vance et al., 2000) (Myers et al., 2002)	VLDL-R	Cholesterol metabolism
9q22	linkage linkage linkage	(Kehoe et al., 1999) (Myers et al., 2002) (Blacker et al., 2003)	ABCA1	Brain cholesterol metabolism
10q21-22	linkage linkage linkage	(Kehoe et al., 1999) (Myers et al., 2002) (Blacker et al., 2003)	HHEX	Hematopoietically expressed homeobox
10q24-25	linkage linkage	(Li et al., 2002) (Blacker et al., 2003)	IDE PLAU KNSL1	Degradation of Aβ Degradation of Aβ Thyroid receptor interacting protein
12p11	linkage linkage linkage	(Pericak-Vance et al., 1997) (Kehoe et al., 1999) (Myers et al., 2002)	A2M LRP TFCP2	Aβ clearance Internalization of APP and Aβ Transcription factor
19q13	linkage linkage linkage linkage linkage association	(Kehoe et al., 1999) (Pericak-Vance et al., 2000) (Myers et al., 2002) (Li et al., 2002) (Blacker et al., 2003) (Zubenko et al., 1998)	APOE	Aβ aggregation
Xp21	linkage linkage linkage	(Kehoe et al., 1999) (Myers et al., 2002) (Blacker et al., 2003)	MAOA	Metabolism of neuroactive and vasoactive amines
Xq21-26	linkage linkage association	(Kehoe et al., 1999) (Myers et al., 2002) (Zubenko et al., 1998)	-	

Screenings for additional susceptibility genes have revealed several potential candidates associated with the disease (Table 2, see http://www.alzgene.org/). However, the role of several potential risk genes in AD is controversial, as the numerous studies in different ethnic populations have frequently failed to replicate the initial association results. Ambiguous results may be due to the complex etiology of AD and the variable demographic history of population. The average age of AD patients and the size of sample cohort examined affects to the results, as well. Additionally, it is known that many of the genetic variants that alter the risk for complex diseases are expected to have only a small effect on disease outcome. The power to obtain typical thresholds of P value significance after applying multiple testing correction is limited for such markers, because significance is a function of sample size, allele frequency, and OR (Li et al., 2004). Hence, replication may be a more practical measure of overall significance, especially for markers with small effect sizes and/or when multiple test adjustment for large numbers of markers limits the available power given a fixed sample size.

Table 2. A sample of AD candidate genes studied worldwide. At the day of this writing (7 September 2007) the number of positive and negative results are based on reported case-control studies of Caucasian population in AlzGene database (see www.alzgene.org). Overall conclusion reached by authors of the original publication as follows; "positive" usually indicates significant ($P<0.05$) association in at least one of the performed analyses, and "negative" indicates no evidence for significant association. Population structure may have influenced these results. Na= not available

Gene symbol	Gene	Chromosomal location	Relevance to AD pathogenesis	Number of (-/+) results	Meta-analysis (Association to AD risk; rs number; OR (95% CI))
NCSTN	nicastrin	1q22-23	component of γ-secretase complex	3/4	+ rs17370539 OR 1.38 (1.03-1.83)
SOAT1	sterol O-acyltransferase	1q25	regulate the production of Aβ	2/3	-
CHRNB2	cholinergic receptor, nicotinic, beta 2	1q31.3	beta subunit of neuronal acetylcholine reseptor	2/1	+ rs4845378 OR 0.69 (0.51-0.94)
DHCR24	24-dehydrocholesterol reductase	1p33-p31.1	encodes seladin 1, a cholesterol synthesizing enzyme; protects neurons, participates in Aβ42 formation	-/1	na
TF	transferring	3q22	iron transporter, oxidative stress	5/1	+ rs1049296 OR 1.24 (1.06-1.45)
NEP	neprilysin	3q25	degradation of Aβ	1/2	-
APOD	apolipoprotein D	3q26	lipid metabolism	-/1	na
UBQLN1	ubiquilin 1	9q22	PSEN degradation, APP maturation and intracellular trafficking	10/2	-
TFAM	transcription factor A	10q21	mitochondrial dysfunction	-/4	+ rs2306604 OR 0.78 (0.67-0.91)
PLAU	plasminogen activator	10q22	degradation of Aβ	10/11	-
IDE	insulin degrading enzyme	10q23	degradation of Aβ	18/5	-
BDNF	brain derived neurotrophic factor	11p13	survival of neuronal cells	12/4	+ rs6265 OR 1.1(1.01-1.19)
APBB1	APP binding, family B, member 1	11p15	encodes FE65 protein, APP binding protein	3/2	-
SORL1	sortilin-related receptor	11q23.2-q24.4	APP sorting, Aβ production	-/5	+ rs1010159 OR 1.14 (1.02-1.29) rs1699102 OR 1.13(1.02-1.25) rs2070045 OR 1.26 (1.08-1.46) rs2282649 OR 1.16 (1.04-1.3) rs3824968 OR 1.3 (1.07-1.58) rs661057 OR 0.88 (0.80-0.97)
LRP	low density lipoprotein receptor	12q13	receptor for APOE and A2M	19/8	-
A2M	alpha 2 magroglobulin	12p13	clearance of Aβ	35/6	-

Table 2. continued

Gene symbol	Gene	Chromosomal location	Relevance to AD pathogenesis	Number of (-/+) results	Meta-analysis (Association to AD risk; rs number; OR (95% CI))
PSEN1	presenilin 1	14q24.3	component of γ-secretase complex	23/11	-
CYP46	cytochrome P450 family 46, cholesterol 24S-hydroxylase	14q32.1	elimination of cholesterol	9/8	-
CYP19	cytochrome P450 family 19	15q21.1	encodes aromatase protein, regulate estrogen synthesis	-/3	na
MAPT	microtubule-associated protein tau	17q21	forms PHF of NFTs	11/5	+ rs 2471738 OR 1.3 (1.01-1.67)
ACE	angiotensin converting enzyme	17q23	degradation of Aβ	16/13	+ rs1800764 OR 0.79 (0.68-0.92) rs4291 OR 0.82 (0.7-0.96) rs4343 OR 0.83 (0.72-0.96)
LDLR	low density lipoprotein receptor	19p13.3	binds APOE, transport cholesterol	5/4	-
APOE	apolipoprotein E	19q13.31	Aβ aggregation	8/25	+ rs405509 OR 0.73 (0.68-0.78) rs440446 OR 0.56 (0.48-0.64) rs449647 OR 0.74 (0.62-0.8)
PCK1	phosphoenolpyruvate carboxykinase 1	20q13.31	regulation of gluconeogenesis	2/4	+ rs 8192708 OR 1.29 (1.09-1.52)
CST3	cystatin C	20p11	protease inhibitor, Aβ fibril formation	6/5	+ 5`UTR-72 OR 1.28 (1.05-1.56) rs5030707 OR 1.28 (1.05-1.56)
APP	amyloid precursor protein	21q21.3	precursor of Aβ	1/2	na

2.3.1. APP and PSEN mutations

The APP, PSEN1 and PSEN2 genes include over 160 mutations (http://www.molgen.ua.ac.be/ADMutations/). Mutations in the APP gene, that are the reason for FAD, are located around α-, β- and γ-secretase cleavage sites. To date 25 different mutations in APP have been found in AD families with early onset and autosomal dominant inheritance from different parts of the world. Mutation V717I known as London mutation is the most frequent APP mutation found in 23 AD families, while other mutations have been found only in one to three families (Figure 4). The direct consequence of APP mutations is increased production of Aβ42, a variant of Aβ that is more prone to aggregate into fibrils. APP mutations in amino acid positions 714 to 717 at the γ-secretase site increase the ratio of Aβ42: Aβ40 (De Jonghe et al., 2001), and the Swedish double mutation K670N/M671L at the β-secretase cleavage site increases both Aβ40 and Aβ42 deposits (Citron et al., 1992). Mutations may also affect the amyloid fibrils formation. Aβ with the Arctic mutation (E693G) forms protofibrils at a much higher rate and in larger quantities than wild-type Aβ (Nilsberth et al., 2001). This may lead to rapid protofibril formation of Aβ leading to accelerated buildup of insoluble Aβ both intra- and extracellularly. Additionally, Arctic mutation has been shown to favor intracellular Aβ production by making APP less available to α-secretase (Lord et al., 2006, Sahlin et al., 2007).

Some APP mutations have also been shown to decrease Aβ40 and Aβ42 levels. Recent data from APPV715F substitution, which has been previously shown to dramatically decrease levels of Aβ40 and Aβ42 while increasing the amount of Aβ38, does not affect in vitro generation of AICD (Tesco et al., 2005). Additionally, APPL720P substitution, which has been previously shown to prevent in vitro generation of AICD, completely prevents Aβ generation. Both substitutions significantly increase the distance between the N- and C-terminus of PSEN1, which has been proposed to contain the catalytic site of γ-secretase. Data suggest that γ-segretase activity can be modulated by conformational changes. Additionally, it has also been shown that Aβ42-lowering non-steroidal anti-inflammatory drugs increase the distance between the PSEN1-N and C-termini in favoring the production of Aβ1-38 (Lleo et al., 2004)

Gene dosage alterations can also be involved in the etiology of neurodegenerative

disorders caused by protein accumulation. It has been shown that APP locus duplication causes autosomal dominant EOAD with cerebral amyloid angiopathy (CAA) and that the duplication region varies in size and localization between the reported families (Rovelet-Lecrux et al., 2006). Additionally, Cabrejo et al. (2006) showed that the EOAD/CAA phenotype was not dependent on the size of the duplication and that there was no clinical feature of Down`s syndrome (Cabrejo et al., 2006). A Finnish study confirmed that an increase in APP gene dosage is linked to a phenotype consisting of early onset dementia and CAA and that the phenotype does not depend on the size of the duplicated region, but may vary between families and between the genders (Rovelet-Lecrux et al., 2007). These findings support the hypothesis that the APP gene is located in a region of increased recombination.

To date 155 mutations in PSEN1 gene (encodes PSEN1 protein) have been found in AD families with early onset and autosomal dominant inheritance from different parts of the world. The mutations in PSEN1 are located primarily within the transmembrane domains and in the N-terminal portion of the cytoplasmic loop, close to the endoproteolytic cleavage site. PSEN1 FAD mutations have also been shown to affect the intracellular levels of longer forms of Aβ in a distinct manner. For example, PSEN1M146L mutation led to an increase in the levels of Aβ1-48, while PSEN1G384A evoked a decrease in the levels of Aβ1-48 (Qi-Takahara et al., 2005). Ten pathogenic mutations have been found in the PSEN-2 gene with somewhat later onset ages and greater variability in penetrance than with PSEN1 mutations.

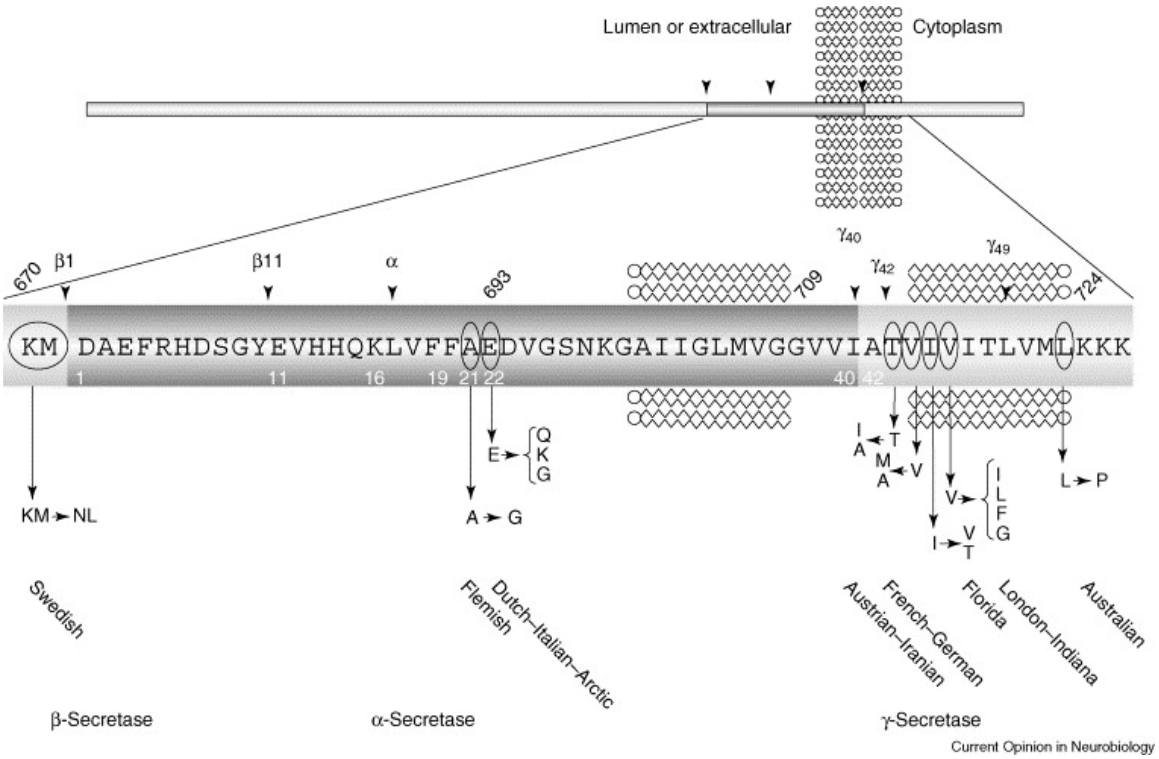


Figure 4. The mutations causing FAD in APP surround the cleavage sites of the three secretases. The amino acid sequence of the region encompassing Aβ is given; amino acids mutated in familial AD are circled, and the corresponding amino acid changes (KM->NL: KM mutated in NL) are indicated. The country from where the families originate is also mentioned in the figure. Amino acid numbering of APP is written in black and of Aβ in white. Arrowheads indicate the cleavage sites of α-, β- and γ-secretase. Notice, the novel ζ-cleavage site locate in the same site as the London mutation (V717I) (Wilquet and De Strooper, 2004).

2.3.2. APOE ε4 allele and AD

The APOE ε4 allele has been associated with an increased risk of developing both EOAD and LOAD (Roses, 1997). APOE ε4 allele was the first identified and still the only clear genetic risk factor for LOAD. However, the APOE ε4 allele is estimated to account for about 50% of the genetic background of the LOAD suggesting that additional susceptibility genes must exist (Saunders et al., 1993a, Combarros et al., 2002, Bertram and Tanzi, 2004a, Raber et al., 2004). There are three alleles in APOE that encode three different isoforms of apolipoprotein E (ε2, ε3 and ε4). In most Caucasian populations, ε3 is the most common allele (78%) compared to

ε2 (8%) and ε4 (14%) occurrence, although the allele distribution varies in different ethnic populations (Utermann et al., 1980). The allele code for APOE proteins differs at amino acid position 112 and 158 so that ε2 has a cysteine in both positions, ε3 has cysteine and arginine and ε4 has two arginines (Zannis et al., 1993). Inheritance of one APOE ε4 allele increases the likelihood of developing late-onset AD by 2 to 5-fold, and the inheritance of two alleles elevates the risk by 4 to 10-fold or more (Mahley and Rall, 2000). Although APOE ε4 allele is an established risk factor for AD, the exact role of APOE in the pathogenesis of AD still remains to be elucidated. One hypothesis is that APOE may act as a chaperone for Aβ by binding the peptide and altering its conformation, thereby influencing its clearance and ability to aggregate.

2.4. Genes encoding for proteins influencing to Aβ clearance

Accumulation of Aβ in the brain is considered the initial pathological event leading to AD (Hardy and Selkoe, 2002). An imbalance between Aβ production and clearance may trigger AD. An overview of genes encoding for proteins influencing the Aβ life cycle is presented in Table 3 (Tanzi and Bertram, 2005). Recently, insulin degrading enzyme (IDE), neprilysin (NEP), endothelin-converting enzyme 1 (ECE1), ECE2 and plasmin have been postulated as candidate enzymes, responsible for the degradation and clearance of Aβ (Tanzi and Bertram, 2005). Particularly the IDE and NEP are interesting. The finding that IDE can degrade Aβ prompted a search for possible genetic linkage and/or allelic association of late-onset AD to the IDE region on chromosome 10, with the first positive evidence being reported by Bertram and colleagues (Bertram et al., 2000). Subseguently several laboratories have reported evidence of genetic linkage and/or allelic association of the IDE and NEP to AD and some not (see www.alzgene.org and discussion). Several studies have also indicated that there are reduced IDE and NEP levels in AD patients (Perez et al., 2000, Akiyama et al., 2001, Yasojima et al., 2001a, Yasojima et al., 2001b, Cook et al., 2003, Wang et al., 2003, Caccamo et al., 2005). It has also been shown that overexpression of IDE and NEP in a transgenic AD mouse model could prevent plaque formation, secondary pathology and premature death, and that there may be possible cross-regulation between these two proteases (Leissring et al., 2003). Taken together, the available genetic evidence and the functional data suggest that IDE

and NEP could have a role of regulating Aβ levels as presented in Figure 5.

Table 3. Overview of genes encoding for proteins influencing the Aβ life cycle.

Gene (Location [Mb])[a]	**Functional Relevance to AD**
A2M (12p13 [9])	Aβ clearance
ADAM9 (8p11 [39])	α-secretase
ADAM10 (15q21 [57])	α-secretase
ADAM17 (2p25 [10])	α-secretase
APH1A (1q21 [147])	γ-secretase
BACE1 (11q23 [117])	β-secretase
BACE2 (21q22 [42])	β-, α-secretase
ECE2 (3q27 [185])	Aβ degradation
HTRA1	Aβ degradation
IDE (10q23 [94])	Aβ/AICD degradation
LRP1 (12q13 [56])	Aβ clearance
NCSTN (1q23 [157])	γ-secretase
NEP (3q25 [156])	Aβ degradation
PEN2 (19q13 [41])	γ-secretase
PLAT (8p11 [42])	Aβ degradation (via plasmin)
PLAU (10q22 [75])	Aβ degradation (via plasmin)
PLG (6q26 [161])	Aβ degradation (via plasmin)

[a] Location according to "UCSC Human Genome Browser," May 2004 assembly (URL: http://genome.ucsc.edu/cgi-bin/hgGateway). For a more formal summary of studies for any specific gene, including meta-analysis, visit the "AlzGene" database (URL: http://www.alzgene.org)(revised from (Tanzi and Bertram, 2005). Proteases with proposed α-secretase function belong to the ADAM (a disintegrin and metalloproteinase domain) family of proteins; these include ADAM 9, ADAM 10 and ADAM 17. Gamma-secretase is a complex of PSEN, NCSTN, APH-1 and PEN-2 proteins (Vassar et al., 1999, Kimberly et al., 2003, Vetrivel et al., 2006). A2M; alpha-2-macroglobulin, ADAM; disintegrin and metalloprotease, APH1A; anterior pharynx defective 1 homolog A, BACE; beta-site APP-cleaving enzyme, ECE; endothelin-converting enzyme, HTRA1; HtrA serine peptidase 1, IDE; insulin degrading enzyme, LRP1; low density lipoprotein-related protein 1, NCSTN; nicastrin, NEP; neprilysin, PEN2; presenilin enhancer 2 homolog, PLAT; plasminogen activator, PLAU; plasminogen activator urokinase, PLG; plasminogen.

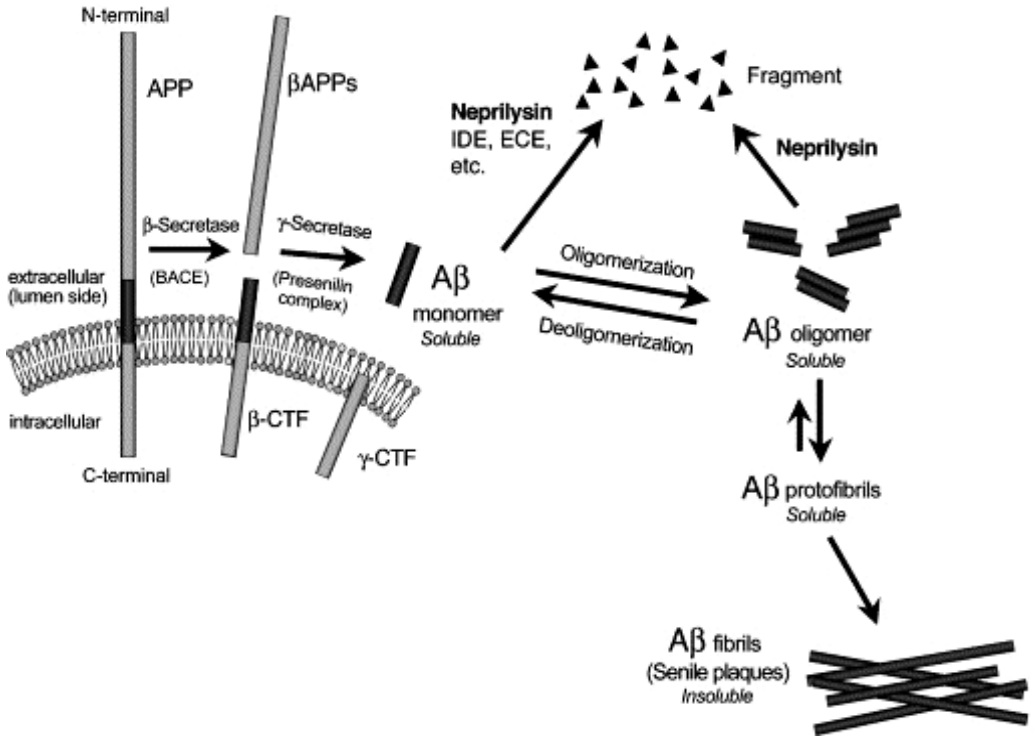


Figure 5. Schematic pathway of Aβ metabolism and formation of Aβ fibrils in the brain (Iwata et al., 2005).

The clearance of soluble Aβ from the brain is a complex process consisting of at least three major mechanisms. First, receptor-mediated flux of Aβ across the blood brain barrier (reviewed in (Zlokovic, 2004)), second, intracellular and extracellular proteolytic degradation (Iwata et al., 2001, Morelli et al., 2002) and third, the interstitial fluid bulk flow into the CSF (Silverberg et al., 2003). Newly generated Aβ is rapidly cleared from the brain, suggesting that Aβ-degrading proteases could play an important role in regulating cerebral levels of the peptide (Savage et al., 1998, Iwata et al., 2000). IDE and NEP proteases may contribute to overall Aβ degradation in the brain by complementing each other. It has been postulated that deposition of Aβ may begin intracellularly, and because IDE appears to be more effective at degrading intracellular Aβ, changes in the relative expression or activity status of IDE may have severe consequences on the pathogenesis underlying the AD (Sudoh et al., 2002). NEP has been shown to reduce not only extracellular but also cell-associated Aβ-levels, suggesting that NEP may degrade Aβ through a secretory pathway and possibly act on the cell surface (Hama et al., 2004). Moreover, human NEP is capable of degrading Aβ not only in the

monomeric form but also the pathological oligomeric form (Kanemitsu et al., 2003). It seems likely that both enzymes affect Aβ-clearance mechanisms. Upregulation of IDE and NEP proteases could represent a potential novel therapeutic approach to reduce the steady-state Aβ levels and prevent the pathogenesis of AD.

2.4.1. Insulin degrading enzyme (IDE)

The human IDE gene is located in chromosome 10 (10q23-q25) and it spans more than 120 kb. IDE gene encodes IDE protein (E.C. 3.4.24.56), also known as insulysin and insulinase which is a 110-115 kDa zinc metallopeptidase capable of degrading a number of peptides including insulin, glucagon, atrial natriuretic factor, transforming growth factor α (TGFα), amylin, Aβ and AICD (Kurochkin and Goto, 1994, Duckworth et al., 1998, Edbauer et al., 2002). IDE has been highly conserved during evolution and its substrate specificity is very selective (reviewed in (Kurochkin, 2001)). IDE is expressed in various tissues including many regions in brain (Farris et al., 2005). Although IDE is primary located in the cytosol (Duckworth et al., 1998), it is also found in endosomes (Hamel et al., 1991), in peroxisomes (Authier et al., 1995), in mitochondria (Leissring et al., 2004), in brain microvessels (Morelli et al., 2004), while a small fraction of the enzyme can be found on the plasma membrane (Seta and Roth, 1997, Vekrellis et al., 2000) as well as being secreted (Qiu et al., 1998).

It is proposed that 3'UTRs play an important role in regulation of mRNA stability, control of mRNA subcellular localization, and efficiency of mRNA translation (Conne et al., 2000). Six distinct human IDE transcripts were identified and much of the variability of mRNA size is due to alternative polyadenylation sites: four with a wide tissue distribution (~3.5, ~3.8, ~5.5, ~9 kb) and two exclusive to testis (~2.0, ~4.0 kb) (Farris et al., 2005). All four of the multitissue IDE variants mRNA contains the catalytic site sequence which lies in exon 3. Additionally, one IDE protein isoform from an alternative splicing has been described. This novel isoform is widely expressed at low levels in multiple tissues, including in several brain regions and interestingly, it has a decreased ability to degrade insulin and Aβ. Moreover, Leissring et al. 2004 reported the novel IDE isoform generated by translation at an in-frame initiation codon 123 nucleotides upstream of the canonical translation start site (=the first initiation codon), which contain additional 41 amino acids in N-terminus and is targeted to

mitochondria (Leissring et al., 2004). When the second initiation codon is used, IDE localizes predominantly to the cytosol. It is suggested that IDE degrades Aβ via endoplasmic reticulum-associated degradation, in which the endoplasmic reticulum-localized Aβ is translocated into the cytosol where it is degraded by IDE (Schmitz et al., 2004). Interestingly, it is believed that the accumulation of Aβ leads to oxidative stress and the formation of reactive oxygen species. Shinall et al. (2005) suggested that these reactive oxygen species would lead to inactivation of IDE and NEP (Shinall et al., 2005). Decrease of IDE and NEP would in turn lead to an increase in Aβ due to a reduction in its degradation.

Epidemiological studies suggest that insulin resistance as a consequence of type 2 diabetes mellitus (DM2; a non-insulin dependent form of diabetes mellitus, characterized by glucose intolerance, obesity and hyperinsulinemia) is associated with a 2- to 3-fold increased relative risk for AD (Ott et al., 1996, Leibson et al., 1997). The IDE region of chromosome 10 has been genetically linked to both AD and DM2 (Bertram et al., 2000, Wiltshire et al., 2001). It has been proposed that there might be competition between insulin and Aβ for degradation of IDE. Watson et al. (2003) has shown that boosting insulin levels in human subjects increases Aβ42 levels in cerebrospinal fluid, suggesting that insulin may modulate Aβ42 levels acutely in humans (Watson et al., 2003). Interestingly, it has also been shown that insulin-resistance caused by a high-fat diet is associated with reduced IDE levels and increased amyloidosis in an AD animal model (Ho et al., 2004). Additionally, missense mutations in IDE that cause partial loss of function, result in decreased catalytic activity of IDE and a deficit in the degradation of both insulin and Aβ, possibly to increase the risk of DM2 and AD, respectively (Fakhrai-Rad et al., 2000, Farris et al., 2004). These findings provide compelling support for the hypothesis that there is co-morbidity between AD and DM2 and in some cases this may be attributable to impairment in IDE function.

2.4.2. Neprilysin (NEP)

The human NEP gene (NEP, MME) is located in chromosome 3 (3q25.1–q25.2) and it spans more than 104 kb. NEP (EC 3.4.24.11), also known as neutral endopeptidase, enkephalinase, CD10 and common acute lymphoplastic leukemia antigen (CALLA), is a 97 kDa type II membrane metalloendopeptidase with an active site containing a zinc-binding motif at the

extracellular carboxyl terminal domain. NEP exists on the plasma membrane as a noncovalently associated homodimer.

NEP, functioning at the cell surface, acts mainly on peptides smaller than 5 kDa (5–40 amino acid residues in length) based on the size of known substrate peptides. With regards polypeptide length, $A\beta_{1-42}$ is the longest known NEP substrate. NEP may play a role in degrading a variety of neuropeptides, such as enkephalin, somatostatin, atrial natriuretic peptide, substance P, neurokinins, cholecystokinin, nociceptin, and corticotrophin-releasing factor (reviewed in (Iwata et al., 2005). However, at present, Aβ is the only physiological substrate peptide for which degradation in the brain has been proven to be regulated by NEP. The active site of NEP faces the extracellular side, where Aβ should be released, indicating that it would have access to physiological Aβ in vivo. The overexpression of NEP led to significant decreases in secreted and membrane-associated Aβ in primary cortical neurons (Hama et al., 2001). NEP is also capable of degrading oligomeric forms of Aβ (Kanemitsu et al., 2003) although IDE cannot degrade the oligomers (Walsh et al., 2002, Morelli et al., 2003).

The expression of NEP is transcriptionally regulated in a tissue-specific manner (Figure 6) (Iwata et al., 2005). The 5`-untranslated region including exons 1, 2 and 3 is alternatively spliced, resulting in 4 types of mRNA transcripts. From these transcripts, NEP protein with the same amino acid sequence is translated. Neurons predominantly express the type 1 transcript containing exon 1, and oligodendrocytes express the type 3 transcript starting at exon 3, while type 2a and 2b are major forms in peripheral tissues (Li et al., 1995). The sequences in alternatively spliced 5`-untranslated regions might affect the mRNA translation rate or stability and the protein expression levels. It is possible that at least some of the mutations or polymorphisms in 5`-untranslated regions could influence the expression of NEP in a neuron-specific manner and consequently alter the Aβ levels in the brain. Interestingly, NEP mRNA levels are known to be much higher, while APP695 and PSEN1 mRNA levels are known to be much lower in the periphery than in the brain (Yasojima et al., 2001b). It seems that in plaque prone regions of the AD brain, the capacity to degrade Aβ is weak, while the capacity to generate Aβ is high.

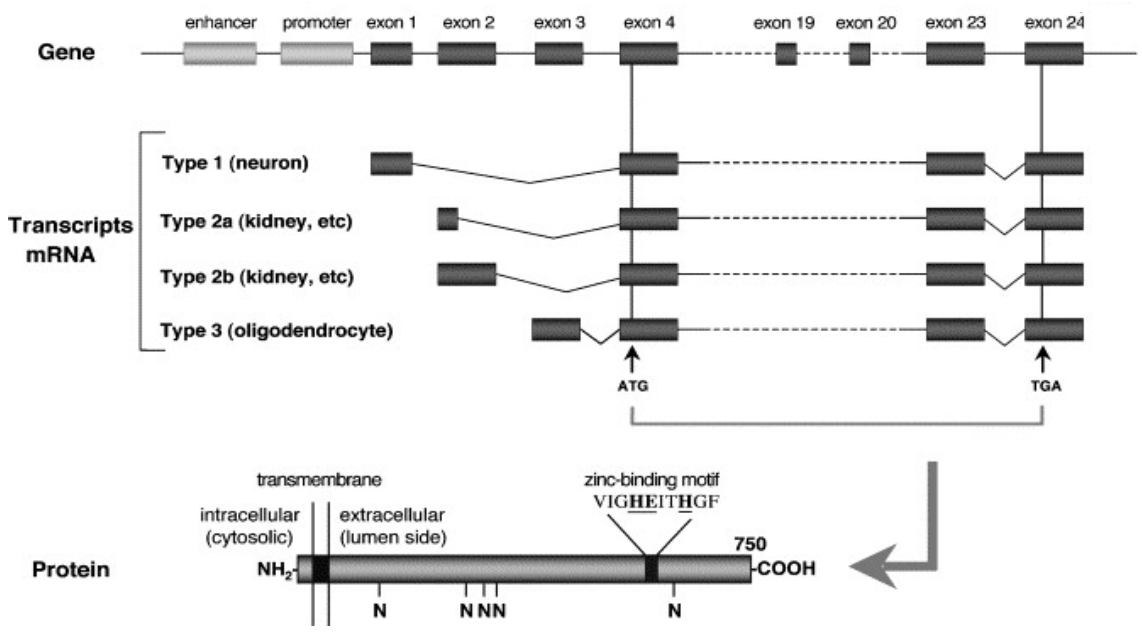


Figure 6. Diagrams of NEP gene, transcripts and protein. Four types of mRNA transcripts with different structures at the 5′-noncoding region are generated from the NEP gene in a tissue-specific manner by alternative splicing. From these transcripts, NEP protein of the same amino acid sequence is translated (Iwata et al., 2005).

In neurons, the synaptic and axonal localization of NEP suggests that after synthesis in the soma, NEP is axonally transported to the presynaptic terminals, where Aβ degradation likely takes place (Figure 7.) (Fukami et al., 2002). APP also undergoes axonal transport to presynaptic terminals, while Aβ is generated through sequential cleavages by β- and γ-secretases and is released to extracellular space. Aβ released from presynaptic sites contributes to extracellular amyloid deposits, as demonstrated by in vivo experiments (Lazarov et al., 2002, Sheng et al., 2002). Degradation of Aβ by NEP is likely to take place at or near synapses and may also proceed inside the secretory vesicles during axonal transport if both APP and NEP are co-localized in the same vesicles. Thus, the sites for release and degradation of Aβ seem to be closely related to each other. In fact, the synaptic localization of NEP is of particular importance in supporting a link of this enzyme with AD pathology. Moreover, the expression of NEP is observed in reactive astrocytes surrounding amyloid plaques of aged APP Tg mice but not in microglial cells (Apelt et al., 2003, Iwata et al., 2005).

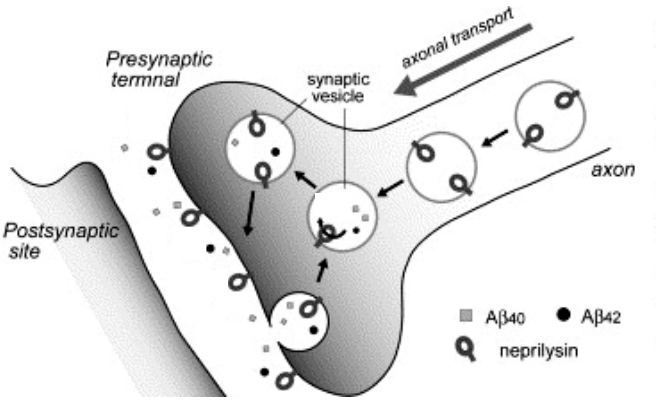


Figure 7. Aβ is produced from APP, which undergoes axonal transport, by proteolytic cleavage, and is released into the extracellular spaces and, in part, to the intravesicular side. NEP, a transmembrane-integrated protein, is taken into secretory vesicles after synthesis in the soma and axonally transported to the presynaptic terminals, where it degrades Aβ (Iwata et al., 2005).

It has been shown that presenilin-deficient cells fail to degrade Aβ and exhibit a drastic reduction in the transcription and activity of NEP (Pardossi-Piquard et al., 2005). NEP activity is restored by transient expression of PSEN1, PSEN2 or AICD. In addition, NEP gene promoters are transactivated by AICD but not by Aβ. This presenilin-dependent regulation of NEP, possibly mediated by AICD, provides a physiological means to modulate Aβ levels. In contrast, Hébert et al. (2006) showed that the significant decrease or even the absence of endogenous AICD production was not reflected in systematic changes in the levels of expression of NEP (Hebert et al., 2006). Similarly, Chen et al. (2007) were unable to provide evidence that NEP levels and activity would be regulated by presenilin-mediated processing of APP (Chen and Selkoe, 2007). Interestingly, Pardossi-Piquard et al. (2006) also observed that nicastrin, a member of the γ-secretase complex, deficiency could drastically lower neprilysin expression, membrane-bound activity and mRNA levels (Pardossi-Piquard et al., 2006). Nicastrin restores NEP activity and expression in nicastrin-deficient, but not presenilin-deficient fibroblasts, indicating that the control of NEP necessitates the complete γ-secretase complex harboring its four reported components. Since it is crucial to understand the proceses involved in NEP regulation, as inhibition of Aβ generation by γ-secretase inhibitors would affect such a putative signaling pathway, more studies are needed to clarify this point.

The ageing of the human brain is a cause of cognitive decline in the elderly and the major risk factor for AD. Interestingly, it has been shown that NEP mRNA and protein levels are decreased with aging (Iwata et al., 2002, Apelt et al., 2003). It is also known that in human brains, the expression of neuropeptide, somatostatin (SST) significantly declines after the age of 40 (Lu et al., 2004). Based on the down-regulation of SST and NEP occurring with aging and the finding that SST influences brain Aβ42 level by modulating the activity of NEP (Saito et al., 2005), one hypothesis is that the down-regulation of SST expression in the human brain at the early stage of aging initiates a gradual decline in NEP activity, resulting in a corresponding elevation in the steady-state levels of Aβ. When this goes on for it leads to Aβ accumulation and this then triggers the AD pathological cascade (Figure 8). Moreover, Wang et al (2003) showed that NEP in both controls and AD human brains can be modified by HNE but the ratio of oxidized form to total NEP protein is greater in the brains of AD patients than in age-matched control brains (Wang et al., 2003). If NEP undergoes inactivation by such oxidative modification, in addition to down-regulation, age-related cerebral amyloidosis would be further exacerbated in AD.

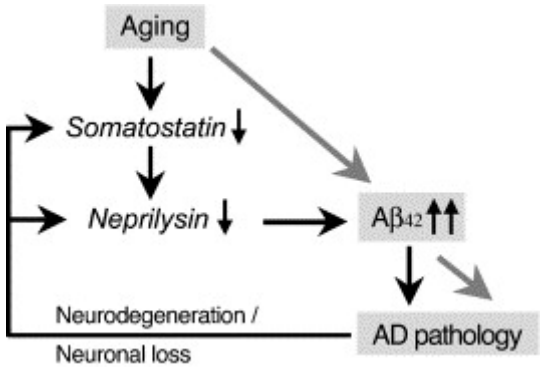


Figure 8. The age-dependent reduction of the neuropeptide, SST, specifically suppresses neuronal NEP activity triggering the accumulation of Aβ (Iwata et al., 2005).

2.5. SST, BDNF and TrkB as a possible risk genes for AD

2.5.1. Somatostatin (SST)

SST is one of the most selective neuropeptides in the mammalian central nervous system (CNS) that consistently decreases in the brain and cerebrospinal fluid of AD patients suggesting that genetic determinant(s) may underlie the altered SST expression or stability (Reichlin, 1983, Soininen et al., 1984, Epelbaum, 1986, Reinikainen et al., 1987, Bissette and Myers, 1992). SST is important hypothalamic hormone with one of its many functions being to inhibit the secretion of growth hormone, thyrotrophin, glucagon, insulin and gastric acid. SST deficiency displays a close association with senile plaques and neurofibrillary tangles in AD brain (Davis et al., 1999). Additionally, in AD brain tissue, loss of SST-like immunoreactivity in the cortex is directly linked to poor cognitive function and memory impairment (Grouselle et al., 1998). Saito et al. also demonstrated that SST may be associated with AD via modulation of NEP as discussed in the previous chapter (Saito et al., 2005).

SST is located on chromosome 3q27.3 (spans 2308 bp), close to a linkage region with late-onset AD (Hiltunen et al., 2001a). Two other family-based studies of individuals from different ethnic backgrounds have also identified the same locus as possibly harboring a genetic variant that increases late-onset AD susceptibility (Hahs et al., 2006, Lee et al., 2006). Two endogenous bioactive forms of SST, (SST-14 and SST-28, reflecting their amino acid chain length) can be synthesized. Both forms of SST are generated by proteolytic cleavage of prosomatostatin, which itself is derived from preprosomatostatin (Patel and O'Neil, 1988, Kumar, 2005). The biological actions of SST are mediated by a family of G-protein coupled receptors and five known subtypes of these receptors have been identified.

2.5.2. BDNF and TrkB

Neurotrophins are neuronal growth factors, which regulate a variety of functions in the nervous system. Neurotrophins can promote both either survival or the death of neurons, induce differentiation of specific neuronal populations and modulate neuronal activity and synaptic events related to plasticity (Huang and Reichardt, 2001). Brain-derived neurotrophic

factor (BDNF) is a pro-survival factor induced by cortical neurons that is necessary for survival of neurons in the brain. The BDNF gene is located in chromosome 11p13-p14 (~68 kb) and encodes BDNF protein (13.5 kDa). Tyrosine kinase receptor B (TrkB, 145 kDa transmembrane glycoprotein) has been shown to function as a receptor for BDNF (Klein et al., 1991). The TrkB gene is located in chromosome 9q22.1 (376 kb). Since BDNF, via activation of its TrkB receptor, regulates a wide variety of cellular processes in the nervous system, genetic alterations in these genes may confer an increased risk for neurodegenerative changes, such as those seen in AD. Additionally, previous studies have shown that one polymorphism in 5` pro-region of the human BDNF gene is associated with AD (Kunugi et al., 2001, Riemenschneider et al., 2002) and another affects activity-dependent secretion of BDNF and human memory and hippocampal function (Egan et al., 2003). Data from transgenic mice models suggest that lack or improper BDNF signaling is accompanied by seriously impaired learning and memory function, which is also one of the earliest clinical symptoms in patients with AD (Linnarsson et al., 1997, Saarelainen et al., 2000) and that, conversely, enhanced TrkB signaling improves learning and memory (Koponen, 2004). Reduced gene expression of BDNF has also been observed in the post-mortem hippocampus and temporal cortex of AD patients (Phillips et al., 1991, Connor et al., 1997). Additionally, a selective decline has been detected in protein expression of the BDNF/TrkB signaling pathway components in the frontal cortex and hippocampus in AD (Ferrer et al., 1999). According to these data, BDNF and its receptor TrkB genes may affect the AD pathogenesis.

2.6. Mapping of novel AD genes

In the past two decades, many genes that were implicated in single gene (Mendelian) diseases have been identified by using genetic linkage and positional cloning methods. Less success has been enjoyed in trying to use family-based linkage analysis to study complex diseases without any clear Mendelian inheritance patterns. Complex diseases are more likely to be caused by many genes, each with a small overall contribution and relative risk leading to the situation where the pathological pathway may involve a wide spectrum of biological mechanisms. An individual phenotype results from the sum of the total effects of all the numerous contributing loci (Risch, 2000). Additionally, complex diseases typically vary in

severity of symptoms and age of onset, which results in difficulty in defining an appropriate phenotype and selecting the best population to study (Mayeux, 2005). Thus, in family-based linkage analysis, in addition to the heterogeneity difficulties are related to the limited number of affected members in different generations available for genetic and phenotype analyses and to the possibility that carriers of the disease causing mutation or polymorphisms die before the appearance of the symptoms of late-onset disease. It has been suggested that in complex and heterogenic disorders, the loci associated with the disease under study may be identified more readily by studies of association and linkage disequilibrium (LD) (Jorde, 1995, Risch, 2000, Emahazion et al., 2001).

2.6.1. Association studies

Linkage refers to co-segregation of a genetic marker with a given phenotype in a set of families, whereas association denotes the correlated occurrence of two investigated factors, typically a DNA sequence and a phenotype, in a group of cases as compared to those of controls (Emahazion et al., 2001). Because closely linked sequences are less likely to be separated by the DNA reshuffling that takes place during meiotic recombination than distantly spaced sequences, markers that tend to co-segregate with the disease provide pointers to the approximate chromosomal location of the underlying disease gene. In association analysis, rather than focusing merely on the position of a tested marker, instead one determines whether a particular allele of a marker, a genotype (set of alleles for one marker in an individual) or a haplotype (a particular series of marker alleles along a stretch of DNA), are associated with diseased individuals when compared to unaffected controls. Allelic enrichment implies that the associated marker is causally involved in disease predisposition, or that nearby (within a few kb) pathogenic sequences exist that are themselves nonrandomly mixed (in linkage disequilibrium (LD)) with alleles of the associated marker.

One clear advantage of association studies compared to linkage-based approaches is the large number of cases and controls that are more easily collected compared to trying to locate families with multiple affected members (Kwon and Goate, 2000). In particular, candidate-gene approaches are useful for studying complex genetic traits. Rather than relying on markers that are evenly spaced throughout the genome without regard to their function or

context in a specific gene, candidate-gene studies focus on genes that are selected because of a priori hypothesis about their role in disease pathogenesis. Another approach is to choose the candidate gene based on its localization in genomic region that has been linked to the disease in a genome wide scan. The most promising positional candidate genes for AD are those genes that are also biological susceptibility genes for AD.

For association analysis the current trend is to use polymorphic DNA markers such as single nucleotide polymorphisms (SNP). SNPs are sites in the human genome where individuals differ in their DNA sequence, by a single base. SNPs are the most frequently detected type of genetic polymorphisms, with an estimated 11 million SNPs present in the human genome. Markers tested for association must either be the causal allele or be highly correlated (in LD) with the causal allele (Jorde, 2000). SNPs with very low allele frequencies would need to have very large relative risk associated with them to be detected in a candidate gene study, and alleles with very high relative risk would have been detected using linkage analysis. Therefore, SNPs with frequencies of at least 5% are generally more likely to be useful in a candidate gene study (Risch, 2000). Recently, there has been increased attention on the effects of polymorphisms in transcriptional promoters and regions of DNA that regulate the expression of genes (Drysdale et al., 2000). It is reasonable to place a high priority on such a sequence variants when SNPs are being selected.

2.6.2. Linkage disequilibrium (LD) and haplotype blocks

Another important consideration in selecting SNPs for an association study is whether there is significant LD in the candidate gene among the study population. LD is the non-random association of alleles at adjacent loci and is found when alleles of different loci are inherited together more often than would be expected based on their frequencies (Ardlie et al., 2002). The ability to use LD to map genes that increase the risk of diseases depends critically on the distance at which LD extends around the disease and the marker loci. In a Caucasian population LD may extend over an unexpectedly large region (Dunning et al., 2000). For example, it was recently shown that it is possible to detect allelic association with SNPs lying 40 kb from the APOE polymorphism (Martin et al., 2000). Most of the genome falls into segments of strong LD and it has been suggested that the human genome can be arranged into

a block-like pattern of LD, referred to as haplotype blocks (Daly et al., 2001). Studies have also shown that most of the common SNPs in the genome have groups of neighbours that are all nearly perfectly correlated to each other. Once the pattern of LD is known for a given region, a few SNPs (tagging SNPs) can be chosen such that, individually or in multimarker combinations (haplotypes), they will be able to capture most of the common variation within that region. However, due to enormous variation in the extent of LD from one region of the genome to another, relying on the average extent of LD when designing an association mapping study might be considered as risky (Ardlie et al., 2002). A higher density of SNPs must be characterized to comprehensively survey the fraction of the genome that exhibits low LD.

Several variables are used as measures of LD. The D-value is common with most other measures of LD and it quantifies disequilibrium as the difference between the observed frequency of a two-locus haplotype and the frequency it would be expected to show if the alleles were segregating at random (Ardlie et al., 2002). The numerical value of D is of little use for measuring the strength of and comparing levels of LD. This is due to the dependence of D on allele frequencies. As a result, several alternative measures based on D have been proposed. One good candidate is the D' value. The absolute value of D' is determined by dividing D by its maximum possible value, given the allele frequencies at the two loci. For example, D' is 1 if two SNPs have not been separated by recombination during the history of the sample.

2.6.3. Genome-wide association studies

Genome-wide association studies require knowledge about common genetic variation and the ability to genotype a sufficiently comprehensive set of variants in a large patient sample. Due to the expense and labour involved, the extension of candidate-gene studies to a genome-wide approach has not, until now, been feasible. Genotyping technology has considerably improved and became cheaper in recent years. Emerging technologies make it possible to genotype hundreds of thousands of SNPs simultaneously. By using GeneChips technology, it is now possible to analyze over 500 000 taq SNPs on a single slide per sample (Carlson, 2006, Pe'er et al., 2006). LD patterns on a genome-wide scale through HapMap project (2003) will be

useful for methods that use markers selected on the basis of LD. The use of tag SNPs improves the power of association studies as only a subset of loci needs to be genotyped though still maintaining the same information and power as if one had genotyped a larger number of randomly chosen SNPs.

2.6.4. The International HapMap project

The International HapMap Project is a multi-country effort to identify and catalog genetic similarities and differences in human beings (2003). The Project is collaboration among scientists and funding agencies from Japan, the United Kingdom, Canada, China, Nigeria, and the United States. The aim of the International HapMap Project is to determine the common patterns of DNA sequence variation in the human genome in DNA samples from populations with ancestry from parts of Africa, Asia and Europe. More than one million SNPs for which accurate and complete genotypes have been obtained in 269 DNA samples extracted from four populations (2005). These data document the generality of recombination hotspots, a block-like structure of linkage disequilibrium and low haplotype diversity, leading to substantial correlations of SNPs with many of their neighbours. By making this information freely available, the project provides tools that allow the association approach to be applied readily to any functional candidate gene in the genome, to any region suggested by family-based linkage analysis, or ultimately to the whole genome for scans for disease risk factors.

2.6.5. Population choice in complex disease gene mapping and study examples from Finnish population

Genetic isolates with a history of a small founder population, long-lasting isolation and population bottlenecks can modify gene pools significantly, and may render some populations better suited than others for linkage disequilibrium (LD) based gene mapping strategies (Peltonen, 2000). The Finnish population has been frequently cited as an example of a gene pool which demonstrates the effect of multiple bottlenecks in its population history, and LD has been used successfully in mapping of genes responsible for numerous rare diseases whose frequency has been enriched in this population (Peltonen et al., 1999, Varilo et al., 2003).

There are two consequences of founder effects and subsequent isolation of Finnish population i.e. reduced disease disposing alleles and fewer influencing genes. Population isolates, starting from a small founding population, have higher overall levels of LD than outbred populations, and they have fewer regions of very low LD (Peltonen et al., 1999, Jorde et al., 2000, Peltonen, 2000, Service et al., 2006). LD observed in disease alleles adds power to linkage and association analysis and helps to define the exact location of disease loci on the genetic map. In this respect, descendants derived from subpopulations founded in the late settlement regions of Finland during the 16th and 17th centuries are believed to represent an ideal population also for association studies of complex diseases (Peltonen et al., 1999, Jorde et al., 2000). As an example, in an attempt to identify new risk genes associated to LOAD, genome-wide LD mapping studies were carried out aiming at finding novel susceptibility loci for LOAD (Hiltunen et al., 1999, Hiltunen et al., 2001a). Forty-seven patients with LOAD and 51 age-matched control subjects were carefully chosen from the same geographic area in eastern Finland, where the population is descended mainly from a small group of original founders. These subjects were initially genotyped with 379 polymorphic microsatellite markers, and a follow-up analysis was performed with additional microsatellite markers for those chromosome loci found to be associated with AD. In these studies, a total of 22 chromosomal loci were found to be associated with AD and screening of the LD regions with additional microsatellite markers revealed 8 chromosomal loci where more than one microsatellite marker was associated with AD (1p36.12, 2p22.2, 3q28, 4p13, 10p13, 13q12, 18q12.1 and 19p13.3). The candidate genes in close proximity to these loci and other genes which are biologically related to AD provide targets for genetic association and functional studies of AD. Many moderate susceptibility genes have been already found in the Northern Savo population by using association analysis (Table 4). More studies are still needed to estimate the disease risk genes and to clarify the pathological mechanisms of AD.

Table 4. A sample of studied susceptibility genes in the Northern Savo population.

Gene	Chr location	Relevance to AD pathogenesis	Association with AD in un-stratified population	OR (95%CI) for risk genotypes	Study[a]
ACT	14q32	found in Aβ plaques	Negative		(Helisalmi et al., 1997)
BCHE	3q26	associate to NFT and Aβ	Negative [b,c]		(Hiltunen et al., 1998)
APOE	19q13.32	Aβ aggregation	Positive [c]	ε4-allele 5.85 (3.29-0.41)	(Helisalmi et al., 1999)
PSEN1 E318G	14q24.3	component of γ-secretase complex	Negative [b,c]		(Helisalmi et al., 2000a)
NPY	7p15	affects total and LDL cholesterol levels	Negative		(Helisalmi et al., 2000b)
MPO	17q23	found in activated migroglia surrounding amyloid plaques and in the plaques themselves	Negative [b,c]		(Reynolds et al., 2000)
A2M	12p13-12	clearance of Aβ	Negative [b,c]		(Pirskanen et al., 2001)
IL1A	2q14	proinflammatory gene	Negative		(Pirskanen et al., 2002)
HSPG2	1p36.12	promotes tau phosphorylation, binds to Aβ and accelerate Aβ fibril formation	Negative [b,c]		(Iivonen et al., 2003)
ICAM1	19p13	contribute to inflammatory responses	Negative		(Mattila et al., 2003)
NCSTN	1q22-23	component of γ-secretase complex	Negative [b,d]		(Helisalmi et al., 2004a)
APOD	3q26	lipid metabolism	Negative [b,d]		(Helisalmi et al., 2004b)
CYP19	15q21.1	encodes aromatase protein, regulate estrogen synthesis	Positive [c,d]	CT+TT 1.49 (1.05-2.12) [1] CT+TT 1.49 (1.10-2.02) [2] TT 1.40 (1.06-1.84) [3] GA+GG 1.51 (1.11-2.06) [4] TA+TT 1.46 (1.07-1.98) [5] CT+TT 1.41 (1.02-1.94) [6]	(Iivonen et al., 2004)
ESR2	14q22	mediate the effects of estrogens	Negative [b,c,d]		(Pirskanen et al., 2005)
IL6	7p21	inflammatory response	Negative [b,c]		(Koivisto et al., 2005)
CYP46	14q32.1	elimination of cholesterol	Positive [c]	CC 1.38 (1.01-1.89) [7]	(Helisalmi et al., 2006)
PPARγ	3p25	increases BACE gene promoter activity, regulate the inflammatory responses	Negative		(Koivisto et al., 2006)

[a] Populations do not fully overlap; [b] Association with AD in stratified population; [c] Association to a single nucleotide polymorphism; [d] Association to a haplotype; [1] rs1008805; [2] rs767199; [3] rs727479; [4] rs1065778; [5] rs1143704; [6] rs10046; [7] rs754203

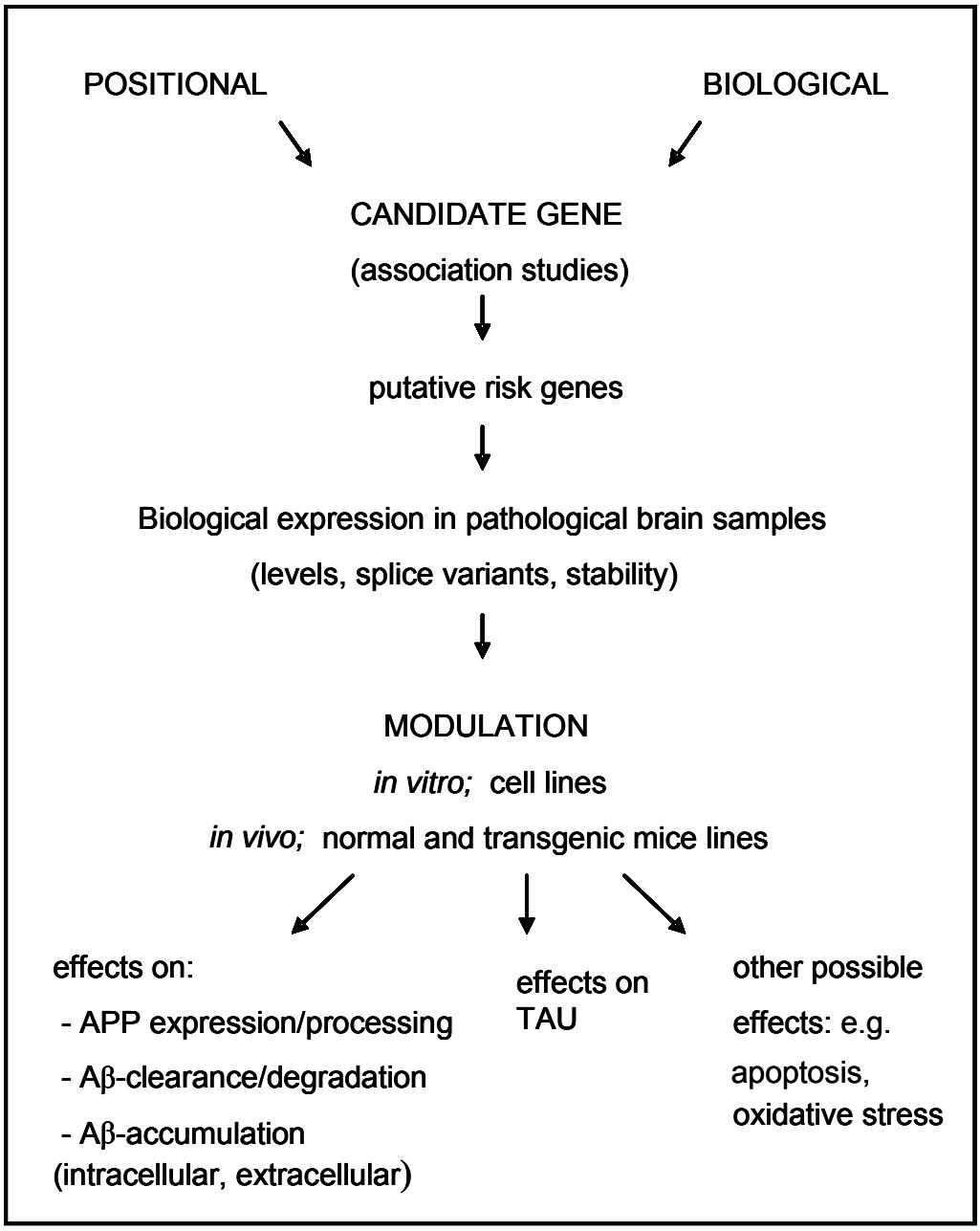
Abbreviations: Chr = chromosomal, ACT; alpha 1-antichymotrypsin, BCHE; buturylcholinesterase, APOE; apolipoprotein E, PSEN1; presenilin 1, NPY; neuropeptide Y, MPO; myeloperoxidase, A2M; alpha-2-macroglobulin, IL1A; interleukin 1 alpha, HSPG2; heparan sulfate proteoglycan 2, ICAM 1; intercellular adhesion molecule 1, NCSTN; nicastrin, APOD; apolipoprotein D, CYP19; cytochrome P450 family 19, ESR2; estrogen receptor 2, IL6; interleukin 6, CYP46; cytochrome P450 family 46,PPARγ; peroxisome proliferators-activated receptor gamma 3p25, DHCR24; 24-dehydrocholesterol reductase, LDLR; low density lipoprotein receptor, SOAT1; sterol O-acyltransferase.

2.7. Functional studies

Genetics is a powerful tool to identify factors contributing to different abnormal conditions and can be used as a first step toward unraveling mechanisms involved in biological processes of interest. The most important challenge for the future is moving from risk genes of AD to proteins, to elucidate what happens in the organism normally and in response to the presence of altered genetic information. Putative risk genes found by using genetic tools should be characterized further by using functional methods. Since expression levels and processing of APP clearly influence AD pathogenesis, it is essential to clarify how expression levels of putative AD risk genes can affect APP expression and/or processing. Figure 9 shows an example how putative candidate genes could be characterized further.

In living cells, the regulation of function is based on many regulatory events. Regulatory systems are like three-dimensional networks where many factors are influencing each others in different regulatory levels. By affecting one gene, it may have severe effects on several biological processes. It is essential to gain a more profound knowledge of the endogenous regulation of gene expression and the association of altered expression to cellular changes for understanding the pathogenesis of the disease and identifying molecular targets for drug development.

Figure 9. Workflow from association studies to functional studies.



2.8. Aims of the study

The present work was designed to study putative risk genes in AD. Mutations in APP, PSEN1, and PSEN2 genes cause the autosomal dominant form of EOAD while the APOE ε4 allele has been associated with an increased risk of developing both EOAD and LOAD. APOE ε4 allele accounts for about 50% of the genetic background of the LOAD suggesting that additional susceptibility genes exist. Identifying new genes involved in the pathogenesis of AD could open new avenues for development of rational treatment and prevention for AD. The aim was to use the homogenous eastern Finnish population to identify new candidate genes for AD and to clarify the function of putative risk genes.

The specific aims of the study were:

1) To study whether polymorphisms in the BDNF and TrkB genes are associated with AD in the Finnish population (Study I).
2) To study whether polymorphisms in the NEP gene are associated with AD in the Finnish population (Study II).
3) To study whether polymorphisms in the IDE gene and the HHEX gene at its vicinity are associated with AD in the Finnish population (Study III).
4) To study whether polymorphisms in the SST gene are associated with AD in the Finnish population (Study IV).
5) Based on the findings of studies II and III, the aims were
   a) To study whether the pathological accumulation of Aβ modify its own degradation.
   b) To study the age and brain region specific alterations in expression levels of IDE and NEP (Study V).

3. MATERIALS AND METHODS

3.1. Materials for studies I to IV

All AD patients and controls derived from eastern Finland and were examined at Kuopio University Hospital. The Ethics Committee of the Kuopio University Hospital and Kuopio University approved the study. According to the NINCDS-ADRDA, all patients fulfilled clinical criteria for probable AD (McKhann et al., 1984, Lehtovirta et al., 1996a). EOAD patients with an onset age of ≤ 65 years had been screened previously and did not carry any known mutations in APP, PSEN1 or PSEN2 genes. Control subjects had no signs of dementia according to interview and neuropsychological testing. The neuropathological investigation was carried out according to standard procedures, and the diagnosis of AD was based on the CERAD criteria (Mirra et al., 1991). The demographic data of the AD patients and controls is presented in Table 5.

Table 5. Demographic data of the AD patients and controls for studies I to IV

	Study I (BDNF, TrkB)		**Study II (NEP)**		**Study III (IDE, HHEX)**		**Study IV (SST)**	
	AD	CO	AD	CO	AD	CO	AD	CO
Number of subjects	375	460	390	468	370	454	424	466
[1]Age	72±7	70±5	72±7	70±5	72±7	70±5	71±7	70±5
Gender (% women)	69	60	70	61	69	61	67	60
APOE ε4 n (%)	277 (74%)	126 (27%)	283 (73%)	129 (28%)	273 (74%)	125 (28%)	312 (74%)	129 (28%)
OR for APOE ε4 allele	*4.9 (95% CI 3.9-6.2)		*4.7 (95% CI 3.8 -5.9)		*4.8 (95% CI 3.8-6.1)		*4.8 (95% CI 3.8-5.9)	
Number of subjects ≤65 years	60	119	63	112	61	116	70	112

Populations are mostly overlapping. AD=Alzheimers`s disease, CO=controls, [1] =AD, mean age at onset; CO, mean age at examination or death. *P<0.001 in Pearson`s χ^2 test.

3.2. Materials for study V

Transgenic mice (Tg) expressing either the human PSEN1 (A246E mutation) or a chimeric mouse/human APP695 (K595N, M596L, Swedish mutation; APPswe) (Borchelt et al., 1997) were backcrossed to C57BL/6J mice for 6 generations, and then the lines were crossed together to generate double Tg mice co-expressing both transgenes. Double Tg mice were used in this study and age-matched non-transgenic (NT) littermates served as controls. The numbers of mice in use in the study were as follows: 4-month-old mice, Tg (n=15), NT (n=13); 11-month-old mice, Tg (n=15), NT (n=17), 12-month-old mice, Tg (n=9), NT (n=9) and 17-month-old mice, Tg (n=17), NT (n=5). For verification of significant results APPswe/PSEN1(dE9) mice were used. The APPswe/PSEN1(dE9) founder mice were obtained from Johns Hopkins University, Baltimore, MD, USA (D. Borchelt and J. Jankowsky, Dept. Pathology) and a colony was established in the University of Kuopio. In brief**,** mice were created by co-injection of chimeric mouse/human APPswe (mouse APP695 harboring a human Aβ domain and mutations K595N and M596L linked to Swedish familial AD pedigrees) and human PSEN1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. The two transgenes co-integrated and co-segregated as a single locus (Jankowsky et al., 2004). This line was originally maintained in a hybrid C3HeJ x C57BL6/J F1 background, but the mice of the present study derived from backcrossing to C57BL6/J for 5-6 generations. The numbers of mice in use in the study were Tg (n=11) and NT (n=7); 9-month-old mice. The Aβ accumulation is more severe in these APPswe/PSEN1(dE9) mice and the first plaques develop around 4 months of age. The housing conditions (National Animal Center, Kuopio, Finland) were controlled (temperature +21˚C, light from 7:00 AM to 7:00 PM; humidity 50-60%), and food and fresh water were freely available. The experiments were conducted according to the Council of Europe (Directive 86/609) and Finnish guidelines, and approved by the State Provincial Office of Eastern Finland. For the reverse transcription polymerase chain reaction (RT-PCR) and protein analysis, animals were killed by decapitation, the brains were quickly removed, and cortex and cerebellum were dissected on ice. In addition, the hippocampus was dissected for total Aβ40 and Aβ42 measurements by an ELISA method. Brain samples were frozen in liquid nitrogen and then stored in -70 ºC until further examinations.

3.3. Gene analysis for studies I to IV

3.3.1. DNA extraction and sequence analysis

Genomic human DNA was extracted from peripheral leucocytes using a phenol-chloroform-isoamyl alcohol method (Vandenplas et al., 1984). The polymerase chain reaction (PCR) products and polymorphisms sites of studies I to IV were ascertained with cycle-sequencing using automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The PCR primers used are listed in Table 6. PCR products were purified using QIAguick™ PCR Purification Kit (Qiagen) and the sequencing was conducted with the Ready Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase (Applied Biosystems). PCR products were cycle-sequenced with the genetic analyzer and the data were analyzed by using ABI PRISM Sequencing Analysis Software (Applied Biosystems).

3.3.2. SNaPshot procedure

SNPs were selected according to the frequency of their minor allele (>0.1) among the eastern Finnish population as well as their location in the gene. Primers used for PCR reactions are presented in Table 6. PCR amplification was followed by Shrimp alkaline phosphatase (SAP, Amersham Pharmacia Biotech) and ExoI (New England Biolabs Inc.) enzymatic treatment (Table 7). SAP/ExoI treated PCR products were subjected to SNaPshot Multiplex PCR in a 10 µl reaction volume containing 1.25 µl SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems) and appropriate amount of each SNaPshot primer (primers SS in Table 6). The SNaPshot multiplex reaction was followed by enzymatic SAP treatment. The samples were analyzed using ABI 3100 Genetic Analyzer (Applied Biosystems) and the allele determination was carried out with the Genotyper 3.7 program or GeneMapper™ 3.0 software (Applied Biosystems).

Table 6. SNPs and primers used in association studies I to III.

Study (Gene)	SNP`s rs-number[1]	Variation	Primers (F=forward primer, R=reverse primer, SS=SNaPshot primer)
I (BDNF)	2030324	C/T	F:TCCAAACATCACACAGCCTAA R:TGGTCAAAAGGGATGTGAGA SS: CATTGAATCAGATGAAAGATGAACT20
I (BDNF)	2049045	C/G	GCAACAGAGGGAACCAACTC AAGCCTGAACAGTGGACCAG SS: AAATTGGGTTACCTCCTGGGAAT28
I (BDNF)	6265	A/G	F:ACTCTGGAGAGCGTGAATGG R:ACGTGTACAAGTCTGCGTCCT SS: TGGCTGACACTTTCGAACACT36
I (BDNF)	2203877	A/G	F:AGTTCCTCACCTGCAAAGGA R:CATTTCACCCTGGCTTCAGT SS: GGATCTCTTCCAGGAGTCTGAATAT37
I (BDNF)	*	A/G	F:TGCTGATCACCTGCTCTCAC R:AAGTTGCTTTCCCTGACTGC SS: CACAGGTGAATGAAAATGTCCACT44
I (TrkB)	1212171	A/G	F:TCTGTCCAAAGGACAAACTGC R:CATGTCAATGCGGTATGGAG SS: CGGTGGTTTGTACACCAGAAGAT56
I (TrkB)	1187326	A/G	F:AGTGCCTCTCGGATCTGGT R:TGTCACACTTCTCCCTGCAC SS: TGGCCAGCTCGGGCCCTAT66
I (TrkB)	1187327	A/G	F:GAGCAGGCGTGATACATACC R:AGCTGAAGTTACGTGCGTCT SS: AATGTCCCACTATTTGTGTTCTTAGT64
II (NEP)	989692	C/T	F: TCCCCCTTACTCCCCTCTTA R: GCAAAACGAATGAACACATGA SS: T37AATAGATACTGGTATCAGTAG
II (NEP)	2196521	C/T	F: AAAGCCTACCATCAGGGACA R: CATGGACCACGCATTGAATA SS: T19CTCACCTCTGATTAATCCCTT
II (NEP)	1025192	C/T	F: CACTCCATTTTCCAATGTGCT R: TCCACTGTCGGACTTATCACC SS: C26TCCACTATAAAAAATTCATC
II (NEP)	1816558	C/T	F: GTAGAGACGGGGTTTCACCA R: AACCAACACCTTTCCCCAAT SS: C43AAAACTAATAATCTTACAC
II (NEP)	3773885	C/T	F: CTTTACACACATTTCCATTTTTGC R: CCTAGCATAATTAAGCCAACCATT SS: T59AAGCTTAAAGGCTTTGTTTTC
II (NEP)	3773882	C/T	F: GCTCCAAGGGGTTTAGAAAGTT R: GCCTTCCCAAGAAGAAGAAAAT SS:C30ATCAAAAAAGAAAAAATTTC
II (NEP)	3736187	A/G	F: TCTGTGATGAGGAGGGATGAC R: GATACACCATGCACTGGGATT SS:C54TTTTACTTAAATAAATATATTA
III (IDE)	3758505	A/C	F:CCACTAGGAGGCTTGCCATA R:TTGCACCCACGAACAACTTA SS:CAAAAACGGTGTCCTTAGTCCAT51
III (IDE)	4646953	C/T	F: CCCTCACAGTCAGACACACG R: AGCTGCGGAGAGGTAGCTG SS:CCTCGGCTGTCCGCGGTCT59
III (IDE)	4646955	C/T	F:GTAGGCCTGAAGCCTCATTT R: CATGAACACCTAGAAGGTGC SS:TGAATTATCCAACTTTGTGTACTTAT75
III (IDE)	SNP5	A/G	F:TTCCACATTTTCACCTAACACTG R:TCCTCTGGATGGCTCATTTC SS:ATTACATACATGTCAATAAAGAGG(T83)

1 The NCBI SNP Cluster ID (rs-number)

* (gi: 37541814, position 26429483)

Table 7. Overview of SNaPshot procedure:

1) Template preparation
   - amplify genomic DNA
   - remove dNTPs and primers (SAP, enzyme inhibitor; Exo1, primer inhibitor)
   - obtain purified template
2) Reaction preparation
   - prepare sample reaction (combine: template, SNaPhot primer, SNaPshot™Multiplex ready reaction mix)
4) Thermal Cycling (Post-Extension treatment)
   - perform thermal cycling (denature the sample, anneal unlabeled primer, extend primer with target complementary ddNTP)
   - Remove unincorporated ddNTPs (with SAP)
4) GeneScan Analysis
   - Electrophorese samples (for example with ABI Prism 3100 instrument)
   - Analyze data (for example with Genemapper)

3.3.3. High-throughput fluorescence detection system

High-efficiency fluorescence polarization (rs1544210 and rs2251101) detected single-base extension on a Criterion Analyst AD high-throughput fluorescence detection system (Molecular Devices).

3.3.4. Allelic discrimination

SNPs were selected for screening using SNPbrowser™ software 3.0 and genotyping was done using TaqMan® Pre-Developed Assay Reagents for Allelic Discrimination (Applied Biosystems, Foster City, CA). In allelic discrimination assays, the PCR assay includes specific, fluorescent, dye-labeled probes for each allele. The probes contain different fluorescent reporter dyes to differentiate the amplification of each allele. During the PCR, each probe anneals specifically to complementary sequences between the forward and reverse primer sites. AmpliTaq Gold® DNA polymerase can cleave only probes that hybridize to the

allele. Cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter dye. Thus, the fluorescence signal(s) generated by PCR amplification indicate(s) the alleles that are present in the sample. The TaqMan genotyping reaction was amplified on a MJ Research PTC-200 Cycler (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min), and fluorescence was detected on a ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA).

3.3.5. Determination of APOE allele forms

For detection of the APOE allele forms (ε2, ε3 and ε4), PCR and HhaI (New England Biolabs Inc.) digestions of the PCR products were carried out with primers and conditions as previously described (Tsukamoto et al., 1993) or using SNaPshot procedure as previously described (Lehtovirta et al., 1996b).

3.4. Gene and protein analysis for study V

3.4.1. RNA extraction

Total RNA was extracted from 50-100 mg mouse tissue using TRIzol reagent (Gibco BRL, Grand Island, NY, USA). To avoid coamplification of genomic DNA, DNase treatment of the total mouse RNA samples was performed using RNase-Free DNase Set (QIAGEN GmbH, Hilden, Germany). The quality of RNA was determined by electrophoresis in ethidium bromide stained agarose gels.

3.4.2. cDNA synthesis

The reverse transcription was performed to generate cDNA using TaqMan® Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA, USA) with random hexamers in 50 µl reaction volumes from 1µg of total RNA. The samples were incubated for 10 min at 25°C, the reverse transcription step was 2 h at 37°C, following the inactivation of reverse transcriptase for 5 min at 95°C. Efficiency of cDNA synthesis was confirmed by

analysing triplicate cDNA samples using RT-PCR. The minus RT reaction was performed to detect possible co-amplification of genomic DNA.

3.4.3. Relative mRNA quantification

Relative mRNA quantification of mouse IDE and NEP was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. Real-time quantification of IDE and NEP relative mRNA levels was carried out using ABI PRISM® 7000 or 7500 Sequence Detection System with FAM dye-labeled fluorescent mouse assays (Assays-On-Demand™ Gene Expression Products, Applied Biosystems), and the endogenous control gene, GAPDH with VIC dye-labeled TaqMan® Rodent GAPDH Control Reagents kit (Applied Biosystems). Amplifications were performed in multiplex or singleplex reactions according to the manufacturer's instructions. In multiplex reactions, the concentrations of primers used in the study were limited to avoid primer competition.

The relative quantification was performed using the relative standard curve method or the comparative Ct –method depending on the study. In standard curve methods, the R^2 for the standard curves varied from 0.988 to 0.997 in NEP mouse study. In the GAPDH studies, R^2 for the standard curves varied from 0.989 to 0.997. For each experimental sample, the amounts of IDE, NEP and GAPDH were determined from the appropriate standard curves. The IDE or NEP amount was then divided by the GAPDH amount to obtain a normalized target value. A calibrator was included in each assay run, and each normalized target value was divided by the calibrator to generate the relative mRNA levels. The comparative Ct method is similar to the standard curve method, except that it uses arithmetic formulas to achieve the same results for relative quantitation. The benefit of resorting to the Ct method is to eliminate the use of standard curves for relative quantitation. The PCR efficiencies of mouse IDE and rodent GAPDH were measured to ensure that they were equivalent. In the Ct method, the IDE amount of target, normalized to GAPDH and relative to a calibrator which was included in each assay run, is given by $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = \Delta Ct$ target $- \Delta Ct$ calibrator and ΔCt is the difference in threshold cycles for IDE and GAPDH. Control reactions were performed to verify that no amplification would occur without cDNA. The means calculated from three parallel PCR reactions were used in the statistical analyses.

3.4.4. Western blotting

Western blotting was performed to measure protein levels of IDE, NEP, APP-FL, C-terminal fragments of APP-FL; APP-C99 and APP-C83, GAPDH and α-tubulin from the cortex of 12-month-old APP/PSEN1 (A246E) Tg mice and NT littermates (n=9 in both groups) and from the cortex of APP/PSEN1(dE9) Tg (n=11) mice and NT littermates (n=7). Proteins were extracted with T-Per solution (Pierce, Biofellows Oy) according to the manufacturer's instructions. The following rabbit polyclonal antibodies were used: Anti-NEP (AB5458, Chemicon International, Temecula, CA), anti-IDE (AB25970, Abcam, Cambridge, UK) and anti-APP C-terminal (A8717, Sigma, St.Louis, USA). The following mouse monoclonal antibodies were used: anti-GAPDH (AB8245, Abcam, Cambridge, UK) and anti-α-tubulin (T5168, Sigma, St.Louis, USA). Signals were detected with ECL+ system (Amersham Biosciences, Buckinghamshire, UK) and proteins were quantified from scanned membranes with Quantity One software (Bio-Rad). Protein levels were assessed by using GAPDH as an endogenous control.

3.4.5. Aβ40 and Aβ42 ELISAs

To analyze total Aβ levels, the mouse hippocampus was homogenized in a guanidine buffer (5.0 M guanidine-HCL/50 mM Tris-HCL, pH 8.0) in proportion to their weight. The samples and Aβ-peptides used as standards were prepared to contain 0.5 M guanidine-0.5% BSA-1 mM AEBSF in the final composition. The levels of transgenic human Aβ40 and Aβ42 were quantified using the Signal Select^{TM} Beta Amyloid ELISA Kits (BioSource International Inc.) according to the manufacturer`s protocol. The total Aβ40 and Aβ42 levels were standardized to brain tissue weight and expressed as ng Aβ/g (brain tissue).

3.5. Statistical analyses

3.5.1. Studies I to IV:

Standardized D' values, which were used to measure pair-wise linkage disequilibrium (LD), were calculated using the 2LD program (http://www.iop.kcl.ac.uk/IoP/Departments/PsychMed/GEpiBSt/software/2ld.stm). The significance of linkage disequilibrium as well as the Hardy-Weinberg distribution of genotypes among the AD and control groups was assessed using GenePop option 2 and 1 forms, respectively (http://wbiomed.curtin.edu.au/ genepop/genepop_op1.html.). Single locus allele and genotype frequencies were compared by using two-tailed Pearson´s χ^2 test with SPSS 11.5 software. Odds ratios (OR) for combined genotype data were calculated using uni- and multivariate logistic regression with 95% confidence intervals (CI). For stratified data, the Bonferroni correction was applied by multiplying P values with the number of tests. The Expectation-maximization (EM) algorithm was used to obtain maximum-likelihood estimates of haplotype frequencies of genes SNPs with Arlequin ver. 2.000 software (http://anthro.unige.ch/arlequin). Haplotype frequencies with absolute chromosome numbers were compared between AD and control groups using the RxC program that employs the Metropolis algorithm to obtain unbiased estimates of exact P-values with standard errors (SE) (Raymond ML and Rousset F, 1995). Haplotype block predictions were performed by using the Haploview program which is based on D` value, the standardized disequilibrium parameter. The level of statistical significance was defined as $P < 0.05$.

3.5.2. Study V:

All statistical analyses were performed using SPSS 11.5 software applying non-parametric Mann-Whitney U-test, Kruskall-Wallis H-test and Spearman's correlation analysis. All data are expressed as means ± standard error and the level of statistical significance was defined as $P<0.05$.

4. RESULTS

4.1. BDNF and TrkB polymorphisms; Study I

Five SNPs in BDNF gene and 3 SNPs in TrkB genes promoter region were used in this study. Locations of BDNF SNPs are indicated in Figure 10. The Hardy-Weinberg equilibrium was tested separately among stratified and un-stratified AD and control groups for all SNPs, with no deviations from equilibrium being observed. The distribution of APOE ε2/3/4 alleles differed as expected for the 375 AD cases from the 460 control subjects (Table 5).

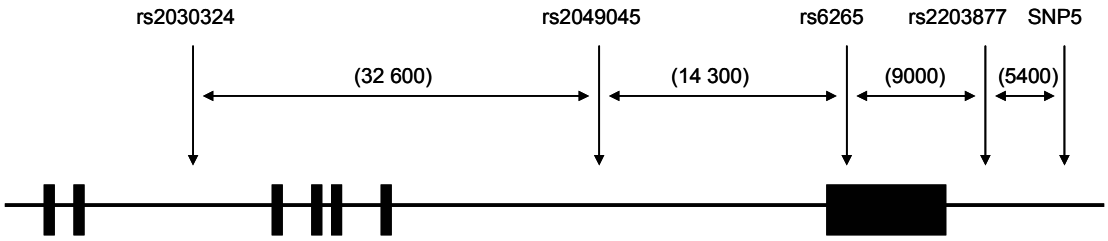


Figure 10. Schematic map of BDNF. The numbers in brackets indicate distances between analyzed SNPs (kb). Boxes represent exons and lines introns. Exon orientation (1→7) from contig NT_086780.

When AD patients and controls were analyzed together in a pairwise LD analysis (1670 alleles), a strong LD was observed between all five SNPs in the BDNF gene as well as between all three SNPs in the TrkB gene (Table 8). The allele and genotype frequencies of the BDNF and TrkB SNPs did not differ significantly between un-stratified AD and control groups (Table 9). In addition, no significant differences were found in single or overall haplotype distributions.

Table 8. Pairwise LD (D`above diagonal) and statistical significance (P-value below diagonal) for BDNF and TrkB SNPs.

BDNF SNP	1	2	3	4	5
1		1.000	1.000	0.960	0.960
2	<0.001		0.995	0.905	0.905
3	<0.001	<0.001		0.905	0.905
4	<0.001	<0.001	<0.001		1.00
5	<0.001	<0.001	<0.001	<0.001	

TrkB SNP	1	2	3
1		0.994	0.997
2	<0.001		1.000
3	<0.001	<0.001	

Since the AD group consisted of both early- and late- onset patients, the sample cohorts were analyzed separately using 65 years as the cutoff age. Nonetheless, comparison of the allele, genotype and haplotype distribution of BDNF and TrkB SNPs between these subgroups did not reveal any significant differences. In addition, even after stratification according to gender or APOE status, there were no major differences in allele, genotype or haplotype frequencies of BDNF and TrkB SNPs between cases and controls. When BDNF data was stratified according to familial status, significant differences were observed in allele frequencies of four SNPs between sporadic AD and control populations (Table 10). Three haplotypes out of seven (CCGGG, TGAAA and TCGAA), as well as in un-stratified material exceeded the previously considered threshold haplotype frequency of 0.05 (Xu et al., 2002). Association testing of individual haplotype distribution revealed that the haplotype CCGGG was significantly over represented among the sporadic AD group before Bonferroni correction (frequencies in sporadic AD; 0.51, 0.13 and 0.33 and in controls; 0.44, 0.17 and 0.35; P=0.04; SE=0.006; Bonferroni corrected P value: P=0.8). The distribution of APOE ε2/3/4 alleles was 0.04/0.80/0.15 and 0.02/0.55/0.43 in control and sporadic AD groups ($P < 0.001$), respectively. The APOE ε4-allele was significantly more common in the sporadic AD group compared to the control group ($P < 0.001$, OR = 4.24; 95% CI 3.19-5.64). Stratification of TrkB polymorphisms according to familial status did not reveal any significant results.

Table 9. Allele, genotype and haplotype frequencies of the BDNF polymorphisms for patient and controls.

BDNF	Allele	Allele frequency		P value for Allele[a]	Genotype	Genotype frequency		P value for Genotype[a]
		Control (N =920)	AD (N =750)			Control (N =460)	AD (N=375)	
rs2030324	C T	0.53 0.47	0.51 0.49	0.35	CC CT TT	0.27 0.52 0.21	0.24 0.53 0.23	0.62
rs2049045	C G	0.87 0.13	0.86 0.14	0.72	CC CG GG	0.75 0.23 0.02	0.75 0.23 0.02	0.65
rs6265	G A	0.87 0.13	0.86 0.14	0.71	GG GA AA	0.75 0.24 0.02	0.74 0.23 0.02	0.65
rs2203877	G A	0.52 0.48	0.49 0.51	0.18	GG GA AA	0.26 0.51 0.23	0.23 0.51 0.26	0.39
SNP5	G A	0.52 0.48	0.49 0.51	0.18	GG GA AA	0.26 0.51 0.23	0.23 0.51 0.26	0.39
TrkB								
rs1212171	G A	0.36 0.64	0.38 0.62	0.46	GG GA AA	0.14 0.45 0.42	0.16 0.45 0.40	0.74
rs1187326	G A	0.19 0.81	0.20 0.80	0.94	GG GA AA	0.05 0.30 0.66	0.04 0.31 0.65	0.86
rs1187327	G A	0.64 0.36	0.62 0.38	0.49	GG GA AA	0.41 0.45 0.14	0.40 0.45 0.16	0.71

BDNF Haplotype[a]	Control (N =920)	AD (N =750)	P-value[b]
CCGGG	0.507	0.477	0.237±0.021
TGAAA	0.125	0.132	0.717±0.012
TCGAA	0.334	0.350	0.448±0.026
Others	0.033	0.040	0.522±0.010
Overall			0.641±0.023

TrkB Haplotype[a]	Control (N =920)	AD (N =750)	P-value[b]
GGA	0.193	0.196	0.892±0.006
GAA	0.165	0.181	0.565±0.019
AAG	0.638	0.623	0.520±0.021
Others	0.003	-	0.256±0.002
Overall			0.473±0.036

Table 10. Allelic and genotypic frequencies of the polymorphism of the BDNF gene in patients with sporadic AD (n=160) and controls (n=460).

BDNF locus	Allele	Allele frequency		P-value for Allele[a]	Geno-type	Genotype frequency		P-value for Geno-type[a]
		CO (n=920)	AD (n=320)			CO (n=460)	AD (n=160)	
rs2030324	C T	0.53 0.47	0.46 0.54	**0.04** 0.8*	CC CT TT	0.27 0.52 0.21	0.22 0.49 0.29	0.09
rs2049045	C G	0.87 0.13	0.83 0.18	0.06	CC CG GG	0.75 0.24 0.02	0.69 0.28 0.04	0.12
rs6265	G A	0.87 0.13	0.82 0.18	**0.04** 0.8*	GG GA AA	0.75 0.24 0.02	0.68 0.28 0.04	0.06
rs2203877	G A	0.52 0.48	0.45 0.55	**0.04** 0.8*	GG GA AA	0.26 0.51 0.23	0.22 0.47 0.31	0.09
SNP5	G A	0.52 0.48	0.45 0.55	**0.04** 0.8*	GG GA AA	0.26 0.51 0.23	0.22 0.47 0.31	0.09

[a] P-values calculated using two-tailed Pearson´s χ^2 test
[b]P-values calculated using RXC program
* Bonferroni corrected p-values for 20 tests

4.2. NEP polymorphisms; Study II

Seven intronic SNPs in NEP gene were selected for screening (Figure 11). All SNPs were in Hardy-Weinberg equilibrium in both cases and controls (P>0.05). The distributions of APOE e2/e3/e4 alleles differed as expected for the 390 AD cases compared with the 468 control subjects (Table 5). In a single locus analyses among 7 studied SNPs 2 SNPs rs989692 and rs3736187 differed significantly between the study groups (Table 11). In carriers of the SNP 1 T-allele and the SNP 7 A-allele, an age and sex adjusted odds ratio for the risk of AD was 1.32 (95% CI 1.00 to 1.74; P=0.05) and 4.06 (95% CI 1.14 to 14.4; P=0.03), respectively. The age and sex adjusted odds ratio for the risk of AD in homozygous carriers of the SNP 1 T-allele and the SNP 7 A-allele was 1.85 (95% CI 1.17 to 2.94; P=0.01) and 1.46 (95% CI 1.08 to 1.97; P=0.01), respectively. No significant differences were found in the APOE stratified groups.

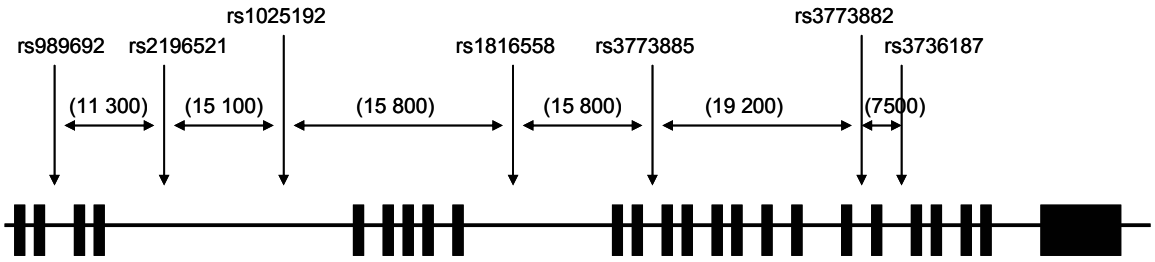


Figure 11. Schematic map of NEP gene. The numbers in brackets indicate distances between analyzed SNPs (kb). Boxes represent exons and lines introns. SNP rs989692 is in different intron compared to what we earlier reported. This is due to the fact that the amount of new genomic data is increasing continuously which leads to changes in intron/exon locations and numbering. Exon orientation (1→24) from contig NT_086780.

Table 11. Allelic and genotypic frequencies of the polymorphism of NEP gene for AD patient (n=390) and controls (n=468). [a] P-values calculated using two-tailed Pearson´s χ^2 test, [b]P-values calculated using RXC program. Bonferroni corrected values are given in parenthesis.

NEP rs-number	Alleles	Allele frequency		[a]P-value	Geno-types	Genotype frequency		[a]P-value
		CO n=936	AD n=780			CO n=468	AD n=390	
rs989692 (SNP1)	C **T**	0.70 0.30	0.64 0.36	**0.01** (0.14)	CC CT TT	0.48 0.45 0.07	0.41 0.46 0.13	**0.01** (0.14)
rs2196521 (SNP2)	C T	0.12 0.88	0.14 0.86	0.36	CC CT TT	0.03 0.19 0.78	0.03 0.22 0.75	0.54
rs1025192 (SNP3)	C T	0.52 0.48	0.53 0.47	0.92	CC CT TT	0.28 0.49 0.23	0.28 0.49 0.23	0.98
rs1816558 (SNP4)	C T	0.41 0.59	0.39 0.61	0.50	CC CT TT	0.18 0.46 0.36	0.17 0.44 0.39	0.73
rs3773885 (SNP5)	C T	0.68 0.32	0.68 0.32	1.00	CC CT TT	0.46 0.44 0.10	0.47 0.41 0.12	0.62
rs3773882 (SNP6)	C T	0.78 0.22	0.78 0.22	1.00	CC CT TT	0.59 0.37 0.04	0.61 0.34 0.05	0.62
rs3736187 (SNP7)	G **A**	0.19 0.81	0.14 0.86	**0.01** (0.14)	GG GA AA	0.03 0.31 0.66	0.01 0.25 0.74	**0.01** (0.14)

NEP Haplotype	AD frequency n= 780 alleles	Control frequency n= 936 alleles	[b]P-value
CTTTTCA	0.12	0.17	NS
CTCCCCG	0.07	0.12	NS
CTCCCCA	0.10	0.09	NS
CTCCCTA	0.06	0.07	NS
CTTTCCA	0.07	0.07	NS
TTCCCC**A**	0.09	0.06	**0.02**

When AD patients and controls were analyzed together in a pair-wise LD analysis (1716 alleles) 62% of SNP pairs demonstrated LD (Using a threshold D' value of 0.30). With the likelihood ratio test of LD, 67% of SNP pairs had p <0.01. Haplotypes with frequencies above 5% were included into the statistical tests (Table 11). Haplotype TTCCCCA includes T and A alleles for rs rs989692 and rs3736187, which were also significantly overrepresented in the AD cohort in the single locus analyses.

Haplotype blocks as determined with the use of Haploview software on the basis of the strength of the linkage disequilibrium (using a threshold D´value >0.95, Confidence interval (Gabriel et al., 2002)) showed two separate blocks in analyzed NEP gene region (Figure 12). In block two, three haplotypes were observed (Table 12). Haplotype CA was overrepresented in the AD cohort compared to controls. Conversely, haplotype CG was underrepresented among the AD patients.

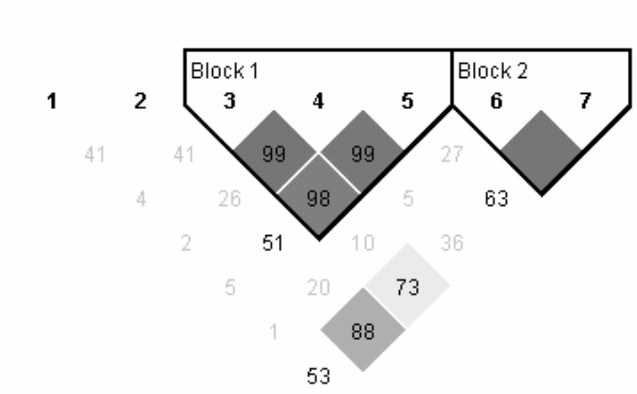


Figure 12. Haplotype blocks as determined with the use of Haploview software on the basis of the strength of the linkage disequilibrium. A) Numbers 1 to 7 indicate SNPs rs989692, rs2196521, rs1025192, rs1816558, rs3773885, rs3773882, rs3736187, respectively. Shades of gray represent decreasing degrees of LD between pairs of SNPs; dark gray represent strong LD.

Table 12. Estimated haplotype frequencies for NEP polymorphisms, rs 3773882 and rs3736187 based on Haploview software.

NEP Haplotype	AD frequency n= 780 alleles	Control frequency n= 936 alleles	P-value
CA	0.64	0.59	**0.02**
TA	0.22	0.23	NS
CG	0.14	0.19	**<0.01**

4.3. IDE and HHEX polymorphisms; Study III

Five IDE SNPs and 1 HHEX SNP were selected for screening. Locations of IDE SNPs are indicated in Figure 13. All SNPs were in Hardy-Weinberg equilibrium in both cases and controls (P>0.1). The APOE ε4 allele was significantly overrepresented among 370 AD patients when compared to 454 control subjects (Table 5). Genotyping of the IDE and HHEX SNPs revealed that the minor allele frequency of IDE intron 20 SNP (12973709) was ≤ 0.01 in both AD and control cohorts, which led us to exclude this SNP from further analysis.

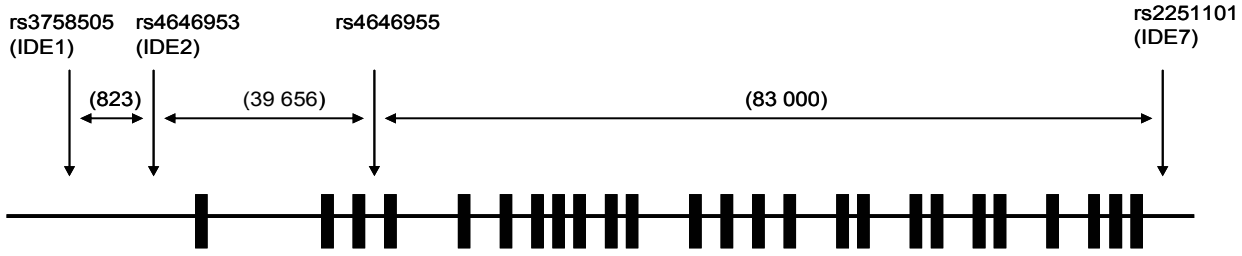


Figure 13. Schematic map of IDE gene. The numbers in brackets below arrows indicate distances between analyzed SNPs (kb). Boxes represent exons and lines introns. Exon orientation (1→25) from contig NT_086775.1.

Comparison of the genotype and allele distribution of SNPs between AD and control cohorts revealed that IDE SNPs rs4646953 and rs4646955 showed nominal evidence of association with AD (Table 13). When the sample was stratified based on onset age using 65

years as cut off, significant genotype and allele association of IDE SNPs rs4646953 (P<0.03 and P<0.03, respectively) and rs4646955 (P<0.03 and P<0.03, respectively) were observed with late-onset, but not with early-onset AD patients. Stratification according to gender and APOE status did not reveal any major differences between subgroups. IDE SNPs rs4646953 and rs4646955 conferred an approximately two-fold increased risk for AD in logistic regression analyses (Table 13). The risk effect of IDE SNPs rs4646953 and rs4646955 appeared to be somewhat stronger among the LOAD patients when compared to whole cohort.

Table 13. Single locus, uni- and multivariant logistic regression test results for IDE and HHEX SNPs.

NCBI rs-number and location (kb)[a]	Allele	Allele frequency		P-value	Geno-type	Genotype frequency		P-value
		CO (n=908)	AD (n=740)			CO (n=454)	AD (n=370)	
rs1544210[b] HHEX (0)	A G	0.566 0.434	0.535 0.465	0.21	AA AG GG	0.322 0.476 0.192	0.300 0.446 0.232	0.32
rs3758505 IDE 5'-UTR (153)	G T	0.052 0.948	0.053 0.947	0.93	GG GT TT	0.004 0.095 0.901	- 0.105 0.895	0.48
rs4646953 IDE 5'-UTR (154)	C T	0.338 0.662	0.284 0.716	0.02 (0.10)	CC CT TT	0.126 0.425 0.449	0.076 0.416 0.508	0.04 (0.20)
rs4646955 IDE Intron 3 (194)	C T	0.380 0.620	0.327 0.673	0.03 (0.15)	CC CT TT	0.152 0.456 0.392	0.095 0.465 0.441	0.04 (0.20)
rs2251101 IDE 3'UTR (276)	C T	0.177 0.823	0.174 0.826	0.87	CC CT TT	0.030 0.289 0.678	0.027 0.295 0.678	0.87

Uni-and multivariant logistic regression for significant IDE SNPs	OR (95% CI) Univariate		OR (95% CI) Adjusted for age, gender and APOE	
	Whole	> 65 years	Whole	> 65 years
rs4646953 CT+TT vs CC	1.75(1.09-2.82) *	2.21(1.29-3.80) **	2.08(1.20-3.60) **	3.00(1.58-5.69) *
rs4646955 CT+TT vs CC	1.72(1.11-2.64) *	2.08(1.29-3.36) **	1.95(1.18-3.23) **	2.58(1.45-4.57) **

[a]Distance from HHEX SNP rs1544210. All SNPs were in Hardy-Weinberg equilibrium in both cases and controls (p>0.1). [b]AD patients n = 362, Controls n = 449. [c]Allele and genotype frequencies were compared using two-sided Pearson´s χ^2 and Fischer's exact tests, respectively. P-values were corrected for multiple testing using Bonferroni correction (in parenthesis). * P<0.05, ** P<0.01.

Haplotype estimation analysis was performed using IDE SNPs rs3758505, rs4646953 and rs4646955 as these SNPs were shown to be a part of same haplotype block in the 5'-prime end of the IDE gene (Figure 14). Four haplotypes were identified in the AD and control cohorts (Table 14). Assessment of individual haplotype distributions showed that the TTT may be significantly overrepresented in the AD cohort and conversely, haplotype TCC was underrepresented among the AD patients. Haplotype TTT includes T alleles for rs4646953 and rs4646955, which were also significantly overrepresented in the AD cohort in the single locus analyses. The frequencies of the TCC and TTT haplotypes were similar in the early- and late-onset AD patients when compared to the whole cohort, but the distribution of these haplotypes was significantly different from cases only in the late-onset AD patients when compared to age-matched controls (TCC, p = 0.03 and TTT, p = 0.03).

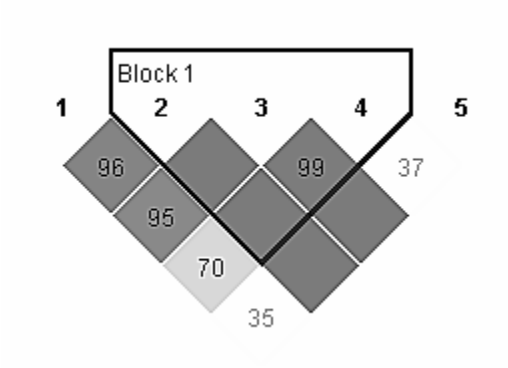


Figure 14. Haplotype blocks as determined with the use of Haploview software on the basis of the strength of the linkage disequilibrium. Numbers 1 to 5 indicate SNPs rs1544210, rs3758505, rs4646953, rs4646955, rs2251101, respectively. Shades of gray represent decreasing degrees of LD between pairs of SNPs; dark gray represent strong LD.

Table 14. Estimated haplotype frequencies for IDE gene. P-values calculated using RXC program.

IDE haplotypes	Control frequencies n=740 alleles	AD frequencies n=908 alleles	P-value
TCC	0.34	0.28	**0.02**
TTT	0.57	0.62	**0.03**
GTT	0.05	0.05	NS
TTC	0.04	0.04	NS

4.4. SST polymorphisms; Study IV

The distributions of APOE ε2/ε3/ε4 alleles was 0.04/0.80/0.16 in control (n=466) and 0.02/0.51/0.47 in sporadic AD (n=424) groups, respectively, (P<0.001) and were consistent with those previously described in the same population (Table 5) (Lehtovirta et al., 1996b). All SNPs were in Hardy-Weinberg equilibrium in cases and controls (P>0.05).

The allele and genotype frequencies did not differ significantly between whole AD and control groups (Table 15). However, an increased frequency of C-allele carriers of SNP rs4988514 can be seen in AD cases (0.31) compared to controls (0.26). By using a binary logistic regression analysis, we found that in whole study group, an age, sex and APOE adjusted OR for the risk of AD in C-allele carriers of the rs4988514 was 1.42 (95% CI 1.02-2.00; P=0.04). When AD patients and controls were analyzed together in a pair-wise LD analysis (1780 alleles), a strong LD was observed between all three SNPs in the SST gene (D'=1.0 in all pairs, P<0.01). The frequencies of all observed haplotypes were above 0.05 and they all were included into statistical tests. The association testing of individual haplotype distributions in whole samples showed that a major haplotype TTG was less common for cases compared to controls (Table 15). No other significances were found in whole samples.

It is known that the SST protein levels are significantly lower in AD patients carrying the APOE ε4 allele (Grouselle et al., 1998). To study the genetic background of the SST gene in this particular AD group, we stratified the data according to APOE. In the APOE ε4 allele group, the allele and genotype frequencies of rs4988514 differed significantly between AD cases (n=312) and controls (n=129) (Table 15) and the age and sex adjusted OR for the risk of AD in C-allele carriers of the rs4988514 was 2.05 (95% CI 1.23-3.41; p=0.006, Bonferroni corrected p=0.018). As in the whole group, the major haplotype TTG in the APOE ε4 allele group was underrepresented for cases compared to controls. Additionally, 2-fold overrepresentation of TCG haplotype in APOE ε4 samples for AD cases was significantly overrepresented among cases compared to the remaining haplotypes (Table 15). When all haplotype combinations were compared between the study groups, a significant difference was detected in APOE ε4 samples with P=0.012. We did not find any significant differences in APOE ε4 negative subjects between the study groups.

Table 15. Genotype, allele and haplotype frequencies of the polymorphism of SST gene for whole and APOE ε4 (+) samples. [a] P-values calculated using GenePop option, [b]P-values calculated using RXC program. Bonferroni corrected P-values are given in parenthesis.

SST		**Whole Samples**		**[a]P-value**	**APOE ε4 (+)**		**[a]P-value**
		AD	**CO**		**AD**	**CO**	
SNP	**Genotypes**	AD (n=424)	CO (n=466)		AD (n=312)	CO (n=129)	
rs7624906	TT	0.73	0.78	0.134	0.71	0.71	0.828
	CT	0.25	0.21		0.27	0.28	
	CC	0.02	0.02		0.02	0.01	
rs4988514	TT	0.69	0.74	0.079	0.69	0.81	**0.002** (0.024)
	CT	0.29	0.25		0.29	0.19	
	CC	0.02	0.01		0.03	0	
rs3864101	GG	0.62	0.66	0.166	0.05	0.04	0.656
	GT	0.34	0.30		0.35	0.33	
	TT	0.04	0.04		0.60	0.63	
SNP	**Alleles**	AD (n=848)	CO (n=932)		AD (n=624)	CO (n=258)	
rs7624906	C	0.14	0.12	0.127	0.15	0.15	0.839
	T	0.86	0.88		0.85	0.85	
rs4988514	C	0.16	0.14	0.078	0.17	0.09	**0.004** (0.012)
	T	0.84	0.86		0.83	0.90	
rs3864101	G	0.79	0.81	0.160	0.78	0.79	0.661
	T	0.21	0.19		0.22	0.21	

Sample and Haplotype	**AD frequency (n=alleles)**	**CO frequency (n=alleles)**	**P-value[b]**	**OR (95% CI)**
Whole Sample:	AD (n=848 alleles)	CO (n=932 alleles)		
TTG	0.622 (528)	0.678 (632)	**0.012±0.004**	**0.78 (0.64-0.95)**
TTT	0.069 (59)	0.067 (62)	0.854±0.006	1.05 (0.73-1.52)
TCG	0.164 (139)	0.135 (126)	0.110±0.009	1.25 (0.97-1.63)
CTT	0.143 (122)	0.120 (112)	0.136±0.008	1.23 (0.93-1.62)
Overall			0.073±0.011	
APOE ε4:	AD (n=624 alleles)	CO (n=258 alleles)		
TTG	0.609 (380)	0.702 (181)	**0.012±0.004**	**0.66 (0.49-0.91)**
TTT	0.067 (42)	0.058 (15)	0.652±0.007	1.17 (0.64-2.15)
TCG	0.169 (106)	0.093 (24)	**0.004±0.001**	**2.00 (1.25-3.19)**
CTT	0.154 (96)	0.147 (38)	0.839±0.005	1.05 (0.70-1.58)
Overall			**0.012±0.002**	

To investigate the possible interaction between SST and APOE genes, ORs were calculated for SST and APOE genes separately and in combination, using cases and controls who had neither APOE ε4 nor SST C-alleles of SNP rs4988514 as the reference (Table 16). The results indicated that the SST C allele carriers with APOE ε4 allele had an increased risk of AD. Additionally, logistic interaction analysis supported the hypothesis that APOE ε4 and SST C alleles interact with AD (P=0.045; OR 2.0 with 95% CI 1.01-4.04). Both analyses showed that the SST C allele was not independently associated with AD risk, whereas APOE ε4 was an independent risk factor

Table 16. OR of AD cases when taking the subjects with neither APOE ε4 nor SST C alleles as reference.

[a] APOE ε4+	[a] SST rs4988514 C+	AD	Control	OR (95% CI)	[b]P-value	
-	-	80	240	reference		
-	+	32	97	0.99 (0.62-1.59)	1.0	
+	-	214	105	6.11 (4.33-8.63)	<0.001	[b]P<0.01;OR 2.0 (95% CI 1.21-3.32)
+	+	98	24	12.25 (7.33-20.46)	<0.001	

[a] The minus sign indicates cases/controls who lack these genotypes; plus indicates number of cases with these genotypes.
[b] Pearson x2 test.

4.5. Expression analysis of IDE and NEP; Study V

4.5.1. Aβ levels in AD like Tg mice and NT littermates

The cortical and cerebellar samples from APPswe/PSEN1(A246E) Tg mice and NT littermates at 4, 11 and 17 months of ages were used in this study. In APP/PSEN1(A246E) Tg mice, in agreement with published results (Borchelt et al., 1997) the formation of Aβ plaques

began in the hippocampus and soon extended to all cortical areas but the cerebellum remained free of plaques (Figure 15). During the studied period of time (from 4 month to 17 month), the total hippocampal levels of Aβ42 in APP/PSEN1(A246E) Tg mice increased exponentially from 15 to 5455 ng/g (Spearman's correlation r=0.79, P<0.01, Figure 15A) and those of Aβ40 from 70 to 4325 ng/g (Spearman's correlation r=0.81, P<0.01). No Aβ deposition or detectable Aβ levels were observed in NT littermates at any age.

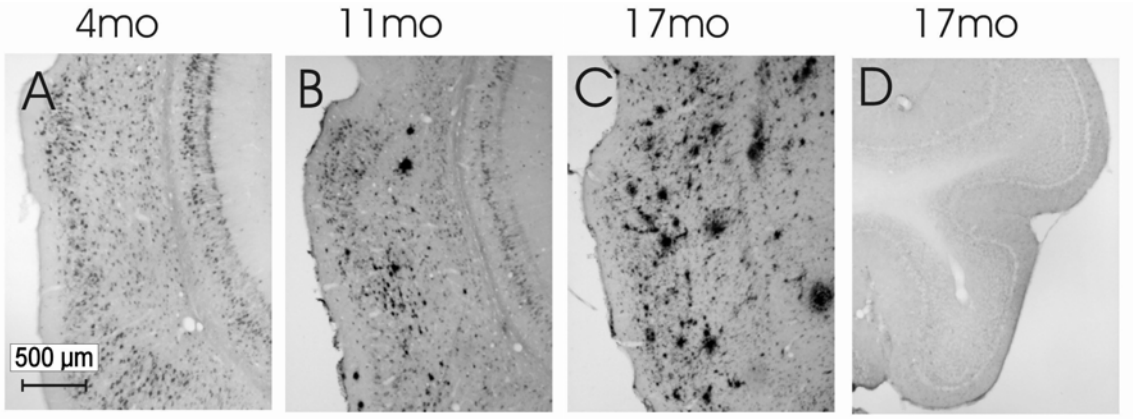


Figure15. Low power photomicrographs (A-D) to demonstrate the pattern of Aβ labeling with increasing age in the entorhinal cortex (A-C) and cerebellum (D) (mo=month) of APP/PSEN1(A246E) mouce brain. Note that cerebellum remains free of plaques. Scale bar in A equals 500 µm and applies to all (Thomas van Groen).

4.5.2. IDE and NEP mRNA levels in cortex of AD like Tg mice and NT littermates

A statistically significant positive correlation between Aβ40 or Aβ42 and relative mRNA-levels of IDE in cortex was observed (Spearman's r = 0.33, p = 0.030 and r = 0.35, p = 0.018, respectively). The IDE mRNA-levels in NT mice showed an age-related decrease from 4 to 11 months of age (Figure16B). Consequently, the IDE mRNA-levels in the cortex differed significantly between APPswe/PSEN1(A246E) Tg mice and NT littermates at the age of 11 months (p=0.002, Figure 16B). The cortical NEP mRNA-levels decreased age-dependently in both APPswe/PSEN1(A246E) Tg mice and NT littermates. However, the cortical NEP mRNA-levels were higher in APPswe/PSEN1(A246E) Tg mice compared to NT littermates

from 11 to 17 months of age (Figure 16C) although the difference did not reach statistical significance. Interestingly, a significant positive correlation was observed also between IDE and NEP mRNA-levels in cortex of 17-month-old mice (Spearman's correlation r=0.51, $p<0.05$).

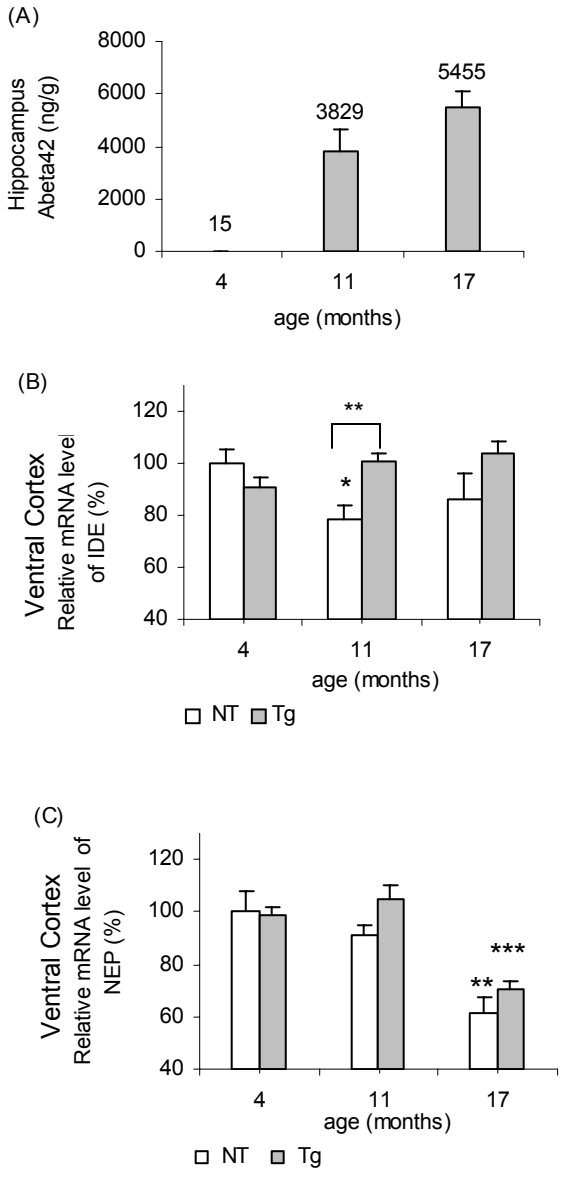


Figure 16. The relative mRNA-levels of IDE and NEP in the cortex compared to total hippocampal levels of Aβ in mice brain. In panel A the total Aβ42 levels were standardized to brain tissue weight and expressed as ng Aβ/g brain tissue (values above column). APPswe/PSEN1 (A246E) Tg mice exhibited an exponential increase in hippocampal Aβ42 levels from 4 to 17 months of age (Spearman's correlation r=0.79, $p<0.01$). In panels B and C, mRNA levels of IDE and NEP are expressed as percentage changes relative to the levels of 4-month-old NT mice. The IDE mRNA-levels differed significantly between APPswe/PSEN1 (A246E) Tg mice and NT littermates at the age of 11 months (p-value: ** <0.01, panel B). Age related changes are presented by comparing each time point to 4-month-old NT or APPswe/PSEN1 (A246E) Tg mice (p-values: *<0.05, **<0.01, ***<0.001). Note that the age-related increase in IDE mRNA-levels paralleled the age-related Aβ accumulation in APPswe/PSEN1 (A246E) Tg mice. The numbers of male mice used in the study were as follows: 4-month-old APPswe/PSEN1 (A246E) Tg (n=15) and NT (n=13), 11-month-old APPswe/PSEN1 (A246E) Tg (n=15) and NT (17) and 17-month-old APPswe/PSEN1 (A246E) Tg (n=17) and NT (n=5).

4.5.3. IDE and NEP mRNA levels in cerebellum of AD like Tg mice and NT littermates

The relative mRNA-levels of IDE in the cerebellum did not differ between APPswe/PSEN1(A246E) Tg mice and NT littermates at any age (Figure 17A). An age-dependent increase of mRNA-levels of IDE was seen in both APPswe/PSEN1(A246E) Tg mice and NT littermates from 4 to 11 months ($p<0.001$, Figure 17A). As was the case in the cortex, mRNA-levels of NEP in the cerebellum decreased age-dependently in APPswe/PSEN1(A246E) Tg mice from 4 to 17 months ($p<0.001$, Figure 17B). The same trend of an age-dependent decrease in the mRNA-levels of NEP was also seen in NT mice. Interestingly, the NEP mRNA-levels in the cerebellum were significantly higher in APPswe/PSEN1(A246E) Tg mice compared to the NT littermates at the age of 11 months ($p= 0.047$). Additionally, the mRNA-levels of IDE were significantly higher in the cerebellum compared to the cortex at all ages (p-values for both APPswe/PSEN1(A246E) Tg mice and NT littermates: 4 months: $p<0.001$, 11 months: $p<0.001$, and 17 months: $p<0.05$; the average increase being 2.4-fold in NT littermates and 1.8-fold in APPswe/PSEN1(A246E) Tg mice). Opposite results were observed for NEP, such that NEP mRNA levels were significantly lower in the cerebellum than in the cortex at all ages (p-values for both APPswe/PSEN1(A246E) Tg mice and NT littermates: 4 months: $p<0.001$, 11 months: $p<0.001$, and 17 months: $p<0.01$; the average decrease being 4.0-fold in NT littermates and 3.9-fold in APPswe/PSEN1(A246E) Tg mice).

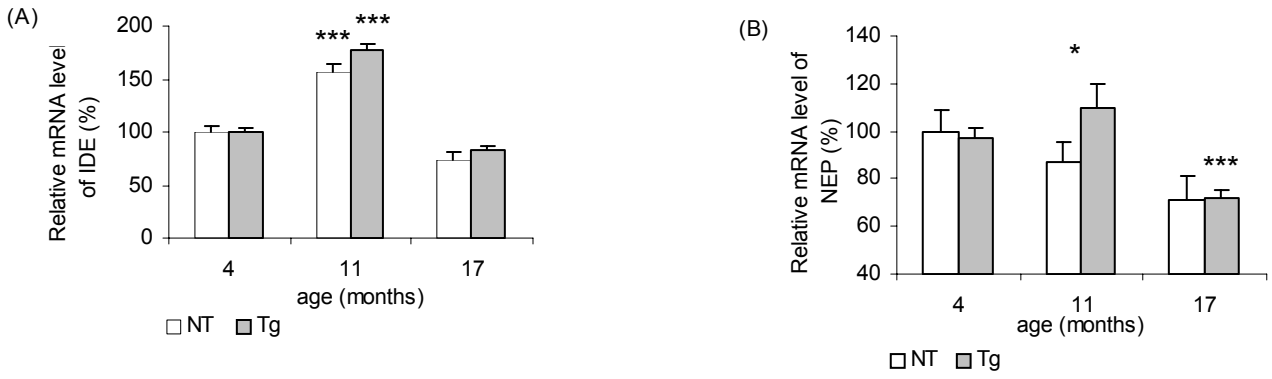


Figure 17. The relative mRNA-levels of IDE and NEP in the cerebellum. The mRNA levels of IDE and NEP in panels A and B are expressed as percentage changes relative to the levels of 4-month-old NT mice. The NEP mRNA-levels differed significantly between APPswe/PSEN1(A246E) Tg mice and NT littermates at the age of 11 months (p-value: * <0.05, panel B). Age related changes are illustrated by comparing each time point to 4-month-old NT or APPswe/PSEN1(A246E) Tg mice (p-values: * <0.05, ** <0.01, *** <0.001). The numbers of male mice used in the study were as follows: 4-month-old APPswe/PSEN1(A246E) Tg (n=15) and NT (n=13), 11-month-old APPswe/PSEN1(A246E) Tg (n=15) and NT (17) and 17-month-old APPswe/PSEN1(A246E) Tg (n=17) and NT (n=5).

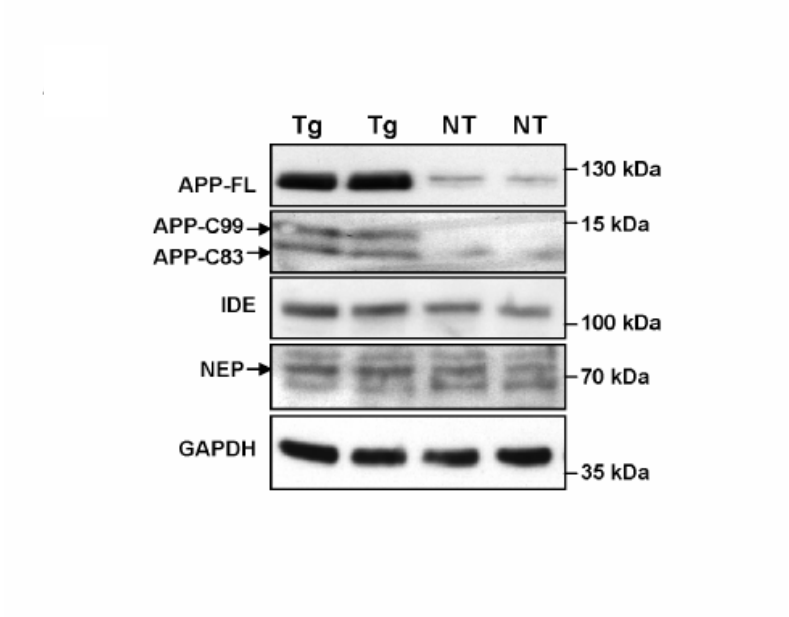
4.5.4. IDE and NEP protein levels in cortex of AD like Tg mice lines and NT littermates

Since the IDE mRNA-levels differed significantly between 11-month-old APPswe/PSEN1(A246E) Tg mice and NT littermates, IDE and NEP protein levels were measured in the cortex from additional mice of closely matching ages (APPswe/PSEN1(A246E) Tg mice n=11 and NT littermates n=7, 12 months of age; APPswe/PSEN1(dE9) Tg mice n=11 and NT littermates n=7, 9 months of age). In addition, protein levels of full-length APP (APP-FL) and C-terminal fragments of APP, APP-C83 and APP-C99 were measured.

GAPDH normalized protein levels were measured from the cortex of 12 month old APPswe/PSEN1(A246E) Tg mice which develop the first signs of amyloid plaques around 9 months of age. Protein levels of APP-FL in APPswe/PSEN1(A246E) Tg mice were on average 5.3-fold higher (range 3.5-7.5-fold) than in their NT littermates ($p<0.001$, Figure 18A and B). Similarly, levels of APP-C83 were significantly higher in APPswe/PSEN1(A246E) Tg

mice compared to NT littermates ($p<0.01$, Figure 18A and B). Levels of APP-C99 were below the detection level in NT littermates but the fragment was clearly seen in APPswe/PSEN1(A246E) Tg mice (Figure 18A). Interestingly, in agreement with the mean mRNA-levels of IDE and NEP, the protein levels of IDE and NEP in APPswe/PSEN1(A246E) Tg mice were higher than in NT littermates (11% and 16% increase, respectively), although the difference did not reach statistical significance (Figure 18A and C). We also calculated the mean protein levels of IDE and NEP by normalizing the data for α-tubulin and both results were consistent (data not shown). To verify these results, α-tubulin normalized protein levels were measured from the cortex of 9 month old APPswe/PSEN1(dE9) mice which develop plaques already around 4 months of age. Similarly, protein levels of APP-FL in APPswe/PSEN1(dE9) Tg mice were on average 2.8-fold higher (range 1.7-4.0-fold) than in their NT littermates ($p<0.001$) and protein levels of APP-C83 were significantly higher in APPswe/PSEN1(dE9) Tg mice compared to NT littermates ($p<0.001$, Figure 19A and B). Protein levels of APP-C99 were below detection level in NT mice but the protein was clearly seen in APPswe/PSEN1(dE9) Tg mice (Figure 19A). Interestingly, protein levels of IDE were significantly higher in APPswe/PSEN1(dE9) Tg mice compared to NT littermates (58 % increase, $p<0.01$, Figure 19A and B). Protein levels of NEP were higher in APPswe/PSEN1(dE9) Tg mice compared to NT littermates, although the increase was not significant (37 % increase).

A)



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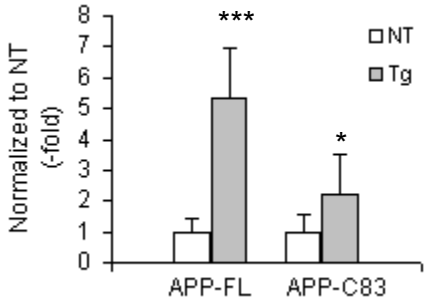


C)

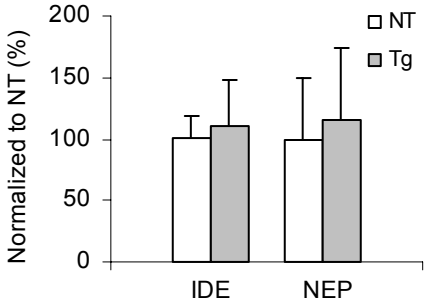
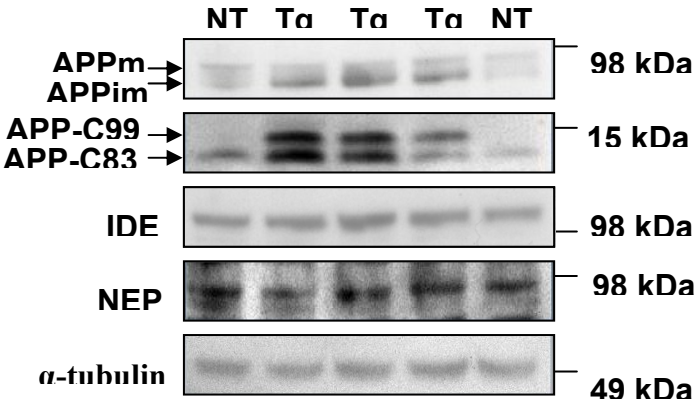
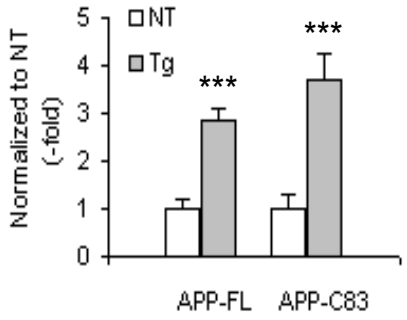


Figure 18A) Protein levels of APP-FL, APP-C83, APP-C99, IDE and NEP were analyzed from the cortex of 12-month-old APPswe/PSEN1(A246E) Tg mice (n=9) and NT littermates (n=9). B) APP-FL and APP-C83 protein levels were significantly higher in APPswe/PSEN1(A246E) Tg mice compared to NT littermates and similarly C) IDE and NEP protein levels of cortex in APPswe/PSEN1(A246E) Tg mice were slightly higher compared to NT littermates. GAPDH normalized levels are indicated in figures.

A)



B)



C)

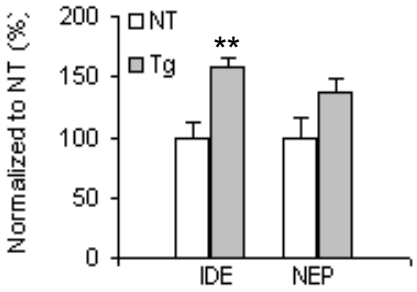


Figure 19A) Protein levels of APP-FL, APP-C83, APP-C99, IDE and NEP were analyzed from the cortex of 9-month-old APPswe/PSEN1(dE9) Tg mice (n=11) and NT littermates (n=7). B) APP-FL and APP-C83 protein levels were significantly higher in APPswe/PSEN1(dE9) Tg mice compared to NT littermates and similarly C) IDE and NEP protein levels of cortex in APPswe/PSEN1(dE9) Tg mice were higher compared to NT littermates. Note that the increase was significant in IDE study (58% increase, $p<0.01$). Alpha-tubulin normalized levels are indicated in figures.

Since a significant positive correlation was observed between IDE and NEP mRNA-levels in 17-month-old mice, we tested whether we could detect a correlation between these two proteases at the protein level by using APPswe/PSEN1(A246E) Tg mice and their NT littermates. Interestingly, a significant positive correlation was found between protein levels of

IDE and NEP (Spearman's correlation r=0.50, p<0.05, Figure 20A). In addition, the protein levels of IDE correlated with the protein levels of APP-FL (Spearman's correlation r=0.54, p<0.05, Figure 20B). The correlation between the protein levels of NEP and APP-FL did not reach a significant value (Spearman`s correlation r=0.30, p=0.23). In addition, the protein levels of APP-FL correlated with the protein levels of APP-C83 (Spearman's correlation r=0.5, p<0.05). In APPswe/PSEN1(A246E) Tg mice the protein levels of APP-FL did not correlate with the protein levels of APP-C99. In contrast, levels of the control protein GAPDH or α-tubulin did not correlate with any of the measured protein levels. Similar findings were observed from APPswe/PSEN1(dE9) mice study. A significant positive correlation was found between IDE and NEP protein levels (Spearman's correlation r=0.75, P<0.001, Figure 20C). Similarly, the protein levels of IDE nominally correlated with protein levels of APP-FL (Spearman's correlation r= 0.53, p<0.05, Fig 19 D). We did not find a significant correlation between the protein levels of NEP and APP-FL (Spearman`s correlation r=0.26, p=0.31). In addition, the protein levels of APP-FL correlated with the protein levels of APP-C83 (Spearman's correlation r=0.71, p<0.01). In APPswe/PSEN1(dE9) Tg mice the protein levels of APP-FL did not correlate with the protein levels of APP-C99. In contrast, levels of the control protein α-tubulin did not correlate with any of the measured protein levels.

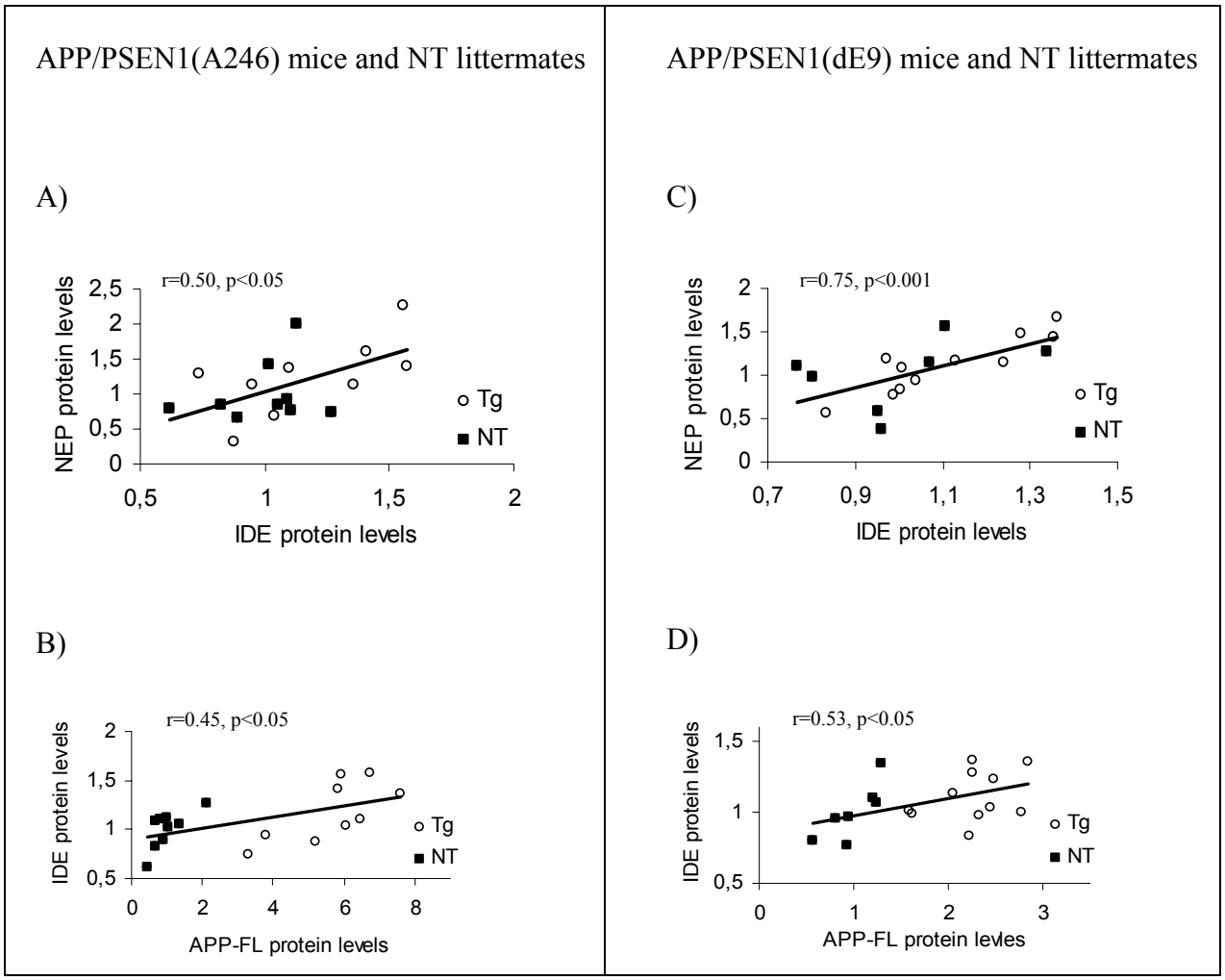


Figure 20. A and C) A significant correlation was found between IDE and NEP protein levels from the cortex region in both studied mice lines. B and C) A nominally significant correlation was also found between protein levels of IDE and APP-FL in both studies. Protein levels are indicated in arbitrary units. When Tg and control groups of APP/PSEN1(A246) study were analyzed separately, a significant correlation was found between protein levels of IDE and APP-FL in Tg group ($r=0.68$ $p<0.05$) but not in the control group ($r=0.63$ $p=0.07$). When Tg and control groups of APP/PSEN1(dE9) study were analyzed separately, a significant correlation was found between protein levels of IDE and APP-FL in the control group ($r=0.86$ $p<0.05$) but not in the Tg group ($r=0.32$ $p=0.34$).

5. DISCUSSION

5.1. Methodological considerations of association studies I to IV

There have been enormous advances in genetics studies resulting from the increased knowledge about the human genome. New data allows the use of novel powerful methods to find putative risk genes of complex diseases. Population isolates are increasingly being used in attempts to map the genes underlying complex diseases. Variations in demographic histories between populations generate substantial differences in the extent and distribution of LD and therefore cause variability between populations in the number of SNPs required for association studies. It has been proposed that population isolates have longer stretches of LD than outbred populations and may require fewer markers for genome-wide association studies. It has been also proposed that the Finnish population is particularly suitable for association studies because of its homogenous genetic background (Peltonen et al., 1999, Jorde et al., 2000, Peltonen, 2000, Service et al., 2006). Service et al. (2006) concluded that association analysis in samples from Finland would require at least 30% fewer markers than studies in outbred populations (Service et al., 2006). On the contrary, it has been speculated that isolated populations of constant size will be more useful for mapping genes underlying complex disorders than populations (such as that of Finland) that have recently expanded in size (Terwilliger et al., 1998). Further studies are needed to clarify the utility of constant-sized populations for association-based mapping of common disease genes.

The strength of the present study is in the homogeneity of the patients and control subjects. Both study patients and controls were derived from a geographically restricted area in northern Savo or Kuopio city. There were an adequate number of subjects in the whole study population to have detected true differences between the compared groups. We estimated power in our whole dataset (450 cases and 450 controls) and found that our study sample have >80% power to detect a moderate odds ratio 1.5 at a significance level of less than 0.05. The power calculations imply that our data is sufficient to detect relatively low genetic risk genes between all AD cases and controls. Power calculations were carried out using the Genetic Power Calculator (Purcell et al., 2003). Additionally, the diagnostic procedure was carried out by experienced personnel. The diagnosis of AD was accepted only if made according to the globally used DSM-IIIR criteria for dementia (APA, 1987) and

NINCDS-ADRDA criteria for AD (McKhann et al., 1984). In this thesis, the candidate genes in close proximity to loci which have been shown to associate with AD and other genes which are biologically related to AD represented the targets for genetic and functional studies of AD.

Although huge advances have been made in recent years association studies related to complex disease aetiology are known to be complicated. It is more difficult to unravel the aetiology of complex diseases, which are more likely to be caused by many genes, each making a small overall contribution and slightly elevating relative risk. If a disease is not lethal, then disease-causing mutations may persist in populations for a long time (Jorde et al., 2000). In this case, the mutation may persist for such a long period of time that disequilibrium will dissipate to undetectable levels. In the case of a minor risk, multiple testing issues reduce the statistical power to obtain significant results. Genetic heterogeneity, population stratification, clinical diagnosis of used samples and sample sizes affect the observed association results as well. Due to these facts, association studies between a putative risk gene and different ethnic populations can give rise to a spectrum of results (see www.alzgene.org). It has been claimed that a better way is to perform independent replication studies rather than using correction of data that might lead to false negative results. For example, Oda et al. have corrected their results of the NEP study by using Bonferron`s method and reported a negative result. It is possible that there are false positive and false negative results concerning the particular gene. In following chapters, the results of this thesis work are discussed in the light of the published literature. The numbers of positive and negative results are based on reported case-control studies in AlzGene database before April 2007 (see www.alzgene.org).

5.2. BDNF and TrkB polymorphisms; Study I

We genotyped multiple SNPs among AD patients and controls originated from eastern part of the Finland in order to examine the genetic association of the BDNF and TrkB genes with AD. Although BDNF, via activation of its receptor TrkB, is considered as a plausible candidate for contributing to AD we did not find any association between the unstratified Finnish AD population and the polymorphisms of BDNF and TrkB.

The functional polymorphism in the coding region of BDNF gene (rs6265), 196A/G, Met66Val) has recently been investigated in many studies (see www.alzgene.org). In

Caucasian populatios rs6265 has been claimed to associate to sporadic AD in 2 study populations but negative results have been observed in 11 other studies. Another well studied polymorphic site is C270A which is located near to the rs6265 polymorphic site (74 bp downstream). The minor allele frequencies of this SNP are low, especially in the control population and thus it is only marginally informative. In Caucasian populations, there are two positive and seven negative findings concerning this polymorphic site. Riemenschneider et al. (2002) concluded that the BDNF C270T polymorphism is a relevant risk factor for AD, particularly in patients lacking the APOE ε4 allele in German population. Olin et al (2005) also detected an association of this same C270T polymorphism between AD and controls in US population but their results were independent of the APOE ε4 allele. Interestingly, in an Asian population, rs6265 and C270T polymorphisms have been shown to associate to AD in 4 studies with one negative result being reported. The meta-analysis of rs6265 by using all published AD association (case-control) studies showed no significant risk effect but interestingly, when only Caucasian AD association studies were used, a nominal OR was observed (OR 1.1; 95 CI 1.01-1.19, data from date 18.04.2007; see www.alzgene.org). Meta-analysis of C270T by using all or only aCaucasian population did not show any risk effect.

In our study, the data showed that BDNF and TrkB genes did not contribute any significant risk effect among Finnish AD patients. We also studied the BDNF SNP rs6265 and no association was found to AD. Our results are in line with another study from Finland (Saarela et al., 2006). Additionally, we studied four other SNPs which cover the whole BDNF gene region. None of these SNPs or haplotypes revealed any signal for association. These results are in line with the majority of results obtained from a Caucasian population. Stratification according to familial status revealed a possible allele and haplotype association of the BDNF gene with AD among the sporadic group instead of the familial AD group. However, this result should be viewed with caution since multiple testing issues limit the power to find conclusive evidence of an association and independent replication studies are needed to verify this finding in other populations.

In the TrkB study we had SNPs from the promoter region and since TrkB is a large (~356 kb) gene, the lack of association in promoter region does not exclude the possibility that an alteration in other parts e.g. the coding or non-coding region of the TrkB gene, could increase the risk for AD. Our data suggest that the BDNF and TrkB genes do not contribute a

significant risk effect among the Finnish AD population. However, it is still possible that regulation of BDNF transcription or translation may be altered in AD (Connor et al., 1997, Garzon and Fahnestock, 2007). Interestingly, Aβ oligomers have been shown to decrease the mRNA levels of BDNF in human neuroblastoma cells (Garzon and Fahnestock, 2007). Perhaps it is time to focus on factors affecting transcriptional or translational regulation of BDNF rather than studying genetic factors related to BDNF gene itself.

5.3. NEP polymorphisms; Study II

NEP is an Aβ degrading enzyme expressed in the brain, and accumulation of Aβ is the neuropathological hallmark in AD. Our candidate gene based association study with the NEP gene in a large clinic based series of AD and control subjects originating from the eastern part of Finland implicated NEP as a risk gene for AD. SNPs rs989692 and rs3736187 had significantly different allelic and genotypic frequencies between AD and control subjects and the haplotype analysis reinforced this observation. Others have also conducted the same kind of studies in independent populations (see www.alzgene.org). In addition to our study, one positive and one negative study have been reported in Caucasian populations. In Asian populations two positive and two negative studies have been published. The data is controversial. However, we and Shi et al (2005) have studied several SNPs which allow the use of powerful haplotype analysis. Both of these studies were positive which strengthens the idea of association between NEP polymorphisms and AD. It is possible that NEP gene contributes to the pathogenesis of AD in both populations but additional independent replication studies are clearly needed. According to our results, polymorphisms in the NEP gene confer an increased risk for AD among un-stratified Finnish AD cases. Additionally, because of its biological function, NEP is a highly relevant candidate gene for AD. Based on the genetic findings it would be interestingto study what happens in the organism because of the altered genetic information. Additionally, based on the biological function of NEP, functional studies to clarify the regulation of NEP expression are needed.

In study II, we reported one haplotype block in the studied NEP gene region. However, based on the Haploview program two haplotype blocks can be identified. In study II, we used

a threshold D´value of 0.30 (Iivonen et al., 2004) which was the common way to do the analysis at that time. However, threshold D´value >0.95 (Confidence interval) is more accurate and the Haploview software uses these parameters (Gabriel et al., 2002). Nonetheless, in both methods, single locus analysis gave consistent results (data not shown). Additionally, based on the Haploview software, the haplotype CA comprising of SNPs rs3773882 (SNP6) and rs3736187 (SNP7) were significantly over-represented in AD cases compared to controls. In study II, the significantly over-represented haplotype TTCCCCA in AD cases contains this CA haplotype. Based on the results from Haploview software, single SNP rs989692 (SNP1) was also significantly over-represented in AD cases compared to controls. Interestingly, the data may indicate that there is another risk region in the NEP gene.

5.4. IDE and HHEX polymorphisms; Study III

IDE has been demonstrated to play a key role in Aβ degradation in vitro and in vivo and is selective for the Aβ monomer (Vekrellis et al., 2000, Farris et al., 2003, Farris et al., 2004). IDE is located on chromosome 10q24 close to a region for which linkage and association with late-onset AD have been previously described (Bertram et al., 2000). In addition, genetic linkage was simultaneously reported on chromosome 10 in a region approximately 30 Mb from IDE (Ertekin-Taner et al., 2000, Myers et al., 2000). Numerous subsequent case-control studies have reported genetic association between IDE and AD, though some others failed to observe significant effects (see http://www.alzgene.org). To assess the genetic contribution of IDE in the Finnish population, we genotyped six non-coding SNPs in the 276 kb genomic region spanning the IDE and HHEX in a large clinic-based series of AD patients and control subjects. HHEX gene is located next to the IDE gene and by analyzing one SNP from that region we could study more precisely the interval region. Two IDE SNPs, rs4646953 and rs4646955 were significantly associated with AD conferring an approximately two-fold increased risk for AD. Additionally, the risk effect appeared to be somewhat stronger among the LOAD patients when compared to whole cohort.

Strong evidence of linkage to LOAD has been observed on chromosome 10, which implicates a wide region and at least one disease-susceptibility locus (Bertram et al., 2000, Ertekin-Taner et al., 2000, Myers et al., 2000). Although significant associations with several

biological candidate genes including IDE on chromosome 10 have been reported, these findings have not been consistently replicated and they remain controversial. Grupe et al. (2006) found a novel locus on chromosome 10 showing a strong association with LOAD (Grupe et al., 2006). This observed locus is located relatively close to linkage peaks identified earlier. However, the most consistently associated SNP marker, rs498055, is located in a gene annotated as an RPS3A homologue in the Entrez Gene database. RPS3A is an important player in the early phase of apoptosis, a feature observed in AD-affected brains. They conclude that the results point to a role of RPS3A homologue gene, adjacent genes, or other functional variants (e.g. noncoding RNAs) in the pathogenesis of AD. This new region should be further characterized and independent replication studies are needed to verify this finding in other populations.

A few case-control studies have reported that genetic variants in IDE increase the risk for AD (see www.alzgene.org). Until April 2007, in Caucasian populations a total of four positive and 18 negative association studies had been previously described. Meta-analysis of commonly studied SNPs rs3758505 (IDE1), rs4646953 (IDE2) and SNP rs2251101 (IDE7) showed no significant risk effect (see www.alzgene.org). There are 3 negative studies in Asian populations. The conflicting results in different populations may be due to genetic heterogeneity, population stratification, and a small genetic effect confounded by sample sizes insufficient to replicate the initial reports. However, in our sample, polymorphisms in the IDE gene confer an increased risk for AD in these Finnish patients. Additionally, IDE is interesting because of its biological background and further functional studies are clearly warranted.

5.5. SST polymorphisms; Study IV

The SST gene is located in close proximity (~ 500 kb) to microsatellite marker D3S2436 on chromosome 3q28 which we have previously shown to associate with late-onset AD (Hiltunen et al., 2001a). Two other family-based studies of individuals from different ethnic backgrounds have also identified the same locus as possibly harboring a genetic variant that increases late-onset AD susceptibility (Hahs et al., 2006, Lee et al., 2006). We evaluated whether genetic variations across the SST gene could modify the risk for AD in our Finnish population. As far as we are aware, this is the first genetic association study between SST and

AD.

It is known that the SST protein levels are significantly lower among AD patients carrying the APOE ε4 allele (Grouselle et al., 1998). Because of this biological relevance we wanted to study the genetic background of the SST gene in this particular AD group and stratified the data according to APOE status. We found that in APOE ε4 carrier group, an age and sex adjusted OR for the risk of AD in C-allele carriers of the rs4988514 was 2.05 (95% CI 1.23-3.41; P<0.01) and the haplotype data reinforced this observation. Strong LD was detected between all three SNPs spanning the whole genomic region (~ 2300 bp) pointing to one haplotype block. Risk haplotype TCG encompassed the C allele for SNP rs4988514, which was also significantly overrepresented among the AD APOE ε4 group in the single locus analyses. Additionally, SNP rs4988514 may interact with the APOE ε4 allele to increase the risk of AD. Due to the smaller sample sizes in the APOE stratified study groups, independent replication studies will be needed to verify these significant findings in other populations.

The observation that SST and the APOE ε4 allele may interact was interesting. The APOE ε4 allele is the major genetic risk factor for both EOAD and LOAD (Saunders et al., 1993, Chartier-Harlin et al., 1994, Farrer et al., 1997). It has been shown to be associated with higher amyloid-plaque burden in the brain and decreased levels of CSF Aβ42 (Corder et al., 1993b, Schmechel et al., 1993, Galasko et al., 1998, Prince et al., 2004, Sunderland et al., 2004). Interestingly, SST protein levels are known to be significantly lower in AD patients carrying the APOE ε4 allele (Grouselle et al., 1998). Moreover, SST is believed to regulate the metabolism of Aβ in the brain by modulating the protein turnover and cellular localization of Aβ degrading enzyme NEP (Saito et al., 2005). One possible hypothesis is that the down-regulation of SST expression in the human brain due to the possible altered genetic information initiates a gradual decline in NEP activity, resulting in a corresponding elevation in the steady-state levels of Aβ, and when this continues for a decade or more it ultimately evokes Aβ accumulation that triggers the AD pathological cascade in AD patients carrying the APOE ε4 allele. It is not known whether the observed association reflects a functional alteration in the SST gene leading to lower SST expression levels in APOE ε4 AD patients; clarification will require further studies.

The haplotype TTG was significantly under-represented among the entire study population of AD patients and also when stratified according to APOE ε4 allele. Interestingly,

this major haplotype TTG may provide a protective effect against AD. Consistently, the protective haplotype TTG does not include the risk allele C of SNP rs4988514.

The three genotyped markers for SST covered ~10 kb and were located ~1.4 kb upstream, in the 3`UTR region and ~7kb downstream of the gene and were found to be in complete LD with each other. According to the HapMap database, they belong to a 40 kb haplotype block as defined by the Confidence intervals of LD (Gabriel et al., 2002) from Utah residents with ancestry from northern and western Europe population (CEU) encompassing the whole genomic region of SST. No other genes are known to exist within this haplotype block, reinforcing the hypothesis that SST may be a putative risk gene for AD. Additionally, the SST gene is located a mere ~500 kb away from the microsatellite marker D3S2436 associated with LOAD (Hiltunen et al., 2001a). This workflow is a good example of how a particular locus region can be found and how it is further characterized. The next step will be to study the functional role of SST and to elucidate what happens in the organism because of possible altered genetic information.

5.6. Methodological considerations of expression analysis of IDE and NEP; Study V

There are many benefits of using real-time PCR in preference to other methods to quantify mRNA levels. It is highly sensitive, can be highly sequence-specific, has little to no post-amplification processing, and is amenable to increasing sample throughput. However, to obtain an optimal benefit from these advantages, is required a clear understanding of the many options available for running a real time PCR experiment (Wong and Medrano, 2005). One important step is the sample preparation. Samples must be taken avoiding contamination, frozen in liquid nitrogen and transferred immediately to the freezer (at least -70 °C) after sampling. RNA isolation, DNase treatment if needed and cDNA synthesis needs also extreme care to avoid contamination and to obtain a sufficient amount material for further analysis (Bustin, 2002). By verifying each sample preparation step, the material will be valid and the QPCR method is easy to perform. Another important step is to validate the best endogenous control gene for analysis. The significance of the obtained results strongly depends on how the data is normalized to compensate for differences between the samples. The most widely used approach is to use endogenous reference genes (=housekeeping genes) as internal standards

(Thellin et al., 1999, Wong and Medrano, 2005). This approach is heavily debated in the literature because none of the reference genes are stably expressed throughout all biological samples. The ideal control gene should be expressed in an unchanging fashion regardless of experimental conditions, including different tissue or cell types, developmental stage, or sample treatment. Ribosomal RNA is another possible reference gene for normalization. However rRNAs are transcribed with a different polymerase than mRNA and changes in polymerase activity may not affect both types of RNA expression equally. Gene expression measurements may be normalized also against total RNA concentrations. However, total RNA levels are affected by cellular processes, reverse transcription efficiency is not considered and normalization is only as accurate as the RNA quantification.

The use of housekeeping genes as internal controls needs to be examined critically; otherwise they can be lead to misleading quantification results. There are voices favouring or criticising the use of GAPDH as an endogenous control gene (Bustin, 2002, Gilsbach et al., 2006). Based on Thellin et al. (1999) study, GAPDH can be used as internal standard without any problems when comparing the mRNA results of cerebellum, brain cortex, brain stem or subcortical structures of murine brain (Thellin et al., 1999). Since we used samples of cortex and cerebellum from mice, it is believed that the use of GAPDH as an internal control should give accurate results. Moreover, according to our analysis, GAPDH showed a stable occurrence between samples. The inclusion of one particular sample, which is called the calibrator, in each run, improves the comparison between different runs.

We used Ct method and standard curve method to calculate relative values. The benefit of resorting to the Ct method is that it eliminates the need for standard curves for relative quantitation. However, it does require more verification than the standard curve method and in some cases it may not be suitable to use. The standard curve method is easier to perform and it does give accurate data. For statistical testing we used nonparametric tests because of the small sample number and the abnormal distribution of the values.

At the cellular level, regulatory elements form a wide network by affecting each other and the final effect is the sum of inhibitory and activator effects. Nature regulates genes function in many ways e.g. by controlling histone acetylation, transcription, translation, post modifications and activity. When gene function is being studied, it is important to clarify the expression levels at different regulatory levels. High levels of mRNA do not necessarily lead

to high levels of protein and vice-versa, since mRNA stability might play important roles in the translation process. Even more importantly, since Aβ is degraded by IDE and NEP, it is the specific activity of these enzymes, rather than either their mRNA or protein levels, that plays a key role in Aβ degradation. However, mRNA and protein levels of IDE and NEP proteases are indicative. We have measured the protein levels of IDE and NEP by using standard western blotting. We are currently setting up activity measurements of IDE and NEP to clarify the function of these proteins.

5.7. Expression analysis of IDE and NEP; Study V

The availability of Tg mice which develop Aβ plaques in the brain with ageing represents a unique experimental approach to study how the pathological accumulation of Aβ can modify its own degradation (Borchelt et al., 1997). We examined the mRNA levels of IDE and NEP proteases from the cortex and cerebellum of APPswe/PSEN1(A246E) double Tg mice and their non-transgenic (NT) littermates at three ages by using real-time PCR quantification (Schmittgen, 2001). Protein levels of IDE and NEP were also measured from 12 month old APPswe/PSEN1(A246E) mice as well as from 9 month old APPswe/PSEN1(dE9) mice.

APPswe/PSEN1(A246E) mice carried mutated APPswe and PSEN1-A246E genes. The Tg strain exhibits neuropathological changes such as accumulation of amyloid plaques in the brain starting around the age of 9 months (Borchelt et al., 1997). The formation of amyloid plaques is primarily detectable in the subiculum and caudal cortex, and extends later to hippocampus and other cortical areas. At the age of 12 months, Tg mice exhibit significant deficits in water maze learning when compared to wild type littermates (Puolivali et al., 2002). According to our APP-FL protein measurements, the used APPswe/PSEN1(A246E) mice line showed approximately 5.3 times higher (range 3.5-7.5-fold) APP-FL expression levels of cortex region in Tg mice compared to NT-littermates at the age of 12 month. C-terminal fragments of APP, C83 and C99, were also clearly upregulated in Tg mice compared to NT-littermates. However, these mice do not show cholinergic cell loss in the basal forebrain at an adult age. Experiments were performed with APPswe/PSEN1(A246E) 4-, 11- and 17-month-old mice that are representatives of different age groups according to the following age criteria: 4 months; young adults without Aβ plaques, 11 months; Aβ have started to

accumulate to plaques around 9 months of age and 17 months; mice with high levels of Aβ plaques. Additionally 12 month old APPswe/PSEN1(A246E) mice and 9 month old APPswe/PSEN1(dE9) mice were used for protein quantitation. The Aβ accumulation is more severe in these APPswe/PSEN1(dE9) mice, so that the first plaques develop around 4 months of age. Protein levels of APP-FL in APPswe/PSEN1(dE9) mice were on average 2.8-fold higher (range 1.7-4.0-fold) than in their NT littermates ($p<0.001$). Protein levels of APP-FL are not as high as those found in APPswe/PSEN1(A246E) mice but the range is more stable. Levels of APP-C83 were significantly higher in APPswe/PSEN1(dE9) mice compared to their NT littermates. Levels of APP-C99 were below the detection level in NT mice but the fragment was clearly seen in APPswe/PSEN1(dE9) mice.

As mentioned above, the APPswe/PSEN1(A246E) mice develop the first signs of amyloid plaques around 9 months of age (Borchelt et al., 1997). Interestingly, soon after that critical age, mRNA levels as well as protein levels of IDE and NEP became elevated in Tg mice compared to the NT littermates. Additionally, in 9 month old APPswe/PSEN1(dE9) mice, protein levels of IDE and NEP became even more elevated in Tg mice compared to the NT littermates. It is noteworthy, that in these APPswe/PSEN1(dE9) mice, the first plaques develop around 4 months of age. These results may reflect up-regulation of the IDE/NEP levels in response to the permanently elevated amounts of APP or its derivatives (Aβ/AICD) as well as Aβ accumulation during aging. However, the degree of up-regulation of Aβ-degrading proteases does not appear to be sufficient to compensate for the severe ongoing Aβ accumulation.

Up-regulation of IDE mRNA levels may be controlled by Aβ40 and Aβ42 production, since hippocampal Aβ40 and Aβ42 levels exhibited a positive correlation with mRNA levels of IDE but not with those of NEP in the cortex. Additionally, protein levels of APP-FL and IDE correlated in both studied mouse lines. However, when Tg and control groups were analyzed separately, we found significant correlation between protein levels of IDE and APP-FL in two groups instead of four (see Figure 20 legends). The number of cases could have influenced these results. In the light of these results, regulation of IDE through APP-FL or its derivatives other than Aβ such as AICD remains a possible alternative. Currently the possible regulation of NEP by APP-FL or its derivatives is a subject of debate. First, Pardossi-Piquard et al. (Pardossi-Piquard et al., 2005) showed that the regulation of NEP expression is

presenilin-dependent and mediated by AICD. However, in a recent attempt to replicate that study, Chen and Selkoe (Chen and Selkoe, 2007) failed to observed any such a regulation. Our results present additional evidence for the hypothesis that the increased expression of IDE and NEP may be a response to the continually elevated levels of APP or its derivatives. Further studies to clarify the exact regulation mechanisms are warranted.

In our study, an interesting finding was the possible cross-regulation of IDE and NEP protein levels. This was seen in both Tg and NT mice. A recent paper indicated that APP derived AICD possibly regulate the NEP protein levels (Pardossi-Piquard et al., 2005). Further studies will be needed to clarify whether APP or some unknown factor(s) is the link to IDE and NEP cross-regulation.

In summary, our data suggest that age- and region-specific changes in the proteolytic degradation of Aβ make an important contribution to the pathogenic mechanisms in AD. IDE and NEP expression levels may be regulated through APP or its derivatives and there may be cross-regulation between IDE and NEP. Upregulation of IDE and NEP proteases represents a potential novel therapeutic approach to reduce the steady-state Aβ levels, but this would require a more profound knowledge of the endogenous regulation of IDE and NEP expression.These findings emphasize the need for further studies to clarify the exact regulation mechanisms involved in IDE and NEP expression.

6. CONCLUSION

The main focus in this study was to find new risk genes for AD among the Finnish population. Six gene regions were studied and particularly polymorphisms in three genes, IDE, NEP and SST showed a significant risk effect among Finnish AD patients. Studied genetic variants in IDE, NEP and recently investigated APOE (Helisalmi et al., 1999) and CYP19 (Iivonen et al., 2004) genes confer increased risk for AD among un-stratified Finnish AD patients. Additionally, the first genetic association study between SST and AD indicates that genetic variations in the SST gene may modify the risk for AD among Finnish AD subjects with the APOE ε4 allele. We also demonstrated that the studied polymorphisms in HHEX, BDNF and its receptor TrkB gene are not contributing a significant risk effect among Finnish AD patients. In addition, because of the genetic findings and biological function of IDE and NEP, we clarified the expression status of these genes in AD mouse model. Our data indicate that age- and region-specific changes in the proteolytic degradation of Aβ may contribute to pathogenic mechanisms in AD. Additionally, our data suggest that accumulation of the pathological Aβ peptide may modify its own degradation. In particular, expression levels of IDE may be regulated through APP or its derivatives and there may be cross-regulation between IDE and NEP.

AD is a complex disorder and among the other risk factors many genes affect its pathogenesis. One important aspect to be considered is the degradation of Aβ, the main constituent of senile plaques. In this thesis work, we provided evidence that genetic variants of two Aβ degrading genes, IDE and NEP increase the risk of AD. Additionally, genetic variants of the SST gene which is known to modulate NEP expression increase the risk of AD in APOE ε4 carriers. In conclusion, the factors which affect Aβ degradation have particular relevance in AD and further studies to clarify their roles are warranted. One important challenge for the future is moving from genes to proteins, to elucidate what happens in the organism as a result of altered genetic information. This progress may also represent the key to identifying novel disease process modifying treatments.

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APPENDIX: ORIGINAL PUBLICATIONS (I-V)

I

Genetic analysis of BDNF and TrkB gene polymorphisms in Alzheimer`s disease

Vepsäläinen S, Castren E, Helisalmi S, Iivonen S, Mannermaa A, Lehtovirta M, Hänninen T, Soininen H, Hiltunen M

Journal of Neurology 2005; 252:423-428

II

Polymorphisms in neprilysin gene affect the risk of Alzheimer`s disease in Finnish patients

Helisalmi S, Hiltunen M, Vepsäläinen S, Iivonen S, Mannermaa a, Lehtovirta M, Koivisto A M, Alafuzoff I, Soininen H

Journal of Neurology, Neurosurgery and Psychiatry 2004; 75:1746-1748

Cited in Journal of Medical Genetics 2005;42:158

III

Insulin degrading enzyme is genetically associated with Alzheimer`s disease in the Finnish population

Vepsäläinen S, Parkinson M, Helisalmi S, Mannermaa A, Soininen H, Tanzi R, Bertram L, Hiltunen M

Journal of Medical Genetics 2007; 44(9):606-608

IV

Somatostatin genetic variants modify the risk for Alzheimer`s disease among Finnish patients

Vepsäläinen S, Helisalmi S, Koivisto A M, Tapaninen T, Hiltunen M, Soininen H

Journal of Neurology (in press)

V

Increased expression of Aβ degrading enzyme IDE in the cortex of transgenic mice with Alzheimer`s disease-like neuropathology

Vepsäläinen S, Hiltunen M, Helisalmi S, Wang J, van Groen T, Tanila H, Soininen H

Submitted for publication

PUBLICATIONS
SERIES OF REPORTS, DEPARTMENT OF NEUROLOGY

1. **Juhani Partanen (1978):** Time-locked phenomena of human motor unit potentials. An electromyographic study of satellites and doubles.
2. **Eeva Leino (1981):** Clinical and biochemical studies on progressive myoclonus epilepsy.
3. **Hilkka Soininen (1981):** Senile dementia. A clinical, neurochemical and etiological study.
4. **Rolf Danner (1982):** Adverse effects of anticonvulsive treatment on peripheral nerve conduction and posterior dominant EEG rhythm.
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6. **Juhani Sivenius (1982):** Studies on the rehabilitation, epidemiology and clinical features of stroke in East Central Finland.
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9. **Kari Reinikainen (1988):** Neurotransmitters in Alzheimer's disease.
10. **Tapani Keränen (1988):** Epilepsy in adults. An epidemiologic study in Eastern Finland.
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14. **Asla Pitkänen (1989):** Somatostatin in experimental and human epilepsy.
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16. **-**
17. **Paavo Riekkinen Jr (1990):** Animal models of age-related degeneration of subcortical regulatory systems. With special reference to cholinergic, noradrenergic and serotonergic systems.
18. **Toivo Halonen (1990):** Neurotransmitter amino acids in epileptic convulsions and during vigabatrin treatment.
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