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Genetic and Expressional Studies of Alzheimer's Disease Candidate Genes

Emphasis on CYP19, Seladin-1 and HSPG2 Genes

Doctoral dissertation

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ABSTRACT

The aging of the population as result of elongated life expectancy will increase also the number of individuals affected by dementing disorders leading to loss of independence and ability to cope with the problems of everyday life. Alzheimer's disease (AD) is the most common form of dementia, affecting up to 20 % of population aged over 90 years. Most AD cases take place in late life, but a minority is affected as early as the age of 30 years, these being termed early-onset AD cases. These rare cases are often attributable to autosomal dominant mutations in the amyloid precursor protein (APP) and presenilin (PSEN) 1 and 2 genes that cause the earlier onset of symptoms like memory loss and confusion and neuropathological changes including neuronal loss, intracellular neurofibrillary tangles and extracellular amyloid deposits. Nonetheless, the mystery behind a substantial amount of AD cases, i.e. the late-onset cases, remains unsolved, since the only widely established susceptibility gene, the APOE, accounts for only approximately half of the late-onset AD cases.

In the present series of studies, the objective was to identify new susceptibility loci for late-onset AD and to study their expressional changes in AD or in AD models using molecular biological approaches such as single nucleotide polymorphisms and reverse transcription polymerase chain reaction methods. Genetic loci recognized earlier as susceptibility loci for late-onset AD in whole genome scan at 1p36.12 and 15q21.1 encompassing HSPG2 and CYP19 genes were studied. The expressional changes in the CYP19 gene encoding an enzyme involved in estrogen metabolism, a hormone with controversial effects on AD, was studied in a mouse model of estrogen deprivation and replacement therapy. Also, the expression of seladin-1, an enzyme protective in apoptosis, was studied in AD and Lewy body dementia cases, and in an AD mouse model.

A cluster of CYP19 SNPs was found to form a risk haplotype that was independent of sex, age and APOE polymorphism. The HSPG2 A allele in conjunction with APOE ε4 allele doubled the risk of AD compared to the APOE ε4 allele alone, and the tau pathology was more prominent in HSPG2 A and APOE ε4 carriers than in APOE ε4 allele carriers alone, suggesting that HSPG2 and APOE might interact at the cellular level impacting more on tau pathology rather than influencing Aβ pathology. The down-regulation of seladin-1 expression was specific for AD, and it was associated with hyperphosphorylation of tau protein. These genes associated with AD in the present studies may represent potential targets for further genetic and functional analysis in the field of AD therapeutics.

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Medical Subject Headings: Alzheimer disease/genetics; Apolipoproteins E; Apoptosis; Aromatase; Chromosomes; Gene Expression; Heparan Sulfate Proteoglycan; Polymorphism, Single Nucleotide; Risk Factors; Oxidoreductases; tau Proteins

To Pasi and Joonas

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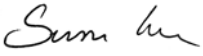

Susan Iivonen

ABBREVIATIONS

Aβ	β-amyloid
AChE	acetylcholinesterase
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease family
ApoE	Apolipoprotein E
APH-1	Anterior pharynx-defective
APLP	Amyloid precursor-like proteins
APP	Amyloid precursor protein
AR	Androgen receptor
ATP	Adenosine triphosphate
BACE1	β-secretase 1
bp	Base pair
B4-CTF	ErbB4 C-terminal fragment
B4-ICD	ErbB4 intracellular domain
CBP	CREB binding protein
cDNA	Complementary DNA
CERAD	The Consortium to Establish a Registry for Alzheimer's Disease
CI	confidence interval
CSF	Cerebrospinal fluid
cSNP	coding SNP
C_t	threshold cycle
CYP19	Cytochrome P450 19
ΔE9	Exon 9 deletion
D'	A measure of linkage disequilibrium
DCC	deleted in colorectal cancer
DHCR24	3β-hydroxysterol Δ^{24}-reductase
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosteronesulphate
DHT	dihydrotestosterone
DLB	Lewy body dementia
DSM-III-R	Diagnostic and Statistical Manual of Mental Disorders, 3rd edition, revised
ELISA	enzyme linked immunosorbent assay
ER	Estrogen receptor
ERT	Estrogen replacement therapy
FAD	familial Alzheimer's disease
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSK-3β	glycogen synthase kinase-3β
HPRT	hypoxanthine phosphoribosyltransferase
HSPG	Heparan sulphate proteoglycan
htSNP	haplotype tagging SNP
IDE	Insulin degrading enzyme
IR	Insulin receptor
kDa	kilodalton
LD	Linkage disequilibrium
LDLR	low density lipoprotein receptor
LRP	LDL receptor related pathway
MNAR	modulator of non-genomic estrogen receptor

mRNA	messenger RNA
NEP	Neprilysin
NEXT	Notch extracellular truncation
NCSTN	Nicastrin
NICD	Notch intracellular domain
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association
NFT	Neurofibrillary tangle
NP	Neuritic plaque
OKA	Okadaic acid
OR	Odds ratio
OVX	Ovariectomy
PCR	Polymerase chain reaction
PEN-2	Presenilin enhancer 2
PHF	Paired helical filament
PI3K	phosphatidylinositol 3-kinase
PSEN	Presenilin
RT	Reverse transcription
SAP	Shrimp alkaline phosphatase
sAPP	soluble APP
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error
SEM	standard error of mean
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for Social Sciences
TACE	tumour necrosis factor alpha converting enzyme
UTR	Untranslated region
VLDL	very low density lipoprotein

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-IV.

I Iivonen S, Corder E, Lehtovirta M, Helisalmi S, Mannermaa A, Vepsäläinen S, Hänninen T, Soininen H, Hiltunen M. Polymorphisms in the CYP19 gene confer increased risk for Alzheimer's disease. Neurology 2004; 62:1170-1176.

II Iivonen S, Heikkinen T, Puoliväli J, Helisalmi S, Hiltunen M, Soininen H and Tanila H. Effects of ovariectomy and estrogen treatment on spatial learning and hippocampal CYP19, estrogen alpha and estrogen beta receptor expression in female mice. *Submitted to Neuroscience.*

III Iivonen S, Hiltunen M, Alafuzoff I, Mannermaa A, Kerokoski P, Puoliväli J, Salminen A, Helisalmi S, Soininen H. Seladin-1 transcription is linked to neuronal degeneration in Alzheimer's disease. Neuroscience. 2002; 113: 301-310.

IV Iivonen S, Helisalmi S, Mannermaa A, Alafuzoff I, Lehtovirta M, Soininen H, Hiltunen M. Heparan sulfate proteoglycan 2 polymorphism in Alzheimer's disease and correlation with neuropathology. Neurosci Lett. 2003; 352: 146-150.

CONTENTS

1. INTRODUCTION

The most common form of dementia among aging people is Alzheimer's disease (AD), which involves the parts of the brain controlling thought, memory and language. The disease usually begins after the age of 60 years, and the risk increases with age, affecting 0.6% of population for ages 65-69 years, but up to 22.2% of the population over 90 years of age (Lobo et al. 2000). Thus age is the most important known risk factor for AD.

Despite decades of intensive research in the field of AD, the causes of the disease still remain a mystery. There are a multitude of pathological hallmarks and biological changes in AD, indicating that there probably is not one single cause, instead several genetic, environmental and lifestyle factors contribute to the disease development, affecting each person differently. To recognize the risk factors and genes for such a multifactorial, complex disease is extremely challenging and laborious compared to single gene disorders, where defects in a single gene result in a characteristic disease phenotype.

The most important known risk factor for AD is age. Family history is another risk factor, with genetics playing a considerable role in familial early-onset AD cases, a rare form of AD (< 5%) that usually occurs between the ages of 30 and 60 years. The more common form of AD is known as late-onset AD and this occurs later in life, but here no obvious inheritance pattern is seen. One form of a cholesterol carrying protein apoE, the ε4 allele, lowers the age of onset in AD, being the only late-onset AD susceptibility gene currently recognized in different ethnic populations. One APOE ε4 allele decreases the age of onset by five years, and two alleles accelerate the age of onset by nine years in late-onset AD cases (Tsai et al. 1994). However, there probably are several other genetic variants which may interact with each other, either predisposing to the disease or protecting against it, but they remain to be discovered.

The most reasonable approach to search for factors contributing to the disease development is to aim at elucidating the mechanisms underpinning the pathophysiology of the disease. The hallmark pathologic lesions of AD are the extracellular amyloid

plaques and intracellular neurofibrillary tangles (NFT). Amyloid plaques are mainly composed of β-amyloid (Aβ) peptides, and their production is most clearly increased when amyloid precursor protein (APP) or presenilin (PSEN) genes carry mutations that affect the processing of APP, usually leading to the early-onset forms of AD (Goate et al. 1991, Levy-Lahad et al. 1995, Sherrington et al. 1995). NFTs, on the other hand, consist of paired helical filaments, that are formed by the microtubule-associated protein tau (Kosik et al. 1986). The Braak staging based on the NFT pathology, describes the severity of AD according to the distribution of NFT pathology in different brain areas (Braak and Braak 1995). In addition, nerve cells are lost in brain areas vital to memory and other mental abilities, and the levels of the neurotransmitter acetylcholine are lower in the AD brain (Davies and Maloney 1976, Ball 1977, Davies 1979). Glucose metabolism is also impaired in AD brains (Adolfsson et al. 1980), but the main question remains: are these observed changes involved in the disease process predisposing to AD or are they merely a consequence of some still unknown factor that actually is behind the disease?

In addition to genetics and APOE, the roles of education, diet and environment in the development of this disease has been investigated. Increasing evidence shows that some of the risk factors for heart disease and stroke, such as high blood pressure (Skoog et al. 1996), high cholesterol (Kalmijn et al. 1997, Notkola et al. 1998, Kivipelto et al. 2001), and low levels of the vitamin folate (Joosten et al. 1997), may predispose individuals to AD. However, evidence is gathering for physical, mental, and social activities as protective factors against AD (Scarmeas et al. 2001, Seidler et al. 2003).

The lifetime risk of a genetically influenced multifactorial disorder like AD, is estimated to be 60% in western populations (Baird et al. 1988). Common clinical disorders have significant genetic components, as defined by the attributable risk (Lander and Schork 1994), and the objective of the present series of studies was to search for genes that might affect the risk of suffering AD, and thus possibly elucidate the mechanisms behind the disease. The basis for the majority of the present studies is the previously conducted genome-wide linkage mapping, that identified multiple loci associated with AD (Hiltunen et al. 2001). Many moderate susceptibility effects have been found in the Northern Savo population used also in the present series of studies

(table 1). It is important to identify the unmapped susceptibility loci if one wishes to understand the mechanisms behind the pathophysiology of the disease and to estimate the disease risk, and thereafter to be able to explore and design effective drug therapies and means to prevent or delay the disease initiation and progression.

Table 1. Candidate genes studied in Northern Savo population**.**

Gene	Chromosomal location	Association with AD	Study[a]
AACT	14q32	Negative	(Helisalmi et al. 1997)
BCHE	3q26	Positive[b]	(Hiltunen et al. 1998)
APOE -491A/T	19q13.32	Positive[b]	(Helisalmi et al. 1999)
PSEN1 E318G	14q24.3	Positive[b]	(Helisalmi et al. 2000a)
NPY	7p15	Negative	(Helisalmi et al. 2000b)
MPO	17q23	Positive[b]	(Reynolds et al. 2000)
A2M	12p13-12	Positive[b]	(Pirskanen et al. 2001)
IL1A	2q14	Negative	(Pirskanen et al. 2002)
HSPG2	1p36.12	Positive[b]	(Iivonen et al. 2003)
ICAM1	19p13	Negative	(Mattila et al. 2003)
NCSTN	1q22-23	Positive[c]	(Helisalmi et al. 2004a)
APOD	3q26	Positive[c]	(Helisalmi et al. 2004b)
NEP	3q25	Positive[b,c]	(Helisalmi et al. 2004c)
CYP19	15q21.1	Positive[b,c]	(Iivonen et al. 2004)
BDNF	11p13	Negative	(Vepsäläinen et al. 2005)
TRKB	9q22	Negative	(Vepsäläinen et al. 2005)

[a] Populations do not fully overlap.
[b] Association to a single nucleotide polymorphism.
[c] Association to a haplotype.

2. REVIEW OF THE LITERATURE

2.1. Alzheimer's disease

AD is a progressive, neurodegenerative disease leading to dementia, and eventually to death. It is the most common form of dementia in the elderly with prevalence of 0.6% for individuals aged 65 to 69 years, and 22.2% for those aged over 90 years (Lobo et al. 2000). AD and its brain pathology were first described in 1907 by the German physician Alois Alzheimer in a 51-year-old woman. AD is characterized by memory loss and confusion, related to a distinctive combination of neuropathological changes including neuronal loss, intracellular neurofibrillary tangles (NFT) and extracellular amyloid plaques. Amyloid plaques and NFTs are also found in normally aged brains, although in reduced numbers, and therefore, they are considered as age-related changes.

AD is subdivided into early (< 65 years) and late onset (>65 years) forms. About 10% of AD cases are familial with an autosomal dominant inheritance and these cases have often early onset. In these families, single point mutations in amyloid precursor protein (APP), or presenilins 1 and 2 (PSEN 1/2) result in cleavage of APP to neurotoxic β-amyloid peptides (Aβ) which is deposited in the core of neuritic plaques (Goate et al. 1991, Levy-Lahad et al. 1995, Sherrington et al. 1995). In addition to these cases, 30% of AD cases have a positive family history of AD, but insufficient evidence of autosomal dominant inheritance (van Duijn et al. 1991). Therefore, up to 60% of AD cases, termed as sporadic, do not have a family history of AD. The early onset cases appear similar to sporadic cases with the exception of more severe symptoms and pathology, and an earlier age of onset. Exploring the genetic background behind the sporadic late onset cases, has revealed apolipoprotein E (ApoE) to be a risk factor of AD (Corder et al. 1993, Saunders et al. 1993). Since then, almost 100 genes have been tested for association with AD throughout the world (table 2), but none of them has consistently conferred susceptibility to AD (Bertram and Tanzi 2004). Nevertheless, a number of studies have identified several additional AD susceptibility loci in addition to APOE, in both linkage and association studies, pointing to the existence of novel AD genes on several chromosomes.

Table 2. A sample of AD candidate genes studied worldwide.

Gene	Chromosomal location	Relevance to AD pathogenesis	Study
Early-onset			
PSEN2	1q31-42	Essential for γ-secretase activity (?)	(Levy-Lahad et al. 1995)
PSEN1	14q24.3	Componen of γ-secretase complex	(Sherrington et al. 1995)
APP	21q21.3	Precursor of Aβ	(Goate et al. 1991)
Late-onset			
HSPG2	1p36	Component of Aβ plaques?	(Rosenmann et al. 2004)
MTHFR	1p36	Homocysteine metabolism	(Chapman et al. 1998)
NCSTN	1q22-23	Component of γ-complex	(Dermaut et al. 2002)
IL1A	2q14	Inflammatory response	(Grimaldi et al. 2000)
IL1B			
IL1RN			
TF	3q22	Iron transporter, oxidative stress	(Namekata et al. 1997)
NEP	3q25	Degradation of Aβ	(Oda et al. 2002)
APOD	3q26	Lipid metabolism	(Desai et al. 2003)
SNCA	4q21	Component of Aβ plaques	(Xia et al. 1996)
TNFA	6p21	Inflammation	(Tarkowski et al. 2000)
HLA-A2	6p21	Inflammation	(Small et al. 1991)
ESR1	6q25	Mediator of estrogen effects	(Brandi et al. 1999)
IL6	7p21	Nerve cell differentation	(Papassotiropoulos et al. 1999)
NOS3	7q36	Synthesis of nitric oxide, apoptosis	(Dahiyat et al. 1999)
LPL	8p22	Plasma lipid transportation	(Baum et al. 1999)
VLDL-R	9p24	Cholesterol metabolism	(Chen et al. 1998)
UBQLN1	9q22	PSEN biosynthesis and degradation	(Bertram et al. 2005)
PLAU	10q22	Plasminogen activator, degradation of Aβ	(Finckh et al. 2003)
CTNNA3	10q22	Binds β-catenin, Wnt signalling	(Ertekin-Taner et al. 2003)
IDE	10q23	Degradation of Aβ	(Abraham et al. 2001)
CTSD	11p15	Lysosomal aspartyl protease, cleavage of APP?	(Papassotiropoulos et al. 2000)
FE65	11p15	APP binding protein	(Cousin et al. 2003b)
BCHE	11p15	Component of Aβ plaques	(Lehmann et al. 1997)
BDNF	11p13	Survival of neruronal cells	(Nishimura et al. 2005)
A2M	12p13	Degradation of Aβ	(Blacker et al. 1998)
LRP	12q13	Receptor for APOE and A2M	(Lendon et al. 1997)
NOS1	12q24	Neurotransmitter properties in brain	(Liou et al. 2002)
DLST	14q24	Enenrgy metabolism	(Cruts et al. 1995)
AACT	14q32	Component of Aβ plaques	(Kamboh et al. 1995)
5HTT	17q11	Serotonin transport	(Li et al. 1997)
BLMH	17q11	Cysteine protease, BACE activity?	(Montoya et al. 1998)
MAPT	17q21	Forms PHF of NFTs	(Crawford et al. 1999)
ACE	17q23	Hypertension	(Chapman et al. 1998)
MPO	17q23	Phagocytosis, inflammation	(Reynolds et al. 1999)
ICAM1	19p13	Inflammation	(Pola et al. 2003)
APOE	19q13.32	Aβ aggregation	(Saunders et al. 1993)
PRNP	20p13	Oxidative stress?	(Casadei et al. 2001)
CST3	20p11	Protease inhibitor, Aβ fibril formation	(Parfitt et al. 1993)
CYP2D6	22q13	Drug metabolism	(Saitoh et al. 1995)
MAOA	Xq21-26	Metabolism of monoamine neurotransmitters	(Takehashi et al. 2002)

The grossest hallmark lesion of AD brain is atrophy in the cerebral hemispheres, medial temporal lobes, hippocampus and amygdala, although this is not always apparent in AD.

Severe, generalized atrophy is more common in a minority of early onset AD cases than in cases developing AD later in life. In family members with AD mutations, cerebral atrophy can be detected even before the onset of psychological or clinical deterioration (Fox et al. 1996). The microscopic pathology of AD brain consists of argyrophilic plaques and NFTs, along with neuron loss, activated microglia, neuropil threads, granuovascular degeneration, Hirano bodies and amyloid angiopathy. NFTs are abnormal intracellular structures composed mainly of bundles of hyperphosphorylated tau proteins forming paired helical filaments (PHF). Argyrophilic plaques are extracellular deposits mainly composed of Aβ, and they are divided into diffuse and neuritic plaques. Neuritic plaques have a heterogenous appearance with dense central core consisting of amyloid fibrils. Around the core, microglial cells, glial and neuritic processes, amyloid P component, complement components, apolipoproteins E and J, acetylcholinesterase, α1-antichymotrypsin, proteoglycans, growth factors and their receptors, can be found (Esiri 2001). Diffuse plaques, on the other hand, are mainly composed of Aβ, and it has been proposed that the diffuse plaques represent the early form of neuritic plaques in the disease development.

The clinical diagnostic criteria most widely used for AD are those of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS/ADRDA) (McKhann et al. 1984). The criteria are divided into possible, probable and definite, the last one requiring a histological diagnosis together with the clinical picture. This is one characteristic feature of AD; postmortem neuropathology provides the only method to definitely diagnose AD on a person that has clinically been diagnosed as probable AD during his/her lifetime. The histopathological diagnosis is generally based on the criteria defined by the Neuropathology Task Force of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra et al. 1991).

In the past few years, brain imaging techniques have also been used in AD diagnostics. Functional imaging techniques reveal the changes in metabolism, while structural imaging allows the detection of atrophy and ischemic changes, offering better sensitivity and specificity of diagnosis than functional imaging, but still they provide only an aid to diagnosis (Fox et al. 2001). Tau-protein and Aβ1-42, the main

constituents of NFTs and amyloid plaques, are the most promising candidates as biological markers of AD. Along with brain imaging techniques, the biochemical testing of tau-protein and Aβ1-42 in cerebrospinal fluid (CSF) has been used in AD diagnostics. CSF-tau levels are significantly increased in patients with AD compared to subjects with other neurological diseases and controls (Arai et al. 1995), while Aβ1-42 in CSF is decreased (Motter et al. 1995). The CSF-tau measurement can be used alone or in combination with Aβ1-42 measurement to detect AD in its early stages, and to rule out some dementing disorders resembling AD (Arai et al. 2001).

2.2. Amyloid precursor protein

2.2.1. Amyloid precursor protein metabolism

The main component of the AD-associated amyloid plaques is the 39-43 amino acid amyloidogenic peptide Aβ, which is derived by the proteolytic processing of amyloid precursor protein (APP) (figure 1). APP is a type 1 membrane protein encoded in chromosome 21 by 18 exons, three of which are alternatively spliced resulting in a variety of isoforms. The isoform of 695 amino acids is the primary APP present in neuronal tissue, while isoforms APP751 and APP770 are widely expressed in non-neuronal cells (Neill et al. 1994). The three alternatively spliced exons encode a domain of unknown function that has homology to Kunitz protease inhibitor domain, and a domain which when included in APP transcript, disrupts a consensus sequence site used for heparin sulphate modification of the protein (Tanaka et al. 1988, Clarris et al. 1997, Wasco 2001).

APP functions as a cell surface receptor potentially performing physiological functions relevant to neurite growth, neuronal adhesion, cell survival, neuroprotection, synaptogenesis, synaptic plasticity and axonogenesis (Mattson 1997). In addition, it can inhibit Notch signalling and regulate transcription by forming a transcriptionally active complex with the adaptor protein Fe65 and the histone acetyltransferase Tip60 (Cao and Sudhof 2001). After cleavage near the membrane, APP is in the form of a secreted protein (sAPP) that has the same functions as the full-length APP. APP is a member of an evolutionarily conserved gene family, and two mammalian amyloid precursor-like proteins, ALPL1 and ALPL2 have been identified (Sprecher et al. 1993, Wasco et al.

1993, Slunt et al. 1994). The overall structures as well as structural and functional domains are extremely well conserved in these proteins, with the exception of the Aβ domain, which is found only in APP.

APP is processed by several enzymes. It is predominantly cleaved by α-secretase, resulting in the secretion of the N-terminal portion of APP (sAPPα) and inhibition of Aβ formation. The membrane bound C-terminal fragment of 83 amino acids (C83) is internalized and degraded, leading to the formation of a 3 kDa fragment (p3) during the process (Esch et al. 1990). Two members of ADAMs family (a disintegrin and metalloprotease family) of proteases, tumour necrosis factor alpha converting enzyme (ADAM17 or TACE) (Buxbaum et al. 1998) and ADAM10 (Lammich et al. 1999) have α-secretase activity. In addition, a minor portion of APP is cleaved by β-secretase, resulting in the formation of the N-terminal secreted APPβ (sAPPβ) and a C-terminal peptide of 99 amino acids (C99). The C99 contains the neurotoxic Aβ fragment, and subsequent to α- and β-secretase cleavage, C83 and C99 can be cleaved by γ-secretase to form p3 or Aβ. The most abundant forms of Aβ consist of 40 or 42 amino acids, of which the Aβ42 is more amyloidogenic peptide associated with AD. In addition to cleavage at amino acids 40 and 42, a new γ-secretase cleavage site at amino acid 49 was identified and termed as the ε-cleavage site (Sastre et al. 2001).

β-secretase is an aspartyl protease known as BACE or Asp2 (Vassar et al. 1999), whereas γ-secretase is a complex of a minimum four proteins (Kimberly et al. 2003b). Transgenic mice over-expressing human APP, but lacking BACE1 gene, can be rescued from Aβ-dependent hippocampal memory deficits, and cholinergic dysfunction (Ohno et al. 2004). Moreover, BACE overexpression is not sufficient to produce beta-amyloid plaques, but simultaneous expression of BACE1 and its substrate (SweAPP) leads to accelerated amyloid plaque formation (Mohajeri et al. 2004). BACE activity increases with aging in human brains, suggesting that an age-related increase of BACE activity contributes to the increased production and accumulation of brain Aβ, potentially predisposing to Alzheimer's disease in humans (Fukumoto et al. 2004). In sporadic AD patients, this may actually happen since in these individuals the increased BACE activity is associated with an elevated Aβ load (Li et al. 2004). On contrary, sAPPα has neuroprotective effects since it reduces the neurotoxicity of glutamate and Aβ (Mattson

et al. 1993, Goodman and Mattson 1994), and sAPPα is ~ 100-fold more potent than sAPPβ in this function (Furukawa et al. 1996). This may be due to heparin binding domain VHHQK that is present in sAPPα, but not in sAPPβ. The secreted forms of APP have also memory enhancing properties (Meziane et al. 1998).

The γ-secretase cleavage of APP is similar to γ-secretase cleavage of Notch, whose intracellular domain (NICD) activates transcription factors by interacting with nuclear transcription factors (Selkoe and Kopan 2003). γ- and ε-cleavage of APP produces an intracellular fragment named the APP intracellular domain (AICD or CTF-γ) that is a potent transactivator of transcription when coupled to a transmembrane region. Membrane-tethered AICD recruits Fe65 that is then released for nuclear translocation by γ-cleavage together with the AICD. Thus the transcriptional transactivation by APP and Notch may involve distinct mechanisms; whereas NICD directly functions in the nucleus, the AICD acts indirectly by activating Fe65 (Cao and Sudhof 2004).

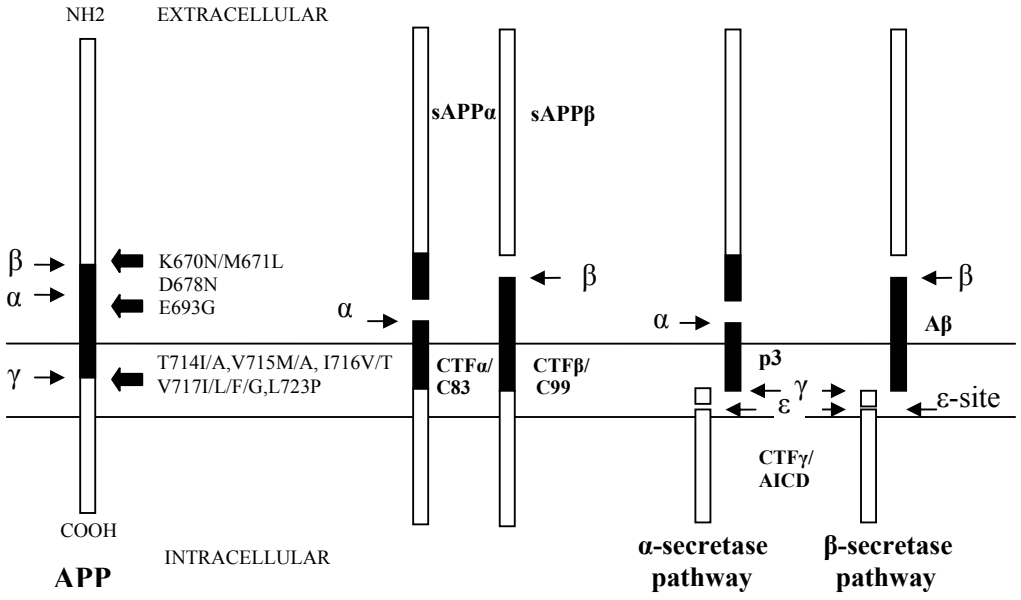

Figure 1. FAD-linked APP mutations and metabolism of APP via α- and β-secretase pathways. Black segment represents Aβ.

The progressive deposition of Aβ is considered to be fundamental to the development of neurodegenerative pathology of AD, and moreover, the aggregation state of the Aβ peptide is probably a key factor in determining the toxicity of Aβ. Fibrillized Aβ is

known to be neurotoxic, whereas soluble Aβ peptides exhibit only little toxicity (Simmons et al. 1994, Howlett et al. 1995). Soluble Aβ monomers are used during the formation of protofibrils, an intermediate in the formation of fibrillar β-amyloid and this is also the rate limiting step in the formation process of amyloid fibrils. The protofibrils provide the nidus that accelerates conversion to fibrils and thence to plaques. The fibrillization may be regulated by many factors such as, acetylcholinesterase (AChE) (Inestrosa et al. 1996), metal ions Fe^{2+}, Cu^{2+} and Zn^{2+} (Atwood et al. 1998), apoE, proteoglycans and glucose. Aβ deposition in AD may be a delicate balance involving enhanced production of Aβ, decreased clearance and the absence or presence of factors regulating the fibrillization process. Inhibitors of Aβ fibrillization that bind to the hydrophobic core region of the peptide, a fundamental component of its ability to aggregate, might provide one mean to halt or at least slow down the progression of AD (Howlett et al. 2001).

2.2.2. Amyloid precursor protein mutations

Mutations in APP gene, that are behind early-onset familial AD (FAD), are located around α-, β- and γ-secretase cleavage sites. To date, 14 pathogenic AD mutations in nine different loci have been identified (Alzheimer Disease & Frontotemporal Dementia Mutation Database; http://www.molgen.ua.ac.be/ADMutations/default.cfm). 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.

Presumably, the position of mutations influences the metabolism of the APP. Possibly, the AD mutations affect the cleavage of one of the three enzymes and consequently, the Aβ40 and Aβ42 levels in the brain. 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 β-secretase cleavage site increases both Aβ40 and Aβ42 deposits (Citron et al. 1992). β-secretase has a 100-fold activity with an APP substrate containing Swedish double mutation compared to wildtype APP (Vassar et al. 1999), and in this case, more C99 is available for γ-secretase to produce both Aβ peptides. Mutations in α-secretase site are linked to cerebral hemorrhages with amyloidosis of Dutch and Flemish type, where the Aβ peptide deposits, consisting mainly of Aβ40, are found in non-neuronal cells of the cerebral blood vessel walls

(Levy et al. 1990, Hendriks et al. 1992). Aβ with the Arctic mutation (E693G) forms protofibrils at a much higher rate and in larger quantities than wild-type Aβ, which may reflect a pathogenic mechanism for AD involving rapid Aβ protofibril formation leading to accelerated buildup of insoluble Aβ both intra- and/or extracellularly (Nilsberth et al. 2001).

2.3. γ-secretase components and pathway

γ-Secretase is an intramembrane-cleaving aspartyl protease that catalyzes the proteolysis of APP, Notch and other type I transmembrane receptors, indicating that it normally serves as a mediator of diverse signaling pathways (Kimberly et al. 2003a). γ-Secretase is a membrane protein complex comprising of PSEN, nicastrin (NCSTN), anterior pharynx-defective-1 (APH-1) and presenilin enhancer-2 (PEN-2) (figure 2), and it functions as a key step in the pathogenesis of AD, cleaving APP into Aβ. This unusual protease has a PSEN heterodimer in its catalytic site and a highly glycosylated NCSTN associated with it. γ-secretase components assemble into the proteolytically active γ-secretase complex in the Golgi/trans-Golgi network compartment (Baulac et al. 2003), and they are expressed in a coordinated manner (Steiner et al. 2002); the over-expression of PSEN and NCSTN is not sufficient for increased γ-secretase activity (Yang et al. 2002), but requires both APH-1 and PEN-2 to circumvent the tight regulation of γ-secretase activity in mammalian cells (Francis et al. 2002, Kimberly et al. 2002). The impact of co-expression of γ-secretase components on APP cleavage and Aβ production has not been shown in mammalian cells, because the co-expression of γ-secretase components affects the endogenous APP expression levels of APP transfected cells thus preventing the accurate quantification of endogenous Aβ (Kimberly et al. 2003b). However, co-expression of *Drosophila* APH-1, PEN-2, and NCSTN increases levels of PSEN fragments and γ-secretase cleavage of APP (Takasugi et al. 2003), and similar results have been obtained using mammalian γ-secretase components in yeast (Edbauer et al. 2003).

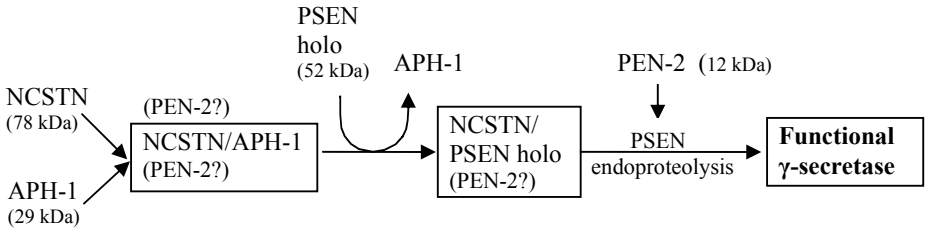

Figure 2. Hypothetical γ-secretase assembly pathway according to Hu and Fortini. NCSTN and APH-1 associate in an early subcomplex, leading to stabilization of APH-1. The subcomplex regulates stabilization of PSEN holoprotein and its incorporation into a complex with NCSTN and possibly PEN-2. PEN-2 is required for endoproteolysis of PSEN holoprotein, although the point at which PEN-2 enters the pathway is unknown.

There may be at least three distinct PSEN complexes; a ~150 kDa NCSTN-APH-1 complex, a ~440 kDa γ-secretase complex, which contains additional components (PSEN-1 and PEN-2), and a high mass (≥670 kDa), low abundance heteromeric complex associated with high γ-secretase activity. The ~440 kDa complex is a non-functional intermediary complex stably and relatively abundantly present. The prevalence, although in low amounts, of the high molecular weight ≥670 kDa complex under conditions associated with functional γ-secretase activity, suggests that this complex is the species that contains full enzymatic activity. The lower mass (~150 kDa and ~440 kDa) complexes might then represent initial and intermediary complexes, while the high mass complex (≥670 kDa) could represent a functional complex that contains either additional components or the four known components in a higher order stoichiometry (e.g. 2:2:2:2) (Gu et al. 2004).

2.3.1. Presenilin-1/2

A member of the γ-secretase complex, PSEN-1 encoded by 12 exons in chromosome 14q24, is a 52 kDa transmembrane protein with eight transmembrane domains. PSEN-2 in chromosome 1q31 has 67% amino acid identity with PSEN-1 resulting in a 90 kDa transmembrane protein encoded by 10 exons. PSEN proteins are aspartyl proteases (Xia et al. 2000) that form the catalytic subunit of γ-secretase complex responsible for Notch and APP cleavage (Kimberly et al. 2003a). The full-length PSEN proteins are rapidly cleaved in the cytoplasmic loop into C- and N- terminal fragments (CTF and NTF), with only a small portion found as full-length PSEN proteins, suggesting that the heterodimers formed by CTF and NTF are the active form of the protein (figure 3). The endoproteolysis is tightly regulated, and consequently over-expression of PSEN leads to

the appearance of full-length PSEN, but does not increase in the levels of the fragments (Thinakaran et al. 1996). In addition to interacting with γ-secretase complex proteins, PSENs interact with signaling proteins, β- and Δ-catenins, and with glycogen synthase kinase-3β (GSK-3β) responsible for tau phosphorylation (Zhou et al. 1997, Takashima et al. 1998, Zhang et al. 1998).

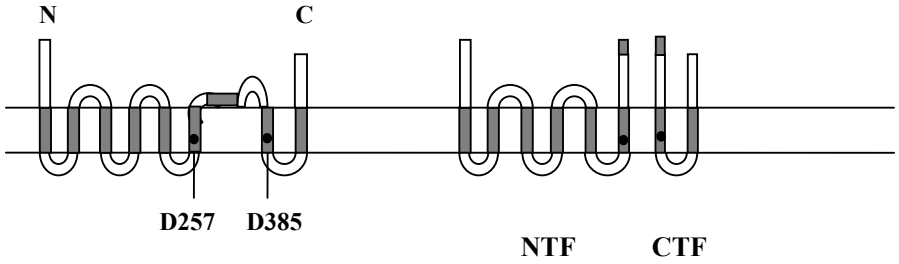

Figure 3. Structure of PSEN proteins and the endoproteolytic site. Functionally essential aspartate residues are shown by dots.

The PSEN genes were linked to AD in 1995, when various missense mutations were localized in several families (Levy-Lahad et al. 1995, Rogaev et al. 1995, Sherrington et al. 1995). Defects in PSEN-1 cause the most severe form of AD, familial AD with complete penetrance and early onset age occurring as early as 24 years of age (Moehlmann et al. 2002), and to date altogether 142 pathogenic mutations have been found in the PSEN-1 gene (http://www.molgen.ua.ac.be/ADMutations/default.cfm) (Coleman et al. 2004). PSEN-1 mutations are mainly missense mutations, leading to an amino acid change, and many of the mutations occur in single kindreds only. A few mutations include insertions or deletions leading to codon insertions/deletions or frame-shift with premature stop codons or deletions of whole exons from the transcript. The mutations in PSEN-1 are located primarily within the transmembrane domains and in the N-terminal portion of the cytoplasmic loop, close to the endoproteolytic cleavage site. All PSEN-1 mutations analyzed to date affect the metabolism of the APP, increasing the levels of Aβ42 deposited in senile plaques (Selkoe 1999). Altogether ten pathogenic mutations have been found in the PSEN-2 gene, with somewhat later onset ages, wide range of onset age and greater variability in penetrance than with PSEN-1 mutations. All PSEN-2 mutations are missense mutations leading to an amino acid change (http://www.molgen.ua.ac.be/ADMutations/ default.cfm).

The biological function of PSEN mutations was known even before the genes were cloned; PSEN mutations increase Aβ1-42 production (Scheuner et al. 1996). In addition to this toxic gain of misfunction, mutations may result in loss of function of γ-secretase activity (Wolfe et al. 1999) and Notch signaling (Berezovska et al. 2000, Moehlmann et al. 2002), when they occur in either of two transmembrane aspartate residues (Asp257 or Asp385). Mutations in aspartate residues prevent the endoproteolysis of PSEN-1, and subsequently the formation of active PSEN-1.

Presenilins interact also with glycogen synthase kinase-3β (GSK-3β) responsible for tau phosphorylation (Takashima et al. 1998). PSEN-1 mutations inhibit phosphatidylinositol 3-kinase (PI3K) and its downstream effector Akt activation, thus promoting GSK-3β activity and tau over-phosphorylation. PSEN-1 may thus prevent development of AD pathology by activating the PI3K/Akt signaling pathway, but in contrast, PSEN-1 mutations may promote AD pathology by inhibiting this pathway (Baki et al. 2004).

Mutations in PSEN-1 gene leading to deletion of exon nine (ΔE9) prevent the endoproteolysis of PSEN-1 protein. The primary effect of this mutation may be the deletion of exon nine, which in its immature form inhibits the activation of γ-secretase and occupies the substrate-binding site. However, when the exon is deleted, the inhibition of γ-secretase activation is also abolished (Knappenberger et al. 2004), leading to extremely high rates of Aβ1-42 production (Houlden et al. 2000). In contrast, deletion of the PSEN-1 gene produces developmental abnormalities indicating a loss in Notch signaling (Wong et al. 1997), and decreased γ-secretase processing of APP, resulting in dramatic declines in Aβ levels (De Strooper et al. 1998).

2.3.2. Nicastrin

Nicastrin (NCSTN) is a type I transmembrane glycoprotein that forms high molecular weight complexes with presenilins (Yu et al. 2000). NCSTN encoded in chromosome 1q22 is a component of the γ-secretase complex probably representing a stabilizing cofactor required for the assembly of the γ-secretase complex. The Caenorhabditis elegans orthologue of NCSTN, APH-2, is essential for Notch signalling during early embryogenesis (Goutte et al. 2002); however, the exact role of APH-2/NCSTN is not

known. Recent studies revealed that NCSTN and APH-1 initially form a subcomplex to bind and stabilize PSEN, and then PEN-2 confers the γ-secretase activity and facilitates endoproteolysis of PSEN (LaVoie et al. 2003, Shirotani et al. 2004). A region at the C-terminal end of NCSTN, encompassing the last 50 residues of the transmembrane domain, and the cytoplasmic domain are important for mediating interactions between human PSEN-1, APH-1, and PEN-2 (Morais et al. 2003).

NCSTN in γ-secretase complex is highly glycosylated, and mutations inhibiting the glycosylation and stability of NCSTN halt the binding with PSENs (Tomita et al. 2002). NCSTN appears to be critical for the trafficking of PSENs and APP to the cell surface (Kaether et al. 2002, Li et al. 2003), and suppression of NCSTN expression results in phenotypes similar to PSEN or Notch1 suppression (Li et al. 2003). NCSTN binds C-terminal derivatives of APP, C83 and C99, and modulates the production of the Aβ of these derivatives. Missense mutations in conserved hydrophilic domain of NCSTN increase Aβ1-42 and Aβ1-40 secretion, whereas deletions in this domain inhibit Aβ production (Yu et al. 2000). However the reports of genetic studies associating NCSTN to early onset AD are conflicting (Dermaut et al. 2002, Confaloni et al. 2003, Cousin et al. 2003a, Orlacchio et al. 2004), though an association with a subpopulation of late onset AD has been found (Helisalmi et al. 2004a).

2.3.3. APH-1

Anterior pharynx-defective-1 (APH-1) is a highly conserved membrane protein with seven transmembrane domains and it topologically resembles the seven-transmembrane domain receptors. Similar to the loss of PSEN or NCSTN, inactivation of endogenous APH-1 decreases presenilin levels, accumulates γ-secretase substrates (APP C-terminal fragments), and reduces γ -secretase products (Aβ and intracellular domains of APP and Notch). These data indicate that APH-1 probably is a functional component of the γ-secretase complex required for intramembrane proteolysis of APP and Notch (Francis et al. 2002), involved in early stages of γ-secretase complex assembly, and required for proper cell-surface localization of NCSTN (Goutte et al. 2002). In addition, APH-1 is involved in the stabilization and glycosylation of NCSTN, and in scaffolding NCSTN to the immature γ-secretase complex which stabilizes PSEN holoprotein (LaVoie et al. 2003).

APH-1 is processed by several endoproteolytic events, and one of these cleavages is strongly up-regulated by co-expression of NCSTN generating a stable C-terminal fragment of APH-1 that associates with NCSTN (Fortna et al. 2004). The association between NCSTN and APH-1 may occur shortly after APH-1 synthesis, and the interaction between these two proteins is mediated by transmembrane domains of APH-1. In C. elegans, a mutation of the conserved transmembrane Gly123 in aph-1 leads to a Notch/glp-1 loss-of-function phenotype. The corresponding mutation in mammalian APH-1a (G122D) disrupts the physical interaction of APH-1a with immature NCSTN and PSEN holoprotein as well as with mature NCSTN, PSEN and PEN-2, and reduces γ-secretase activity in intramembrane proteolysis of Notch. Conserved transmembrane residues Gly122, Gly126, and Gly130 in the fourth transmembrane region of mammalian APH-1a are part of the membrane helix-helix interaction GXXXG motif and are essential for the stable association of APH-1a with PSEN, NCSTN, and PEN-2. These findings suggest that APH-1 plays a GXXXG-dependent scaffolding role in both the initial assembly and in the subsequent maturation and maintenance of active γ-secretase complex (Lee et al. 2004)**.**

Over-expression of APH-1 facilitates PEN-2-mediated PSEN-1 proteolysis, resulting in a significant increase in PSEN-1 fragments (Luo et al. 2003), whereas down-regulation of APH-1 alleviates the accumulation of full-length PSEN-1. This suggests that APH-1 is an essential cofactor for stabilizing nascent full-length PSEN-1, but in the absence of APH-1, PSEN-1 holoprotein is diverted to rapid protein degradation pathway (Ratovitski et al. 1997) rather than to the endoprocessing pathway, which generates PSEN-1 NTF and CTF. Nevertheless, it is still possible that APH-1 may be involved in stabilizing PSEN-1 fragments. PSEN-1 over-expression destabilizes APH-1 expression, whereas NCSTN stabilizes APH-1 expression. Because PSEN prevents the over-accumulation of APH-1 by NCSTN, it is possible that stabilization of PSEN holoprotein by APH-1 leads ultimately to the removal and degradation of APH-1 from the maturing complex (Hu and Fortini 2003) (figure 2).

Mutations in aph-1 cause defects in Notch signalling at embryogenesis seen as a marked defect in the ability to localize the Notch pathway component aph-2/ncstn, similar to mislocalization of aph-2/ncstn seen in inactivation of PSEN or NCSTN (Goutte et al.

2002, Hu and Fortini 2003), indicating that APH-1 is required for Notch signalling throughout development. In a genetic study with an Italian AD population, no association was found between APH-1 and sporadic AD (Poli et al. 2003).

2.3.4. PEN-2

Presenilin enhancer-2 (PEN-2) is a transmembrane protein that was found through strong interactions with sel-12 and aph-2, C. elegans orthologies to human PSEN and NCSTN (Francis et al. 2002). Human PEN-2 is a 101-amino acid protein containing two putative transmembrane domains (Crystal et al. 2003). PEN-2 is a component of the γ-secretase complex, and it probably represents the last maturation step of the γ-secretase complex, facilitating endoproteolysis of presenilin and conferring γ-secretase activity (figure 2).

The human PEN-2 and APH-1 proteins interact closely with each other and with PSEN. The N-terminal domain of PEN-2 is important for interactions with PSEN-1 (Crystal et al. 2003). APH-1, PEN-2, and NCSTN are required for the activity and accumulation of γ-secretase; inactivation of APH-1, PEN-2, or NCSTN reduces γ-secretase cleavage of APP and Notch substrates and levels of processed PSEN (Francis et al. 2002). Down-regulation of PEN-2 expression abolishes the endoproteolysis of PSEN-1, whereas over-expression of PEN-2 promotes the production of PSEN-1 fragments, indicating a critical role for PEN-2 in PSEN-1 endoproteolysis (Luo et al. 2003). PEN-2 is also required for the stabilization of the PSEN fragment heterodimer within the γ-secretase complex following PSEN endoproteolysis, and this function critically depends on the PEN-2 C-terminus. Thus, PEN-2 is generally required for γ-secretase complex maturation independent of its activity in PSEN-1 endoproteolysis (Prokop et al. 2004).

PSEN-1 regulates the subcellular localization of PEN-2: in the absence of PSEN, PEN-2 is sequestered in the endoplasmic reticulum (ER) and not transported to post-ER compartments, where the mature γ-secretase complexes reside. PSEN deficiency also leads to destabilization of PEN-2, which is alleviated by proteasome inhibitors. PEN-2 is ubiquitylated prior to degradation and presumably retrotranslocated from the ER to the cytoplasm. In other words, failure to become incorporated into the γ-secretase complex leads to degradation of PEN-2 through the ER-associated degradation-

proteasome pathway (Bergman et al. 2004). PSEN regulates PEN-2 levels posttranslationally by preventing PEN-2 degradation by the proteasome. Thus, the amount of PEN-2 protein is effectively titrated by its PSEN binding partner, and the rapidity with which PEN-2 is degraded in the absence of PSEN interactions, could provide a mechanism to tightly regulate γ-secretase complex assembly (Crystal et al. 2004).

2.3.5. Notch and other γ-secretase substrates

The Notch receptor is a membrane-bound transcription factor that is released to the nucleus by a two-step cleavage mechanism in response to ligand binding. Notch is involved in cell fate decisions during both development and adulthood, and the core Notch signalling pathway involves complex proteolytic processing. The full-length Notch protein synthesized as a 300 kDa precursor protein is a short-lived species, which is mainly cleaved at cleavage site 1 (S1) by furin-like convertase (Blaumueller et al. 1997, Logeat et al. 1998). These two parts reassemble into a heterodimer that forms the Notch receptor found in the plasma membrane. DSL (Delta and Serrate/Jagged in Drosophila and vertebrates, Lag-2 in C. elegans) ligands interact with the extracellular domain rendering region proximal to the membrane susceptible to cleavage by metalloproteases of the ADAM family, which include ADAM17/TACE (TNF-alpha converting enzyme) in vertebrates (Brou et al. 2000). The cleavage site is referred to as S2, and the resulting C-terminal fragment is called NEXT (Notch extracellular truncation). NEXT undergoes further cleavage at a third site (S3) located within its transmembrane domain by γ-secretase resulting in proteolytic release of the Notch intracellular domain (NICD), which translocates into the nucleus and interacts with DNA-binding proteins affecting transcriptional regulation by switching on a DNA-bound co-repressor CSL (CBF1/RBPjk in vertebrates, suppressor of hairless in Drosophila, Lag-1 in C-elegans) into an activating complex (Greenwald 1998, Artavanis-Tsakonas et al. 1999, Kopan and Goate 2002).

Recently, it was found that Notch ligands Delta1 and Jagged2 are also subject to PSEN-dependent, intramembranous γ-secretase processing, resulting in the production of soluble intracellular derivatives. The ectodomain of mammalian Jagged is cleaved by an ADAM17-like activity. Notch stimulates the ectodomain shedding of the ligand and

yields membrane-tethered C-terminal fragments (CTFs) of Jagged and Delta that accumulate in cells expressing the mutant form of PSEN, indicating that a similar molecular apparatus is responsible for intramembranous processing of Notch and its ligands (Ikeuchi and Sisodia 2003). PSEN forms stable complexes with Delta and Jagged and with their respective CTFs. PSEN/ γ-secretase then mediates the cleavage of CTFs to release the Delta and Jagged intracellular domains, portions which can enter the nucleus. The ligand CTFs compete with an activated form of Notch for cleavage by γ-secretase and are thus able to inhibit Notch signaling in vitro (LaVoie and Selkoe 2003). The cytoplasmic derivative of Delta1 was shown to stimulate nuclear transcription, and the soluble Jagged intracellular domain activated gene expression via the transcription factor AP1, suggesting that Delta1 and Jagged2 play dual roles as activators of Notch receptor signaling and as receptors that mediate nuclear signaling events via γ-secretase-generated cytoplasmic domains (Ikeuchi and Sisodia 2003). Notch and its cognate ligands are also processed by the same molecular machinery and may antagonistically regulate each other's signaling (LaVoie and Selkoe 2003)**.**

CD44 is a major adhesion molecule for extracellular matrix components and it is implicated in a wide variety of physiological and pathological processes including regulation of tumor cell growth and metastasis. CD44 undergoes sequential proteolytical cleavages in extracellular and transmembrane domains, and the cleavage product derived from CD44 intramembranous cleavage acts as a signal transduction molecule. Murakami and coworkers (2003) showed recently that the underlying mechanism of the intramembranous cleavage of CD44 is performed by γ-secretase. These findings suggest that γ-secretase may have an important role in CD44-dependent signal transduction and in functional regulation of adhesion molecules (Murakami et al. 2003).

Nectin-1 is a member of the immunoglobulin superfamily and a Ca^{2+}-independent adherens junction protein involved in synapse formation. Nectin-1α interacts with N-terminal fragment of PSEN-1. It undergoes intramembrane proteolytic processing analogous to that of APP, mediated by γ-secretase-like activity, resulting in generation of a 24-kDa nectin-1α intracellular domain. Similar to nectin-1, other members of the

immunoglobulin superfamily involved in synapse formation may also serve as substrates for γ-secretase-like intramembrane proteolytic activity (Kim et al. 2002).

Epithelial (E-) and neural (N-)-cadherins are cell-cell adhesion receptors involved in the development, maintenance and function of most tissues, and they play important roles in cell signaling, proliferation and differentiation (Steinberg and McNutt 1999, McNeill 2000, Tepass et al. 2000). E- and N-cadherins are cleaved with γ-secretase-like activity. The γ-secretase cleavage dissociates E-cadherins, β-catenins and α-catenins from the cytoskeleton, promoting the disassembly of the E-cadherin-catenin adhesion complex. Cleavage releases cytoplasmic E-cadherin to the cytosol and increases the levels of soluble β- and α-catenins, thus increasing the cytosolic pool of β-catenins, a key regulator of Wnt signaling (Marambaud et al. 2002). γ-Secretase cleavage of N-cadherin releases the C-terminal fragment that promotes degradation of transcriptional co-activator CREB binding protein (CBP), and downregulates CBP function (Marambaud et al. 2003). In the same study, it was found that PSEN-1 mutations of familial AD fail to generate the C-terminal fragment of N-cadherin leading to up-regulation of CRE-dependent transactivation. FAD-mutation induced transcriptional abnormalities may be causally related to the dementia in FAD (Marambaud et al. 2002).

ErbB4, a transmembrane tyrosine kinase receptor for neuregulins regulating cell proliferation and differentiation, is a substrate for presenilin-dependent γ-secretase (Ni et al. 2001). Constitutive ectodomain shedding of full-length ErbB4 yields an approximately 80 kDa membrane-associated C-terminal fragment (B4-CTF). Subsequent intramembrane cleavage of B4-CTF into a soluble intracellular domain of ErbB4 (B4-ICD) is inhibited in cells devoid of functional presenilins or by treatment of cells with a γ-secretase inhibitor, leading to enhanced accumulation of B4-CTF. The soluble B4-ICD functions in the nucleus presumably at the transcriptional level, and thus ErbB4 represents the first receptor tyrosine kinase found to undergo intramembrane proteolysis and it may mediate a novel signaling function independent of its canonical role as a receptor tyrosine kinase (Lee et al. 2002).

Deleted in colorectal cancer (DCC), p75 and low density lipoprotein receptor-related protein (LRP) are also substrates for PSEN-dependent γ-secretase cleavage, resulting in

release of their intracellular domains (ICD) (May et al. 2002, Jung et al. 2003, Kanning et al. 2003, Taniguchi et al. 2003). DCC may perform dual roles first as a cell surface receptor that modulates intracellular signaling pathways and second as a transcriptional coactivator that relies on γ-secretase-dependent production and nuclear translocation of cytoplasmic domain. γ-Secretase-mediated p75 proteolysis plays a role in the formation/disassembly of the p75-TrkA receptor complex by regulating the availability of p75 transmembrane domain that is required for this interaction (Jung et al. 2003). LRP is a multifunctional cell surface receptor that interacts through its cytoplasmic tail with adaptor and scaffold proteins that participate in cellular signaling. The γ-secretase cleavage resulting in the release of cytoplasmic domain is indicative of a novel role for the intracellular domain of LRP that may involve subcellular translocation of preassembled signaling complexes from plasma membrane (May et al. 2002).

2.4. Susceptibility genes involved in AD

2.4.1. Apolipoprotein E gene (APOE)

Apolipoprotein E (ApoE) is a polymorphic lipid transport protein present in liver-synthesized very low density lipoproteins (VLDL) and high density lipoprotein (HDL) (Weisgraber 1994). ApoE mediates the cellular uptake of lipoprotein particles through binding to low density lipoprotein receptor (LDLR) and LDLR-related protein (Mahley 1988). ApoE protein exists in three common isoforms (E2, E3 and E4) encoded by three alleles (ε2, ε3 and ε4) of the APOE gene in chromosome 19q13.2 (Emi et al. 1988). In most Caucasian populations, ε3 is the most common allele (78%), while ε2 (8%) and ε4 (14%) are considered variants (Utermann et al. 1980), although the allele distribution varies in different ethnic populations. The alleles code for apoE proteins differing at amino acid positions 112 and 158; ε2 has a cysteine in both positions, ε3 has cysteine and arginine, and ε4 has two arginines (Zannis et al. 1993)**.**

The ε4 allele of APOE gene is a putative susceptibility factor for aggressive multiple sclerosis (Evangelou et al. 1999, Chapman et al. 2001) and coronary heart disease (Wilson et al. 1994). The ε4 allele was also found to be a risk factor for early- and late-onset familial and sporadic AD acting in a dose-dependent manner, and it is linked to an earlier onset of AD (Corder et al. 1993, Saunders et al. 1993, Chartier-Harlin et al.

1994, van Duijn et al. 1994, Rao et al. 1996). Brain apoE is synthesized in astrocytes and microglia, and in AD, apoE immunoreactivity is detected in senile plaques, NFTs and cerebrovascular amyloid (Namba et al. 1991). APOE ε4 carriers have more senile plaques (Rebeck et al. 1993), NFTs (Ohm et al. 1995) and more severe cholinergic deficits (Soininen et al. 1995a, Soininen et al. 1995b) than non-carriers. The APOE genotype specific effects on Alzheimer's disease vary according to age and sex; the ε4 allele has a stronger risk effect in men, and the ε2 allele confers a protective effect only in less elderly people (Qiu et al. 2004).

In response to neuronal damage, apoE production increases to allow a redistribution of lipid breakdown products and cholesterol during neuronal regeneration (Ignatius et al. 1986, Boyles et al. 1989), and the ε4 allele seems to be detrimental to efficient recovery after head trauma as well as after intracerebral haemorrhage, since ε4 allele carriers have higher mortality and poorer recovery rate than ε3 carriers (Alberts et al. 1995). ApoE4 may also impair cognition in stroke patients (Slooter et al. 1997).

Although APOE is an established risk factor for AD, the exact role of apoE in the pathogenesis of AD still remains to be unraveled. There are two popular hypotheses concerning the role of apoE in the aetiology of AD. According to the intracellular model, apoE4 may not bind tau as well as apoE3, leading to microtubule destabilization and formation of NFTs. The extracellular model hypothesizes that apoE4 may bind Aβ more readily than apoE3, and this facilitates the deposition of amyloid in plaques.

The extracellular model is supported by the finding that human apoE forms complexes with synthetic Aβ peptide, and moreover, complex formation occurs within minutes with apoE4 and Aβ, compared to the hours needed with apoE3 and Aβ (Strittmatter et al. 1993). ApoE3 and apoE4 both interact with Aβ peptide to form novel monofibrillar structures, although the Aβ monofibrils appear earlier with apoE4 than with apoE3, which is consistent with the biochemical and genetic association between apoE4 and Alzheimer's disease (Sanan et al. 1994). Furthermore, a body of evidence suggests that one major mechanism underlying the connection between apoE and both AD and cerebral amyloid angiopathy (CAA) is related to the ability of apoE to interact with Aβ peptide, influencing its clearance, aggregation, and conformation. In addition to a

number of in vitro studies supporting this concept, in vivo studies with APP transgenic mice indicate that apoE and a related molecule, clusterin (apolipoprotein J), have profound effects on the onset of Aβ deposition, as well as on the local toxicity associated with Aβ deposits both in the brain parenchyma and in cerebral blood vessels (Holtzman 2004). One convincing piece of evidence favoring the extracellular model is the observation that lack of apoE dramatically reduces Aβ deposition in a transgenic model of AD (Bales et al. 1997).

The intracellular model is supported by findings according to which apoE4 is associated with microtubule depolymerization and apoE4 also inhibits neurite outgrowth (Nathan et al. 1995), whereas apoE3 promotes neurite sprouting and extension and binds tau regions which appear to promote self-assembly of tau into PHF. The binding of apoE3 to the microtubule-binding repeat region of tau possibly prevents tau phosphorylation and incorporation into NFTs (Strittmatter et al. 1994, Nathan et al. 1995). In addition, C-terminal-truncated forms of apoE occurring in AD brains and cultured neurons induce intracellular NFT-like inclusions in neurons. Furthermore, apoE4 is more susceptible to truncation than apoE3, resulting in a much greater intracellular inclusion formation, suggesting that apoE4 preferentially undergoes intracellular processing, and creates a bioactive fragment that interacts with cytoskeletal components and induces NFT-like inclusions in neurons (Huang et al. 2001).

On the other hand, apoE4 may lack the properties of apoE3, and it has been suggested that is not the presence of apoE4, but rather the lack of the other apoE isoforms that leads to the increased predisposition to AD (Strittmatter et al. 1994). Consistent with this, apoE3 is hypothesized to provide a dose-dependent protective effect against Aβ deposition (DeMattos 2004).

2.4.2. Insulin degrading enzyme and neprilysin: Aβ degrading enzymes

One hypothesis suggested to be behind the process of AD, is that there is an imbalance between Aβ production and clearance (Hardy and Selkoe 2002). Therefore, reducing Aβ production or accelerating its clearance from brain have been proposed as therapeutic targets against AD (Glabe 2000, Iwata et al. 2000). Insulin degrading enzyme (IDE) and neprilysin (NEP) are novel genes associated to AD – these proteins

possess the biological function of degrading Aβ, and overexpression of either of these two genes retards or completely prevents amyloid plaque formation in transgenic mouse models, indicating that they may represent an efficacious therapeutic approach in preventing the pathological changes characteristic of AD (Leissring et al. 2003).

NEP or membrane metallo-endopeptidase (MME, CD10) in chromosome three is a zinc metallopeptidase constitutively expressed in various tissues. NEP is important in destruction of opioid peptides by cleaving Gly-Phe bonds. It also cleaves at amino groups of hydrophobic residues in insulin, casein, hemoglobin, and a number of other proteins and polypeptides. In addition, NEP has recently been shown to degrade Aβ (Iwata et al. 2000), and its expression is down-regulated in neurons of wildtype and transgenic, APPswe expressing mice as a result of aging (Iwata et al. 2002, Apelt et al. 2003) as well as in affected areas of AD brains (Akiyama et al. 2001). Inhibiting the peptidase function of NEP leads to deposition of Aβ42 in the brain (Iwata et al. 2000). Injecting Aβ42 into the brains of young mice leads to an up-regulation of endogenous NEP expression, reduction of Aβ levels and delayed amyloid plaque deposition (Mohajeri et al. 2002), while in aged mice, NEP expression is increased but not capable of reducing brain Aβ concentrations or removing pre-existing brain amyloid plaques (Mohajeri et al. 2004). Therefore, NEP may be effective early in the course of AD, when brain Aβ is found at a higher degree in soluble rather than in aggregated form (Mohajeri et al. 2004). Thus, the biological activity of NEP might be affected by amyloidogenic factors during aging. In most familial early-onset AD cases, Aβ accumulation is caused by an increase in Aβ42 production (Scheuner et al. 1996, Hardy 1997), while in sporadic late-onset AD there does not seem to be any overproduction of Aβ42 (Scheuner et al. 1996) and therefore, aging-associated reduction of Aβ clearance is a candidate mechanism accounting for the accumulation of Aβ in aging brain. In support of this theory, late-onset AD has been associated with a repeat polymorphism in the promoter region of NEP gene (Sakai et al. 2004), which might cause or be in linkage disequilibrium with another polymorphism with a functional effect on the NEP transcription rate.

IDE or insulysin, a cytosolic metalloendoprotease that degrades insulin, glucagon and other polypeptides, is involved in insulin metabolism. In 1994, IDE was found to degrade Aβ (Kurochkin and Goto 1994), thus preventing the accumulation of amyloidogenic derivatives (McDermott and Gibson 1997). The interaction between insulin and Aβ was demonstrated by the observation that excess insulin inhibited the degradation of Aβ nearly completely, and Aβ inhibited insulin degradation in a dose-dependent manner. In addition, IDE activity is considerably lower in the cytosolic fraction of AD brains compared to controls (Perez et al. 2000), possibly reflecting the findings of stronger neuronal IDE immunostaining in AD brains compared to controls. The staining is particularly found in neurons associated with senile plaques (Bernstein et al. 1999), indicating the abnormal localization of IDE with insoluble compartments (Perez et al. 2000). At the same time, IDE activity in insulin degradation is less extensively decreased than in Aβ degradation, suggesting that the metabolic abnormalities in AD cells change the affinity of IDE for its substrates (Perez et al. 2000, Hoyer 2002). Alternatively, the decreased activity of IDE may be related to the cell loss in AD brains. Therefore, it is a matter of debate whether the lower IDE activity in AD brain is a primary defect or a result of neurodegenerative processes (Perez et al. 2000). IDE interacts with Aβ residues 17-24 (McDermott and Gibson 1997), which are also crucial to the formation of amyloid fibrils (Soto et al. 1995). 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, sufficient to cause type 2 diabetes mellitus, and possibly, to increase the risk of AD (Farris et al. 2004).

Impaired glucose and energy metabolism as such are characteristic for sporadic AD, and it has even been suggested that formation of amyloidogenic derivatives and hyperphosphorylation of tau are merely a consequence of reduced neuronal activity deriving from the deficit of neuronal glucose and energy metabolism (Hoyer 2002). The abnormal neuronal glucose metabolism is supposedly caused by a disturbance in the insulin signal transduction, whereas in AD, insulin receptor density is upregulated, indicating desensitization of neuronal insulin receptors (Frolich et al. 1998, Hoyer 1998). Impaired cerebral glucose metabolism leads to a decline in acetyl-CoA (Perry et al. 1980) and in acetylcholine concentrations (Sims et al. 1983), which in turn leads to a

degeneration of the cholinergic system correlating with the progress of disturbed mental capabilities in AD patients (Baskin et al. 1999, Davis et al. 1999). ATP production is also diminished, leading to an energy deficit that progresses during the course of disease (Hoyer 1992). Reduction of insulin and ATP attenuates the secretion of neuroprotective sAPP (Gasparini et al. 1997, Gasparini et al. 1999, Hoyer 2002), increases the secretion of Aβ (Gabuzda et al. 1994), and activity of protein kinases that hyperphosphorylate tau (Roder and Ingram 1991, Bush et al. 1995, Hong and Lee 1997). In addition, insulin resistance has recently been shown to promote amyloidosis in a mouse model of AD (Ho et al. 2004), and moreover, decreased hippocampal glucose metabolism and spatial memory impairment are associated with Aβ deposition in APP/PSEN-1 transgenic mice (Sadowski et al. 2004). The decreased insulin receptor (IR) mediated transduction, as a consequence of insulin resistance, might mediate this function through decreased IR beta subunit autophosphorylation and reduced phosphatidylinositol-3 kinase to increase GSK-3α activity, which in turn promotes Aβ generation. Thus, insulin resistance may be connected to mechanisms responsible for developing AD, and IR signaling may underlie the Aβ production in the brain (Ho et al. 2004).

Based on functional grounds, IDE is a noteworthy candidate gene of AD. Furthermore, IDE is within or near the chromosome 10 locus identified in family-based linkage-studies of AD (Bertram et al. 2000, Myers et al. 2000). Subsequently IDE has been associated to AD in numerous SNP and haplotype studies (Ait-Ghezala et al. 2002, Prince et al. 2003, Ertekin-Taner et al. 2004), and in particular to AD in patients without the APOE ε4 allele (Edland et al. 2003, Edland 2004). The associated polymorphisms in the promoter region might affect the expression levels of IDE or perhaps intronic variants can affect the post-transcriptional processing and expression levels of splice variants (Edland et al. 2003). Alternatively, the associated polymorphisms may be in linkage disequilibrium with another, yet unidentified, variant of IDE or on a neighboring gene (Prince et al. 2003).

2.5. Mapping of novel AD genes

2.5.1. Population choice in complex disease gene mapping

In order to map genes underlying complex diseases like AD, genetic isolates with a history of a small founder population, long-lasting isolation and population bottlenecks have been increasingly used, since they are valuable resources in the identification of disease genes (Peltonen 2000). The main advantage of genetic isolates is that in genes behind complex diseases there should be less variability (fewer disease disposing alleles and fewer influencing genes), and therefore, the isolates could be useful in association-based mapping studies (Jorde et al. 2000). The Finnish population has been especially notable in the application of linkage disequilibrium approaches, largely because of its small founding size and limited admixture (Jorde et al. 2000) shown by extreme locus and allelic homogeneity of monogenic Finnish disease heritage disorders (Peltonen 2000). Particularly the Finnish population in the late settlement area in the eastern and northern part of Finland is only 300-400 years, or 15-20 generations of age (Peltonen 2000), and the relatively young age of Finnish population means that this the population is useful in the initial localization of disease-causing genes to relatively large chromosomal regions due to larger regions of linkage disequilibrium (Jorde et al. 2000). The age of the Finnish population is also young enough for disease-causing mutation to be associated with a specific background haplotype, but old enough for recombinations to decrease disequilibrium between the mutation and less closely linked markers (Jorde 1995).

2.5.2. Single nucleotide polymorphisms and candidate gene association studies

More than five million single nucleotide polymorphisms (SNPs) (one per 600 bp) with a minor allele frequency greater than 10% are expected to exist in the human genome (Kruglyak and Nickerson 2001). Compared to mutations, whose frequency is below 1% in a population, SNPs are very polymorphic and because of their polymorphic nature, SNPs can be utilized in the search of genes predisposing to multifactorial diseases. SNPs are likely more useful for population association studies than microsatellite markers as a result of their much higher density in the genome and much greater mutational stability (Ardlie et al. 2002). On the other hand, instead of single SNPs or SNP haplotypes, highly polymorphic microsatellite markers can provide much greater

power for detecting intermarker LD, emphasizing the importance of careful marker selection when designing a LD study (Varilo et al. 2003).

In the search of susceptibility genes for single gene disorders, linkage analysis or positional cloning, where the whole genome of affected family members is scanned, have been widely applied (Kwon and Goate 2000, Daly and Day 2001). A candidate gene approach, where the association between an allele or alleles of a candidate gene and a disease is studied, has been suggested to be better suited for detecting genes underlying common complex disorders where the risk associated with a given candidate gene is relatively small (Risch and Merikangas 1996). One clear advantage of association studies compared to linkage-based approaches is the large number of cases and controls that are more easily obtained compared to trying to locate families with multiple affected members (Kwon and Goate 2000).

Choosing a potential candidate gene involves an educated guess and the understanding of the mechanisms underlying the disease studied. Another approach is to choose candidate gene based on its localization in genomic region that has been linked to the disease in a genome wide search. A polymorphic site chosen within the gene may represent a possible functional SNP causing a change in protein function via amino acid substitution, in gene activity by affecting gene transcription, RNA splicing, mRNA stability and mRNA translation or it may be associated to deletion or insertion resulting in a frameshift in the coding region and complete deletion of the gene (Daly and Day 2001). Possibly, the SNP has no known functional significance, however, noncoding SNP in intron or outside the gene may also be associated with altered phenotype, if it is located close to functional SNP (typically within 200 kb) or other change in nearby genomic region, and is inherited together or is in linkage disequilibrium with the functional SNP (Kwon and Goate 2000).

Polymorphisms with functional consequences should be considered as high priority, because most likely they affect the function of the protein, and therefore might be involved in disease aetiology (Tabor et al. 2002). However, such polymorphisms are expected to have low allele frequencies and, in fact, the majority of coding region SNPs (cSNPs) that change an amino acid have allele frequencies below 5% (Cargill et al. 1999, Halushka et al. 1999). SNPs with very low allele frequencies should have very

large relative risks associated with them, if they are to be detected in an association study, and therefore, SNPs with allele frequencies of at least 5% are generally more likely to be useful in association studies. Thus using polymorphisms with less severe functional effects might be advisable (Tabor et al. 2002). The estimates that there are approximately 30,000 genes in the human genome (Ewing and Green 2000, Roest Crollius et al. 2000, Lander et al. 2001), point to the existence of a total of approximately 120,000 cSNPs. Of these, 40% are expected to change an amino acid (Cargill et al. 1999, Halushka et al. 1999). These 50,000 non-synonymous cSNPs, together with an unknown number of regulatory and other non-coding but functional polymorphisms, comprise the bulk of common molecular variation with potential phenotypic consequences (Kruglyak and Nickerson 2001). In the future, genotyping this complete set of functional variants will be a minimal requirement for direct association studies (Lander 1996, Risch and Merikangas 1996, Collins et al. 1997).

Candidate gene association studies have pitfalls that should be considered when designing any study. A major drawback in testing candidate SNPs directly is the fact that lack of association with candidate SNP does not rule out functionally important changes at nearby SNPs, except those that are in LD with the candidate SNP (Carlson et al. 2004b). A problem of case-control design is the inappropriate matching of cases and controls with respect to demographic factors, such as age, gender and genetic background which may result in false associations (Kwon and Goate 2000, Daly and Day 2001). From the statistical viewpoint, genotyping a number of candidate genes and polymorphisms in the same population of cases and controls increases the chance of false associations due to multiple testing. Bonferroni correction (multiplying the p-value with the number of tests performed) has been applied, but the conservative nature of this correction may also lead to loss of genuine associations. The requirement of Bonferroni correction has been debated in cases where the candidate gene has a clear biological relevance to the disease and the chosen polymorphism has a proven functional significance (Perneger 1998, Daly and Day 2001). Another problem arises from interactions between polymorphisms in multiple loci that have been modelled with several possible epistatic models, such as synergistic, antagonistic, balanced or permissive epistatic interactions. The number of gene-gene or gene-environment interactions is enormous, and because of the sheer number of potential interactions, no

comprehensive search has the power to detect them all. There are several reasonable approaches to reduce the interactions analyzed for example, to limit analysis to biologically plausible interactions between genes in related pathways or to markers with an appreciable single-locus effect, given that risk interactions are more reasonable between genes in a physical interaction, in the same pathway or in the same regulatory network (Carlson et al. 2004a).

2.5.3. Linkage disequilibrium

The genetic complexity underlying common diseases is largely unknown, and the unmapped susceptibility genes behind common complex disorders with genetic components have only a scant prospect of being identified using current technologies such as linkage and linkage disequilibrium analysis (Wright et al. 1999). Linkage analysis using independent pedigrees has a limited power to detect genes with intermediate or small effects on common diseases, while association methods have a greater power to detect such effects (Jorde 1995, Risch and Merikangas 1996). Linkage disequilibrium (LD) is found when alleles of different loci are inherited together more often than would be expected based on their frequencies, and it measures the co-segregation of alleles in a population, when linkage measures the co-segregation in a pedigree. The key factors influencing the LD are the recombination frequency and the number of generations after introduction of the mutation or the age of the mutation. LD around an allele arises because of selection or population history, small population size, genetic drift or population mixture, and decays owing to rapid population growth reducing the genetic drift and recombination breaking down ancestral haplotypes (Hartl and Clark 1997).

LD mapping relies on the assumption that a single ancestral mutation is responsible for a large proportion of disease cases in the present-day population, and LD mapping in the population has an increased power to detect genes with intermediate or small effects on common diseases, but the power is limited by the amount of allelic heterogeneity (Terwilliger and Weiss 1998).

As a measure of LD, several variables are used (Lewontin 1964, Devlin and Risch 1995). One of the earliest measures of LD, symbolized by *D*, quantifies disequilibrium

as the difference between the observed frequency of a two-locus haplotype (P_{AB}, for alleles A and B) and the frequency it would be expected to show if the alleles were segregating at random ($P_A \times P_B$).

$D = P_{AB} - P_A \times P_B$.

Alternative measures of LD are the absolute values of r^2 and D' defined by

$D' = D/D_{max}$.

$D' = 1$ is known as complete LD, when two markers have not been separated by recombination, and values of $D' < 1$ indicate that the complete ancestral LD has been disrupted during the history of the sample.

Considerable variation has been found in the pattern and extent of LD, resulting in a debate concerning the average extent of levels of LD useful for association mapping (Ardlie et al. 2002). For northern European populations, a range of 10-30 kb of LD has been proposed, but due to a tremendous 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).

Full-genome linkage and association analysis have been used to detect novel susceptibility regions for AD, and in future we will know whether any candidate gene of these susceptibility regions shows consistent evidence for AD association in various populations (table 3).

2.5.4. Haplotype blocks

LD patterns vary across the human genome with some regions of high LD interspersed by regions of low LD (Daly et al. 2001, Johnson et al. 2001, Patil et al. 2001, Dawson et al. 2002, Gabriel et al. 2002). Thus it has been suggested that the human genome can be arranged into block-like patterns of LD, referred to as haplotype blocks, with low between block recombination frequency (Daly et al. 2001, Jeffreys et al. 2001, Reich et al. 2001, Wang et al. 2002). In these regions of strong LD, only a few common haplotypes (the arrangement of genetic polymorphisms within a single chromosome) are found. The haplotype blocks may extend up to 100 kb regions in which almost no

Table 3. Linkage and association regions observed in full-genome screens, and potential candidate genes locating at the regions according to Bertram and Tanzi, 2004.

Chromosomal location	Study	Candidate genes	Relevance to AD pathogenesis
Early-onset loci			
11q25	(Blacker et al. 2003)	*BACE*	β-cleavage of Aβ
19q13	(Blacker et al. 2003)	*PEN-2/ GSK3A*	Component of γ-secretase complex/ APP processing and tau phosphorylation
Late-onset loci			
1p36	(Kehoe et al. 1999, Hiltunen et al. 2001, Myers et al. 2002)	*MTHFR*	Homocysteine metabolism
4q35	(Li et al. 2002, Blacker et al. 2003)	*SNCA*	Tau fibrillization, component of Aβ plaques
5p13-15	(Kehoe et al. 1999, Pericak-Vance et al. 2000, Hiltunen et al. 2001, Myers et al. 2002, Blacker et al. 2003)	-	
6p21	(Kehoe et al. 1999, Hiltunen et al. 2001, Myers et al. 2002, Blacker et al. 2003)	*TNFA* *HLA-A2*	Inflammation and apoptosis Inflammation
6q22	(Pericak-Vance et al. 1997, Kehoe et al. 1999, Myers et al. 2002)	*ESRA*	Estrogen mediated actions
9p21	(Kehoe et al. 1999, Pericak-Vance et al. 2000, Myers et al. 2002)	*VLDL-R*	Cholesterol metabolism
9q22	(Kehoe et al. 1999, Myers et al. 2002, Blacker et al. 2003)	*ABCA1*	Brain cholesterol metabolism
10q21-22	(Kehoe et al. 1999, Myers et al. 2002)	*HHEX*	Hematopoietically expressed homeobox
10q24-25	(Li et al. 2002, Blacker et al. 2003)	*IDE* *PLAU* *KNSL1*	Degradation of Aβ Degradation of Aβ ? Thyroid receptor interacting protein
12p11	(Pericak-Vance et al. 1997, Kehoe et al. 1999, Myers et al. 2002)	*A2M* *LRP* *TFCP2*	Aβ catabolism Internalization of APP and Aβ Transcription factor
19q13	(Zubenko et al. 1998, Kehoe et al. 1999, Pericak-Vance et al. 2000, Myers et al. 2002, Blacker et al. 2003)	*APOE*	Aβ aggregation
Xp21	(Kehoe et al. 1999, Myers et al. 2002, Blacker et al. 2003)	*MAOA*	Metabolism of neuroactive and vasoactive amines
Xq21-26	(Zubenko et al. 1998, Kehoe et al. 1999, Myers et al. 2002)	-	

- No potential candidate gene assessed.

recombination takes place, being separated by recombination hot-spots (Patil et al. 2001). Therefore, the human genome can be partitioned into blocks with limited

haplotype diversity, so that only a small fraction of the SNPs captures most haplotypes (Zhang et al. 2002). Using these haplotype tagging SNPs (htSNPs) that are sufficient to capture most of the haplotype structure of the human genome with a block structure (Johnson et al. 2001, Patil et al. 2001) makes the genome-wide association studies more feasible (Daly et al. 2001, Johnson et al. 2001). Using htSNPs forming only 25% of all the SNPs, the power to find an association is reduced by 4%, compared to an approach using a comparable number of randomly chosen SNPs, where the power loss is 12%. At least two human chromosomes (chromosomes 21 and 22) seem to show this kind of block-like structure (Patil et al. 2001, Dawson et al. 2002).

Although htSNPs are superior to randomly selected SNPs, LD-selected tagSNPs describe more comprehensively common patterns of variation with more straightforward interpretation of the results (Carlson et al. 2003). To genotype only the minimal amount of SNPs, rational selection of SNP sites providing maximal information about common variations in genomic region is possible on the basis of the observed pattern of LD between common SNPs. In Americans originating from Europe, covering all genomic regions would mean genotyping of 4.2 tagSNPs per 10 kb, and extrapolating this to 35 000 genes with an average of 27 kb, genotyping of 400 000 SNPs would be required if one wished to resolve over 80% of all existing haplotypes (Carlson et al. 2004b). However, because an appreciable fraction of all common variants is either private or common only in a single population, a subset of SNPs required to detect disease associations should be selected specifically for each population with a different demographic history (Wang et al. 2002, Carlson et al. 2003).

2.6. CYP19

2.6.1. Aromatase enzyme

Aromatase enzyme, a product of the CYP19 gene, catalyzes the formation of estrone and estradiol from C_{19} androgens, androstenedione and testosterone (figure 4) in a series of reactions leading to formation of the phenolic A ring characteristic of estrogens. In most mammals, aromatase is expressed only in gonads and brain, whereas primates express the gene in additional extragonadal sites (Simpson et al. 1994). Estrogen synthezised in extragonadal sites acts predominantly at the local tissue level in a

paracrine or intracrine fashion (Labrie et al. 1998). The total amount of estrogen synthezised at these sites may be minute, but the local tissue concentrations achieved are probably high and exert biological influence locally. Thus extragonadal estrogen biosynthesis plays an essential but thus far mostly unrecognized, physiological and pathophysiological role (Simpson and Davis 2001).

In brain, the aromatase enzyme is expressed in neurons, astrocytes and glial cells (Zwain and Yen 1999, Ishunina et al. 2005), and it is present mainly in brain regions involved in the control of reproductive functions, such as the hypothalamus, the preoptic area and the limbic system (Roselli and Resko 1993, Abdelgadir et al. 1994), where aromatization of androgens is required for the sexual differentation of the developing brain (MacLusky and Naftolin 1981), and for the display of male sexual behavior (Clancy et al. 1995). In primates, aromatization mediates the effects of testosterone on gonadotrophin secretion as a part of the feedback mechanism (Ellinwood et al. 1984), but in addition to this effect, the local formation of estrogens in adult brain may be involved in modulating cognitive processes or altering aging by mechanisms currently unknown (Lephart 1996).

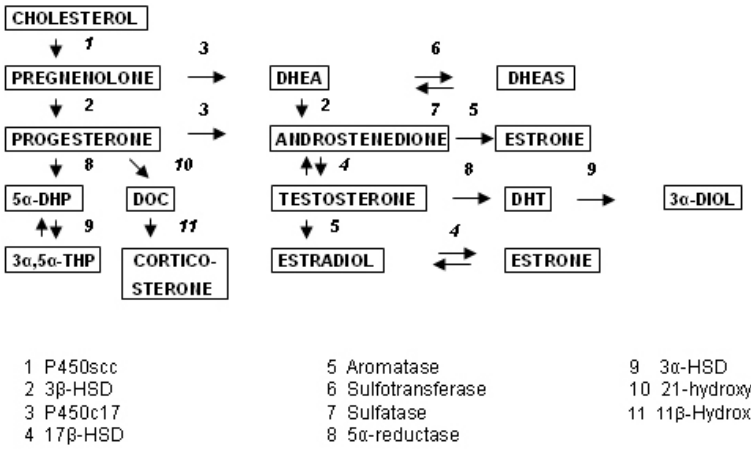

Figure 4. Current knowledge concerning the biochemical pathways of neurosteroidogenesis. Biochemical reactions indicated by arrows have been demonstrated by measuring the biological activity of the enzyme in neural tissue and the localization of the enzyme by immunohistochemistry or its mRNA in situ hybridization in the nervous system with the exception of 21-hydroxylase for which only expression of its mRNA has been shown. (Zwain and Yen 1999, Stoffel-Wagner 2001).

Aromatase enzyme of P450 cytochrome superfamily is encoded by CYP19 gene on chromosome 15q21.1. The coding region of CYP19 gene is 30 kb in size spanning nine

exons of which exon II is the first translated exon. Upstream of exon II are a number of alternative first exons that are spliced into the 5'-untranslated region of the transcript in a tissue-specific fashion. For example, placental transcripts contain at their 5'-end a distal exon I.1 that is driven by powerful promoter upstream of exon I.1 (Means et al. 1991). On the other hand, transcripts of ovaries, testes, adipose tissue and endometric plaques contain promoter II-specific exonic sequence, which is immediately upstream of the translational start site (Zeitoun et al. 1999). Osteoblast transcripts contain yet another distal exon (I.4) downstream of exon I.1 (Mahendroo et al. 1993), and in addition, brain have their own first exon (I.f) (Honda et al. 1994). Splicing of these untranslated exons to form the mature transcript occurs at a common 3'-splice junction that is upstream of the translational start site. The presence of regulatory region of 93 kb with a number of alternative first exons indicates that although transcripts of different tissues have different 5'-termini, the coding region and thus the protein expressed in various tissue sites is always the same. However, the promoter regions upstream of each of the several untranslated first exons have different cohorts of response elements, and so the regulation of aromatase expression and estrogen biosynthesis in each tissue is unique (Simpson et al. 1997).

The brain-specific exon I.f promoter region contains a potential androgen/glucocorticoid binding site (Honda et al. 1994), and furthermore, aromatase mRNA may contain an estrogen responsive element, that might stabilize the aromatase mRNA and increase CYP19 gene expression (Harada et al. 1993, Bourguiba et al. 2003). Indeed, there is large body of evidence that aromatase is regulated by both its substrates, aromatizable androgens, and by its endproduct, estradiol, at least partly at the translational level (Wagner and Morrell 1996, Balthazart et al. 2003). Furthermore, aromatase activity and mRNA are influenced by castration and ovariectomy and aromatizable androgens and estrogen (Yamada et al. 1993, Dellovade et al. 1994, Roselli et al. 1998, Balthazart et al. 2003). Tissue-specific and brain region-dependent expression of aromatase may be regulated by different tissue- and site-specific promoters employing different signalling pathways and different transcription factors including more than sex steroids (Simpson and Davis 2001, Ishunina et al. 2005). Additionally, locally produced neurosteroids may diffuse from other brain regions and contribute to the regulation of aromatase expression (Balthazart et al. 2003).

2.6.2. Estrogens and AD

A gender-related difference has been related to the risk of AD; prevalence rates of AD are higher in women than men, irrespective of age (Fratiglioni et al. 1997). In premenopausal women, the ovaries are the principal source of estradiol, which functions as a circulating hormone to act on distal target tissues. However, in postmenopause the ovaries cease to produce estrogens with the plasma estrogen levels decreasing dramatically (Van Beek et al. 2004), and under these circumstances, estradiol is no longer solely an endocrine factor; instead it is produced in a number of extragonadal sites and acts locally as a paracrine or intracrine factor (Labrie et al. 1998). The plummeting estrogen levels might increase the risk of developing AD in postmenopausal women compared to age-matched men (Paganini-Hill and Henderson 1994). This hypothesis is also supported by a study which measured lower estrogen levels in women with AD compared to age-matched controls (Honjo et al. 1989). The increased lifetime risk of dementia for women reflects both the higher life expectancy of women and the higher dementia risk at very old age (Ott et al. 1998).

Estrogens have a variety of beneficial effects on brain functioning; estrogens induce neurite outgrowth (Ferreira and Caceres 1991), increase dendritic spine density and regulate synaptogenesis (Gould et al. 1990, Murphy and Segal 1996), reduce the accumulation of β-amyloid (Xu et al. 1998) and protect neurons from oxidative stress (Behl et al. 1995, Goodman et al. 1996). Estrogens may also promote the activity of the cholinergic system, reduce cardiovascular risk, and ERT has been claimed to protect against cognitive decline and dementia in the elderly (Skoog and Gustafson 1999). In addition, estrogens have improved some forms of learning and memory in animal studies conducted mainly with mice and rats mimicking the postmenopausal state by ovariectomy (O'Neal et al. 1996, Packard and Teather 1997, Heikkinen et al. 2002, Li et al. 2004)

The cellular actions of estrogens are mediated through estrogen receptors (ERs) α and β that are expressed throughout the brain, including the brain areas important for learning and memory functions, such as the hippocampus (Mitra et al. 2003). ERs are ligand-activated enhancer proteins that function both as signal transducers and transcription factors. The ER subtypes are expressed differentially and tissue-specifically in response

to estrogen (Carley et al. 2003, Matthews and Gustafsson 2003). The classical nuclear receptor mechanism of gene transcription modulation involves the binding of the hormone to its cognate receptor protein, conformational changes of the receptor site and altered composition of interacting proteins, allowing the ligand-occupied ER dimer to translocate to the nucleus and to bind specific sequences in the promoter region of target genes (Beato and Klug 2000). In the direct binding mechanism, the estradiol-ER complex binds directly to a specific sequence, the so-called estrogen responsive element, and interacts directly with coactivator proteins and components of the RNA polymerase II transcription initiation complex resulting in enhanced transcription (Klinge 2000). During tethering, the ER interacts with another DNA-bound transcription factor stabilizing the transcription factor and recruiting coactivators to the complex without ER binding to DNA (Li et al. 2001). The timescale of these actions varies from one to several hours.

In addition to the genomic action, there is a growing body of evidence that estrogens can mediate physiological effects through non-genomic or non-classical signaling mechanisms that are characterized by their rapid onset from seconds to minutes, and their insensitivity to transcriptional inhibitors. These effects are likely to be mediated by receptors integrated or associated with the plasma membrane and by an activation of distinct intracellular signaling cascades (Falkenstein et al. 2000, Kuppers et al. 2001). It is possible that the classical ERα associates with neuronal plasma membrane via MNAR (modulator of non-genomic estrogen receptor) protein (Wong et al. 2002) capable of phosphorylating members of the MAP kinase family, and mediating these rapid non-genomic estrogen actions (Beyer et al. 2003).

2.6.3. Estrogen replacement therapies in AD

Numerous case-control, cross-sectional and prospective studies (Henderson et al. 1994, Paganini-Hill and Henderson 1994, Tang et al. 1996, Kawas et al. 1997, Baldereschi et al. 1998, Jacobs et al. 1998, Harwood et al. 1999, Steffens et al. 1999, Waring et al. 1999) have reported a lower risk of AD and dementia for women receiving postmenopausal ERT compared to women without ERT, whereas several prospective observational studies (Barrett-Connor and Kritz-Silverstein 1993, Brenner et al. 1994, Matthews et al. 1999, Henderson et al. 2000, Mulnard et al. 2000, Wang et al. 2000)

have found no protective effect of estrogen on cognition, or the incidence of dementia. Four recently conducted meta-analyses assessing the risk of AD and dementia, found a protective effect for estrogen use (Yaffe et al. 1998, Hogervorst et al. 2000, LeBlanc et al. 2001, Nelson et al. 2002), although the methodological problems of the studies and the harmful effects of estrogen use on breast cancer and stroke risk were also emphasized. However, a recently conducted randomized, placebo-controlled clinical trial demonstrated an adverse effect of the combination of estrogen-progestin on the risk of dementia, with no effect on mild cognitive impairment (Shumaker et al. 2003). The risk of stroke also increased in women using the combined estrogen-progestin preparation, and the hypothesis could be that the increase in the risk of developing dementia may primarily be due to an increase in vascular-related dementia, since vascular disease often coexists with AD (Snowdon et al. 1997), and silent brain micro-infarcts increase the risk of dementia (Vermeer et al. 2003). The combination of estrogen/progestin may also illustrate the possibility of increased risk of dementia, since when used in combination, the synthetic medroxyprogesterone acetate used in the study (Shumaker et al. 2003), appears to reverse the positive effects of estrogen alone (Nilsen and Brinton 2002a, b, 2003), as also demonstrated in a prospective observational study (Rice et al. 2000). Disappointingly, on February 2004, a large randomized, placebo-controlled clinical trial of estrogen use was also stopped 13 months prior to planned study end, because of the increased risk of stroke and no apparent protective effect on coronary heart disease (http://www.wfubmc.edu/whims/docs/E-alonePress2-27-04finalSR.pdf). Until the final results of this study concerning the risks of AD and mild cognitive impairment are available, estrogen and progestin in combination should not be prescribed with the expectation of enhancing cognitive performance in postmenopausal women, since the risks clearly outweigh the benefits (Shumaker et al. 2003).

2.6.4. Androgens and AD

Androgens are synthesized in ovary, testes and adrenal gland, and aging diminishes the production and serum concentrations of androgens in both genders (Ravaglia et al. 1996, Burger et al. 2000, Schatzl et al. 2003, Van Beek et al. 2004). In women, aging affects more than menopause (Burger et al. 2000, Van Beek et al. 2004), however, postmenopausal ERT reduces DHEAS and testosterone concentrations in serum, and in

addition, increases circulating steroid hormone binding protein levels, resulting in an even more prominent decline of free testosterone (Casson et al. 1997).

The impact of androgens on cognitive decline in the elderly and molecular mechanisms of neurodegeneration are still unclear because of conflicting data (Hogervorst et al. 2001, Weill-Engerer et al. 2002, Bimonte-Nelson et al. 2003, Tan and Pu 2003, Wolkowitz et al. 2003). The information regarding the testosterone levels of men with AD compared to healthy control subjects is also controversial (Hogervorst et al. 2001, Pennanen et al. 2004). Nevertheless, androgen supplementation by testosterone has been shown to improve cognition and visual-spatial skills in hypogonadal aging men (Tan and Pu 2003), and working memory of aged male rats (Bimonte-Nelson et al. 2003), but the testosterone precursor, dehydroepiandrosterone (DHEA), is incapable of improving cognitive performance of subjects with AD (Wolkowitz et al. 2003). Androgen receptors and cognitive function have also been associated in a genetic study (Yaffe et al. 2003).

The benefits of androgens on characteristics relevant to AD are clear; androgens prevent hyperphosphorylation of tau, a component of NFTs (Papasozomenos 1997, Papasozomenos and Shanavas 2002), attenuate β-amyloid toxicity (Pike 2001), stimulate the α-secretase pathway resulting in increased sAPPα production (Goodenough et al. 2000) and reduced β-amyloid peptide production (Gouras et al. 2000, Ramsden et al. 2003), increase hippocampal spine synapse density (Leranth et al. 2004) and protect neurons from oxidative stress (Bastianetto et al. 1999), excitotoxicity (Veiga et al. 2003) and against apoptosis (Hammond et al. 2001). However, the importance of testosterone aromatization on these effects is still controversial. Several studies support the hypothesis of androgens affecting directly through androgen receptors (Papasozomenos 1997, Hammond et al. 2001, Pike 2001, Papasozomenos and Shanavas 2002, Ramsden et al. 2003), while others have suggested that it is the androgens which are aromatized to estrogens that underlie the positive effects of androgens (Goodenough et al. 2000, Bimonte-Nelson et al. 2003, Veiga et al. 2003) or that both pathways may be involved (Leranth et al. 2004). Androgens have many similar neuroprotective effects as estrogens, and it is possible that in postmenopausal brain, androgens acting via androgen receptors are the neuroprotective hormones.

Testosterone also inhibits early atherogenesis via its aromatization to estradiol (Nathan et al. 2001). The risk of coronary artery disease is greater in men than women during the reproductive years, but after female menopause, the risk is equal among women and men (Nathan and Chaudhuri 1997), implicating that the gender-difference might be caused by the attenuating effect of estrogen on atherogenesis (Krempler et al. 1995, Rosano and Fini 2002). Since the clinical indicators of atherosclerosis are more frequent in individuals with AD than in nondemented individuals (Hofman et al. 1997), the conversion of androgens to estrogens might also protect from dementia via the protection against atherogenesis.

2.6.5. CYP19 and AD

Epidemiological studies suggest a neuroprotective role for estrogen in AD and therefore the genetic role of components of estrogen metabolism, as well as α and β estrogen receptors, have been studied extensively (Brandi et al. 1999, Forsell et al. 2001, Lambert et al. 2001). In brain, local expression of aromatase is involved in regulating neural differentiation, plasticity, and sexual behavior through estrogen synthesis (Hutchison 1991, Lephart 1996, Mellon et al. 2001, Mellon and Vaudry 2001). Aromatase enzyme deficiency in mice increases the vulnerability of hilar neurons to neurotoxic agents (Azcoitia et al. 2001), whereas brain injury in mice and rats rapidly up-regulates aromatase expression in glial cells at the injury site suggesting that aromatase may be involved in the protection of injured neurons through increased estrogen levels (Garcia-Segura et al. 1999a, Garcia-Segura et al. 1999b). In songbirds, neural aromatization increases the size of hippocampal neurons and improves learning (Oberlander et al. 2004). Recent studies also suggest that the brain aromatase encoded by CYP19 gene may serve as a target for novel approaches for the treatment of neurodegenerative diseases and ischemia (Azcoitia et al. 2001, Azcoitia et al. 2003, McCullough et al. 2003).

The estrogen production in extragonadal sites, such as brain, is dependent on an external source of C_{19} androgenic precursors because these tissues are incapable of converting cholesterol to C_{19} steroids. In postmenopausal women, the circulating testosterone levels are an order of magnitude greater than circulating estradiol levels suggesting that circulating testosterone levels might be more important for maintaining local estrogen

levels in extragonadal sites than are circulating estrogens (Simpson and Davis 2001). Moreover in men, circulating testosterone levels are an order of magnitude greater than those in postmenopausal women. Thus, the uninterrupted sufficiency of circulating testosterone in men throughout life supports the local production of estradiol by aromatization of testosterone, affording an ongoing protection against the so-called estrogen deficiency diseases, i.e. protecting the bones against mineral loss and possibly contributing to the maintenance of cognitive function and prevention of Alzheimer's disease (Simpson et al. 2000).

A genome-wide LD mapping study conducted with late-onset AD patients and control subjects (Hiltunen et al. 2001) revealed a region of interest on chromosome 15q21.1 (table 4). Another recent study found also an association between chromosome 15q22 and AD (Scott et al. 2003). Based on the relatively short distance from the associated microsatellite marker D15S659 (Hiltunen et al. 2001) and the possible neuroprotective role of estrogens in AD (Paganini-Hill and Henderson 1996, Costa et al. 1999, Waring et al. 1999), the CYP19 gene encoding for aromatase could be regarded as a susceptibility candidate for AD.

Table 4. Evidence for linkage/association in chromosome 15q near CYP19 locus observed in full genome screens.

Study	Chromosomal location	Marker	Linkage/association	Distance to CYP19 (cM)
Hiltunen et al. 2001	15q21.1	D15S659	P = 0.036	2
Scott et al. 2003	15q22	D15S1507	LOD = 2.8	15
Scott et al. 2003	15q22	D15S153	LOD = 3.1	17
Blacker et al. 2003	15q26	D15S642	MLS = 1.3	77

LOD, Logarithm of odds score.
MLS, Multi-point lod score.

2.7. *Seladin-1*

2.7.1. Seladin-1 or DHCR24

Selective Alzheimer's disease indicator-1 (seladin-1) or 3β-hydroxysterol Δ24-reductase (DHCR24) gene is located on chromosome 1p31.3-p33, and contains nine exons. It encodes a 60.1 kDa protein that belongs to the flavin adenine dinucleotide (FAD)-binding oxidoreductase family (Waterham et al. 2001). Seladin-1 catalyzes the reduction of the delta-24 double bond in a series of enzymatic reactions converting

lanosterol to the final product of isoprenoid/cholesterol biosynthesis pathway, cholesterol, in endoplasmic reticulum (figure 5).

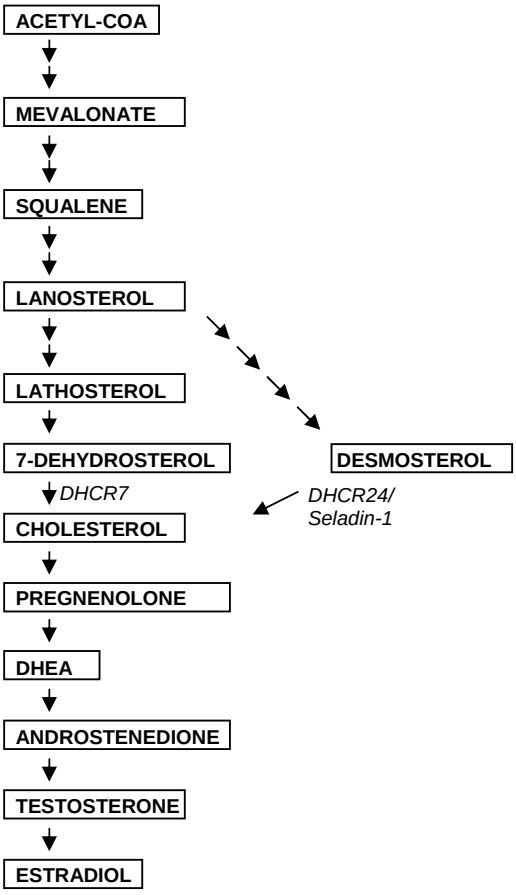

Figure 5. Cholesterol and steroid biosynthesis pathways, and localization of seladin-1 enzyme in the cholesterol synthesis pathway (Waterham and Wanders 2000).

The sequence of the various enzyme reactions required for converting lanosterol to cholesterol may vary, but two major routes, having 7-dehydrocholesterol or desmosterol as the ultimate precursors of cholesterol, have been proposed (Bae and Paik 1997, Waterham and Wanders 2000). Seladin-1 is highly expressed in brain and adrenal gland with moderate expression in liver, lung, spleen, prostate and spinal cord. Defects in seladin-1 can lead to desmosterolosis, a rare autosomal recessive disorder characterized by multiple congenital anomalies and elevated levels of the cholesterol precursor desmosterol in plasma, tissue, and cultured cells (Waterham et al. 2001). Human seladin-1 gene was identified by its sequence similarity with the plant enzyme

DIMINUTO/DWARF catalyzing partially similar reaction in steroid/sterol biosynthesis of plants (Waterham et al. 2001).

2.7.2. Seladin-1 in neuronal degeneration and apoptosis

In normal brain, seladin-1 is strongly expressed in neuronal cells, but in brains affected by Alzheimer's disease, seladin-1 expression in the temporal lobe is substantially reduced compared to the frontal cortex. In normal control subjects, neuronal expression of seladin-1 is identical in frontal and temporal cortices (Greeve et al. 2000), indicating that there is a loss of seladin-1 expression in brain regions vulnerable in AD, due to the reduced amounts of the transcript in the remaining neurons and not simply reflecting the neuronal cell loss (Greeve et al. 2000). This is in line with previous findings indicating lower activities of antioxidant enzymes in temporal cortices of AD brains compared to the temporal cortices of normal brain (Marcus et al. 1998).

Differential activity of antioxidant enzymes in nerve cell populations may be one cause for the selective resistance of specific cells against degeneration caused by toxic factors such as Aβ. In cell culture, overexpression of seladin-1 protects cells from apoptosis induced by Aβ, and a high expression of seladin-1 is associated with resistance against Aβ-induced toxicity (Greeve et al. 2000). Seladin-1 protects cells from oxidative stress by reducing caspase-3 activity, which activates the caspase cascade responsible for apoptosis execution, and this might prevent or delay the neurodegeneration in AD (Greeve et al. 2000). Seladin-1 probably is a component of the cellular machinery protecting cells from oxidative stress, but when the stress becomes overwhelming, seladin-1 is cleaved and probably inactivated. Under these conditions, the non-covalent FAD-binding domain of seladin-1 locating within the caspase cleavage motifs is cleaved in cells undergoing apoptosis (Greeve et al. 2000). Transcriptional activation of antioxidant enzymes has been attributed to resistance of cultured cells against Aβ toxicity (Behl et al. 1994, Sagara et al. 1996, Sagara et al. 1998), and seladin-1 may function in concert with these enzymes in protecting cells from oxidative stress and Aβ.

2.8. HSPG2 or perlecan

2.8.1. Proteoglycans, HSPGs and perlecan

Proteoglycans are a heterogenous superfamily of proteoglycan macromolecules, composed of linear sulphated polysaccharides, glucosaminoglycans (GAGs), covalently attached to a core protein. Proteoglycans are associated with all kinds of amyloid deposits in the human body. These complex macromolecules have also been implicated in several features of AD, including the genesis of senile plaques, cerebrovascular amyloid and neurofibrillary tangles. Heparan sulphate proteoglycans are a subgroup of proteoglycans with heparan sulphate as the polysaccharide moiety, and they are the most important group of proteoglycans associated with amyloid deposits (van Horssen et al. 2003).

Heparan sulphate proteoglycan 2 (HSPG2) or perlecan is the largest extracellular matrix-associated HSPG, and an integral component of basement membranes. Perlecan has a 400 – 470 kDa core protein composed of five domains with homology to other extracellular matrix and adhesive proteins (figure 6). Domain I is unique for the proteoglycan since it shares no significant homology with any other protein, and contains three Ser-Gly-Asp sequences that act as attachment sites for heparan sulphate GAGs. Domains II to V contain repeats similar to low density lipoprotein receptor, laminin, epidermal growth factor and neural cell adhesion molecule (Noonan et al. 1991)**.** The perlecan core protein is post-translationally modified by attachment of N- and O-linked oligosaccharides forming the heparan sulphate chains. Perlecan has a strong tendency to aggregate in dimers or stellate structures, and it interacts with other basement membrane components such as laminin, prolargin and collagen type IV. It is responsible for the fixed negative electrostatic charge, and is involved in the charge-selective ultrafiltration properties, as well as serving as an attachment substrate for cells. Defects in HSPG2 cause two types of skeletal disorders: the mild Schwartz-Jampel syndrome, which is characterized by permanent myotonia and skeletal dysplasia (Nicole et al. 2000)**,** and the lethal neonatal dyssegmental dysplasia Silverman-Handmaker type (Arikawa-Hirasawa et al. 2001). Both diseases prove that perlecan plays an important part in neuromuscular function and cartilage formation.

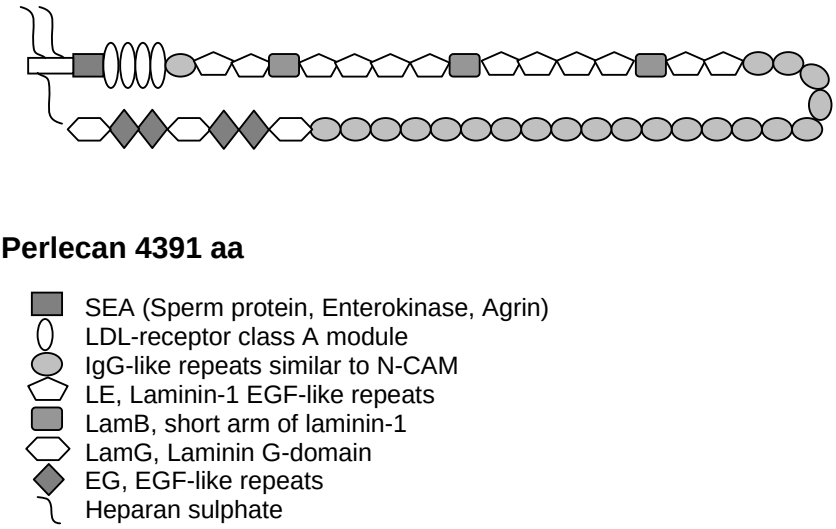

Figure 6. Schematic representation of perlecan protein (van Horssen et al. 2003).

2.8.2. HSPGs and tau

Heparan sulphate and hyperphosphorylated tau coexist in nerve cells of AD brain at the earliest known stages of neurofibrillary pathology, and moreover heparan sulphate and heparin stimulate the formation of paired helical-like filaments (Goedert et al. 1996). Furthermore, the presence of sulphated GAGs increases the ability of several kinases to phosphorylate tau (Mawal-Dewan et al. 1992, Brandt et al. 1994, Yang et al. 1994), causing tau to dissociate from microtubules (Goedert et al. 1996). When highly sulphated GAGs bind to the microtubule-binding repeat region of tau, their binding to microtubules is prevented. Thus GAGs might cause a conformational change in tau promoting microtubule disassembly and polymerization of tau molecules, which ultimately leads to the formation of insoluble paired helical filaments (Perry et al. 1991, Hasegawa et al. 1997, Hernandez et al. 2002). The magnitude of the GAG effects on tau hyperphosphorylation is proportional to their degree of sulphation; the more sulphated GAGs like heparin are more potent at evoking tau hyperphosphorylation (Hasegawa et al. 1997).

2.8.3. HSPGs and β-amyloid

The localization of HSPGs in neuritic plaques has been widely debated. Studies from a decade ago reported the presence of perlecan in neuritic plaques of AD and Down's syndrome (Snow et al. 1988, Snow et al. 1990). When infused together to rat brain,

perlecan and β-amyloid were found to form amyloid deposits similar to the amyloid plaques present in human AD brain (Snow et al. 1994). There are multiple interactions between perlecan and Aβ; perlecan binds Aβ proteins, accelerates Aβ fibril formation and maintains Aβ fibril stability (Castillo et al. 1997). The sulphate moieties on the GAGs are critical for enhancement of Aβ fibril formation, and other sulphated GAGs may also participate in the fibril formation (Castillo et al. 1999). To date, perlecan and agrin are currently the only proteoglycans known to directly interact with Aβ (Castillo et al. 1997, Cotman et al. 2000).

The question remains whether deposition of HSPGs precedes the accumulation of Aβ and hyperphosphorylated tau or vice versa. In Down's syndrome brains, HSPGs are found in diffuse primitive senile plaques, and the accumulation of proteoglycans seems to be an early event in the formation of senile plaques (Snow et al. 1990). Diffuse senile plaques primarily composed of non-fibrillar Aβ are thought to gradually transform into classic senile plaques with a central core of fibrillar Aβ (Selkoe 1991, Dickson 1997). Perlecan and agrin promote the conversion of non-fibrillar Aβ into fibrillar Aβ, and contribute to increased neurotoxic properties of Aβ (Snow et al. 1994, Castillo et al. 1997, Verbeek et al. 1997).

HSPGs may also protect Aβ against proteolytic degradation (Gupta-Bansal et al. 1995) by functioning as chaperones that form a protective shield against extracellular proteases. Simultaneously $A\beta_{1\text{-}40}$ possibly inhibits the activity of heparanases, and thereby prevents the proteolytic breakdown of the proteoglycans (Bame et al. 1997), which may indirectly contribute to the stability and persistence of plaques. Heparin also increases the secretion of APP, and moreover the secretion of sAPPβ, indicating that highly sulphated GAGs may promote the amyloidogenic pathways of APP metabolism (Leveugle et al. 1997). In contradiction, recent data revealed that heparan sulphate could inhibit β-secretase (BACE1) activity and it decreased the amount of sAPPβ and Aβ (Scholefield et al. 2003), probably by binding to BACE1 and thus blocking its access for APP.

The role of HSPG2 on β–amyloid pathology has been debated, since novel studies using monoclonal antibodies have questioned the earlier reports concerning the localization of

perlecan in senile plaques and neurofibrillary tangles. Recently, heparan sulphate GAG and agrin, syndecans and glypican have been detected in senile plaques and neurofibrillary tangles, whereas perlecan has not been detected (van Horssen et al. 2003).

2.8.4. HSPGs and ApoE

HSPGs have been linked to ApoE through several biochemical findings. ApoE binds heparin with high affinity (Cardin et al. 1986), stimulates HSPG2 mRNA and protein production as well as heparan sulphate production *in vivo* (Paka et al. 1999a, Paka et al. 1999b). The ApoE4 isoform also stimulates sulphation of GAG chains more efficiently than the ApoE3 isoform (Bonay and Avila 2001), which in turn is associated to more efficient tau hyperphosphorylation and Aβ fibril formation (Hasegawa et al. 1997, Castillo et al. 1999). In the presence of lipoproteins, apoE4 secreting cells have reduced neurite outgrowth, branching and extension compared to apoE3-secreting cells (Nathan et al. 1994, Bellosta et al. 1995), and the inhibitory effect of apoE4 on neurite outgrowth is associated with microtubule depolymerization (Nathan et al. 1995).

ApoE exhibits neurotoxicity in an isoform-specific manner (Marques et al. 1996, Jordan et al. 1998), and the neurotoxic properties appear to be mediated by an HSPG/LDL receptor related pathway (Tolar et al. 1997, Mahley and Ji 1999). ApoE-containing lipoproteins interact with HSPG before internalization through the HSPG/LDL receptor related pathway to neurons and other cells (Ji et al. 1993), but HSPG is also able to bind and internalize VLDL independent of this endocytic activity (Wilsie and Orlando 2003). HSPG appears also to function alone as a receptor and displays unique handling properties for specific isoforms of apoE, with apoE3 containing lipoproteins accumulating in cells more rapidly than apoE4 lipoproteins (Mahley and Ji 1999).

2.8.5. Glucosaminoglycan therapies

HSPGs have been linked to several characteristics of AD, including senile plaques composing of fibrillar Aβ, tau protein of NFTs and APOE. Altogether, these findings point to a noteworthy effect of the sulphation of heparan sulphate moieties of the HSPG glycoprotein rather than through the core protein itself. In that sense, these mechanisms and factors involved in regulating heparan sulphate synthesis might themselves become

important. Domain I of HSPG2 contains three Ser-Gly-Asp sequences that can accept heparan or chondroitin sulphate side chains. The acidic residues, aspartic and glutamic acid, primarily determine the targeting of GAGs in serine residues of HSPG2. The SEA module in the C-terminal region of domain I and the presence of multiple acceptors in close proximity can enhance heparan sulphate synthesis at targeted sites (Dolan et al. 1997). This may indicate that sequences in non-GAG-binding regions of the core protein can influence the utilization of GAG attachment sites.

Proteoglycans bind Aβ leading to fibril and finally to plaque formation (Castillo et al. 1997, Dickson 1997, Verbeek et al. 1997). A binding site in 13-16 region of Aβ peptide represents a unique target site for inhibitors of Aβ fibril formation (Fraser et al. 1992), and drugs inhibiting the binding of Aβ peptide to proteoglycans might have therapeutic value via increased turnover of Aβ peptide by natural proteases (van Horssen et al. 2003). In *in vivo* and *in vitro* studies, small polysulphated compounds protect against Aβ induced effects (Kisilevsky and Szarek 2002). In mouse model, these low-molecular-weight sulphate compounds reduce the progression of inflammation-associated amyloid (Kisilevsky et al. 1995), and low-molecular-weight heparin derivatives interfere with the binding of heparin to APP and inhibit the β-secretase cleavage of APP (Leveugle et al. 1998). In addition to potential use in AD, therapeutic inhibitors of amyloid formation may be valuable also in other amyloid disorders.

Another basis for glycosaminoglycan therapies comes from inhibition of apoE-related neurotoxicity. ApoE exhibits neurotoxicity in an isoform-specific manner (Marques et al. 1996, Jordan et al. 1998), and all toxic apoE species include both receptor-binding and heparin-binding regions (Weisgraber et al. 1983, Libeu et al. 2001). The neurotoxic properties appear to be mediated by an HSPG/LDL receptor related pathway (Tolar et al. 1997, Mahley and Ji 1999), and therefore compounds disrupting the binding of apoE to HSPG could be effective in inhibiting the neurotoxic properties of apoE. Sulphated oligosaccharides, as well as heparin and heparan sulphate, prevent the neurotoxicity caused by apoE peptides. The synthetic compounds have better protective effects but also cause clumping and detachment of cells (Bazin et al. 2002).

Whether targeted against Aβ or apoE neurotoxicity or tau phosphorylation and microtubule disassembly, small polysulphated compounds mimicking sulphated GAGs can be considered as a promising therapeutic strategy against AD and disorders with the related pathological hallmarks. The CSF concentrations of HSPGs or GAGs might also serve as indicators of their accumulation in AD brains. No link has been found between AD severity and CSF concentrations of HSPGs (Briani et al. 2002), but studies in larger patient populations detecting specific proteoglycan subtypes might offer a basis for converting biochemical analysis of CSF into a useful diagnostic tool (van Horssen et al. 2003).

2.8.6. HSPG2 and AD

A genome-wide linkage disequlibrium (LD) mapping study (Hiltunen et al. 1999, Hiltunen et al. 2001) revealed chromosomal loci 1p36.12 to be associated with late-onset AD (table 5). The HSPG2 gene resides in this chromosomal region, and thus it might be regarded as a susceptibility candidate for AD. The HSPG2 gene consists of 97 exons, and it is expressed in several tissues including the brain. Although HSPG2 has been considered as a promising candidate in the AD pathogenesis, there is only one report available concerning the possible genetic involvement of HSPG2 in AD (Rosenmann et al. 2004).

Table 5. Evidence for linkage/association in chromosome 1p36 near HSPG2 locus observed in full genome screens.

Study	Chromosomal location	Marker	Linkage/association	Distance to HSPG2 (Mb)
Hiltunen et al. 2001	1p36	D1S552	P = 0.0006	2.9
Hiltunen et al. 2001	1p36	D1S2644	P = 0.072	3.1
Blacker et al. 2003	1p36	D1S1597	MLS = 1.1	8.4
Kehoe et al. 1999	1p36	D1S1675	MLS = 2.67	92.5
	1p36	D1S548		14.5
Myers et al. 2002	1p36	D1S1675	TLS = 1.72	92.5

MLS, Multi-point LOD score.
TLS, Two-point LOD score.

3. AIMS OF THE STUDY

The aim of the study was to find new candidate genes that might affect the risk of developing AD. Genetic studies so far have identified three genes that cause autosomally inherited familial Alzheimer's disease, and often the gene mutations behind these familial forms can predispose the affected individuals to AD at a rather early age. However, the majority of people suffering from AD are affected at a later age, around 80 years of age. Quite a few genes have been identified that predispose an individual to the late-onset form of AD, with APOE being the most widely acknowledged.

Our study population consisted mainly of late-onset AD patients and their age-matched controls. The purpose was to use the homogenous eastern Finnish population to identify new candidate genes for AD from chromosomal regions that were found to be in linkage disequilibrium with AD in a whole genome scan of this population, and also to study their possible mechanisms of action using gene expression methods. The specific aims were:

1) To study the role of CYP19 gene polymorphisms in a sporadic AD population (I).

2) To determine the effect of CYP19 gene expression in estrogen deprivation and estrogen replacement (II)

3) To compare the expression of seladin-1 gene in AD and normal, aged brains and to determine the possible changes in seladin-1 expression during aging in wildtype and AD disease model mice, and in neuronal cells during apoptosis (III).

4) To study the role of HSPG2 gene polymorphism in a sporadic AD population (IV).

4. SUBJECTS, MATERIALS AND METHODS

4.1. Subjects

The study subjects were from eastern Finland, and they were examined in the Kuopio University Hospital. The studies were approved by the ethical committee of Kuopio University Hospital. Study III was part of a project focusing on risk genes of AD that had been approved by the local ethical committee, and the use of tissue was sanctioned by the Office of Legal Health Care Affairs. The clinically diagnosed patients fulfilled the criteria of probable AD according to NINCDS-ADRDA (McKhann et al. 1984). The patients with onset age < 65 years of age did not carry common mutations in APP, PSEN-1 or PSEN-2 genes. A total of 218 subjects (25% of study I population) was involved in both studies I and IV.

Study I

The case group consisted of 394 AD patients. The mean age at the onset of symptoms was 72 ± 7 years (range 43 to 90 years). The sample was predominantly women (70%), and 45% had a positive family history of AD, i.e. at least two first-degree relatives in two generations were affected, but there was inconclusive evidence of autosomal dominant transmission (Lehtovirta et al. 1996). The control group consisted of 469 subjects who were not demented according to interview and neuropsychological testing. Their mean age was 70 ± 5 years (range 60 to 87 years) at the time of neurologic examination. The majority were women (60%). APOE ε2/3/4 allelic frequencies differed as expected for the case (n = 394; 0.02/0.52/0.46) and control (n = 469; 0.04/0.80/0.16) groups ($p < 0.001$) in the study population. The estimates of relative risks of disease, ORs, were calculated using binary logistic regression with 95% confidence intervals (CI). APOE ε4 was five-fold more common for cases (OR = 4.74; 95% CI 3.79 to 5.94) and associated with a three-year earlier onset age (71 vs 74 years; $p < 0.001$).

Study III

Postmortem samples (Kuopio Brain Bank) obtained since 1991 were evaluated. The AD group consisted of eight patients, the DLB group of six patients and a control group of six subjects. The clinical diagnosis of AD assessed by neurologists was based on the NINCDS-ADRDA criteria (McKhann et al. 1984) and the diagnosis of other dementias on the DSM-III-R criteria (American Psychiatric Association 1987)**.** 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), and the diagnosis of DLB on consensus guidelines produced by an international workshop (McKeith et al. 1996). Temporal and occipital cortices were selected for transcription analysis, as they represented the most severely affected and the most spared cortical region (Arnold et al. 1991, Braak and Braak 1991).

Study IV

The study group consisted of 213 patients with AD and 269 age matched controls from clinical or population-based follow-up studies. The mean onset age of clinical AD patients was 71 ± 7 years (range 48 to 82 years; 59% women). In addition to clinical diagnosis, 85 (40%) cases were autopsied and the diagnosis of AD was confirmed according to the neuropathologic criteria of CERAD (Mirra et al. 1991). The mean onset age of these neuropathologic patients was 73 ± 10 years (range 45 to 92 years; 82% women). The controls were nondemented; 80 (30%) controls were autopsied and neuropathologically confirmed not to demonstrate AD pathology and 189 (70%) were neurologically examined nondemented subjects. The mean age at the time of neurological examination of 189 age matched controls was 71 ± 7 years (range 42 to 87 years; 63% women) and the mean age at the time of death of the 80 control subjects was 78 ± 6 years (range 70 to 98 years; 45% women). As expected, the distribution of APOE ε2/ε3/ε4 alleles in the AD and control groups was 0.02/0.58/0.40 and 0.04/0.79/0.17, respectively, and the APOE ε4 allele was strongly associated with AD, giving an OR of 3.3 (95% CI 2.5 - 4.5; $p < 0.001$).

4.2. Animals and cell lines

The housing conditions of 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. All efforts were made to minimize the animal suffering, and to reduce the number of animals used and to utilize alternatives to *in vivo* techniques. 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 RT-PCR and Aβ analysis, the brain was removed and hippocampi, cortex and olfactory bulb were dissected. Brain samples were frozen in liquid nitrogen and then stored at -70°C until RNA extraction and Aβ analysis.

Study II

Animals and experimental design. This study consisted of two experiments, referred to as *Experiment I* and *Experiment II*. In both experiments, the mice were ovariectomized (OVX) at the age of three months. The OVX mice received estrogen treatment for the last five weeks of their lifetime and the control groups of OVX mice were given a placebo treatment. In *Experiment I*, estrogen was administered via intraperitoneal injection of 20 μg of 17β-estradiol diluted in sesame oil (i.p., one hour prior to behavioural testing). In *Experiment II*, the estrogen was administered via subcutaneously implanted estrogen pellets (IRA, USA) containing 0.18 mg of 17β-estradiol that delivers a continuous supply of estrogen for 90 days. According to the manufacturer, the pellet yields serum estradiol levels of 50-75 pg/ml, which is similar as serum estradiol levels of 35-75 pg/ml reported in mice during proestrus (Nelson et al. 1992, Grasso and Reichert 1996). The OVX mice received sesame oil via subcutaneous injection. The mice were killed at the age of 4.5 months in *Experiment I* and at the age of 11 months in *Experiment II*.

The total number of animals used in the study was 49. Serum estrogen levels were not measured. Instead, the weight of the uterus was used to confirm the success of ovariectomy and of 17β-estradiol treatment. Uterine tissue is very responsive to estrogen, and therefore uterine weight was used as an index of circulating estrogen

levels to assess the efficacy of ovariectomy and hormone delivery (Kelner et al. 1982, Branham et al. 1993).

Study III

Transgenic animals. Female non-transgenic and double transgenic mice coexpressing human PSEN-1 harboring a familial AD-linked A246E mutation and chimeric mouse/human APP695 harboring a human Aβ domain with mutations (K595N, M596L) linked to Swedish familial AD pedigrees (APPswe) (Borchelt et al. 1997) were used in the study. The total number of animals used was 44.

Expression gene constructs. Expression plasmid pHD-WT containing a human wild-type presenilin-1 (PSEN-1) 1.4 kb cDNA under herpes simplex virus thymide kinase promoter and plasmid pHD-ΔE9 encompassing an inframe deletion of codons 290-319 produced from pHD-WT using the site-directed PCR-mutagenesis procedure (ExSite™ PCR-Based Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA) were used. Both pHD-WT and pHD-ΔE9 constructs were sequence analysed using ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Expression plasmid pEGFP-F (Clontech, Palo Alto, CA, USA) encoding for a farnesylated membrane-bound enhanced green fluorescent protein was used to control transfection conditions and efficiency.

Cell cultures and treatment of cultures. Mouse N2a neuroblastoma cells (American Type Culture Collection, CCL 131) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. In apoptosis experiments, exponentially growing N2a cells (plated 18-24 h before transfections, 60-70% confluence) were transfected on 60 mm plates either with wild-type PSEN-1 (pHD-WT), ΔE9 PSEN-1 mutant (pHD-ΔE9), or control vector (pHD) using FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions. In a total volume of 4 ml per plate, 2.5 μg of each plasmid DNA and 7.5 μl FuGENE 6 reagent were used. DNA/reagent complex was removed after 3 or 12 h, and apoptosis was induced with okadaic acid (OKA; Calbiochem-Novabiochem, San Diego, CA, USA) culturing the cells in medium with or without 25 nM OKA for 36 or 45 h.

Okadaic acid is an inducer of apoptosis (Nuydens et al. 1998, Suuronen et al. 2000), and it is known to induce *in vivo* an Alzheimer's type hyperphosphorylation of tau (Harris et al. 1993, Gong et al. 2000, Bennecib et al. 2001). OKA treated and untreated cells were collected 48 h after initiation of transfections and subjected to the analyses of caspase-3 activity (Suuronen et al. 2000) and seladin-1 semi-quantitative reverse transcriptase (RT) PCR analyses. Expression of wild-type and mutant PSEN-1 at 48 h after transfection initiation was controlled using RT-PCR with human specific PSEN-1 PCR primers and Western blotting with N-terminal antibody for PSEN-1.

4.3. Gene analyses

4.3.1. DNA and RNA extractions

Standard procedures were used to extract genomic DNA from leucocytes (Vandenplas et al. 1984) and from paraffin-embedded tissue of postmortem cases (Isola et al. 1994). Total RNA was extracted from human and mouse cortical and hippocampal tissue samples using Trizol reagent and from cultured cells using Trizol LS (Gibco BRL, Grand Island, NY, USA). The total RNA was DNase treated using RNase-Free DNase Set (QIAGEN GmbH, Hilden, Germany). The quality of RNA was determined by electrophoresis through ethidium bromide stained agarose gel. All PCR amplifications and RT reactions were carried out using a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA).

4.3.2. Sequence analyses

The PCR products and polymorphism sites of studies I, III and IV were ascertained with cycle-sequencing using the automated ABI PRISM 310 laser fluorescence sequencer. PCR primers are listed in table 6. PCR products were purified using QIAquick™ PCR Purification Kit (QIAGEN Qiagen) and cycle-sequenced with the genetic analyzer and ABI PRISM Sequencing Analyzis Software (Applied Biosystems). The sequencing was conducted with the dReady Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase FS (Applied Biosystems).

4.3.3. Determination of CYP19 polymorphisms

Nine intragenic SNPs were investigated, each having a minor allele frequency > 0.1, and covering the promoter/5'-untranslated (5'-UTR), coding/intronic and 3'-untranslated (3'-UTR) regions at 6-23 kb intervals (figure 7).

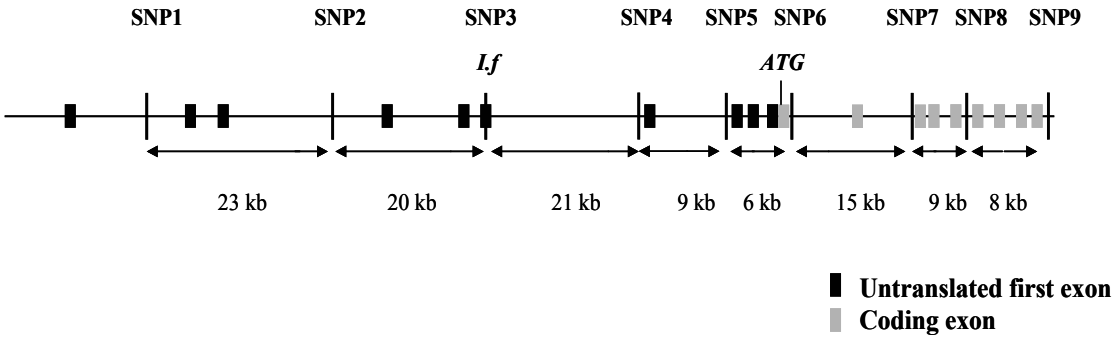

Figure 7. Genomic structure of the *CYP19* gene and the location of nine intragenic SNPs used in the study. *I.f*; brain specific promoter/first untranslated exon (Iivonen et al. 2004).

Amplification of SNPs was performed in two multiplex PCRs with 25 ng of genomic DNA and 4 pmol of primers (Primers F and R in table 6) with AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR amplification was followed by Shrimp alkaline phosphatase (SAP, Amersham Pharmacia Biotech) and ExoI (New England Biolabs Inc.) enzymatic treatment with 4 µl of both multiplex PCR products. SAP/ExoI treated PCR products (4 µl of both) were subjected to SNaPshot multiplex PCR in a 10 µl reaction volume containing 1.25 µl SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems) and 1-3 pmol 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 (Applied Biosystems).

Table 6. SNPs and PCR primers used in association studies I and IV.

Study	SNP	dbSNP[a]	Location in gene	Variation	Primers[b]	PCR product (bp)	Annealing temperature (°C)
I	CYP19/1	1004984	5'UTR	C/T	F: CTGGTTTGAGGACTCTAAGGATGG R: ATCACTGATGGACCCTGTGAGC SS1: CCCATGACTGCCTACTGTT	243	61
	CYP19/2	730154	5'UTR	A/G	F: TAGCCCTTCAGACAGAAGATGC R: CTCATTGCTAATGTGAGCTTGC SS2: CAGCAAAACTTCATGGAGC	245	61
	CYP19/3	1902586	5'UTR	C/T	F: CACAACTGAACCTGATGCAGTGAC R: GAAAACTTGAATGCAGGGTGGAG SS3: CCTGATGCAGTGACAATCA	190	61
	CYP19/4	1008805	5'UTR	T/C	F: TGTCCTTACCGAATCACTACCCT R: CCACTTTACAGATGTGGCTTGG SS4: ACTACCCTTCACCTTCCTG	197	61
	CYP19/5	767199	5'UTR	C/T	F: CAGTGTGTAGCTCCGTGAAGGTT R: GGCAGTCCCAAGCTCTAGTGTCT SS5: AGGTGACATGAATGTGGAG	212	61
	CYP19/6	727479	Intron 2	T/G	F: GCTCAAGATGGGGTGGAGTAAAG R: CCACTATCACCACATTCCCAAGAA SS6: TCCCTTTCACTTTGTTTCC	229	61
	CYP19/7	1065778	Intron 3	A/G	F: TGATTCAACTGGAGAAGGATGTCC R: CTTGAGGACCTGAAAAGACAGGAA SS7: TGCATTTGCTAAGAGAGCT	195	57
	CYP19/8	1143704	Intron 6	A/T	F: TGCTATCGTGGTTAAAATCCAAGG R: TTACCTGAGAGGCCAAGAAAAACA SS8: GGGAAAAAAACCAATCCAA	202	57
	CYP19/9	10046	3'UTR	C/T	F: CTTGCACCCAGATGAGACTAAAA R: GGAGGTATGCCTATAAAATGCC SS9: GAGAAGGCTGGTCAGTACC	210	57
II	HSPG2	3767140	Intron 5	C/A	F: CATGTCCCATGCCCCACGTGTGCT R: ATTGTAGCTGTGGCAGGCAAACTC	241	55

[a] The NCBI SNP Cluster ID (rs-number).
[b] F: Forward primer, R: Reverse primer, SS: SNaPshot primer

4.3.4. Determination of HSPG2 polymorphism

To detect HSPG2 intron 5 polymorphism (C/A), PCR and digestion of PCR products were carried out as previously described (Hansen et al. 1997) (primers in table 6). The PCR product of 241 bp was digested with the BamHI restriction enzyme (New England Biolabs Inc.) and the fragments were analyzed on 2% agarose gel.

4.3.5. Determination of APOE alleles

APOE alleles were analyzed using PCR and HhaI (New England Biolabs Inc.) digestions of the PCR products with PCR primers and conditions as previously described (Tsukamoto et al. 1993). The heterozygote sample APOE ε2/ε4 sample was used as a control in each run.

4.3.6. Expression analyses

Study II

The reverse transcription was performed using TaqMan® Reverse Transcription Reagents Kit (Applied Biosystems) to generate cDNA in a 25 µl reaction volume from 0.5 µg of total RNA. Relative quantifications of mouse CYP19, ERα and ERβ were calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control. Real-time quantification of CYP19, ERα and ERβ gene expressions were carried out using ABI PRISM® 7000 Sequence Detection System with FAM dye-labeled fluorescent 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 singleplex reactions with 10 to 40 ng cDNA in a 15 µl reaction volume with PCR cycling from 40 to 44 cycles. The amplification of GAPDH was performed with 50 nM GAPDH primers and 200 nM GAPDH probe.

The relative quantification was calculated using the relative standard curve method. The R^2 for the standard curves varied from 0.944 to 0.993. For each experimental sample, the amounts of target (CYP19, ERα/β) and housekeeping gene were determined from the appropriate relative standard curves. The target amount was then divided by the endogenous control (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 expression levels. The means calculated from three parallel PCR reactions were used in statistical analysis. Control reactions were performed to verify that no amplification occurs without cDNA.

Study III

Semi-quantitative RT-PCR analysis. Human seladin-1 primers extended from coding exons 5 to 9 of human seladin-1 genomic sequence (table 7), and human nicastrin primers covered exons 6 to 13 of human nicastrin genomic sequence. Amplification of human cytoplasmic β-actin was used as a positive control and internal standard in every sample, and β-actin pseudogene amplification was prevented using β-actin primers described earlier (Raff et al. 1997). Creation of a genomic DNA amplification product of the same size as the reverse-transcribed target was prevented with DNase treatment of the RNA samples and by using PCR-primers covering several exons. In semi-quantitative RT-PCR of mouse seladin-1, hypoxanthine phosphoribosyltransferase (HPRT) was used as a positive control and internal standard in every sample. Mouse seladin-1 primers were designed to extend from coding exons 7 to 9 of C57BL/6J strain mouse seladin-1 mRNA sequence.

RT-PCR amplifications were carried out using QIAGEN OneStep RT-PCR Kit (QIAGEN). RT-PCR parameters were selected for both seladin-1 and control gene amplification reactions to be in a linear range in a multiplex reaction, when product intensities were logarithmically related to cycle numbers and equally strongly amplified. Control reactions were performed to verify that no genomic DNA amplification occurred without reverse transcriptase enzyme using Taq-Polymerase (Promega, Madison, WI, USA). AlphaImager™ 1200 and AlphaEase™ Software (Alpha Innotech Corporation, Catalina, CA, USA) were used in spot density calculations to quantificate RT-PCR products. All RT-PCR reactions were duplicated and the means calculated from individual reactions were used in statistical analysis.

Northern blot analysis. A total of 15-20 μg total RNA from human and mouse brain tissues and cells was separated on 1% formaldehyde-agarose gel and blotted on Magna Graph nylon membrane (Osmonics Inc., Minnetonka, MN, USA). Northern blots were hybridised with [^{32}P]-labeled mouse seladin-1 probe. The seladin-1 probe was amplified using MSelF1 and MSelR primers with QIAGEN OneStep RT-PCR Kit from mouse total RNA. The probe sequence was 90% identical with the corresponding human sequence.

Table 7. Expression primers used in study III.

Primer	Chr.	Primer sequence 5'-3'	PCR product (bp)	Number of PCR cycles	Annealing temperature (°C)
Human seladin	1p33-p31.1				
SelF		TCGTGGAAGGGCTGCTCTAC	674	28	55
SelR		GTACAAGGAGCCATCCATCTC			
Human β-actin	7p15-p12				
AktβF		CCTCGCCTTTGCCGATCC	626	28/30	55
AktβR		GGATCTTCATGAGGTAGTCAGTC			
Human nicastrin	1q22-q23				
HNicF		AACCCAGAAATCGTCTGTGACC	770	30	55
HNicR		TACAGAGCACGTCCCAGCAC			
Mouse seladin	4				
MSelF1		TCATCGTCCCACAAGTATGGCC	889	22	58
MSelF2		CAACAACCCCATCTTCCGCTAC	373	22	58
MSelR		ATAACATTTGGAACCCGTGCACA			
Mouse HPRT	X				
HPRTF		TGCTGACCTGCTGGATTACA	288	22	58
HPRTR		GTCCTTTTCACCAGCAAGCT			

4.4. Neuropathology

Studies III and IV

NFTs and NPs were quantified on Bielschowsky silver stained sections under light microscopy with a 100x magnification as described earlier (Mölsä et al. 1987, Alafuzoff et al. 1999). The visualization and the quantification of Aβ aggregates, paired helical filament tau (PHF-τ) and α-synuclein were carried out as described earlier (Alafuzoff et al. 1999, Parkkinen et al. 2001). For immunohistochemical techniques, antibodies to human βA4, at a dilution of 1:100 (M872, DAKO, Glostrup, Denmark), to human PHF-τ at a dilution of 1:100 (BR-03, Innogenetics, Ghent, Belgium), to rat-synuclein-1 (ZYMED, San Francisco, CA, USA) at a dilution of 1:1000, and the streptavidin–alkaline phosphatase system (Histomark Kit, 71–00-39) were used. The reaction

product, the streptavidin-biotin complex, was visualized with Vector-Red (Vector Labs, SK-5100).

4.5. Protein and enzyme activity analyses

4.5.1. Aβ40 and Aβ42 analysis

For Aβ40 and Aβ42 assay the hippocampi were homogenised in guanidine buffer in proportion to their weight. The levels of Aβ40 and Aβ42 were quantified using the Signal Select™ Beta Amyloid ELISA Kits (Biosource International Inc., Camarillo, CA, USA) according to the manufacturer's protocol. The Aβ40 and Aβ42 levels were standardised to brain tissue weight and expressed as ng (Aβ)/g (brain tissue).

4.5.2. Western blot analysis

Western blot analysis of PSEN-1 was carried out with a standard procedure. Transfected N2a cells were lysed with RIPA lysis buffer and 40 μg of total protein per lane was subjected to 12% SDS-PAGE gel. After electroblotting, the nitrocellulose filters were blocked and probed with PSEN-1 N-terminal specific rabbit serum antibody R222 (1:1600; a gift from Dr. P. D. Mehta, Department for Basic Research in Developmental Disabilities, Department of Immunology, Staten Island, NY). After washings and secondary antibody probing with anti-rabbit HRP conjugate (1:4000; Amersham Pharmacia Biotech, Uppsala, Sweden), enhanced chemiluminescence detection was performed.

4.6. Statistical analyses

The level of significance was defined as $p < 0.05$. Hardy-Weinberg distributions of genotypes were assessed by using the GenePop option 1 form (http://wbiomed.curtin.edu.au/). Equality of variances in one-way anova was analyzed using Levene's test.

Study I

Disease association. The case and control frequencies of alleles and genotypes at each locus were compared using two-tailed Pearson´s χ^2 test and Fischer's exact tests. Differences in onset ages between *APOE* ε4+ and ε4- cases were studied using a log-rank statistic. The relative risk of AD was estimated from logistic odds ratios (OR) and 95% confidence intervals (CI) adjusting for age and sex in multivariate analysis.

LD within the CYP19 gene. Pair-wise linkage disequilibrium (LD) of SNPs was determined by estimating standardized D' values for all subjects (1726 total chromosomes) using the 2LD program (http://www.iop.kcl.ac.uk/IoP/Departments/ PsychMed/GEpiBSt/ software.shtml).

Haplotype analyses. Maximum likelihood estimates of haplotype frequencies were obtained using the Expectation-Maximization algorithm (Arlequin 2.0 software) after ruling out deviations from Hardy-Weinberg equilibrium. Haplotype frequency comparisons between AD and control groups with absolute chromosome numbers were carried out using RxC program employing the Metropolis algorithm to obtain unbiased estimates of exact p values with standard errors (SE) (http://bioweb.usu.edu/mpmbio/). Haplotype blocks were identified by pair-wise EM haplotype estimation analysis and then applying the four gamete test to identify obligate recombination between loci (Wang et al. 2002).

Latent class analysis. The latent class analysis was performed to identify risk haplotypes and to verify that risk was not strongly related to age, sex or APOE genotype. Information on genotypes for the nine SNPs was jointly used to identify two groups differing in genotypic frequencies (Grade of Membership Software, Center for Demographic Studies, Duke University, Durham, NC (1987)) (Manton et al. 1994). Age, sex, APOE and disease status frequencies were calculated for each latent SNP group. One χ^2 test was used to compare disease status for subjects who matched the latent SNP groups, i.e. multiple comparisons were avoided.

Study II

All gene expression data are expressed as percentages of expression in OVX group. All statistical analyses for RT-PCR and uterine weights were performed using SPSS 11.5 software applying T-test and Spearman's correlation analysis. Univariate general linear model was applied in covariance analysis of gene expression comparisons using the housekeeping gene GAPDH as covariate. Values of $p < 0.05$ were considered to be significant.

Study III

All statistical analyses were performed using SPSS 9.0 software applying independent samples two-tailed t-test, nonparametric Mann-Whitney test and bivariate correlation analysis.

Study IV

Statistical analyses for comparing allele and genotype frequencies were carried out using two-sided Pearson´s χ^2 and Fischer's exact tests with SPSS 10.0 software. Odds ratios (OR), as the estimates of relative risk of disease, were calculated using binary logistic regression with 95% confidence intervals (CI). An interaction model in logistic regression analysis was used to study the interaction between HSPG2 A and APOE ε4 alleles. The RxC program employing the Metropolis algorithm was used for analysis of contingency tables to obtain unbiased estimates of exact p-values with standard errors (SE) (http://bioweb.usu.edu/mpmbio/). For APOE4 stratified data, the Bonferroni correction was applied by multiplying the P values with the number of stratifications. PHF-tau expression and Aβ load were analysed using one-sided, nonparametric Mann-Whitney test based on the hypothesis that the A allele of HSPG2 impairs the pathology in brain.

5. RESULTS

5.1. CYP19 polymorphisms (Study I)

Allelic and genotypic frequencies for adjacent SNPs 5 to 7 were significantly associated with AD (table 8). Flanking SNPs 4 and 8 demonstrated smaller and non-significant associations with AD. Hardy-Weinberg equilibrium tests were separately performed among AD and control groups for each locus and no deviations from equilibrium were observed.

Table 8. Allele and genotype distributions of the CYP19 SNPs 1 to 9 (Iivonen et al. 2004).

SNP	Allele	Allele frequency		p[a]	Genotype	Genotype frequency		p[a]
		Control (N = 938)	AD (N =788)			Control (N = 469)	AD (N = 394)	
SNP 1	C	0.77	0.77	0.92	CC	0.58	0.59	0.56
	T	0.23	0.23		CT	0.37	0.35	
					TT	0.05	0.06	
SNP 2	G	0.16	0.15	0.64	GG	0.03	0.02	0.72
	A	0.84	0.85		GA	0.25	0.26	
					AA	0.72	0.72	
SNP 3	C	0.91	0.91	0.97	CC	0.83	0.83	1.00
	T	0.09	0.09		CT	0.16	0.16	
					TT	0.01	0.01	
SNP 4	C	0.46	0.41	0.05	CC	0.22	0.17	0.11
	T	0.54	0.59		CT	0.47	0.49	
					TT	0.31	0.34	
SNP 5	C	0.55	0.50	0.05	CC	0.32	0.25	0.04
	T	0.45	0.50		CT	0.46	0.51	
					TT	0.22	0.24	
SNP 6	G	0.35	0.30	0.009	GG	0.12	0.09	0.04
	T	0.65	0.70		GT	0.46	0.41	
					TT	0.42	0.50	
SNP 7	G	0.45	0.51	0.03	GG	0.22	0.25	0.04
	A	0.55	0.49		GA	0.46	0.51	
					AA	0.32	0.24	
SNP 8	T	0.46	0.50	0.06	TT	0.23	0.25	0.07
	A	0.54	0.50		TA	0.46	0.51	
					AA	0.31	0.24	
SNP 9	C	0.51	0.47	0.10	CC	0.27	0.22	0.13
	T	0.49	0.53		CT	0.47	0.55	
					TT	0.26	0.28	

[a] P-values were calculated using two-tailed Pearson´s χ^2 test (alleles) and Fischer's exact tests (genotypes).

Logistic regression models constructed to estimate the relative risk of AD related to the risk genotype(s) found at the locus compared to other genotype(s) revealed that age and

sex did not affect relative risks. No significant p-values were observed with the early-onset (onset age < 65 years) AD patient group. Stratification according to gender and APOE status did not reveal any major differences between subgroups. The risk associated with adjacent SNPs 4 to 9 suggested that they might represent a high-risk unit, or haplotype. The possibility of a high-risk haplotype was supported by the strong pairwise LD between SNP loci (all subjects; 1726 chromosomes). Using a threshold D' value of 0.30, 75% of SNP pairs demonstrated LD, and LD diminished with physical distance between SNP pairs (Pearson's correlation value = - 0.613; p = 0.01).

Six haplotype blocks were initially identified for the study subjects with an average block size of 22 kb (figure 8). When allowing for a low frequency of recombination (< 3%) to occur between two loci, only two haplotype blocks defined by SNPs 2 to 4 and 5 to 9 (> 40 kb in size) were identified.

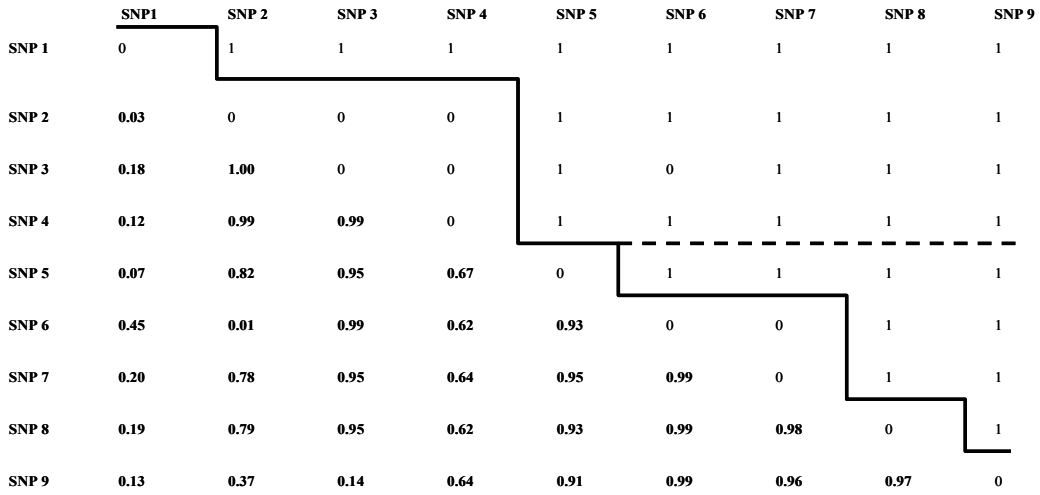

Figure 8. Identification of haplotype blocks within the *CYP19* gene region. Solid and dotted lines represent the configurations when no recombination and < 3% proportion of recombination are allowed between two loci, respectively. Four gamete test results performed between pair-wise loci: 0 and 1 indicate the absence and presence, respectively, of all four gametes estimated according to EM-based haplotype analysis. D' values for SNP pairs are indicated for the comparison of the haplotype block arrangement and the LD across the *CYP19* region (Iivonen et al. 2004).

Haplotypes displaying frequencies > 0.05 were included in haplotype association analyses that revealed the overall distribution of haplotypes derived from SNPs 1 to 9 to differ between AD and control groups (p = 0.039 ± 0.008)(table 9). Haplotype A1 including risk alleles for SNPs 5 to 7 was over-represented among AD patients.

Conversely, haplotype A2 (the complement of A1 for SNPs 4 to 9) was less common in the AD cases. Haplotype frequencies for the SNP block defined by SNPs 5 to 9 differed for AD and control groups ($p = 0.039 \pm 0.010$), whereas the frequencies for the SNP block defined by SNPs 2 to 4 did not differ ($p = 0.109 \pm 0.012$).

Table 9. Estimated haplotype frequencies for SNP 1 to 9 (Iivonen et al. 2004).

Haplotype[a]	Control (N = 938)	AD (N = 788)	p[b]
CACT**TTG**TT (A1)	0.300	0.350	0.021 ± 0.001
CACCCGAAC (A2)	0.270	0.214	0.011 ± 0.004
CACCC**T**AAC (A3)	0.055	0.064	0.555 ± 0.011
TACC**TTG**TT (A4)	0.054	0.060	0.675 ± 0.009
Others	0.321	0.312	0.532 ± 0.024
Overall			0.039 ± 0.008

[a] EM estimated haplotypes displaying frequencies ≥ 0.05 are indicated in table. Nucleotides indicated in bold were significantly over-represented in the AD group in single-locus analyses.
[b] P-values calculated using RxC program (± S.E).

SNP latent class analysis distinguished the haplotypes into two genetic groups I and II based on genotype information for the CYP19 SNPs and APOE. The groups were similar for SNPs 1 to 3 and differed for SNPs 4 to 9. APOE genotypic frequencies did not appreciably differ for the two genetic groups, nor did age or sex distributions. The genetic groups differed in AD risk: Group II had a 49% AD frequency, compared to 38% for group I or group II had a 60% increased risk of AD (OR = 1.6, 95% CI 1.1 to 2.2).

5.2. CYP19 expression in ovariectomized mice (Study II)

5.2.1. Uterus weight

The efficacies of ovariectomy and estrogen treatments were monitored by measuring the fresh weight of uterus immediately after killing the mice. All OVX mice had uterine weights below 30 mg, and in both experiments, estrogen treated mice had significantly increased uterine weights ($p < 0.001$) compared to OVX mice. When comparing only ERT mice, uterine weights of mice receiving tonic ERT in *Experiment II* were higher compared to those receiving phasic ERT in *Experiment I* ($p = 0.003$). Therefore, gene expressions in both studies were reanalyzed after normalizing uterine weights of the ERT groups by excluding three to four mice with the lowest uterine weights in both experiment groups, to test whether the differences in brain aromatase and ERs gene

expressions between phasic and tonic ERT groups persisted after the groups have been equalized with respect to the peripheral effect of estrogen. After the normalization, the statistical differences remained unchanged (figure 9).

5.2.2. Normalization with GAPDH

The target gene expressions were compared without GAPDH normalization (normalized with total RNA amount), with GAPDH normalization and with GAPDH normalization using covariance analysis. Covariance analysis, using housekeeping gene expression as a covariant to extract its effect from target gene expression, gave similar results in group comparisons with the GAPDH normalized data, whereas gene expression comparisons without normalization with GAPDH led to differing results from the aforementioned methods.

The approximate C_t values values with 40 ng of total RNA converted to cDNA for CYP19, ERα and ERβ were 35, 30 and 33, respectively, indicating that genes were expressed in the order ERα > ERβ > CYP19 in mouse hippocampus.

5.2.3. CYP19, ERα and ERβ expression in ovariectomized mice

In *Experiment I*, the phasic ERT decreased significantly CYP19 expression by 51 % ($P = 0.003$) and ERα expression by 47 % ($P < 0.001$), whereas the small change in ERβ expression was not significant ($P > 0.05$) (figure 9A). In *Experiment II*, the tonic ERT increased significantly CYP19 expression by 69 % ($P < 0.001$), ERα expression by 86 % ($P < 0.001$) and ERβ expression by 51 % ($P < 0.001$) (figure 9B). In *Experiment I*, the phasic ERT decreased the ERα/ERβ ratio by 42 % ($P = 0.04$) compared to the OVX group, but the ratio remained almost the same in OVX mice with or without tonic ERT (*Experiment II*) ($P > 0.05$). The gene expressions considered to reflect brain estrogen status correlated strongly with each other. Furthermore, uterine weights, that reflect the peripheral response to estrogen, correlated weakly but significantly with the gene expressions in the brain.

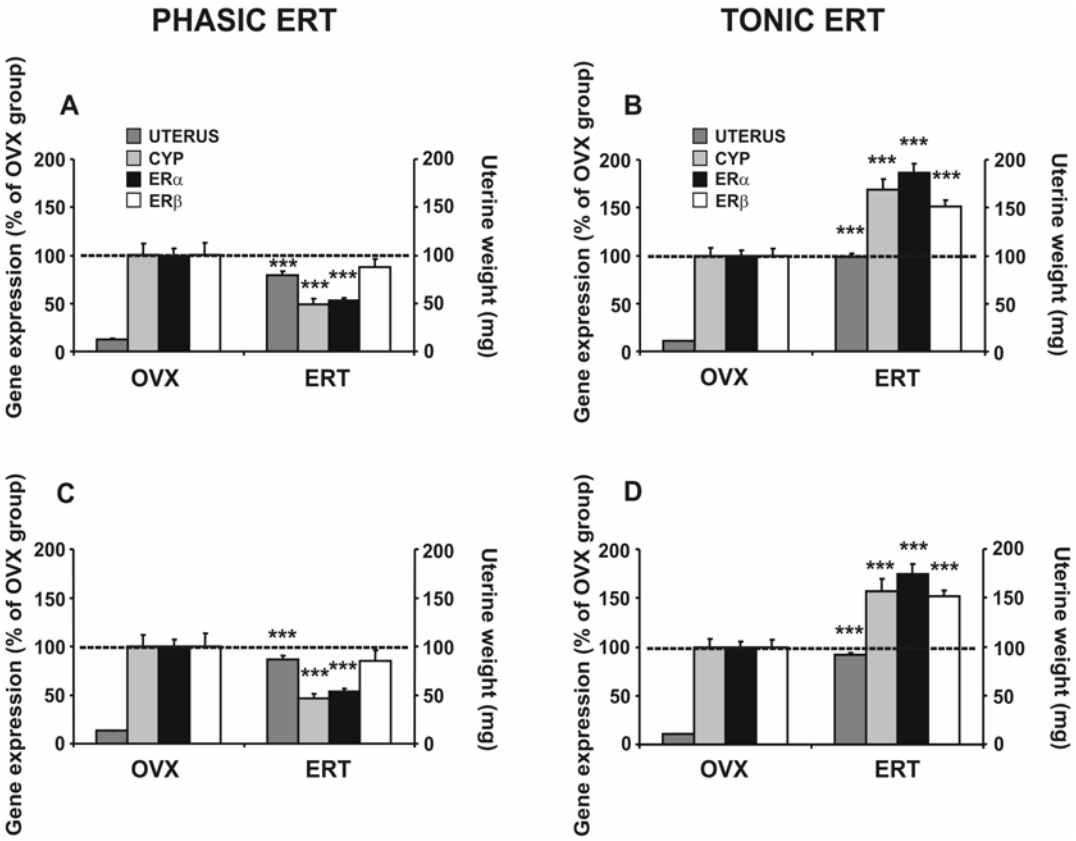

Figure 9. CYP19, ERα and ERβ gene expressions and uterine weights in ovariectomized (OVX), and estrogen treated mice. The bars represent mean gene expressions as percentages of expression in OVX group, or uterine weights (mg), and error bars represent SEMs. A and B describe the values for all the subjects, whereas C and D describe the values for phasic and tonic ERT, respectively, after normalizing the data according to the uterine weights of ERT mice. The gene expressions and uterine weights, injection (AC) and pellet groups (BD) were compared to OVX group, and the P values calculated with T-test are displayed (*** < 0.001).

5.3. Seladin-1 transcription and neuronal degeneration (Study III)

5.3.1. Seladin-1 transcription in human brain tissue

The relative transcription of seladin-1 was downregulated by 27% in demented subjects (n = 14), and moreover, by 33% in definite AD subjects (n = 9) compared to nondemented individuals (n = 6). In half of the AD patients, the relative transcription of seladin-1 was lower in temporal cortex compared to occipital cortex, whereas in nondemented subjects, the transcription of seladin-1 was higher or equal in temporal cortex compared to the occipital cortex. Subjects with DLB lacking AD pathology (n = 3) had equal relative transcription of seladin-1 to that found in nondemented subjects. Transcription analysis of another AD-associated gene, nicastrin, did not reveal any

differences in the relative transcription between demented and nondemented subjects (table 10).

The severity of AD pathology correlated with downregulation of seladin-1 transcription; the relative transcription of seladin-1 was significantly lower in subjects with NFTs or NPs compared to subjects with no AD hallmark lesions (table 11), which was also seen as a negative correlation between these pathological hallmarks and seladin-1 transcription (NFT: R = - 0.499; P = 0.025, and NP: R = - 0.521; P = 0.019). Subjects with numerous fibrils both in cortical and hippocampal areas had also lower relative seladin-1 transcription than subjects with positive PHF-τ labeling in hippocampal regions. This difference was also seen as a negative correlation between PHF-τ labeling score and seladin-1 transcription (R = - 0.516; P = 0.020), whereas no correlation between seladin-1 transcription and Aβ or α-synuclein pathology was found.

Table 10. Statistical analysis of seladin-1 and nicastrin transcription data in human temporal versus occipital cortex.

Status	N	Relative transcription (Mean ± SD)	Minimum	Maximum	P-value[a]
Seladin-1					
Nondemented	6	1.47 ± 0.48	1.00	2.29	
Demented	14	1.06 ± 0.37	0.53	1.77	0.069
DefAD[c]	9	0.98 ± 0.32	0.53	1.56	0.045
All ADs[c]	13	1.01 ± 0.32	0.53	1.56	0.044
All DLBs[d]	6	1.13 ± 0.45	0.67	1.77	0.200
Pure DLBs[d]	3	1.49 ± 0.31	1.15	1.77	0.796
Nicastrin					
Nondemented	6	1.11 ± 0.32	0.82	1.57	
Demented	14	1.01 ± 0.33	0.53	1.68	0.509
DefAD[c]	9	1.01 ± 0.36	0.53	1.68	0.556
All ADs[c]	13	1.03 ± 0.33	0.53	1.68	0.661
All DLBs[d]	6	0.92 ± 0.32	0.53	1.43	0.337
Pure DLBs[d]	3	1.06 ± 0.36	0.71	1.43	0.796

[a] P-values calculated using nonparametric Mann-Whitney test.
[c] Definite and all AD cases according to CERAD classification.
[d] All demented cases having DLB pathology and pure DLB cases without AD pathology.

Table 11. Relative transcription of seladin-1 compared in subjects with none or mild neuropathology and subjects with more extended and severe stage of neuropathology.

Stage of neuro-pathology	Location of neuropathology	N	Total scoring of neuropathology (Mean±SEM)[a]	Relative transcription of seladin-1 (Mean±SEM)	P-value[a]
NFT-	cortex	9	-	1.50 ± 0.15	
NFT+	cortex	11	17.2 ± 2.6	0.95 ± 0.09	0.004
NP-	cortex	6	-	1.63 ± 0.19	
NP+	cortex	14	15.7 ± 2.5	1.01 ± 0.08	0.006
PHF-τ+	hippocampus	8	1.4 ± 0.2	1.49 ± 0.16	
PHF-τ+	hippocampus &cortex	12	9.4 ± 0.8	1.00 ± 0.09	0.016
Aβ-	cortex	4	-	1.53 ± 0.27	
Aβ+	cortex	16	4.6 ± 0.7	1.11 ± 0.10	0.148
α-synuclein-	substantia nigra, gyrus cinguli & hippocampus	11	-	1.20 ± 0.10	
α-synuclein+	substantia nigra, gyrus cinguli & hippocampus	9	4.0 ± 0.6	1.19 ± 0.19	0.656

[a] P-values calculated using nonparametric Mann-Whitney test.

5.3.2. Seladin-1 transcription in APPswe/PSEN1 transgenic mice

APP/PSEN-1 double transgenic mice display detectable Aβ40 and Aβ42 levels at the age of 4 months, and amyloid levels increase thereafter dramatically with age. Time points before (4 months), at early phase (11 months) and at severe phase (16-17 months) of amyloid accumulation were selected to study the changes in seladin-1 transcription during aging and in the course of amyloid deposition. Parietal cortex was chosen to represent more affected and olfactory bulb as less affected tissue in mice. The relative transcription of mouse seladin-1 fluctuated during aging. A 15% decrease in seladin-1 transcription was seen between the ages of 4 and 11 months both in control and transgenic mice, but the decrease was significant only in transgenic mice ($P = 0.042$). Between the ages of 11 and 16 months the seladin-1 transcription increased by 28%, which was seen in both animal groups, but this was significant only in transgenic mice ($P = 0.002$). No correlation between the relative transcription of seladin-1 and hippocampal Aβ40 and Aβ42 levels was found.

5.3.3. Seladin-1 transcription in apoptosis model

Mouse N2a neuroblastoma cells transfected with human wildtype or ΔE9 PSEN-1 constructs were used to study the possible effect of PSEN-1 transcription on endogenous seladin-1 transcription in apoptosis. The induction of apoptosis with okadaic acid was established by measuring the caspase-3 activity from cell lysates.

After 36 h exposure to okadaic acid, caspase-3 activity was highly elevated, and after 45 h exposure, caspase-3 activity was already 14-fold higher when compared to untreated N2a cells. After 36 h exposure to okadaic acid, the caspase-3 activity was lowest in ΔE9 PSEN-1 group, but after 45 h, the caspase-3 activity was significantly higher than after 36 h ($P = 0.016$). In control (pHD) and wild-type PSEN-1 transfected groups, the caspase-3 activities were essentially the same after 36 and 45 h.

The relative transcription of seladin-1 increased in time, or it was higher after 45 h than after 36 h of exposure in all treatment groups. Seladin-1 up-regulation was most striking in the wild-type PSEN-1 transfected, okadaic acid treated group, in which the seladin-1 transcription increased by 69% from 36 h to 45 h, whereas the increase in okadaic acid treated ΔE9 PSEN-1 group was only 7%.

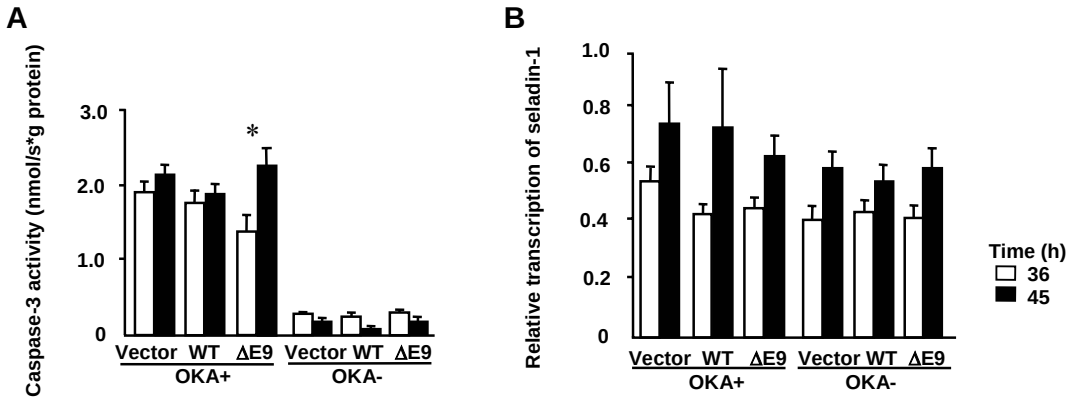

Figure 10. Caspase-3 activity and seladin-1 transcription in mouse N2a cells induced to apoptosis. Bars show means of six individual experiments, error bars show SEM. (A) Caspase-3 activation in vector, wild-type and ΔE9 PSEN-1 transfected N2a cells 36 and 45 h after induction of apoptosis with 25 nM okadaic acid (OKA+) and in control cells (OKA-). The asterisk indicates significant difference ($P < 0.05$) between 36 h and 45 h ΔE9 PSEN-1 transfected, OKA+ cells. (B) The relative transcription seladin-1 in vector, wild-type and ΔE9 PSEN-1 transfected N2a cells 36 and 45 h after induction of apoptosis with 25 nM okadaic acid and in control cells. The transcription of seladin-1 was related to the transcription of the HPRT gene (Iivonen et al. 2002).

5.4. HSPG2 polymorphism (Study IV)

Allele and genotype distributions of HSPG2 did not differ between AD and control populations, and HSPG2 genotypes were in Hardy-Weinberg equilibrium in both populations. No modulatory effect of gender was observed. However, both genotype

and allele frequencies differed between AD and control populations carrying the APOE ε4 allele (table 12).

Table 12. HSPG2 genotype and allele distributions (Iivonen et al. 2003).

Group		n[a]	Genotypes			P[b]	Alleles		P[b]
			CC	CA	AA		C	A	
All	AD	213	0.53	0.39	0.08		0.72	0.28	
	C	269	0.57	0.37	0.06	0.465	0.76	0.24	0.266
APOE4-	AD	81	0.49	0.40	0.11		0.69	0.31	
	C	191	0.51	0.42	0.07	0.481	0.72	0.28	0.535
APOE4+	AD	132	0.55	0.39	0.06		0.74	0.26	
	C	78	0.72	0.26	0.02	0.043	0.85	0.15	0.014

[a]n, number of Alzheimer's disease patients (AD) and controls (C).
[b]Fisher's exact test for genotype data and Pearson's χ^2 test for allele data.

In ε4 carriers, the combined CA+AA genotype (OR = 2.1; 95% CI 1.2 - 3.9) and the A allele (OR = 1.9; 95% CI 1.1 - 3.1) frequencies were higher among the AD than in the control population. The results pointed out an interaction or an additive effect between the HSPG2 A and APOE ε4 alleles, and logistic regression revealed that HSPG2 A allele was not an independent risk factor for AD (OR 1.1), whereas in conjunction with the ε4 allele, it doubled the risk for AD (from OR 3.1 to OR 6.6) when compared to APOE ε4 alone (table 13). The interaction between APOE and HSPG2 was studied using an interaction model in the logistic regression analysis. No significant interaction was observed between the two genes (OR = 2.0; 95% CI 0.9 – 4.4; p = 0.086) indicating that this represents alternatively an additive effect of HSPG2 on APOE ε4 carriers.

Table 13. Odds ratios of the risk for AD when taking the subjects with neither APOE ε4 nor HSPG2 A alleles as the reference (Iivonen et al. 2003).

APOE 24/34/44[a]	HSPG2 CA/AA[a]	AD n (%)	Control n (%)	OR (95% CI)[b]	P[b]
-	-	40 (19)	97 (36)	Reference	
-	+	41 (19)	94 (35)	1.1 (0.6-1.8)	0.832
+	-	72 (34)	56 (21)	3.1 (1.9-5.2)	<0.001
+	+	60 (28)	22 (8)	6.6 (3.6-12.2)	<0.001

[a]The minus sign indicates subjects lacking these genotypes; plus indicates subjects with these genotypes.
[b]Binary logistic regression analysis.

In the neuropathologically defined group, the OR for AD in HSPG2 A allele carriers with ε4 allele was 8.3 (95% CI 2.8 - 24.3; p < 0.001) and in non-carriers with ε4 allele 5.3 (95% CI 1.8-15.3; p = 0.003) when compared to the reference group without these

alleles. In the clinical group, the equivalent ORs were 5.3 (95% CI 2.5 - 11.5; $p < 0.001$) and 2.8 (95% CI 1.5 - 5.0; $p = 0.001$).

The neuropathological markers of AD, Aβ and PHF-tau, were studied in relation to the HSPG2 C/A polymorphism. AD cases with ε4 and HSPG2 A alleles had a 17% higher PHF-tau expression compared to ε4 carriers without the HSPG2 A allele ($p = 0.046$; 95% CI 0.041 - 0.052), whereas in the control group, no significant difference was observed ($p = 0.130$; 95% CI 0.121 - 0.139). In Aβ load, there was no significant difference between the stratified groups in AD population, although a 12% increase in the Aβ load among the ε4 carriers with HSPG2 A allele compared to those without A allele was detected. Furthermore, no differences were found in the control population.

6. DISCUSSION

In the present series of studies, a cluster of CYP19 SNPs associated with AD, forming a haplotype with a 60% increase in the risk of AD. In an animal model of female postmenopause and ERT, the CYP19 expression responded differently to tonic and phasic hormone replacement, with tonic ERT increasing the expression of CYP19 and also having beneficial memory effects. Downregulation in seladin-1 transcription in AD was associated with hyperphosphorylation of tau protein which indicated that seladin-1 may also be downregulated in other dementias involving hyperphosphorylation of tau protein. Also, the HSPG2 gene encoding for perlecan protein of basement membranes was associated with a subpopulation of late-onset AD cases, indicating that HSPG2 may be involved together with APOE ε4 allele in the pathophysiology of AD. The methodological aspects and results of these studies are discussed in this chapter.

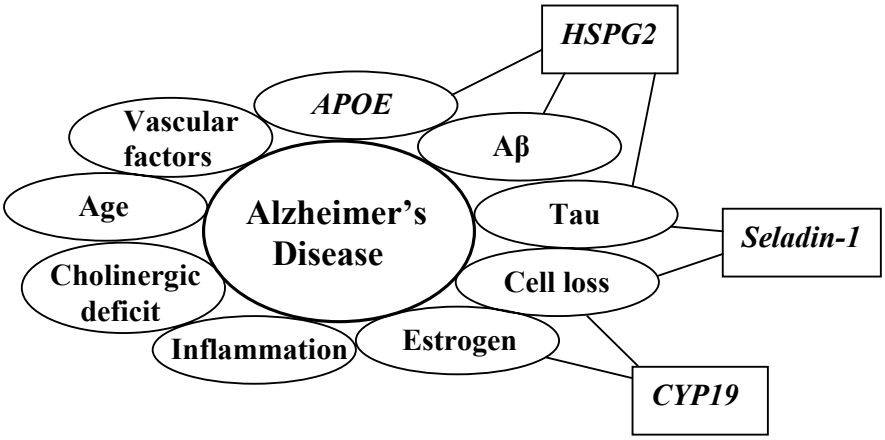

Figure 11. Factors implicated in Alzheimer's disease and the association of the genes of this study to them.

6.1. Methodological considerations

6.1.1. Study populations

Although Finland, and especially the Kuopio region are considered as isolated populations, they do not differ from larger admixed populations, possibly due to the larger founding population than previously believed (Wright et al. 1999, Jorde et al. 2000). In the North Savo population, which is part of the late settlement region in Finland, the regions of LD have diminished when compared to isolated subpopulations

like Kuusamo (Varilo et al. 2003). For polygenic disorders, the frequencies of underlying disease-causing mutations may be high enough so that even a small founder population will contain multiple mutations on multiple haplotype backgrounds, obscuring associations between a disease phenotype and a linked marker (Kruglyak 1999). This addresses the need for careful characterization of population to ascertain that they are true isolates. Nevertheless, a rapidly growing population with decaying LD provides an efficient tool for finer-scale gene localization.

The drawback of using isolated populations in association-based mapping is the relatively low number of chromosome copies not closely related to one another, when the sample size may become an issue (Bonne-Tamir et al. 1997). Decreased heterozygosity of polymorphic markers may also decrease the power of association studies in isolated populations, as does their relatively short histories that allow a limited resolution of the location of the disease gene. The Finnish population may be one of the few populations, in which relative isolation has persisted long enough to allow fine-scale mapping of Mendelian disorders affected by recent mutations, but it remains to be seen whether this population will be useful for more complex disorders (Jorde et al. 2000). Isolated populations of constant size might be more useful for mapping genes underlying complex diseases than populations recently expanded in size, of which Finland is a good example. In constant-sized populations, genetic drift can produce new disequilibrium more quickly than recombinations, but in rapidly expanding populations, genetic drift is inhibited and the accumulation of new risk alleles continues (Hartl and Clark 1997). The constant-sized Ainu and Saami populations show increased LD, and they may be more appropriate for mapping genes for complex traits caused by older mutations, whereas recently expanded populations are convenient for mapping single disease genes affected by recent mutations (Laan and Paabo 1997, Lonjou et al. 1999).

6.1.2. Candidate gene association studies

Selecting a right candidate gene is the first challenge in performing a candidate gene association study. For example, for neuronal disorders, all genes expressed in brains may be considered as potential candidate genes. When a gene and polymorphism(s) have been chosen, and the study has been performed, there is a chance that in case of

negative association, the real association in nearby SNP may have been missed due to negative linkage disequilibrium. To avoid this possibility, and because of the high variability in the extent of linkage disequilibrium across the genome, a whole-genome linkage disequilibrium map should be available if one wishes to carry out a successful association study.

A systematic genome-wide linkage disequilibrium mapping using tens of thousands of candidate-SNPs in a case-control association study, could avoid this problem, but the extremely high number of SNPs needed and the resulting high costs hamper the performance of such studies. DNA pooling has been introduced as a solution, but the accurate interpretation of SNP allele frequencies might prove to be problematic (Baron 2001).

Furthermore, statistical issues puzzle researchers performing association studies. When setting the p-value criteria critically to avoid false-positive associations, there may never be enough power to detect modest gene effects with confidence (Terwilliger and Weiss 1998). The search for susceptibility genes of complex diseases may require complex solutions, and joint linkage and association studies (Baron 2001) might be efficient to reliably and cost-efficiently map susceptibility genes with both large and intermediate effects on the risk of developing disease in reasonable-sized sample populations.

6.1.3. Real-time RT-PCR

The real-time quantitative PCR offers a new dimension in sensitivity when compared to older reverse transcription methods, but this sensitivity also places higher demands on understanding and meeting the methodological demands. The quantification of real-time PCR can be performed using a standard curve or comparative C_t method that requires approximately equal efficiencies for target and housekeeping gene PCR amplifications (Giulietti et al. 2001). In study II, the requirement for equal PCR amplification efficiencies was not met, and the relative standard curve method was applied. The benefits of the standard curve method are the standard dilutions that are included in each PCR run providing an extra control and correction for PCR efficiency, making the interassay comparison more reliable (Giulietti et al. 2001).

A reliable quantitative RT-PCR method requires correction for variations in individual reverse transcription and PCR efficiencies. Using real-time PCR, these problems are minor, since the quantification is based on C_t values, which are determined very early in the exponential phase of the reactions as opposed to end-point analysis. The normalization to a housekeeping gene is currently the most acceptable method to correct for minor variations in RNA input amount or reverse transcription efficiency (Giulietti et al. 2001). When analyzing the results, it is important to consider the possible differences in housekeeping gene expressions in order to avoid making false positive or negative conclusions.

It has been suggested that the unadjusted values of target genes without normalization with a housekeeping gene, might actually be the best estimates of the real change in gene expression (Bond et al. 2002). Even without housekeeping gene expression normalization, gene expressions are normalized by the amount of total RNA used in reverse transcription and PCR amplification, since the same amount of RNA converted to cDNA is used in real-time PCR for all samples. According to our results, this may not be sufficient, indicating that the reverse transcription or PCR amplification efficiencies vary between the samples. Bond et al. (2002) has recently proposed the use of covariance analysis in gene expression studies. Analyzing target gene expressions using housekeeping gene expression as a covariate is recommended, if the housekeeping gene expression is altered by the treatments (Bond et al. 2002). According to present study, the univariate general linear model using GAPDH expression as a covariate gave similar results with the GAPDH normalized results. Although the requirement for equal variances was not met, covariance analysis gave identical results with nonparametric Kruskall-Wallis test that does not require either normal distribution or equal variances. Thus, the normalization by the amount of total RNA does not seem to be adequate, but both normalizing by calculating target gene to housekeeping gene expression relation, or using housekeeping gene expression as a covariate might assist in drawing more realistic conclusions from the data in question.

6.1.4. Statistical analyses

Expression studies are usually conducted with rather small sample numbers because of the expense involved in expression analysis and the complicated statistics needed

following the analysis. As a consequence of small sample numbers, the observations seldom are normally distributed, and the use of nonparametric tests is recommended. Nonparametric tests rank the individual values and analyses are based on the distribution of ranks resulting in loss of original information regarding the individual observations and group means. Nonparametric tests are less powerful than the parametric tests, meaning that p-values tend to be higher, rendering it harder to detect real differences as being statistically significant.

In study III, nonparametric tests were used because of the small sample number and the abnormal distribution of the values. In this study, nonparametric tests proved powerful in detecting significant differences, and since the differences were substantial, they are also considered to be accurate.

6.2. CYP19

LD and haplotype blocks. CYP19 gene displayed considerable LD between SNP pairs up to 40 kb, but at greater distances, the standardized D' values diminished. The majority of chromosomes were accounted for by two major haplotypes (high-risk and low-risk), and the third haplotype found had a low (5%) frequency for both case and control groups. To detect haplotype blocks, a four-gamete test application was used (Wang et al. 2002). When a small proportion of recombination (< 3%) was allowed to occur between two loci, two haplotype blocks defined by the SNPs 2 to 4 and 5 to 9 within the CYP19 gene region were found. SNP 1 locating at the 5' prime end of the CYP19 gene was considered to belong to an undefined block flanking the gene region. The distribution of proposed blocks suggested that the recombination crossover site was located between SNPs 4 and 5, following basically the promoter/5'-UTR and coding/intron/3'-UTR organization. SNP pairs between the blocks displayed also considerable LD, indicating that the recombination frequency at the crossover region is low. A second implication of the strong LD observed at the region is that EM algorithm estimates of SNP haplotype frequencies should be accurate (Xu et al. 2002). Several studies have proposed that the human genome is arranged into block-like patterns of LD that may be useful in defining disease susceptibility genes (Daly et al. 2001, Jeffreys et al. 2001, Reich et al. 2001, Wang et al. 2002), and the low haplotype diversity of

CYP19 gene supports this notion with its genomic region arranged into two haplotype blocks with low between-block recombination frequency.

Disease associations. CYP19 was implicated as a susceptibility gene for AD in this candidate gene-based association study of a large clinic-based series of AD patients and nondemented subjects, these results being particularly supported with the existence of a common high-risk haplotype. This haplotype was over-represented in cases using two analytic approaches which both estimated ~60% excess risk for the high-risk haplotype. The independence of CYP19 polymorphism from APOE genotype in both single-locus analyses and latent SNP groups suggested that CYP19 polymorphism is probably an independent risk factor for AD. Moreover, the risk related to CYP19 was likely to be similar for men and women and operated over a wide range of age: raw and age-sex adjusted logistic estimates of risk were essentially the same, and the low-risk and high-risk latent SNP groups had similar age-sex distributions.

The functional significance of haplotype block of SNPs 5 to 9 associated to AD remains to be determined. The SNPs significantly associated with AD were located more to the intron and 3'-UTR parts of the gene than to the large 5'-UTR region. Thus, it would be more likely that the functional alteration responsible for disease association, and which probably is in LD with the SNPs 4 to 9, would be at the coding/intron or 3' regulatory region of the CYP19 gene. It is also possible that some other susceptibility gene located at the 3' flanking region of CYP19 gene is actually responsible for the disease-association seen, but the nearest 3' flanking gene is located more than 70 kb from the end of CYP19 gene, and though it is improbable that the observed association would be accounted for by this unknown gene. Two novel studies identified an association between CYP19 polymorphisms and aromatase activity or estradiol levels (Berstein et al. 2004, Dunning et al. 2004) supporting the possible relevance of CYP19 polymorphisms on AD by their ability to modify estrogen levels.

The single locus genotype and allele frequencies of SNPs were only moderately different between cases and controls, but there are two aspects that support the idea of a true association rather than false positive result. A series of associated SNPs across the intron/3'-UTR parts of the gene, which are basically also located into a single haplotype block support the idea of true association (Terwilliger et al. 1997). Second, two different

statistical approaches, that are also less sensitive to multiple testing as SNPs were considered jointly not individually and they also implicated CYP19 as a susceptibility gene for AD. In other large genome screening studies, chromosome 15 has not attracted much attention (Pericak-Vance et al. 1998, Kehoe et al. 1999, Pericak-Vance et al. 2000, Myers et al. 2002) or the linkage region found has been located far (15q26, 79 cM) from the CYP19 gene (Blacker et al. 2003) suggesting that the CYP19 possesses only a moderate effect in AD and thus this gene region may have been missed in previous linkage-based genome screenings. Recently, however, one study identified genetic markers 15 and 17 cM distally to CYP19 that were associated with AD (Scott et al. 2003) supporting the relevance of this genomic region as an AD susceptibility region.

CYP19, ERα and ERβ expression in estrogen deprivation and ERT. The down-regulation of CYP19 gene expression by phasic ERT is consistent with previous findings on mouse diencephalon (Yamada et al. 1993). Phasic ERT may possibly decrease the production of testosterone and dehydroepiandrosterone sulfate via the hypothalamic/pituitary axis feedback (Casson et al. 1997), and the subsequent decrease in the amount of aromatase substrate androgens might explain the down-regulation of CYP19 gene expression. In addition, steroid hormone binding globulin, which binds steroid hormones slowing hormone degradation and excretion, is increased during ERT, further decreasing the amount of free testosterone in plasma (Casson et al. 1997). These events together may cause the overall decline of free plasma testosterone levels in ERT explaining the downregulation of *CYP19* gene expression. In contrast to ERT with injections, the tonic estrogen therapy used in this study upregulated CYP19 expression. The opposite response may result from the exposure to prolonged, tonically high hormone levels that may dampen the regulation of hypothalamic/pituitary axis feedback or cellular transcription machinery.

The regulation of CYP19 gene expression is complex. In addition to being a substrate of the aromatase enzyme, androgens may regulate CYP19 gene expression, since CYP19 mRNA contains androgen and estrogen responsive elements, that may stabilize the CYP19 mRNA and increase the CYP19 gene expression (Harada et al. 1993, Lephart 1996, Bourguiba et al. 2003). In addition, the promoter region of human brain specific

CYP19 mRNA contains potential androgen and glucocorticoid binding sites (Honda et al. 1994). Gonadal hormones affect CYP19 expression; testosterone induces and estradiol inhibits the activity of this enzyme. The regulation of brain aromatase expression may also be affected by other gonadal hormones since the effects of testosterone and estradiol are more substantial in ovariectomized mice than in intact animals (Yamada et al. 1993). The effects are inhibited by androgen and estrogen receptor antagonists, but ER antagonists do not prevent the effects of testosterone on aromatase expression. This suggests that these hormones use different signaling pathways to regulate aromatase expression (Bourguiba et al. 2003). According to another theory, exogenous androgen can be aromatized to estrogen which would act through ER, while the remaining androgen acts through the AR. Together these two receptors may modulate the CYP19 gene expression (Balthazart et al. 2003). This view is supported by findings of co-localization of aromatase and androgen receptor on the one hand (Balthazart et al. 1998), and aromatase and ERα on the other hand (Balthazart et al. 1991).

The expression profile of ERα in response to estrogen manipulations was very similar to CYP19. One previous study reported ERα up-regulation in rat cerebral cortex after OVX and down-regulation to normal level after ERT (Mohamed and Abdel-Rahman 2000). In the same study, the expression of ERα was down-regulated in rat cerebellum after OVX and up-regulated after ERT, similarly to our study. The discrepancies of ERα expressions in response to OVX and ERT may be attributed to different brain areas studied, since ERs are probably expressed cell and tissue-specifically (Carley et al. 2003, Matthews and Gustafsson 2003). ERs show also tissue-specific autoregulation by estrogen (Lee and Gorski 1998), and moreover, according to this study, the delivery route and dose kinetics may affect the regulation of ER expression.

ERβ expression in mouse brain has been less studied than Erα expression. A previous study has reported down-regulation of ERβ in the rat brain after estrogen treatment (Shima et al. 2003). According to our results, hippocampal ERβ expression is not changed in mice after phasic ERT, but it is up-regulated after tonic ERT. It has been suggested that the main function of the ERβ isoform may be to modulate or inhibit the effects of ERα (Hall and McDonnell 1999, Weihua et al. 2000, Matthews and

Gustafsson 2003). Thus it would be reasonable for ERβ expression to remain constant during hormonal changes, while ERα expression would fluctuate more extensively. The relative expression level of the two ER isoforms has been proposed to be a key determinant of the cellular responses to estrogen agonists and antagonists (Hall and McDonnell 1999), and in our study the expression of ERα was approximately eight-fold compared to ERβ expression in mouse hippocampus. Although the ERβ gene expression seemed unchanged by phasic ERT, ERβ protein may still respond to estrogen at posttranscriptional level.

In contrast to phasic injected estrogen, tonic estrogen delivery from an implanted pellet up-regulated all three genes in ovariectomized female mice. Nevertheless, the effect on uterine weight was comparable between 20 µg of injected estrogen and the estrogen pellet. A similar uterine response to the pellet that releases 2 µg per day and a daily injection of a 10-fold estrogen dose is rather surprising at first glance. However, the hormone levels in the pellet mice are constant around the clock, whereas the hormone levels after injection peak once daily reaching supraphysiological concentrations. The regulation of mouse uterine ER expression has been shown to be dependent on hormone levels; physiological estrogen levels up-regulate ER mRNA, while supraphysiological levels evoke no increase in ER mRNA compared to OVX mice (Bergman et al. 1992). Our results suggest that brain ERs respond similarly, since ERα and ERβ expressions were up-regulated in pellet treated mice, but downregulated or unaffected in injected mice.

Androgens have also many of the neuroprotective effects of estrogen (Papasozomenos 1997, Hammond et al. 2001, Pike 2001, Papasozomenos and Shanavas 2002, Ramsden et al. 2003, Tan and Pu 2003, Leranth et al. 2004), and it is possible that androgens acting through androgen receptors are the neuroprotective hormones in the estrogen deprived brain. Supporting this theory, a recent report has shown that estrogen and androgens share a common downstream target, heat shock protein 70 that protects neurons from Aβ1-42 induced toxicity (Zhang et al. 2004). One interesting, new finding suggests that the maintenance of hippocampal spine synapses depends on hippocampal estrogen synthesis, but exogenous estrogen has no effect on these synapses (Kretz et al. 2004). In addition, the concentration of estradiol in hippocampus is manifold compared

to plasma estradiol levels, indicating that in hippocampus, neurosteroids modulate interneuronal communication acting in a paracrine manner (Hojo et al. 2004). Endogenously synthesized neurosteroids might also be essential for the other neuroprotective functions of estrogen.

In conclusion, the present findings indicate that ERT not only affects brain by directly delivering estrogen across the blood-brain barrier, but also indirectly regulates the estrogen synthesis in the brain by modulating the expression of the aromatase gene. Furthermore, estrogen has been reported to stimulate aromatase enzyme activity without affecting mRNA levels, pointing to posttranscriptional effects of estrogen on aromatase activity levels (Roselli et al. 1997). Whether the direct estrogen delivery to the brain or the modulation of aromatase activity is more important for the outcome of ERT remains to be explored in further studies.

The present study also demonstrated that the pattern of estrogen delivery may dramatically change the ERT effect on the brain. Interestingly, tonic ERT with an implanted pellet that had beneficial memory effects also increased the level of CYP19 and ERα gene expression, whereas injected estrogen with the same peripheral effect had the opposite influence on the brain. This finding suggests that the brain effects of peroral estrogen may also differ from the effect of transdermal estrogen.

6.3. Seladin-1

Human seladin-1 transcription is linked to neuronal degeneration. The expression study with a semi-quantitative RT-PCR method revealed that transcription of seladin-1 was significantly decreased in temporal cortices of AD cases compared to nondemented subjects, whereas in pure DLB cases, no differential transcription of seladin-1 could be seen. Interestingly, DLB cases carrying AD pathology had a lower transcription of seladin-1 in temporal cortex indicating that the differential transcription of seladin-1 in DLB/AD cases could be attributed to AD rather than DLB pathology. The decline in the relative transcription of seladin-1 was related to the presence of NFTs, NPs and PHF-τ fibrils but not to Aβ. Therefore, the decrease in transcription level of seladin-1 in AD could be considered as a marker of the neuronal degeneration occurring in AD seen as NFTs, NPs and neuropil threads. However, due to the limited number of cases in each

study group, no firm conclusions concerning the specificity of the seladin-1 mRNA downregulation to AD but not to other neurological diseases should be made on the ground of this study alone.

To minimize the variation between samples attributable to differences in tissue conditions (e.g. pH) and handling, transcription of seladin-1, calculated as the proportion with respect to the transcription of the control gene (β-actin), was compared between two different brain areas, temporal and occipital cortex, in every individual case. Nicastrin, which served as reference data, was processed in a similar way. Nicastrin expressed both in neuronal and nonneuronal cells (Satoh and Kuroda 2001), was found to be remarkable constantly transcripted in nondemented and demented cases, in both temporal and occipital cortex. This confirms the belief that the decrease in seladin-1 transcription in temporal cortex of AD cases is due to factors other than cell loss alone, which was also shown previously by *in situ* hybridization (Greeve et al. 2000).

Mouse seladin-1 transcription fluctuates during aging. To study whether the decrease in seladin-1 transcription precedes the accumulation of amyloid deposits or is a consequence of amyloid accumulation, double transgenic APP/PSEN-1 mice were used. The amyloid deposits begin to develop around the age of 9 months in these mice, and the amyloid formation increases dramatically between 10 and 12 months of age. Amyloid deposits appear first in the hippocampus, subiculum, frontal and occipital cortices subsequently spreading to other cortical areas. Many of the Aβ deposits are associated with dystrophic neurites (PHF-ι negative) and reactive astrocytes (Borchelt et al. 1997). Different cortical regions are similarly affected by the amyloid deposits while the olfactory bulb is only slightly affected (T. van Groen, Department of Neuroscience and Neurology, University of Kuopio, personal communication).

The results indicated that the relative transcription of mouse seladin-1 fluctuates during aging, but the transcription pattern of seladin-1 was similar in control and APP/PSEN-1 transgenic mice, indicating that changes in seladin-1 transcription were attributable more to the general aging process than to the pathological changes occurring in transgenic mice. However, the changes were significant only between transgenic mice

groups, indicating that the APP/PSEN-1 mutations could enhance the effects of seladin-1 transcription in aging. As in humans, the Aβ accumulation in animals did not show any relationship with seladin-1 transcription. A decline in seladin-1 transcription seen at the age of 11 months could have been potentiated by the amyloid deposition. On the other hand, the same phenomenon, although not significant, was seen in littermates of the same age that did not develop amyloid deposits. The increase in the seladin-1 transcription at the age of 16-17 months compared to the levels at 11 months might be an attempt by the cells to compensate for the aging process and amyloid deposit formation. The absence of apoptotic cell loss in the transgenic mice could partly explain why age-matched transgenic and littermate mice did not differ in seladin-1 transcription during aging. This indicates that instead of Aβ accumulation, other factors such as tau pathology or cell loss might be needed to affect the seladin-1 transcription in AD brain.

Induction of apoptosis increases seladin-1 transcription in N2a cells. Since PSEN-1 and seladin-1 have been linked to apoptosis (Guo et al. 1997, Greeve et al. 2000), an *in vitro* study with mouse N2a cells stimulated to undergo apoptosis was performed to examine the possible consequences of wild-type PSEN-1 and ΔE9 PSEN-1 mutation in relation to seladin-1 in apoptosis. Seladin-1 overtranscription protects cells from oxidative stress and apoptosis via caspase-3 activation (Greeve et al. 2000), however, in intense oxidative stress, seladin-1 is presumably inactivated mainly by caspase-6 which cleaves seladin-1 into a 40 kDa derivative. In addition to this derivative that possibly possesses a proapoptotic attribute, decreased transcription of seladin-1 may also be a death signal to cells leading to increased caspase-3 activation and subsequent cascade of apoptosis. PSEN-1 is highly expressed in nerve cell populations known to be targeted in AD, such as cells in hippocampus, amygdala and neocortex (Lee et al. 1996).The ΔE9 mutant PSEN-1 increases cell vulnerability to apoptosis, a characteristic not seen in the case of wildtype PSEN-1 (Guo et al. 1997), and ΔE9 PSEN-1 also affects Aβ42 peptide production (Mehta et al. 1998).

Caspase-3 activity, a measure of apoptosis, was significantly higher in all okadaic acid treated groups than in untreated groups, indicating that apoptosis had been initiated. Stalled entering of the ΔE9 PSEN-1 transfected N2a cells into the apoptotic phase that was seen as delayed increase of caspase-3 activity from 36 to 45 h time point when

compared to wildtype and vector transfected groups, could be related to the PSEN-1 mutation involvement in arrested cell cycle (Janicki et al. 2000).

The relative transcription of seladin-1 increased in all groups from the 36 to 45 h time point, suggesting that induction of apoptosis also increased the seladin-1 transcription. Interestingly, seladin-1 transcription in the ΔE9 PSEN-1 group was lower than in vector and wild-type PSEN-1 transfected groups after 45 h exposure to okadaic acid, and corresponded approximately to the transcription level of untreated control cells. Since seladin-1 is known to be able to inhibit caspase-3 (Greeve et al. 2000), this might explain why this group exhibited higher caspase-3 activity; this could be a compensation mechanism that increases apoptotic signals and also modulates to a certain extent the transcription of protective factors such as seladin-1.

6.4. HSPG2

Even though HSPG2 can be considered as a promising candidate in AD pathogenesis, no previous reports have been available concerning the possible genetic involvement of HSPG2 in AD, until recently, when an association study found no association between HSPG2 and AD, irrespective of APOE ε4 status (Rosenmann et al. 2004). In the present study, HSPG2 polymorphism alone did not associate with AD, but in APOE ε4 allele carriers, HSPG2 A allele did associate with AD. Although the association was introduced only after a stratification of the whole data, a technique which also increases the risk of false positive results, the observed effect between the HSPG2 A and APOE ε4 alleles is consistent with the current biological data. ApoE binds heparin with high affinity (Cardin et al. 1986), stimulates HSPG2 mRNA and the HSPG2 protein production as well as heparan sulphate production *in vivo* (Cardin et al. 1986, Paka et al. 1999a, Paka et al. 1999b). The ApoE4 isoform also stimulates sulphation of GAG chains more efficiently than the ApoE3 isoform (Bonay and Avila 2001). These findings, together with the well-known linkage of APOE to AD, indicate that HSPG2 might be associated with AD through the sulphation of heparan sulphate moieties of the glycoprotein rather than through the core protein itself.

The sulphation of heparan sulphate moieties is also linked to tau protein and Aβ. Heparan sulphate and heparin stimulate the formation of paired helical-like filaments by

promoting phosphorylation of tau (Goedert et al. 1996), and this effect is influenced by the amount of sulphation of GAG chains, with the more extensively sulphated heparan sulphates being more active (Hasegawa et al. 1997). Consistent with this, we were also able to show more abundant PHF-tau expression in neuropathologically confirmed AD patients carrying both APOE ε4 and HSPG2 A alleles when compared to ε4 carriers lacking the HSPG2 A allele. The sulphate moieties on the GAGs are also critical for enhancement of Aβ fibril formation (Castillo et al. 1999), and in addition, HSPG2 has been found to bind Aβ proteins, accelerate Aβ fibril formation and maintain Aβ fibril stability (Castillo et al. 1997). Overall, these findings highlight the noteworthy effect of HSPG2 sulphation on the association of HSPG2 with AD.

In summary, this data suggests that some functional change in the HSPG2 gene, which is probably in linkage disequilibrium with the A allele, may cause changes in the expression or splicing status of the HSPG2 gene, and lead to an altered number of heparan sulphate attachment sites. In parallel to this process, the APOE4 isoform sulphates more efficiently the heparan sulphate disaccharide-repeating units in the glycoprotein (Bonay and Avila 2001), which in turn can stimulate tau phosphorylation and consequently lead to tau filament formation. The same mechanism may also underlie the Aβ fibril formation. Unfortunately, the multiple testing limits the power to find conclusive evidence of association, and the p-values should be viewed with caution. Elucidating the biological relevance of HSPG2 and APOE in AD would provide more definite proof of a true association.

In the future, detailed genetic studies in different ethnic populations and expressional analyses using animal models of AD might offer more precise information regarding the conceivable role of CYP19 region in AD. In the case of seladin-1, SNP and haplotype analysis using SNPs covering the gene coding for seladin-1 and the nearby regions, together with correlation analysis with attributes of tau pathology might be worth more detailed examination. Also the possible downregulation of seladin-1 in other tauopathies could strengthen the linkage between seladin-1 and tau protein. A more detailed SNP analysis of HSPG2 gene region would be essential in order to confirm the authenticity of the association. Also the biological relevance of HSPG2 and APOE in AD might be an interesting objective of further functional analysis. In summary, the present series of

studies provide initial clues about the importance of several proteins in AD pathogenesis but additional research will be needed to confirm their relevance and their potential application in future AD therapies.

7. SUMMARY AND CONCLUSIONS

The aim of the study was to find new candidate genes that might affect the risk of developing Alzheimer's disease and to study the possible changes in their expression levels using gene expression methods. Genetic studies so far have identified only a few genes to be involved in early-onset age, familial forms of Alzheimer's disease, whereas the majority of individuals suffering from Alzheimer's disease are affected at a rather later age, as the disease usually begins after the age of 60 years. Only a few genes are identified that predispose to late-onset form of AD, with APOE being the most widely acknowledged.

Our study population consisted mainly of late-onset AD patients and their age-matched controls. The purpose was to use the homogenous eastern Finnish population to identify new candidate genes for AD and also to study their possible mechanisms of action using gene expression methods.

1. In the CYP19 gene, a cluster of SNPs with an increased risk of Alzheimer's disease were found. These SNPs together formed a risk haplotype that was independent of sex, age and APOE polymorphism.

2. CYP19 and ER gene expressions were found to be affected by the hormonal status of mice. Depending on the delivery route and dosage of estrogen, the expression of CYP19 and ERα were modified, while ERβ expression was rather constant. Linking this to Alzheimer's disease, one could postulate that postmenopausal women, who have an increased risk of developing Alzheimer's disease compared to men or premenopausal women, might gain greater benefit from replacement of hormone delivered at constant low levels as opposed to oral administration of a single, high hormone dose every day. An assessment of the optimal delivery route of the hormone may also be worthy of consideration.

3. The transcription of seladin-1 was selectively downregulated in brain areas affected with the hallmark lesions of AD, and the decrease in seladin-1 transcription was linked to neuronal degeneration in AD, including PHF-τ, NFTs and NPs, whereas

no linkage could be found in human or transgenic animal studies between seladin-1 transcription and Aβ accumulation. Furthermore, cell culture studies indicated that seladin-1 transcription increases in apoptotic cells, with apoptotic signals possibly modulating the transcription of seladin-1. In conclusion, the decrease in the transcription of seladin-1 found in AD was considered to be a marker for a specific neuronal degeneration including PHF-τ formation, rather than being a marker for apoptosis or the accumulation of Aβ protein.

4. HSPG2 was identified to be a possible, additive risk factor for Alzheimer's disease in APOE ε4 allele carriers. In genetic analysis, the HSPG2 A allele in conjunction with the APOE ε4 allele doubled the risk compared to carrying the APOE ε4 allele alone. The tau pathology in HSPG2 A and APOE ε4 allele carriers was more prominent than in APOE ε4 allele carriers, suggesting that HSPG2 and APOE might interact at the cellular level impacting more on tau pathology rather than on Aβ pathology.

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