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Timo Sarajärvi

Alzheimer's Disease-Related Genes and Pathways

Special Emphasis on Seladin-1, δ-Opioid Receptor and BACE1

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ABSTRACT

Alzheimer's disease (AD) is a complex and heterogeneous disorder with an evident genetic background. So far, mutations in three genes have been implicated in the familial, autosomal dominant form of AD with an early age of onset (< 65 years). These genes are *APP* encoding amyloid precursor protein and *PSEN1* and *PSEN2* encoding presenilin-1 or presenilin-2 proteins, respectively. On the other hand, a naturally occurring polymorphism, the ε4 allele in the apolipoprotein E gene, (*APOE* ε4), represents the major genetic risk factor for the development of both early- (EOAD) and late-onset (LOAD) forms of AD. Causative mutations in *APP* and *PSEN* genes explain less than 1% of all AD cases, while the vast majority of cases represent LOAD with a complex genetic inheritance. LOAD is considered to be caused by a combination of genetic, acquired, and environmental risk factors. Since the early nineties, nearly 700 individual susceptibility genes with low risk effects have been proposed as being to be associated with AD in various ethnic populations. Nonetheless, the underlying biological mechanisms of many of the risk genes in AD pathogenesis remain to be determined. This thesis aimed at exploring the molecular mechanisms of specific genes that have been postulated to genetically and/or functionally play a role in the pathogenesis of AD. The effects of these genes on the key pathways and processes relevant for AD pathogenesis, such as generation and accumulation of β-amyloid (Aβ), were investigated in different *in vitro* and *in vivo* models.

In this thesis, the risk effects of the common risk gene polymorphisms were assessed in the Eastern Finnish sample cohort consisting of ~1300 AD patients and controls as well as in the familial AD sample set originating from USA. In addition, the role of different factors believed to be involved in AD pathogenesis, namely seladin-1, δ-opioid receptor (δOR) and β-site APP cleaving enzyme 1 (BACE1), were examined in cultured human neuronal and non-neuronal cells as well as in the rat thalamus under conditions partially mimicking the cellular environment of the AD brain.

In study I, single nucleotide polymorphisms (SNPs) in the candidate genes were selected for genotyping on the basis of the meta-analyses retrieved from the publically accessible AlzGene database. This study revealed SNPs in the tumor necrosis factor α (*TNF*) and interleukin 1B (*IL1B*) genes, which genetically associated with AD in the Finnish case-control cohort and which also affected cerebrospinal fluid biomarker levels in AD patients.

Study II revealed that under *in vitro* stress conditions, reduced seladin-1/DHCR24 expression resulted in enhanced GGA3 depletion in neuronal cells. GGA3 is a well-characterized BACE1 trafficking protein, which regulates the lysosomal degradation of BACE1. GGA3 depletion subsequently led to augmented post-translational stabilization of

BACE1 and increased β-amyloidogenic processing of APP. This is an important mechanistic finding since the expression of seladin-1 has been consistently shown to be down-regulated in brain regions affected in AD.

Study III elucidated the genetic and functional role of δOR in AD pathogenesis. Previous data had shown that the agonist-induced activation of δOR increased the activity of β- and γ-secretases, which are responsible for Aβ generation. It was observed that the cysteine variant in the codon 27 of δOR (δOR-Cys27) differentially affected APP processing through altered endocytic trafficking in non-neuronal and neuronal cells as compared to the phenylalanine variant (δOR-Phe27). Furthermore, genetic assessment of the *OPRD1* gene encoding for δOR in two different AD sample cohorts indicated that the heterozygosity of δOR-Phe27Cys variation may increase the risk of AD. As intervention approaches focusing on the formation and trafficking of the δOR/β- and γ-secretase complex have been suggested as novel strategies against AD, it is possible that patients with the δOR-Cys27 variant may respond to δOR antagonist treatments differentially when compared to the δOR-Phe27 homozygotes.

In study IV, a non-selective calcium channel blocker and β-secretase inhibitor, bepridil, was used to treat ischemic rats after transient middle cerebral artery occlusion (MCAO). In these MCAO rats, Aβ and calcium accumulate in the ipsilateral thalamus. Thus, MCAO represents a feasible *in vivo* model with which to study whether bepridil can modify Aβ and calcium pathology. Daily treatment of MCAO rats for 27 days with bepridil (50 mg/kg *p.o.*) starting two days after the operation decreased Aβ40 and Aβ42 and calcium levels in the ipsilateral thalamus as compared to vehicle-treated MCAO rats. Furthermore, seladin-1 mRNA levels were significantly decreased by an average of 40% in the ipsilateral thalamus of vehicle-treated, but not in the bebridil-treated MCAO rats. Significant inverse correlations between seladin-1 mRNA and calcium levels as well as with the levels of seladin-1 and insoluble Aβ42 in the ipsilateral thalamus after MCAO were observed. Collectively, these findings suggest that in rats bepridil mitigates Aβ and calcium pathology and that seladin-1 is an indicator of neuronal survival.

AD-related genes and pathways studied in this thesis may prove to be potential molecular targets and used as novel biomarkers for risk assessments, early diagnosis, and monitoring disease progression. Furthermore, the results obtained in this thesis may be applied in the development of novel intervention approaches to interrupt the progression of AD. Finally, the genetic results emphasize the importance of knowing the genetic profile of a person when introducing new potential pharmacological approaches in the therapeutics of AD.

National Library of Medical Classification: WT 155, QU 470, QU 500, QU 55.7
Medical Subject Headings: Alzheimer Disease/etiology; Genes; Genetics; Genotype; Polymorphism, Genetic; Polymorphism, Single Nucleotide; Risk; Nerve Tissue Proteins; Receptors, Opioid, delta; Tumor Necrosis Factor-alpha; Interleukin-1beta; Cerebrospinal Fluid; Biological Markers; Apoptosis; Amyloid beta-Peptides; Protein Transport; Disease Models, Animal; Infarction, Middle Cerebral Artery; Bepridil; Calcium; Thalamus; Cohort Studies; Case-Control Studies; Finland

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TIIVISTELMÄ

Alzheimerin tauti (AT) on geneettisesti monimuotoinen sairaus. Tällä hetkellä tunnetaan kolme geeniä (amyloidiprekursoriproteiini (*APP*) -geeni sekä preseniliini 1 ja 2 (*PSEN1* ja *PSEN2*) –geenit), joissa tapahtuvat mutaatiot aiheuttavat varhaisella iällä (< 65-vuotiaana) alkavan perinnöllisen AT:n. Lisäksi kromosomissa 19 sijaitsevan apolipoproteiini E epsilon 4 (*APOE* ε4) alleelin on todettu merkittävissä määrin lisäävän riskiä sairastua AT:iin. Tautia aiheuttavat geenimutaatiot selittävät kuitenkin vain pienen osan varhaisiän AT-tapauksista, ja näin ollen suurin osa AT-tapauksista onkin myöhäisiän tautimuotoa, jonka taustalla on sekä geneettisiä että ympäristöön liittyviä riskitekijöitä. Viimeisten kahdenkymmenen vuoden aikana on löydetty satoja uusia ehdokasgeenejä, joiden on raportoitu joko altistavan tai suojaavan AT:lta lukuisissa eri etnisissä väestöryhmissä. AT:n patogeneesin kannalta keskeisiä ovat APP:n pilkkoutumisessa tapahtuvat muutokset, jotka vaikuttavat amyloidi-β (Aβ)-peptidin tuotantoon ja kertymiseen aivokudoksessa. Tästä huolimatta taudin perimmäisiä syntymekanismeja ei vielä ole täysin pystytty selvittämään ja siksi riskigeenien toiminnallinen tutkimus on ehdottoman tärkeää etsittäessä keinoja taudin ennaltaehkäisemiseksi. Tämän väitöskirjan tarkoituksena oli selvittää jo tunnettujen geenimuutosten riskivaikutuksia suomalaisilla AT-potilailla. Tämän lisäksi tavoitteena oli tutkia AT:n patogeneesiin liittyvien tekijöiden geneettisiä sekä molekyyli- ja solutason vaikutusmekanismeja erilaisissa solu- ja eläinmalleissa.

Riskigeenimuutosten vaikutuksia itäsuomalaisessa tautipopulaatiossa tutkittiin käyttämällä SNP-pohjaista assosiaatioanalyysiä, jossa riskigeenialueiden valinta perustui julkiseen AlzGene tietokantaan. Itäsuomalainen tapaus-verrokkiaineisto koostui noin ~1300 myöhäisiän AT:a sairastavasta potilaasta ja kontrollihenkilöistä. Geneettisissä jatkotutkimuksissa käytettiin yhdysvaltalaista Alzheimer-perheaineistoa varmentamaan saatuja löydöksiä. Tämän lisäksi tutkittiin mm. seladiini-1:n, δ-opioidireseptorin (δ-OR) sekä β-sekretaasiproteaasin (BACE1) molekulaarisia vaikutusmekanismeja AT:lle keskeisissä solutason tapahtumissa käyttäen erilaisia solu- ja eläinmalleja.

Ensimmäisessä osatyössä AlzGene -tietokantaan pohjautuvien kandidaattigeenien SNP-pohjainen kartoitus osoitti, että muutokset *TNF*- (tumor necrosis factor α) sekä *IL1B*- (interleukin 1B) geeneissä vaikuttivat AT:n riskiin itäsuomalaisessa tapaus-verrokkiaineistossa. Kyseiset geenimuutokset vaikuttivat merkittävästi AT-potilaiden selkäydinnesteen merkkiaineiden, kuten Aβ42:n ja tau-proteiinin tasoihin.

Toisessa osatyössä osoitettiin, että seladiini-1-proteiinin tasojen lasku SH-SY5Y-neuroblastoomasoluissa apoptoottisen stressin yhteydessä aiheutti GGA3-proteiinin lisääntyneen pilkkoutumisen, mikä puolestaan johti BACE1:n post-translationaaliseen stabiloitumiseen endosomeihin. Aikaisemmissa tutkimuksissa on

osoitettu, että GGA3 toimii BACE1:n kuljetusproteiinina, joka säätelee BACE1:n lysosomaalista hajotusta. Kyseisissä olosuhteissa BACE1:n stabiloitumisen puolestaan osoitettiin lisäävän Aβ-peptidin tuotantoa. Tämä on merkittävä mekanistinen havainto AT:n patogeneesin kannalta, sillä seladiini-1:n tasojen on useissa aiemmissa tutkimuksissa raportoitu alentuneen juuri niillä aivoalueilla, jotka eniten altistuvat AT:n tyypillisille patologisille muutoksille.

Kolmannessa osatyössä selvitettiin δOR:n merkitystä AT:n patogeneesissä. Aikaisemmin on näytetty, että δOR:n agonistivälitteinen aktivaatio lisää β- ja γ-sekretaasien aktiivisuutta lisäten näin Aβ-peptidin muodostusta. Tutkimuksessamme havaittiin, että kodonissa 27 esiintyvä δOR:n kysteiinivarianttimuoto (δOR-Cys27) lisäsi APP:n prosessointia muuttamalla sen endosytoottista kuljetusta sekä ei-neuronaalisissa että neuronaalisissa soluissa verrattuna reseptorin fenyylialaniinimuotoon (δOR-Phe27). Lisäksi δOR:a koodaavan geenin *OPRD1*:n SNP-pohjainen assosiaatioanalyysi kahdessa eri AT-potilasaineistossa osoitti, että δOR:n kysteiini- ja fenyylialaniinivarianttien heterotsygoottimuoto (δOR-Phe27Cys) saattaa lisätä AT:n riskiä. δOR-antagonistihoitoja on esitetty potentiaaliseksi AT:n terapiavaihtoehdoksi. Tämän väitöskirjatutkimuksen mukaan on mahdollista, että AT-potilaat, jotka kantavat heterotsygootisti δOR-Phe27Cys variaatiota, reagoivat δOR-antagonistihoitoihin APP prosessoinnin ja Aβ-tuotannon osalta eri tavalla verrattuna δOR-Phe27-homotsygootteihin.

Viimeisessä osatyössä tutkittiin ei-selektiivisen kalsiumkanavasalpaajan, bepridilin, roolia koe-eläinmallissa, jossa rotan toiseen aivopuoliskoon aiheutettiin kohdennettu aivoiskemia (MCAO). Aikaisemmat tutkimukset ovat osoittaneet, että MCAO aiheuttaa rotilla Aβ-peptidin ja kalsiumin tasojen merkittävän lisääntymisen talamuksen alueella tarjoten näin mahdollisuuden tutkia AT:n patogeneesissä keskeisten tekijöiden roolia. Koska bepridilin on todettu myös inhiboivan APP:n β-sekretaasivälitteistä pilkkoutumista, tutkimuksessa pyrittiin selvittämään voidaanko Aβ- ja kalsiumpatologiaan vaikuttaa bepridil-käsittelyllä MCAO:n jälkeen. Rottia lääkittiin 27 vuorokauden ajan annoksella 50 mg/kg alkaen kaksi päivää MCAO-leikkausoperaation jälkeen. Tutkimuksessa havaittiin, että bepridil-käsittely laski MCAO-rottien kohonneita Aβ40- ja Aβ42- sekä kalsiumtasoja talamuksessa verrattuna kontrollieläimiin. Tämän lisäksi havaittiin, että seladiini-1:n lähetti-RNA-tasot talamuksessa olivat merkittävästi laskeneet kontrollirotilla, kun taas bepridil-lääkityillä rotilla seladiini-1:n lähetti-RNA-tasoissa ei nähty merkittäviä muutoksia. MCAO-rottien talamuksen seladiini-1:n ja kalsiumtasojen välillä havaittiin lisäksi merkittävä käänteinen korrelaatio, samoin kuin seladiini-1:n ja liukenemattoman Aβ42:n tasojen välillä. Tämän tutkimuksen havainnot osoittavat, että bepridil voi vaikuttaa rottien aivojen Aβ- ja kalsiumpatologiaan ja että seladiini-1:n ilmentymistasot heijastelevat mahdollisesti hermosolujen selviytymistä tilanteessa, jossa nähdään AT:lle tyypillisiä patologisia muutoksia.

Tässä väitöskirjassa tutkitut geenit sekä molekyyli- ja solutason mekanismit liittyen AT:n patogeneesiin tarjoavat potentiaalisia kohteita uusien biomarkkereiden kehittämiseen, joita voidaan hyödyntää AT:n riskikartoituksessa sekä varhaisemmassa diagnostiikassa. Lisäksi saadut tulokset voivat edesauttaa uusien AT:n terapiavaihtoehtojen kehittämistä. Tässä yhteydessä potilaiden geneettisen profiilin tuntemisella on tämän väitöstutkimuksen valossa merkittävä osa.

Luokitus: WT 155, QU 470, QU 500, QU 55.7
Yleinen Suomalainen asiasanasto: Alzheimerin tauti; molekyylilääketiede; patogeneesi; geenit; genetiikka; genotyyppi; riskitekijät; proteiinit; reseptorit; aivo-selkäydinneste; merkkiaineet

To my beloved ones, Johanna and Niilo:

You are the light of my life!

x

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Kuopio, November 2011

Timo Sarajärvi

List of the original publications

This dissertation is based on the following original publications:

I Sarajärvi T, Helisalmi S, Antikainen L, Mäkinen P, Koivisto AM, Herukka SK, Haapasalo A, Soininen H, Hiltunen M. An association study of 21 potential Alzheimer's disease risk genes in a Finnish population. *Journal of Alzheimer's Disease 21(3): 763-7, 2010.*

II Sarajärvi T, Haapasalo A, Viswanathan J, Mäkinen P, Laitinen M, Soininen H, Hiltunen M. Down-regulation of seladin-1 increases BACE1 levels and activity through enhanced GGA3 depletion during apoptosis. *Journal of Biological Chemistry 4; 284(49): 34433-43, 2009.*

III Sarajärvi T, Tuusa JT, Haapasalo A, Lackman JJ, Sormunen R, Helisalmi S, Roehr JT, Parrado AR, Mäkinen P, Bertram L, Soininen H, Tanzi RE, Petäjä-Repo UE, Hiltunen M. Cysteine 27 variant of the δ-opioid receptor affects amyloid precursor protein processing through altered endocytic trafficking. *Molecular and Cellular Biology 31(11): 2326-40, 2011.*

IV Sarajärvi T, Lipsanen A, Mäkinen P, Peräniemi S, Soininen H, Haapasalo A, Jolkkonen J, Hiltunen M. Bepridil decreases soluble Aβ and calcium levels in the thalamus after middle cerebral artery occlusion in rats. *Submitted.*

Throughout the overview, these papers will be referred to by their Roman numerals.

Contents

Abbreviations

Aβ	β-amyloid
ABCA7	ATP-binding cassette, sub-family A (ABC1), member 7 **(gene)**
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease
ADNI	Alzheimer's Disease Neuroimaging Initiative
AICD	APP intracellular domain
A2M/*α2M*	α-2-macroglobulin **(gene)**
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APH	Anterior pharynx-defective
APLP	APP-like protein
APOE	Apolipoprotein E
APOE ε2/3/4	Apolipoprotein E (ε2/3/4) **(gene)**
APOJ	Apolipoprotein J / clusterin
APP	Amyloid precursor protein
APPim	APP immature
APPm	APP mature
ARF	ADP ribosylation factor
β_2AR	Beta 2 adrenergic receptor
BACE1	β-site APP cleaving enzyme 1
BBB	Blood-brain barrier
BChE	Butyrylcholinesterase **(gene)**
BSA	Bovine serum albumin
CAA	Cerebral amyloid angiopathy
CALHM1	Calcium homeostasis modulator 1 **(gene)**
Cav1.2	Voltage gated L type calcium channels
ChAT	Choline acetyltransferase
ChE	Cholinesterase
CD2AP	CD2-associated protein **(gene)**
CD33	CD33 molecule **(gene)**
CDK	Cyclin-dependent kinase
CDR	Clinical Dementia Rating
cDNA	Complementary DNA
CERAD	Neuropathology Task Force of the Consortium to Establish a Registry for Alzheimer's Disease
CLU	Clusterin **(gene)**
CNS	Central nervous system
CR1	Complement component (3b/4b) receptor 1 (Knops blood group) **(gene)**
CSF	Cerebrospinal fluid
CT	Computed tomography

CTF	C-terminal fragment
DDM	*n*-Dodecyl-*β*-D-maltodextrine
DHCR24	24-dehydrocholesterol reductase **(gene)** (also known as seladin-1)
DMEM	Dulbecco's modified eagle medium
DMS-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
DMSO	Dimethyl sulfoxide
δOR	Delta (δ) opioid receptor
δOR-Phe27Cys	phenylalanine-to-cysteine amino acid substitution at position 27 of δ-opioid receptor
DPBS	Dulbecco's phosphate buffered saline
DS	Down's syndrome
ECE1,2	Endothelin-converting enzyme 1, 2
ECL	Enhanced cheminoluminescence
EGFP	Enhanced green fluorescent protein
eIF2α	Translational initiation factor eIF-2, alpha subunit
eMCI	Early-stage MCI
ELISA	Enzyme-linked immunosorbent assay
EOAD	Familial early-onset Alzheimer's disease
EPHA1	EPH receptor A1 **(gene)**
ER	Endoplastic reticulum
ERK	Extracellular signal-regulated kinase
EYFP	Enhanced yellow fluorescent protein
FAD	Familial Alzheimer's disease
FBS	Fetal bovine serum
FDG-PET	Fluorodeoxyglucose PET
Fe65	APP-binding protein, family B, member 1
FL	Full-length
FTD	Frontotemporal dementia
FTDP-17	Frontotemporal dementia with Parkinsonism linked to chromosome 17
GAPDH	Glyceraldehyde-3-phosphatase dehydrogenase
GGA3	Golgi-localized, γ-ear-containing ADP-ribosylation factor-binding protein 3
GDS	Global Deterioration Scale
gp330/megalin	Glycoprotein receptor gp330/megalin precursor protein
GPRC	G protein coupled receptor
GRN	Granulin **(gene)**
GSK	Glycogen synthase kinase
GSK3β	Glycogen synthase kinase 3 beta **(gene)**
GWAS	Genome-wide association study
Hrs	Hepatocyte growth factor-regulated substrate
IDE	Insulin-degrading enzyme
IL1B	Interleukin 1, β **(gene)**
IRβ	Insulin receptor β-subunit

KPI	Kunitz-type protease inhibitor domain
LAR	Leukocyte common antigen-related receptor tyrosine phosphatase
LDLR	Low density lipoprotein receptor **(gene)**
LEL	Late endosomal/lysosomal compartment
LMCI	Late-stage MCI
LOAD	Late-onset Alzheimer's disease
LRP	Low density lipoprotein receptor-related protein **(gene)**
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MAPT	Microtubule-associated protein tau **(gene)**
MARK	MAP/microtubule affinity-regulating kinase
MDC-9	Metalloprotease/disintegrin/cysteine-rich protein (or meltrin γ)
MCAO	Middle cerebral artery occlusion
MCI	Mild cognitive impairment
MMSE	Mini-Mental State Examination
MPR	Mannose 6-phosphate receptor
MRI	Magnetic resonance imaging
MS4A6A/MS4A4E	Membrane-spanning 4-domains, subfamily A, member 6A/4E **(gene)**
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVB	Multivesicular body
nAChR	Nicotinic acetylcholine receptor
NEP	Neprilysin
NFT	Neurofibrillary tangle
NIMH	National Institute of Mental Health
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease Related Disorders Association
NMDA	N-methyl-D-aspartate
NP	Neuritic plaque
NTF	N-terminal fragment
NPH	Normal pressure hydrocephalus
OPRD1	Opioid receptor, δ1 **(gene)**
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEN-2	Presenilin enhancer-2
PET	Positron emission tomography
PHF	Paired helical filament
PI3K	Phosphatidylinositol-3-kinase
PiB	Pittsburgh compound B
PICALM	Phosphatidylinositol-binding clathrin assembly protein **(gene)**
PKA	Cyclic AMP–protein kinase A
PKC	Protein kinase C
PP2A,B	Protein phosphatase 2 A, B
PSEN1,2	Presenilin 1, 2 **(gene)**

p-tau	Hyperphosphorylated tau protein
qPCR	Quantitative PCR
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
sAPPα,β	Secreted APP α, β
sAPPtot	Secreted APP total
Seladin-1	Selective Alzheimer's disease indicator 1
SNP	Single nucleotide polymorphism
SORCS1	Sortilin-related VPS10 domain-containing receptor 1 **(gene)**
SPECT	Single-photon emission computed tomography
STAM	Signal-transducing adaptor molecule
STS	Staurosporine
TACE	Tumour necrosis factor α-converting enzyme
TBI	Traumatic brain injury
TfR	Transferrin receptor
TGN	*Trans*-Golgi network
Tip60	Histone acetyltransferase, a 60 kDa HIV-Tat-interactive protein
TMD	Transmembrane domain
TNF	Tumour necrosis factor α **(gene)**
TNFα	Tumour necrosis factor α
t-tau	Total tau protein
TTR	Transthyretin
tPA	Tissue-type plasminogen activator
uPA	Urogenase-type plasminogen activator
UTR	Untranslated region
VGCC	Voltage-gated calcium channel
VPS27	Vacuolar protein sorting 27
VHS	VPS27/Hrs/STAM complex

1 Introduction

Alzheimer's disease (AD) is an irreversible progressive neurodegenerative brain disease affecting learning and memory. It is the most common form of dementia among the elderly and the fourth leading cause of death in Western countries. In Finland, the prevalence of dementia is approximately 2% in individuals aged 65–67 years, increasing to 35% at the age older than 85 (Viramo and Sulkava, 2010). Around 13 000 new patients with dementia are annually diagnosed, and of these patients approximately 65-70% are estimated to suffer from AD (Viramo and Sulkava, 2010; Pirttila *et al.*, 2010). A recent report suggested that the prevalence of dementia will double every 20 years worldwide, reaching an alarming 120 million by 2050 (World Alzheimer's Report 2010).

The histopathological hallmarks of AD include widespread neuronal degeneration, neuritic plaques (NPs) containing β-amyloid (Aβ) and neurofibrillary tangles (NFTs) (Selkoe 2001; Iqbal *et al.*, 2009). Amyloid plaques are primarily composed of aggregates of Aβ while NFTs are intracellular aggregates of hyperphosphorylated tau protein. These aggregates are distributed throughout the brain of patients with AD, particularly in the neocortex (Braak and Braak, 1991) and are believed to trigger a series of neurodegenerative processes. A number of other mechanisms appear to contribute to the neurodegenerative process as well, including alterations in calcium homeostasis in the endoplasmic reticulum, which contribute to neuronal apoptosis and excitotoxicity. Mitochondrial dysfunction may also be linked to neurodegenerative diseases through a variety of different pathways, including free-radical generation, impaired calcium buffering, and the mitochondrial permeability transition (Beal, 1999). Synapse loss is an early ubiquitous feature in AD and there is a strong correlation between the extent of synapse loss and the severity of dementia (Selkoe, 2001; Shankar and Walsh, 2009). Synaptic dysfunction is caused by diffusible oligomeric assemblies of the Aβ protein, and there is mounting evidence to suggest that these maky evoke a subtle alteration of hippocampal synaptic efficacy prior to frank neuronal degeneration (Selkoe, 2002).

AD can be roughly grouped into two forms, the familial and sporadic form. In the early-onset familial form, mutations in the amyloid precursor protein (*APP*), presenilin-1 (*PSEN1*), and presenilin-2 (*PSEN2*) genes are responsible for rare autosomal dominant form of the disease, which usually appears before the age of 65 years (Goate *et al.*, 1991; Sherrington *et al.*, 1995; Levy-Lahad *et al.*, 1995; Rogaev *et al.*, 1995). These causative familial early-onset AD (EOAD) mutations, however, explain less than 1% of all early-onset AD cases. Thus, the vast majority of EOAD patients cannot be explained by such simple genetics. The sporadic late-onset form of AD (LOAD) is considered to be caused by a combination of genetic, acquired, and environmental risk factors, although it is believed that up to 60-80% of this form of AD results from complex genetic inheritance (Gatz *et al.*, 2006). Moreover, environmental and epigenetic factors likely make an important contribution in determining an individual's risk. However, the precise nature and mechanisms underlying this nongenetic component remain largely elusive, in part because it is difficult to assess these factors experimentally (Traynor and Singleton, 2010). In the search of genetic causes of LOAD, apolipoprotein E (*APOE*) was introduced approximately two decades ago as a major risk factor for complex forms of AD (Strittmatter *et al.*, 1993; Saunders *et al.*, 1993; Raber *et al.*, 2004). Subsequently, *APOE* has represented the major risk gene for LOAD, showing a strong association to AD in several independent replication

studies in a number of different ethnic populations (Bertram *et al.*, 2007). Importantly, *APOE* ε4 allele has been associated with an increased risk of developing both EOAD and LOAD (Tanzi and Bertram, 2005).

Mutations in the *PSEN1*, *PSEN2* or *APP* genes as well as genetic alterations in some sporadic AD susceptibility genes result in an increase in the production of Aβ1-42, the 42-amino acid long Aβ peptide (Suzuki *et al.*, 1994; Lemere *et al.*, 1996; Borchelt *et al.*, 1996; Scheuner *et al.*, 1996; Ertekin-Taner *et al.*, 2001). In addition, genetic variation in some genes may influence either Aβ clearance or aggregation (Murakami *et al.*, 2003; Tsubuki *et al.*, 2003). The identification of the familial EOAD genes has significantly improved our understanding of the pathogenetic mechanisms underlying neurodegeneration in AD. The knowledge of the identified pathogenic mechanisms has been widely utilized in developing *in vitro* and *in vivo* AD models for investigating the role of LOAD genes in disease pathogenesis.

Altered APP processing leading to increased production of Aβ is a commonly accepted key feature in the pathogenesis of AD. APP processing is initiated by either α-secretase (non-amyloidogenic pathway) or β-secretase (amyloidogenic pathway) to generate APP C-terminal fragments (CTFs). These CTFs are then cleaved by γ-secretase, leading to the formation of p3 (from αCTF) or Aβ (from βCTF) peptides (Tanzi and Bertram, 2005). The γ-secretase mediated cleavage of the βCTF generates Aβ peptides of different lengths, but the Aβ1-42 (42 amino acids), the Aβ1-40 (40 amino acids) (Thinakaran and Koo, 2008) as well as Aβ1-43 (43 amino acids) (Saito *et al.*, 2011) forms are the most prevalent. Changes in the above-mentioned APP processing steps alter the production of Aβ. In the context of AD, however, it should be noted that the events controlling the degradation of Aβ through the Aβ-degrading enzymes (Insulin-degrading enzyme (IDE), neprilysin (NEP), endothelin-converting enzyme 1 (ECE1), and ECE2) are equally as important as those involved in the production of Aβ (Miners *et al.*, 2008). Nonetheless, decreasing the activity of β- or γ-secretase have been shown to inhibit the production of Aβ peptides. Therefore, identification of proteins or compounds that influence the activity of these secretases is a major goal in AD research as it might yield potential new therapies.

Ever since the *APOE* was identified, the LOAD candidate gene approach has spurred a large number of genetic association studies assessing other loci of potential relevance based on functional, mostly Aβ-centered, hypotheses. These studies have resulted in the identification of a number of AD susceptibility genes, including *A2M* (encoding α-2-macroglobulin) (Blacker *et al.*, 1998; Saunders *et al.*, 2003), *BChE (*the K-variant of butyrylcholinesterase; Lehmann *et al.*, 1997), and *LRP* (Low density lipoprotein receptor-related protein; Lendon *et al.*, 1997) genes, as well as more recently *LDLR* (Low density lipoprotein receptor; Lamsa *et al.*, 2008), *CALHM1* (Calcium homeostasis modulator 1; Bertram *et al.*, 2008), *CLU* (Clusterin; Harold *et al.*, 2009; Lambert *et al.*, 2009), *PICALM* (Phosphatidylinositol-binding clathrin assembly protein; Harold *et al.*, 2009), and *CR1* (Complement component (3b/4b) receptor 1; Lambert *et al.*, 2009) genes. The consistency, however, have been poor in different cohorts. In fact, out of the nearly 700 candidate AD genes investigated over the past 30 years, only a few have shown significant risk effects when data from all available studies are combined (Bertram and Tanzi, 2008). Still, identification of new potential candidate genes is important, because functional studies on these genes can help to better understand the molecular and cell biological events influencing AD pathogenesis. This functional approach provides essential information about the biological mechanisms underlying the disease pathogenesis and progression, offering the opportunity to learn from and, eventually, overcome AD.

Despite the growing number of risk genes identified, only a few of the studies have employed functional approaches to determine the biological consequences of the genetic alteration. It is evident that the candidate genes may influence certain key events in AD, such as Aβ production and degradation. Therefore, in the initial functional assessments, it is reasonable to focus on how the observed variations in the identified candidate genes affect the already known factors involved in AD pathogenic mechanisms, such as the secretases (e.g. β-secretase), Aβ-degrading enzymes, or disrupted calcium homeostasis.

This thesis focuses on screening some of the most prominent AD candidate genes and assessing their disease association among Eastern Finnish case-control cohort. Furthermore, it aims to investigate the functional influences of some of the key factors – such as delta opioid receptor (δOR), seladin-1 and BACE1 – hypothezised to play a role on cellular events relevant for AD pathogenesis.

2 Review of the literature

2.1 CLINICAL AND NEUROPATHOLOGICAL CHARACTERISTICS IN ALZHEIMER'S DISEASE

2.1.1 Clinical aspects and diagnostic criteria of AD

The clinical features in AD are very heterogeneous. Typically, the earliest clinical sign is an impairment of recent memory function and attention, followed by a failure in language skills, visual–spatial orientation, abstract thinking, and judgement. As the disease progresses, alterations of personality start to accompany these defects (Förstl and Kurz, 1999; Bouchard and Rossor, 2001; Cummings, 2003). The diagnosis of AD is based on the neuroimaging and biomarker quantification, and the patient's clinical status. Mental status questionnaires are useful in providing a global quantification of the deficits and a means of characterizing the stage of the patient's cognitive decline as well as other dementia-associated abnormalities. The Mini-Mental State Examination (MMSE) (Folstein *et al*., 1975) is the most widely used mental status questionnaire (Cummings, 2003). It includes 30 questions assessing orientation, learning, attention, serial subtraction/attention, recall, naming, repetition, comprehension, reading, writing, and copying a complex figure. However, it is generally insensitive to the cognitive abnormalities present in the earliest phases of AD, and also loses applicability in the final phases of the disease (Cummings, 2003). Another example of a cognition measuring test is the Alzheimer's Disease Assesment Scale (ADAS) (Rosen *et al*., 1984). In order to characterize the patient's cognitive, functional and behavioural aspects of the disorder more extensively, global assessment scales for diagnosis and stage of severity are employed. These kinds of global assessment measures include The Clinical Dementia Rating (CDR) (Hughes *et al.*, 1982; Berg, 1988), and The Global Deterioration Scale (GDS) (Reisberg *et al.*, 1982). CDR measures the performance in six domains: memory, orientation, judgement and problem-solving, community affairs, home and hobbies, and personal care. It was last revised in 1993 (Morris, 1993; Sahadevan *et al.*, 2001). A comprehensive evaluation may involve both cognitive tests and global assessment. By performing all of the three latter tests – MMSE, GDS, and CDR –, one may categorize AD into three different stages according to respective scores: mild (MMSE 18-26, GDS 3-4, CDR 0.5-1), moderate (MMSE 10-22, GDS 4-6, CDR 1-2), and severe (MMSE 0-12, GDS 6-7, CDR 2-3) (Pirttila and Erkinjuntti, 2002).

Prior to the appearance of significant clinical symptoms, the neuropathological changes in AD are thought to begin primarily in the entorhinal and transentorhinal cortex, hippocampus, and then to progress to the association cortices of the temporal, parietal and frontal lobes (Braak and Braak, 1991). In particularly, the hippocampal formation is thought to be a major location of the memory impairment seen in AD (de Leon *et al.*, 1999). The primary sensory and motor cortices and most subcortical structures are relatively well spared (Figure 1). Consistent with these widespread neuropathologic changes, the primary clinical manifestation of AD is a progressive dementia syndrome that usually begins in later life. Braak and Braak's model (1991) devides AD into six subcategories: Transentorhinal (I-II), limbic (II-IV), and neocortical (V-VI) stages (Figure 1). The progression of the clinical symptoms runs in tandem within these stages (Pirttila and Erkinjuntti, 2002). In the final stages (V-VI), typically about 8-10 years after the initial diagnosis of AD, patients are completely bedridden and unable to communicate effectively. Perpetual confusion,

incontinence, and an inability to execute even the most basic cognitive functions are the hallmarks of this final stage of AD (Table 1) (Sweatt, 2003). The diagnosis of the disease is confirmed when there are frequent NPs using the criteria developed by the Consortium to Establish a Registry for AD (CERAD) (Mirra *et al.*, 1991), and NFTs in the medial temporal-lobe structures graded by the Braak and Braak approach as stage V-VI (Braak and Braak, 1991).

Table 1. Different stages of AD progression.

AD stage	Areas first affected	Symptoms
I, II	Trans-entorhinal region Entorhinal cortex Hippocampal CA1 area	Can be clinically silent: Subtle loss of episodic memory and difficulty in excecuting complex progressive tasks; anecdotal repetition; some spatial disorientation
III, IV	Entirety of hippocampus	Early-stage AD: loss of episodic memory; difficulty in spatial reasoning and recognition, difficulty in coherent speech and planning
V, VI	Neocortex	Fully developed AD: pronounced decline in cognition; frank dementia, can include psychosis or depression; ultimately a complete inability to communicate or care for themselves

Adapted from Sweatt, 2003.

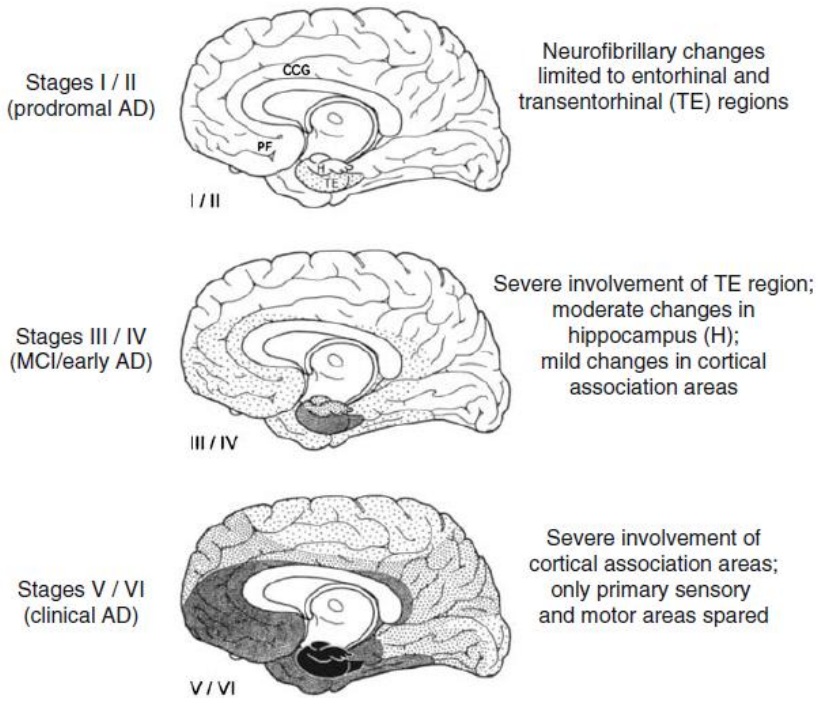

Figure 1. Evolution of neurofibrillary tangle (NFT) pathology as originally conceived by Braak and Braak (1991). Before the first clinical symptoms appear, neurofibrillary changes begin to accumulate in the entorhinal and transentorhinal cortex (Transentorhinal stage; I and II) and may appear surprisingly early in life (e.g., in the individual's thirties or forties). Mild clinical symptoms are believed to coincide with increasing appearance of NFTs in the medial temporal lobe and surrounding association cortices (Limbic stage; III and IV), followed by clinically apparent Alzheimer's disease and the severe involvement of medial temporal and cortical association areas (Neocortical stage; V and VI). (From Bondi et al., 2008)

Pathologically, the severity of the characteristic findings in AD differs considerably among individual patients and this diversity complicates accurate diagnosis. Thus, clinical definitions and diagnostic criteria are needed to advance research in AD, improve the treatment of AD, and identify non-AD causes of dementia. There are three widely used criteria based approach in the diagnosis of AD: the International Classification of Diseases, 10th revision (ICD-10), the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV), and the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease Related Disorders Association (NINCDS-ADRDA) Work Group criteria (McKhann *et al.*, 1984; Cummings and Khachaturian, 2001; Cummings, 2004). The characteristics used in each approach are presented in Table 2. The NINCDS-ADRDA criterion takes a different approach than the other two by dividing the disease states into definite, probable and possible AD. The definite criteria require that the patient meets both probable clinical criteria and has histopathological evidence of AD obtained by biopsy or autopsy.

Since NINCDS-ADRDA and DSM-IV criteria have long been the prevailing diagnostic standards in research, some revisions have been proposed. Distinctive and reliable biomarkers of AD are now available through structural magnetic resonance imaging (MRI), molecular neuroimaging with positron emission tomography (PET), and cerebrospinal fluid analyses. This progress provides an additional support for revised diagnostic criteria for AD (Bouwman *et al.*, 2010). The criteria have been devised to capture both the earliest stages before full-blown dementia as well as the full spectrum of the illness, and they are centered on a clinical core of early and significant episodic memory impairment. According to the new putative guidelines, there must be at least one or more abnormal biomarker(s) among structural neuroimaging with MRI, molecular neuroimaging with PET, or cerebrospinal fluid analysis of Aβ or tau proteins, to support the diagnosis suggested by the cognitive tests (Dubois *et al.*, 2007).

Table 2. Main characteristics and comparison of three commonly used criteria for the diagnosis of Alzheimer's disease.

Criteria	Main characteristics
ICD-10	Deterioration in both memory and thinking, which is sufficient to impair personal activities of daily living.
	The registration, storage, and retrieval of new information are impaired.
	Deficits in thinking and reasoning in addition to the memory disturbances.
	Presence of insidious onset, slow deterioration of cognition.
	Absence of clinical or laboratory evidence of systemic illness or brain disease that can induce dementia.
	Absence of sudden onset. Both early (< 65-years) and late-onset subtypes recognized as well as atypical and mixed types.
DSM-IV	Multiple cognitive deficits including memory impairment.
	At least one of the following: aphasia, apraxia, agnosia, or disturbance in executive functioning.
	Social and occupational impairment. Other neurological disorders, systemic conditions or substance abuse excluded.
	Absence of other major psychiatric disorder (e.g. schizophrenia).
	Deficits not limited to delirious period. Subtypes of both early (< 65-years) and late-onset forms of AD recognized.
NINCDS-ADRDA	Probable, possible, and definite AD recognized.
• **Probable**	Dementia established by clinical examination and documented by mental status questionnaire.
	Dementia confirmed by neuropsychological testing.
	Deficits in two or more areas of cognition.
	Progressive worsening of memory and other cognitive functions.
	No disturbances of consciousness.
	Onset between ages 40 and 90.
	Absence of systemic disorders or other brain diseases capable of producing a dementia syndrome.
• **Possible**	Presence of a systemic disorder or other brain disease capable of producing dementia but not thought to be a cause of the dementia.
	Gradually progressive decline in a single intellectual function in the absence of any other identifiable cause (e.g. memory loss or aphasia).
• **Definite**	Clinical criteria for probable AD and histopathological evidence of AD (autopsy or biopsy).

Adapted from Cummings and Khachaturian, 2001.

One of the main features of AD is the age of onset, according to which the disease may be divided into three categories (Terry *et al.*, 1983; Ertekin-Tanner, 2007). First, the most common form is the LOAD accounting for approximately 90% of all AD cases and occurring usually at the age of 65 years or more. Age is the most common risk factor of AD. This is supported by the fact that the prevalence of AD is below 1% in individuals aged 60

to 64 years, but it shows an almost exponential increase with age and in people aged 85 years or older, the prevalence is around 30% (Pirttila and Erkinjuntti, 2002; Hebert, 2003). In longitudinal studies exploring inheritance of AD subjects and their offspring, it is evident that both LOAD and EOAD have a transmissible pattern. However, the genetic component in LOAD is likely to have a more complex form of inheritance compared to that of EOAD with minor evidence of autosomal dominant transmission component (Ertekin-Taner, 2007). Despite the fact that LOAD is often referred to as "sporadic AD", it is important to understand that up to 60 to 80% of this form of AD is claimed to be genetically determined (Gatz *et al.*, 2006). Second, EOAD can be divided into two different subgroups: those patients with no Mendelian inheritance status, and the ones with clear transmissible component. EOAD is exceedingly rare. Its prevalence is estimated to range between 6 to 7% of all AD cases, and autosomal dominant form of EOAD accounts for 13% of all EOAD cases. The mutations in *APP*, *PSEN1* or *PSEN2* can explain up to 71% of the autosomal dominant transmission pattern in EOAD. These genes account for about 0.5% of all AD cases (Ertekin-Tanner, 2007; Lehtovirta, 2002). However, one must understand that this traditional categorization of AD into LOAD and EOAD (including the familial EOAD subgroup with rare *APP*, *PSEN1*, and *PSEN2* genes) is overly simplistic. There are cases of EOAD without evidence for Mendelian transmission. Conversely, LOAD is frequently observed with a strong familial clustering, sometimes resembling a Mendelian pattern (Bertram *et al.*, 2010).

2.1.2 Neuropathology in AD

Neuroimaging studies and subsequent post-mortem correlations have shown that AD features a loss of neurons and synapses in the cerebral cortex and certain subcortical regions. This loss results in gross atrophy of the affected regions, including degeneration in the temporal and parietal lobes, and parts of the frontal cortex and cingulate gyrus (Wenk, 2003). The brain tissue, especially the cortex, becomes atrophied and a visual examination reveals the microscopic appearance of diffuse NPs and NFTs (Braak and Braak, 1991; Bondi *et al.*, 2008). In addition, degeneration in the basal forebrain results in a major reduction in the levels of acetylcholine, a central neurotransmitter (Whitehouse *et al.*, 1982). Loss of white matter, congophilic (cerebral) amyloid angiopathy (CAA), inflammation and oxidative damage are also present in AD brain (Querfurth and LaFerla, 2010).

NPs have a central core consisting of Aβ peptide, which is surrounded by astrocytes, microglia, and dystrophic neurites containing paired helical filaments (Cummings and Back, 1998; Cummings, 2003). Plaque densities are typically at their highest in the temporal and occipital lobes in the AD brain (Cummings, 2003). NFTs are intra-axonal deposits consisting of paired helical filaments of abnormally hyperphosphorylated, microtubule-associated tau protein (Grundke-Iqbal *et al.*, 1986). These filaments disrupt normal intracellular transport and result in cell death (Cummings, 2003). The toxicity of hyperphosphorylated tau is based on its ability to inhibit microtubule assembly and disrupt microtubules. Furthermore, hyperphosphorylated tau is able to sequester also normal tau to the tau aggregates (Alonso *et al.*, 1994). Both plaques and tangles are considered to be the leading causes of neurotoxicity. Aβ oligomers and plaques are potent synaptotoxins: synapses and axons are known to degenerate when surrounded by filamentous, star-shaped plaques (Selkoe, 2002; Haass and Selkoe, 2007; Walsh and Selkoe, 2007).

In addition to the two classical histopathological features, the brains of patients with AD also feature a reduction in synaptic density and loss of neurons in the hippocampal neurons

(Cummings and Back, 1998). Hence, AD may also be considered as a disorder characterized by synaptic failure. In the early stages of AD, there can be up to a ~35% decrease in the density of synapses in the cortex, and a ~15 to 35% decrease in the number of synapses per cortical neuron, as estimated in by brain biopsy (Selkoe, 2002). At the advanced stages of the disease, this loss of synapses has been reported to correlate better with cognitive decline than the numbers of plaques or tangles, degree of neuronal loss, or extent of cortical gliosis (DeKosky and Scheff, 1990; Terry *et al*., 1991; Selkoe, 2002).

The majority of AD patients suffer from vascular lesions, such as cerebral amyloid angiopathy (CAA; affecting more than 90% of patients with AD) (Greenberg *et al*., 2004), and exhibit capillary abnormalities, disruption of the blood-brain-barrier (BBB), and large-vessel atheroma (Roher *et al*., 2004). CAA is characterized by amyloid depositions in the cerebral vasculature leading to fibroid necrosis of the vessel wall and formation of microaneurysms. These predispose the vessels to rupture or leakage, thus causing haemorrhage in the brain (Vasilevko *et al*., 2010). Approximately 60 to 90% of patients are affected by ischemic disease mainly due the vascular lesions. Conversely, one third of putative cases of vascular dementia display coincidental pathological features of AD (Querfurth and LaFerla, 2010). Additionally, individuals with familial EOAD mutations in *PSEN1*, Down Syndrome (DS) patients, and carriers of *APOE* ε4 allele have increased Aβ deposition in the cerebral vasculature (Yamada *et al*., 1997; Yamada, 2004; Belza and Urich, 1986; Chalmers *et al*., 2003). Furthermore, polymorphisms in *PSEN1* and *NEP* genes, which are important in the context of AD, have also shown an association with CAA (Yamada *et al*., 1997; 2003).

Neuronal loss in the nuclei responsible for the maintenance of different neurotransmitter systems results in a reduction in the levels of several major neurotransmitters, including acetylcholine (ACh) (Hardy *et al*., 1985; Cummings and Back, 1998). Several postmortem studies have reported a loss of cholinergic neurons in the basal forebrain (Cullen and Halliday, 1998; Geula *et al*., 2008; Lehericy *et al*., 1993; Vogels *et al*., 1990) and a decline in cortical choline-acetyltransferase (ChAT) and cholinesterase (ChE) activity (Gil-Bea *et al*., 2005; McGeer *et al*., 1984; Ruberg *et al*., 1990) in patients with AD compared to nondemented control subjects. Moreover, the reduction of ChAT activity in the cortex has been shown to correlate with the density of plaques (Perry *et al*., 1978) as well as with the loss of cholinergic neurons in AD (Joynt and Shoulson, 1985). The cholinergic system appears to play a role in memory and cognition. The loss of cholinergic neurotransmission in the cerebral cortex caused by degeneration of these nerve cells in the basal forebrain might be responsible for the significant decline in the cognitive abilities so common among subjects with AD (Bartus *et al*., 1982; Coyle *et al*., 1983). Thus, this has become the basis for a symptom-based treatment currently available for AD: inhibition of cholinesterase (ChE), the enzyme responsible for breaking down acetylcholine in the synapse. Today, three ChE inhibitors, donepezil, rivastigmine, and galantamine, are used to delay the onset of the symptoms, but so far no treatment exists, which could cure AD or even stop the disease progression.

Other than findings from histopathology of AD brain, there is also data emerging from experimental *in vivo* and *in vitro* studies that have been important in characterizing typical pathophysiological changes in AD. It has been described that long-term potentiation (LTP) shows a strong correlation with learning and memory as well as synaptic plasticity in different experimental *in vitro* and *in vivo* models (Haass and Selkoe, 2007). Several electrophysiological studies in APP-transgenic mice carrying human AD mutations have revealed significant deficits in basal synaptic transmission and/or LTP. These deficits were shown to occur in the hippocampus well before the appearance of any detectable Aβ

deposits. Additionally, the LTP deficit in older mice was associated with impaired performance in a spatial working memory task, but not with the loss of certain synaptic markers, suggesting that functional – not structural – synaptic changes were responsible for the cognitive deficits (Selkoe, 2002). There is now compelling evidence to suggest that the maintenance of hippocampal LTP is inhibited by synthetic human Aβ-derived diffusible ligands (ADDLs) (Lambert *et al.*, 1998) and soluble, low-number oligomers of naturally secreted human Aβ (Walsh *et al.*, 2002; Kamenetz *et al.*, 2003). Aβ oligomers impair synaptic plasticity by altering the balance between LTP and long-term depression (LTD) and by reducing the number of dendritic spines. Furthermore, excess build-up of Aβ and Aβ oligomers can induce neurotransmitter receptor internalization [such as N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors] and inhibition of voltage-gated calcium channels (VGCC), and nicotinic acetylcholine receptors (nAChR) (Querfurth and LaFerla, 2010).

2.1.3 Neuroimaging and CSF biomarkers

Recently, more emphasis has been placed on attempts to diagnose AD at an earlier phase, commonly referred to as mild cognitive impairment (MCI) (Petersen, 2004). In this phase, the patient experiences mild cognitive deficits, but exhibits no functional decline. One important challenge is to try to identify the disease at the pre-dementia stage and enhance the specificity of the clinical diagnosis by using imaging and other biomarkers. Additionally, from a therapeutic perspective, it is essential to reliably identify individuals as early as possible during the disease course: the earlier the intervention takes place, presumably, the better the chance to effectively intervene in the disease process.

Computed tomography (CT) and magnetic resonance imaging (MRI) techniques, among others, can be used to reveal areas of neuronal death and brain atrophy caused by the accumulation of plaques and tangles. CT or MRI are often used to detect intracranial lesions or diseases that might cause (e.g. tumour or hydrocephalus) or contribute (e.g. cerebrovascular disease) to dementia syndromes (Waldemar *et al.*, 2007). For example, semi-quantitative structural MRI has been successfully used to identify differences in medial temporal lobe atrophy between patients with AD and age-matched control individuals with sensitivity and specificity greater than 85%, (Waldemar *et al.*, 2007). There is some evidence that assessment of medial temporal atrophy could also be used to identify patients with MCI that will eventually progress to AD. However, differential diagnosis in medial temporal atrophy between AD and non-AD dementias is difficult, and the differences in brain structure may well be too small for diagnostic purposes in individual patients (Ballard *et al.*, 2011).

Novel quantitative techniques, such as volumetric imaging, three-dimensional mapping of the hippocampus, and cortical thickness measurement are promising markers in terms of AD diagnostics (Querbes *et al.*, 2009), and are being investigated as part of the AD Neuroimaging Initiative (ADNI) multicentre study (Ballard *et al.*, 2011). Radioisotopic scans can measure blood flow with single-photon emission CT (SPECT) to distinguish AD from frontotemporal dementia (FTD) (Ballard *et al.*, 2011). Various ligands have also been developed that visualise specific neurotransmitter systems with SPECT, which help to distinguish AD from dementia with Lewy bodies and Parkinson-type of dementia (McKeith *et al.*, 2007). PET with fluorodeoxyglucose (FDG-PET) measures glucose metabolism and has shown good accuracy in distinguishing patients with probable AD from both normal control individuals and patients with non-AD dementias. This imaging

method has been approved in the USA for diagnostic purposes and is sensitive and specific for detection of AD in its early stages (Ballard *et al.*, 2011). A reduction of glucose metabolism in bilateral temporal parietal regions and in the posterior cingulate cortex is the most commonly described diagnostic criterion for AD (Ballard *et al.*, 2011). One of the most promising novel imaging techniques is PET with Aβ ligands, such as ^{11}C-labelled Pittsburgh compound B (PiB) (Ikonomovic *et al.*, 2008; Klunk *et al.*, 2004) or other radioligands with longer half-lives (for example florbetaben and ^{18}F-AV-45) (Rowe *et al.*, 2008; Choi *et al.*, 2009). These can be used to directly visualise Aβ *in vivo* (Nordberg, 2004). There is evidence that PiB binding as assessed with PET is significantly associated with the amount of fibrillar Aβ measured *post-mortem* in most patients with AD. PiB binding also significantly correlates with CSF Aβ biomarkers (Svedberg *et al.*, 2009; Forsberg *et al.*, 2010). Despite the advancement in imaging techniques, clinical accuracy for patients with AD versus control individuals has shown wide variation between studies, and constant development of the technology is still needed in order to obtain reliable and sensitive diagnostic tools for AD.

Over the past two decades, it has become possible to obtain *in vivo* evidence of the specific neuropathology of AD by using validated and disease-specific biomarkers (Fagan *et al.*, 2006; Jagust *et al.*, 2009; Mintun *et al.*, 2006). These can be measured from the cerebrospinal fluid (CSF) or by different brain imaging techniques. The concept of using biomarkers for early diagnostic purposes has a long history, with many studies showing that AD biomarkers can be used to predict conversion from MCI to AD (Jack *et al.*, 2010). Laboratory and neuroimaging biomarkers display high correlation coefficients with the neuropathological lesions of AD (Buerger *et al.*, 2006; Clark *et al.*, 2003; Ikonomovic *et al.*, 2008). These biomarkers can be divided into pathophysiological and topographical markers (Table 3) (Dubois *et al.*, 2010).

Table 3. Categorization of the current, most-validated AD biomarkers.

	Pathophysiological markers	**Topographical markers**
Cerebrospinal fluid		
• $A\beta_{42}$	Yes	No
• Total tau, phosphorylated tau	Yes	No
PET		
• Amyloid tracer uptake	Yes	No
• Fluorodeoxyglucose	No	Yes
Structural MRI		
• Medial temporal atrophy	No	Yes

Adapted from Dubois et al., 2010.

Pathophysiological biomarkers reflect the two aetiological degenerative processes that characterize AD pathology: the amyloidosis path to generate NPs and the tauopathy path resulting in NFTs. They include reduced concentrations of Aβ, increased total tau (t-tau), and increased phosphorylated tau (p-tau) in the CSF (Buerger *et al.*, 2006; Clark *et al.*, 2003; Strozyk *et al.*, 2003; Tapiola *et al.*, 2009; Blennow *et al.*, 2010; Mattsson *et al.*, 2009), and

amyloid PET scanning with PiB or other radioligands (e.g. ^{18}F). CSF biomarkers (low Aβ and, specifically, an abnormal ratio of tau to Aβ) (Li *et al.*, 2007) are associated with very high rates of progression from amnestic MCI to AD dementia (Ewers *et al.*, 2007; Mattsson *et al.*, 2009). These markers also have shown a consistently high sensitivity and specificity in predictive models, such as assessing the value of CSF t-tau/Aβ1-42 ratio or CSF p-tau levels in prediction of MCI (Li *et al.*, 2007; Ewers *et al.*, 2007). High mean cortical binding values for PiB-PET have been predictive of cognitive decline and development of AD clinical signs in cognitively normal elderly individuals (Morris *et al.*, 2009; Resnick *et al.*, 2010). *In vivo* pathophysiological markers correlate very well with their respective neuropathological lesions. For example, CSF Aβ and PiB-PET correlate well with the numbers of senile plaques (Ikonomovic *et al.*, 2008; Strozyk *et al.*, 2003; Tapiola *et al.*, 2009; Blennow *et al.*, 2010), and t-tau and p-tau with NFTs (Buerger *et al.*, 2006; Clark *et al.*, 2003; Tapiola *et al.*, 2009; Blennow *et al.*, 2010).

Topographical markers are used to assess the less specific and downstream brain changes that correlate with the regional distribution of Alzheimer's pathology. These include medial temporal lobe atrophy (Teipel *et al.*, 2010; de Leon *et al.*, 2006; Hampel *et al.*, 2008) and reduced glucose metabolism in temporo-parietal regions detected by FDG-PET (Patwardhan *et al.*, 2004). These markers are valuable indicators because structural brain changes accurately map to the areas of the Braak stages of neurofibrillary tangle deposition (Whitwell *et al.*, 2008). MRI and PET markers have been consistently shown to predict the development of AD dementia in MCI cohorts (de Leon *et al.*, 2007; Morris *et al.*, 2009; Okello *et al.*, 2009; Wolk *et al.*, 2009) and to correlate with disease severity (Jack *et al.*, 2002; Nordberg *et al.*, 2010).

In 2004, The Clinical Core of the Alzheimer's Disease Neuroimaging Initiative (referred subsequently as ADNI 1) programme was launched as a collaboration between academic and industrial investigators. The goal of ADNI 1 was to clarify the relationships between demographic, genetic, clinical, cognitive, neuroimaging, and biochemical measures throughout the course of AD pathogenesis, and to facilitate the development of effective therapies. The project has delivered insights into disease mechanisms, and has provided guidance for drug development programs based primarily on the use of standardized biomarkers in longitudinal follow-up studies. Subsequently, ADNI-GO was established in 2010 focusing on imaging techniques and biomarkers in blood and CSF to identify AD at the pre-dementia stage. ADNI 2 is an extension of the original ADNI 1 study and ADNI-GO. ADNI 2 aims to understand the progression of AD and addresses the possible utilization of neuroimaging and other biomarkers at the stage of early clinical symptom presentation (eMCI).

ADNI and ADNI-GO are using neuroimaging and biomarker measurements to track the changes taking place in the brains of approximately 1300 elderly people. These individuals are either free of symptoms or have been diagnosed with early and/or late-stage mild cognitive impairment (eMCI and LMCI, respectively) and EOAD, a condition that may progress to AD. Based on these projects, a hypothetical model has been created to illustrate the power of biomarkers relative to progression of AD (Figure 2) (Aisen *et al.*, 2010). First, accumulation of Aβ42 is detected by molecular imaging techniques, such as PET scanning using an amyloid-specific ligand or by measuring CSF Aβ42 levels. The next step in the disease progression involves neuronal injury and dysfunction. This may be detected by FDG-PET as altered regional metabolic activity and as elevated levels of CSF t-tau, indicative of neuronal damage, and p-tau reflecting the worsening tangle pathology, and as MRI volumetric changes. When a threshold of neuronal dysfunction is reached, cognitive and then functional manifestations of AD appear with increasing frequency. It is believed

that early intervention is preferable compared to treating individuals only after the full-blown dementia syndrome is present (Aisen *et al.*, 2010; Jack *et al.*, 2010). Figure 2 shows that amyloid biomarker abnormalities are already present during the asymptomatic stage. Ideally, one would hope to intervene at this point to have the greatest preventive effect on subsequent neuronal damage.

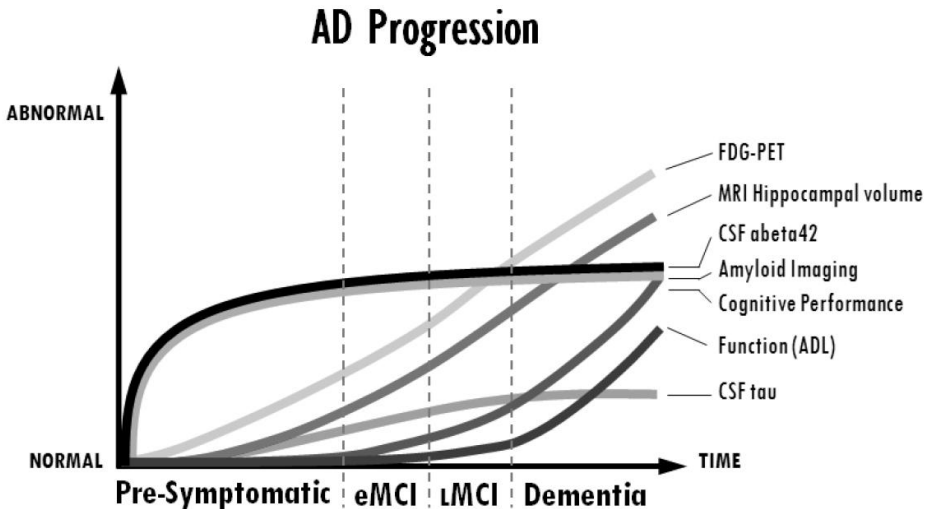

Figure 2. Different biomarkers during the progression of AD. This hypothetical graph is designed to illustrate the following points: (1) CSF Aβ42 and amyloid PET, reflecting amyloid accumulation in brain, move in tandem. (2) Amyloid accumulation precedes cognitive and functional decline by years, and changes only gradually after symptoms develop. (3) CSF tau, MRI volumes, and FDG-PET are more dynamic biomarkers of disease progression across the spectrum of AD pathogenesis than CSF Aβ42 and amyloid PET. (4) Cognitive decline becomes evident at the onset of eMCI, and accelerates as the disease progresses. (5) Functional decline becomes evident at the onset of dementia, and accelerates as the disease progresses. (6) All of these points are conjectural to varying degrees and they require confirmation by long-term longitudinal follow-up in ADNI 2 and beyond. LMCI, late stage of MCI; eMCI, early stage of MCI. (Adapted from Aisen et al., 2010)

2.2 MOLECULAR MECHANISMS IN ALZHEIMER'S DISEASE PATHOGENESIS

2.2.1 APP and Aβ processing

2.2.1.1 APP

AD is pathologically characterized by the deposition of amyloid in the central nervous system as neuritic plaques and in the cerebral vasculature. Glenner and Wong first identified the major protein component of vascular amyloid, which was a low-molecular-weight, 4-kDa polypeptide, now referred to as β-amyloid (Aβ) (Glenner and Wong, 1984).

Subsequent studies established that the same protein was the major component of amyloid plaques (Masters *et al.*, 1985). The complete amino acid sequence of Aβ led to the identification of its precursor, the amyloid precursor protein, APP (Kang *et al.*, 1987). Despite extensive efforts toward understanding the function of APP, its physiological role(s) still remains poorly defined. A physiological role for APP has been suggested in neurite outgrowth and synaptogenesis, neuronal protein trafficking along the axon, transmembrane signal transduction, cell adhesion, calcium metabolism, cell migration and synapse remodeling (Zheng and Koo, 2006).

The human *APP* gene is a gene with 19 exons which is located on the long arm of chromosome 21 (Kang *et al.*, 1987; Goldgaber *et al.*, 1987; Tanzi *et al.*, 1987; Yoshikai *et al.*, 1991). It is a member of a larger gene family, the amyloid precursor-like proteins (APLPs) (Slunt *et al.*, 1994), which display substantial homology, both within the large ectodomain and particularly within the cytoplasmic tail, but are largely divergent in the Aβ region. APP comprises a heterogeneous group of ubiquitously expressed polypeptides in all mammals (Selkoe *et al.*, 1988). This heterogeneity arises first of all from alternative splicing of the *APP* gene, which yields three major isoforms of 695, 751, and 770 amino acid residues (APP770 is illustrated in Figure 3). Moreover, APP can be modified by a variety of post-translational modifications, including the addition of N- and O-linked sugars (glucosylation), sulfation, and phosphorylation (Oltersdorf *et al.*, 1990; Walter *et al.*, 1997; Weidemann *et al.*, 1989). The APP splice forms containing 751 or 770 amino acids are widely expressed in non-neuronal cells throughout the body and also occur in neurons (Tanaka *et al.*, 1989). However, neurons predominantly express the 695-residue isoform, which occurs at very low abundance in nonneuronal cells (Haass *et al.*, 1991). The difference between the 751/ 770- and 695-residue isoforms is due to the presence of an exon that encodes a 56-amino acid motif homologous to the Kunitz-type of serine protease inhibitors (KPI) specifically in the longer isoform (Figure 3) (Tanzi *et al.*, 1988), suggesting one potential function for the longer APP isoforms (Selkoe, 2001). According to the Alzheimer Disease & Frontotemporal Dementia Mutation Database (http://www.molgen.ua.ac.be/ADMutations; retrieved on July 1st, 2011), 32 pathogenic mutations have been identified within the *APP* gene from 89 EOAD families. The effects of many of these mutations on APP processing have been assessed in subsequent functional studies (Theuns *et al.*, 2006).

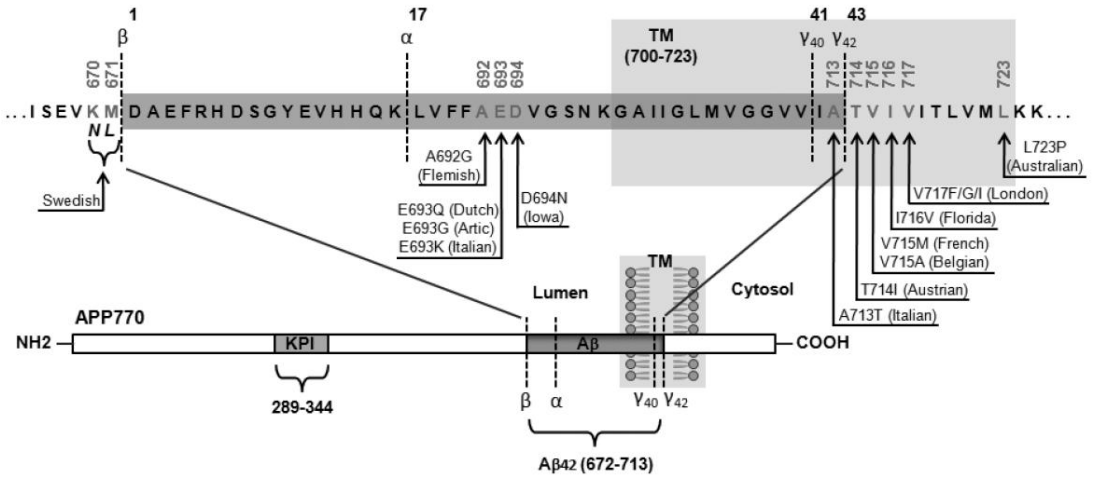

Figure 3. Schematic representation of the domain structure of APP770 isoform and APP mutations genetically linked to familial AD or related disorders. APP is a type I membrane protein with an extracellular N-terminus and cytosolic C-terminus. Cleavage at positions 672 and 713 by β- and γ-secretases, respectively, generates Aβ peptide. The sequence within APP that contains the Aβ and transmembrane region is expanded and shown by the single-letter amino acid code. The residues highlighted in grey represent the Aβ1–42 peptide (amino acids 672-713). The area shaded in light grey indicates the location of the transmembrane domain (TM). The bold letters in light gray designated with arrows highlight the currently known missense mutations identified in certain patients with FAD and/or hereditary cerebral hemorrhage with amyloidosis. Three-digit numbers above these letters refer to the residue number according to the APP770 isoform. The black numbers above the figure (1, 17, 41 and 43) indicate amino acids of Aβ domain at the corresponding secretase (β-, α-, and γ40/γ42) cleavage sites. In some APP isoforms, such as APP770, there is an alternatively spliced exon that is homologous to the Kunitz-type serine protease inhibitor (KPI) family (grey box) located between amino acids 289-344.

APP is a single-pass, type I cell membrane glycoprotein which has a large ectodomain containing the N-terminus and a small cytoplasmic domain containing the C-terminus (Figure 3) (Lazarov *et al.*, 2005). During maturation, the nascent immature APP (N-glycosylated in the endoplasmic reticulum) moves to the Golgi complex through the constitutive secretory pathway where complete maturation occurs (O-glycosylated and sulfated) (Thinakaran and Koo, 2008). The majority of APP localizes in the Golgi and TGN or is degraded in the endoplasmic reticulum, and only a small fraction (~10%) enters the secretory pathway (Kuentzel *et al.*, 1993; Citron, 2000). Once APP reaches the cell surface, it may be cleaved to sAPP or become rapidly reinternalized via clathrin-coated pits to endosomes and lysosomes (Small and Gandy, 2006; Koo *et al.*, 1996). Amyloidogenic processing of APP yielding the Aβ peptides mostly takes place in these subcellular compartments. Some APP accumulates in the secretory transport vesicles leaving the late Golgi compartment and returns to the cell surface (Huse *et al.*, 2000). In non-neuronal cells, APP is internalized within minutes of its arrival at the cell surface due to the presence of the YENPTY internalization motif near the C-terminus of APP (residues 682–687 of the APP695 isoform). Subcellular sorting and targeting of APP upon endocytosis appears to be critical in Aβ production and AD etiology. The retrieval of APP from endosomes to TGN (Figure 4,

pathway no. 3) was recently found to involve the retromer complex and intermediate clathrin-coated endocytic vesicles that carry the early endosomal Rab GTPase 5 (Rab5) marker (Vieira *et al.*, 2010). It was shown that APP phosphorylation at residue S655 within the APP 653YTSI656 basolateral motif enhances APP retrieval via the retromer-mediated process. Additionally, the experimental phosphomimetic APP S655E mutant displayed decreased APP lysosomal targeting, enhanced half-life, and a decreased tendency towards Aβ production. Downregulation of VPS35, a central component of the retromer complex, impairs the phosphorylation dependent APP retrieval to the TGN, and decreases APP half-life (Vieira *et al.*, 2010). The pathways of APP trafficking are depicted in Figure 4.

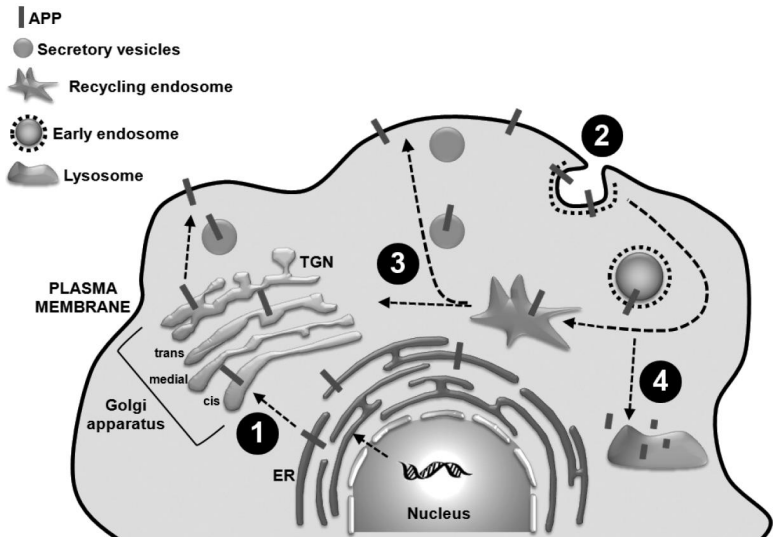

Figure 4. Intracellular trafficking of APP. Nascent APP molecules (grey bars) mature on the constitutive secretory pathway (1). Once APP reaches the cell surface, it is rapidly internalized (2) and subsequently trafficked through endocytic and recycling compartments back to the cell surface (3) or degraded in the lysosome (4). Some APP molecules also undergo retromer-mediated recycling from the endosomes back to the TGN. Non-amyloidogenic processing occurs mainly at the cell surface, where α-secretases are present. Amyloidogenic processing involves transit through the endocytic organelles, where APP encounters β-and γ-secretases. (Adapted from Thinakaran and Koo, JBC, 2008)

APP can be processed at the cell surface by α-secretase-mediated cleavage, resulting in the shedding of the sAPPα ectodomain (Sisodia, 1992). Activation of protein kinase C (PKC) increases sAPPα secretion by mechanisms involving the formation and release of secretory vesicles from the TGN, thus enhancing APP (and possibly α-secretase) trafficking to the cell surface (Thinakaran and Koo, 2008). Alternatively, the APP ectodomain may be shed at a different site by BACE1. This initiates the amyloidogenic processing pathway and leads to Aβ release by γ-secretase-mediated cleavage. BACE1 is predominantly localized in the late Golgi/TGN and endosomes, consistent with the amyloidogenic cleavage of wild-type APP during endocytic/recycling steps (Koo and Squazzo, 1994; Vassar, 2009). The γ-secretase complex is present and enzymatically active in multiple compartments, including the ER,

ER-Golgi intermediate compartment, Golgi, TGN, endosomes, and plasma membrane. Studies conducted in non-neuronal and neuroblastoma cell lines have revealed that Aβ is generated mainly in the TGN and endosomes as APP is trafficked through the secretory and recycling pathways (Figure 4) (Small and Gandy, 2006). Evidence emerging from a number of studies also suggests that amyloidogenic processing of APP occurs in cholesterol- and sphingolipid-enriched membrane raft microdomains of intracellular organelles (Riddell *et al.*, 2001; Ehehalt *et al.*, 2003; Vetrivel *et al.*, 2004, 2005).

2.2.1.2 Aβ generation, degradation, and clearance

In the context of AD, the metabolism of APP occurs via a complex process involving the activity of three enzymes termed α-, β- and γ-secretases (LaFerla *et al.*, 2007). The secretases cleave only a small portion of the total pool of APP, leaving the majority as intact full-length APP (De Strooper and Annaert, 2000). Intact APP has been postulated to mediate neuronal and synaptic functions, such as neurite outgrowth and synaptogenesis, neuronal protein trafficking along the axon, cell migration, and synapse remodeling (Zheng and Koo, 2006). In the non-amyloidogenic pathway, APP is cleaved protelytically at its extracellular domain by the α-secretase at a site 83 amino acids from the carboxy (C) terminus, producing a large amino (N)-terminal ectodomain (sAPPα), which is secreted into the extracellular milieu, having a 83-amino-acid long C-terminal fragment, C83 (Allinson *et al.*, 2003; LaFerla *et al.*, 2007) (Figure 5). C83 is subsequently cleaved by the γ-secretase, producing a short 3-kDa fragment p3 and APP intracellular domain (AICD) (Haass *et al.*, 1993; De Strooper and Annaert, 2000). Importantly, cleavage by the α-secretase occurs within the Aβ region, thereby precluding formation of Aβ.

The amyloidogenic pathway is an alternative cleavage pathway for APP, which leads to Aβ generation. The initial proteolysis is mediated by the β-secretase or BACE1 at a position located 99 amino acids from the C-terminus. BACE1 initiates Aβ generation by cleaving APP at the Asp^{+1} residue of Aβ N-terminus (Figure 5) (Vassar, 2004). This cut results in the release of sAPPβ into the extracellular space, and leaves the 99-amino-acid C-terminal stub (C99, or β-C terminal fragment, β-CTF) within the membrane, with the newly generated N-terminus corresponding to the first amino acid of Aβ (Haass *et al.*, 1993). In addition, BACE1 can also cleave within the Aβ domain between Tyr^{10} and Glu^{11} (β′-cleavage site) producing the C89 C-terminal fragment. Subsequent cleavage of C99 (between residues 38 and 43) by the γ-secretase liberates an intact Aβ1–x peptide and AICD (Golde *et al.*, 1992). In contrast, γ-secretase cleavage of C89 would result in the production of Aβ11–x. Most of the full-length Aβ peptide produced is 40 residues in length (Aβ40), whereas a small proportion (~10%) is the 42-residue variant (Aβ42). The Aβ42 isoform is more hydrophobic and more prone to fibril formation than Aβ40 (Jarrett *et al.*, 1993), and it is also the form that is predominantly found in cerebral plaques (Younkin, 1998). AICD, in combination with Fe65, is delivered to the nucleus by retrograde transport (Goodger *et al.*, 2009), where it binds to histone acetyltransferase Tip60. This complex can facilitate transcription of specific genes, e.g. *NEP* (Belyaev *et al.*, 2009), *APP*, *BACE*, and *GSK3β* (Konietzko *et al.*, 2010; von Rotz *et al.*, 2004) genes (Figure 5). The amyloidogenic processing involves APP transit through the endocytic organelles, in which APP encounters the β- and γ-secretases (Small and Gandy, 2006).

It depends on the cell type which pathway – amyloidogenic or non-amyloidogenic – is preferentially utilized. Amyloidogenic processing is the favoured pathway of APP metabolism in neurons, largely due to the greater abundance of BACE1, whereas the non-

amyloidogenic pathway is predominant in all other cell types (Thinakaran and Koo, 2007). Non-amyloidogenic processing of APP occurs mainly in the late Golgi compartment or in caveolae (plasma membrane invaginations) (Ikezu *et al.*, 1998). It destroys the Aβ sequence and thus prevents Aβ formation. Commitment of these pathways can be differentially modulated by the activation of cell-surface receptors such as the serotonin 5-hydroxytryptamine (5-HT_4) receptor, metabotrobic glutamate receptors, glutamate receptors, muscarinic acetylcholine receptors, and the platelet-derived growth factor receptor (Thinakaran and Koo, 2007). In addition, in many instances, an increase in non-amyloidogenic APP metabolism is coupled to a reciprocal decrease in the amyloidogenic processing pathway, and vice-versa, as the α- and β-secretase moieties compete for APP substrate (Cole and Vassar, 2007).

Aβ42 is more neurotoxic as a result of its higher hydrophobicity, which causes more rapid oligomerization and aggregation (Blennow, 2006). Therefore, Aβ42 is essential for the initiation of AD pathogenesis. Thus far, various longer Aβ species, such as Aβ43, have been qualitatively described in the brains of individuals with Alzheimer's disease (Miravalle *et al.*, 2005). Further quantitative studies have revealed that Aβ43 is deposited more frequently than Aβ40 in both LOAD and EOAD (Iizuka *et al.*, 1995; Parvathy *et al.*, 2001; Welander *et al.*, 2009).

Previous studies (Qi-Takahara *et al.*, 2005**;** Takami *et al.*, 2009) have demonstrated that it is the positions of γ-secretase cleavage that control which Aβ forms are produced. Aβ43, generated from Aβ49 via Aβ46, is subsequently converted to Aβ40, whereas Aβ42 is independently generated from Aβ48 via Aβ45. It has also been reported that Aβ43 appears to be as prone to aggregate *in vitro* as Aβ42 (Jarrett *et al.*, 1993), leading to faster formation of oligomers than with Aβ40 (Bitan *et al.*, 2003). Recently, it was reported that Aβ43 could contribute to AD pathology in an *in vivo* experiment with knock-in mice containing a pathogenic PS1 R278I mutation that caused overproduction of Aβ43. This led to short-term memory loss before plaque formation and considerably accelerated amyloid pathology in these mice (Saito *et al.*, 2011).

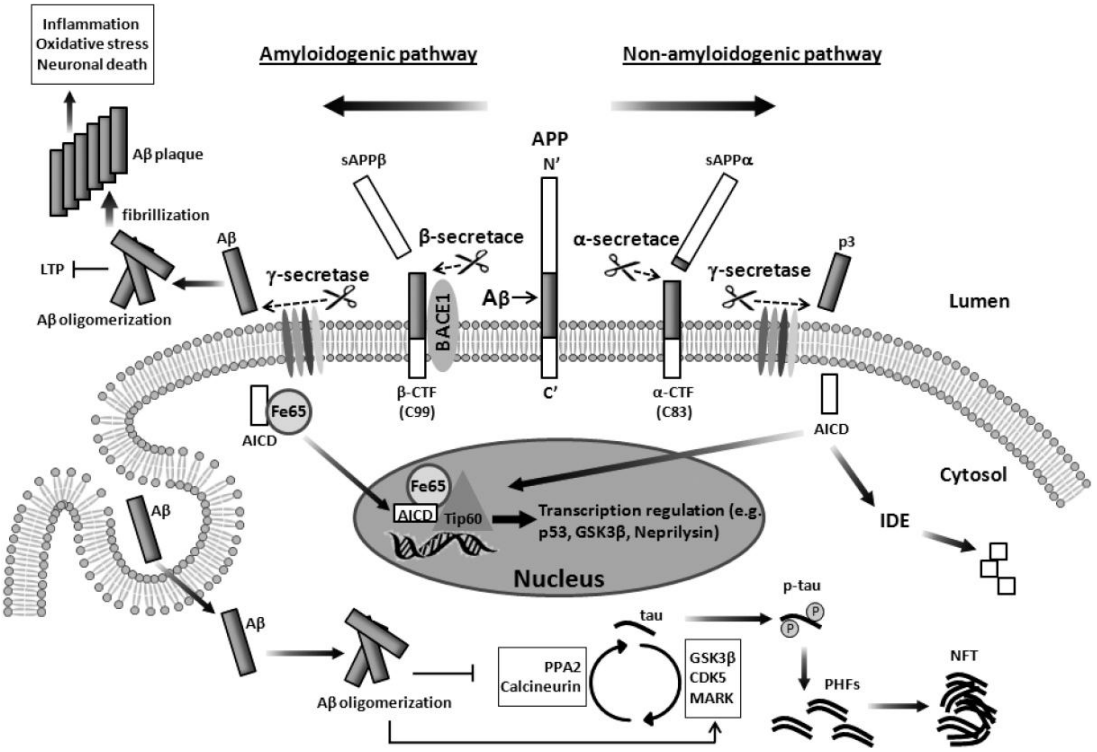

Figure 5. Schematic representation of the processing of APP through amyloidogenic (left) and non-amyloidogenic (right) pathways. Non-amyloidogenic processing of APP refers to sequential processing of APP by membrane-bound α- and γ-secretases. α-Secretases (ADAM9, ADAM10 and ADAM17; LaFerla et al., 2007) cleave within the Aβ-domain, thus precluding the generation of intact Aβ peptide. After α-secretase cleavage, sAPPα is released, leaving C83 on the membrane. Subsequent γ-secretase cleavage generates extracellular p3 fragment and APP intracellular domain (AICD). AICD, in combination with Fe65, is delivered to the nucleus by retrograde transport, where it binds to histone acetyltransferase Tip60. This complex can facilitate gene transcription e.g. of the NEP, APP, BACE, and GSK3β genes. Amyloidogenic processing of APP is carried out by sequential action of membrane-bound β- (BACE1) and γ-secretases releasing sAPPβ and generating C99. β-Secretase-mediated cleavage occurs mainly in acidic intracellular compartments, such as in endosomes (not shown in the figure) (Haass et al., 1993). Extracellular Aβ forms oligomers known to inhibit LTP, a phenomenon indicating synaptic strengthening. Oligomers can further aggregate to form protofibrillis and Aβ plaques. Aβ oligomers and plaques are synaptotoxic and induce inflammation and neuronal death. Aβ monomers may be internalized, where cytotoxic oligomers are also formed. In functional neurons, Aβ, Aβ oligomers, and AICD are degraded by IDE and NEP. The intraneuronal presence of Aβ oligomers has many adverse consequences, including the facilitation of tau pathology. Tau is abnormally hyperphosphorylated, leading to the formation of paired helical filaments (PHFs) and neurofibrillary tangles (NFT). Numerous kinases and phosphatases have been implicated in the regulation of tau hyperphosphorylation, including GSK3β, CDK5, MARK, and PP2A (Iqbal et al., 2005).

Aβ degradation

The accumulation of Aβ is thought to be central to the pathogenesis of AD (Hardy, 1997). Consequently, factors involved in modulating Aβ levels, and in particular Aβ42, have been most intensely studied during the last decade. The majority of these studies have focused on processes involved in Aβ production, particularly on the activities of β-, and γ-secretase, which cleave APP to liberate Aβ. However, the mechanism of Aβ accumulation in the case of LOAD is less clear. There is evidence that degradation and clearance of Aβ might serve an equally, if not even more, important role in Aβ accumulation (Mawuenyega *et al.*, 2010). Several candidate Aβ peptidases that enzymatically degrade Aβ are expressed both neuronally and within the cerebral vasculature.

The two major endopeptidases involved in Aβ degradation are zinc metalloendopeptidases, referred to as insulin-degrading enzyme (IDE) or insulysin (Selkoe, 2001), and neprilysin (NEP). IDE is a 110-kDa enzyme that hydrolyzes several regulatory peptides (Duckworth *et al.*, 1998), including insulin, glucagon, atrial natriuretic factor, transforming growth factor α, β-endorphin, amylin, Aβ, and AICD. Most of the IDE is localized in the cytosol, but unlike NEP, only a small fraction resides in the plasma membrane. Farris *et al.* demonstrated that IDE knockout mice had increased endogenous levels of Aβ and AICD in the brain (Farris *et al.*, 2003). Additionally, heterozygous $IDE^{+/-}$ mice exhibited Aβ levels that are intermediate between wild-type controls and homozygous $IDE^{-/-}$ mice. It also has been shown that primary neuronal cultures derived from these mice were less efficient at degrading radiolabeled Aβ *in vitro*. In another study, chronic transgenic overexpression of IDE (2-fold compared to endogeneous levels) decreased both soluble and insoluble AβX-40 and AβX-42 levels by 50% in mice overexpressing human APP717 and APP670/671 missense mutations (Leissring *et al.*, 2003). Conversely, a 50% reduction in plaque burden was observed in these APP/IDE double transgenic mice, and there was a significant reduction in the rate of premature death as compared to the situation in age-matched APP transgenic littermates.

The other major peptidase responsible for the degradation of Aβ is NEP. This is a type II membrane protein and, like IDE, hydrolyzes circulating biologically active peptides, including enkephalin, cholecystokinin, neuropeptide Y, and substance P (Turner *et al.*, 2001). NEP enzyme functions mostly in the brain where its localization on the plasma membrane with its catalytic site exposed extracellularly makes this peptidase a prime candidate for peptide degradation at extracellular sites of amyloid accumulation i.e., Aβ-containing diffuse deposits and neuritic plaques (Miners *et al.*, 2008). A recent study noted that NEP was able to decrease ipsilateral cortical amyloid deposits by 50% when human NEP was injected intracerebrally via a lentiviral vector in a transgenic mouse model of cerebral amyloidosis (Marr *et al.*, 2003).

There are other potential metalloendopeptidase candidates, such as endothelin-converting enzyme or ECE, as well as properly positioned circulating serine proteases, such as urokinase-type plasminogen activator (uPA), tissue plasminogen activator (tPA), and plasmin, which have been suggested to be able to degrade Aβ. The latter three – uPA, tPA, and plasmin – are anticipated to have a more significant impact on cerebrovascular amyloid deposits or congophilic amyloid angiopathy (CAA) (Selkoe, 2001). Moreover, the proteasome may play a key role in degrading intracellularly localized Aβ, for example, as part of the process of endoplasmic reticulum (ER)-associated proteolytic degradation (Schmitz *et al.*, 2004). One potential molecule in Aβ proteolysis is transthyretin (TTR), which has been shown to bind to Aβ in the CSF and thus decrease Aβ aggregation (Schwarzman *et al.*, 1994). Aβ cleavage by TTR was inhibited in the presence of sAPPα peptide containing

the KPI domain but not in the presence of sAPPα derived from the APP isoform 695 without the KPI domain. TTR is able to proteolytically cleave Aβ at multiple sites (Costa *et al.*, 2008) and its over-expression was shown to protect APP23 AD mice from Aβ toxicity and also from the subsequent behavioral impairment (Buxbaum *et al.*, 2008). Overall, the literature emphasizes the importance of the major Aβ-degrading enzymes, IDE and NEP, *in vivo* and one could postulate that even moderate increases in IDE activity could lead to dramatic changes in steady-state Aβ levels as well as in the overall amyloid plaque burden. Further support for the role of IDE in AD pathogenesis stems from reports of its genetic association with LOAD (Tanzi *et al.*, 2004).

Aβ clearance

There is substantial evidence showing that the low-density lipoprotein receptor-related protein (LRP) and the receptor for advanced glycation end products (RAGE) are involved in receptor-mediated flux of Aβ across the blood-brain barrier (BBB) (Zlokovic, 2004). Both LRP and RAGE are cell surface receptors. LRP mediates the efflux of Aβ from the brain to the periphery, whereas RAGE is responsible for Aβ influx back into the CNS. Additionally, there is some evidence to suggest that 10 to 15% of Aβ can pass into the CSF from the brain interstitial fluid and onward into the bloodstream (Shibata *et al.*, 2000).

LRP is a lipoprotein receptor with multifunctional signaling and scavenging properties on the capillary endothelium. It can bind a variety of ligands, such as APP, APOE, α-2-macroglobulin (α2M), and Aβ (Herz, 2003). LRP antagonists have been shown to specifically reduce the efflux of Aβ from brain by up to 90% (Shibata *et al.*, 2000) in mice injected with radiolabeled Aβ40. In another study, a 2-fold increase in cerebral amyloid load was observed in AD animal models expressing low levels of LRP at the BBB (Van Uden *et al.*, 2002). In the process of LRP-mediated clearance, Aβ can be transported across the BBB and exported out of the brain into the bloodstream by direct binding to LRP (Deane *et al.*, 2004) or by first forming a complex with the LRP ligands, α2M or apoE on the abluminal (=directed away from the lumen of a tubular structure) side of the endothelium. The latter Aβ/chaperone complex then binds to LRP and becomes internalized. LRP-ligand complexes are then internalized to late endosomes, after which they can either be delivered to lysosomes for subsequent degradation or be targeted for transcytosis across the BBB into the plasma (Herz, 2003). On the other hand, fibrillogenic forms of Aβ, which accumulate in AD brain, may be difficult to remove from the CNS via LRP, because LRP favors the clearance of Aβ40 over the more detrimental Aβ42 (Deane *et al.*, 2004). The same study also showed that Aβ can promote the proteasome-dependent degradation of LRP, which may explain the relatively low LRP activity in brain microvessels in AD patients and mutant APP mouse models of β-amyloidosis (Bertram and Tanzi, 2004).

Once Aβ enters the bloodstream, it can gain re-entry to brain via RAGE, which is the receptor most probably responsible for transport of Aβ through the BBB (Zlokovic, 2004). RAGE is a multifunctional receptor belonging to the immunoglobulin superfamily. Like LRP, it can bind a large array of different ligands (Yan *et al.*, 2000), one of which is Aβ. RAGE has been implicated in the regulation of neurite outgrowth and cell migration (Huttunen *et al.*, 2002, Srikrishna *et al.*, 2002). In animal studies, it has been shown that down-regulation of RAGE inhibits the influx of Aβ from the periphery into the CNS (Deane *et al.*, 2003). Furthermore, the binding of Aβ to RAGE is able to stimulate cellular responses that are potentially pathological, including expression of proinflammatory cytokines and decreased cerebral blood flow (Zlokovic, 2004). gp330/Megalin is another protein that has been reported to transport circulating plasma Aβ back into the brain in a complex with

APOJ/clusterin (Zlokovic *et al.*, 1996). However, since gp330/megalin is normally saturated by abundant amounts of plasma APOJ/clusterin under physiological conditions, RAGE is the most probable receptor responsible for the transport of Aβ back into the brain (Zlokovic, 2004).

2.2.2 Secretases processing APP

2.2.2.1 α- and γ-secretase

Several zinc metalloproteinases, such as TACE (TNFα converting enzyme)/ADAM17 (a disintegrin and metalloprotease 17), ADAM9, ADAM10 and MDC-9 [metalloprotease/disintegrin/cysteine-rich protein, also known as meltrin γ (Koike *et al.*, 1999)], and the aspartyl protease BACE2 can cleave APP at the α-secretase cleavage site within the Aβ domain between Lys^{16} and Leu^{17} (Koike *et al.*, 1999; Lammich *et al.*, 1999; Slack *et al.*, 2001). The cleavage at this site precludes the generation of Aβ (Allinson *et al.*, 2003). α-Secretase cleavage generates sAPPα, which has neurotrophic and neuroprotective properties (Furukawa *et al.*, 1996; Meziane *et al.*, 1998; Stein *et al.*, 2004; Hiltunen *et al.*, 2009). A recent *in vitro* study using a new, sAPPα-specific antibody and two different cell lines as well as primary neurons expressing endogenous APP, strongly suggests ADAM10, but not ADAM9 or ADAM17, is the main α-secretase for constitutive α-cleavage in neurons (Kuhn *et al.*, 2010).

γ-Secretase is composed of four essential subunits: presenilin-1 or -2 (PSEN1 and PSEN2), nicastrin, anterior pharynx-defective 1 (Aph-1), and presenilin enhancer (PEN-2) (Figure 6) (Iwatsubo *et al.*, 2004; Kaether *et al.*, 2006). γ-Secretase attacks at multiple sites within the transmembrane domain of APP, generating Aβpeptides of different lengths and releasing AICD (Selkoe and Wolfe, 2007). Nearly 90% of secreted Aβ ends in residue 40, whereas Aβ42 accounts for approximately 10% of the secreted Aβ (Thinakaran and Koo, 2008). Moreover, minor amounts of longer (such as Aβ43) as well as shorter (e.g. Aβ38 and Aβ37) Aβ peptides are also generated. Interestingly, familial AD-linked mutations in *PSEN1* and *PSEN2* genes influence the specificity of the γ-secretase cleavage site by favoring breakdown at position 42 relative to that at position 40, thus increasing the Aβ42/40 ratio (Selkoe and Wolfe, 2007). In addition to APP, γ-secretase has been shown to metabolize a large number of other proteins as well. This suggests that in addition to its pathophysiological role in AD, γ-secretase plays an important role in the normal physiological signaling of a variety of proteins (Haapasalo and Kovacs, 2011).

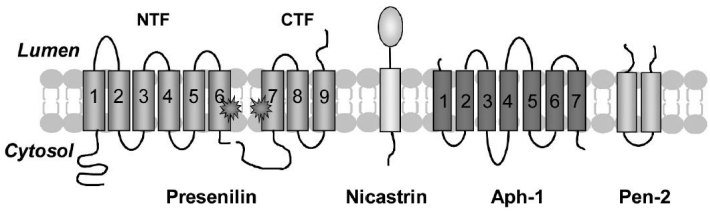

Figure 6. Schematic representation of membrane bound γ-secretase complex illustrating its essential components Presenilin, Nicastrin, Aph-1 and Pen-2. Presenilin contains 9 transmembrane domains (TMDs; numbered) and the catalytic aspartic acid residues are marked with stars in TMDs 6 and 7. N- and C-terminal fragments (NTFs and CTFs, respectively) of presenilin are formed after proteolysis at the large cytoplasmic loop. Aph-1, anterior pharynx-defective 1; Pen-2, presenilin enhancer 2. (Courtesy of A. Haapasalo).

2.2.2.2 BACE1

BACE1 (β-site APP-cleaving enzyme 1), also known as Asp2 (novel aspartic protease 2) or memapsin2 (membrane aspartic protease/pepsin 2), is a transmembrane aspartyl protease and the major neuronal β-secretase. BACE1 mediates the first critical rate-limiting step in Aβ formation by creating the free N-terminus of Aβ (Vassar *et al.*, 1999; Vassar, 2002, 2009; Citron, 2000). The BACE1 cleavage site is located at the N-terminus of Aβ at Asp^{1}. BACE1-mediated cleavage generates a secreted N-terminal fragment, sAPPβ, and a membrane-bound Aβ-containing C-terminal fragment, CTF-β (also referred to as C89 and C99). sAPPβ seems to have a proapoptotic function (Nikolaev *et al.*, 2009). Generation of the N-terminus is followed by a C-terminal cleavage of the remaining CTF-β by γ-secretase to release the final Aβ-product. In addition, BACE1 can also cleave within the Aβ domain between Tyr^{10} and Glu^{11} (β'-cleavage site). *BACE1* gene is located in chromosome 11 (Vassar, 2002).

BACE was independently discovered by five different laboratories approximately ten years ago (Vassar *et al.*, 1999; Yan *et al.*, 1999; Sinha *et al.*, 1999; Hussain *et al.*, 1999; Lin *et al.*, 2000). There are two major forms of the enzyme, BACE1 (501 amino acids) and BACE2 (518 amino acids). Their coding regions are 45% identical (Yan *et al.*, 1999) and coding sequences 75% homologous, but they show no similarity in their promoter regions (Sun *et al.*, 2005). In another study using the GrowTree algorithm comparison (Wisconsin Sequence Analysis Package), BACE1 was shown to share 64% amino acid similarity with BACE2 (Bennett *et al.*, 2000). BACE2 interestingly maps in the long arm of chromosome 21 in the obligatory Down syndrome region (Saunders *et al.*, 1999). It also cleaves APP, but BACE1 has a much greater preference for APP than BACE2 (Farzan, *et al.*, 2000; Hussain *et al.*, 2000). Two aspartic protease active site motifs in the sequence DTGS (residues 93-96) and DSGT (residues 289-292) are present in BACE1, and mutation of either aspartic acid renders the enzyme inactive (Figure 7) (Hussain *et al.*, 1999; Bennett *et al.*, 2000; Vassar, 2009). BACE1 has an N-terminal signal peptide sequence (SP) (residues 1-21) and a propeptide domain (Pro) (residues 22-45) that are removed post-translationally, with the mature enzyme beginning at residue Glu46. Importantly, BACE1 has a single transmembrane domain (TM) near to its C-terminus (residues 455-480) (Vassar et al., 2009). Thus, BACE1 is a type I membrane protein with a lumenal active site, which are features predicted for the β-secretase. The position of the BACE1 active site within the lumen of intracellular compartments suggests that BACE1 possesses the correct topological orientation to undertake cleavage of APP at the β-secretase

site. As observed with other aspartic acid proteases, BACE1 has six luminal cysteine residues that form three intramolecular disulfide bonds and several N-linked glycosylation sites (Haniu *et al.*, 2000).

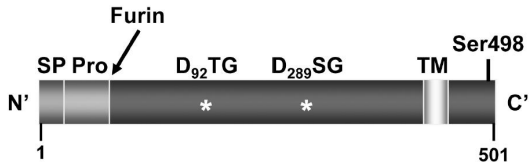

Figure 7. Structure of the 501 amino acid long BACE1 protein showing the signal peptide (SP), propeptide domain (Pro), and active site motifs D_{92}TG and D_{289}SG (asterisks). Furin is a major BACE1 proprotein convertase. The arrow indicates the N-terminal position (Glu46) where the mature BACE1 is cleaved in the Golgi apparatus before targeting via the secretory pathway to the plasma membrane. BACE1 is phosphorylated by casein kinase 1 on Serine 498 (Ser498), and this phosphorylation together with a C-terminal dileucine motif (adjacent to Ser498) regulates BACE1 recycling between the cell surface and endosomal compartments (Huse et al., 2000; Walter et al., 2001). The numbers below indicate the first and the last amino acid of BACE1. C'/N', C-/N-terminal domain; TM, transmembrane domain.

BACE1 primarily localizes in the Golgi apparatus and late endosomes, which is consistent with its optimal activity at low pH (Vassar *et al.*, 1999). BACE1 is highly expressed in brain and pancreas, but is also found in other organs although at low levels (Vassar *et al.*, 1999; Marcinkiewicz and Seidah, 2000). In contrast, BACE2 is present at varying levels in most peripheral tissues with the highest levels being found in the kidney (Bennett *et al.*, 2000). Within the brain, BACE2 is believed to reside mostly in astrocytes, whereas BACE1 is largely neuronal (Basi *et al.*, 2003; Irizarry *et al.*, 2001). BACE2 cleaves APP between Phe^{19} and Phe^{20} (Cole and Vassar, 2007) which abolishes the Aβ production. A recent study has indicated that BACE2 has a role in insulin receptor trafficking, which is a critical step in insulin signaling and sensitivity. Therefore BACE2 might function as a sensor in the plasma membrane to signal mobilization of the insulin receptor β-subunit (IRβ) (Casas *et al.*, 2010).

BACE1 has its maximal enzymatic activity at acidic pH, since agents that elevate intracellular pH also inhibit β-secretase activity (Haass *et al.*, 1993). Moreover, β-secretase activity is highest in the acidic subcellular compartments of the secretory pathway, including the Golgi apparatus and endosomes (Vassar, 2002). These data suggest that the active site of β-secretase is located within the lumen of acidic intracellular compartments. Site-directed mutagenesis of the amino acids surrounding the cleavage site in APP has demonstrated the sequence specificity of the β-secretase (Citron *et al.*, 1995). Substitutions with a large hydrophobic amino acid (such as Leu found in the Swedish FAD mutation) at the Met residue at P1 site before the BACE1 cleavage site in APP improve the efficiency of the β-secretase-mediated cleavage (Figure 5). Conversely, substitution with a small hydrophobic amino acid, Val, at the same position inhibits the cleavage. Many other substitutions at this site or at surrounding positions decrease cleavage activity evidence that the β-secretase cleavage is highly sequence-specific.

2.2.2.3 Intracellular trafficking and post-translational modifications of BACE1

Attempts to gain insights into the regulation of BACE1 and to identify potentially therapeutic targets in the β-secretase pathway must be based on an understanding of the cell biology of BACE1 protein, particularly its post-translational modifications and intracellular trafficking. BACE1 is initially synthesized in the endoplasmic reticulum (ER) as an immature precursor protein (proBACE1) with a molecular mass of ~60 kDa (Haniu *et al.*, 2000; Huse *et al.*, 2000). ProBACE1 is short-lived and undergoes rapid maturation into a ~70 kDa form in the Golgi apparatus. This process involves addition of complex carbohydrates and removal of the propeptide domain (Figure 7). The mature enzyme is quite stable, having a half-life of over 9 h in cultured cells. Unusually, the propeptide domain does not suppress protease activity, and proBACE1 may cleave APP to generate a potentially toxic intracellular Aβ pool (Cole and Vassar, 2007). BACE1 is glycosylated at four N-linked sites, but does not exhibit O-glycosylation (Haniu *et al.*, 2000). The N-glycosylation of BACE1 affects its protease activity, since abolishing two out of four N-linked sites by site-directed mutagenesis significantly reduces β-secretase activity (Charlwood *et al.*, 2001). Glycosylation may not directly affect BACE1 activity, but it may promote proper folding, and modify the solubility, or stability of the BACE1 protein. Mature N-glycosylated moieties of BACE1 are sulfated. Furthermore, three cysteine residues within the cytosolic tail of BACE1 can be palmitoylated, which might influence BACE1 intracellular localization or trafficking (Benjannet *et al.*, 2001). Although the majority of BACE1 in cells is produced as an integral membrane protein, a small fraction of BACE1 undergoes ectodomain shedding, a process that is suppressed by palmitoylation. Co-overexpression of APP and the soluble ectodomain of BACE1 in cells appears to enhance the generation of Aβ, suggesting that BACE1 ectodomain shedding may increase the amyloidogenic processing of APP (Benjannet *et al.*, 2001).

Related to intracellular trafficking of BACE1, a recent study demonstrates that BACE1 is sorted to early endosomes via a route controlled by the small GTPase ADP ribosylation factor 6 (ARF6) (Sannerud *et al.*, 2011). Alterations in ARF6 levels or its activity affects endosomal sorting of BACE1, and consequently results in altered APP processing and Aβ production. Furthermore, sorting of newly internalized BACE1 from ARF6-positive towards Rab5-positive early endosomes depends on its C-terminal short acidic cluster-dileucine motif. This ARF6-mediated sorting of BACE1 is confined to the somatodendritic compartment of polarized neurons in agreement with Aβ peptides being primarily secreted from this site. This study demonstrated a spatial separation between APP and BACE1 during surface-to-endosome transport, pointing to subcellular trafficking as a regulatory mechanism for this proteolytic processing step (Sannerud *et al.*, 2011).

BACE1 is localized in cholesterol-rich lipid rafts, and specific lipids stimulate BACE1 activity (Vetrivel *et al.*, 2009). Although BACE1 homodimers exhibit enhanced catalytic activity, the protease interacts with several other proteins (e.g. reticulon/Nogo proteins, SorLA/LR11) that modulate the BACE1-APP interaction and/or BACE1 activity (Cole and Vassar, 2007). Such interactions may provide clues for the therapeutic strategies to inhibit BACE1. Signals, including the acid cluster-dileucine motif in the C-terminal tail, control BACE1 trafficking and subcellular localization. Members of the GGA (Golgi localized γ-ear-containing ADP-ribosylation factor (ARF) -binding) protein family interact with BACE1 to regulate this process (discussed further in chapter 2.2.2.5) (Tesco *et al.*, 2007).

2.2.2.4 BACE1 functions in the amyloidogenic pathway

Interestingly, the mutations in APP that cause familial AD are all located near to the secretase cleavage sites and they directly affect the efficiency or position of the cleavages. For example, the Swedish mutation is the amino acid substitution LysMet→AsnLeu at the P2-P1 positions immediately N-terminal to the β-secretase cleavage site in APP (Mullan *et al.*, 1992) (Figures 3 and 8). This mutation makes APP a much better substrate for β-secretase and dramatically increases the efficiency of cleavage at the β-secretase site leading to increased production of Aβ. Several other familial AD mutations have been identified near the γ-secretase cleavage site and these mutations shift the position of γ-secretase cleavage and specifically lead to increased generation of Aβ42 (Hutton *et al.*, 1998). Familial AD mutations also exist near the α-secretase site. These mutations appear to reduce the efficiency of α-secretase cleavage, thus providing more APP for β-secretase-mediated cleavage and potentially leading to enhanced production of Aβ (Vassar, 2004). Moreover, high level neuronal expression of BACE1 preferentially channels APP through the amyloidogenic processing pathway in the brain (Thinakaran and Koo, 2008).

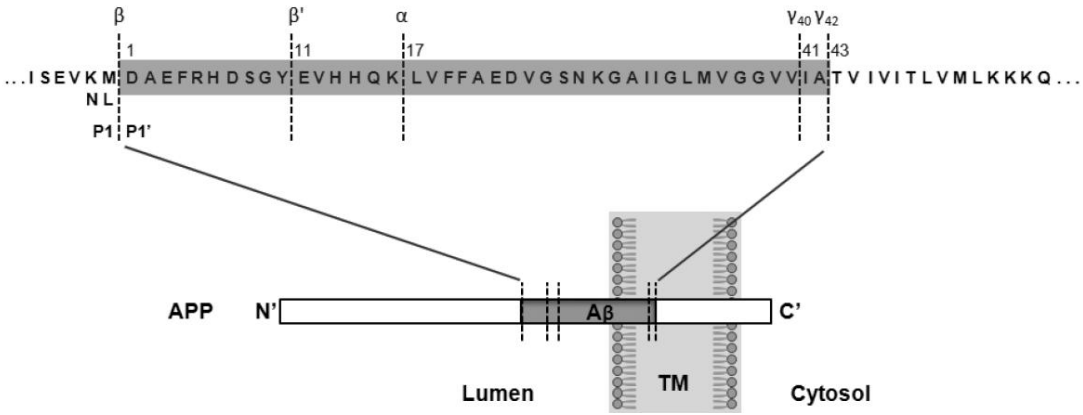

Figure 8. APP cleavage sites of α-, β-, and γ-secretase. Schematic structure of APP is shown with Aβ domain shaded in grey and enlarged. The sites of cleavage by α-, β-, and γ-secretases are indicated along with Aβ numbering from the N-terminus of Aβ (Asp1). Another BACE cleavage site (β') is also indicated. TM, transmembrane domain.

Evidently, BACE1 is critical for Aβ generation, and normal neuronal Aβ production indicates that the peptide may have physiological function(s). Indeed, the complete absence of Aβ in BACE1$^{-/-}$ mice caused a specific memory impairment, suggesting that Aβ is required for normal memory (Ohno *et al.*, 2004). This is consistent with the involvement of Aβ also in non-pathogenic neuronal function (Kamenetz *et al.*, 2003). Although neuronal BACE1 can cleave a large proportion of APP, APP is predominantly broken down by α-secretase in non-neuronal tissues. Recent studies have shown that several other putative BACE1 substrates exist in addition to APP (Cole and Vassar, 2007), implicating BACE1 in the regulation of different physiological functions. The identification of neuregulin-1 and the sodium channel β2-subunit as BACE1 substrates implicates BACE1 function in myelination and neuronal activity, respectively (Cole and Vassar, 2007; Kim *et al.*, 2005,

2007; Willem *et al.*, 2006; Wong *et al.*, 2005). Furthermore, many of these novel putative BACE1 substrates are involved in the response to stress and/or injury (Cole and Vassar, 2007). The fact that BACE1 levels are elevated under stressful conditions suggests that BACE1 functions as a stress-response protein and that cleavage of specific BACE1 substrates may facilitate recovery after acute stress/injury. It has also been hypothesized that elevated BACE1 levels become pathogenic and facilitate harmful Aβ formation during chronic stress or injury (Cole and Vassar, 2007; Hiltunen *et al.*, 2009).

Recent findings propose that BACE1 is able to cleave APP efficiently in both lipid raft and non-raft microdomains (Vetrivel *et al.*, 2009). Moreover, it seems that cholesterol is not only important in β-amyloidogenic processing of APP, but it is also able to modulate Aβ-induced neurotoxicity in AD (Fernández *et al.*, 2009). In addition to this, BACE1 expression is regulated in a transcriptional (Guglielmotto *et al.*, 2009) and translational manner (O'Connor *et al.*, 2008) during different stress conditions. Energy deprivation induces phosphorylation of the translation initiation factor eIF2α leading to increased BACE1 levels both *in vitro* and *in vivo* (O'Connor *et al.*, 2008). This study showed that BACE1 is translationally controlled by its complex 5'-untranslated region encompassing three upstream open reading frames, which regulate the translation of BACE1 mRNA depending on the eIF2α phosphorylation status.

Since its discovery almost a decade ago, there have been major advances in the understanding of BACE1 function and regulation. However, despite the promising reports of BACE1 inhibitor development, routine clinical use of such therapeutic agents for AD treatment is yet to become a reality. Although there is good reason to believe that BACE1 is the principle β-secretase responsible for the production of Aβ in the brain, its physiological role in the brain and in AD is not fully understood. Several studies support the concept that regulation of BACE1 expression in AD is a complex process, which likely involves several regulatory mechanisms simultaneously affecting BACE1 levels and activity.

2.2.2.5 BACE1 and GGA3

GGA1, 2, and 3 (Golgi-localized γ-ear-containing ARF binding proteins) are monomeric adaptor proteins that are recruited to the TGN by the ARF1-GTPase. Recent studies have indicated that GGA proteins are key regulators of BACE1 levels in cells (Tesco *et al.*, 2007). GGAs consist of four distinct segments: a VHS [VPS27 (Vacuolar protein sorting protein 27), Hrs (Hepatocyte growth factor-regulated substrate), and STAM (Signal-transducing adaptor molecule)] domain that binds the acidic dileucine sorting signal DXXLL; a GAT (GGA and Tom1) domain, which binds ARF:GTP; a hinge region which recruits clathrin; and a GAE (γ-adaptin ear homology) domain, which exhibits sequence similarity to the ear region of γ-adaptin and recruits a number of accessory proteins (Figure 9) (Tesco *et al.*, 2007). GGAs are necessary for the sorting of acid hydrolases to the lysosomes. Newly synthesized acid hydrolases modified with mannose 6-phosphate groups bind to mannose 6-phosphate receptors (MPRs). MPRs bind to the VHS domain of GGAs via the DXXLL motif (Bonifacino, 2004). GGAs are likely involved in the transport of proteins containing the DXXLL signal from the Golgi complex to the endosomes (Kang *et al.*, 2010) and also in the delivery of endosomal cargoes to lysosomes (Tesco *et al.*, 2007). However, recent studies have shown that cargo proteins can be recruited in the GGA pathway not only by the di-leucine sorting motif but also by ubiquitin (Puertollano and Bonifacino, 2004; Scott, *et al.*, 2004; Lauwers *et al.*, 2009). It has been shown that RNAi-mediated silencing of GGA1, GGA2, and GGA3 significantly increases the levels of BACE in endosomes (He *et al.*, 2005).

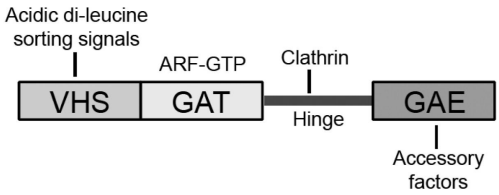

Figure 9. A schematic representation of GGA3 (Golgi-localized γ-ear-containing ARF binding protein 3) illustrating its four distinct domains: a VHS (VPS27, Hrs, and STAM) domain that binds the acidic di-leucine sorting signal, DXXLL in the BACE1 C-terminal fragment; a GAT (GGA and Tom1) domain that binds ARF:GTP; a hinge region which recruits clathrin; and a γ-adaptin ear homology domain that recruits a number of accessory proteins.

GGA3 is responsible for targetting BACE1 to the lysosomes for degradation. Previously Tesco *et al.* demonstrated that RNAi-mediated depletion of GGA3 resulted in impaired degradation of BACE1, consequently increased levels and activity of BACE1 and elevated Aβ levels *in vitro* (Figure 10) (Tesco *et al.*, 2007). Importantly, both *in vitro* and *in vivo* experiments have revealed that GGA3 is cleaved by activated caspase-3 and that the depletion of GGA3 resulted in increases in BACE1 levels and activity through post-translational stabilization. This finding is important in the context of AD, because apoptosis induced by oxidative stress plays an essential role in the disease pathogenesis (Greeve *et al.*, 2000). Moreover, it emphasizes the importance of post-translational regulation of BACE1 levels and activity. Furthermore, it has been shown that GGA3 regulates BACE1 degradation via binding to the ubiquitin sorting machinery, and this regulation does not require an interaction with the VHS/di-leucine motif (Kang *et al.*, 2010) corroborating the fact that GGA3 is an important mediator in post-translational regulation of BACE1 levels.

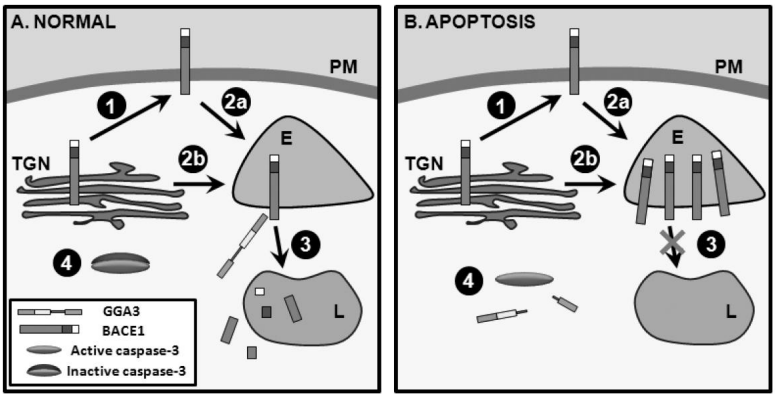

Figure 10. Model of BACE1 stabilization during apoptosis. (A) Under normal conditions, BACE1 is transported (1) from the trans-Golgi network (TGN) to the plasma membrane (PM). There it undergoes endocytosis (2a) into endosomes (E). Alternatively, BACE1 can directly traffic to the endosomes from TGN (2b). In the endosome, GGA3 binds to the cytosolic tail (dileucine motif) of BACE1 and targets it (3) to the lysosome (L) for degradation. Caspase-3 is inactive under normal conditions (4). (B) During apoptosis, caspase-3 is activated. As a result, BACE1 sorting to the lysosome is reduced (3), thereby stabilizing BACE1 in the endosome where it accumulates and elevates Aβ generation. TGN, trans-Golgi network; E, endosome; L, lysosome; PM, plasma membrane. (Adapted from Vassar, Neuron, 2007).

2.2.3 Tau hyperphosphorylation

Another histopathological hallmark in AD is the formation of intraneuronal filamentous inclusions or neurofibrillary tangles (NFT) that are composed of aggregated hyperphosphorylated tau protein (Buee *et al.*, 2000). Tau is transcribed from the *MAPT* (Microtubule-associated protein tau) gene on chromosome 17q21. Tau proteins belong to the family of microtubule-associated proteins (MAP) (Grundke-Iqbal *et al.*, 1986). The six tau isoforms that are generated by alternative splicing of the *MAPT* gene are differentially expressed in neurons (Buee *et al.*, 2000). In the normal brain, tau stabilizes axonal microtubules, modulates signal transduction, interacts with the actin cytoskeleton, modulates neurite outgrowth, anchors enzymes such as kinases and phosphatases, and regulates intracellular vesicle transport (Hernandez and Avila, 2007). The abnormal tau modifications found in AD include hyperphosphorylation, glycation, ubiquitination, oxidation, and truncation and these typically enhance tau aggregation and interfere with the normal functions of tau (Hernandez and Avila, 2007).

In AD, tau undergoes abnormal hyperphosphorylation. This may result from either increased activity of the kinases that phosphorylate tau (such as GSK3β, CDK5, and MARK; see also figure 5) or decreased activity of phosphatases, such as PP1, PP2A and PP2B, that dephosphorylate tau (Iqbal *et al.*, 2009). Phosphorylated tau may be degraded by the ubiquitin-proteasome system (Iqbal *et al.*, 2009). However, tau hyperphosphorylation leads to tau accumulation and aggregation and consequently to destabilization of microtubules. This can cause an impairment in axonal transport and neuronal dysfunction. Self-assembly of hyperphosphorylated tau results in the formation of NFTs (Iqbal *et al.*, 2009).

There is an increasing amount of evidence suggesting that Aβ may induce tau pathology by a variety of mechanisms. For example, inflammation triggered by the accumulation of extracellular Aβ deposits alters the cytokine profile in the brain, creating an environment that is favourable for the formation of tau pathology (Kitazawa *et al.*, 2005). Interestingly, different forms of Aβ may differentially induce tau pathology. Extracellular Aβ plays a role in the inflammation-mediated induction of pathogenic tau hyperphosphorylation, but oligomeric intraneuronal Aβ alters CHIP [C-terminus of Hsc70 (heat-shock cognate 70)-interacting protein] levels and thus induces pathogenic tau hyperphosphorylation (Oddo *et al.*, 2008). In a recent study, Jin *et al.* used extracts which had been purified directly from the brains of AD patients, and demonstrated for the first time that these isolated Aβ dimers of human origin were able to induce tau hyperphosphorylation at AD-relevant epitopes and evoke neuritic degeneration in primary cultures of rat neurons (Jin *et al.*, 2011). This study strongly supports not only the validity of amyloid cascade hypothesis but also the idea according to which Aβ deposits are in a close relationship to neurofibrillary tangle formation in AD pathogenesis.

The *MAPT* gene encoding tau is not genetically linked to AD, but tau mutations cause FTDP-17 (Frontotemporal dementia with Parkinsonism linked to chromosome 17) (Hutton *et al.*, 1998). The identification of disease-causing mutations in tau supports the hypothesis that tau dysfunction is involved in the neurodegeneration (Hutton *et al.*, 1998). The lack of genetic association to AD, however, further corroborates the suspicion that tau lies downstream of Aβ in the neurodegenerative cascade (LaFerla and Oddo, 2005).

2.2.4 Aβ-associated toxicity

Much AD research has focused upon determining the mechanisms underlying Aβ-associated toxicity. One major area of research has centered around calcium-mediated neurotoxicity. Aβ peptides can (i) increase calcium influx through voltage-gated calcium channels (N- and L-type channels); (ii) reduce magnesium blockade of NMDA receptors to allow increased calcium influx, and (iii) form a cation-selective ion channel after Aβ peptide incorporation into the cellular membrane (Pearson *et al.*, 2000; Kawahara and Kudora, 2000; Zhu *et al.*, 2000). Cation channels are induced by soluble Aβ peptides (Bhatia *et al.*, 2000). Thus, Aβ peptides may be toxic even prior to fibril formation. There is recent evidence to suggest that copper and zinc may modulate the structure of the Aβ peptides to allow either their pore formation or their precipitation (Curtain *et al.*, 2001).

It is evident that there is a reciprocal interaction between calcium and Aβ pathology (Supnet and Bezprozvanny, 2010; Demuro *et al.*, 2010). Aβ plaques have been shown to impair calcium homeostasis in the mouse models of AD (Kuchibhotla *et al.*, 2008), while sustained increase in cytosolic calcium levels induced by KCl-mediated depolarization in neuronal cultures triggered intraneuronal Aβ1-42 production and neuronal death (Pierrot *et al.*, 2004). Furthermore, due to the interaction of soluble Aβ oligomers with certain lipids and receptors at the plasma membrane, increased calcium influx could well be an outcome of disrupted membrane lipid integrity, Aβ-mediated pore formation, or modulation of certain ion channels, such as NMDA and AMPA receptors (Demuro *et al.*, 2010). In addition, it was recently shown that APP could regulate intracellular trafficking of voltage-gated L-type calcium channels (Cav1.2) by retrieving Cav1.2 into intracellular compartments away from the plasma membrane (Yang *et al.*, 2009). This means that alterations in APP expression or its processing can affect the levels of Cav1.2 at the plasma membrane and thus modulate calcium influx.

Many neurodegenerative disorders are characterized by the formation of insoluble inclusions. However, recent evidence indicates that these deposits might not be directly responsible for the primary neurotoxic effects and memory loss in these disorders. The inclusions might function as a reservoir for soluble oligomers, which are able to diffuse through the brain parenchyma and into synaptic clefts. Insoluble deposits and diffusible oligomers in different diseases are composed of diverse amyloidogenic proteins, such as Aβ tau, prion protein (PrP), α-synuclein, or huntingtin. These might share common structural epitopes and mechanisms of neurotoxicity and memory impairment (Kayed *et al.*, 2003). The identification of soluble, diffusible oligomers of Aβ that are capable of interfering with synaptic function and integrity could provide an important new perspective for understanding the basis of memory loss in AD (Haass and Selkoe, 2007).

Other major areas of research regarding Aβ toxicity include the enhancement of excitotoxicity via reduction of inhibitory signals, induction of inflammatory responses, and free radical formation. Aβ peptides induce the release of pro-inflammatory cytokines that trigger an inflammatory cascade resulting in neuronal death (Manelli *et al.*, 1995; Leonard *et al.*, 2001). Production of reactive oxygen species (ROS), including nitric oxide, is regulated by multiple factors including calcium, inflammatory cascades, and mitochondrial processes (Law *et al.*, 2001; Leonard *et al.*, 2001).

2.2.5 Amyloid cascade hypothesis

A series of genetic and experimental findings form the basis of the amyloid cascade hypothesis that was first introduced in 1991 to describe the progression of AD pathogenesis (Hardy and Allsop, 1991; Hardy and Selkoe, 2002; Tanzi and Bertram, 2005). This hypothesis represents a causality model, in which the increased Aβ levels are the prime pathogenic driver leading to a series of molecular downstream events (e.g. tau hyperphosphorylation) and, subsequently, histological and clinical features of AD (Figure 7). In familial EOAD, mutations in the *APP*, *PSEN1* and *PSEN2* genes increase Aβ production. Additionally, Aβ levels are strongly elevated in the brain of DS patients due to increased *APP* gene dose. The mechanism by which excessive Aβ accumulation occurs in LOAD remains unclear. Reduced Aβ clearance and/or degradation is one potential mechanism leading to increased cerebral Aβ levels in AD. However, it is also possible that minor increases in Aβ production over time may tip the balance toward Aβ accumulation (Cole and Vassar, 2007). The histological and clinical phenotypes of EOAD and LOAD, however, are rather similar, and thus the amyloid cascade hypothesis has been proposed to underlie all forms of AD. In particular, the increased production of Aβ oligomers is linked to synaptic toxicity, Aβ aggregation, and amyloid plaque formation (Figure 11 and 12; see also Figure 5), which eventually initiates inflammatory response by activating microglia and astrocytes. The increased production of ROS affects the activation of different kinases and phosphatases, leading to tau hyperphosphorylation and formation of PHFs and NFTs.

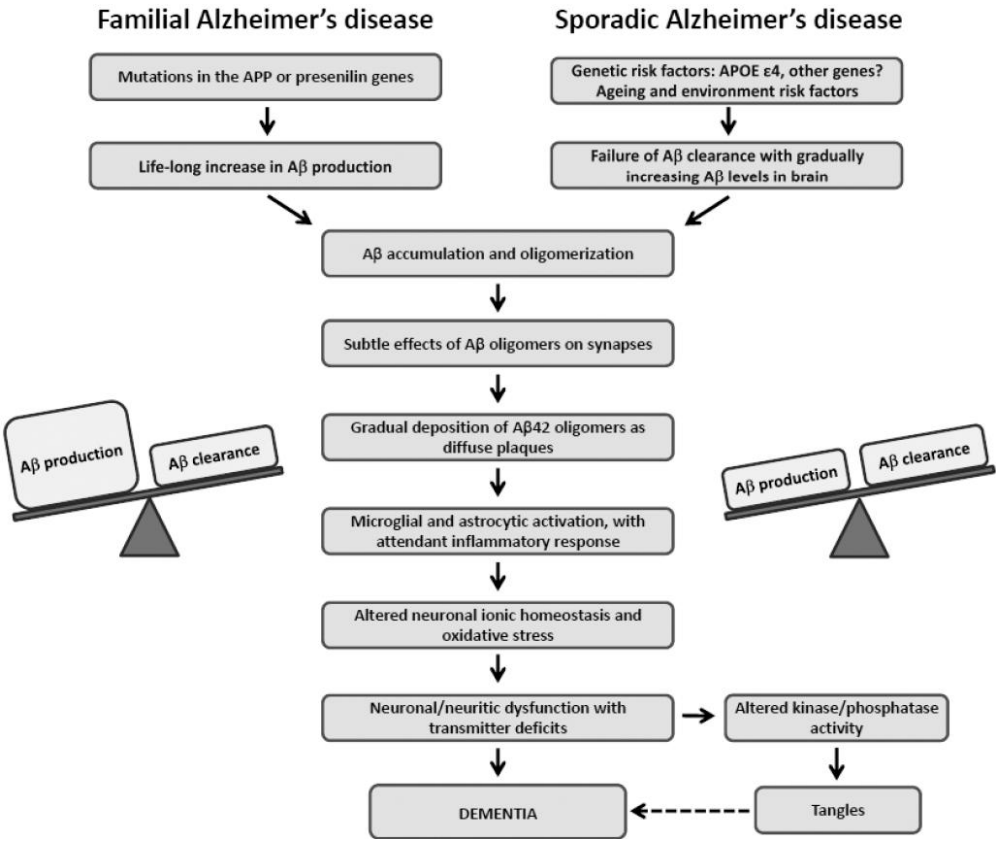

Figure 11. Amyloid cascade hypothesis. According to this hypothesis, the central event in the disease pathogenesis is an imbalance between Aβ production and clearance. In FAD, there is increased Aβ production, whereas Aβ clearance is decreased in sporadic disease. Aβ oligomers could directly inhibit hippocampal long-term potentiation and impair synaptic function, while aggregated and deposited Aβ causes inflammatory reaction and oxidative stress. These processes impair neuronal and synaptic function and consequently result in deficits in neurotransmission and cognitive symptoms. Tau pathology with tangle formation is regarded as a downstream event, but it could contribute to neuronal dysfunction and the cognitive symptoms (Tanzi et al., 2004). (Adapted from Blennow et al., 2006, and Hardy and Selkoe, 2002).

Since the introduction of the original amyloid cascade hypothesis more than two decades ago, several additional molecular players have been postulated to affect the disease pathogenesis. Related to this, a novel dual hypothesis was created (Small and Duff, 2008). This hypothesis links Aβ and tau as drivers of LOAD through independent but parallel mechanisms (Figure 12). Several lines of evidence favour this alternative hypothesis. First, the major risk gene in AD, *APOE,* has been shown to associate with an increased risk of developing both EOAD and LOAD (Tanzi and Bertram, 2005). Second, *APOE* has been shown to affect both Aβ and misfolded tau formation via separate mechanisms (Brecht *et al.*, 2004; Harris *et al.*, 2003; Huang *et al.*, 2001). *APOE* ε4 allele was proposed to be linked to decreased Aβ clearance (Bales *et al.*, 2002; DeMattos *et al.*, 2004; Dodart *et al.*, 2005). On

the other hand, the same allele was observed to regulate GSK3 activation – an important kinase responsible for tau phosphorylation (Cedazo-Minguez *et al.*, 2003; Hoe *et al.*, 2006; Ohkubo *et al.*, 2003). Finally, the recent studies demonstrate that Aβ and phosphorylated tau induce neurotoxicity in a different, possibly synergistic, manner through microglial activation (Hauptmann *et al.*, 2006; Reddy *et al.*, 2008). Moreover, Ittner *et al.* have recently demonstrated a molecular mechanism that links tau to Aβ toxicity occurring at the synapse (Ittner *et al.*, 2010). In that study it was shown that tau – having a previously unknown role in the dendrites – could target the Src kinase Fyn to the NMDA receptor. This allowed tau to mediate Aβ-induced excitotoxicity at the synapse. When tau is deleted or mistargeted in an AD model mouse, survival and memory improve to similar levels to the wild-type mice, although plaque burden and Aβ levels do not change. Other studies also support the hypothesis that tau likely to play a role in mediating Aβ toxicity in synapses (Rapoport *et al.*, 2002; Vossel *et al.*, 2010).

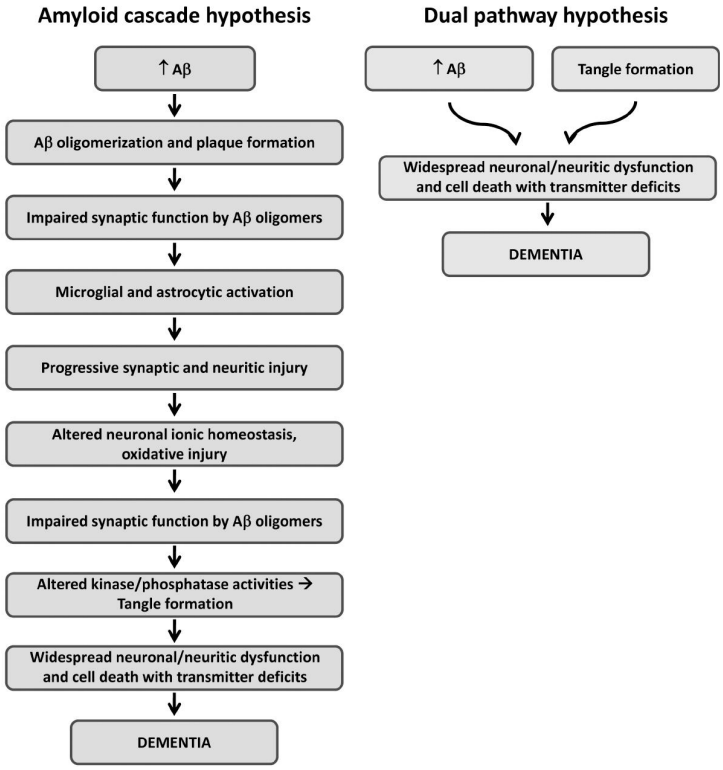

Figure 12. Amyloid cascade hypothesis and a newly hypothesized dual pathway model linking core features of AD. The traditional amyloid hypothesis (left) assumes a serial model of causality, whereby abnormal elevations in Aβ drive tau hyperphosphorylation and other downstream manifestations of the disease. According to the dual pathway hypothesis, Aβ elevations and tau hyperphosphorylation can be linked by separate mechanisms driven by a common upstream molecular defect. (Adapted from Citron et al., 2004, and Small and Duff, 2008)

2.3 GENETICS OF AD

2.3.1 Identification of AD-related genes

To facilitate the design of novel intervention approaches for AD, it is important to identify and functionally characterize genetic alterations, which play a role in AD pathogenesis. Initially, most of the genetic reports came from linkage analyses and case-control association studies that had examined the association of single-nucleotide polymorphisms (SNPs), usually in a single candidate gene. These studies were conducted in the case-control cohorts varying from hundreds to a couple of thousand subjects. In addition, the selection of a candidate gene was based on the function and relevance of its encoding protein in AD pathogenesis, as in the case of BACE1 (Murphy *et al.*, 2001). This study represents a good example of a situation in which polymorphisms within a gene do not necessarily display a risk association with AD despite the fact that BACE1 was identified as a key enzyme in the amyloidogenic pathway.

Over the past decade, hundreds of genetic variants have been suggested as possible risk factors, mostly for the LOAD. Unfortunately, quite often replication studies were not able to replicate the promising results of the initial study. This led to the idea of creating an AD-related database, in which researchers could evaluate the risk effects of a particular candidate. For that purpose, the AlzGene database (http://www.alzgene.org) was launched in 2004 (Bertram *et al.*, 2007). At present, after systemic meta-analysis of almost 1400 papers covering nearly 3000 polymorphisms in approximately 700 genes, the most viable susceptibility gene candidates are now identified and published in the database (Bertram *et al.*, 2007). This publicly available databank represented a useful tool for researchers all over the world to examine which genes might be worth exploring in greater detail.

It was soon realized that new AD risk genes can be found by applying the genome-wide association (GWA) approach. The development of public databases to catalog variation between individuals (SNPs) and advances in high-throughput, high-density genotyping technology together with the recent decline in the costs have led to a massive increase in the number of GWA studies. The clear benefit of GWA studies compared to the candidate gene approach is that the GWA technique simultaneously tests a very large number of genetic markers, normally several hundreds of thousands, in a largely hypothesis-independent fashion (Bertram and Tanzi, 2008). The markers in a GWA array consist of SNPs which are chosen based on their ability to cover common variation in the human genome. GWA studies have emerged as an increasingly effective tool for identifying genetic contributions to complex diseases such as AD, and understanding the underlying etiologic, biological, and pathologic mechanisms associated with the disease. The number of subjects in GWA studies is usually around several thousands, which also favours the advantage of this method in terms of statistical power.

SNPs can also be analyzed in another way. Haplotype analysis is the method that specifies the genetic information descending through a pedigree. In AD gene mapping, a set of haplotypes from individuals who have AD can be used to predict the locus of a disease susceptibility gene. In addition, a set of haplotypes from healthy controls is utililzed. A haplotype for a given certain individual and a set of loci are defined as the set of alleles inherited, one per locus, from the same parent (Figure 13). Haplotype patterns shared by (or over-represented in) the cases that predict the gene to be close to the best patterns are

searched. Haplotype association analysis is useful in population isolates where individuals carrying the mutated gene have potentially inherited it from a relatively recent common ancestor, because they most likely share common haplotype patterns around that gene. The disadvantages of haplotype analysis are the weak effects of genes, diagnostic problems, gene-gene interactions, gene-environment interactions, and small data sets.

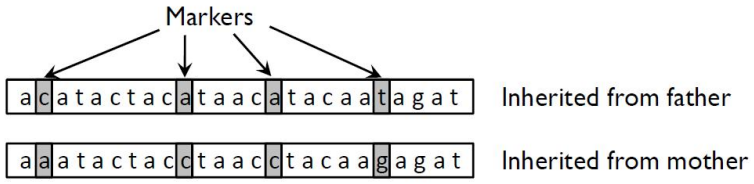

Figure 13. Illustration of a haplotype. Nucleotides, or alleles, in a gene of interest (grey boxes) inherited from the father and the mother in each individual form a particular variant in a marker (e.g. c/a or 1/2). Haplotype (H) is a series (two or more) of alleles along a single chromosome: H_{father} *= (c, a, a, t),* H_{mother} *= (a, c, c, g).*

2.3.1.1 Causative gene mutations in AD

APP gene

During the early 1980's it was discovered that the "amyloid protein" isolated from the brains of AD patients had the same biochemical properties and amino acid sequence as that isolated from DS brains (Glenner *et al.*, 1984; Masters *et al.*, 1985). Because of the clinical, histopathological, and biochemical association of DS with AD and the existence of an extra copy of chromosome 21 in DS, researchers started to focus on chromosome 21 as the possible location for an AD susceptibility gene. In 1987, a linkage to a locus on chromosome 21 was found in extended families with the autosomal dominant form of EOAD (St George Hyslop *et al.*, 1987). Soon after this two related articles reported the mapping of the *APP* gene found in the brains of AD and DS patients to a region on chromosome 21 that was near to the newly identified AD locus (Goldgaber *et al.*, 1987; Tanzi *et al.* 1987). At the end of the 1980's, Goate *et al.* first found evidence of linkage to chromosome 21 in autosomal dominant EOAD families (Goate *et al.*, 1989), and then identified a missense mutation in the *APP* gene segregating with AD in some of the families they analyzed (Goate *et al.*, 1991). The mutation occurred in exon 17 of the *APP* gene and led to a valine to isoleucine change at amino acid 717 (Val717Ile) located in the transmembrane domain of the protein (Figure 3). It was thus predicted that this first familial EOAD mutation (London mutation) would lead to AD through its effects on Aβ. Since then, according to the Alzheimer Disease & Frontotemporal Dementia Mutation Database (http://www.molgen.ua.ac.be/ADMutations; retrieved in July 1st, 2011), 32 pathogenic mutations have been identified within the *APP* gene in 89 EOAD families. Many of these mutations have been functionally assessed for their effects on APP processing. The mutations are strategically located either immediately before the β-secretase cleavage site, shortly after the α-secretase site, or slightly COOH-terminal to the γ-secretase cleavage site (Figure 3). These missense mutations lead to AD by altering APP proteolytic processing at

the three secretase sites in subtly different ways (Selkoe, 2001). This has been supported by several studies analysing each of the mutations in transfected cells or primary cells from patients and then in transgenic mouse models (Selkoe, 1997). Families harboring *APP* missense mutations generally have the onset of the disorder before age 65, often even in their 50s (Selkoe, 2001).

Presenilins (PSEN1, PSEN2)

After identification of mutations in *APP*, it soon became apparent that these mutations would account for only a minor fraction of all familial EOAD cases. In 1995, *PSEN1* and *PSEN2* genes were reported as novel familial EOAD genes on chromosomes 14 and 1, respectively (Levy-Lahad *et al.*, 1995, Rogaev *et al.*, 1995; Sherrington *et al.*, 1995). The PSs are serpentine proteins believed to have eight transmembrane domains (TMD) and large hydrophilic, cytoplasmic loops. However, the precise topology has not been unequivocally determined, some studies have found nine TMDs (Laudon *et al.*, 2005; Kornilova *et al.*, 2006). Of these TMDs, the third cytoplasmic loop between TMDs 6 and 7 undergoes regulated endoproteolytic cleavage to produce PSEN N- and C-terminal fragments (Thinakaran *et al.*, 1997). *PSEN1* missense mutations cause the most aggressive form of AD, commonly leading to onset of symptoms before the age of 50 and death of the patients in their 60s. Unlike the case with *PSEN1*, mutations in *PSEN2* are rare. To date, 182 rare, autosomal-dominant mutations have been found in *PSEN1*, and 13 in *PSEN2* (http://www.molgen.ua.ac.be/ADMutations; retrieved in July 1st, 2011).

2.3.1.2 Risk genes in AD

Among the several genetic factors, which have been implicated in AD, mutations in only a few genes (e.g. *APP*) are thought to be causative for the disease. In the majority of sporadic AD cases, genetic alterations seem to act as predisposing factors that increase the risk, but do not directly cause the disease. The risk genes probably exert their pathogenic effect by acting together with environmental or other pathogenic factors. They may also interact between themselves to further enhance the probability of inducing the disease (synergistic effect) (Rocchi *et al.*, 2003).

A number of putative risk genes have been considered as possible candidates in the association studies on the basis of their function. The typical approach to evaluate the genetic contribution of a particular gene to the risk for AD is to compare the frequency of the allelic variation at polymorphic sites of the gene in cases and controls. Many of the potential candidiate genes are enzymes, plasma proteins, growth factors, or membrane receptors, which may exist as different allelic variants. Some polymorphic variants have particular properties that could explain their role as genetic risk factors. For example, polymorphism in a gene may lead to an altered biological function (e.g. transcription), which may contribute to the pathogenesis of AD. Many of the potential risk genes are have been proposed to be involved in APP processing and/or in the degradation and clearance of Aβ (Rocchi *et al.*, 2003).

In 1993 it was reported that the ε4 allele in *APOE* gene leads to a remarkable increase in AD risk by ~4-fold as compared to non-carriers (Schmechel *et al.*, 1993; Strittmatter *et al.*, 1993). This has been replicated in essentially all independent follow-up studies showing that *APOE* is thus far the strongest known risk factor for LOAD (Bertram *et al.*, 2007). In Finnish

population, the risk effect has been seen both in EOAD and LOAD (Lehtimaki *et al.*, 1995). Since the identification of *APOE*, more than 1400 genetic studies employing SNPs or other genetic variations have revealed hundreds of potential candidate genes and polymorphisms that are associated with AD worldwide. The latest GWA studies have identified several susceptibility genes in large case cohorts and in different populations that associate to AD, such as *ABCA7*, *MS4A6A*/*MS4A4E*, *EPHA1*, *CD33*, *CD2AP*, *CLU*, *CR1*, (Lambert *et al.*, 2009; Hollingworth *et al.*, 2011; *Naj et al.*, 2011). According to the Alzgene database, with the exception of *APOE*, the majority of the most commonly identified candidate genes have a relative risk effect of 1.1–1.3 (Table 4).

Table 4. List of the current top 10 risk genes in AD (Retrieved and adapted from Alzgene database, 18th of April, 2011). Chromosomal locations are according to NCBI gene database (http://www.ncbi.nlm.nih.gov).

Rank	Gene	Chromosomal location	Odds ratio [95% CI] *	Protein
1.	*APOE ε2/3/4*	19q13.2	3.69 [3.30, 4.11]	Apolipoprotein E
2.	*BIN1*	2q14	1.17 [1.13, 1.20]	Bridging integrator 1
3.	*CLU*	8p21-p12	0.88 [0.86, 0.91]	Clusterin
4.	*ABCA7*	19p13.3	1.23 [1.18, 1.28]	ATP-binding cassette, sub-family A (ABC1), member 7
5.	*CR1*	1q32	1.17 [1.11, 1.20]	Complement component (3b/4b) receptor 1 (Knops blood group)
6.	*PICALM*	11q14	0.88 [0.86, 0.9]	Phosphatidylinositol-binding clathrin assembly protein
7.	*MS4A6A*	11q12.1	0.90 [0.88, 0.93]	Membrane-spanning 4-domains, subfamily A, member 6A
8.	*CD33*	19q13.3	0.89 [0.86, 0.93]	CD33
9.	*MS4A4E*	11q12.2	1.08 [1.05, 1.11]	Membrane-spanning 4-domains, subfamily A, member 4E
10.	*CD2AP*	6p12	1.12 [1.08, 1.16]	CD2-associated protein

** Odds ratio was calculated from all studies including the initial one.*

2.3.1.3 Seladin-1/DHCR24

Although not ranked in the top ten list of candidate genes (table 4), *seladin-1* (selective AD indicator 1) or *DHCR24* is an interesting candidate since the studies from the last decade reporting its association to AD. *Seladin-1* gene was first described by Greeve *et al.* in 2000 (Greeve *et al.*, 2000). Seladin-1 expression was observed to be downregulated in large pyramidal neurons in specific regions in AD brain and thus suggested to be a cause for selective vulnerability in AD. Furthermore, previous studies have shown that decreased seladin-1 mRNA levels inversely correlate with hyperphosphorylated tau levels in AD brain (Iivonen *et al.*, 2002) and that specific *seladin-1*/*DHCR24* gene variants associate with increased AD risk (Lamsa *et al.*, 2007).

Functional characterization has revealed that seladin-1 confers specific protective properties against Aβ-mediated and oxidative stress-induced apoptosis by inhibiting caspase-3 activity (Greeve *et al.*, 2000; Luciani *et al.*, 2005; Di Stasi *et al.*, 2005). The protective function of seladin-1 is postulated to be mediated by its C-terminal domain harboring scavenging activity toward ROS in the ER (Lu *et al.*, 2008) or through a p53-dependent mechanism (Kuehnle *et al.*, 2008), possibly by preventing Ras-induced senescence (Wu *et al.*, 2004).

Seladin-1 also has specific enzymatic activity. It catalyzes the reduction of the Δ24 double bond of desmosterol, the immediate precursor of cholesterol, to form cholesterol (Waterham *et al.*, 2001). This implies that seladin-1 has a role in the formation of cholesterol-rich detergent-resistant membrane domains, also known as lipid rafts (Abad-Rodriguez *et al.*, 2004). On the other hand, lipid rafts have been found to affect β- and γ-secretase-mediated APP cleavage and Aβ generation *in vitro* and *in vivo* (Crameri *et al.*, 2006; Abad-Rodriguez *et al.*, 2004). Furthermore, ~50% reduction in seladin-1 levels was shown to decrease cholesterol levels, which was accompanied by a disorganization of lipid raft domains (Crameri *et al.*, 2006) in heterozygous seladin-1 knockout mouse brain. This led to an increase in APP C99 and Aβ production, suggesting that β-secretase-mediated cleavage of APP was enhanced in the brain of seladin-1-deficient mice. However, another study did not identify a similar role of the lipid raft domains in Aβ production. The authors demonstrated that APP processing and Aβ generation were unaffected in cells stably expressing a BACE1 (BACE1-4C/A) mutant with an alanine substitution in its cysteine residues. The mutant BACE1 failed to undergo S-palmitoylation, a reversible post-translational modification responsible for targeting BACE1 to the lipid raft microdomains. In contrast, the authors observed an increase in the levels of APP CTFs in detergent-soluble fractions of cells expressing BACE1-4C/A as compared with cells expressing wild-type BACE1. These data suggest that BACE1 is able to efficiently cleave APP in both lipid raft and non-raft microdomains (Vetrivel *et al.*, 2009). Similarly, it is also possible that the protective effect of seladin-1 does not necessarily depend on its association to lipid rafts organization (or cholesterol), but it may be mediated through some other still unknown mechanism.

The nomenclature of seladin-1 protein and genes has varied in the publications. According to NCBI gene database (http://www.ncbi.nlm.nih.gov/gene) the official name for the gene is *DHCR24*, and *seladin-1* is one of the aliases. The general protein information prefers the name delta-24 (Δ24)-sterol reductase for the encoding protein, but also other names are used, such as seladin-1, 3-β-hydroxysterol Δ24-reductase, and diminuto/dwarf1 homolog. In this thesis, "*seladin-1*/*DHCR24*" is used for the gene, and "seladin-1" for the protein.

2.3.1.4 G protein-coupled receptors (GPCRs) - δ-opioid receptor 1

GPCRs

GPCRs are important in many fundamental physiological functions and mediate the actions of the majority of known neurotransmitters and hormones (Waldhoer *et al.*, 2004). Ligand-bound GPCRs activate heterotrimeric G proteins, inducing the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP). This leads to the formation of a GTP-bound G_{α} subunit and the release of the $G_{\beta\gamma}$ dimer. The G protein subunits then activate specific effector molecules, such as adenylyl cyclase (AC), phospholipase C (PLC), or phospholipase A2 (PLA2), leading to the generation of second messengers and the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), Janus

kinase (JAK) or phosphatidylinsitol-3-kinase (PI3K). The activation of certain GPCRs is known to modulate the α-secretase-mediated cleavage. In the case of the M1 muscarinic acetylcholine receptor (M1 mAChR), the group I metabotropic glutamate receptors (mGluRs) and the 5-hydroxytryptamine receptors 5-$HT_{2A/2C}$R and 5-HT_4R, agonist stimulation leads to an increase in sAPP release, a decrease in Aβ generation, a decrease in tau phosphorylation, and/or an alleviation of cognitive deficits in a mouse model of AD (Thathiah and Strooper, 2011). Conversely, agonist stimulation of the Group II mGluRs evokes an increase in Aβ42 generation and tau phosphorylation, and an exacerbation of the cognitive deficits in AD mice. In the case of the 5-HT6 receptor, antagonism of the receptor leads to an improvement in cognition (Thathiah and Strooper, 2011).

Recently, several studies have shown compelling evidence implicating GPCRs in the pathogenesis of AD and in the processing of APP. GPCRs affect the cleavage of APP by α-, β-, and γ-secretases and thus the extent of Aβ generation. On the other hand, Aβ can directly or indirectly affect GPCR function (Thathiah and Strooper, 2011). With respect to the GPCRs that regulate APP processing, the δ-opioid receptor (δOR) and the adenosine A_{2A} receptor (A_{2A}R) have been shown to modulate β-secretase-mediated cleavage of APP (Teng *et al.*, 2010; Dall'Igna *et al.*, 2003, 2007) In addition, δOR, β2 adrenergic receptor (β_2AR), G protein-coupled receptor 3, and CXC-chemokine receptor 2 modulate γ-secretase-mediated cleavage of C99 or C83 (Ni *et al.*, 2006; Teng *et al.*, 2010, Thathiah *et al.*, 2009; Bakshi *et al.*, 2008, 2011).

The α-secretase-mediated cleavage of APP is regulated by PKC (Buxbaum *et al.*, 1990; Caporaso *et al.*, 1992), cyclic AMP–protein kinase A (PKA) (Xu *et al.*, 1996), sequential activation of MAPK and extracellular signal-regulated kinase (ERK) (Mills *et al.*, 1997), and PI3K (Solano *et al.*, 2000). Specifically, activation of these signalling cascades shifts APP metabolism towards the α-secretase-mediated pathway and away from β-secretase-mediated Aβ generation (Buxbaum *et al.*, 1993; Ishida *et al.*, 1997; Hung *et al.*, 1993). Conversely, a recent study has suggested that chronic, rather than acute, activation of PKC could differentially regulate the PKCα and PKCε isozymes, leading to increased Aβ generation (da Cruz e Silva *et al.*, 2009). Nevertheless, numerous studies have claimed that many GPCRs and activation of their downstream signalling cascades can increase the non-amyloidogenic processing of APP.

δ-Opioid receptor (δOR)

δOR is a GPCR with a typical seven-transmembrane helix (7TM) topology. δORs have been implicated in the presynaptic modulation of synaptic function and in the regulation of pain and mood (Bie *et al.*, 2010; Zhang and Pan, 2010). Furthermore, assessments of postmortem brain samples have revealed that different opioid receptor populations (such as δOR and κOR) are differentially altered in distinct areas of postmortem brain specimens (Mathieu-Kia *et al.*, 2001). The only non-synonymous SNP T80G (rs1042114) in the coding region of *OPRD1*, leading to Phe27Cys amino acid substitution in δOR, has been reported to associate with opioid dependence (Zhang *et al.*, 2008), although another study found no association (Xuei *et al.*, 2007). Like other GPCRs, δORs not only act as monomers but can form homomeric complexes with other opioid receptor subtypes and heteromeric complexes with other GPCRs, creating new receptors with novel pharmacological properties (van Rijn *et al.*, 2010).

It has been previously shown that the presence of cysteine at position 27 affects the maturation and subcellular localization of δOR in non-neuronal cells (Leskela *et al.*, 2009;

Petaja-Repo *et al.*, 2000). More specifically, the δOR-Cys27 variant displayed a low maturation efficiency, and lower stability at the cell surface than the δOR-Phe27 variant. These features of the δOR-Cys27 receptor resulted in the retention of receptor precursors in the ER and enhanced turnover of mature cell surface receptors. Thus, it was proposed that the δOR-Cys27 variant may cause a gain-of-function phenotype with possible pathophysiological consequences due to the intracellular accumulation of the receptor (Leskela *et al.*, 2009).

δOR, which plays important roles in learning and memory, is deregulated in specific regions of the AD brain (Mathieu-Kia *et al.*, 2001). There is evidence that both δOR and β_2AR can promote the γ-secretase-mediated cleavage of C99 (Ni *et al.*, 2006). A more recent study by the same group suggested that activation of the δOR promotes the translocation of a complex consisting of δOR, β-secretase and γ-secretase from the cell surface to the late endosomes and lysosomes (LEL). This resulted in enhanced β- and γ-secretase-mediated proteolysis of APP (Teng *et al.*, 2010). In a mouse model of AD, administration of natrindole, a selective δOR antagonist, improved spatial learning and reference memory and reduced the amyloid plaque burden (Teng *et al.*, 2010). Similarly, *in vivo* knockdown of the δOR reduced Aβ40 accumulation in the hippocampus of AD mice. However, there was no effect on the more hydrophobic (and therefore more toxic) Aβ42 (Teng *et al.*, 2010). Overall, the above-mentioned studies hint that δOR may play a significant role on AD pathogenesis.

2.4 ALZHEIMER'S DISEASE-RELATED CO-MORBIDITIES

Several studies have shown that AD and several other brain disorders, such as cerebral ischemia, traumatic brain injury (TBI) and normal pressure hydrocephalus (NPH), share similar neuropathological features. These features include altered APP processing, and accumulation of calcium and Aβ levels (Abe *et al.*, 1991, Pluta *et al.*, 1994; Koistinaho *et al.*, 1996; Blasko *et al.*, 2004). Importantly, the common factor for the above-mentioned disorders is that they all show increase in expression and activity of BACE1. Identification of these overlapping factors is valuable for achieving a better understanding of the key molecular mechanisms in AD and AD-related co-morbidities. In the following chapters, central findings in other brain injuries and disorders similar to those in AD are introduced.

2.4.1 Traumatic brain injury (TBI)

There is solid evidence that traumatic brain injury (TBI) is a risk factor for AD and that disturbances in the balance between the production and degradation of Aβ play a crucial role in the disorder (Blasko *et al.*, 2004). Post-mortem analysis of the brains of long-term survivors as well as patients surviving only a few days after brain trauma have demonstrated a widespread deposition of Aβ peptides (Graham *et al.*, 1995; Jellinger *et al.*, 2001; Roberts *et al.*, 1991). Increased Aβ levels in the CSF (Emmerling *et al.*, 2000; Raby *et al.*, 1998) and marked overproduction of sAPPβ in neurons of injured humans or experimental animals have also been observed (Blasko *et al.* 2004). Moreover, activity of BACE1, which is known to be activated in stress, has been shown to increase after TBI (Blasko *et al.*, 2004).

2.4.2 Cerebral ischemia

Cerebral ischemia has been reported to significantly increase the risk of AD (Zlokovic, 2002; Honig *et al.*, 2003). Moreover, individuals diagnosed with severe cognitive decline or possible AD are at increased risk for ischemic events in the brain (Koistinaho and Koistinaho, 2005). Cerebral ischemia leads to transient up-regulation and accumulation of APP and its proteolytic fragments in the cortex adjacent to the boundary of the ischemic lesion and white matter (Abe *et al.*, 1991; Koistinaho *et al.*, 1996; Lin *et al.*, 1999; Pluta *et al.*, 2006, 2009), pointing to an interplay between cerebrovascular and amyloid pathology.

It is well-established that astrogliosis and the expression of proinflammatory cytokines are induced in cerebral ischaemia (Fan *et al.*, 1996; van Groen *et al.*, 2005; Sriram *et al.*, 2006). In addition, middle cerebral artery occlusion (MCAO) in *in vivo* animal models promotes secondary degeneration in the thalamus (Iizuka *et al.*, 1990; Ross and Ebner, 1990; Wei *et al.*, 2004, van Groen *et al.*, 2005) and enhances APP processing and Aβ generation (van Groen *et al.*, 2005; Makinen *et al.*, 2008; Hiltunen *et al.*, 2009), and BACE levels and activity (Wen *et al.*, 2004). Lastly, Aβ-degrading enzyme NEP expression was decreased in the rat model of four-vessel occlusion ischemia (Nalivaevaa *et al.*, 2004).

2.4.3 Normal pressure hydrocephalus (NPH)

Abnormalities in CSF production and turnover in normal pressure hydrocephalus (NPH) and in AD may be important causes of amyloid retention in the brain in these two diseases (Silverberg *et al.*, 2006). Furthermore, CSF circulatory failure in AD and NPH may result in reduced CSF clearance and accumulation of tau protein (Silverberg *et al.*, 2003). Patients with clinical and radiographic evidence of NPH also show typical pathological changes of AD, including plaques and tangles (Silverberg, 2004). There are also AD-like lesions seen in the brains of NPH patients (Bech *et al.*, 1999). Furthermore, in a large follow-up study, Leinonen *et al.* assessed the Aβ and p-tau protein levels of a total of 433 cortical biopsy samples from the brains of elderly patients with suspected NPH and revealed that the presence of Aβ and p-tau associated with the presence or later development of clinical AD (Leinonen *et al.*, 2010).

3 Aims of the study

To facilitate the design of novel biomarkers and the design of novel intervention approaches for AD, it is important to identify and functionally characterize genetic alterations involved in AD pathogenesis. The aim of this thesis was to investigate the genetic and mechanistic roles of specific risk genes linked to AD. The study population consisted of LOAD patients and age-matched control subjects originating from Eastern Finland. In addition, the familial AD sample set originating from USA was used as a replication cohort. Special emphasis was laid on elucidating the underlying molecular mechanisms of seladin-1, δOR and BACE1 in the pathogenesis of AD. Furthermore, a non-selective calcium channel blocker and β-secretase inhibitor, bepridil, was used to treat ischemic rats after MCAO. In these MCAO rats, Aβ and calcium accumulate in the ipsilateral thalamus. Therefore, they provide a feasible *in vivo* model to study drug effects on Aβ and calcium pathology, which are among key pathogenic features of AD.

The specific aims were:

1. To assess the risk effects of candidate gene polymorphisms selected on the basis of meta-analyses performed in AlzGene database among the Eastern Finnish case-control cohort (Study I).

2. To study the genetic and/or functional role of seladin-1, BACE1, and δOR in the processes relevant for AD pathogenesis, such as generation and accumulation of Aβ in different *in vivo* and *in vitro* models (Studies II-IV).

3. To study the effects of novel β-secretase inhibitor, bepridil, on the thalamic Aβ and calcium pathology in MCAO rats (Study IV).

4 Subjects, materials and methods

4.1 SUBJECTS (STUDIES I AND III)

4.1.1 AD patients and controls (Studies I and III)

In the case-control studies I and III, all subjects originated from Eastern Finland and were examined at the Department of Neurology, Kuopio University Hospital (Table 5). Patients fulfilled the NINCDS-ADRDA criteria for probable AD (McKhann *et al.*, 1984). Early-onset AD patients (onset age ≤65 years) did not display any conclusive evidence of autosomal dominant transmission (Lehtovirta *et al.*, 1996) and there were no *APP* or *PSEN1/2* mutations. Control subjects had no signs of dementia according to the interview and neuropsychological testing (Hänninen *et al.*, 2002). Ethical approval was provided by the Ethics Committee of the Kuopio University Hospital.

Table 5. Subject parameters in Eastern Finnish case-control cohort (studies I and III).

	AD patients			**Controls**			**All**
Descriptive	*Age: 43 to 89 years* *[1]Mean: 71±7 years*			*Age: 37 to 87 years* *[1]Mean: 69±6 years*			
	Women	**Men**	**Total**	**Women**	**Men**	**Total**	
Study I							
N (% in status)	434 (67.6)	208 (32.4)	**642**	411 (60.3)	271 (39.7)	**682**	**1324**
Study III							
N (% in status)	368 (69.0)	165 (31.0)	**533**	401 (59.8)	270 (40.2)	**671**	**1204**

[1]Mean age of onset of AD patients and at the time of neuropsychological examination of controls.

4.1.2 Family-based sample set (study III)

An AD family sample set previously used for GWAS was used in study III (Bertram *et al.*, 2008). The National Institute of Mental Health (NIMH) Genetics Initiative Study (Blacker *et al.*, 1997) sample contained 1376 individuals across 410 AD families, in which all members were of self-reported European ancestry.

4.2 GENOTYPING (STUDIES I AND III)

DNA samples were isolated from peripheral lymphocytes using the Blood Prep™ Chemistry kit (Applied Biosystems).

Study I

DNA samples were randomly placed on 384 well plates and genotyped using Sequenom iPLEX platform (Sequenom®, Hamburg, Germany). Genotyped SNPs were selected on the basis of meta-analyses retrieved from AlzGene database (http://www.alzgene.org/) (Table 6). Sequencing around the rs760678 site in *NEDD9* gene was performed from 47 randomly selected DNA samples using BigDye™ Terminator sequencing kit (Applied Biosystems, Foster City, USA) and ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA). *APOE* genotyping was performed as described previously (Tsukamoto *et al.*, 1993).

Table 6. Summary of 21 genes, SNPs, and their allelic variation among Finnish AD patients and controls analyzed in study I.

Gene	Polymorphism	[1]Allele Variation	AD patients (N)	Controls (N)
ACE	rs4343	G/A	553	579
CH25H	rs13500	T/C	617	666
CHRNB2	rs4845378	T/G	573	592
DAPK1	rs4878104	T/C	624	661
ENTPD7	rs911541	G/A	500	546
GAB2	rs2373115	T/G	621	672
GALP	rs3745833	C/G	621	665
GAPDHS	rs4806173	G/C	567	590
GWA_14q32.13	rs11622883	A/T	615	654
hCG2039140	rs1903908	T/C	613	663
IL1B	rs1143634 (+3953)	T/C	620	668
IL33	rs7044343	C/T	567	590
LOC651924	rs6907175	A/G	607	653
MAPT/STH	rs2471738 (intron 9)	T/C	562	680
MTHFR	rs1801133 (C677T)	T/C	617	662
NEDD9	rs760678	G/C	528	548
PGBD1	rs3800324	A/G	560	592
SORCS1	rs600879	A/G	620	668
TF	rs1049296 (P570S)	T/C	506	587
TFAM	rs2306604	C/T	622	667
TNF	rs1800629 (-308)	A/G	563	591

[1]Major/minor, minor allele based on information from Alzforum (wwwl.alz.org).

Study III

Four tag SNPs (rs678849, rs760588, rs4654323 and rs529520) within the *OPRD1* gene were selected from the Applied Biosystems database (Human Pre-Designed Assays) using the SNPBrowser™ 3.0 software and one SNP (rs1042114) was designed with Custom TaqMan®

SNP Genotyping Assay Design Tool (Table 7). The selected SNPs captured all the genetic information within *OPDR1* gene according to the HapMap database (http://www.hapmap.org/) in CEU population. SNP genotyping was performed by using TaqMan Allelic Discrimination Assays (Applied Biosystems). The TaqMan PCR reactions were done on a Research Peltier Thermal Cycler (PTC 200) and the fluorescence was detected on an ABI Prism 7000 sequence detector (Applied Biosystems). *APOE* genotyping was performed as previously described (Lehtovirta *et al.*, 1996). Genotyping of the NIMH Genetics Initiative Study sample set was completed with the GeneChip Human Mapping 500K Array Set from Affymetrix (Bertram *et al.*, 2008). Hardy-Weinberg equilibrium for each SNP in AD patient and control cohorts was calculated using the GenePop program (http://genepop.curtin.edu.au/genepop/genepop_op1.html).

Table 7. SNP's in OPDR1 gene used in study III.

[a]SNP and location (kb)	Allele polymorphisms (major → minor)
rs1042114 (0)	T → G
rs678849 (6.2)	T → C
rs706588 (23.6)	A → G
rs4654323 (30)	G → T
rs4654323 (30)	C → A

[a]*Locations of SNPs are indicated in 5' to 3' orientation with respect to SNP rs1042114.*

4.3 IMPUTATION ANALYSIS (STUDY III)

Markers for the NIMH families were generated as part of a GWAS as previously described (Bertram *et al.*, 2008). Since SNP rs1042114 in study III was not directly genotyped on the Affymetrix 500K SNP array, we inferred genotypes at this site via imputation using IMPUTE v2.0 (Marchini *et al.*, 2007). As reference panels, the precompiled HapMap 3 and 1000 Genomes Project "CEU TSI" panels from the IMPUTE website (obtained on 2 June 2010) were used. These contain HapMap 3 data (from release 2 of February 2009) and 1000 Genomes Project data from Pilot 1 genotypes (released in August 2009) for autosomal SNPs.

4.4 CELL CULTURE EXPERIMENTS (STUDIES II AND III)

4.4.1 siRNA and plasmid constructs (Studies II and III)

In study II, SMARTselection™ pre-designed ON TARGETplus SMARTpool small interfering RNAs (siRNAs) (Thermo Scientific Dharmacon) targeted to human *seladin-1/DHCR24* gene (GGAGUACAUUCCCUUGAGA, AGAACUAUCUGA-AGACAAA, GCACAGGCAUCG-AGUCAUC and GAAAUGAGGCAGAGCUCUA) were used to down-regulate seladin-1 (siSel). Negative control siRNA (siCtrl; Thermo Scientific Dharmacon) Duplex with 3' AlexaFluor488 (Qiagen) was used as a control in RNAi

experiments. cDNAs for human APP751 isoform and BACE1 (3' tagged with Myc; BACE1-Myc) cloned into pcDNA 3.1 vector were used to create stable cell lines.

The Myc-δOR-Flag-pFT-SMMF and HA-δOR-pcDNA5/FRT/TO constructs (study III), encoding the human δOR-Cys27 and δOR-Phe27 variants, have been described previously (Leskela *et al.*, 2007, 2009). All δOR constructs contained a cleavable HA signal peptide and either an N-terminal Myc tag and a C-terminal Flag tag or an N-terminal HA tag with a native C-terminus. The enhanced yellow fluorescent protein (EYFP)-Golgi compartment, enhanced green fluorescent protein (EGFP)-Rab7 (a marker for LEL), EGFP-Rab9 (a marker for lysosomes), and EGFP-Rab11 (a marker for recycling endosomes) constructs have been described previously (Choudhury *et al.*, 2002).

4.4.2 Stable cell lines (studies II and III)

SH-SY5Y human neuroblastoma cells were transfected with APP751 and BACE1-Myc plasmid constructs using Effectene transfection reagent (Qiagen) to create stable cells lines overexpressing APP751 (SH-SY5Y-APP751) (Studies II and III) and BACE1-Myc (SH-SY5Y-BACE1-Myc) (Study II). The BACE1-Myc cell line lacked an endogenous promoter and 5'-UTR regions of BACE1, thus making it possible to elucidate the underlying molecular mechanisms of seladin-1 knockdown on the post-translational stability of BACE1 in study II. After the transfections, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (DMEM-C). Two days after the transfection, 800 μg/ml G418 (G-418, Geneticin®; Invitrogen) was added to induce selection of APP751- and BACE1-Myc overexpressing cell clones. Three weeks after selection, clones overexpressing either APP751 or BACE1-Myc were identified using APP-, BACE1- and Myc-specific antibodies in Western blotting.

In study III, HEK293 cells over-expressing AP (alkalic phosphatase) and APP695 fusion protein (HEK293-AP-APP) were grown as described previously (Lichtenthaler *et al.*, 2003). The AP ectodomain was fused to the N-terminus of the full length APP695 lacking a signal peptide (Neumann *et al.*, 2006). Preparation and maintenance of stable HEK293$_i$ cell lines with inducible expression of the epitope tagged δOR cysteine 27 and phenylalanine 27 (δOR-Cys27 and δOR-Phe27, respectively) variants have been described earlier (Leskela *et al.*, 2007, 2009).

4.4.3 Transfections (Studies II and III)

Selected clones were subsequently cultured in DMEM-C containing 200 μg/ml G418. siRNA and plasmid constructs were transfected into SH-SY5Y-APP751 (studies II and III), SH-SY5Y-BACE1-Myc (study II), and HEK293-AP-APP (study III) cells using Lipofectamin 2000™ reagent (Invitrogen) according to the manufacturer's instructions. δOR expression was induced for 24-48 h by adding 0.5 μg/ml tetracycline (Invitrogen) to the culture medium. Opioid ligands in study III were added in the concentrations and for the incubation periods indicated in Table 8.

Table 8. Ligands and inhibitors/activators used in study III.

Ligand	Concentration (μM)	Induction period (h)	Role	Provider
Naltrexone (NTX)	10	48	δOR antagonist	Tocris
Naltriben (NTB)	10	48	δOR antagonist	Tocris
ICI-174,864	5	48	δOR inverse agonist	Tocris
SNC-80	10	48	δOR agonist	Tocris
Bisindolyl-maleimide I (Bis I)	1-10	4-24	protein kinase C (PKC) inhibitor	Enzo Life Sciences, Inc.
Protein phosphatase 2 (PP2)	1-5	24	specifc inhibitor of Src family kinases	Enzo Life Sciences, Inc.
Phorbol-12-myristate-13 acetate (PMA)	1	4	protein kinase C (PKC) activator	Sigma

4.4.4 Metabolic labeling (Study III)

HEK293$_i$-δOR-Cys27 cells were cultivated in a medium lacking cysteine and methionine, supplemented with FBS, penicillin, streptomycin, tetracycline, and 100 μCi/ml EXPRE^{35}S^{35}S labeling mix (Perkin-Elmer). Fresh label (50 μCi/ml) was added after 24-h incubation for 60 min to ensure efficient labeling of newly synthesized proteins. Sequential immunoprecipitation with anti-FLAG M2 and C-terminal anti-APP antibodies (Table 10) was performed from mixed cellular extracts and samples were analyzed by SDS-PAGE and fluorography.

4.5 RNA EXTRACTION AND REAL-TIME PCR (STUDIES II AND IV)

In study II, total RNA was extracted from transfected cells using TRIzol® Reagent (Invitrogen). In study IV, tissue samples from the ipsilateral and contralateral thalamus of MCAO- or sham-operated rats were first weighed and mechanically homogenized in 400 μl of Dulbecco's phosphate buffered saline (DPBS, Lonza) in an ice bath. Approximately 10-fold excess of PBS/mg tissue was used. Then, the tissue homogenate was mixed with 500 μl of TRIzol® reagent for total RNA extraction. RNA sample quality was controlled with NanoDrop 2000 (Thermo Scientific) by calculating the A260/280 ratio. Moreover, RNA profile for 18S and 28S RNAs was assessed in 1.5% agarose gel electrophoresis before the cDNA synthesis. To conduct real-time PCR in studies II and IV, equal amounts of total RNA samples were subjected to cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen). Subsequently, SYBR Green Master PCR Mix (Applied Biosystems) and target specific PCR primers (Table 9) were used for amplification of cDNA samples by using real time quantitative PCR (qPCR) machine (7500 Fast Real Time PCR System, Applied Biosystems). PCR primers were designed to anneal to sequences at least in two different exons on opposite sides of an intron. The qPCR cycles were set as 40 repetitions and annealing temperature 55°C. The mRNA levels of each gene were normalized to those of GAPDH from the same samples.

Table 9. PCR primers used in studies II and IV.

Study	Primer	Primer sequence 5'-3'	
		F	R
II, IV	**Human Seladin-1**	CAAGCCGTGGTTCTTTAAGC	CATCCAGCCAAAGAGGTAGC
II	**Human GAPDH**	GATCATTCAGCTCAGCAAACA	GTGAGGGTCTCTCTCTTCCT
IV	**Mouse GAPDH**	CGTCCCGTAGACAAAATGGT	TCTCAATGGTGGTGAAGACA
IV	**Mouse TNF**	CGAGTGACAAGCCTGTAGCC	GTGGGTGAGGAGCACGTAGT
IV	**Mouse LTCC**	TTCGATGTGAAGGCACTGAG	TATGCCCTCCTGGTTGTAGC
IV	**Mouse IDE**	TCCATACCACTGCTCTGTGC	TGGAGTGCTCACAGCTGAAT
IV	**Mouse NEP**	TCTTATCATCAGTGCCAACAAAA	AGGCGGACAACCTCTACTCA

4.6 PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS (STUDIES II, III AND IV)

Brain tissue samples in study IV were mechanically homogenized in 400 µl DPBS (Lonza) solution. Total proteins from both homogenized brain cells in study IV and human secondary cells in studies II and III were extracted by using the TPER extraction buffer (Pierce) containing the EDTA-free protease inhibitor cocktail (Thermo Scientific) or lysed in DDM buffer [0.5% *n*-dodecyl-β-*D*-maltodextrine (DDM; Alexis)] (study III). The DDM solubilized samples in study III were further incubated for 60 min at 4°C with constant rotation and the insoluble material was removed by 11000 × *g* centrifugation for 30 min, and membrane extracts were prepared as described earlier (Tuusa *et al.*, 2007).

Total protein concentrations of the lysates were quantified using either BCA (studies II, III and IV) or DC (study III) protein assays (Pierce and Bio-Rad, respectively) and, subsequently, 10 to 50 µg of each lysate were subjected to 4-12% Bis-Tris polyacrylamide gel electrophoresis (PAGE; Invitrogen) (studies II, III and IV) or to 10 or 14% SDS PAGE (Bio-Rad) (study III) and blotted onto Immun-Blot (Bio Rad) or Immobilon P (Millipore) polyvinylidene fluoride membranes. Primary antibodies used in studies II, III and IV are indicated in Table 10. After incubation with appropriate horse radish peroxidase (HRP) conjugated secondary antibodies (GE Healthcare), enhanced chemiluminescence substrate (Amersham Biosciences) was applied to membranes and detection of protein bands was performed with ImageQuant RT ECL camera (studies II and III) or by ECL hyperfilm (study III) (both GE Healthcare) scanned with a Umax Powerlook 1120 color scanner using the Image Master 2D Platinum 6.0 software (Amersham Bioscience). The band intensities were quantified using Quantity One (Bio Rad) (studies II and III) or Image J 1.41 (public domain, National Institute of Health) (study III) software. The housekeeping protein GAPDH was used to normalize the protein levels in each sample. In study IV, human SH-SY5Y-APP751 cell lysates were used as a control in protein fragment visualization.

4.6.1 Aβ and sAPP measurements

Aβ40 levels in the SH-SY5Y-APP751 cell culture media in study II and III were determined by using the human Aβ40 enzyme-linked immunosorbent (hAmyloid β40) ELISA assay kit (The Genetics Company). In study IV, soluble or insoluble Aβ x-40 and Aβ x-42 (Aβ40 and Aβ42) levels were measured from the total protein suspended in DPBS containing EDTA-free protease and phosphatase inhibitors (1:100). Aliquots in study IV were first ultracentrifuged (100000 × *g*, 50.4 Ti rotor; Beckman) for two hours at +4°C, and the supernatant (= soluble fraction) was collected. Subsequently, the remaining pellet was resuspended in guanidine buffer (5 M guanidine-HCl/50 mM Tris-HCl, pH 8.0), incubated for 2 hours at room temperature on a shaker, and diluted 1:50 in BSAT-DPBS [5% BSA (Bovine serum albumin)/0.03% Tween-20 in DPBS, pH 9.0] (BSA from Sigma) containing protease and phosphatase inhibitors. Finally, the suspension was centrifuged for 20 minutes at 15700 × *g* and the supernatant (= insoluble fraction) was collected for Aβ measurements. Both insoluble and soluble Aβ40 and Aβ42 levels were determined using monoclonal and HRP-conjugated antibody-based Human/Rat β Amyloid 40 (294-62501) and β Amyloid 42 (High-Sensitive; 290-62601) ELISA Kit (Wako). After a thirty-minute incubation at room temperature, the reaction was terminated and the absorbance was measured at 450 nm with an ELISA microplate reader (Wallac). Aβ40 levels in studies II and III were normalized to the total protein levels, which were determined from the lysates of the same cells from which the culture media had been initially collected. In study IV, Aβ concentrations were normalized to the tissue weights in each sample.

Soluble APPα (sAPPα; studies II, III and IV) (sAPPα; α-secretase cleaved N-terminal APP fragment) as well as total sAPP (sAPPtot = sAPPα + sAPPβ; β-secretase cleaved N-terminal APP fragment) levels were detected either from the tissue protein homogenates suspended in DPBS containing EDTA-free protease and phosphatase inhibitors (1:100; study IV) or from SH-SY5Y-APP751 cell culture media (studies II and III) using Western blot analysis with 6E10 (Signet Laboratories) and 22C11 (Mab348, Millipore) antibodies, respectively (Table 10). In study III, sAP-APP measurements from HEK293 AP-APP cells were performed as previously described (Hiltunen *et al.*, 2006). sAPP (studies II and III) and sAP-APP (study III) levels were subsequently normalized to the total protein levels, which were determined from the lysates of the same cells from which the culture media had been initially collected. In study IV, GAPDH normalized sAPP levels in the ipsilateral thalamus were compared relative to the contralateral thalamus (100%).

Table 10. Antibodies used in Western blotting in studies II, III and IV.

Study	Primary ab	Recognizes	[1]Clonality	Cat. no	[2]Dilution		Provider
					WB	IF	
II	seladin-1/ DHCR24 (C59D8)	seladin-1/ DHCR24	M	2033	1:800		Cell Signalling
II, IV	GGA3	GGA3	M	612310	1:2500		BD Transduction Labs
II, III	c-Myc (4A6)	c-Myc	M	05-724	1:1000	1:100 and 1:200	Millipore
III	A-14	c-Myc	P	sc-789	1:1000		Santa Cruz
III	9E10	c-Myc	M	sc-40	1:10000		Santa Cruz
III	M2	FLAG	M	F3165	0.5 µg/mL		Sigma
II	BACE1	BACE1	P	PA1-757	1:1000		ABR/ Thermo
II	Caspase-3	Caspase-3	P	9662	1:1000		Cell Signalling
II, III, IV	Alzheimer precursor protein (A8717)	APP C-terminus, C83, C89, C99, AICD	M	A8717	1:2000	1:250 and 1:1000	Sigma
III	AC-15	β-actin	M	A5441	1:15000		Sigma
III	Alzheimer precursor protein A4 (22C11)	APP N-terminus, sAPPtot	M	MAB348	1:1000	1:100	Millipore
III	β-amyloid (6E10)	sAPPα	M	SIG-39320	1:1000		BioSite (Signet)
II	GAPDH (6C5)	GAPDH	M	ab8245	1:15000		Abcam
IV	GFAP	Glial fibrillary acidic protein	M	G3893	1:1000		Sigma Aldrich
IV	Insulin-degrading enzyme	IDE C-terminus	P	ab32216	1:500		Abcam
IV	Neprilysin (AB5458)	NEP	P	ab5458	1:1000		Chemicon
IV	LRP (EPR3724)	Low density LRP	M	ab92544	1:1000		Abcam
II, III	LAMP2a	Lysosomes	P	ab18528		1:200	Abcam
II, III	EEA1 (clone 14)	Early endosomes	M	610456		1:200	BD Biosciences
III	Calnexin (clone C37)	Calnexin protein in ER	M	610523		1:100	BD Biosciences
III	LBPA (clone 6C4)	Lysobis-phosphatidic acid, MVB	M	Z-SLBPA		1:100	Echelon Biosciences
II, III	Transferrin (H68.4)	Transferrin receptor	M	13-6800		1:200	Zymed

[1]M, mouse monoclonal; P, rabbit polyclonal; [2]Dilutions for Western blotting (WB) and immunofluorescence (IF); ER, endoplasmic reticulum; MVB, multivesicular bodies.

4.7 IMMUNOPRECIPITATIONS (STUDY III)

Whole cell extracts containing equal amounts of protein were supplemented with 0.1% BSA and pre-cleared with mouse-IgG agarose. The pre-cleared samples were immunoprecipitated with the anti-FLAG M2 antibody affinity gel (Sigma). They were washed once with DDM buffer, twice with the same buffer containing 300 mM NaCl, twice with DDM buffer, and finally three times with DDM buffer containing 0.1%, instead of 0.5%, DDM. Bound proteins were eluted with 200 μg/ml FLAG peptide (Sigma). In the extract mixing experiments, the anti-FLAG M2 antibody affinity gel was used for the first immunoprecipitation step. After an overnight incubation, the resin was washed five times with DDM buffer, and the bound proteins were eluted with 1% SDS, 25 mM Tris-HCl pH 7.5, at +95°C for 5 min. The eluted samples were diluted 10-fold with DDM buffer supplemented with 0.1% BSA and used for a second immunoprecipitation with the anti-APP-CTF antibody (A8717) coupled to Protein A Sepharose (GE Healthcare), followed by washes as described above and elution with the SDS-PAGE sample buffer. Aliquots of immunoprecipitates and of whole cell extracts containing equal amounts of protein were separated in 10% SDS-PAGE and analyzed by Western blotting or fluorography as described previously (Tuusa *et al.*, 2007).

4.8 CALCIUM MEASUREMENT (STUDY IV)

Tissue homogenate samples (20 μl) were digested (CEM MDS 2000 microwave digestor, Akribis Metrology Ltd) in 100 μl suprapur nitric acid (Merck). After digestion, 100 μl 2% lanthanum solution (Riedel de Haen) was added and the samples were diluted to a volume of 1 ml with Milli Q water. Calcium measurements were carried out on a ZEEnit 700 atomic absorption spectrometer (Analytik Jena AG) with a calcium hollow cathode lamp at wavelength 422.7 nm using an air-acetylene flame and SFS6 injection module. Calcium concentrations were normalized to the tissue weights of each sample.

4.9 ENZYME ACTIVITY ASSAY (STUDIES II AND IV)

In study IV, α- and β-Secretase Activity Kits (FP001 and FP002, respectively; R&D Systems) were used to measure α- and β-secretase activities from the total protein lysates of thalamic tissue homogenates suspended in DPBS (without protein or phosphatase inhibitors) according to the manufacturer's instructions. Briefly, equal amounts of membrane protein fractions were incubated at +37°C for two hours with the secretase specific substrate peptides conjugated to fluorescent reporter molecules EDANS and DABCYL. Subsequently, emitted light (510 nm) was detected on a fluorescence microplate reader (Wallac) after EDANS excitation at 355 nm.

γ-Secretase activity was measured in study IV from the thalamic tissue homogenates as previously described (Farmery *et al.*, 2003). In brief, 60 μg of solubilized membrane protein preparation was incubated at 37°C overnight in 150 μl of assay buffer containing 50 mM Tris-HCl, pH 6.8, 2 mM EDTA, 0.7% CHAPSO (w/v), and 8 μM fluorogenic γ-secretase substrate (NMA-GGVVIATVK(DNP)- D R D R D R-NH 2, Cat nr. 565764, Calbiochem). At the same time, γ-secretase inhibitor L685,458 (Sigma) was used to validate the specificity of the γ-secretase activity assay. After incubation, the incubates were centrifuged at 15700 × *g*

for 10 min and transferred to a 96 well plate. Fluorescence was measured using a plate reader (Fluorstar Galaxy) with excitation wavelength at 355 nm and emission wavelength at 440 nm. Background fluorescence from substrate samples was subtracted in the final analysis.

In study II, caspase-3 activity assay was performed in total protein lysates in reaction mixtures containing 20 mM HEPES (pH 7.5), 10% glycerol, 2 mM dithiothreitol and Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) Fluorogenic Substrate (BD Pharmigen). After 60 min of incubation at +37 °C, production of AMC (7-amino-4-methylcoumarin), as an indication of caspase-3 activity, was measured at thirty-minute intervals for 60 min in a Victor 1420 multilabel counter 96-well plate ELISA reader (Wallac) using excitation wavelength of 380 nm, and emission wavelength of 460 nm. The data are shown as relative fluorescence units (RFU).

4.10 *IN VITRO* AICD GENERATION ASSAY (STUDY III)

For the *in vitro* AICD generation assay, a protocol similar to that described by Pinnix *et al.* was used (Pinnix *et al.*, 2001). Briefly, SH-SY5Y-APP751 cells were plated at a density of 200000 cells/cm^2. The cells were scraped in buffer A (50 mM HEPES, 150 mM NaCl, 5 mM 1,10-phenanthroline, pH 7.4) and homogenized by passing through a 25G 5/8 needle 10 times. The homogenates were centrifuged at 10000 × *g* for 15 min. The resulting membrane fractions (P10) were washed once with buffer A and centrifuged at 10000 × *g*. Total protein concentrations were measured in the P10 fraction, and the same amounts of protein in each sample were incubated in 30 µl of buffer B (50 mM HEPES, 150 mM NaCl, 5 mM 1,10-phenanthroline, pH 7.0 and the protease inhibitor cocktail; Roche) for 2 h on ice (as a negative control), or at +37°C to allow the release of AICD. The samples were then centrifuged at 10000 × *g* for 15 min. The supernatants and solubilized pellets were analyzed by Western blot using the anti-APP-CTF antibody (A8717) to detect APP C83 and AICD, respectively (Table 10).

4.11 STAUROSPORINE TREATMENT AND CELL VIABILITY ASSAY (STUDY II)

SH-SY5Y-APP751 and SH-SY5Y-BACE1-Myc cells were induced with apoptosis by treating the cells for 6 and/or 10 hours with 1 µM staurosporin (STS; Sigma). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Invitrogen) assay was used to assess cell viability after treatment of the cells with STS. After the addition of MTT, cells were incubated for 60 min at +37°C, after which the formazan granules were solubilized in 99.7% DMSO (Dimethyl sulfoxide, Hybri Max™, Sigma). Finally, cell viability was determined as the optical density (OD) at 595 nm with a Victor 1420 ELISA reader and expressed as percentage of untreated control cells.

4.12 FLOW CYTOMETRY (STUDY III)

Cell surface receptors expressed in stably transfected HEK293$_i$ cells after a 48-h induction were analyzed by flow cytometry as described earlier (Petaja-Repo *et al.*, 2006) using 1 μg/ml anti-c-Myc 9E10 antibody and 1 μg/ml phycoerythrin-conjugated secondary antibody (BD Biosciences). To assess constitutive internalization, the protocol devised by Markkanen and Petaja-Repo was applied (Markkanen and Petaja-Repo, 2008). Briefly, cell surface receptors on stably transfected HEK293$_i$ cells were labeled on 12-well plates (2 x 10^6 cells/well) with the anti-c-Myc 9E10 antibody (1 μg/ml), washed and pulsechased for 4 h at +37°C after a 24-h induction period. The c-Src inhibitor PP2 (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine) (4 μM) (see also Table 8) was added together with the antibody and was maintained throughout the incubations. The controls were treated with a vehicle (DMSO). The remaining antibody-labeled receptors at the cell surface were stained with the phycoerythrin-conjugated secondary antibody (2 μg/ml). The fluorescence of live cells was measured in a Becton-Dickinson FACSCalibur flow cytometer and analyzed by the CellQuestPro 6.0 software (both from BD Biosciences) as described previously (Markkanen and Petaja-Repo, 2008).

4.13 MICROSCOPY (STUDIES II AND III)

4.13.1 Confocal microscopy (Studies II and III)

In study II, SH-SY5Y-BACE1-Myc cells were plated on sterile coverslips coated with 100 μg/ml poly-D-lysine (Sigma), transfected with siRNA (*seladin-1*/*DHCR24* or control) and treated with or without STS. Similarly in study III, SH-SY5Y-APP751 cells were transfected with δOR-Cys27, δOR-Phe27 constructs, control pcDNA, or left untransfected. In order to inhibit PKC activity in study III, SH-SY5Y-APP751 cells were treated with 1 μM Bis I for 4 h. In the APP subcellular localization experiments (study III), SH-SY5Y-APP751 cells were plated on chamber slides (Lab-Tek) and transfected with EYFP-Golgi, EGFP-Rab7, EGFP-Rab9, or EGFP-Rab11 constructs (Choudhury *et al.*, 2002).

Next in both studies II and III, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature. After incubation in blocking and permeabilization buffer with 5% (also 0.5%, study III) BSA and 0.1% Triton X-100 (BHD Laboratory Supplies) in PBS (Invitrogen), the cells were stained with primary antibodies as indicated in Table 10 (see "Dilution" for IF). Alexa Fluor® 488 goat anti mouse, Alexa Fluor® 568 goat anti rabbit (Invitrogen; 1:250 or 1:500), Cy3 sheep anti-mouse (C 2181, Sigma; 1:500) (studies II and III), or Cy5 goat anti rabbit (Jackson ImmunoResearch Laboratories; 1:1000, study III), were used as secondary antibodies. Single optical *z*-sections were examined in a Nikon Eclipse TE300 microscope together with the Ultra VIEW laser scanning confocal unit (Perkin Elmer) or, alternatively with Carl Zeiss LSM 700 or LSM510 confocal laser scanning microscopes. The images were processed with Adobe Photoshop™ CS4 software (version 11.0; Adobe Systems Inc., USA).

4.13.2 Electron microscopy (study III)

Induced (24 h) or mock-induced HEK293$_i$-hδOR-Cys27 cells were rinsed with phosphate buffered saline and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer containing 2.5% sucrose. The cryosectioning, immunolabeling and imaging were done as described

earlier (Apaja *et al.*, 2006) with minor modifications. Antibodies against LBPA and APP C-terminus (Table 10) were used for immunodetection, followed by incubations with Protein A-conjugated 5 nm and 10 nm of gold prepared as described by Slot and Geuze, respectively (Slot and Geuze, 1985). The sections were embedded in methylcellulose and examined in Philips CM100 (FEI company). Images were captured by Morada CCD camera (Olympus Soft Imaging Solutions GMBH).

4.14 ANIMAL EXPERIMENTS (STUDY IV)

4.14.1 Animals and MCAO

In study IV, thirty-one male Wistar rats (age 2-3 months) from Laboratory Animal Centre, Kuopio, were induced by either MCAO, (n=23) or sham operation (n=8). The rats were housed under 12:12 h light/dark conditions in a temperature-controlled environment (20±1 °C). Food and water were available ad libitum. All experimental procedures were conducted in accordance with the directives of Commission of the European communities COM(2008)543 and the study was approved by the National Animal Experiment Board (Licence nr. ESLH-2008-07622/Ym-23).

Focal cerebral ischemia was induced by the intraluminal filament technique (Longa *et al.*, 1989). Anesthesia was induced in a chamber using 5% halothane in 30% O_2/ 70% N_2O. In the surgery, the filament was inserted into the internal common carotid artery and held in place with a tightened suture and microvascular clip around the artery. After 120 min of MCA occlusion, the filament was removed and the external carotid artery was permanently closed. The sham-operated rats were treated in a similar manner excluding the filament insertion.

4.14.2 Drug treatment and behavioral experiments

Two days after surgery, MCAO animals were assigned to behaviorally equivalent experimental groups: MCAO (vehicle treatment, n = 7), MCAO+BEP (50 mg/kg bepridil treatment, n = 5) and SHAM (vehicle treatment, n = 6) for 27 days. Animals were subjected to three different behavioral tests as described in Figure 13.

i) Limb-placing test
The modified version of the limb-placing test was used to assess hindlimb and forelimb responses to tactile and proprioceptive stimulation (Jolkkonen *et al.*, 2000; Puurunen *et al.*, 2001). The rats were habituated for handling and tested before ischemia induction and then again on postoperative days 2, 4, 7, 10, 14, 21 and 28.

ii) Cylinder test
The cylinder test was used to assess imbalances between the impaired and nonimpaired forelimb use (Karhunen *et al.*, 2003). The number of contacts by both forelimbs and by either impaired or unimpaired forelimb was counted. The cylinder test was carried out on the day before the MCAO operation and on postoperative days 14 and 28.

iii) Tapered/ledged beam walking test

Sensorimotor functions of hindlimbs were tested using a tapered/ledged beam (Zhao *et al.*, 2005). The rats were pretrained for 3 days to traverse the beam before ischemia induction. The beam walking apparatus consisted of a tapered beam with underhanging ledges on each side to permit foot faults without falling. Each rat's performance was analyzed by calculating the slip ratio for the impaired (contralateral to lesion) forelimb and hindlimb. The test was carried out one day before the MCAO operation and on postoperative days 14 and 28.

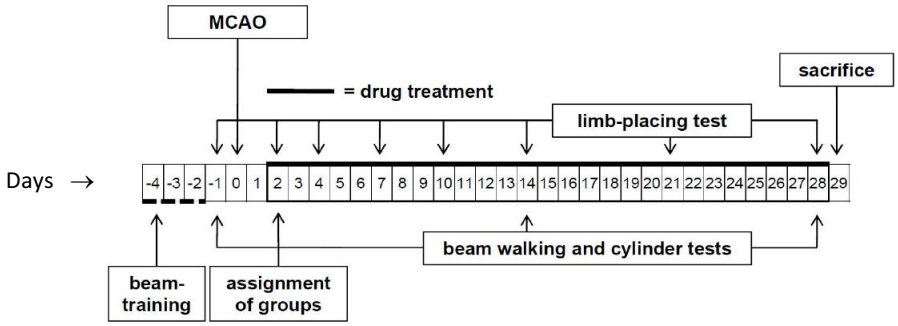

Figure 13. Study design in study IV.

4.15 STATISTICAL ANALYSES

Genetic analyses, studies I and III

Single locus allele and genotype frequencies utilizing two-sided Pearson X^2 and Fisher's exact tests, logistic regression, and Kaplan-Meier survival analyses were performed to assess disease association and age of onset effects with the studied SNPs among the unstratified case-control cohort. Additionally, multivariate logistic regression analysis was applied to assess age, gender and *APOE* ε4 adjusted risk effects. In study I, non-parametric Mann-Whitney *U* test (equal variances not assumed) was used to investigate possible differences in CSF Aβ42, t-tau, or p-tau levels with respect to the studied SNPs. Bonferroni correction was not applied in study I, since this study was based on the previously described risk gene polymorphisms studied in the context of AD (see more in Discussion).

In study III, Hardy-Weinberg equilibrium of SNPs in the AD patient and control cohorts was calculated using the GenePop program (http://genepop.curtin.edu.au/genepop/genepop_ op1.html). Pairwise LD values and estimation of haplotypes and haplotype blocks were performed with the HaploView program (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview). Association analyses in the NIMH GWAS data set in study III were conducted using PBAT v3.6 as previously described (Bertram *et al.*, 2008). Effect sizes in the family data were estimated in SAS v9.2 using conditional logistic regression stratified by family as described previously (Schjeide *et al.*, 2009). Meta-analyses were based on random-effect models and were performed in R programming

language, version 2.10.0, using rmeta, version 2.16. Final results are based on the meta-analyzed data combined from two independent, familial and case-control sample sets.

Functional quantifications, studies II, III and IV

The GraphPad Prism 4.01 software was used for one-way analysis of variance (ANOVA) in study I, followed by Bonferroni's post-hoc test, in experiments with more than one variable. In all other studies, the SPSS/Win statistical program (version 14.0.1) was used to perform statistical analyses. In experiments with only one variable, paired *t* tests, independent-sample *t* tests (equal variances assumed), or Mann-Whitney *U* tests (equal variances not assumed) were used to test statistical significance between the sample groups. The values are reported as means ± standard deviations (SD). The level of statistical significance was defined as $p < 0.05$.

5 Results

5.1 META-ANALYSIS-BASED ASSESSMENT OF RISK GENES IN FINNISH CASE-CONTROL COHORT: STUDY I

Over the past 30 years, nearly 1400 studies have assessed almost 700 different genes as potential risk factors for AD, mostly using the candidate gene-based approach (Bertram *et al.*, 2007; Bertram and Tanzi, 2008). However, with the exception of one genetic variant, the ε4 allele of *APOE* gene (Strittmatter *et al.*, 1993; Saunders *et al.*, 1993), none of these candidates has been proven to consistently influence the disease risk or onset age in different populations. As most of the AD cases represent the more complex, LOAD form of the disease and *APOE* ε4 alone is estimated to explain only a fraction of this form, further efforts are needed to reveal other possible genetic risk factors for the disease.

To assess the risk gene effects in Finnish AD case-cohort cohort, 21 candidate gene polymorphisms were selected for genotyping on the basis of the meta-analyses retrieved from the AlzGene database. The distribution of *APOE* ε2/3/4 alleles was 0.04/0.80/0.16 and 0.01/0.54/0.45 in control and AD groups ($p < 0.001$), respectively, indicating that the *APOE* ε4 allele was significantly over-represented among the AD patients ($p < 0.001$, OR = 6.00; 95% CI 4.73 to 7.61). All the genotyped SNPs were found to be in Hardy-Weinberg equilibrium (HWE) in both AD and control groups, except for the SNP rs760678. As a control measure, ~4% of the randomly selected DNA samples were cycle-sequenced at the rs760678 site and a subsequent comparison of the results did not reveal discrepancies in the genotype distribution between two genotyping approaches.

The results indicated that there was an association especially of two additional genes with AD. A statistically significant genotype and allele association with AD was observed with rs1800629 in the *TNF* gene. *APOE*, gender and age-adjusted logistic regression analysis revealed a protective effect for the A allele carriers of rs1800629 ($p = 0.02$; OR = 0.69, 95% CI 0.49-0.95). In addition, a significant difference we found in genotype but not in allele distribution between the AD and control groups with rs1143634 in the *IL1B* gene.

Next, it was decided to assess the possible effects of the studied SNPs on CSF Aβ42, t-tau, or p-tau levels among a subset of AD patients. Interestingly, there was a significant decrease in the CSF Aβ42 levels (p=0.002) among AD patients carrying the A allele (GA+AA vs. GG) of rs1800629 in *TNF* gene, while the T allele carriers (CT+TT vs. CC) of rs1143634 in *IL1B* gene associated with increased CSF p-tau levels ($p = 0.05$). Moreover, AD patients carrying the A allele (GA+AA vs. GG) of rs600879 in *SORCS1* gene exhibited a statistically significant increase in the CSF Aβ42 levels ($p = 0.03$), but *SORCS1* did not show statistically significant allele or genotype association with AD. Kaplan-Meier survival analyses with the studied SNPs did not reveal significant age of onset effects among AD patients.

In summary, AlzGene-based top hit screening in study I revealed two proinflammatory genes, *TNF* and *IL1B*, which genetically associated with AD and altered CSF biomarker levels among Finnish population. In addition to being important players in the innate inflammatory response induced by factors, such as Aβ, the current study points to the potential underlying role of genetic alterations in the *TNF* and *IL1B* genes in affecting the susceptibility for AD.

5.2 DOWN-REGULATION OF SELADIN-1 INCREASES BACE1 LEVELS AND ACTIVITY THROUGH ENHANCED GGA3 DEPLETION DURING APOPTOSIS: STUDY II

Seladin-1 is a neuroprotective protein selectively down-regulated in brain regions affected in AD. Seladin-1 is also known to protect cells against Aβ42- and oxidative stress-induced apoptosis activated by caspase-3, a key mediator of apoptosis. Study II utilized RNA interference (RNAi) to assess the molecular effects of seladin-1 down-regulation on the BACE1 function and APP processing in SH-SY5Y human neuroblastoma cells in both normal and apoptotic conditions.

Down-regulation of seladin-1 in SH-SY5Y-APP751 cells (siSel) showed an average of a 1.3-fold increase in APP C-terminal fragment (C83 and C99) levels, although the levels of secreted APP (sAPPα and sAPPtot) and Aβ40 remained unchanged. During staurosporin (STS)-induced apoptosis, down-regulation of seladin-1 mRNA levels by approximately 60% resulted in augmented caspase-3 activity, as indicated by the 1.5 to 2.0-fold increase in active caspase-3 cleavage fragments (~14 and 17 kDa) This, in turn, correlated with 30 to 40% decrease in full-length GGA3 protein levels compared with those in control cells.

Since GGA3 is known to target BACE1 to the lysosomes for degradation, it was decided to assess whether the decrease in GGA3 levels affected BACE1 protein levels and activity in SH-SY5Y-BACE1-Myc cells stably over-expressing BACE1. Similar to SH-SY5Y-APP751 cells, STS treatment led to augmented caspase-3 activity as well as decreased full-length GGA3 levels in cells with seladin-1 down-regulation compared to the levels in cells transfected with control siRNA (siCtrl). Importantly, these alterations coincided with an average of 1.5-fold increase in BACE1 levels. Furthermore, no differences in BACE1 or full-length GGA3 protein levels were found between siSel- and siCtrl-transfected cells under normal growth conditions. Moreover, cleaved caspase-3 fragments were not observed under normal conditions.

Next, it was to investigated whether the observed increase in BACE1 levels led to enhanced amyloidogenic APP processing under apoptotic conditions. It was discovered that seladin-1 knockdown increased the production of APP β-CTF and Aβ40 levels in SH-SY5Y-BACE1-Myc cells, while C83 levels remained undetectable. Furthermore, a significant decrease in cell viability (~30%) was observed after seladin-1 knockdown as compared to siCtrl-transfected cells.

Finally, confocal microscope analysis showed that more BACE1-Myc was co-localizing with TfR (transferrin receptor), a marker for early endosomes and the plasma membrane, in siSel-transfected cells than in siCtrl-transfected cells. This colocalization was observed in the subcellular compartments near or at the plasma membrane. A similar result was also obtained using co-staining with an antibody against EEA1, another marker for early endosomes (data not shown). In addition, some co-localization of BACE1-Myc with LAMP2a (a lysosomal marker) was detectable in intracellular structures, most likely lysosomes, in cells transfected with siCtrl. This is in line with previous reports on BACE1 subcellular localization of BACE1 (Huse *et al.*, 2000). However, co-localization of BACE1-Myc with LAMP2a appeared to decrease during apoptosis. In addition, more BACE1-Myc seemed to reside near or at the plasma membrane in siSel- than in siCtrl-transfected cells under apoptotic conditions. Hence, the depletion of GGA3 levels due to seladin-1

knockdown may have affected the BACE1 subcellular localization in SH-SY5Y-BACE1-Myc cells during apoptosis.

Taken together, these findings in study II suggest that even a moderate decrease in seladin-1 expression may increase caspase-3 activity. This appears to sensitize cells to apoptosis and consequently to augment BACE1 levels and activity through depletion of the BACE1-sorting protein, GGA3. Therefore, seladin-1 may play an important role in the early phases of the disease and prove to be a potential biomarker for AD.

5.3 CYSTEINE 27 VARIANT OF δ-OPIOID RECEPTOR AFFECTS APP PROCESSING THROUGH ALTERED ENDOCYTIC TRAFFICKING: STUDY III

There is recent evidence indicating that agonist-induced activation of δOR can contribute to AD pathogenesis by affecting Aβ generation *in vitro* and *in vivo* (Ni *et al.*, 2006; Teng *et al.*, 2010). Moreover, it has been demonstrated that two different forms of δOR – the δOR-Phe27 variant and the less common δOR-Cys27 variant – significantly differ in receptor maturation and intracellular localization (Leskela *et al.*, 2009). Hence, study III examined whether these two variants differentially affect APP processing and Aβ generation when expressed in agonist-independent conditions in *in vitro*. In this way, the genetic role of *OPRD1* in AD could be clarified by assessing the risk effect of T80G variation (rs1042114) in both case-control and familial AD sample sets.

Transient overexpression of the N- and C-terminally tagged δOR-Cys27 variant led to a robust accumulation of APP C83 and AICD, while total sAPP and particularly Aβ40 levels were decreased. Nevertheless, the increase in APP C83 levels was not related to the augmented α-secretase activity. As total protein-normalized Aβ40 levels were significantly decreased in the δOR-Cys27-transfected cell medium, the *in vitro* AICD generation assay did not reveal any changes in the γ-secretase activity in SH-SY5Y-APP751 cells after δOR transfection. There were no significant alterations in sAPPα and APP C99 CTF levels, suggesting that δOR-Cys27 overexpression does not lead to any general enhancement of APP processing. In addition to these findings, confocal microscope analysis of SH-SY5Y-APP751 cells showed a markedly augmented intracellular localization of the δOR-Cys27 variant as compared to δOR-Phe27 variant. This is in line with the previous observations that the δOR-Cys27 variant undergoes constitutive internalization (Leskela *et al.*, 2009). The co-immunoprecipitation assay did not detect evidence for the formation of a stable complex between APP and δOR variants in HEK293i cells, suggesting that APP and δOR may not be interacting directly, but instead could reside in the same complexes via an interaction with another protein(s).

As the δOR variants significantly differed in receptor maturation when expressed in SH-SY5Y-APP751 cells, the next experiment assessed whether the observed changes in APP processing in the δOR-Cys27-expressing cells were related to the inefficient receptor maturation. After inducing HEK293$_i$-δOR-Cys27 cells with saturating concentrations of different pharmacological δOR ligands, it was found that 48-h treatment with some ligands (naltrexone and ICI 174,864) significantly suppressed the δOR-Cys27-induced increase in APP C83 levels. However, this occurred in a manner that was not directly related to their ability to enhance receptor maturation.

The observed APP processing phenotype in the δOR-Cys27-overexpressing cells appears to be related to the possibly altered nature of mature receptors that have reached the cell surface. Thus, it was next examined whether specific δOR-linked signal transduction pathways might be involved. After screening of several GPCR inhibitors, we found that the c-Src tyrosine kinase inhibitor PP2 was found to strongly suppress the increase of APP C83 levels and the constitutive receptor internalization in HEK293$_i$-δOR-Cys27 cells. In addition, flow-cytometry revealed that PP2 treatment had increased the number of the δORs at the cell surface.

Similar to pH-neutralizing conditions (Vingtdeux *et al.*, 2007), inhibition of PKC is known to alter the endocytic trafficking of internalized cell surface proteins to the perinuclear area (Kermorgant *et al.*, 2003). Therefore, it was interesting that in the screen of inhibitors of GPCR signaling molecules, Bis I, a specific inhibitor of all PKC isoforms, was found to change APP processing in a manner that closely mimicked that evoked by δOR-Cys27 overexpression. Exposure of uninduced HEK293$_i$-δOR-Cys27 cells and parental HEK293$_i$ cells to increasing concentrations of Bis I led to the accumulation of APP CTFs and AICD in a concentration-dependent manner without an increase in the sAPPα levels. As expected, an increased sAPPα secretion was observed when PKC was activated by PMA (Racchi *et al.*, 2003). These results suggest that the δOR-Cys27 expression-induced, agonist-independent, increase in the APP C83 levels is related to disturbances in a constitutive cellular process(es) rather than to PKC-dependent or -independent induction of the nonamyloidogenic APP processing as described for several other GPCRs upon agonist-mediated activation (Camden *et al.*, 2005; Nitsch *et al.*, 1992, 1993).

Confocal microscopy revealed that inhibition of PKC caused a robust dispersion of the APP-containing vesicles to the cell periphery in the SH-SY5Y-APP751 cells, as detected by an antibody specifically recognizing APP C-terminus. Hence, it is conceivable that the APP processing phenotype in cells expressing δOR-Cys27 under agonist-independent conditions might be related to impairment in PKC-dependent cellular trafficking. This is in line with the concept that altered processing and impaired trafficking of APP may be coupled.

APP C83 has previously been shown to accumulate under pH-neutralizing conditions. This was proposed to take place because of reduced degradation of C83 in the LEL compartment as well as through partial α-secretase-mediated conversion of C99 to C83 (Waldron *et al.*, 2008). Therefore, it was decided to elucidate whether overexpression of the δOR-Cys27 variant could affect the subcellular localization of APP CTFs in SH-SY5Y-APP751 cells. To results of confocal microscopy using an antibody against APP C-terminus found that APP CTFs mostly localized in a compact juxtanuclear structure with a partial localization at or near the plasma membrane in the δOR-Phe27-expressing and untransfected cells. In the δOR-Cys27-overexpressing cells, however, APP was localized ubiquitously, suggesting that APP had dispersed away from the juxtanuclear compartment(s) in δOR-Cys27-transfected cells. Co-staining of the untransfected cells with APP N- and C-terminal antibodies confirmed the suspicion that the APP staining in the juxtanuclear structure had originated mainly from the APP CTFs and not from the full-length form of APP. Subsequent characterization revealed that APP CTFs predominantly co-localized with LEL in untransfected SH-SY5Y-APP751 cells. Partial colocalization was also observed with a marker of the Golgi compartment. Quantification showed that there was on average 60% less colocalization of APP with δOR-Cys27 than with the δOR-Phe27 variant ($p < 0.05$) in the juxtanuclear structures. In δOR-Cys27-transfected cells, APP CTFs were also found to partially co-localize with the lysosomal marker LAMP2a and the multivesicular body (MVB) marker lysobisphosphatidic acid (LBPA). Moreover, immunoelectron microscopy in HEK293 cells showed an increased number of LBPA-positive vesicle clusters with or

without the MVB-type outer membrane in cells expressing δOR-Cys27 compared to control cells. Interestingly, APP CTFs were abundantly concentrated in these vesicles. These vesicles may represent intermediates which have failed to mature and/or move to the LEL/Golgi compartments. Taken together, the δOR-Cys27variant appears to interfere with the normal subcellular trafficking of APP, possibly via affecting PKC-mediated intracellular signaling.

Finally, it was decided to assess whether or not genetic variations in *OPRD1* affect the risk for AD in case-control and family-based (GWAS) AD cohorts. According to the allele and genotype association analyses of SNPs (rs1042114, rs678849, rs706588, rs4654323, and rs4654323), only rs1042114 showed a borderline genotype association with AD ($p = 0.07$). This was caused by TG heterozygotes (δOR-Phe27Cys), who were marginally more frequent in AD cases vs. controls [$p = 0.07$; OR = 2.5, 95% CI (0.94-6.50)] (Table 11). The age of onset and cerebrospinal fluid Aβ42 analyses with respect to the rs1042114 variation did not reveal any statistically significant changes among the AD patients. In an attempt to independently replicate the results observed with rs1042114, it was decided to extract this SNP from a GWAS dataset of Caucasian AD families from the National Institute of Mental Health (NIMH) Genetics Initiative Study (Bertram *et al.*, 2008). Since the rs1042114 variant was not included in the Affymetrix 500K GWAS array, genotypes of this SNP were inferred by imputation via IMPUTE v2.0 using HapMap CEU and 1000 Genomes Project pilot release data. In these independent, family-based data observed an overtransmission of the heterozygous TG genotype was detected in the affected individuals ($p = 0.021$). Using conditional logistic regression stratified on family, this translated into an OR of 1.62 (95% CI: 1.00-2.64). Combining these data with the case-control results via random-effects meta-analysis yielded an OR 1.77 (95% CI: 1.14-2.74; $p = 0.011$). Collectively, these data suggest that the Phe27Cys variation in δOR may play a role in AD.

Table 11. Summary of allele and genotype frequencies of OPRD1 SNPs among Finnish AD patients and controls.

[a]SNP and location (kb)	Allele Frequency				Genotype Frequency			
	Allele	Controls	AD	[b]p-value	Genotype	Controls	AD	[b]p-value
rs1042114 (0)		n=1324	n=1002			n=662	n=501	
					GG	0.024	0.012	
	T	0.875	0.865	0.47	TG	0.201	0.246	0.07
	G	0.125	0.135		TT	0.775	0.743	
rs678849 (6.2)		n=1340	n=1062			n=670	n=531	
					TT	0.209	0.271	
	T	0.534	0.518	0.44	TC	0.515	0.493	0.54
	C	0.466	0.482		CC	0.276	0.235	
rs706588 (23.6)		n=1340	n=1064			n=670	n=532	
					AA	0.460	0.438	
	A	0.678	0.675	0.88	AG	0.436	0.474	0.36
	G	0.322	0.325		GG	0.104	0.088	
rs4654323 (30)		n=1340	n=1062			n=670	n=531	
					TT	0.090	0.079	
	G	0.701	0.699	0.88	TG	0.418	0.444	0.60
	T	0.299	0.301		GG	0.493	0.476	
rs4654323 (30)		n=1342	n=1066			n=671	n=533	
					CC	0.306	0.287	
	C	0.569	0.540	0.17	AC	0.526	0.507	0.24
	A	0.431	0.460		AA	0.168	0.206	

[a]*Location of SNPs are indicated in the 5' to 3' orientation with respect to SNP rs1042114.*
[b]*Allele and genotype frequencies were compared using two-sided Pearson´s $\chi 2$ test. All the studied SNPs were in Hardy-Weinberg equilibrium in both cases and controls ($p < 0.05$).*

5.4 BEPRIDIL DECREASES Aβ AND CALCIUM LEVELS IN THALAMUS AFTER MIDDLE CEREBRAL ARTERY OCCLUSION IN RATS: STUDY IV

AD and cerebral ischemia share similar features in terms of altered APP processing and Aβ accumulation. It was previously shown that Aβ and calcium deposition and β-secretase activity are robustly increased in the ipsilateral thalamus after transient MCAO in rats (van Groen *et al.*, 2005; Hiltunen *et al.*, 2009). Since there exists an intimate link between Aβ and calcium in AD pathology (Supnet and Bezprozvanny, 2010; Demuro *et al.*, 2010), it was decided to investigate in study IV whether the non-selective calcium channel blocker bepridil could affect thalamic accumulation of Aβ and calcium and in turn improve the functional recovery in rats subjected to MCAO. Interestingly, bepridil is also able to inhibit β-secretase cleavage of APP *in vitro* and *in vivo* (Mitterreiter *et al.*, 2010).

Consistent with the previous findings (van Groen *et al.*, 2005; Makinen *et al.*, 2008; Hiltunen *et al.*, 2009), soluble Aβ40 and Aβ42 levels were significantly increased in the ipsilateral thalamus of MCAO rats. Bepridil treatment decreased both soluble Aβ40 and Aβ42 levels in the ipsilateral thalamus by 1.8- and 2.3-fold, respectively, when compared to vehicle-treated MCAO rats. However, bepridil treatment did not affect the levels of soluble Aβ40 and Aβ42 or insoluble Aβ42 in the contralateral thalamus when compared to vehicle-treated or sham-operated rats.

Enhanced accumulation of Aβ has been shown to coincide with increased calcium levels in the ipsilateral thalamus of MCAO rats (Makinen *et al.*, 2008; Hiltunen *et al.*, 2009). Here, a robust increase was also observed in the calcium levels in the ipsilateral thalamus when compared to the contralateral side in MCAO rats. Importantly, calcium levels in the ipsilateral thalamus were significantly reduced (~3-fold) in MCAO rats treated with bepridil. When compared to the sham-operated rats, however, calcium levels in the ipsilateral thalamus of bepridil-treated MCAO rats were still significantly higher, indicating that bepridil treatment was not able to fully normalize the calcium levels. Conversely, no difference in calcium levels was observed in the contralateral thalamus between vehicle- and bepridil-treated or sham-operated rats. Correlation analysis revealed a positive correlation between Aβ42 and calcium ($r = 0.85$, $p < 0.001$) and Aβ40 and calcium ($r = 0.74$, $p < 0.001$) levels in the ipsilateral thalamus.

It was next investigated whether bepridil-related changes were linked to altered expression and/or processing of APP in the ipsilateral thalamus. Consistent with the previous findings (Hiltunen *et al.*, 2009), a significant increase (~1.4 fold) in CTF levels was observed in the ipsilateral thalamus when compared to contralateral thalamus after MCAO. Moreover, total APP (=APP695 immature + APP695 mature) levels were significantly decreased. In the bepridil-treated MCAO rats, however, there were no significant APP-related alterations in the ipsilateral thalamus. In addition, the GGA3 protein levels declined by about half concomitantly with an increase (~1.8-fold) in BACE activity in the ipsilateral thalamus of both vehicle- and bepridil-treated MCAO rats. Similarly to the apoptotic conditions in study II and data from Tesco *et al.* (Tesco *et al.*, 2007), the reduction of full-length GGA3 protein levels coincided with the increase in GGA3-specific cleavage product of ~50 kDa in both vehicle- and bepridil-treated samples. This suggests that full-length GGA3 had been proteolytically cleaved by some yet unknown protease(s). These changes were not observed in the ipsilateral thalamus of sham-operated rats. α- and γ-secretase activities were not significantly altered in the ipsilateral thalamus of vehicle- or bepridil-treated MCAO rats.

The levels of sAPP were measured to further assess possible APP-related alterations. Total sAPP levels were significantly increased, particularly in the ipsilateral thalamus of vehicle-treated MCAO rats (~2.3 fold), but also in the bepridil-treated MCAO rats (~1.8 fold). Moreover, sAPPtot levels showed a positive correlation with BACE activity in the ipsilateral thalamus ($r = 0.55$, $p < 0.05$; data not shown). Since a cell culture medium sample from SH-SY5Y-APP751 cells was used as a control in the Western blots, it was possible to explore the isoform expression profiles of sAPPtot in the thalamic samples. In addition to the expected elevation of total sAPP695, in the ipsilateral thalamus of vehicle- and bepridil-treated MCAO rats there was the appearance of an APP-specific band, which was similar in size as the total sAPP751 in the SH-SY5Y-APP751 control sample. This is in line with the previous findings showing an up-regulation in the APP751 isoform-specific expression in the ipsilateral thalamus after 30 days of MCAO (Hiltunen *et al.*, 2009). Taken together, these results suggest that the treatment of MCAO rats with bepridil had affected APP processing in the ipsilateral thalamus in a manner that may not have involved alterations in α-, β-, or γ-secretase activities.

Apart from the altered APP processing and Aβ production, it is also possible that alterations in Aβ degradation and/or clearance account for the observed decrease in Aβ levels in bepridil-treated MCAO rats. To address this possibility, it was decided to investigate the protein levels of IDE, NEP, and LRP, which are involved in the degradation and clearance of Aβ. Similar to our previous observations (Hiltunen *et al.*, 2009), IDE levels were significantly increased in the ipsilateral thalamus of vehicle-treated MCAO rats.

Bepridil treatment, however, did not affect IDE levels. NEP and LRP levels were unchanged in the ipsilateral thalamus of vehicle- and bepridil-treated MCAO rats as well as sham-operated rats.

Since MCAO promotes astrogliosis in the rat thalamus (Hiltunen *et al.*, 2009; van Groen *et al.*, 2005), it was decided next to assess the effects of bepridil treatment on the inflammatory response by determining the levels of GFAP and TNFα expression in the ipsilateral thalamus. Western blot analysis revealed a strong ~10 fold increase in GFAP protein expression in the ipsilateral thalamus of vehicle-treated MCAO rats. Similarly, a robust ~3.5 fold increase in the GAPDH-normalized TNFα mRNA levels was observed in the ipsilateral thalamus of vehicle-treated MCAO rats. However, bepridil did not prevent the up-regulation of these markers of astrogliosis and inflammation after MCAO. Taken together, these results suggest that bepridil treatment does not affect the expression of well-known regulators of Aβ degradation or clearance nor alleviate astrogliosis or TNFα activation in the ipsilateral thalamus of MCAO rats.

Given the putative neuroprotective role of seladin-1 in study II (see also chapter 2.3.1.3), it was next decided to assess whether seladin-1 mRNA levels were altered in the ipsilateral thalamus of MCAO rats. Quantitative PCR analysis revealed a significant ~40% decrease in seladin-1 mRNA levels in the ipsilateral thalamus of MCAO rats. Interestingly, no similar kind of decline in seladin-1 mRNA levels was observed in bepridil-treated MCAO rats. Instead, a trend towards a statistically significant difference ($p = 0.057$) in seladin-1 mRNA levels in the ipsilateral thalamus was observed in the bepridil-treated MCAO rats as compared to vehicle-treated rats. Correlation analyses were conducted between seladin-1 and calcium levels as well as seladin-1 and Aβ levels; there was a significant inverse correlation between seladin-1 mRNA and calcium ($r = -0.70$, $p < 0.01$) as well as with insoluble Aβ42 levels ($r = -0.64$, $p < 0.05$), suggesting that the reduction in seladin-1 expression was associated with the thalamic pathology emerging after MCAO.

To elucidate the underlying molecular mechanisms related to the reduced calcium levels in bepridil-treated MCAO rats, it was decided to determine the mRNA levels of voltage-dependent L-type calcium channel subunit α-1C (LTCC) in the thalamus. The rationale for this experiment originated from a previous study, which showed that bepridil treatment could change the LTCC expression in canine atria (Nishida *et al.*, 2007). A significant decrease in LTCC mRNA levels was observed in the ipsilateral thalamus of rats with MCAO but there was no difference between vehicle- and bepridil-treated MCAO rats. Collectively, these data suggest that bepridil treatment could increase seladin-1 but not LTCC levels in the ipsilateral thalamus of MCAO rats. Thus, increased seladin-1 expression could be a reflection of improved neuronal survival in the ipsilateral thalamus of MCAO rats after bepridil treatment.

Sensorimotor impairment and recovery of function after MCAO was assessed in three behavioral tests (see 4.14.2). There was no significant difference between the treatment goups in forelimb use. However, ANOVA for repeated measures showed a significant group × time interaction ($p < 0.01$), indicating that sensorimotor recovery did differ between the groups. A more detailed analysis revealed that bepridil-treated MCAO rats increased the use of the impaired forelimb on post-operative day 28 compared to vehicle-treated MCAO rats. The limb-placing test was used to assess forelimb and hind limb responses to tactile and proprioceptive stimulation. The test showed that MCAO rats were initially severely impaired, but this was followed by partial recovery. The significant overall group effect was explained by the difference between sham-operated and MCAO rats throughout the follow-up ($p < 0.01$). Test results in the vehicle treated and bepridil-treated MCAO rats

did not differ from each other. Tapered/ledged beam-walking test was used as a measure of hind limb function. The special feature of the test is that the ledge allows the rat to display a deficit normally hidden by compensatory adjustment. All rats showed an increase in slips made (foot faults) with no apparent recovery after MCAO. A significant overall group effect was reflected in the MCAO rats making significantly more slips compared to sham-operated rats ($p < 0.05$). There was no difference between vehicle- and bepridil-treated MCAO rats during the follow-up. All in all, the behavioral tests demonstrated a slight functional improvement in MCAO rats after treatment with bepridil that may have been associated with reduced thalamic pathology.

6 Discussion

6.1 AN ASSOCIATION STUDY OF AD RISK GENES IN A FINNISH POPULATION: STUDY I

Study I revealed polymorphisms in two inflammatory response-related genes, *TNF* and *IL1B*, which significantly associated with AD and altered CSF biomarker levels. Several top AlzGene hits were also studied, such as *ABCA7, APOE*, *BIN-1, CALHM1*, *CLU*, *CR1*, *CST3*, *GRN*, *LDLR*, *MS4A6A*/*MS4A4E*, and *PICALM* among the Finnish population (Lehtovirta *et al.*, 1996; Lamsa *et al.*, 2008; Bertram *et al.*, 2008; Lambert *et al.*, 2009; Helisalmi *et al.*, 2009; Viswanathan *et al.*, 2009; Hollingworth *et al.*, 2011). The current study on AlzGene-based top hit screening of 21 potential risk genes emphasized that genetic alterations in *TNF* and *IL1B* genes may play an important role in AD. This was an interesting finding considering that the cytokines TNFα and interleukin 1β are key mediators of inflammation and apoptosis during neurodegeneration in AD. They also modulate synaptic memory mechanisms, such as LTP (Tarkowski, 2002; Rowan *et al.*, 2007).

Since the variation rs1800629 in *TNF* gene displayed a significant variation in all the parameters of the study [i.e. difference in genotype and allele frequency between patients and controls, as well as in risk ratio (OR) among AD patients], we examined this gene more closely in the context of AD. The rs1800629 polymorphism in *TNF* has been indicated to associate with AD risk also in other studies. The mechanism of how this particular polymorphism may affect *TNF* gene function could be mediated by transcriptional regulation, since the polymorphic site of rs1800629 is located at the promoter region (−308) of the *TNF* gene. In the genetic analysis, the A allele carriers of *TNF* showed protective effect against AD. In another study, the A allele carriers were shown be more likely subjects with reduced *TNF* mRNA levels during severe sepsis (O'Dwyer *et al.*, 2008). Furthermore, it was recently shown that high serum TNF levels accompanied by systemic inflammatory events (SIE) in AD patients associated with a more pronounced cognitive decline during a 6-month follow-up period as compared to patients with low levels of serum TNF and the absence of SIE (Holmes *et al.*, 2009). Thus, it is possible that the A allele at position −308 or a nearby allele, which is in linkage disequilibrium with the A allele, exerts a protective effect against AD through reduced transcription of the *TNF* gene. However, based on the AlzGene data (http://www.alzgene.org), it should be emphazised that the A allele of rs1800629 has been shown to significantly associate with both increased and decreased risk of AD in different populations.

The biomarker analysis detected a significant decrease in CSF Aβ42 levels among AD patients carrying the A allele (GA+AA vs. GG) of rs1800629 in *TNF* gene, while the T allele carriers (CT + TT vs. CC) of rs1143634 in *IL1B* gene associated with increased CSF p-tau levels. Moreover, AD patients carrying the A allele (GA+AA vs. GG) of rs600879 in *SORCS1* gene showed a statistically significant increase in the CSF Aβ42 levels ($p = 0.03$) even though the SNP data from genotype and allele frequency as well as logistic regression analysis did not show any significant association with AD. Collectively, these results confirm the findings in genotype and allele frequency analyses as well as logistic regression, and support the idea that biomarkers in CSF are indicative for pathogenic mechanisms in AD.

Since this study was based on the previously identified risk gene polymorphisms in AD, significant *p*-values were not corrected for multiple testing. However, it should be noted that after Bonferroni correction, significant *p*-values did not remain statistically significant. This means that further genetic studies in different sample sets are still needed to establish the role of *TNF* and *IL1B* in AD.

In conclusion, the AlzGene-based top hit screening revealed genetic variations in two candidate genes, *TNF* and *IL1B*, which genetically associated with AD and altered CSF biomarker levels among Finnish population. Apart from being important players in the innate inflammatory response induced by factors such as Aβ, the current study points to the underlying role of genetic alterations in the *TNF* and *IL1B* genes in AD by thus far unknown mechanisms.

6.2 DOWN-REGULATION OF SELADIN-1 INCREASES BACE1 LEVELS AND ACTIVITY DURING APOPTOSIS: STUDY II

In study II, the effects of seladin-1 down-regulation on BACE1-mediated processing of APP in SH-SY5Y human neuroblastoma cells were studied. The results show that down-regulation of seladin-1 expression under apoptotic conditions resulted in augmented BACE1 levels and activity. This was mediated by increased caspase-3 activation and subsequently enhanced depletion of the BACE1-sorting protein GGA3, which regulates trafficking of BACE1 to the lysosomes for degradation (Tesco *et al.*, 2007). Depletion of GGA3 after seladin-1 knockdown appeared to associate with an altered subcellular localization of BACE1. More BACE1 accumulated in early endosomes and near to the plasma membrane in apoptotic conditions as compared to normal conditions. These observations suggest that lysosomal sorting and thereby degradation of BACE1 is reduced under apoptosis. This leads to increased levels and activity of BACE1 by post-translational stabilization. Stabilization of BACE1 resulted in increased BACE1-mediated cleavage of APP leading to elevated production of APP β-CTFs and Aβ as well as decreased viability of SH-SY5Y cells over-expressing BACE1 under apoptosis. The above-mentioned cascade may take place also during AD pathogenesis due to the fact that seladin-1 is down-regulated in the temporal cortex of AD patients by ~40% (Greeve *et al.*, 2000; Iivonen *et al.*, 2002). Decreased seladin-1 mRNA levels also correlated with increased phosphorylated tau levels in AD brain (Iivonen *et al.*, 2002). Furthermore, a recent genetic study revealed that genetic alterations in the *seladin-1*/*DHCR24* gene increased the risk of AD, suggesting that genetic predisposition may also contribute to the observed down-regulation of seladin-1 expression in AD brain (Lamsa *et al.*, 2007).

Collectively, these results implicate that seladin-1 plays an important role in AD pathogenesis. Thereby, even a moderate decrease in seladin-1 expression due to genetic and/or environmental factors (oxidative stress-induced apoptosis, ER stress, etc.) may initiate a vicious cascade resulting in increased BACE1 activity and ultimately augmented Aβ production (Figure 4). Increased production of APP β-CTFs and Aβ may further reinforce seladin-1 down-regulation and/or caspase-3 activation, possibly augmenting the vicious cycle of pathogenic events.

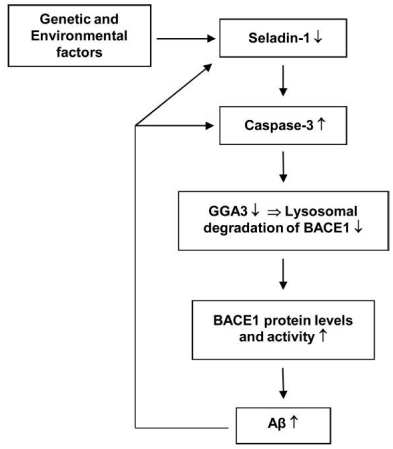

Figure 14. Suggested mechanism of the effects of reduced seladin-1 expression on BACE1 stabilization and BACE1-mediated processing of APP under apoptosis.

Strong evidence from several functional studies suggest that seladin-1 is protective against Aβ-mediated and oxidative stress-induced apoptosis by inhibiting caspase-3 activity (Greeve *et al.*, 2000; Luciani *et al.*, 2005, Di Stasi *et al.*, 2005). The protective function of seladin-1 could be partially associated with its C-terminally located scavenging activity toward ROS in the ER (Lu *et al.*, 2008). Alternatively, seladin-1-mediated neuroprotection could be p53-dependent (Kuehnle *et al.*, 2008), possibly preventing the Ras-induced senescence (Wu *et al.*, 2004). In addition to its effects in neuroprotection, seladin-1 also has a specific enzymatic activity, which catalyzes the reduction of the Δ24 double bond of desmosterol to form cholesterol (Waterham *et al.*, 2001). This function implies that seladin-1 has a role in the formation of cholesterol-rich detergent-resistant membrane domains, also known as lipid rafts (Abad-Rodriguez *et al.*, 2004). Lipid rafts have been found to affect β- and γ-secretase-mediated APP cleavage and Aβ generation *in vitro* and *in vivo* (Crameri *et al.*, 2006; Abad-Rodriguez *et al.*, 2004). Interestingly, ~50% reduction in seladin-1 levels was shown to decrease cholesterol levels, which was accompanied by a disorganization of lipid raft domains (Crameri *et al.*, 2006) in seladin-1 heterozygous knockout mouse brain. This defect led to an increase in APP C99 (and a moderate increase in APP C83) and Aβ production in the brain of seladin-1-deficient mice. Similar to this study, a moderate increase (~1.3-fold) occurred in the levels of APP C99 as well as an augmentation of the APP C83 levels after seladin-1 knockdown in SH-SY5Y-APP751 cells. This suggests that APP processing in general was moderately enhanced rather than there being exclusively a change in the β-secretase-mediated cleavage of APP. In contrast to findings from Crameri *et al.*, we did not detect any increase in Aβ levels which may be related to differences between the *in vitro* and *in vivo* knockdown models of seladin-1 in general.

Interestingly, recent findings propose that BACE1 is able to efficiently cleave APP in both lipid raft and non-raft microdomains (Vetrivel *et al.*, 2009). Moreover, it appears that cholesterol is not only important in β-amyloidogenic processing of APP but it is also able to modulate Aβ-induced neurotoxicity in AD (Fernández *et al.*, 2009). Together these data imply that there are different plausible mechanisms by which down-regulation of seladin-1 during apoptosis might be harmful. However, the present study suggests that it is more likely that the pro-apoptotic effects of seladin-1 down-regulation are linked to the

scavenging activity of seladin-1 protein rather than depletion of *de novo*-synthesized cholesterol. This proposal is based on the fact that the serum-containing cell culture medium contained extracellular cholesterol and thereby supplemented the potentially reduced *de novo* synthesis of cholesterol during the acute STS treatment (6 h).

It is well known that BACE1 is a stress-induced protease, e.g. its activity has been shown to increase after TBI (Blasko *et al.*, 2004; Loane *et al.*, 2009), oxidative stress (Tong *et al.*, 2005), and cerebral ischemia (Tesco *et al.*, 2007; Wen *et al.*, 2004; Hiltunen *et al.*, 2009). In addition, it has been recently shown that BACE1 expression is regulated in a transcriptional (Guglielmotto *et al.*, 2009) and translational manner (depending on the eIF2α phosphorylation status; O'Connor *et al.*, 2008) during different stress conditions. In the present study, however, translational regulation of BACE1 was not expected to take place because a BACE1-Myc cDNA construct was used, which did not include the native 5′ or 3′ UTRs important in the regulation of BACE1 translation. This made it possible to specifically examine the effects of seladin-1 knockdown on post-translational stability of BACE1.

The possible molecular mechanisms related to seladin-1 down-regulation were elucidated in the AD brain. These findings suggest that seladin-1 down-regulation exacerbates caspase-3 activity under staurosporine-induced stress conditions. This sensitizes cells to apoptosis and consequently augments BACE1 levels and activity through depletion of the BACE1 sorting protein, GGA3. Although the initial cause(s) for seladin-1 down-regulation in AD is still elusive, this data support the idea that seladin-1 is a plausible target for disease-modifying therapies for AD as well as other pathologic conditions involved in oxidative-stress-induced apoptosis, such as cerebral ischemia.

6.3 CYSTEINE 27 VARIANT OF THE δ-OPIOID RECEPTOR AFFECTS APP PROCESSING: STUDY III

It was recently demonstrated that GPCRs, such as β2-AR and δOR, are linked to AD pathogenesis by affecting Aβ generation *in vitro* and *in vivo* (Ni *et al.*, 2006; Teng *et al.*, 2010). This was indicated by the findings that agonist-induced stimulation of β2-AR or δOR led to their endocytosis and consequent trafficking of γ-secretase to late endosomes and lysosomes and enhanced Aβ production (Ni *et al.*, 2006). A subsequent study showed that δOR directly forms a complex with both BACE1 and γ-secretase, which results in the co-endocytic sorting of δOR and the secretases and increased APP processing (Teng *et al.*, 2010). Keeping in mind this intimate link, it was decided to assess whether the phenylalanine-to-cysteine substitution at position 27 of δOR (δOR-Cys27Phe) plays a functional role in APP metabolism *in vitro*.

It was previously shown that Cys27 substitution affects the maturation and subcellular localization of δOR in non-neuronal cells (Leskela *et al.*, 2009; Petaja-Repo *et al.*, 2000). δOR-Cys27 variant exhibited a decreased mature/precursor receptor ratio, which was related to the retention of receptor precursors in the ER and enhanced turnover of mature receptors at the cell surface (Leskela *et al.*, 2009). Similarly, study III demonstrated that the δOR-Cys27 variant underwent less efficient maturation compared to δOR-Phe27 in human SH-SY5Y-APP751 neuroblastoma cells. This indicated that the receptor maturation phenotypes are similar in neuronal and non-neuronal cells. Furthermore, a robust accumulation of APP C83 and AICD was observed, but not of APP C99, in both SH-SY5Y and HEK293 cells expressing δOR-Cys27. Total APP levels remained unchanged in these cells. These effects were not evident in cells expressing δOR-Phe27. The similar results

obtained in the cells expressing exogenous or endogenous APP confirmed that the observed changes in APP processing were not caused by APP over-expression *per se*. Furthermore, sAPPα levels were not altered, while total sAPP and Aβ40 levels were significantly decreased in δOR-Cys27-expressing cells. These data suggest that cellular events affecting APP processing, such as the altered trafficking of newly synthesized APP in the secretory pathway, do not cause the observed changes in δOR-Cys27-expressing cells. No signs of enhanced α- or γ-secretase activity were detected, which excludes the possibility that C83 accumulation would have been caused by increased secretase-mediated cleavage of APP.

Confocal microscopy results suggested that in δOR-Cys27-expressing cells, the endocytic trafficking of APP CTFs to LEL was reduced. This led to the accumulation of APP CTFs due to its inefficient degradation, as indicated by the strongly increased levels of C83 detected in the Western blots. Moreover, the δOR-Cys27 variant expression resulted in similar alterations in APP processing to those previously observed under pH-neutralizing conditions (Waldron *et al.*, 2008), where C83 accumulated at the cost of C99 without any increase in total APP levels or α-secretase activity. Consistent with this finding, it was recently shown that C99 was processed by α-secretase (ADAM10) to C83 (Kuhn *et al.*, 2010). Furthermore, it has also been reported that alkalizing drugs, such as bafilomycin A1, could induce the accumulation of APP C83 and AICD in luminal MVB vesicles (Vingtdeux *et al.*, 2007). MVBs are transport intermediates between early and late endosomes involved in receptor recycling and protein degradation processes (Gruenberg and Stenmark, 2004). They are also suggested to play a role in AD pathogenesis (Takahashi *et al.*, 2002) as well as in a transgenic mouse model of amyloidosis (Langui *et al.*, 2004). These similarities between the alkalization and δOR-Cys27 variant phenotypes in terms of altered APP processing point to the involvement of overlapping molecular mechanisms, which are related to changes in the endocytic trafficking pathway.

When investigating the difference in constitutive downstream signaling between the δOR variants, it was found that c-Src and possibly PKC activity may regulate the molecular events underlying the altered endocytic trafficking of APP CTFs in cells expressing the δOR-Cys27 variant. Thus, it could be proposed that the altered APP processing caused by δOR-Cys27 expression is different from the classical non-amyloidogenic APP processing and that it is related to constitutive δOR internalization and altered endocytic trafficking.

The fact that the δOR-Cys27 variant robustly affected the endocytic trafficking of APP prompted us an investigation into the genetic variants of *OPRD1* in an unbiased manner in two independent AD cohorts. Interestingly, this data suggest that the rs1042114 variation may play a role in AD as the meta-analysis of combined case-control and familial data pointed to a statistically significant risk effect for TG heterozygotes. The T→G allele change at the rs1042114 site leads to a phenylalanine-to-cysteine substitution in the N-terminal domain of δOR. It is therefore possible that this variation itself rather than some other proximal genetic alteration, which is in LD with rs1042114, may have functional relevance in AD.

The present results have significant relevance in the context of AD pathogenesis, as the intervention approaches focusing on the formation and trafficking of the GPCR/β- and γ-secretase complexes have been suggested as a novel strategy against AD (Ni *et al.*, 2006; Teng *et al.*, 2010). Since ~25% of AD patients are heterozygous for the δOR-Phe27Cys variation, it is possible that these patients could well respond to δOR antagonist treatments differently from the δOR-Phe27 homozygotes. These facts emphasize the importance of determining the genetic background of the patients with respect to their δOR-Phe27Cys genotype. Moreover, little is known what is the functional role of δOR heteromers

composed of the two different δOR variants in receptor signaling and other events, such as APP processing *in vitro* and *in vivo*. Taken together, these results underscore the importance of taking into account the genetic profile of the δOR-Phe27Cys SNP when developing potential δOR-related therapeutic strategies against AD.

6.4 BEPRIDIL DECREASES Aβ AND CALCIUM LEVELS IN THE THALAMUS AFTER MIDDLE CEREBRAL ARTERY OCCLUSION IN RATS: STUDY IV

It has previously been shown that APP and Aβ or their fragments are present and aggregate in the dense plaque-like deposits and that altered APP processing and expression of Aβ-degrading enzymes take place in the ipsilateral thalamus of rats subjected to transient MCAO (van Groen *et al.*, 2005; Hiltunen *et al.*, 2009). These alterations coincided with significantly augmented calcium levels, depletion of GGA3 protein, and increased BACE activity in the ipsilateral thalamus. Based on these findings, it could be postulated that the observed alterations related to Aβ accumulation were linked to disrupted calcium homeostasis, which initiated secondary degeneration in the thalamus known to take place after MCAO (Iizuka *et al.*, 1990; Ross and Ebner, 1990; Wei *et al.*, 2004). Considering the intimate link between calcium and Aβ in AD pathology (Supnet and Bezprozvanny, 2010; Demuro *et al.*, 2010), drugs, which modulate both calcium and Aβ dyshomeostasis, are potentially interesting therapeutic candidates. One such drug candidate is bepridil, a non-selective calcium channel blocker (Flaim *et al.*, 1985; Yatani *et al.*, 1986; Van Amsterdam *et al.*, 1989), which has been used as an anti-anginal drug (Hollingshead *et al.*, 1992). Bepridil was recently shown to also inhibit BACE-mediated cleavage of APP both *in vitro* and *in vivo* by increasing the membrane-proximal, endosomal pH (Mitterreiter *et al.*, 2010). Interestingly, bepridil also modulated γ-secretase function of APP independently of endosomal alkalization (Mitterreiter *et al.*, 2010). Thus, it is possible that bepridil can modulate both calcium and Aβ pathology in the thalamus of MCAO rats.

It was found that chronic treatment of MCAO rats with bepridil significantly decreased both soluble Aβ40 and Aβ42 levels as well as insoluble Aβ42 levels in the ipsilateral thalamus without affecting the Aβ42/40 ratio. This ratio was significantly higher in the ipsilateral than in the contralateral thalamus of MCAO rats, confirming the previous findings that MCAO robustly increased Aβ production. Furthermore, bepridil treatment significantly decreased the calcium levels and this decline strongly correlated with the reduction in Aβ42 and Aβ40 levels in the ipsilateral thalamus. As shown previously after MCAO (Hiltunen *et al.*, 2009), it was observed that a significant increase in APP CTF production could be found in vehicle-treated MCAO rats in the ipsilateral thalamus. In contrast, bepridil-treated MCAO rats did not exhibit any statistically significant changes in APP CTF levels. Consistent with the increased APP CTF production, sAPPtot levels measured from the soluble protein fraction were also significantly increased in the vehicle-treated MCAO rats. An increase in sAPPtot levels was also observed in the ipsilateral thalamus of bepridil-treated rats, but this increase was ~30% less extensive. BACE1 activity was increased ~2.0-fold in the ipsilateral thalamus following MCAO, consistent with previous findings (Hiltunen *et al.*, 2009). However, this change was not affected by bepridil treatment and BACE1 activity remained elevated. There were no statistically significant changes in α- or γ-secretase activities in either vehicle- or bepridil-treated MCAO rats. However, it should be noted that the *in vitro* activity assays were performed using a procedure in which tissue homogenates were incubated at +37°C together with secretase-specific substrate peptides. Given the fact that BACE activity in HEK293-AP-APP cell

lysates was not blocked by bepridil according to a previous *in vitro* activity assay (Mitterreiter *et al.*, 2010) it may be impossible to detect bepridil-mediated changes in BACE activity in tissue homogenates. Taken together, these data suggest that bepridil treatment could affect APP processing in the ipsilateral thalamus of MCAO rats, which may have been the mechanism mediating the reduction in Aβ levels. However, since calcium levels were simultaneously decreased, it is difficult to dissect whether the changes in calcium homeostasis contributed to the production of Aβ or whether the BACE cleavage of APP was directly inhibited by bepridil. Alternatively, it is also possible that these two independent mechanisms were both modulating the levels of Aβ.

In addition to the alterations in APP processing and Aβ production, it is possible that changes in Aβ degradation or clearance also contributed to the present data. To address this question, the expression levels of IDE and NEP were determined; these are the enzymes/proteases intimately involved in Aβ degradation in the brain (Miners *et al.*, 2008). It was found that IDE protein levels were increased in ipsilateral thalamus of both vehicle- and bepridil-treated MCAO rats, while no alterations were observed in the levels of NEP. The effect of MCAO on IDE levels in the ipsilateral thalamus is in line with a previous study (Hiltunen *et al.* 2009). In addition, there were no significant changes in the expression of LRP, which is a key component in the receptor-mediated clearance of Aβ (Tanzi *et al.*, 2004), in vehicle- or bepridil-treated MCAO rats. These results suggest that bepridil treatment does not affect the expression of well-known regulators involved in the degradation and clearance of Aβ in the ipsilateral thalamus of MCAO rats. However, owing to the fact that there are several other enzymes or proteases within the brain that are capable of degrading or clearing Aβ (Miners *et al.*, 2008), one cannot rule out the possibility that some of these components may have been affected by bepridil treatment after MCAO.

It is well-established that astrogliosis and proinflammatory cytokine expression are induced in cerebral ischemia (Fan *et al.*, 1996; van Groen *et al.*, 2005; Sriram *et al.*, 2006) and that MCAO promotes secondary degeneration in the thalamus (Iizuka *et al.*, 1990; Ross and Ebner, 1990; Wei *et al.*, 2004, van Groen *et al.*, 2005). Here, an increase in GFAP and TNFα expression levels ware also detected in the ipsilateral thalamus of both vehicle- and bepridil-treated MCAO rats, indicating that bepridil did not mitigate the astrogliosis or the inflammatory response occurring after MCAO. The reduced seladin-1 mRNA levels are believed to be linked to neurodegeneration in AD (Greeve *et al.*, 2000; Iivonen *et al.*, 2002). Interestingly here seladin-1 mRNA levels were significantly reduced in the ipsilateral thalamus of vehicle-treated MCAO rats, but not in the bepridil-treated MCAO rats. Furthermore, correlation analysis revealed that there was a significant inverse correlation between seladin-1 mRNA and calcium levels as well as seladin-1 mRNA and insoluble Aβ42 levels. This suggests that seladin-1 mRNA expression is down-regulated in response to calcium and Aβ dysregulation in the ipsilateral thalamus of MCAO rats in a similar manner to the affected brain regions in AD (Greeve *et al.*, 2000; Iivonen *et al.*, 2002). Previous studies have revealed that the over-expression of seladin-1 could protect neuronal cells from Aβ-induced toxicity *in vitro* (Greeve *et al.*, 2000; Kuehnle *et al.*, 2008). Study II noted that the down-regulation of seladin-1 sensitized neuronal cells to apoptosis. On the basis of these data, these results suggest that increased seladin-1 mRNA expression in the ipsilateral thalamus of bepridil-treated MCAO rats could be a reflection of imncreased neuronal survival. This in turn could be linked with the improved functional recovery in the bepridil-treated MCAO rats.

It is evident that there is a reciprocal interaction between calcium homeostasis and Aβ pathology (Supnet and Bezprozvanny, 2010; Demuro *et al.*, 2010). Aβ plaques have been shown to impair calcium homeostasis in mouse models of AD (Kuchibhotla *et al.*, 2008). On

the other hand, KCl-mediated depolarization induced a sustained increase in cytosolic calcium levels in neuronal cultures, which triggered intraneuronal Aβ1-42 production and neuronal death (Pierrot *et al.*, 2004). Furthermore, soluble Aβ oligomers interact with critical lipids and receptors at the plasma membrane. Thus, it has been suggested that the increased calcium influx could be an outcome of disrupted membrane lipid integrity, Aβ-mediated pore formation, or modulation of receptor-gated ion channels, such as NMDA and AMPA receptors (Demuro *et al.*, 2010). In addition, it was recently shown that APP could regulate intracellular trafficking of voltage-gated L-type calcium channel Cav1.2 by retrieving the channel into intracellular compartments away from the plasma membrane (Yang *et al.*, 2009). This means that alterations in APP expression or its processing can affect the levels of Cav1.2 at the plasma membrane and thus modulate calcium influx. Bepridil treatment also affected the mRNA expression of α-1C subunit of Cav1.2 in a canine model of atrial tachycardia (Inoue *et al.*, 2006). Here it was found that the mRNA levels of α-1C subunit were reduced in the ipsilateral thalamus of vehicle-treated MCAO and that bepridil treatment of MCAO rats did not affect these levels in the thalamus. This finding indicates that there may be a feedback mechanism that down-regulates Cav1.2 expression when calcium homeostasis is impaired in the thalamus. It is also possible that bepridil does not exert its effects through the inhibition of voltage-gated calcium channels but instead via inhibition of sodium-calcium exchangers (Stys and Lopachin, 1998). This concept is supported by the findings in anoxic myelinated CNS axons, in which the influx of calcium was blocked by bepridil but not by specific inhibitors of L-type calcium channels, suggesting that the calcium entry occurring during CNS anoxia takes place primarily through reverse operation of sodium-calcium exchange (Stys and Lopachin, 1998).

Reduction of Aβ and calcium levels by bepridil also appeared to affect behavioral recovery in MCAO rats. Improved limb-placing scores and particularly improved sensorimotor performance in the cylinder tests in MCAO rats were observed on postoperative day 28. These data are consistent with the organization and integration of sensory, motor, and cognitive pathways within the thalamus (Briggs and Usrey, 2008). Furthermore, the data agree with the pharmacological evidence showing that drugs that prevent or mitigate secondary pathology in the thalamus can improve behavioral performance in a delayed manner (Hiltunen and Jolkkonen, in press).

In conclusion, this data highlight the close interplay between calcium and Aβ pathology. These findings emphasize the importance of therapeutic approaches targeted at preventing calcium dyshomeostasis in diseases, such as AD. In this context, bepridil is a feasible candidate worth further testing due to its multifunctional effects on both calcium- and BACE1-related events.

7 Methodological considerations related to studies I-IV

Study I: In this study, candidate genes were selected on the basis of the meta-analyses performed in the AlzGene database. Altogether, 21 major risk gene polymorphisms, which were not previously analyzed among the Finnish population, were selected for genotyping. The AlzGene database assembles previously published risk gene polymorphism findings in AD, which means that these genetic alterations have been already studied in at least one ethnic sample cohort. Therefore, *p*-values obtained from different analyses in study I were not corrected for multiple testing. This is a common procedure in the field of genetics related to replication studies. In this context, however, it should be noted that Bonferroni-corrected *p*-values were not statistically significant ($p > 0.05$) in study I. Moreover, it is a well-established fact that sample size is a crucial determinant in genetic association analyses, such as conducted in study I. Based on the previously conducted power analyses with the case-control cohort used for study I, the population size was shown to be large enough to detect a 1.5-fold risk effect with 80% probability. Conversely, statistical power was lower among the CSF sample set owing to the smaller number of AD patients. This may have thus affected the likelihood of detecting significant changes in the biomarker levels. Collectively, the results obtained here with *TNF* and *IL1B* warrant further genetic, epigenetic, and molecular studies in the context of AD.

Study II: This study concentrated on elucidating the molecular effects of seladin-1 down-regulation on pathways relevant for AD using cellular models. Commonly accepted molecular, cellular, and biochemical approaches were used to assess the interplay between seladin-1 and BACE1 in human SH-SY5Y neuroblastoma cells under apoptotic conditions. In the individual experiments, different parameters, such as number of replicated samples, condition of cells, transfection time, and transfection efficiency were carefully controlled in each experiment to ensure reproducible and reliable results. As a proof of this, GAPDH-normalized seladin-1 levels after siRNA transfections were similarly down-regulated throughout the study. Importantly, the main findings e.g. related to the effects of seladin-1 down-regulation on caspase-3 activity after the induction of apoptosis were studied using two different biochemical assays (substrate-based activity- and Western blot assays). The results from these two independent assays provided a similar outcome in terms of caspase-3 activity. All the studied protein levels and Aβ values were normalized to the housekeeping protein (GAPDH) or total protein levels, respectively.

Study III: All the experiments conducted in cellular models, such as transfection time and efficiency were carefully controlled in a similar way as in study II. The results obtained with δOR variants were studied in two different cell lines (HEK293 and SH-SY5Y), which expressed either exogenous or endogenous APP. Results from the different cell lines in terms of alter APP processing were similar, indicating that the observed key outcome measures were reproducible and that they were not dependent on the expression status of APP (over-expression vs. endogenous APP). In addition, over-expression and/or maturation status of δOR-Cys27 and δOR-Phe27 variants were carefully assessed in the biochemical and histological experiments. In the genetic part of the study, power analyses with the case-control cohort revealed that the population size was large enough to detect a 1.5-fold risk effect with 80% probability (see also study I). Since only significance was observed with the δOR-Cys27 variant among the Finnish case-control cohort ($p = 0.07$), an independent familial AD sample set from USA was used as a replication cohort to assess

the risk effect of δOR-Cys27 variant in a different ethnic population. Subsequently, meta-analysis was performed using these two AD sample cohorts with regard to the δOR-Cys27 risk effect. This is a commonly accepted protocol in genetic studies when possible risk gene effects are being evaluated. Nevertheless, it should be pointed out that both of these sample sets showed only a moderate risk effect and thus it is of the utmost importance to confirm the observed findings in additional sample cohorts. The *OPRD1* gene region has not shown any significant association with AD in recent GWA studies after adjusting the *p*-values for multiple comparisons

Study IV: Successful cerebral occlusion was controlled by performing behavioral tests in MCAO rats. The number of MCAO rats was decreased by ~10% due to unsuccessful operation or post-operational mortality. Commonly accepted molecular, cellular, biochemical and functional approaches were applied to ensure reliable outcome measures. In general, the sample size was sufficient for these analyzes (n = 5-7 animals/group). However, it is evident that the preliminary results obtained in study IV should be replicated in a larger sample set. Bepridil treatment at the 50 mg/kg/day dose was chosen on the basis of previous studies conducted in bepridil-treated rodents. However, in the subsequent experiments, it would be important to assess the outcome effects of different doses of bepridil on the behavior and AD-related pathology as well as on the bio-availability of the drug in the brain. Bepridil has been used as an anti-anginal drug in humans, suggesting that it is a safe candidate for pre-clinical studies in animals. Related to this, the weights of the rats were monitored during the entire treatment procedure to ensure animal wellbeing. Although study IV concentrated on thalamic changes mimicking some of the pathological changes seen in AD brain (accumulation of Aβ and changes in calcium), it should be recognized that there are also differences in terms of AD-related pathogenic processes between AD and rat brain. It is also noteworthy that in the MCAO model, the short term insult initiates rapid pathological changes, whereas AD is a slowly progressing disease, in which the clinical symptoms are usually observed years after the first pathological brain changes. Nevertheless, the MCAO rat model used in study IV is already a well-established model to examine the molecular interplay between Aβ and calcium. Similar robust calcium changes are not typically detected in other animal models of AD, which makes the MCAO rat model a good way to investigate the molecular mechanisms underlying Aβ and calcium pathology.

8 Summary and future perspectives

Several candidate gene-based association studies in various ethnic populations have confirmed that common variants in different genes affect the risk of AD. These genetic variations individually make only a minor contribution to AD risk, but in combination with other genetic or environmental factors, they may have a significant impact on disease pathogenesis over time. In complex diseases, such as in AD, the identification and functional characterization of risk genes is an important task, because it may shed light on the disease pathogenesis. Molecular studies related to these proteins and pathways enable better understanding of the disease pathophysiology and may ultimately lead to the identification of novel biomarkers and therapies. In AD, the molecular players behind the formation of Aβ-containing plaques and tau-containing tangles are fairly well known. Therefore, the initial functional assessments of the candidate genes are often targeted to the key pathological events, such as altered Aβ production, secretases (e.g. BACE) or Aβ degrading enzymes, such as IDE, NEP, and LRP. This thesis focused on the functional characterization of seladin-1; its down-regulation has been linked to neuronal degeneration in AD. Another focus was on δOR, which has been proposed to contribute to AD pathogenesis by affecting Aβ generation upon agonist stimulation. More specifically, this thesis aimed to elucidate the effects of these factors on APP processing and intracellular trafficking as well as on Aβ and calcium pathology in various conditions, such as during apoptosis, disrupted calcium homeostasis, and astrogliosis and inflammation in different *in vitro* and *in vivo* models.

The results obtained in this thesis support the concept that alterations in the intracellular trafficking of APP and BACE1 play an important role in AD pathogenesis. In addition, seladin-1 acts as a neuroprotective factor, as demonstrated in different *in vivo* and *in vitro* models as well as in AD brain. Genetic studies have provided further support for these findings. One of the most important findings in this thesis was that seladin-1 down-regulation was closely connected to the increase in BACE1 levels and activity especially under stress conditions, such as apoptosis (study II). It appears that the decrease in seladin-1 levels could be an early indicator of neurodegeneration before the signs of deleterious Aβ accumulation are detectable. This hypothesis is supported by the findings in the MCAO rat model, where increased seladin-1 mRNA levels correlated with decreased insoluble Aβ and calcium levels after bepridil treatment, suggesting that increased seladin-1 expression could be an indication of improved neuronal survival (study IV). In this study, MCAO was used to introduce secondary degeneration in the thalamus. A similar kind of disruptive degeneration takes place in the brain after stroke and in many neurodegenerative diseases, such AD. The general hallmarks of neurodegeneration include astrogliosis (increased GFAP levels), enhanced Aβ degradation (increased IDE levels), and increased proinflammatory cytokine expression (increased TNFα). *TNF* and *IL1B* genes were also found to genetically affect the AD risk in the genetic screening of AlzGene based risk genes in the Eastern Finnish population (study I). Similarly, a genetic association of *OPRD1* with AD was observed in study III, where the molecular and genetic aspects of the δOR-Cys27Phe variant were elucidated in cells expressing different δOR variants. Study III suggests that those AD patients carrying the heterozygous δOR-Cys27Phe genotype may respond differently to the δOR antagonist treatment as compared to individuals, who are homozygous for δOR-Phe27 variant. These possibilities emphasize the importance of

determining the genetic background of the δOR-Phe27Cys variation on a case-by-case basis in order to provide AD patients with the best possible treatment when evaluating potential δOR-related pharmacological approaches.

As genetic research gradually enters the post-genomic era, future research will focus on epigenetic regulation of risk genes. Examples of such epigenetic modifications are DNA methylation or histone acetylation, two processes which regulate gene expression. These modifications are anticipated to play a central role also in AD pathogenesis and thus a detailed epigenetic assessment of genetically identified risk genes is important in the future. The AD-related genes and pathways studied in this thesis may also prove to be potential molecular targets e.g. as novel biomarkers for risk assessment, early diagnosis, and monitoring disease progression. Furthermore, the results obtained in this thesis may be applicable for the development of novel intervention approaches to interrupt the progression of AD. Finally, the genetic results emphasize the importance of mapping the genetic profile of a person when introducing new potential pharmacological approaches in the therapeutics of AD.

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Timo Sarajärvi

Alzheimer's Disease-Related Genes and Pathways

Special Emphasis on Seladin-1, δ-Opioid Receptor and BACE1

Alzheimer's disease (AD) is a complex disorder with a clear genetic background. Causative mutations explain less than 1% of all AD cases; the vast majority of cases are genetically heterogeneous. Several risk genes are known but their underlying biological mechanisms in AD pathogenesis remain generally unspecified. This thesis aims at exploring the molecular mechanisms of seladin-1 and δ-opioid receptor that have been postulated to genetically and/or functionally play a role in AD. Genes and pathways studied in this thesis may prove to be potential targets as novel biomarkers in early diagnosis, and the results obtained may be applied for the development of novel therapeutic approaches in AD.

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