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MOLECULAR STUDIES OF THE γ-SECRETASE COMPLEX: FOCUS ON GENETIC AND PHARMACOLOGICAL MODULATION

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Dedicated to my grandmother Kerstin,
who bravely fought the disease until the end.

“Everything is hard before it is easy”
Johann Wolfgang von Goethe

ABSTRACT

γ-Secretase is a multi-subunit protease complex, composed of presenilin (PS1 or PS2), Nicastrin, Pen-2 and Aph-1, which generates the Alzheimer disease (AD) related 30-43 amino acid long amyloid β-peptide (Aβ). The complex is also crucial for important cell signaling, such as the Notch receptor pathway. More than 200 different Familial AD (FAD) causing mutations have been identified. They are all restricted to either PS1, PS2 or the amyloid β-precursor protein (APP), from which Aβ is generated, therefore proving how central γ-secretase mediated Aβ production is in AD pathogenesis. A common feature of FAD mutants is an increased Aβ42/Aβ40 ratio production. This results in a more amyloidogenic Aβ product and accelerated oligomerization and plaque formation.

A number of γ-secretase inhibitors have been in clinical trials but so far there have been no major progress, due to mechanism-based side effects that is probably caused by impaired Notch signaling. It is therefore very important to develop novel therapeutic strategies targeting Aβ production without interfering with other crucial γ-secretase signaling pathwats. The aim of my thesis was to i) get a better understanding of the molecular basis behind the heterogeneous activity of γ-secretase resulting in different Aβ peptides, ii) to identify novel ways to target γ-secretase mediated Aβ production in a Notch sparing manner, iii) to explore the impact of a novel class of drugs called γ-secretase modulators (GSMs) on different γ-secretase processes.

In **Paper I**, we specifically investigated whether the membrane integration and/or the active site of PS would be affected by different PS1 FAD mutations, which cause an increased Aβ42/Aβ40 production ratio. We found that while some FAD mutations located in hydrophobic domains around the catalytic site (TMD6, H7 and TMD7) changed the membrane integration of PS1, all FAD mutations studied affected the structure of the catalytic site of γ-secretase. In **Paper II** the large hydrophilic loop of PS1 was examined. Interestingly, by using a deletion mutant strategy, we found that, similar to many FAD mutants, Aβ38, Aβ39 and Aβ40 were dramatically decreased in the absence of the loop, while Aβ42 was affected to a lesser extent, resulting in a net increase in the Aβ42/Aβ40 ratio. Importantly, neither AICD nor NICD formation was impaired, suggesting that the integrity of the loop region is important for proper γ-site cleavage but not for the overall cleavage activity at the ε- site. To further study the mechanism of γ-secretase processing, we reported in **Paper III** the first study describing single residues in a γ-secretase component besides presenilin, such as Nicastrin, that affects the processing of γ-secretase substrates differently. In the final study, **Paper IV**, we studied the pharmacology of different GSMs and found that it is possible to generate *in vivo* potent second-generation γ-secretase-targeting modulatory compounds that are pre-selective for Aβ over Nβ production without affecting NICD formation. These findings may have major implications for the development of GSMs for AD and will be further discussed in the thesis.

SAMMANFATTNING PÅ SVENSKA

Alzheimers sjukdom är den vanligaste demenssjukdomen i dagens samhälle och representerar mer än 70% av alla demensfall. Sjukdomen drabbar främst äldre människor och beror på att nervceller i hjärnan dör allteftersom sjukdomen fortgår. Sjukdomen är uppkallad efter Alois Alzheimer som i början på 1900-talet var den första att beskriva de klassiska histologiska kännetecknen i hjärnan som förknippas med sjukdomen, så kallade *plack* och *neurofibrillära nystan*. De typiska förändringar som kan observeras beror främst på ansamling av två olika proteiner, Amyloid-beta (Aβ) och Tau. Det finns många former av Aβ, den vanligaste är Aβ40. En betydligt ovanligare variant är Aβ42 som är två aminosyror längre och som därför lättare klumpar ihop sig och bildar olika former av aggregat som är giftiga för nervceller. Aβ42-aggregaten är ett mellanstadium innan de slutligen skapar placken. Kvoten Aβ42/Aβ40 har visats vara förhöjd hos vissa Alzheimerpatienter och är en viktig markör för sjukdomsutvecklingen. Till skillnad från placken som skapas omkring nervcellerna så bildar Tau de aggregaten inne i cellerna som kallas för neurofibrillära nystan. Både Aβs och Taus aggregationsförlopp stör cellens funktioner.

Från genetiska studier på patienter med ärftlig Alzheimer har man hittat förändringar i tre gener som kan kopplas till uppkomsten av plack. En av dessa gener bildar proteinet APP (amyloid precursor protein) som är grunden för Aβ-formerna. De andra två generna ger upphov till komponenter i γ-sekretaskomplexet, det enzym som klyver APP i cellens membran för att bilda Aβ. Ett sätt att hindra plackbildning, t.ex. med läkemedel, är att blockera γ-sekretaskomplexets aktivitet och på så vis förhindra klyvningen av APP. Tyvärr klyver γ-sekretaskomplexet även andra viktiga proteiner så bieffekterna från ett sådant läkemedel skulle kunna bli enorma. Många bieffekter beror på störningar i klyvningen av Notch, som är en betydelsefull molekyl under hela livet. I dagsläget finns det ingen effektiv behandling av Alzheimers sjukdom, eftersom nuvarande medicinering inte påverkar sjukdomsförloppet utan endast dämpar symptomen under en tid utan att stoppa nervcellsdöden. Behovet av att utveckla läkemedel som verkligen påverkar sjukdomsförloppet är därför enormt. En prioriterad sjukdomsstrategi är att minska bildandet av Aβ genom läkemedel. Som ovan nämnts är detta dock förenat med komplikationer och vi behöver hitta nya sätt att angripa γ-sekretaskomplexet på. För detta krävs ytterligare förståelse av biologin bakom komplexet.

I den här avhandlingen har jag i fyra studier undersökt skillnader och likheter i hur γ-sekretaskomplexet klyver APP och Notch. Detta för att bättre förstå de mekanismer och faktorer som påverkar valet av vilket protein som ska klyvas och hur APP kan klyvas på ett annat för att på så sätt minska bildandet av Aβ42.

I **studie I** har ärftliga mutationer i presenilin, en av komponenterna i γ-sekretaskomplexet, använts för att kunna förstå anledningen till den förhöjda Aβ42/Aβ40 kvoten. Vissa mutationer som är belägna i närheten av enzymets aktiva del, gav upphov till förändringar av presenilin-proteinets integrering i cellens membran. Dessutom påverkade alla mutationer som undersöktes även strukturen av enzymets aktiva del, något som kan inverka på dess klyvning av APP och därmed leda till en ändrad Aβ42/Aβ40 kvot.

Strukturen av enzymets aktiva del var intressant nog även påverkad när ett annat viktigt område i presenilin studerades i **studie II**. När en specifik del i presenilin togs bort ledde detta till en dramatisk minskning av Aβ40, däremot minskade inte Aβ42 i lika stor utsträckning. Detta har visat sig vara betydelsefullt, eftersom en annan forskargrupp har påvisat samma resultat för möss med denna genförändring och dessa möss hade extremt höga nivåer av plack i hjärnan jämfört med vanliga möss. Vi har identifierat de aminosyror i presenilin som ger upphov till dessa förändringar men som inte påverkar klyvningen av Notch, vilket antyder att denna region kan vara en målregion för nya unika läkemedelsstrategier.

I **studie III** fortsatte vi att undersöka orsakerna bakom skillnader i enzymets aktivitet och kunde som första forskargrupp rapportera att utöver presenilin kan mutationer i en annan komponent av enzymet (Nicastrin), leda till skillnader i klyvningen mellan APP och Notch. Vissa mutationer i Nicastrinmolekylen ledde till en sämre APP-klyvning medan Notch påverkades mindre.

Läkemedel som inte blockerar utan förändrar γ-sekretaskomplexets aktivitet är en mycket lovande behandling av Alzheimers sjukdom. Dock råder fortfarande osäkerhet kring hur ett förändrat enzym påverkar klyvningen av Notch. I **studie IV** studerade vi i detalj hur Notch påverkades av dessa läkemedel. Vi fann att det är möjligt att utveckla läkemedel som är mer selektiva mot att förändra APP-klyvningen än att påverka Notch.

Sammanfattningsvis bidrar de identifierade mekanismerna och kunskaperna från studierna i denna avhandling med ytterligare viktig grundläggande förståelse om γ-sekretaskomplexet och dess klyvningsaktivitet under normala betingelser och i sjukdom. Mer kunskap om γ-sekretaskomplexet och framförallt om hur olika typer av läkemedel påverkar enzymet och dess viktiga processer, såsom klyvningen av Notch, är oerhört viktig för fortsatt utveckling av effektiva och säkra läkemedel för långtidsbehandling av Alzheimers sjukdom.

LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. **Johanna Wanngren***, Patricia Lara*, Karin Öjemalm, Silvia Maioli, Nasim Moradi, Lars O. Tjernberg, Johan Lundkvist, IngMarie Nilsson and Helena Karlström
** contributed equally*
The role of Presenilin 1 FAD-linked mutations for changed membrane integration and catalytic site conformation.
Manuscript

II. **Johanna Wanngren**, Jenny Frånberg, Annelie I Svensson, Hanna Laudon, Fredrik Olsson, Bengt Winblad, Frank Liu, Jan Näslund, Johan Lundkvist and Helena Karlström
The large hydrophilic loop of presenilin 1 is important for regulating γ-secretase complex assembly and dictating the amyloid beta peptide (Aβ) profile without affecting Notch processing.
J. Biol. Chem. ***285****, 8527-8536 (2010)*

III. Annelie Pamrén, **Johanna Wanngren**, Lars O. Tjernberg, Bengt Winblad, Ratan Bhat, Jan Näslund and Helena Karlström
Mutations in Nicastrin protein differentially affect amyloid beta peptide production and Notch protein processing.
J. Biol. Chem **286**, 31153-31158 (2011)

IV. **Johanna Wanngren**, Jan Ottervald, Santiago Parpal, Erik Portelius, Kia Strömberg, Tomas Borgegård, Rebecka Klintenberg, Anders Juréus, Jenny Blomqvist, Kaj Blennow, Henrik Zetterberg, Johan Lundkvist, Susanne Rosqvist and Helena Karlström
Second generation γ-secretase modulators exhibit different modulation of Notch β and Aβ production.
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LIST OF ABBREVIATIONS

Aβ	Amyloid β-peptide
AD	Alzheimer disease
ADAM	A disintegrin and metalloproteinase
AICD	APP intracellular domain
Aph-1	Anterior pharynx defective-1
APLP	APP-like protein
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
APPswe	APP Swedish mutation (K670N/M671L)
BACE	β-site APP cleaving enzyme
BBB	Blood brain barrier
CAA	Cerebral amyloid angiopathy
cDNA	Circular DNA
CSF	Cerebrospinal fluid
CTF	C-terminal fragment
ELISA	Enzyme-linked immunosorbent assay
FAD	Familial Alzheimer disease
FLIM	Fluorescence-lifetime imaging microscopy
GFP	Green fluorescent protein
GSM	γ-Secretase modulator
GSI	γ-Secretase inhibitor
GWAS	Genome-wide association studies
ICD	Intracellular domain
IP	Immunoprecipitation
LTP	Long term potentiation
MALDI	Matrix-associated laser desorption ionization-
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSD	Meso Scale Discovery technology
Nβ	Notch-β
NFT	Neurofibrillary tangles
NICD	Notch intracellular domain
NSAID	Non-steroidal anti-inflammatory drug
NTF	N-terminal fragment
Pen-2	Presenilin enhancer-2
PS/*PSEN*	Presenilin
RIP	Regulated intramembrane proteolysis
sAPP	Soluble APP
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SCAM	Substituted cysteine accessibility method
TMD	Transmembrane domain
TOF	Time of flight

1 INTRODUCTION

1.1 ALZHEIMER DISEASE

Alzheimer disease (AD), recognized as the most common form of dementia among the elderly population, is a progressive neurodegenerative disorder characterized by memory loss and cognitive impairment. In April 2012, WHO estimated that around 35 million people worldwide have dementia and the numbers are expected to increase to 106 million in 2050 (WorldHealthOrganization, 2012). AD represents 50-70% of all dementia cases and the prevalence increases exponentially with age; 1% of individuals between 60-65 years of age are affected and at the age of 85, up to 24-33% (Ziegler-Graham et al., 2008). Due to the increasing life expectancy, the costs of the disease are growing enormously every year. According to a recent report, the estimated worldwide cost was $422 billion in 2009, an increase with 34% from 2005 (Wimo et al., 2010). Thus, besides tragically affecting the lives of AD patients and their close relatives by taking away their memory, awereness and language, AD is both a major public health problem and an economical concern. Therefore, efficient pharmacological treatment is an urgent matter.

The clinical features of AD initially start with a subtle progressive impairment of the episodic memory and orientation, which later develop to affect attention and executive functions, leading to impairments in decision-making and processing of information. As the cognitive dysfunctions continue, personality and behavioural alterations occur, such as paranoia, delusions, apathy and declined language function. Inevitably, these symptoms affect the cognitive and functional status further. After diagnosis, the patient usually lives 5-15 years and the cause of death is typically due to secondary illnesses such as pneumonia, other infections or malnutrition.

1.1.1 Neuropathology

The brains of AD patients are characterized by a decreased volume due to cortical atrophy along with enlargement of sulci and ventricles. The temporal and parietal lobes along with areas of the frontal cortex and the cingulate gyrus are particularly vulnerable. However, the sensory and motor regions as well as the occipital lobe are mainly unaffected. The atrophy, first observed in the hippocampus and entorhinal cortex, is due to; synaptic loss, degenerating neurites and a decreased number of neurons (Terry et al., 1991). A striking feature is the specific susceptibility of the cholinergic neurons, which provide the major cholinergic input to neocortex and cerebral cortex (Davies and Maloney, 1976; Whitehouse et al., 1981).

The major neuropathological hallmarks of AD were described in 1906 by Dr. Alois Alzheimer. He examined a post-mortem brain from the first documented case of AD, a 51-year-old patient named Auguste D (Alzheimer, 1907). Under the microscope, he observed intracellular neurofibrillary tangles (NFTs) composed of the hyperphosphorylated tau protein, and extracellular senile plaques mainly consisting of the aggregated amyloid-β peptide (Aβ), as shown in Figure 1 (Glenner and Wong, 1984; Masters et al., 1985; Nukina and Ihara, 1986). Another hallmark of AD is increased inflammation, evidenced by increased presence of activated microglia and

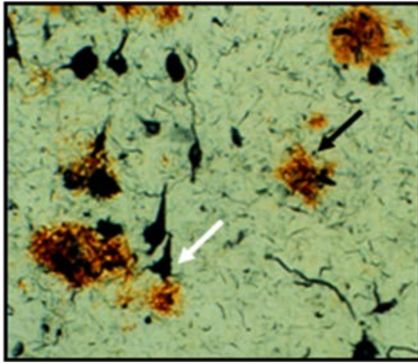

Figure 1. The main hallmarks in AD. Extracellular senile plaques (black arrow) and intracellular neurofibrillary tangles (white arrow). Picture courtesy of Dr. Nenad Bogdanovic.

astrocytes and abnormal levels of pro-inflammatory cytokines. Tau is a microtuble-associated protein mostly localized in the axons of neurons. When tau is hyperphosphorylated, it dissociates from the microtubules, causing impaired axonal transport that finally leads to retrograde degeneration and synapse loss (Iqbal and Grundke-Iqbal, 2005). There are more than 30 phosphorylation sites in tau and in AD tau becomes abnormally hyperphosphorylated probably due to an imbalance between kinase and phosphatase activities (Pei et al., 2008). Once hyperphosphorylated, tau starts to self-assemble into paired filaments that in turn aggregate into the NFTs. There are two major forms of amyloid pathology in the AD brain; neuritic and diffuse plaques that are divided based on their morphology. The neuritic plaques have a fibrillar, compact core mainly composed of Aβ42, a 42 amino acid long Aβ specie, which plays a pivotal role in early plaque formation (Iwatsubo et al., 1994). These plaques stain positive for Congo red, a β-sheet specific dye for amyloid fibrils, in contrast to the amorphous diffuse plaques (Gowing et al., 1994). However, eventhough the diffuse plaques still contain aggregated Aβ42, they lack the dystrophic neurites, activated microglia and reactive astrocytes typically associated with the neuritic plaques. It has been suggested that the diffuse plaques represent an immature precursor state of the neuritic plaques, since they have been found in healthy, aged individuals with normal cognitive function. In addition, studies with transgenic mice have shown that diffuse aggregates form prior to the development of neuritic plaques (Urbanc et al., 1999). This hypothesis is further supported by studies on patients with Down's syndrome. These individuals typically develop AD early in life due to an extra chromosome 21, where the amyloid precursor protein gene (*APP)* is located (Olson and Shaw, 1969). Theses individuals show diffuse deposits already in their teenage years, whereas the neuritic plaques are developed later (Giaccone et al., 1989; Rumble et al., 1989). There is also a rare third form of plaques named "cotton woll plaques" that is predominantly associated with some specific forms of familial AD (FAD) (Shepherd et al., 2009). Cotton wool pathology is also primary composed of Aβ42 but is extensively larger than both diffuse and neuritic plaques. Apart from plaques and tangles, the development of cerebral amyloid angiopathy (CAA) is a frequent pathological observation in AD (Hart et al., 1988). Here, Aβ is deposited into the walls of blood vessels and while Aβ42 is the predominate specie in pathology of the parenchyma; Aβ40 deposition is more common in the vessels (Miller et al., 1993; Suzuki et al., 1994).

The pathogenesis of AD is very complex and so called Braak-staging is a commonly used method to define and evaluate the different stages of the disease. Braak-staging takes both NFT and plaque pathology into account, which differs in regard to the regional distribution. NFT pathology is first observed in the enthorinal cortex, followed by spreading to the limbic structures such as hippocampus and

amygdala and finally occurring in neocortex (Braak and Braak, 1991). The plaque formation starts in the orbitofrontal and temporal cortices before spreading to the parietal cortex and neocortex. The spreading pattern of the pathology can be revealed in the symptoms; the earliest signs are short term memory problems, reflecting the initial damage to the hippocampus area. Later, when executive functions such as planning and initiation of actions are disturbed, the pathology can be observed in the prefrontal cortex and subcortical brain structures that controls these functions. However, the degree of cognitive decline does not correlate well with numbers of plaques, but rather with the extent of Tau pathology or levels of soluble Aβ (Arriagada et al., 1992; Naslund et al., 2000). Importantly, another neuropathological hallmark, which correlates well with disease severity is synapse loss (Terry, 2000; Terry et al., 1991). This is in accordance with that the severity of MRI brain atrophy measurments is well correlated with the conversion of mild cognitive impairment to AD (Jack et al., 2005; Risacher et al., 2009).

1.1.2 Genetics and risk factors of AD

Alzheimer disease is a multi-factorial disease, influenced by both environmental and genetic components. In general, there is no obvious pattern of genetic inheritance, thereby classifying most cases as sporadic AD. In a few percent of all AD patients, an autosomal dominant inheritance of mutations with almost complete penetration can be determined, so called familial AD (FAD). In most FAD cases the age of onset occurs before 65 years of age (early onset), while sporadic AD patients typically present the first symptoms after the age of 65 (late onset).

Genetic studies have revealed mutations in three different genes that cause the familial variant of the disorder. All three proteins encoded by these genes are involved in the generation of Aβ and several mutations in each gene have been identified. To date, 33 mutations in the *APP* gene on chromosome 21, 185 mutations in the presenilin 1 *(PSEN1)* gene on chromosome 14 and 13 mutations in the presenilin 2 (*PSEN2*) gene on chromosome 1 have been reported (http://www.molgen.ua.ac.be/ADMutations, (Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995)). APP is the precursor protein of Aβ, and Presenilin 1 and 2 (PS1, PS2) are members of the γ-secretase complex, which catalyzes intramembrane proteolysis of the APP transmembrane domain (APP TMD) resulting in Aβ production. Identification and characterization of these genes have increased the understanding of the cause of the disease. For instance, many FAD mutations in PS1 and PS2 cause an increase in Aβ42/Aβ40 ratio either by decreasing the production of Aβ40 or by elevating Aβ42 generation (Yu et al., 2000). These observations strongly suggest that the Aβ42/Aβ40 ratio is of high relevance for the molecular pathogenesis of the disease. Regarding APP mutations, most increase the Aβ42/Aβ40 ratio, but some also enhance total Aβ levels. For example, duplication of the *APP* gene is linked to early onset AD with severe CAA (Cabrejo et al., 2006) and patients with Down's syndrome, having a third copy of *APP,* often develop AD starting as early as the age of 35 (Olson and Shaw, 1969; Tyrrell et al., 2001). Thus, besides changes in the Aβ42/Aβ40 ratio, increased levels of Aβ seems to be able to cause the the disease.

Beyond the genes involved in FAD, several susceptibility genes have been suggested to contribute to the risk of developing AD. However, these genes have small effects and therefore larger sample sizes and genome-wide association studies (GWAS) are needed to gain enough power to detect them. Some possible susceptibility genes are; *SORL1* (encoding for sortilin-related receptor), *PICALM1* (phosphatidylinositol-binding clathrin assembly protein), *CR1* (the receptor for the complement C3b protein) and *CLU* (the clustrin gene) (Harold et al., 2009; Lambert et al., 2009). However, the most important and most replicated genetic risk factor for AD is the presence of one or two of the apolipoprotein E (ApoE) ε4 alleles. The *APOE* gene exists in three allelic variants, ε2, ε3 and ε4, encoding the corresponding isoforms of the protein. The only difference between the isoforms is the substitution of Cys to Arg at position 112 and 158. Nevertheless, these small differences can be crucial. The ε4 allele is associated with a dose-related increased risk for AD as well as an earlier onset of the disease (Corder et al., 1993; Saunders et al., 1993; Strittmatter et al., 1993), while the least common allele ε2 has been proposed to be protective (Corder et al., 1994). ApoE is a glycoprotein, whose function is to maintain the lipid and cholesterol homeostasis by transporting lipids and cholesterol (Martins et al., 2009). How the ε4 allele elevates the risk of developing AD is not known, but studies have shown higher Aβ burden in brains from AD patients carrying the allele. The isoforms of ApoE are also believed to differentially affect both the aggregation and clearance of Aβ (Kim et al., 2009), thereby influencing the risk of developing AD in an alternative way.

Besides the most important risk factors for AD, such as age and genetic factors, epidemiological studies have pointed out cerebral infarct and stroke, traumatic brain injury, hypertension and high cholesterol levels at midlife, cardiovascular disease, depression, diabetes mellitus, female gender, low physical and social activity as other influencing factors of AD (Qiu et al., 2009). Fortunately, some positive factors such as; education, physical activity, moderate alcohol consumption, challenging occupation, intake of fish, fruit and vegetables (i.e. omega-3 and anti-oxidants) have also been reported (Eskelinen et al., 2011; Hooshmand et al., 2012; Scarmeas et al., 2009). The impact of some of these factors needs to be further evaluated, but it is very encouraging that there are ways on an individual level to reduce the risk to develop AD. Importantly, very recently it was also found that a mutation in APP, located close to the Aβ encoding sequence is protective against AD as well as cognitive decline in non-demented eldery. The mutation reduces β-secretase activity, which results in less Aβ production, providing further evidence for the pivotal role of Aβ in AD pathogenesis (Jonsson et al., 2012).

1.2 THE AMYLOID PRECURSOR PROTEIN

The Aβ peptide is derived from the amyloid precursor protein (APP) in a physiological normal pathway (Haass et al., 1993). APP is a type I transmembrane protein consisting of a single transmembrane domain, a large extracellular domain and a short cytoplasmic C-terminal region (Kang et al., 1987). It is a member of a conserved gene family including two homologues, APP like proteins 1 and 2 (APLP1 and 2). APP exits as three isoforms, which are 695, 751 and 770 amino acids in length and all variants

contain the Aβ domain. There is a ubiquitous expression of APP throughout the body but in neurons, APP695 is the predominant variant (Weidemann et al., 1989). The physiological function of APP is currently not fully elucidated, since two things complicate the analysis of the *in vivo* function of APP; i) a complex processing of APP generates several products that all are likely to perform specific functions and ii) the APP family share partially overlapping functions. During the processing of APP, the soluble N-terminal ectodomain (sAPP) is released by ectodomain shedding before the remaining membrane bound peptide is cleaved by γ-secretase, generating the Aβ peptide and the APP intracellular domain (AICD). Thus, besides full length APP there are three APP-derived metabolites that could be involved in different aspects of cell signaling.

The APP family shares conserved regions within the ectodomain and the ICD. However, the extracellular juxtamembrane domain within the Aβ region is very divergent and unique for APP. Although APLP1 and 2 lack the Aβ region, they are processed in a similar manner as APP. They undergo ectodomain shedding that releases soluble APLPs (Slunt et al., 1994), before being further processed in a γ-secretase dependent manner, generating Aβ-like fragments and intracellular domains (Eggert et al., 2004; Scheinfeld et al., 2002). In mice, while single knock-outs of the APP, ALPL1 or 2 genes are viable, APP/APLP2 and APLP1/2 double knock-outs as well as the triple APP/APLP1/APLP2 knock-out result in prenatal lethality (Heber et al., 2000; Herms et al., 2004). Surprisingly, the APP/APLP1 double knock-out turned out to be viable and fertile (Heber et al., 2000). Together these data indicate an important role of the APP family in development and a redundancy between APLP2 and the other family members. In agreement with these data, APP and APLP2 are highly expressed in neurons during development and in adult tissues, while APLP1 is primarily found in the nervous system (Lorent et al., 1995). A study using RNA interference has demonstrated a critical role for APP in neuronal migration during development (Young-Pearse et al., 2007). In addition, APP has been implicated to stimulate neuronal outgrowth and to be neuroprotective (Kogel et al., 2005; Milward et al., 1992). However, several other functions have been proposed such as regulating stem cells (Kwak et al., 2006) and also having a role in axonal transport (Kamal et al., 2001). APP has also been reported to serve as a cell adhesion molecule and in trans-cellular interactions (Beher et al., 1996; Breen et al., 1991). This is supported by the observation that extracellular binding of heparin induces APP/APP dimerization (Gralle et al., 2006), mainly via the E1 domain and a GxxGD domain in the transmembrane region (Kaden et al., 2008) and that trans-dimerization of APP family members can promote cell-cell adhesion (Soba et al., 2005).

APP may also function as a cell surface-receptor (Kang et al., 1987), although the ligand has not been found. This is supported by the finding that AICD form a transcriptionally active complex with the adaptor protein Fe65 and the cromatin-remodeling factor Tip60 (Cao and Sudhof, 2001; Cao and Sudhof, 2004). Some downstream target genes of AICD have been suggested, including; neprilysin, p53, APP itself and GSK-3β (Muller et al., 2008). However, there are also data suggesting that the γ-secretase dependent AICD production is not required for the proposed APP signaling, based on the findings that APP signaling could proceed normally in cells

deficient of γ-secretase or in γ-secretase inhibitor-treated cells (Hass and Yankner, 2005). In addition to this controversy, it has even been proposed that Fe65 signaling could be executed independently of APP (Giliberto et al., 2008). Taken together, the role of AICD as a transcription regulator remains controversial.

It has been proposed that the ectodomain of APP is responsible for the majority of the functions of APP, since one protelytic fragment called sAPPα rescues the phenotypes observed in APP deficient mice (Ring et al., 2007). The mice have reduced body and brain weight, consistent with a role of APP for neuritic outgrowth, as well as impairments in learning and spatial memory that are associated with a decrease in long-term potentiation (LTP) (Muller et al., 1994). Moreover, sAPPα has a potent role in neuroprotection as well as in growth promotion and neurotrophic activities. It is thought to promote proliferation of neuronal stem cells in the subventricular zone by acting as a cofactor for epidermal growth factor, EGF (Caille et al., 2004). On the other hand, another proteolytic fragment, sAPPβ has been associated with suppressed neuronal activity and for triggering neuronal death (Furukawa et al., 1996; Nikolaev et al., 2009).

1.2.1 APP processing

APP is transported through the secretory pathway to the plasma membrane. During the transportation, APP is post-translationally modified by N- and O-glycosylations, tyrosin-sulphations and phosphorylations (Weidemann et al., 1989). At the plasmamembrane, if not ectodomain shedded, APP is rapidly internalized due to the presence of the YENPTY internalization motif in the cytosolic domain (Lai et al., 1995). Once endocytosed, APP is trafficked through the endocytotic and recycling pathways back to the cell surface, subjected to proteolytic events or degraded in the lysosomes. The mature protein is subjected to different proteolytic cascades, denoted the amyloidogenic and the nonamyloidogenic pathways, respectively, schematically shown in Figure 2. In the non-amyloidogenic pathway, APP is first cleaved within the

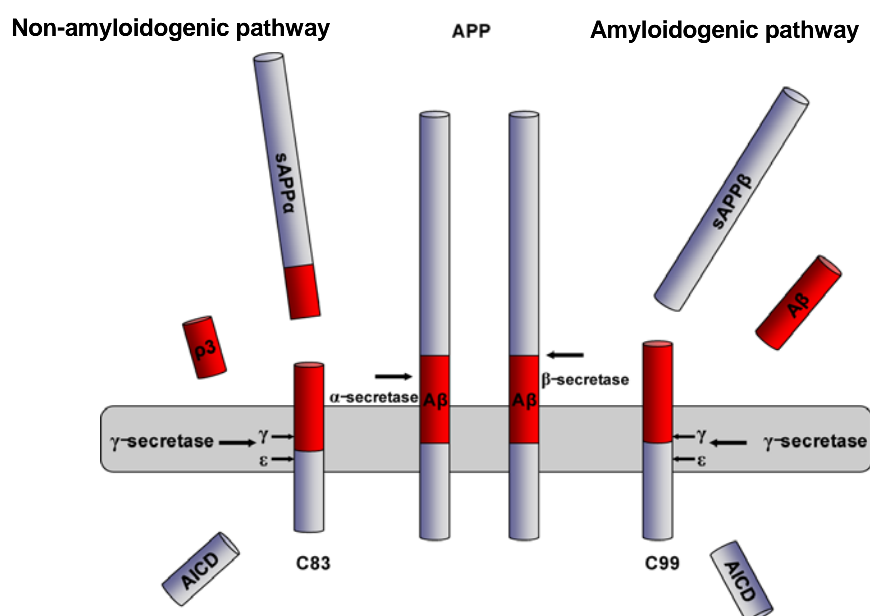

Figure 2. Schematic illustration of APP processing in the non-amyloidogenic and amyloidogenic pathways. APP is cleaved by α-secretase in the non-amyloidogenic pathway, generating C83. C83 is further processed by γ-secretase that results in the release of the p3 peptide and AICD. In the amyloidogenic pathway, APP is processed by β-secretase followed by γ-secretase and Aβ and AICD is formed.

Aβ region by α-secretase, resulting in the soluble APPα fragment, sAPPα, (Esch et al., 1990; Sisodia et al., 1990) and the membrane anchored C-terminal fragment of 83 amino acid residues called C83. The latter fragment is subsequently cleaved by γ-secretase generating non-toxic p3 peptides of different lengths and the APP intracellular domain, AICD (Gu et al., 2001; Haass et al., 1993; Sastre et al., 2001). The amyloidogenic pathway is initiated through the cleavage of APP at the N-termini of Aβ by β-secretase, which generates the secreted extracellular domain denoted sAPPβ, and the 99 amino acid long membrane integral C-terminal fragment, C99. Similar to C83, C99 is then processed by γ-secretase at multiple sites, which results in Aβ peptides of different lengths (γ-cleavage) and the AICD, which is released into the cytosol (Gu et al., 2001; Haass et al., 1993; Sastre et al., 2001). Interestingly, many APP FAD mutations are located in clusters close to the β- and γ-secretase cleavage sites, exerting their pathogenic effect by influencing the activity of these enzymes. For example, the APPswe mutation located just prior to the N-teminus of Aβ (K670N/M671L) (Mullan et al., 1992) increases total Aβ production by providing a better substrate for β-secretase (Cai et al., 1993; Citron et al., 1992). In contrast, the mutations in the C-terminal part of Aβ, such as the London, French, German, Florida and Austrian mutations modulate the γ-secretase complex to produce longer Aβ peptides, resuling in an increased Aβ42/Aβ40 ratio (Ancolio et al., 1999; Campion et al., 1999; De Jonghe et al., 2001; Eckman et al., 1997; Goate et al., 1991; Kumar-Singh et al., 2000). Moreover, mutations within the Aβ domain also affect the primary sequence and structure of Aβ, thereby leading to enhanced aggregation. The Artic mutation (E693G) enhances protofibril formation and has been shown to cause plaque pathology that does not stain Congo red-positive in *post-mortem* brains, while the Osaka mutation (ΔE693) increases oligomerization without fibrilization (Basun et al., 2008; Nilsberth et al., 2001; Philipson et al., 2012; Tomiyama et al., 2008).

The enzymatic mechanism of γ-secretase has been subjected to intense investigations. There is now compelling evidence for a sequential processing model of APP, and likely other substrates, by γ-secretase (Kakuda et al., 2006; Qi-Takahara et al., 2005; Sato et al., 2003). Based on the identification of particular tri- and tetra peptides generated from C99, a model where APP is sequentially processed along two production lines; Aβ49>Aβ46>Aβ43>Aβ40 or Aβ48>Aβ45>Aβ42>Aβ38 has been proposed, see Figure 3 (Takami et al., 2009). Thus, the endoproteolytic cleavage starts at the ε-site, which results in the generation of AICD while Aβ49 or Aβ48 are further processed to shorter Aβ fragments in a precursor product manner. As the consecutive cleavage progresses, less hydrophobic Aβ fragments are formed and increase the probability of their release into the extracellular space or the cytosol. In addition, *in*

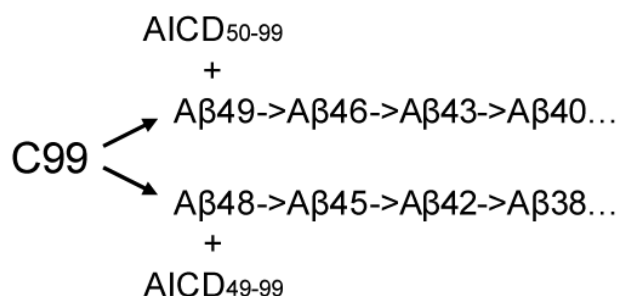

Figure 3. Aβ product lines. APP is suggested to be sequentially processed along two production lines. The product line preference of γ-secretase is determined by the initial position of the ε-cleavage site and releases AICD. Aβ49 or Aβ48 are then further processed to shorter fragments.

vivo experiments have shown that the product line preference of γ-secretase is determined by the initial position of the ε-site (Funamoto et al., 2004), i.e. whether γ-secretase cleaves at position 48-49 or 49-50 in the C99 sequence. This is also supported by a recent study, which reports that FAD mutations in either APP or PS impair the initial cleavage at the ε-site of the predominate Aβ49>>Aβ40 product line and/or impair the fourth cleavage, resulting in decreased Aβ40/Aβ43 and Aβ38/Aβ42 ratios (Chavez-Gutierrez et al., 2012; Golde et al., 2012).

1.2.1.1 α-Secretase

In 1990, it was described that APP was cleaved within the Aβ sequence, thus precluding Aβ formation (Esch et al., 1990), by a protease that was named α-secretase. α-Secretase was identified as a metalloprotease and several candidates for the proteolytic activity exist, all being members of the ADAM family (a disintegrin and metalloprotease), including ADAM9, ADAM10 and ADAM17 (also known as TNFα) (Koike et al., 1999; Lammich et al., 1999; Slack et al., 2001). Proteases of the ADAM family are type I transmembrane proteins that require the addition of a zinc ion in order to be proteolyticly active (Edwards et al., 2008). It was for a long time unclear which candidate protease was responsible for ectodomain shedding of APP, since knock-out and knock-down studies of ADAM9, 10 or 17 gave unclear results. For example; the APP ectodomain shedding was mainly unaltered in cells derived from mice deficient of either ADAM9, 10 or 17 and knock-down studies of the same proteins by RNA interference reduced the shedding process by 20-60% (se review (Lichtenthaler et al., 2011)). Therefore, it was suggested that all three proteases share the α-secretase activity and that proteolytic activity can be rescued by the others proteases if one is absent. In contrast, other substrates to the ADAM family are mainly cleaved by a specific ADAM protease. Recently however, it was shown that ADAM10 is responsible for the α-secretase activity in primary neurons (Jorissen et al., 2010; Kuhn et al., 2010). By using systematic knock-down of ADAM9, 10 and 17 by RNA interference, or cells prepared from conditional ADAM10 knock-out mice, both groups observed an almost complete reduction of sAPPα in the absence of ADAM10. This is in line with a report that ADAM10 and APP show coordinated expression in the human brain (Marcinkiewicz and Seidah, 2000). The proteolytic activity of α-secretase occurs mainly at the plasma membrane (Sisodia, 1992), and apart from APP, ADAM10 sheds more than 30 other membrane bound proteins, including Notch . Notch is an important signaling molecule in cell differentiation during development as well as in adulthood and is cleaved by γ-secretase in a similar manner as APP. In agreement, ADAM10 knock-out mice that die at embryonic day E9.5 display a phenotype reminiscent of mice carrying a loss-of-function of the Notch-allele (Hartmann et al., 2002). Interestingly, ADAM10 itself is a substrate for γ-secretase activity, after being ectodomain shedded by ADAM9 (Tousseyn et al., 2009).

1.2.1.2 β-Secretase

The Aβ initiating and rate limiting enzyme of the amyloidogenic pathway (Vassar, 2004), β-secretase, was cloned and identified by five groups (Hussain et al., 1999; Lin et al., 2000; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). β-Secretase or β-

site APP cleaving enzyme-1 (BACE1) is a membrane bound aspartic protease with its active site in the lumen/ extracellular space (Hong et al., 2000). There are two isoforms of BACE (Vassar, 2004), but the BACE2 protease is not involved in the amyloidogenic pathway, since mice deficient in BACE1 do not produce Aβ (Cai et al., 2001; Roberds et al., 2001). Moreover, when these mice were crossed with APPswe transgenic mice, they gave rise to rescued memory deficits (Ohno et al., 2004). BACE1 is ubiquitously expressed in high levels in the brain, and especially in the neurons, while BACE2 expression is mainly occurring in non-neural cells. APP is highly expressed in the brain as well and these high dual expression levels make the brain the primary tissue for Aβ generation. This is one explanation to why AD is a disease affecting the brain even though APP is expressed throughout the whole body. The function of BACE1 remains unclear, but it has been shown to regulate voltage dependent sodium channels (Kim et al., 2007) and many other substrates such as the neuregulin-1, platelet selectin glycoprotein ligand-1, Type I α-2,6-sialyltransferase, interleukin-like receptor II, APLP1 and 2 and Aβ itself (Dislich and Lichtenthaler, 2012). Very recently, by using a novel method for quantification and identification of secretome proteins, 34 substrates of BACE1 were reported. Importantly, some of these proteins were validated *in vivo* using BACE1 kock-out mice or mice treated with BACE1 inhibitors (Kuhn et al., 2012). Previously, only a few BACE substrates have been validated and associated with a clear biological function. It is of importance to validate BACE1 candidate substrates, since many substrates were identified using over-expression systems that may lead to artificial substrate/protease interactions. The initial reports of the BACE1 knock-out mice showed that they were viable, fertile and had no major behavioral or developmental deficits (Cai et al., 2001; Roberds et al., 2001). However, in more recent studies these mice were shown to display hypomyelination in the peripheral nervous system accompanied by an accumulation of uncleaved neuregulin-1, as well as displayed impaired axonal guidance of olfactory sensory neurons (Rajapaksha et al., 2011; Willem et al., 2006). Thus, some of the physiological functions of BACE1 concerns myelination and in addition, dys-regulation of neuregulin-1, which have also been implicated with schizophrenia (Stefansson et al., 2002; Williams et al., 2003). Interestingly, in accordance with that BACE1 is required for proper axon guidance (Rajapaksha et al., 2011), some of the newly identified BACE1 substrates are associated with synapse formation and neurite outgrowth, indicating a function of BACE1 in the development of the brain (Kuhn et al., 2012).

1.2.2 Aβ

Aβ peptides of various lengths, 30-43 amino acids, are generated by the sequential cleavage of APP by β- and γ-secretase. Aβ is mainly produced in neurons during normal metabolism and is present in the cerebrospinal fluid (CSF) and brain of healthy people during the course of their life (Haass et al., 1992; Seubert et al., 1992; Vigo-Pelfrey et al., 1993). Therefore, the presence of Aβ does not lead to neuronal injury, but the neurodegeneration leading to dementia is instead a cause of the pathological aggregation of Aβ. However, the physiological function of Aβ is not known. Under normal conditions, there is a balance between production and degradation of Aβ since it is rapidly cleared from the brain by different enzymes. Neprilysin and insulin degrading

enzyme are the major Aβ degrading enzymes *in vivo* (Farris et al., 2003; Iwata et al., 2000). In addition, Aβ can also be endocytosed at the synapse, phagocytosed by microglia and astrocytes. Alternative, Aβ can be transported out from the brain, mediated by the low-density lipoprotein receptor related protein (LRP)-1 (Shibata et al., 2000). In the other direction, Aβ can pass the blod-brain-barrier from the systemic circulation by binding to the receptor for advanced glycation end-products (RAGE) (Deane et al., 2003). Interestingly, in AD patients and animal AD-models the LRP1 expression is reduced (Shibata et al., 2000), while RAGE has been reported to be upregulated (Deane et al., 2003; Lue et al., 2001). This suggested a role of these mechanisms in sporadic AD

Aβ40 is the major Aβ specie produced, and is also predominant in human CSF and plasma. Apart from Aβ40, less abundant and shorter Aβ species including Aβ37, Aβ38 and Aβ39 have been found in cell medium by several groups (Qi-Takahara et al., 2005) (Beher et al., 2002; Wang et al., 1996). The longer Aβ42 peptide is very fibrillogenic and prone to aggregate since it is more hydrophobic and is also the form that is particularly important for early plaque formation (Iwatsubo et al., 1994). The length of the C-terminal part of Aβ strongly affects the rate of polymerization, for example Aβ42 forms fibrils very rapidly at lower concentration than Aβ40 (Jarrett et al., 1993). In addition, trace amounts of Aβ42 and Aβ43, the latter recently found in amyloid deposits of human AD brains (Welander et al., 2009), have a seeding effect of other soluble Aβ peptides for the formation of amyloid plaques *in vivo* (Jarrett et al., 1993). Even longer peptides such as Aβ45, Aβ46, Aβ48 and Aβ49 have been identified in both brain homogenates from APP-transgenic mice and cell lysates by combining immunoprecipitation and SDS/urea gel techniques (Qi-Takahara et al., 2005; Yagishita et al., 2006; Zhao et al., 2005). All different peptides are generated by the stepwise cleavage by γ-secretase along the two product lines, as described earlier in section *1.2.1 APP processing*. The longer Aβ peptides are however believed to stay in the membrane due to their hydrophobic properties.

The polymerization process of monomeric Aβ into fibrils and sequentially amyloid plaques is a complex multi-step procedure involving different oligomeric intermediates. The process is not fully understood, partly due to that there is no common experimental description of the different identified Aβ oligomeric species reported. Thus there are difficulties in comparing data and results between different research groups (Benilova et al., 2012). Therefore, it is likely that some of the identified species have similar or overlapping properties. It is also important to note that several of the oligomers have only been found *in vitro*, and consequently, their *in vivo* relevance and properties are uncertain. The *in vitro* oligomeric assemblies of Aβ described in the literature include; dimers and trimers (Podlisny et al., 1995; Shankar et al., 2008; Walsh et al., 2000), a 56-kD Aβ assembly called Aβ*56 (Lesne et al., 2006), Aβ-derived diffusible ligands (ADDLs) (Gong et al., 2003; Lambert et al., 1998), globulomers (Barghorn et al., 2005; Gellermann et al., 2008) and protofibrils (Harper et al., 1997; Walsh et al., 1997). These soluble oligomeric forms are all candidates to be the most toxic pathogens in AD, as described in the next section *1.2.3 The Amyloid cascade hypothesis*.

1.2.3 The Amyloid cascade hypothesis

Alzheimer disease is a complex multi-factorial disease and since a definitive disease mechanism is still not found, many different hypotheses explaining the pathogenesis, resulting in the neurodegeneration, has been proposed. One of the predominant and most supported hypothesis regarding the cause of AD is the amyloid cascade hypothesis. The hypothesis, initially formulated two decades ago, postulates that the major causing event in AD is the pathological production and accumulation of Aβ in the brain, due to abnormalities in amyloid precursor protein (APP) metabolism (Hardy and Higgins, 1992). This drives the AD pathogenesis by initiating cascades of events such as; hyperphosphorylation of tau and formation of NFTs, activation of microglia and astrocytes resulting in oxidative stress, synaptic spine loss and dystrophic neurites culminating in progressive neuronal loss and synaptic dysfunction, leading to dementia, summarized in Figure 4. Two key observations resulted in the initial formulation of the hypothesis. First, Aβ was identified as the main component of neuritic plaques (Glenner and Wong, 1984; Masters et al., 1985) and secondly, mutations in the *APP* and *PSEN* genes were found in families with early onset AD (Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995). Since FAD has a similar phenotype as sporadic AD, except an earlier age of onset, it was believed that the amyloid deposition could explain the pathogenesis of all types of AD. Initially, the main focus was on the insoluble Aβ fibrils in plaques as key mediators of the disease process. However, the focus has shifted and now the soluble Aβ oligomers are considered as the most toxic species (Haass and Selkoe, 2007; Hardy, 2006). In accordance, an update of the amyloid cascade hypothesis was made in 2002 (Hardy and Selkoe, 2002). In agreement, studies have shown that the soluble oligomers are better correlated to the degree of cognitive decline and synaptic loss than the plaques (DaRocha-Souto et al., 2011; McLean et al., 1999; Naslund et al., 2000). Furthermore, rats exposed to Aβ oligomers produced by cultured cells showed inhibited hippocampal LTP, and most importantly, immunodepletion of Aβ in the cell medium prevented the observed effect (Walsh et al., 2002). LTP is a process that correlates learning and memory and it was reported that cell-derived oligomers could interfere with memory functions in rats (Cleary et

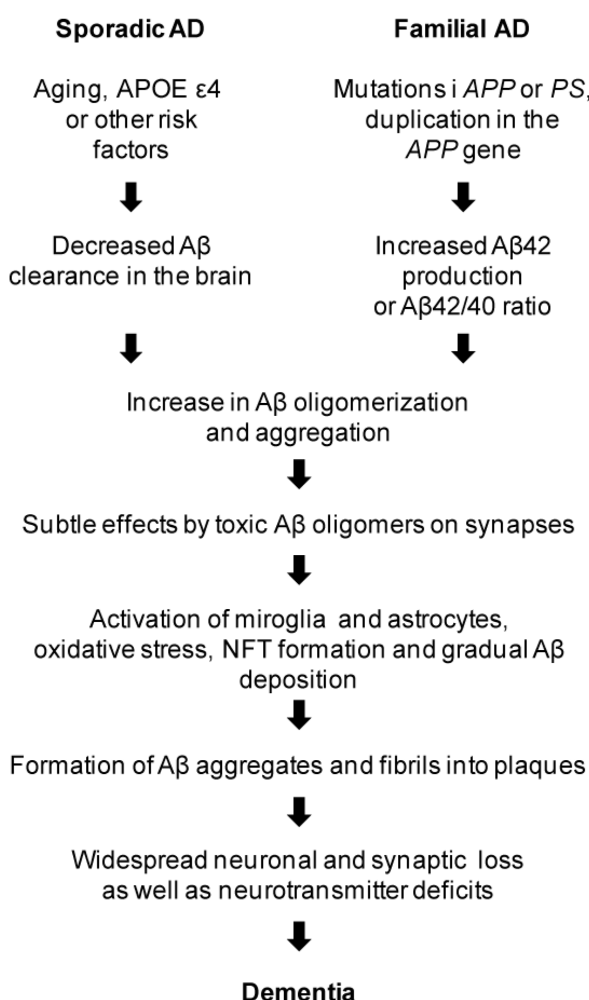

Figure 4. The Amyloid cascade hypothesis.

al., 2005). Finally, studies have shown that dimers and trimers of Aβ secreted from cells or Aβ dimers isolated from AD patients can induce loss of spines and synapses in the hippocampus (Shankar et al., 2007; Shankar et al., 2008) and lead to reduced formation of LTP in hippocampal brain slices (Klyubin et al., 2008).

There is a strong biochemical and genetic support for the amyloid cascade hypothesis. Aβ oligomers have been shown to be toxic to neurons and synapses and are also found to be elevated in brains from individuals with AD (Selkoe, 2002). FAD mutations in the *APP* and *PSEN* genes give rise to either elevated total Aβ levels, an increase in the Aβ42/Aβ40 ratio or enhance the oligomerization of Aβ, leading to early onset AD (St George-Hyslop, 2000). In addition, patients with Down's syndrome typically develop AD-like dementia and pathology, as described in sections *1.1.1 Neuropathology and 1.1.2 Genetics and risk factors of AD*. Furthermore, genes identified in the late onset form of the disease also provide support for the hypothesis. Patients carrying the *APOE ε4* allele, which is the major genetic risk factor, have higher Aβ burden in their brains (Schmechel et al., 1993) and many GWAS have identified genetic variations, which also associate with Aβ related mechanisms (Reitz, 2012). The use of biomarkers in AD has been widely increased during the last decade. Biomarkers include imaging, such as MRI measuring brain atrophy, FDG-PET determining glucose metabolism and PET amyloid imaging as well as CSF analysis of Aβ42, total tau and phosphorylated tau. In 2010, Jack *et al.* proposed a model that relates AD biomarkers to clinical symptom severity (Jack et al., 2010). This model and many other studies have indicated that Aβ accumulation begins decades before the first cognitive signs occur. This was recently confirmed in a longitudinal study using FAD patients. They reported that levels of Aβ42 in CSF declined 25 years before expected symptom onset and that Aβ deposition began 15 years before the first cognitive symptoms were manifested (Bateman et al., 2012)

However, it remains unclear how Aβ induces the formation of NFTs. APP-transgenic mice with reduced levels of endogenous tau could improve Aβ-mediated behavioral deficits (Roberson et al., 2011; Roberson et al., 2007). In addition, tau mutations that cause frontotemperal lobe dementia show tau pathology similar to AD without the appearance of Aβ depositions (Hutton et al., 1998). Nevertheless, patients with Down’s syndrome have Aβ plaques before the occurrence of tangles (Lemere et al., 1996). There are concerns regarding the inconclusive outcomes, such as cognitive improvement, from clinical trials with compounds or antibodies targeting Aβ or components in the amyloid cascade hypothesis (Reitz, 2012). However, these studies are mainly conducted in patients with mild to moderate AD and the disease is very likely to be too advanced in order to observe a disease-modifying effect. Importantly, most studies have not included CSF biomarkers in the trial designs, making it difficult to know whether the substances have hit the proposed target.

1.3 THE γ-SECRETASE COMPLEX

The proteolytic activity of γ-secretase is a key step in the pathogenesis of AD, since it generates the Aβ peptide and a detailed knowledge about this complex will help to understand at least a part of the complex mechanisms of the disease.

The γ-secretase complex is a promiscuous aspartyl protease, responsible for the final intramembrane cleavage of various type I transmembrane proteins, such as APP. It only cleaves the substrate after ectodomain shedding (Struhl and Adachi, 2000). The γ-secretase is a multi-protein complex composed of at least four members; presenilin (PS), Nicastrin, anterior pharynx defective-1 (Aph-1) and presenilin enhancer-2 (Pen-2), as shown in Figure 5 (Edbauer et al., 2003; Kimberly et al., 2003). All components

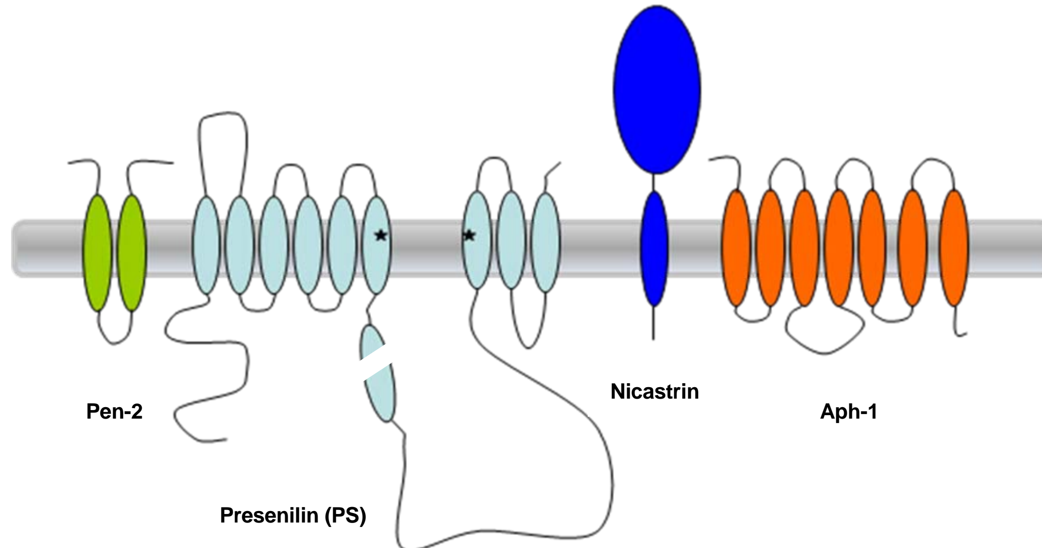

Figure 5. The components of the γ-secretase complex. Stars indicate the catalytic active Asp257 and Asp385 residues.

are initially co-localized in the ER, however the assembly of the complex is tightly regulated to ensure cell and tissue specific levels of active γ-secretase complexes. The PS molecules undergo endoproteolysis upon assembly of all members in ER/early Golgi compartment, generating the active N-terminal and C-terminal fragment (NTF and CTF) (Thinakaran et al., 1996). First, Nicastrin and Aph-1 form a sub-complex to bind and stabilize the PS holoprotein, and then Pen-2 initialize the γ-secretase activity by facilitating the endoproteolysis of PS (reviewed in (Dries and Yu, 2008)). During the transport of the complex through the secretory pathway, the maturation of Nicastrin occurs in the Golgi compartment by complex glycosylation. After further post-translational modifications of Aph-1 and Nicastrin by palmiotylation (Cheng et al., 2008), the mature and active complex reaches its functional sites, on the plasma membrane and in the endosomes/lysosomes. The nature of the active γ-secretase was for a long time a debate. However, multiple lines of evidence strongly suggest that PS, Nicastrin, Aph-1 and Pen-2 are sufficient for γ-secretase activity. For instance, co-expression of these components in *Saccaromyces cerevisiae*, which lack endogenous γ-secretase activity, results in γ-secretase activity (Edbauer et al., 2003). The same has been shown in *Drosophila* and mammalian cells (Hayashi et al., 2004; Kimberly et al., 2003; Takasugi et al., 2003; Zhang et al., 2005a). Active γ-secretase complexes has also been succesfully isolated from post-mortem human brain (Farmery et al., 2003).

1.3.1 γ-Secretase components

The stoichiometry and size of γ-secretase is still under debate. The most accepted model is a 1:1:1:1 stoichiometry, which has got support from various biochemical approaches (Fraering et al., 2004; Sato et al., 2007). However, a dimerization of PS

(Schroeter et al., 2003), thus giving a 2:2:2:2 relationship, has also been proposed. Both suggestions find support in the estimated native molecular weight of the complex, ranging from 250kDa to 2MDa, depending on which method has been used for analysis (Edbauer et al., 2002; Kimberly et al., 2003; Li et al., 2000a; Osenkowski et al., 2009). The difference in the molecular weight could be explained by additional interacting components. For example, a number of proteins have been found to putatively interact with γ-secretase and to affect the Aβ production. These proteins include the transmembrane glycoprotein CD147 and TMP21 a protein involved in protein transport and quality control in ER and Golgi. (Chen et al., 2006; Zhou et al., 2005). In addition, TMP21, the synaptic protein syntaxin 1, NADH dehydrogenase ubiquinone iron-sulfur protein 7 (NDUFS7), a tubulin polymerization promoting protein (TPPP), the contactin-associated protein 1, Erlin-2 and the voltage-dependent anion channel 1 (VDAC1) as well as several other proteins have been associated with active γ-secretase in preparations from rat brain (Frykman et al., 2012; Hur et al., 2012; Teranishi et al., 2012; Teranishi et al., 2010). Members of the tetra spanin web, involved in cell fusion, proliferation, adhesion and migration processes (Levy and Shoham, 2005), were also identified as γ-secretase associated proteins in PS deficient fibroblasts stably expressing epitope-tagged PS1 or PS2 (Wakabayashi et al., 2009). Combined, these findings suggest that γ-secretase activity could be extensively regulated by multiple signaling pathways and interacting proteins.

1.3.1.1 Presenilin

Two human presenilin homologues, PS1 and PS2, were identified in 1995 (Levy-Lahad et al., 1995), (Sherrington et al., 1995) and they are evolutionary conserved between species. PS has nine transmembrane domains (TMDs) (Henricson et al., 2005; Laudon et al., 2005; Oh and Turner, 2005; Spasic et al., 2006) and share an average protein homology of 63%, but up to 95% within the TMDs. The fact that many more pathogenic mutations are found in the PS1 gene than in the PS2 gene suggest a more critical role for PS1 in the onset of the disease. Furthermore, PS1 knock-out mice are embryonic lethal and have dramatically reduced γ-secretase activity, while PS2-/- mice are viable and have preserved γ-secretase activity (De Strooper et al., 1998; Herreman et al., 1999). In addition, PS1 and PS2 complexes show differences in activity and sensitivity to γ-secretase inhibitors (Borgegard et al., 2011; Lai et al., 2003; Zhao et al., 2008). Thus, these proteins appear to possess overlapping but different functions.

After assembly of all γ-secretase components, PS undergoes endoproteolysis, an event that has been proposed to occur via autoproteolysis (Wolfe et al., 1999). Indeed, the domain that contains the PS cleavage site, encoded by exon 9, is hydrophobic and may integrate into the membrane. A major site after amino acid 298 and a minor between amino acid 292/293 were identified as the autoproteolytic cleavage site (Podlisny et al., 1997). In accordance, a recent report observed that endoproteolysis of PS generates tri-peptides (Fukumori et al., 2010), consistent with the stepwise endoproteolysis of APP (Takami et al., 2009). This is also in line with that some PS FAD mutations change the precision of the endoproteolyc site (Fukumori et al., 2010). The PS NTF and CTF constitute the active form of PS by forming a stable heterodimer. Each fragment harbours one of the two catalytic aspartate residues (Asp257 and

Asp385 in PS1) in TMD 6 and 7 (Steiner et al., 1999; Wolfe et al., 1999) that make up the catalytic site of γ-secretase. There are several pieces of evidence that the aspartates exert the catalytic function of the enzyme and thus identify the γ-secretase complex as an aspartyl protease; i) replacement of Asp257 or Asp385 with alanine prevents Aβ formation and abrogates endoproteolysis (Wolfe et al., 1999), ii) γ-secretase transition state inhibitors that bind to the active site of an aspartyl protease directly bind to both NTF and CTF (Berezovska et al., 2000; Esler et al., 2000; Li et al., 2000b; Seiffert et al., 2000) and iii) this binding is abolished by mutations of the aspartate residues (Wrigley et al., 2004).

Most FAD mutations are situated within or flanking the conserved hydrophobic TMDs and are, except for the Δexon9 mutation, missense mutations resulting in single amino acid change or deletion of two amino acid residues. In general, all FAD mutations in PS1 and 2 cause an increase in the Aβ42/Aβ40 ratio, either by decreasing the production of Aβ40 or by increasing the Aβ42 generation (Bentahir et al., 2006; Citron et al., 1997; Kretner et al., 2011; Kumar-Singh et al., 2006; Scheuner et al., 1996). The presence of the mutations in TMDs, their scattered distribution and the overall finding that they change the complex's cleavage preference, i.e. yielding an increase in the Aβ42/Aβ40 ratio, suggests that they may cause a conformational change of PS1. Especially since the topology of a membrane protein is largely dependent on the hydrophobicity of the TMDs (von Heijne, 2006). In addition, inherited mutations in a growing array of membrane proteins frequently lead to improper folding and trafficking (Nakamura and Lipton, 2009). A suggested conformational change of PS1 in the complex could lead to subtle alterations in the presentation of the substrate to the catalytic site or the substrate binding properties, thereby causing the observed shift in the Aβ42/Aβ40 ratio. Nevertheless, how the mutations induce the conformational change of PS remains to be elucidated. Strikingly, there are almost no known mutations in the large cytoplasmic loop of the PS molecule and this domain differ extensively between PS1 and PS2 (Stromberg et al., 2005). The loop is 110 amino acids in length for PS1 and 84 amino acids in length for PS2 and shares only 16% homology when preforming protein blast alignment (www.blast.nvbi.nlm.nih.gov). β-Catenin has been reported to bind to the loop of PS1 but not PS2 (Saura et al., 2000; Yu et al., 1998) and apart from endoproteolysis the loop region is also cleaved by caspases (Kim et al., 1997). Interestingly, some research groups have addressed the role of the large hydrophilic loop with somewhat contradicting results, leaving its function still unclear. The loop has been shown to be dispensable for γ-secretase activity since the lethal phenotype of PS1 deficient mice could be rescued by the introduction of a PS1 molecule lacking the loop (Xia et al., 2002). This is consistent with a study reporting that the Aβ production was not altered by PS1 and 2 FAD mutations when the Aβ levels were measured in cells with mutated PS molecules with or without the loop (Saura et al., 2000). However, a protective role of the loop has been suggested, since knock-in mice with a PS1 molecule lacking most of the hydrophilic loop but with a retained endoproteolytic site, showed reduced Aβ40 generation along with exacerbated plaque pathology (Deng et al., 2006).

1.3.1.2 Nicastrin

Five years after the discovery of PS, another member of the γ-secretase complex, Nicastrin, was identified by PS affinity purification (Yu et al., 2000). Nicastrin is a type I transmembrane protein with a short cytoplasmic tail and a large ectodomain containing multiple glycosylation sites. Nicastrin requires PS for its maturation into a glycosylated protein (Edbauer et al., 2002; Siman and Velji, 2003) but, in contrast, the glycosylation is not essential for γ-secretase activity or complex assembly (Herreman et al., 2003). However, Nicastrin is dependent on PS in order to alter the conformation of its ectodomain, which contains the DYGIS motif that is critical for γ-secretase activity (Shirotani et al., 2003). One part of the Nicastrin ectodomain shows similarity to aminopeptidases and the transferrin receptor superfamily (Fagan et al., 2001), suggesting that Nicastrin could act as a receptor and thus be involved in initial substrate recognition. It was reported that the glutamate residue Glu333 in the Nicastrin ectodomain physically interacted with the N-terminus of APP- and Notch derived γ-secretase substrates (Shah et al., 2005) and mutations of this residue led to reduced APP processing compared to wild type Nicastrin (Dries et al., 2009). The substrate receptor-like role for Nicastrin was later challenged, as both *in vivo* and *in vitro* studies found that the mutation of Glu333 (332 in mouse) was important for the maturation and assembly of the γ-secretase complex rather than the activity (Chavez-Gutierrez et al., 2008). Moreover, another member of the GxGD-type aspartyl proteases, SPPL2b does not require additional co-factors in order to be proteolyticly active. It still displays similar substrate requirements as the γ-secretase, i.e. an ectodomain-shedded substrate (Martin et al., 2009), indicating that substrate selection may not depend on Nicastrin. Thus, it remains unclear whether Nicastrin is involved in substrate selectivity or has a more general role in the stabilization and maturation of the γ-secretase complex (Zhang et al., 2005b).

1.3.1.3 Aph-1 and Pen-2

By performing genetic screening in *C. elegans*, two additional co-factors beyond PS and Nicastrin were identified, Aph-1 and Pen-2 (Francis et al., 2002; Goutte et al., 2002). Aph-1 deficent *C. elegans* lacks the anterior pharynx and therefore, the missing protein was named, anterior pharynx defective-1, Aph-1 (Goutte et al., 2002). There are two homologues of Aph-1 in humans; Aph-1a and b that share 56% of the amino acid sequence. Furthermore Aph-1a can be spliced into two isoforms, generating Aph-1aS (short) and Aph-1aL (long) and in addition there is a third isoform in mice, Aph-1c. Aph-1 is the most stable component of the complex and has a seven transmembrane topology, in which the N-terminus resides in the lumen and the C-terminal part faces the cytosol (Fortna et al., 2004). It has been suggested to function as a scaffold for the complex, thus being important for complex assembly as a conserved GxxxG motif in TMD4 is central for the binding of PS and Pen-2 (Niimura et al., 2005). In addition, two highly conserved histidine residues in TMD5 and TMD6 are important for interaction to the other γ-secretase components and for activity, since mutations in these residues lead to decreased Aβ formation (Pardossi-Piquard et al., 2009b). Pen-2 has a hairpin topology, containing two TMDs with the N- and C-terminus facing the lumen (Crystal et al., 2003). The C-terminus and TMD1 of Pen-2 is vital for endoproteolysis

of PS and thus for activation of the γ-secretase complex as well as stabilizing the generated PS fragments (Kim and Sisodia, 2005b; Prokop et al., 2005; Prokop et al., 2004). In turn the N-terminus is important for the interactions with PS (Crystal et al., 2003).

1.3.2 The structure and active site of γ-secretase

No detailed information about the structure of γ-secretase is available, since the complexity and the numerous TMDs of the γ-secretase complex have made structural analysis such as X-ray crystallography challenging. Nevertheless, recent data from negative stain- and cryo-electron microscopy suggest the formation of a transmembrane barrel-like structure with an aqueous catalytic cavity (Lazarov et al., 2006; Ogura et al., 2006; Osenkowski et al., 2009; Renzi et al., 2011). However, with a low resolution of 12-15Å it is not possible to get an understanding of the structure at a molecular level. More detailed information of the catalytic site has been provided by studies using substituted cysteine accessibility method (SCAM). These studies show that the catalytic pore is formed mainly by PS TMD6 and 7 that face each other (Sato et al., 2006; Tolia et al., 2006) and that both TMD1 and 9 contribute with residues that are water accessible (Sato et al., 2008; Takagi et al., 2010; Tolia et al., 2008). Importantly, both the GxGD motif in TMD7 and the conserved PAL motif between TMD8 and 9, were suggested to be part of the hydrophilic cavity as they were accessible to water (Sato et al., 2006; Sato et al., 2008; Tolia et al., 2006; Tolia et al., 2008). In addition, it was recently reported that the loop domain of Pen-2 was accessible to water from the luminal side, thus contributing to the active site (Bammens et al., 2011). The loop in Pen-2 was also cross-linked to PS1 CTF, suggesting that it is in close proximity to TMD9 that show a comparable accessibility pattern as the loop in Pen-2 (Bammens et al., 2011; Tolia et al., 2008). NMR analysis of the PS1-CTF has been performed suggesting that the TMD7 formed a half helix and the part with the GxGD region had a loose random coil conformation, whereas TMD8 was fully integrated as a helix and the TMD9 had a nicked helix (Sobhanifar et al., 2010). Finally, all these results are supported by previously performed general interaction studies of the subunits, using cross-linking and co-immunoprecipitation techniques. In these studies PS NTF and CTF were shown to interact, PS TMD4 interacted to the hydrophobic domain in Pen-2 and the CTF with Aph-1, which also is in close contact with Nicastrin (Fraering et al., 2004; Kim and Sisodia, 2005a; Steiner et al., 2008; Watanabe et al., 2005).

In contrast to the active site, less information is available for the location of the initial docking site of the substrate. There is, however, compelling evidence for the existence of such site, to where the substrate initially binds before it is passed to the active site for proteolytic processing. For example, by immobilizing a transition-state (TS) analogue inhibitor on an affinity column, it was shown that C99 still can be co-purified with the γ-secretase complex, thus binding a site different from the active site that was presumably interacting with the immobilized inhibitor (Esler et al., 2002). Further, helical peptides that mimic the transmembrane domain of C99 can inhibit γ-secretase processing of APP (Das et al., 2003) by occupying another site different from the transition-state analogue inhibitor binding site (Kornilova et al., 2003). By using affinity labeling of helical peptides, the presenilin NTF-CTF interface has been

identified to contribute to the binding site (Kornilova et al., 2005). Moreover, later both fragments were individually shown to be the target of substrate mimicking peptides (Sato et al., 2008). It has also been implicated that the GxGD and PAL motifs, which are parts of rather flexible structures, are important for substrate interaction as these region are highly vulnerable to mutations when monitoring substrate processing (Perez-Revuelta et al., 2010; Steiner et al., 2000; Wang et al., 2006a; Yamasaki et al., 2006). Interestingly, the exon 9 encoding hydrophobic domain that harbours the endoproteolytic site has been suggested to function as a plug in the catalytic pore. By using an exon 9 mimicking peptide it was demonstrated that the hydrophobic domain did not interact with the active site but rather the docking site, since there were no competition between the TS analogue inhibitor L-685,458 and the exon 9 peptide (Knappenberger et al., 2004). Thus, the intact loop may function as a gatekeeper, keeping PS in its inactive form as a holoprotein and preventing substrates to enter the active site (Fukumori et al., 2010; Knappenberger et al., 2004). This is consistent with a report, where the GlpG, a rhomboid protease in *E.coli* that contain a similar proteolytic chamber as γ-secretase (Wang et al., 2006b), was found to have a flexible loop that sealed the pore and following its removal induced substrate binding (Baker et al., 2007). Interestingly, the PS1 FAD mutation Δexon 9 that is not endoproteolysed due to the lack of the endoproteolytic cleavage site, may be constantly active due to the removal of this regulatory mechanism. As described in section *1.3.1 γ-Secretase components*, PS1 FAD mutations have been proposed to change the conformation of PS1 in the complex, leading to subtle alterations in the presentation of the substrate to the catalytic site or the substrate binding properties and thereby causing the observed shift in Aβ42/Aβ40 ratio. Indeed, induced conformational changes in the catalytic site of PS by certain PS FAD mutations have been reported. By using a FLIM assay, it was shown that PS1 FAD mutations increased the NTF and CTF proximity compared to wild type, thereby leading to a more closed conformation of the complex (Berezovska et al., 2005). A closed conformation of the complex was proposed to stabilize the active site of γ-secretase and C99 in a way that favoured the Aβ42 production. This is supported by observations using pharmacological treatments with substances that either increased or decreased the Aβ42/Aβ40 ratio also led to induction of a closed or open state, respectively (Lleo et al., 2004; Uemura et al., 2009). In addition, genetic manipulation of γ-secretase components gave the same results, thus suggesting that the conformation of the γ-secretase complex correlated with the Aβ42/Aβ40 ratio (Uemura et al., 2009). The finding that PS FAD mutations induce changes in the catalytic site of γ-secretase was further confirmed by another report, which showed that the PS1 FAD mutation L166P and to some extent G384A affected the pull down of active γ-secretase complexes when using a photolabeled transition state analogue inhibitor (Kornilova et al., 2005).

1.3.3 The function of γ-secretase

The γ-secretase is a promiscuous enzyme complex and the list of its substrates still continues to grow. To date, there are more than 90 known substrates for the γ-secretase complex (Lleo and Saura, 2011). There is no identified consensus binding motif in the substrates, but the substrates must have undergone ectodomain shedding, which leads

to a short ectodomain with less than 50 amino acids (Struhl and Adachi, 2000). However, not only ectodomain shedding is a prerequisite for γ-secretase activity. The juxtamembrane domain, the TMD as well as the cytoplasmic domain of the substrates, are important in order to be correctly processed by γ-secretase (Hemming et al., 2008; Ren et al., 2007; Zhang et al., 2002). The growing list of substrates and the poor sequence specificity of γ-secretase have led to the suggestion that the complex acts as a cleaning lady of the membrane, removing transmembrane domains when the full-length proteins have fulfilled their purpose (Kopan and Ilagan, 2004; Lichtenthaler et al., 2011). Indeed, the feature of the γ-secretase complex, hydrolysis of a peptide bond within the transmembrane region, is a highly conserved process called regulated intramembrane proteolysis (RIP) (Brown et al., 2000). There are three classes of proteases that posses the ability to perform intramembranous cleavage; the GxGD-type aspartyl proteases, the S2P-metalloproteases, and the rhomboid serine proteases (Beel and Sanders, 2008). The GxGD-type aspartyl proteases consist of γ-secretase, the signal peptide peptidase (SPP), the SPP-like proteases (SPPL) and the type IV prepilin peptidases. Some intracellular domains (ICD) released by RIP are translocated to the nucleus and are involved in signal transduction, implying a role of initiating signaling for the γ-secretase complex. In fact, during the last decade RIP has emerged as a novel cell signaling mechanism that involves most type I and II transmembrane proteins through a sequential cleavage event. Fore the type I transmembrane proteins, the large soluble extracellular fragment of the substrate is first released by a cleavage in the ectodomain close to the membrane, resulting in a membrane bound CTF fragment. This is referred to as ectodomain shedding and is executed by members of the ADAM family, BACE1, BACE2 and matrix metalloproteases. The RIP process are mostly regulated at the level of shedding and this can either be a constitutive activity or regulated by different stimuli, including ligand binding and subcellular localization (Hayashida et al., 2010). Upon ectodomain shedding, the membrane-associated CTF is subsequently cleaved a second time within the membrane, releasing the ICD. Some substrates also release Aβ-like peptides, such as Notch, APP like protein (APLP) 1 and 2, CD44 and interleukin-1 receptor II (Eggert et al., 2004; Kuhn et al., 2007; Lammich et al., 2002; Okochi et al., 2002; Yanagida et al., 2009).

A well established example of a pathway mediated by RIP is the Notch signaling pathway, schematically shown in Figure 6. The Notch receptors (Notch 1-4) were the next γ-secretase substrates to be discovered after APP (Schroeter et al., 1998). During trafficking through the secretory pathway, Notch is cleaved at the S1 site by furin-like convertase forming a heterodimer (Blaumueller et al., 1997; Logeat et al., 1998). At the plasma membrane, the heterodimer is activated by binding of its ligands Jagged or Delta that is presented by neighbouring cells (Nye and Kopan, 1995), inducing ectodomain shedding of Notch at the extracellular S2 site by ADAM10 (van Tetering et al., 2009) This results in a shorter membrane bound form, NotchΔE or NEXT, which is an immediate substrate for the γ-secretase (De Strooper et al., 1999; Schroeter et al., 1998). γ-Secretase processes NotchΔE in at least two different positions, the S3 and the S4 site. The S3 cleavage is equivalent to the ε-cleavage in APP and generates the Notch intracellular domain (NICD) (Schroeter et al., 1998), while the S4 site corresponds to the γ-site in APP and releases Aβ-like peptides, called Nβ (Okochi et al., 2002). The

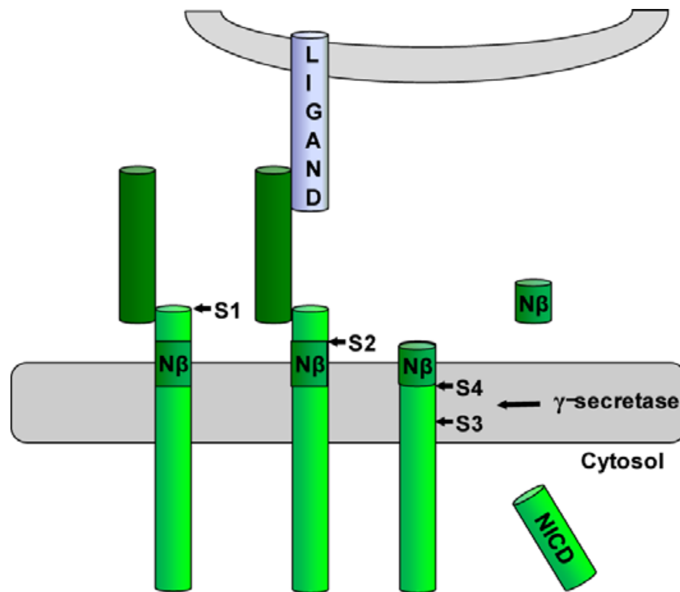

Figure 6. Schematic illustration of Notch processing. During trafficking through the secretory pathway, Notch is cleaved at the S1 site by furin-like convertase forming a heterodimer, which is further transported to the plasma membrane. After ligand binding the heterodimer is ectodomain shedded at the S2 site by ADAM10. Finally, γ-secretase processes NotchΔE at two different positions, the S3 and the S4 site generating NICD and Nβ.

physiological function of Nβ is not known but NICD translocates to the nucleus, where it is involved in transactivation of *HES* (Hairy/Enhancer of split) genes. These genes are involved in non-neural cell fate decisions and are critical for the development of many tissues and organs (Jarriault et al., 1995). This vital signaling pathway is especially assigned the Notch1 receptor and consequently, Notch1 deficiency leads to an embryonic lethal phenotype associated with severe developmental deficits (Huppert et al., 2000; Swiatek et al., 1994). In adults, Notch1 is constitutively expressed, having important roles in various cells and tissues, such as hematopoiesis, the immune system, gastrointestinal tract, pancreas, myogenesis, and self-renewing organs (Groth and Fortini, 2012; Kopan et al., 1994; Pui et al., 1999; Radtke et al., 2004; van Es et al., 2005).

Apart from APP and Notch, other γ-secretase substrates include; the APP homologues APLP1 and 2; the sorting receptors SorlA and sortilin (that are important for intracellular sorting and trafficking of cargo proteins); ligands such as, Jagged and Delta, (which are the Notch ligands); ligands for receptor tyrosine kinase Eph receptors, EphrinB, involved in neuritogenesis and angiogenesis; CD44, E-cadherin and N-cadherin, which are cell adhesion molecules; various receptors including ErbB4, a receptor of growth factors; p75-neurotrophin receptor (p75-NTR) involved in axonal transport and neuronal survival and interleukin-1 receptors that are a part of the immune response (Eggert et al., 2004; Elzinga et al., 2009; Georgakopoulos et al., 2006; Jung et al., 2003; Kanning et al., 2003; Kuhn et al., 2007; Lammich et al., 2002; Marambaud et al., 2002; Marambaud et al., 2003; Ni et al., 2001; Nyborg et al., 2006; Nye and Kopan, 1995; Scheinfeld et al., 2002). Most commonly, it is the ICD that acts as the signaling molecule, either by translocation into the nucleus and inducing the transcription of target genes or by acting as a cytosolic signaling molecule. The first is

in analogy to NICD and includes CD44-ICD that has been shown to increase the activation of the transcription co-activator CBP and the ICD of N-cadherin, which promotes degradation of the transcription factor CREB binding protein (Marambaud et al., 2003; Okamoto et al., 2001). Examples of non-nucleus targeted ICDs are; ephrinB-ICD that leads to the activation of Src-family kinases and the p75-NTR-ICD, which is important for formation/disassembly of receptor complexes (Georgakopoulos et al., 2006; Jung et al., 2003; Kanning et al., 2003). Importantly, most ICDs are rapidly degraded and may not have a signaling function. However, the extracellular fragment released by RIP can also mediate signaling functions, like when the ectodomain from growth factors start a signaling transduction by activating neighbouring cells. E.g. after the cleavage of cytokine tumour necrosis factor α (TNFα) both the ectodomain and the ICD act as signaling molecules, but targeting different cells (Black et al., 1997; Fluhrer et al., 2006).

1.3.4 Regulation of γ-secretase activity

Both PS and Aph-1 exist as homologues, yielding several distinct γ-secretase complexes with the possible combinations of PS and Aph-1 homologues. Due to the presence of PS1 and PS2 homologues and the three Aph-1 isoforms, at least six distinct complexes exist in humans that have different properties and functions (Hebert et al., 2004; Saura et al., 1999; Serneels et al., 2005; Shirotani et al., 2004b). Indeed, both PS homologues co-immunoprecipitate independently with each of the Aph-1 variants (Hebert et al., 2004; Serneels et al., 2005; Shirotani et al., 2004b). Moreover, the multiple glycosylation forms of Nicastrin also contribute to the hypothesis that different γ-secretase complexes exist with distinct functions. So, although γ-secretase is a promiscuous enzyme cleaving many substrates, complexes containing different variants of subunits can have altered activity and substrate specificity. PS1 containing complexes catalyze the majority of the Aβ production (Borgegard et al., 2011; De Strooper et al., 1998) and PS1 and PS2 complexes show differences in activity and sensitivity to some γ-secretase inhibitors (Borgegard et al., 2011; Lai et al., 2003; Zhao et al., 2008). In addition, it has also been reported that cadherins are only processed by PS1 containing complexes, since the catenin-protein-p-120, which binds cadherins and recruits them to the γ-secretase complex for processing, only binds a sequence in PS1 CTF that is not present in PS2 (Saura et al., 2000; Yu et al., 1998). Furthermore, PS1 knock-out mice are embryonic lethal and have dramatically reduced γ-secretase activity, while PS2-/- are viable and have preserved γ-secretase activity (De Strooper et al., 1998; Herreman et al., 1999). Moreover, different cell types may use complexes containing different PS homologues, since it has been shown that there are mainly PS2 containing complexes in microglia (Jayadev et al., 2010), while in fibroblasts only a minor pool of PS2 complexes exist (Franberg et al., 2011). Regarding the Aph-1 isoforms, mice knocked-out of one of the three *APH-1* genes, all display differences in phenotype and γ-secretase activity. In addition, complexes containing Aph-1a are crucial for γ-secretase mediated Notch signaling during development (Ma et al., 2005; Serneels et al., 2005). It has also been reported that Aph-1b/c complexes processes neuregulin as Aph-1b/c knock-out mice have accumulation of neuregulin CTF (Dejaegere et al., 2008) and a phenotype similar to mice deficient of neuregulin

(Stefansson et al., 2002). Furthermore, Aph-1b complexes produce more of the longer Aβ forms in proportion to the shorter forms and also induce a more closed conformation of PS1 (Serneels et al., 2009), similar to the effect of certain PS1 FAD mutations monitored by a FLIM assay (Berezovska et al., 2005). Finally, complexes containing Aph-1b are the major contributors of γ-secretase activity in human brain (Serneels et al., 2009). However, all complexes with combinations of human Aph-1 isoforms together with FAD mutations in PS1 or PS2 can generate Aβ42 (Shirotani et al., 2007). Substrate processing is also controlled by the subcellular localization of the enzyme. For example, glycosylated Nicastrin may promote cell surface localization of γ-secretase (Yang et al., 2002) and thus increase the cleavage of substrates that are mainly expressed at the plasma membrane, such as Notch and cadherins. In contrast, factors that retain or localize γ-secretase to internal compartments, such as the endosomes, facilitate the Aβ production, as the amyloidogenic processing of APP mainly occur there due to high BACE1 activity (Haass et al., 1993; Koo and Squazzo, 1994). Such abilities are for example assigned to i) the Calsenilin protein that inhibits the trafficking of the PS1 CTF fragment and Aph-1 to the cell surface (Jang et al., 2011) ii) two members of the G-protein-coupled receptors, the β2-adrenergic and δ-opioid receptors, which increase the endocytosis and trafficking of γ-secretase to the endosomes by interacting with PS (Ni et al., 2006; Teng et al., 2010). In addition, the ε-cleavage of APP differs in the endosomes and at the plasma membrane (Fukumori et al., 2006), implying subcellular localization dependent properties of the γ-secretase complex that could be due to differences in lipid composition or pH. γ-Secretase interacting protein TMP21 also regulates γ-secretase by binding to PS in the intracellular compartments. In contrast to other factors that retain the complex at a subcellular localization, TMP21 decreases Aβ formation and interestingly not affects the ε-cleavage (Chen et al., 2006; Pardossi-Piquard et al., 2009a). Moreover, there is a growing list of γ-secretase interacting proteins that regulate Aβ generation, as described in section *C.1 γ-Secretase components*. Finally, it is an appealing strategy to pharmacologically regulate the γ-secretase complex with the aim to alter the metabolism of the Aβ42 peptide, which plays a central role in AD pathogenesis. Inhibition and modulation of the γ-secretase complex will be discussed in detail in section *1.4.2 Future treatment strategies*.

1.4 TREATMENT OF AD

More than 100 years have passed since Dr. Alzheimer first described the neuropathological hallmarks of the disease that later got his name. Despite the tremendous research efforts during the last three decades, there is currently no treatment available to cure or to effectively stop the development of the disease. There are only two types of symptomatic drugs approved for treating AD, cholinesterase inhibitors and a N-methyl-D-aspartic acid (NMDA) - receptor antagonist, memantine. However, the knowledge of the neurobiology in AD has increased enormously these past decades and numerous attempts to therapeutically affect the progression of AD are currently under investigation.

1.4.1 Current treatment

The strategy using cholinesterase inhibitors is based on the observation that the cholinergic system is specifically vulnerable and disrupted in AD (Wenk, 2003). The cholinergic system is important for higher cognitive functions in the brain such as memory and attention. Cholinergic neurons in the nucleus basalis of Meynert, which provide the major cholinergic input to cerebral cortex, have been shown to be selectively degenerated in AD (Davies and Maloney, 1976; Whitehouse et al., 1982). Cholinergic signaling between cells is mediated by the neurotransmitter acetylcholine and cholinesterase inhibitors block the degradation of the neurotransmitter. Increasing concentration of acetylcholine in the synaptic cleft prolongs the signaling and thus enhances the activity of the remaining cholinergic neurons. There are three different cholinesterase inhibitors on the market and they are used for treating patients with mild to moderate AD. NMDA receptors can be over-stimulated by glutamate, leading to hyper-excitatory signal transduction and dysfunction that in turn propagates neuronal death (Greenamyre and Young, 1989; Meldrum, 1990). Studies have associated abnormally high glutamate levels with AD (Greenamyre and Young, 1989; Penney et al., 1990). Memantine resets NMDA receptor activity by blocking the effects of excess glutamate, allowing normal physiological transmission to occur (Chohan and Iqbal, 2006).

1.4.2 Future treatment strategies

Epidemiological studies have demonstrated that long-term treatment of various agents including non-steroidal anti-inflammatory drugs (NSAIDs) (aspirin, ibuprofen, naproxen etc), estrogen and cholesterol-lowering drugs reduces the risk of AD (Cote et al., 2012; Henderson, 2008; Li et al., 2010). Similar studies have also associated the intake of Mediterranean food, vitamin B, social and physical activity with decreased risk of developing the disease (Eskelinen et al., 2011; Hooshmand et al., 2012; Scarmeas et al., 2009). Other treatment strategies involve regenerative mechanisms that may increase neurogenesis, which can be induced by drugs, cell transplantation, mitochondrial targets, antioxidants and nerve growth factor (NGF) treatment (Ankarcrona et al., 2010; Eriksdotter-Jonhagen et al., 2012; Marutle et al., 2007; Olson et al., 1992; Pieper et al., 2010). NGF stimulates growth of cholinergic neurons and delivery of NGF through an implantate in the nucleus basalis of Meynert can improve cognition in mild AD patients (Eriksdotter-Jonhagen et al., 2012). However the therapeutic benefits and sometimes the safety of some of these approaches are in general low (Carlsson, 2008; Lukiw, 2012) or still remains to be shown. Beyond these approaches, the current main disease modifying strategies in AD involves; i) drugs targeting Tau, ii) elimination of the aggregation of Aβ and destabilizing Aβ oligomers, iii) improving the clearance of Aβ or iv) compounds that reduce or modulate the Aβ generation (Reitz, 2012). Regarding tau pathogenesis, the main approaches are inhibitors of tau-phosphorylating kinases or drugs that inhibit the aggregation or promote disassembly of aggregated Tau. However, since AD is increasingly considered as a multi-factorial and heterogeneous disease, the use of a drug-cocktail covering multiple targets may be a successful approach.

1.4.2.1 Targeting Aβ

A major research focus of the therapeutic treatment is compounds targeting the Aβ pathway, aiming at reducing Aβ levels in the brain. One approach is the prevention of Aβ aggregation or neutralizing the toxicity of Aβ oligomers by destabilizing them. The concern with these types of compounds often involves difficulties in passing the blood-brain-barrier (BBB) or safety issues. A few compounds have however entered phase II clinical trials. For example, PBT1 was shown to pass into the brain, although in small amounts, in both transgenic mice and human volunteers. In the brain, it binds to amyloid plaques and interferes with the binding of Aβ to copper and zinc and thus reducing Aβ aggregation (Opazo et al., 2006). Unfortunately, the trials were later stopped due to toxicity reasons (Adlard et al., 2008), but a second-generation cousin, PBT2, has better BBB permeability and shows some improvement in cognitive or executive function in animal experiments and mild AD patients (Adlard et al., 2008; Lannfelt et al., 2008). In addition, ELND-005 that modulates Aβ misfolding and aggregation by interacting with Aβ was also reported to have problems with toxicity (Reitz, 2012). Another way of suppressing Aβ aggregation is to enhance its clearance. This could either be accomplished with compounds that increase the activity of Aβ degrading enzymes such as insulin degrading enzyme and neprilysin or by using active or passive immunization.

Immunization as a therapy was developed after an observation that vaccination with Aβ42 in an AD transgenic mouse model prevented plaque formation (Schenk et al., 1999). Antibodies can trigger Aβ clearance in different ways; i) by inducing phagocytosis of Aβ by microglia that bind to Aβ and promote solubilization ii) by binding to peripheral Aβ in the blood and function as a peripheral sink and thus promoting the efflux of Aβ from the brain. Unfortunately, the first vaccination study in human was halted due to severe meningoencephalitis caused by T-cell response in some participants (Gilman et al., 2005). However, most patients developed Aβ-antibody titres that lasted up to 4.6 years and had some cognitive improvement. In addition, *post-mortem* examinations revealed almost complete removal of the amyloid plaques in some subjects even though end-stage dementia symptoms still occurred at the time of death (Vellas et al., 2009). Lately, new vaccines that only target the B-cell epitope have been developed and recently results from the phase II trial with the CAD-106 antigen was reported. No meningoencephalitis was observed, CAD-106 was well tolerated and an immune-response was found in around 70% of all mild-to-moderate AD patients (Winblad et al., 2012). An alternative approach is the use of passive immunization; here the patients are injected with antibodies against Aβ. Several antibodies including; bapineuzumab from Pfizer, Johnson & Johnson and Elan and solanezumab from Lilly, are now being tested and they are in general well tolerated and show some effect on Aβ clearance (Reitz, 2012). The outcome of clinical phase III trials with bapineuzumab and solanezumab are important steps forward in our efforts to address whether anti-Aβ treatment is an effective therapeutic approach in AD. Unfortunately, in August 2012 the trials using intravenous administration of bapineuzumab were discontinued, due to failure to show any impact on cognitive or functional performance in patients with mild to moderate Alzheimer disease. Bapineuzumab, is raised aginst the N-terminal domain of Aβ and targets the plaques.

The failure of this antibody may either high-light the problems of targeting Aβ at later stages of AD, or the antibody was not efficient enough in lowering Aβ. Future analysis of biomarker data from these trials will be very important in fully interpretating the obtained clinical data. Anyhow, solanezumab exhibits quite different properties compared to bapineuzemab and recognizes the mid-part of Aβ and does only bind various forms of soluble Aβ and not plaques. Thus, the solanezumab trials will more specifically address if oligomeric forms of Aβ indeed contributes to the toxicity in AD. Interestinlgy, recent press releases from Eli Lilly suggest that some positive signs from the solanezumab trials have been observed.

1.4.2.1.1 Secretases as a therapeutic target of AD

APP is processed by both α-, β- and γ-secretase and therefore, it is natural to consider all secretases as potential targets in the prevention of AD. In order to reduce Aβ generation, secretases can be targeted either by promoting α- secretase activity or by inhibiting β- and γ- secretase. There are a number of evidence that α- and β-secretase compete for cleavage of APP (Lichtenthaler, 2011), since over-expression of ADAM10 leads to elevated sAPPα in parallel with decreased β-secretase cleavage and subsequently Aβ production (Postina et al., 2004). The same is also observed in experiments using pharmacological stimulation of α-secretase by muscarinic activation (Bandyopadhyay et al., 2007). Moreover, the genetic APPswe mutation results in increased BACE1 cleavage accompanied with reduced sAPPα levels. Thus, increasing α-secretase activity pharmacologically is a valid therapeutic strategy in AD (Fahrenholz, 2007). These approaches involve increased ADAM10 expression that is induced by retinoic acid, which is already used in clinic for psoriasis (Donmez et al., 2010; Tippmann et al., 2009) or by enhancing its activity using M1 muscarinic agonists (Lichtenthaler, 2011). Other alternatives involve treatment with cholesterol-lowering-drugs since these reduces the cholesterol content in certain areas and thus indirectly elevates α-secretase activity that take place outside the cholesterol rich domains (Kojro et al., 2010). Importantly, an increased long-term activation of ADAM10 needs to be thoroughly evaluated as ADAM10 cleaves more than 30 other substrates apart from APP.

β-Secretase initiates the amyloidgenic pathway, thus being an important drug target for Alzheimer disease. However, there have been some key challenges regarding compounds affecting BACE1. The catalytic site of BACE1 is unusually large (Turner et al., 2005), representing a major challenge of designing compounds that are able to block the active site and still effectively penetrate the BBB. Moreover, a growing list of β-secretase substrates raises concerns regarding interference with the physiological functions of BACE1 (Dislich and Lichtenthaler, 2012; Kuhn et al., 2012). The difficulties in developing potent drugs that can cross the BBB have now been over-come and is reflected by that the increasing number of late preclinical and early clinical BACE1 directed inhibitors. The recent results from the first-time-in-man study with a BACE1 non-peptidic inhibitor showed good efficacy as both Aβ and sAPPβ were decreased (May et al., 2011). Unexpectedly, the study had to be halted since a mouse model showed retinal pathology upon long-term treatment. However, the effect was later proven to be unrelated of BACE1 inhibition, since the finding was recapitulated in BACE1 kock-out mice treated with the same compound (May et al., 2011). Similar to

γ-secretase, BACE1 is also subjected to immunization studies. Both active and passive vaccinations studies have reported to inhibit the enzyme in mice models (Atwal et al., 2011; Chang et al., 2007; Zhou et al., 2011). Another strategy involving inhibition of BACE1 is to target its subcellular localization and indirectly reduce its processing activity. These drugs, such as bepridil and amiodarone, raise the membrane-proximal pH in the endosome above pH4.5, which is the optimum pH value for BACE1, and consequently decrease Aβ production (Mitterreiter et al., 2010).

1.4.2.1.2 Inhibition and modulation of the γ-secretase complex

The γ-secretase complex is an appealing drug target when the therapeutic strategy is to alter the metabolism of Aβ. It is directly involved in the Aβ formation and it also determines the pathogenic potential of Aβ. In 1995, the first compounds were reported that could inhibit γ-secretase activity. These compounds were of the peptide aldehyde-type and the following years several peptidomimetic compounds were developed including; MW167, L-685,458, DAPT and helical peptides (Bihel et al., 2004; Das et al., 2003; Dovey et al., 2001; Shearman et al., 2000; Wolfe et al., 1998). Some of these compounds are substrate based, such as the helical peptides that contain the γ-secretase cleavage site of APP, and some are transition-state analogue inhibitors, such as L-685,458 that only bind to the active site. Both these classes of compounds bind the NTF/CTF interface, but at different sites (Esler et al., 2000; Kornilova et al., 2005), consistent with the previous finding of an initial substrate-docking site (Beher et al., 2003; Esler et al., 2002). Interestingly, competition studies with the helical peptides, L-685,458 and DAPT, suggest that DAPT binds to a site distinct from the active site and the docking site (Morohashi et al., 2006) but with some overlap. Thus, DAPT may interact with the substrate transit-path from the docking site to the active site (Wolfe, 2012). Early γ-secretase inhibitors (GSIs) have been very useful as chemical tools for characterizing the γ-secretase complex but have low potency. However, as more potent GSIs have been developed, their Aβ lowering abilities could be confirmed in transgenic mice models (Dovey et al., 2001; Lanz et al., 2004). Unfortunately, severe side effects such as gastrointestinal toxicity and immunosuppression due to interference with the Notch signaling pathway have also been discovered when treatment was performed for longer periods (2 weeks) (Searfoss et al., 2003; Wong et al., 2004). In accordance, side effects assigned blocked Notch signaling and worsening in cognition of treated as compared to placebo-treated patients, caused a recent large clinical phase III trial with the GSI semagacestat to be interrupted in August 2010, reviewed in (Imbimbo and Giardina, 2011; Wolfe, 2012). Since semagacestat has no substrate specificity, this was not unexpected and therefore current investigations attempt to find GSIs with a sufficient therapeutic window between APP and Notch processing. The first Notch-sparing γ-secretase inhibitor Gleevec was reported in 2003. Gleevec is an abl kinase inhibitor, but the Aβ lowering properties are in an abl kinase-independent manner since the selective effect was retained in abl kinase knock-out cells (Netzer et al., 2003). Recently, a γ-secretase-activating-protein (GSAP) that is the target of Gleevec has been identified. GSAP was shown to regulate γ-secretase processing of APP but not Notch as Aβ levels were reduced during knock-down of GSAP in a transgenic mouse model without any observed Notch related side effects (He et al., 2010). Beyond Gleevec, the development of APP selective compounds has been extremely challenging (Lundkvist

and Naslund, 2007) and as a consequence only a few Notch-sparing GSIs with 2-≈190 fold selectivity for APP over Notch have entered clinical trials. Examples of these include; Semagacestat from Lilly, BMS-708163 from Bristol-Myers-Squibb, PF-3084014 from Pfizer, GSI-953 from Wyeth and ELND-006 from Elan. Except BMS-708163, all compounds have been precluded further clinical development, probably due to mechanism-based toxicity as a result of impaired Notch signaling and other pathways discussed above (Gillman et al., 2010; Imbimbo and Giardina, 2011; Lanz et al., 2010; Mayer et al., 2008; Wolfe, 2012).

Given the challenge observed with γ-secretase inhibitors, alternative therapeutic strategies that spare Notch signaling while targeting γ-secretase mediated Aβ production are in progress. For instance, Notch inhibition is avoided using so called γ-secretase modulators (GSMs). Typically, GSMs do not affect the overall rate of Notch, APP (ε and S3 cleavage) or ErbB4 processing (Kukar and Golde, 2008; Weggen et al., 2001). However, by shifting the cleavage preference of the enzyme from producing the amyloid-prone Aβ42 variant to shorter and less toxic Aβ species, GSMs change the proportions of various forms of Aβ peptides (Weggen et al., 2001; Weggen et al., 2003). The first GSMs, subsets of non-steroidal anti-inflammatory drugs (NSAIDs), such as sulindac sulfide and ibuprofen, were identified in 2001 (Weggen et al., 2001). The first GSM entering clinical trials was R-flurbiprofen from Myriad Genetics, showing promising effects in APP transgenic mice (Kukar et al., 2007). Since then, a number of compounds have reached or are approaching clinical trials, including E2212 from Eisai, CHF5074 from Chiesi Farmaceutici, EVP-15962 from EnVivo Pharmaceuticals and NPG-328 from Neuro Genetic Pharmaceuticals (Imbimbo and Giardina, 2011). Unfortunately, R-flurbiprofen failed in phase III (Green et al., 2009), probably due to its low Aβ reducing efficacy and poor brain penetration, a feature likely shared with the other compounds. The last decade, many second-generation GSMs have brought improvement. These compounds are generally structurally distinct from the NSAID family and lack the acidic carboxyl group, associated with the NSAID family of GSMs. Importantly, many of these compounds display a much higher potency and a better BBB penetration, and do therefore lower CNS Aβ production much more efficiently compared to the early GSMs (Kounnas et al., 2010; Oehlrich et al., 2010; Portelius et al., 2010; Tomita and Iwatsubo, 2006). The binding site of GSMs is still under debate. Some studies associate NSAID-based GSMs with the APP-derived C99 peptides (Botev et al., 2011; Kukar et al., 2008; Richter et al., 2010), but other groups have challenged this implied substrate-targeting hypothesis (Barrett et al., 2011; Beel et al., 2009; Clarke et al., 2006; Page et al., 2010). However, during the last year, γ-secretase instead of APP has been identified as the principal target of second-generation GSMs (Borgegard et al., 2012; Crump et al., 2011; Ebke et al., 2011; Jumpertz et al., 2012; Kounnas et al., 2010; Ohki et al., 2011). These studies include pull down of crosslinked photoprobes of the specific GSM, coupling of GSM to a solid support followed by affinity chromatography or autoradiography binding studies with tritium-labeled GSM. Some groups also performed competition experiments with GSIs and /or other GSMs in order to compare binding sites, and although some inconsistent data, the results suggest an allosteric mechanism targeting the enzyme (Borgegard et al., 2012; Ebke et al., 2011; Jumpertz et al., 2012; Ohki et al., 2011).

2 AIMS OF THE THESIS

The proteolytic activity of γ-secretase is a key step in the pathogenesis of AD, since it is directly involved in Aβ formation and also determines the pathogenic potential of Aβ. A detailed knowledge about the γ-secretase complex will help to understand at least a part of the intricate mechanisms of the disease. Since γ-secretase mediates cleavage of many substrates involved in cell signaling, such as the Notch receptor, it is crucial to sustain these pathways when developing γ-secretase targeting drugs for the treatment of AD. Notch signaling, for instance is important for cell differentiation and proliferation processes in many tissues in adulthood, and several clinical trials with different γ-secretase inhibitors have resulted in adverse side effects which are probable due to impaired Notch signaling. (reviewed in (Imbimbo and Giardina, 2011)). It is therefore very important to develop novel strategies to combat Aβ production via γ-secretase and other targets. In order to develop novel, effective and safe γ-secretase targeting strategies, including GSMs, additional knowledge about the mechanisms of γ-secretase processing is required. The general aim of this thesis was to learn more about the molecular basis of γ-secretase heterogeneous γ- and ε-cleavage activity and to characterize and identify differences between γ-secretase mediated APP and Notch processing by using genetic and pharmacological approaches.

The specific aims of this thesis were:

Paper I: To examine how PS1 FAD mutations cause elevated Aβ42/Aβ40 ratio by investigating if they: i) induce an alteration in the membrane integration of PS1 TMDs, ii) influence structural changes in the catalytic site.

Paper II: To study the importance of the large hydrophilic loop of PS1 for γ-secretase complex assembly and processing of APP and Notch.

Paper III: To investigate the role of the Nicastrin ectodomain for APP and Notch processing.

Paper IV: To characterize how first- and second- generation GSMs affect γ-secretase processing of both APP and Notch by examining the modulation of Aβ, Nβ and NICD.

3 COMMENTS ON METHODOLOGIES

Several methods and systems have been used during the course of this thesis project. In this section, benefits and limitations as well as similarities and differences concerning the different models and procedures will be described and discussed. Detailed descriptions of the techniques are found in the respective paper.

3.1 MATERIALS

Alzheimer disease is a complex multi-pathogenic disorder and is therefore challenging to mimic in a suitable model. Human post-mortem brain provides a valuable model for studying the disease state. However, it is only possible to obtain static information at a specific time point and is not reflecting the considerable changes that occur during the course of disease, which progresses over a long time period. Another limitation is the availability of human brain tissues and the often long post-mortem time that needs to be taken into account. Moreover, individual differences between brains can be large. One alternative to human material is to use animal models, which can be used for examining dynamic processes that will gain information about different stages of the disease. In addition, the post-mortem time can also be kept at a minimum. Unfortunately, no species, except humans, develop the disease and thus, there is no true animal model for AD, only transgenic models that mimic different aspects of the disorder. To study a specific process or signaling pathway, the use of cell culture is a very convenient model. The advantages are unlimited numbers, considerably lower individual differences than either human brains or animals and there are less ethical considerations about immortalised cell lines. Moreover, cells are susceptible to modifications such si-RNA knock-down or over-expression of proteins and it is possible to study a mechanism of interest in a less complex and more controlled environment. However, due to their simplified nature and that they have been removed from their physiological context; caution must be taken into account when making comparisons between cellular observation and a human disease.

3.1.1 Cell culture

All studies in this thesis are mainly based on immortalized cell lines and includes; blastocyst-derived embryonic stem cells deficient for PS1 and PS2 (BD8 cells) (**Paper I and II**), mouse embryonic fribroblats lacking Nicastrin (Nct-/- MEF) (**Paper III)** or human embryonic kidney (HEK) cells over-expressing either APP with the swedish mutation or NotchΔE (**Paper IV**). Cell lines deficient of the protein of interest have the advantage of minimizing the influence of endogenous protein and thereby simplifying the interpretation of the result and the effect of over-expressed protein. However, regarding PS1 FAD mutations (**Paper I**), this model does not totally reflect a pathogenic state, which includes a wild type allele and one mutated allele. The studies include both transient and stable over-expression of proteins and both methods have their benefits and disadvantages. Transient expression is a rapid method to express and analyze the protein of interest but since the cDNA is not inserted into the cell genome, it will eventually be degraded. To ensure equal transfection efficiency in the transient

experiments, co-transfection of GFP or a CMV-β-gal vector was used. Stable expression of protein ensures long-term and reproducible protein levels. Nevertheless, for the analysis of multiple mutations it is important to select clones with similar expression levels and one drawback may include decreased expression levels over time, even though the cells are kept under antibiotic selection pressure. Further, long time over-expression of proteins exposes the cells to stress, especially if multiple proteins are over-expressed, which is important to consider when physiological interpretations of results from such cells are made. However, for some methods the use of stably over-expressing proteins is required in order to be able to detect the peptides of interest.

3.1.2 Animals

In **Paper I**, the membrane integration of PS1 TMDs was investigated using dog pancreas rough microsomes in order to get glycosylated samples. The microsomes were a kind gift from Prof. Arthur E. Johnson at the Texas A&M University, USA and ethical permission of the animal tissue was approved in accordance with Animal Welfare Regulations CFR9, Ch. 1, A,2.31 (d)(2)(5) by the University IACUC committee. For examining *in vivo* potency as well as binding site of the AZ4126 compound (**Paper IV**), C57BL/6 mice (Harlan Laboratories) and sagital brain sections from rats were used, respectively. All animal experiments were performed in accordance with relevant guidelines and regulations provided by the Swedish Board of Agriculture and the ethical permission was provided by the Stockholm Södra Animal Research Ethical Board.

3.1.3 cDNA constructs

Several cDNA constructs have been created during this thesis project. Most constructs include single or multiple amino acid changes in PS1 or Nicastrin (**Paper I and III**) and were generated with wild type DNA as a template by using the QuikChange Site-Directed Mutagenesis or QuikChange Multi Site-Directed Mutagenesis (Strategene), respectively. The PS1 TMD segments (**Paper I**) and PS1Δexon 10 construct (**Paper II**) were created using PCR with designated primers and then cloned into desired vectors using conventional cloning techniques. The N-terminal truncated PS1 CTF constructs and introduction of glycosylation acceptor sites on PS1wt and PS1Δexon 10 (**Paper II**) were performed using designated primers according to Quick Change mutagenesis protocol (Stratagene). The DNA sequence of all constructs was verified using the BigDye® Terminator Version 3.1 Cycle Sequencing kit (Applied Biosystems).

3.2 ANALYSIS OF γ-SECRETASE COMPLEX

In this thesis, six methods have been used to study the γ-secretase complex. The techniques were used to cover various aspects of the enzyme and therefore are quite different in their procedures. Though, all methods have in common that all end products of the methods, except the radioligand experiments, were analyzed by immunoblottning using SDS-PAGE. SDS-PAGE is used to separate proteins according to size and the strong detergent SDS is used to reduce the samples, destroying almost all protein interactions. After treatment with antibodies of interest, the proteins are detected using chemoluminescence either by X-ray film or CCD camera. Traditionally,

X-ray film has been used to detect a chemoluminescent signals and have a high sensitivity. However, as it is hard to control when the film reach saturation, the risk of getting over-exposed images can be a problem. The advantage with a CCD camera is its large dynamic range and the possibility to optimize exposure times during the detection to avoid signal saturation. Therefore, images that will be quantified are preferably exposed in a CCD camera but if X-ray films are used, extra caution is needed to avoid saturated bands.

The membrane integration assay of PS1 FAD mutations (**Paper I**) and topology study of the PS1Δexon 10 molecule (**Paper II**) are techniques used to investigate how modifications in PS1 are affecting its integration into the membrane. Both methods are based on the introduction of glycosylation acceptor sites that get glycosylated if they are present in the lumen. As a control, Endoglycosidase H treatment, which deglycosylate the samples were included. Another way to study how alterations affect a protein is to evaluate its stability and half-life using Cycloheximide treatment. Cycloheximide inhibits protein synthesis by interfering with the translation step, blocking the translation of mRNA into protein, and is a convenient method to observe the half-life of a protein without interference from transcription or translation. This treatment was used to study the stability of the PS1Δexon 10 molecule and the most N-terminal truncated CTF fragment, CTF start375 (**Paper II**) as well as Nicastrin containing the C1, C2, C3 or C4 mutants (**Paper III**). The stability of the altered proteins was compared to the wild type proteins.

Co-immunoprecipitation is a commonly used method to examine physiological protein-protein interactions. In this thesis, the method was used to study if alterations in either PS (**Paper II**) or Nicastrin (**Paper III**) decrease or disrupt the interactions to the other components in the γ-secretase complex. It is important to maintain stable interactions throughout the procedure in order to detect the bound proteins and since protein-protein interactions can be sensitive to detergents, analyzing membrane proteins can be challenging. Therefore, a mild detergent (1% CHAPSO) which retains γ-secretase activity (Franberg et al., 2007) was used for studying the interaction within the γ-secretase complex. The interactions between γ-secretase components can also be examined using an affinity capture approach. Here, membrane-prepared γ-secretase complexes were incubated with the L-685,458 coupled to a cleavable biotin group (GCB) followed by incubation with streptavidin beads to capture the active γ-secretase complexes. This method addresses if alterations in γ-secretase components influence structural changes in the catalytic site. Since L-685,458 is a transition state analogue inhibitor, fewer active γ-secretase complexes would be pulled down compared to wild type if the catalytic site is affected. Affinity capture was used to investigate if changes in PS (**Paper I and II**) or Nicastrin (**Paper III**) affect the catalytic site. As the L-685,458-GCB molecule has been modified, it is important to confirm that it has similar characteristics as the L-685,458 inhibitor. L-685,458-GCB has indeed previously been shown to block Aβ production with a similar IC_{50} as L-685,458 (Teranishi et al., 2009). Both affinity capture and co-immunoprecipitation examine interactions between γ-secretase components. However, in contrast to affinity capture that only detects interactions in active complexes, co-immunoprecipitation also detects mature inactive complexes as well as sub-complexes. It is important to secure the specificity of the

detected protein interactions when using pull-down techniques. For affinity capture, L-685,458 was added prior to the biotinylated inhibitor as a negative control and co-immunoprecipitation was also performed with only pre-serum that contains no specific antibody.

To understand how different molecules interact and affect the γ-secretase complex, binding studies in combination with displacement experiments can be performed. In **Paper IV**, radioligand displacement binding experiment with an established tritiated second-generation GSM ([^{3}H]AZ8349) as tracer (Borgegard et al., 2012) was performed to confirm that the novel AZ4126 compound belongs to second-generation GSMs. An alternative to radioligand experiments includes photo-affinity labeling studies, which can be performed on living cells. However, drawbacks with that alternative include possible steric interference of the photo-probe to the binding site and cellular toxicity upon cross-linking with UV-light.

3.3 DETECTION AND QUANTIFICATION OF Aβ AND Nβ

To analyze Aβ production, three methods were used; Meso Scale Discovery (MSD) technology (**Paper I-IV**), Enzyme-linked immunosorbent assay (ELISA) (**Paper IV**) and Immunoprecipitation followed by matrix-associated laser desorption ionization-time of flight mass spectrometry (IP-MALDI-TOF MS) (**Paper IV**). Nβ was also detected using IP-MALDI-TOF MS and the identification of Nβ was confirmed by tandem mass spectrometry (MS/MS) using MALDI-TOF/TOF (**Paper IV**).

MSD technology and ELISA are very similar in their quantification of an antigen, since both are based on a capture and a detection antibody. However, the MSD technology uses ruthenylated-detection antibodies (SULFO-TAG™) that are activated by electricity, in contrast to the more conventional ELISA using detection antibodies coupled to an enzyme, which upon addition of substrate converts it to colour or a fluorescent signal. An advantage with MSD is the high signal-to-noise ratio, since the activation by electricity is decoupled from the electrochemoluminescence signal and also multiple excitation cycles enhances the signal. Moreover, some MSD plates have MULTI-SPOT®, allowing detection of more peptides in one well under exact same conditions. However, MSD technology requires a specific expensive instrument, in contrast to ELISA that is measured in a normal absorbance or fluorescent detecting plate-reader. All sandwich immunoassays have two options for the specific antibody, either as capture or detection antibody. It is preferable to use the specific antibody as the capturing antibody, because if a general antibody that recognizes all isoforms of a peptide is used to capture the peptides, high abundant isoforms may occupy most sites, resulting in low or non-detection of species present in low levels.

By using IP-MALDI-TOF, specific peptides and importantly multiple isoforms of a peptide can be identified/characterized and quantified. Here, the peptides are converted into gas phase ions and then separated according to their mass-to-charge ratios (m/z). Benefits with MALDI-TOF are that it is a very sensitive method, offer high resolution and mass accuracy and the samples are examined relatively fast. Moreover, the technique is quite robust, allowing comparatively high concentrations of salts, buffers and other contaminations in the sample. Unfortunately, it is not possible to couple a liquid chromatography to a MALDI-TOF (Portelius et al., 2008).

Information about the primary structure, i.e. amino acid sequence, of a peptide or protein of interest can be obtained by using MS/MS. Peptides of a specific m/z are selected and isolated from all different peptides entering the mass spectrometer. Next, the peptides are fragmented and analyzed in the mass analyzer.

3.4 DETECTION OF INTRACELLULAR DOMAINS

The detection of ICDs was investigated with a Luciferase-Reporter Gene Assay and the ImageXpress platform that is based on immunocytochemistry. Both AICD and NICD formation was studied with Luciferase-Reporter Gene Assay (**Paper I-III**). γ-Secretase mediated cleavage of C99 and NotchΔE, a truncated form of the Notch1 receptor resembling S2-cleaved Notch, is analyzed with two hybrid constructs containing an incorporated GVP domain. During γ-secretase cleavage, the ICDs with the GVP moiety translocate to the nucleus due to a a nuclear localization signal in the GVP domain. The luciferase-reporter gene (MH100) is activated through the specific binding of GVP to the UAS promoter, thereby enabling an exclusive signal by the GVP domain. The Luciferase-Reporter Gene Assay is both very sensitive and has a high specificity (Karlstrom et al., 2002). In addition, a benefit with this system is that it makes it possible to study the processing of APP and Notch in a similar experimental setup, under the same conditions. Often, APP and Notch processing is investigated in different system, making it harder to evaluate disparities correctly. However, the method is based on the incorporation of a non-physiologic domain into the ICD that interferes with their stability, which needs to be considered. On the other hand, non-manipulated AICD is very unstable, therefore most methods used to investigate AICD involve some kind of manipulation of its stability. Moreover, the results from this technique are compared to the processing of wild type protein under the same conditions, therefore considering relative changes.

Modulation of S3-cleaved NICD was also determined by immunocytochemistry using ImageXpress (**Paper IV**). The system is based on conventional immunocytochemistry but in a 384-well format, having the advantage of allowing massive data collection under the same conditions during one experiment. Therefore, dose-response experiments with AZ GSMs, L-685,458 and vehicle for two antibodies, one recognizing total NICD and the other NICD starting specifically at position V1744 (V1744-NICD), could be performed at the same time. This enables robust data regarding modulation of NICD, by relating V1744-NICD to total NICD. However, the specificity of the method is still dependent on the specificity of the antibodies, but importantly, L-685,458 inhibited V1744-NICD formation with similar potency ($IC_{50Val1744}$=10.0nM) as previously determined for its effect on total NICD formation (IC_{50C20}= 6.0nM (Borgegard et al., 2012)).

4 RESULTS AND DISCUSSION

In this section, the main findings from **Paper I-IV** are summarized and discussed based on the aims of the thesis. To improve the understanding and support the discussion, some figures from the individual papers are also presented. A more detailed discussion of the results and all figures are found in the specific papers.

4.1 THE γ-SECTRETASE COMPLEX CAN BE SUBJECTED TO MANY GENETIC ALTERATIONS AND STILL BE PARTIALLY FUNCTIONAL

In order to gain more knowledge about the mechanisms of γ-secretase processing, to differentiate between γ-secretase mediated APP and Notch processing as well as to gain a better understanding of the molecular basis of the heterogeneous γ- and ε-cleavage activity, both genetic and pharmacological alterations were applied to study γ-secretase in this thesis. Different techniques were used to assess the γ-secretase complex in the papers, although some methods have been used in several studies. Interestingly, one general finding in the thesis is that; the γ-secretase complex can be subjected to many genetic alterations and still be partially functional, although in some cases very vulnerable, when 31 FAD mutations (**Paper I**), the Δexon 10 molecule and N-terminal truncated CTF variants of PS1 (**Paper II**) as well as four Nicastrin mutations (**Paper III**) were examined, schematically shown in Figure 7.

The mechanism(s) by which PS1 FAD mutations cause an elevated Aβ42/Aβ40 ratio is still incompletely understood, but that a conformational change in PS1 is responsible for the Aβ phenotype is one proposed mechanism. In **Paper I**, a membrane integration assay in an *in vitro* transcription/translation system based on N-linked glycosylation was used to evaluate if FAD mutations affect the integration of their respective transmembrane domains. Most mutations did not affect membrane integration, but six mutations were identified that either increased or decreased the

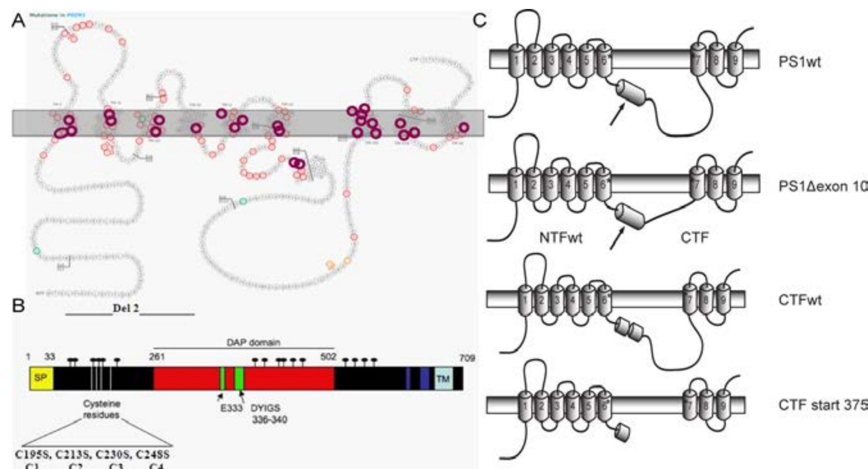

Figure 7. Schematic illustration of the genetic alterations used in the thesis. A) The localization of the PS1 FAD mutations in **Paper I**. Picture modified from http://www.molgen.ua.ac.be/admutations/ B) Schematic presentation of PS1wt, PS1 Δexon 10 and the CTF construct lacking exon10, CTF start 375 in **Paper II**. C) The localization of cysteine residues that are mutated to serines, C195S (C1), C213S (C2), C230S (C3), and C248S (C4) in the Nicastrin protein in **Paper III**.

membrane integration with more than 20% compared to wild-type PS1. Very interestingly, all PS1 FAD mutations that changed the TMD membrane integration are located in hydrophobic domains around the catalytic site (TMD6, H7 and TMD7), suggesting that this area is particularly vulnerable to modifications. In contrast, the PS1Δexon 10 molecule, lacking the large hydrophilic loop between TMD 6 and 7 of PS1 was shown to have unaltered membrane topology (**Paper II**). In addition, co-immunoprecipitation studies showed that PS1Δexon 10 interacts with Nicastrin, Aph-1 and Pen-2. Moreover, affinity capture confirmed that an active site directed transition state analogue inhibitor of γ-secretase can bind the active site of the PS1Δexon 10 molecule and pull down PS1-NTF. This demonstrates that PS1Δexon 10 can assemble into a functional and active γ-secretase complex. Strikingly, not only mutations in PS1 can produce active γ-secretase complexes, three out of four Nicastrin cysteine mutations could interact with the other components and form active complexes, although the C3 mutant gave rise to fewer active complexes (**Paper III**). The fourth mutation, C4 failed to interact with any other component than Aph-1, which has been shown previously (Shirotani et al., 2004a) and explains its lack of activity on both APP and Notch. Moreover, neither Nicastrin cysteine mutations nor the exon 10 deletion in PS1 impaired protein stability as observed by cycloheximide-treated cells (**Paper II and III**). However, even though the NTF of PS1Δexon 10 is stable, apparent lower levels of PS1Δexon 10 NTF were observed, and the endoproteolytic event was found to be 10-fold decreased compared to PS1wt (**Paper II**). It has previously been shown that it is possible to reconstitute γ-secretase activity in the absence of PS endoproteolysis, by co-expressing PS NTF and CTF encoding constructs (Laudon et al., 2004). This experimental setup enabled us to study NTF levels under conditions bypassing endoproteolysis. By generating a PS1 CTF construct lacking exon 10, denoted CTF start 375, and co-expressing it with PS1 NTFwt, we observed lower levels of NTFwt in the presence of CTF start 375 than in the presence of CTFwt. This indicates that the loop, apart from endoproteolysis, is also important for complex formation, since we still noticed lowered NTFwt levels even when bypassing PS endoproteolysis.

Although PS1 can form active complexes in the absence of the large hydrophilic loop, we observed a tendency of decreased binding to the transition state analogue inhibitor for PS1Δexon10 compared to PS1wt. Interestingly, the same phenomenon was also observed when six PS1 FAD mutations as well as the Nicastrin mutant C3 were subjected to affinity capture by the transition state analogue inhibitor (**Paper I and III**). This suggests a conformational change of the active site in these molecules. Indeed, both genetic and pharmacological manipulations, which increase the Aβ42/Aβ40 ratio, are associated with a uniformly conformational change in the catalytic site of PS1 (Berezovska et al., 2005; Lleo et al., 2004; Uemura et al., 2009). Moreover, Kornilova *et al.* have shown that certain PS FAD mutations have reduced photo-labeling by a transition-state analogue (Kornilova et al., 2005). Combined, all experiments indicate that γ-secretase in general can form partially functional complexes although it is subjected to many different genetic alterations. However, the amount of active complexes, and more importantly, the properties regarding their activity differs extensively, as described in the next sections.

4.2 THE RELATIONSHIP BETWEEN ε- AND γ-CLEAVAGE OF APP

One of the aims of this thesis was to understand the relationship between the heterogeneous γ- and ε- cleavage activity on a molecular basis. Consequently, we wanted to compare the Aβ-profile from the six out of 31 investigated FAD mutations that changed the membrane integration of their respective TMDs, with mutations that did not affect membrane integration (**Paper I**). In total 12 mutations were examined. Interestingly, five mutations that did not alter the membrane integration stood out, either by having no effect or by causing a very high increase in the Aβ42/Aβ40 ratio (>4 compared to wild-type). The four mutations; I143T, L166P, Δexon9 and G384A, which resulted in a very high Aβ42/Aβ40 ratio also caused a pronounced decrease in Aβ40. The same phenomenon was observed for the L392P mutation, which caused a lower but still significant increase in the Aβ42/Aβ40 ratio (>3). Besides these similarities, we could also observed some differences in the way these mutants affected APP processing. For instance, while the I143T, L166P, Δexon9 and G384A mutations caused a decrease in AICD formation, the L392P mutation did not. The mechanism between this discrepancy is not known, but recent discoveries by Chavez-Gytierrez and colleagues may shed some light on the underneath molecular mechanism, see Figure 8. By taking on a different approach to explore the effect of FAD mutations on Aβ production, Chavez-Gytierrez and colleagues found two different mechanisms by which FAD mutations could cause an increased Aβ42/Aβ40 ratio. Either the mutation interferes with the processing at the ε-site of the predominant Aβ40 product line ($AICD_{50-99}$ + Aβ49>>Aβ40), causing a net decrease in Aβ40, and/or the mutation impairs the fourth cleavage in any of the two product lines, resulting in either decreased Aβ40 or increased Aβ42 levels (Chavez-Gutierrez et al., 2012). Based on these findings, impaired initial ε-cleavage of the predominant $AICD_{50-99}$ + Aβ49>>Aβ40 product line, would explain the low AICD generation and thus reduced Aβ40 production that we observed for the I143T, L166P, Δexon9 and G384A mutations. For the L392P mutation, which possessed intact AICD formation, damage to the fourth cleavage could explain the obtained results. Either the mutation caused a decrease in the Aβ43>Aβ40-cleavage reaction, leading to lower Aβ40 levels or, alternatively, a decrease in the Aβ42>Aβ38-cleavage, resulting in increased levels of Aβ42. The former mechanism appears most likely for the L392P mutation since it caused a selective decrease in Aβ40 production. For the G384A mutation, which caused a dramatic increase in Aβ42 levels, the latter mechanism makes most sense.

Figure 8. Impairments in the Aβ product lines, as proposed by Chavez-Gytierrez *et al.* 2012.

The product line hypothesis is also to some extent applicable to explain the results obtained from the studies using artificial mutations in nicastrin (**Paper III**) and PS1 (**Paper II**). The nicastrin cysteine mutations; C1, C2 and C3 all displayed a concomitantly reduction of AICD and Aβ40 that might reflect an impairment at the ε-

cleavage of the predominant $AICD_{50-99}$ + Aβ49>>Aβ40 product line. The data obtained from **Paper II**, where we analyzed the impact of the large hydrophilic loop of PS1 on γ-secretase activity, appear more complex. The absence of the loop region resulted in a decrease in total Aβ levels without affecting AICD generation, suggesting that the loop region is important for the γ-secretase activity once processing at the ε-cleavage site has already been initiated. Besides these findings, the Δexon10 mutant also gave rise to a different Aβ pattern, where the formation of the shorter Aβ38, Aβ39 and Aβ40 species were reduced to a much larger extent compared to Aβ42. These observations are not easily explained using the product line hypothesis, but may reflect a general partial loss of function in γ-secretase activity where the Aβ42 product line is impaired to a lesser extent compared to the Aβ40 product line. In our efforts to further understand the role of the loop region in regulating Aβ production, we generated partial deletions starting from the N-terminal towards the C-terminal end of the loop region of PS1 CTF, and co-expressed the different deletions with PS1 NTFwt in PS deficient cells. These deletions resulted in a gradual loss of all Aβ peptides. Interestingly, however, removal of the amino acid closest to the C-terminal end of the loop did not further impair Aβ42 production, but the generation of Aβ38, Aβ39 and Aβ40 was dramatically decreased. The differential effect on Aβ42 versus shorter Aβ peptides would not be compatible with a general loss of function of γ-secretase activity. Rather, the effect of the last C-terminal amino acids of the loop would fit with a selective impairment at the fourth cleavage event of the product line mechanism, resulting in unaltered Aβ42 levels and reduced levels of Aβ38 and Aβ40. Interestingly, the impact of deleting the loop region on Aβ production resembles the Aβ phenotype of many FAD-linked PS1 mutants (**Paper I,** (Bentahir et al., 2006; Chavez-Gutierrez et al., 2012; Kumar-Singh et al., 2006)). This Aβ-phenotype translates to an increased amyloid deposition in both FAD patients and transgenic models expressing FAD-linked PS. The FAD-like Aβ phenotype of PS1Δexon 10 also causes an enhanced amyloidosis in transgenic mice, as recently demonstrated by Deng *et al.* (Deng et al., 2006). Although the removal of Δexon 10 results in an Aβ phenotype similar to many FAD mutants, no FAD causing mutation has so far been identified in this region. Moreover, the similarity between the Aβ profile of the PS1 Δexon10 molecule and many FAD mutations, combined with the fact that many mutations at different loci of the PS gene result in FAD, suggest that minor structural alterations in PS have a major impact on some specific cleavage events. This results in the detrimental imbalance in Aβ40 and Aβ42 production. Basi and colleagues have made a very similar observation. In an effort to identifying amino acids critical for the pharmacology of different γ-secretase inhibitors, they also found that many artificial mutants cause a more severe loss of shorter Aβ variants compared to Aβ42 (Zhao et al., 2008).

A drawback of the hypothesis with impairments in the Aβ product lines and the original tri-/tetra-peptide-rule, is that they do not totally differentiate between the production of Aβ37, Aβ38 and Aβ39. Takami *et al.,* as well as Chavez-Gytierrez and colleagues, associate Aβ38 with the Aβ48>>Aβ42 product line (Chavez-Gutierrez et al., 2012; Takami et al., 2009) and Aβ37 is assumed to be generated from Aβ40, but it has not been detected in the mentioned studies. Moreover, if a strict tri-peptide rule should apply, Aβ39 and not Aβ38 should be generated from Aβ42. However, Takami

and co-workers observed no generation of Aβ39 from Aβ42, only Aβ38 (Takami et al., 2009). The disparities may also reflect on the original α-helical model, which demonstrates that C99 adopts an α-helical structure, where 3.6 residues are needed to complete one turn (Lichtenthaler et al., 1999). Therefore, γ-cleavage cannot always occour every third residue and must sometime be compensated with cleavage after four residues, which would explain the conversion of Aβ42 to Aβ38. This is also in line with reports showing that first generation GSMs of the NSAID class modulate γ-secretase activity by shifting the amino acid cleavage from Aβ position 42 to 38 (Eriksen et al., 2003; Weggen et al., 2001). However, it is more difficult to explain the pharmacology of the second generation GSMs in **Paper IV**, which has a more diverse effect on Aβ peptides. Here, antibody pull-down followed by MALDI-TOF analysis of Aβ peptides was used to study the pharmacological profile of two first- and two second-generation GSMs, as shown in Figure 9. Both common features but also differences were found. First generation GSMs; R-flurbiprofen and sulindac sulfide gave rise to a clear relative decrease in Aβ42 levels and concomitantly elevated Aβ38 levels. In contrast, treatment of both second generation GSMs; AZ1136 and AZ4126 resulted in a very potent reduction in both Aβ42 and Aβ40, consistent with the behavior of second-generation GSMs (Kounnas et al., 2010), and a corresponding increase in Aβ37. These data indicate that these different classes of GSM compounds modulate Aβ production through different mechanisms. Moreover, when treating cells with R-flurbiprofen and sulindac sulfide their pharmacological profiles revealed disparities; R-flurbiprofen primarily increased Aβ37, while sulindac sulfide reduced Aβ39. We also found that the relative levels of Aβ39 were elevated after AZ1136 treatment whereas AZ4126 caused an increase in Aβ38. Thus, besides the differences in Aβ modulation between the first- and second-generation GSMs, clear differences are found within each class of GSMs with regard to Aβ modulation. In summary, the Aβ product line hypothesis could explain the complex nature of Aβ production during normal conditions, in disease and during pharmacological manipulation in a very elegant manner, but additional experiments are needed to fully explain the ethiology of all commonly expressed Aβ peptides during different conditions.

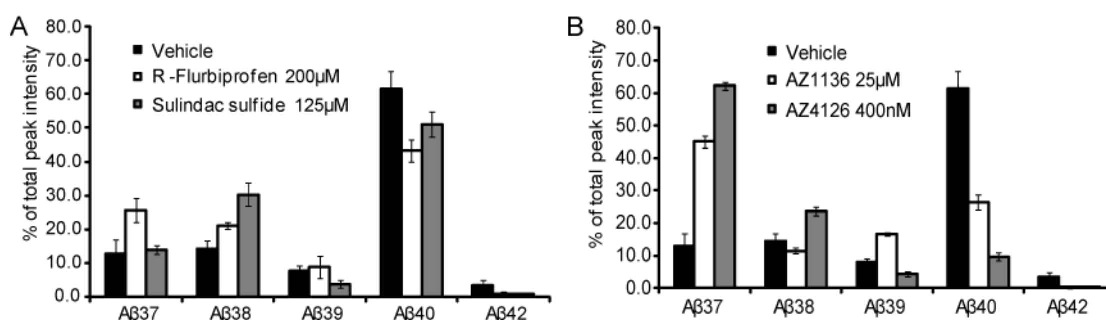

Figure 9. Aβ peak distribution under the influence of A) first- and B) second-generation GSMs, examined by IP-MALDI-TOF. Each Aβ peak is plotted as a percentage of total Aβ (i.e. the sum of Aβ37-42).

4.3 MODULATION OF γ-SECRETASE PROCESSING OF APP AND NOTCH

To develop and evaluate tolerably Notch-sparing compounds, including γ-secretase modulators, it is important to understand and characterize differences between γ-secretase mediated APP and Notch processing. Parts of the DAP-domain in the Nicastrin ectodomain show similarities to the aminopeptidase and transferrin receptor superfamily, spawning the idea that Nicastrin might function as a receptor. However, since studies have reported contradictory results regarding the proposed substrate receptor-like role for Nicastrin (Chavez-Gutierrez et al., 2008; Dries et al., 2009; Shah et al., 2005), it remains unclear if Nicastrin is involved in substrate recognition or not. Interestingly, upstream to the DAP-domain, there are four evenly spaced cysteine residues (at position 195, 213, 230, and 248) that are conserved between human, mouse, *Drosophila* and *C. elegans*. Since cysteine residues in general are involved in protein confirmation and interaction, we wanted to examine the role of these conserved cysteine residues regarding their function in the γ-secretase complex. We found that two mutants of Nicastrin, C213S (C2) and C230S (C3), differentially affected APP and Notch processing (**Paper III**). While both the formation of Aβ and AICD was decreased, the production of NICD was maintained on a high level. This is to our knowledge the first study describing single amino acid residues in a non-PS γ-secretase component which, when mutated, have a differential effect on the processing of different γ-secretase substrates. (Tolia et al., 2006; Walker et al., 2006; Yamasaki et al., 2006). Notably, C3 caused low levels of active γ-secretase complexes and also had less mature Nicastrin, indicating that the residual γ-secretase activity was sufficient to process Notch but not APP to the same extent. This is in line with a previous study, showing that deleted forms of Nicastrin (Δ312–340 and Δ312–369) only gave rise to minor disturbances in NICD signaling, despite the absence of mature Nicastrin (Chen et al., 2001). Furthermore, glycosylation of Nicastrin has previously been demonstrated not to be essential for γ-secretase assembly or γ-secretase activity (Herreman et al., 2003; Shirotani et al., 2003). Nevertheless, the presence of a mature form of Nicastrin seems to be important for proper processing of APP. For example, C3 may induce a conformational alteration in Nicastrin, altering the whole γ-secretase complex so that immature Nicastrin could be part of the active complex, and thereby favoring Notch processing. Alternatively, the C2 and C3 mutations could lead to a change in subcellular localization of the γ-secretase complex, favoring expression at the plasma membrane and thus explain the differential processing of APP and Notch. Indeed, APP processing can be influenced by targeting Nicastrin to different subcellular compartments (Morais et al., 2008).

We also investigated the effect of first- and second-generations GSMs on Nβ production, as shown in Figure 10 (**Paper IV**). Since both the first and second generation GSMs appear selective for Aβ modulation and do not appear to affect total Aβ formation, we were curious to learn whether Nβ would be modulated in a similar manner as Aβ and whether the specificity of the Notch S3 cleavage would be affected by different GSMs. Antibody pull-down followed by MALDI-TOF analysis of Nβ peptides revealed that both AZ1136 and AZ4126, but neither R-flurbiprofen nor sulindac sulfide, affected Nβ production. Similar to their effect on Aβ40 and Aβ42,

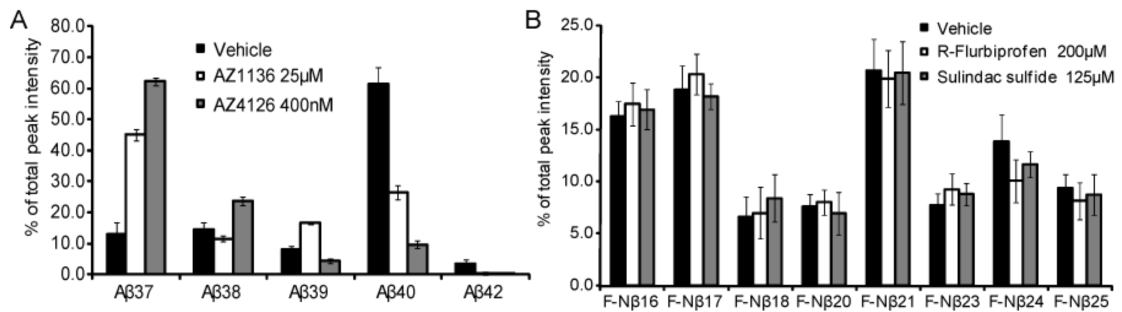

Figure 10. Aβ and Nβ peak distribution under the influence of second-generation GSMs, examined by IP-MALDI-TOF. A) Aβ distribution, each Aβ peak is plotted as a percentage of total Aβ (i.e. the sum of Aβ37-42). B) Nβ distribution, each F-Nβ peak is plotted as a percentage of total F-Nβ (i.e. the sum of F-Nβ16-25).

AZ1136 and AZ4126 reduce both F-Nβ24 and F-Nβ25, but the relative effects are not as discriminating. Strikingly, even though AZ4126 decreases Aβ40 and Aβ42 approximately twice as much as AZ1136 at their respective tested concentration, both compounds appear to have the same efficacy in reducing F-Nβ24 and F-Nβ25. Most of the shorter F-Nβ peptides were unaltered by both AZ GSMs but AZ1136 treatment slightly increased F-Nβ18 and in contrast F-Nβ21 was increased by AZ4126. Thus, the compounds do not affect the same shorter F-Nβ peptides, in contrast to their very clear relative elevation of Aβ37. However, it seems like AZ1136 and AZ4126 share a general pharmacological profile concerning APP and Notch processing, i.e. a decrease in the longer Aβ and F-Nβ peptides (Aβ40/Aβ42 and F-Nβ24/F-Nβ25) and an elevation of some of the shorter species. Nevertheless, AZ GSMs exhibit differences regarding which peptides are being modulated and the efficacy of the process. γ-Secretase-mediated Notch signaling through NICD formation is an extremely important event. Thus, identifying GSMs that do not affect NICD signaling is a key step when developing safe AD therapeutics. GSMs are compounds that are known to spare Notch signaling since they do not affect total NICD levels. However, it may not be only the total amount of NICD generated that ought to be considered, but also the specificity of the γ-secretase cleavage event at the S3 site. Importantly, a recent study showed that there are two physiological forms of NICD with different stability due to variations in their N-terminus, leading to disparities in their signaling intensities (Tagami et al., 2008). Therefore, we analyzed the modulatory effect of second-generation GSMs on the S3 cleavage of Notch. By using immunocytochemistry experiments, we could not find any evidence that would suggest that AZ1136 and AZ4126 modulate Notch S3 cleavage, suggesting that the AZ GSMs are selective for S4 cleavage modulation. Therefore, these data suggest that it is possible to generate γ-secretase-targeting GSMs that are pre-selective for Aβ over Nβ production without affecting NICD formation, a feature that may be important in the development of GSMs for chronic treatment in AD.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The work of this thesis has focused on how genetic (**Paper I-III**) and pharmacological (**Paper IV**) modulations affect the γ-secretase complex. During the last decade the mechanism of γ-secretase processing has started to emerge. Nevertheless, the growing list of γ-secretase substrates and severe side effects of GSIs in clinical trials highlights the needs of considering the importance of these pathways when targeting the γ-secretase complex. Further, a detailed knowledge on how γ-secretase activity is regulated still remains elusive.

In this thesis, it was found that the γ-secretase complex can be subjected to many genetic alterations and still be functional, although to different extents. Moreover, only six out of 31 PS1 FAD mutations changed TMD membrane integration and they were localized in hydrophobic domains around the catalytic site, TMD6, H7 and TMD7 (**Paper I**). In contrast, all tested FAD mutations affected the catalytic site of γ-secretase, the same were also observed for the PS1 Δexon10 molecule and the Nicastrin mutation C3 (**Paper II-III**). This suggests that the catalytic site is vulnerable to genetic alterations but structural changes of the active site often allow the enzyme to be partially functional. This is in line with that three out of four Nicastrin cysteine mutations and PS1Δexon 10 can assemble into a functional and active γ-secretase complex but that the Nicastrin cysteine mutations; C1, C2 and C3 have a concomitantly reduction of AICD and Aβ40. However, the absence of the loop region in PS1 affects Aβ formation but not AICD generation (**Paper II**), implicating that it differentially regulates γ-secretase activity between the γ- and the ε-site. Moreover, the loop region is also important for regulating Aβ production, since the Δexon10 molecule exhibits a large reduction in the formation of shorter Aβ38, Aβ39 and Aβ40 species as well as secreted total Aβ. Importantly, the generation of Aβ42 is only partially impaired under the same conditions. In addition, removal of the amino acids closest to the C-terminal part of the loop did not further impair Aβ42 production but the generation of Aβ38, Aβ39 and Aβ40 was dramatically decreased. Therefore, it appears that these amino acids are more responsible for production of shorter Aβ peptides than for Aβ42. This phenotype resembles the Aβ generation pattern of FAD mutations in **Paper I** and these features fit very well with a recent report on how FAD mutations affect the Aβ product lines (Chavez-Gutierrez et al., 2012).

In **Paper IV**, GSM treatment showed that different classes of GSM compounds modulate Aβ production through different mechanisms. Importantly, besides the differences in Aβ modulation between the first- and second-generation GSMs, clear differences were also found within each class of GSMs with regard to Aβ modulation. Moreover, neither R-flurbiprofen nor sulindac sulfide affected Nβ production. Strikingly, AZ1136 and AZ4126 shared a general pharmacological profile concerning APP and Notch processing, although they exhibited differences regarding which peptides were modulated and the efficacy of the processing. Moreover, these compounds caused no modulation of Notch S3 cleavage, indicating that the AZ GSMs are selective for S4 cleavage modulation. Therefore it is possible to generate γ-secretase-targeting GSMs that are pre-selective for Aβ over Nβ production without

affecting NICD formation. Furthermore, we found that not only pharmacological modulation can induce a diverse activity towards APP and Notch. Two mutants of Nicastrin, C2 and C3 affected APP and Notch processing to different extents (**Paper III**). This is the first study describing single residues in a γ-secretase component besides presenilin that differentially affect the processing of γ-secretase substrates.

The finding that there is a Nicastrin dependent discrepancy in APP and Notch processing is intriguing (**Paper III**). Especially since it seems like low levels of mature Nicastrin and low levels of active γ-secretase complexes, as observed for the C3 mutant, is sufficient to process Notch but not APP to the same degree. One alternative is that the C3 mutation changes the tertiary structure of Nicastrin and thereby induces a conformational change of the whole complex. This could allow immature Nicastrin to be part of an active complex, since complex assembly and activity is not dependent on glycosylation of Nicastrin (Herreman et al., 2003; Shirotani et al., 2003). To investigate this, it would be very interesting to determine the crystal structure of Nicastrin in the presence and absence of the C3 mutant and study their structural differences. Such experiments may gain important knowledge on the overall structural conformation of the enzyme complex. Moreover, the function of Nicastrin whether it is involved in substrate recognition, gating the substrate to the active site, or a more indirect role such as stabilizing the complex is not completely resolved. A study by Futai *et al.* reports that the PS1 double mutant F411Y/S438P is dispensable of Nicastrin for its activity and that the double mutation stabilizes the complex (Futai et al., 2009). This indirectly indicates a stabilizing role of Nicastrin, which is in line with that the SPPL2b GxGD-type aspartyl proteases do not require additional co-factors in order to be proteolyticly active (Martin et al., 2009). However, since our modifications of Nicastrin affect APP and Notch processing differently, these results may indicate a role for Nicastrin in substrate selection. Nevertheless, it would be of great interest to examine the structure of the catalytic site of the PS1 F411Y/S438P mutant in order to further understand the contribution of Nicastrin to γ-secretase processing and activity.

In addition, we and others have gained important information that FAD mutations affect the catalytic site of the γ-secretase complex (**Paper I**, (Berezovska et al., 2005; Kornilova et al., 2005)) and that these as well as pharmacological modulations (Lleo et al., 2004; Uemura et al., 2009) can be linked to an increased Aβ42/Aβ40 ratio. However, it would be very elegant to pin-point the exact structural alteration(s) that give rise to the mechanisms of FAD mutations proposed by Chavez-Gytierrez *et al.*, i.e. leading to impairments in the initial ε-cleavage or harming the fourth cleavage (Aβ43>Aβ40 or Aβ42>Aβ38) (Chavez-Gutierrez et al., 2012). This is naturally a very challenging project, especially since there is no crystal structure of the complex available. Nevertheless, by using the SCAM-technique on the domains that have been suggested to be a part of the hydrophilic cavity (i.e. TMD1, 6, 7, 9, the GxGD and the PAL domains as well as the loop domain of Pen-2) (Bammens et al., 2011; Sato et al., 2006; Sato et al., 2008; Takagi et al., 2010; Takeo et al., 2012; Tolia et al., 2006; Tolia et al., 2008) and compare the results obtained in the presence and absence of selected FAD mutations, parts of this mechanism may be elucidated. Although PS FAD mutations only comprise a few percent of all AD cases, understanding how their mechanism(s) leads to an altered Aβ-profile would gain vital insight into the

knowledge of the γ-secretase complex. This is crucial when developing therapeutical compounds for targeting the complex. In **Paper II**, we observed that the formation of both APP and Notch ICD are intact in the absence of the loop in contrast to the reduced Aβ levels, suggesting a differential cleavage activity between the ε-/S3- and γ-sites. Furthermore, we identified the ten amino acids closest to the C-terminal of the large hydrophilic loop to be of importance for the APP γ-site processing, reducing Aβ38-40 levels substantially more than Aβ42. Interestingly, an array of molecules have been found to bind to the large loop of PS1 including β-catenin and N- and E-cadherins (Georgakopoulos et al., 1999; Kang et al., 1999) and they may influence its processing of substrates, although the loop has been shown to be dispensable for γ-secretase activity (Xia et al., 2002). Moreover, γ-secretase interacting proteins that reduce Aβ levels such as TMP21 or the more recently identified VDAC, Erlin-2 or TPPP (Chen et al., 2006; Frykman et al., 2012; Hur et al., 2012; Teranishi et al., 2012) may also be possible Aβ lowering therapeutic targets. Identifying domains within PS (**Paper II**) or the other components such as Nicastrin (**Paper III**) that are of importance in differentiating between the processing of Aβ species and/or APP compared to Notch can be helpful in the design of GSMs or APP selective GSIs. Disparities in APP and Notch processing may be achieved by selectively targeting distinct γ-secretase complexes that exist due to the presence of PS1 and PS2 homologues and the three Aph-1 isoforms (Hebert et al., 2004; Saura et al., 1999; Serneels et al., 2005; Shirotani et al., 2004b). Moreover, PS1 containing complexes have been reported to catalyze the majority of the Aβ production (Borgegard et al., 2011; De Strooper et al., 1998) and PS1 and PS2 complexes show differences in activity and sensitivity to some γ-secretase inhibitors (Borgegard et al., 2011; Lai et al., 2003; Zhao et al., 2008). Indeed, it seems like GSIs sparing PS2 is a tolerable strategy for lowering Aβ formation (Boregård *et al.,* manuscript under revision)

GSMs do not affect the overall rate of Notch, APP (S3and ε cleavage) or ErbB4 processing (Kukar et al., 2008; Weggen et al., 2001). However, we found that the potent second generation AZ GSMs can modulate Nβ formation although to a much lower level than they affect Aβ formation, in contrast to the effect of first generation NSAIDs, R-flurbiprofen and sulindac sulfide (**Paper IV**). However, modulation by second-generation GSMs may also affect other substrate-releasing Aβ-like peptides, such as APP-like protein (APLP) 1 and 2, CD44 and interleukin-1 receptor II (Eggert et al., 2004; Kuhn et al., 2007; Lammich et al., 2002). Although the biological relevance of the Aβ-like peptides is unclear, it would be of great concern to investigate how this class of compounds affect these peptides. For example, the less amyloidogenic APLP1 Aβ-like peptide, which is present in human CSF, has been suggested to function as a surrogate marker for Aβ42 in response to γ-secretase targeting drugs (Yanagida et al., 2009). Therefore, the selectivity pattern of a given GSM should be a major consideration in biomarker development.

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