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IDENTIFICATION AND INVESTIGATION OF γ -SECRETASE ASSOCIATED PROTEINS FROM BRAIN

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ABSTRACT

Alzheimer disease (AD) is a progressive neurodegenerative disorder and the most common cause of dementia. The pathological hallmarks in the AD brain are extracellular deposition of amyloid plaques, mainly composed of the amyloid β -peptide ($A\beta$), and intracellular neurofibrillary tangles made of hyperphosphorylated tau. Several studies have shown that $A\beta$ aggregation provides the initial insult, and the formation of tangles seems to be a secondary effect. $A\beta$ is generated from the amyloid precursor protein (APP) by cleaving, which is sequentially mediated by β -secretase and γ -secretase. γ -Secretase is a transmembrane protease complex responsible for the processing of a multitude of type 1 transmembrane proteins such as APP and Notch. In clinical trials, treatment with γ -secretase inhibitors often results in Notch-related side effects and, thus, more specific inhibition of APP processing is necessary. Four proteins, presenilin, nicastrin, Aph-1 and Pen-2, are necessary and sufficient to produce an active γ -secretase complex. It has been suggested that γ -secretase associated proteins (GSAPs) could be of importance for substrate selection. Here, we have established an affinity purification method using a γ -secretase inhibitor derivative to isolate the native complex from brain material. We have identified several novel GSAPs from brain and studied their effect on $A\beta$ production and Notch processing.

In Paper I, we designed an efficient and selective method for purification and analysis of γ -secretase and GSAPs. Microsomal membranes were incubated with a γ -secretase inhibitor coupled to biotin via a long linker and an S-S bridge (GCB). After pull-down using streptavidin beads, bound proteins were eluted under reducing conditions and digested by trypsin. The tryptic peptides were subjected to liquid chromatography directly coupled to tandem mass spectrometry analysis, and proteins were identified by sequence data from an MS/MS spectra. All of the known γ -secretase components were identified, as well as the previously reported GSAP TMP21 and the PS-associated protein, syntaxin1. Hence, we suggest that the present method can be used to further study the composition of the γ -secretase complex. **In Paper II**, we investigated novel GSAPs from detergent-resistant membranes (DRMs). Recent studies showed that γ -secretase activity is highly enriched in DRMs. Thus, GSAPs localized to DRMs could be of special interest to study. We employed GCB and identified several novel GSAPs in DRMs from brain. From these identified proteins, silencing of voltage-dependent anion channel 1 (VDAC1) and contactin-associated protein 1 (CNTNAP1) reduced $A\beta$ production. These proteins had a less pronounced effect on Notch processing. We concluded that VDAC1 and CNTNAP1 associate with γ -secretase in DRMs and affect APP processing. **In Paper III**, we investigated novel GSAPs from synaptic membranes and synaptic vesicles prepared from rat brain. Synaptic degeneration is one of the earliest indicators of AD and results in loss of cognitive function. We employed GCB and identified several novel GSAPs in synaptic membranes and synaptic vesicles from brain. From these identified

proteins, silencing of NADH dehydrogenase [ubiquinone] iron–sulfur protein 7 (NDUFS7) resulted in a decrease in A β levels, whereas, silencing of tubulin polymerization promoting protein (TPPP) resulted in an increase in A β levels. These proteins had no effect on Notch processing. Association of TPPP and NDUFS7 with γ -secretase was verified using co-immunoprecipitation and Proximity Ligation Assay. **In Paper IV**, we conduct a large scale affinity purification study of the γ -secretase complex obtained from rat and human brain. Silencing of some of the identified proteins: Probable phospholipid-transporting ATPase IIA (ATP9A), BDNF/NT-3 growth factors receptor precursor (NTRK2), Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2), DnaJ homolog subfamily A member 2 (DNAJA2) and Proton myo-inositol cotransporter (SLC2A13) reduced A β 42 secretion in a siRNA dose-dependent manner. Two of these proteins, SLC2A13 and HCN2, had a relatively lower effect on Notch processing. Interestingly, overexpression of SLC2A13 increased A β 40 generation. The interaction between γ -secretase and ATP9A, NTRK2, HCN2 and SLC2A13 was confirmed by using immunoprecipitation.

In summary, we have established an affinity purification method to isolate the native γ -secretase complex from brain material, and we have identified several novel GSAPs that affect A β processing without affecting Notch cleavage. We suggest that these proteins could be the targets in a strategy to lower A β to treat AD.

LIST OF PUBLICATIONS

- I. **Affinity pulldown of γ -secretase and associated proteins from human and rat brain.**
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- II. **Identification of novel γ -secretase associated proteins in detergent resistant membranes from brain.**
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- III. **Identification of two novel synaptic γ -secretase associated proteins that affect amyloid β -peptide levels without altering Notch processing.**
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- IV. **Identification and functional studies of novel γ -secretase associated proteins in membranes from brain.**
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- V. **Erlin-2 is associated with active γ -secretase in brain and affects amyloid β -peptide production.**
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LIST OF ABBREVIATIONS

AD	Alzheimer disease
ADAMs	A disintegrin and metalloprotease-family
AICD	APP intracellular domain
APLP1	APP-like protein 1
APLP2	APP-like protein 2
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATP9A	Probable phospholipid-transporting ATPase IIA
A β	Amyloid β -peptide
BACE1	β -site APP cleaving enzyme 1
BACE2	β -site APP cleaving enzyme 2
C99	C-terminal fragment consisting of 99 amino acids
CNTN1	Contactin1
CNTNAP1	Contactin-associated protein 1
COX4I1	Cytochrome c oxidase subunit IV isoform 1
DNAJA2	DnaJ homolog subfamily A member 2
DRMs	Detergent-resistant membranes
FA	Formic acid
GCB	γ -Secretase inhibitor coupled to biotin via a cleavable linker
GSAPs	γ -Secretase associated proteins
HCN2	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2
HEK	Human embryonic kidney
HTRF	Homogenous time resolved fluorescence
KO	Knock-out
LC-MS/MS	Liquid chromatography directly coupled to tandem mass spectrometry
LP1	Synaptic membranes
Nct	Nicastrin
NDUFS7	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7
NFTs	Neurofibrillary tangles
NICD	Notch intracellular domain
Notch Δ E	Notch construct with a deleted ectodomain
NTRK2	BDNF/NT-3 growth factors receptor precursor
PION	Pigeon homologue protein
PLA	Proximity Ligation Assay
PS	Presenilin
PS1	Presenilin 1
PS1-CTF	PS1-Cterminal fragment
PS1-NTF	PS1-N-terminal fragment
PS2	Presenilin 2
SA	Streptavidin
sAPP- α	Soluble APP- α
sAPP- β	Soluble APP- β

SLC2A13	Proton myo-inositol cotransporter
SORT1	Sortilin1
STX12	Syntaxin12
SV	Synaptic vesicles
SYP	Synaptophysin
TLC	Thin Layer Chromatography
TPPP	Tubulin polymerization-promoting protein
VDAC1	Voltage-dependent anion channel 1

1 INTRODUCTION

Alzheimer disease

Alzheimer disease (AD) is a progressive, neurodegenerative disorder that accounts for 50–60% of all dementia cases (Mattson 2004). A German psychiatrist, Alois Alzheimer, described, in 1907, a condition associated with pathological changes in the brain tissues of a 51-year-old-female patient known as Auguste D., a condition diagnosed as a progressive dementia (Alzheimer 1907). Episodic memory deficits, associated with poor encoding and rapid forgetting of new material, are early symptoms of AD. Some patients demonstrate marked impairment of visuo-spatial ability, while in others attention or semantic processing deficits predominate. As the disease progresses, AD patients lose the ability to perform activities of daily living and need more care (Hodges 2006).

The estimated prevalence of AD, expressed as a ratio, ranges from 4.4% in the population of persons aged 65 years to 22% of those 90 years-of-age and older (Lobo, Launer et al. 2000). Worldwide, in 2012, nearly 35.6 million people live with dementia. This number is expected to double by 2030 (65.7 million) and more than triple by 2050 (115.4 million) because of the anticipated increase in life expectancy (WHO homepage, <http://www.who.int/mediacentre/en/>). Thus AD will be a major public health problem in the world.

Pathology of AD

Alzheimer disease is characterized by thinning of synaptic density and loss of neurons in the hippocampi and neocortex. Gross atrophy is observed in the affected regions, notably, degeneration in hippocampal, temporal and parietal areas, and in parts of the frontal cortex (Wenk 2003).

The hallmarks of AD brain pathology are extracellular deposition of amyloid plaques and intracellular neurofibrillary tangles (NFTs) (Selkoe 1999). These plaques are usually found in moderate or large numbers in limbic structures and association neocortex (Dickson 1997); and are composed of amyloid fibrils (7–10 nm in diameter), which are formed by the highly amyloidogenic, 42 and 43-amino acid residues, ~~formed in the~~ amyloid β -peptide (A β 42, A β 43) (Welanders, Franberg et al. 2009). A β 42 and A β 43 are normally produced in much lower quantities than the 40-residue form (A β 40), which constitutes approximately 90% of total secreted amyloid β -peptide (A β) ref.

Neurofibrillary tangles are observed in entorhinal cortex, hippocampus, and amygdala; in frontal, temporal and parietal association cortices; and in certain subcortical nuclei that project to these regions. NFTs are intraneuronal cytoplasmic lesions consisting of non-membrane-bound bundles of paired, helically wound, 10-nm filaments (PHF). The subunit protein of the PHF is the microtubule-associated protein, tau. Biochemical studies have shown that tau found in the tangles consists of hyperphosphorylated, insoluble forms (Goedert 1993).

Genetics and risk factors of AD (familial and sporadic AD)

Alzheimer disease occurring in people over the age of 65 is classified as late-onset, most of which are sporadic cases. However, around 5% of AD patients develop the disease phenotype at a much younger age (40 to 50 years old) and are classified as early-onset, and most of these are familial cases (Bettens, Sleegers et al. 2010).

Familial AD is an autosomal dominant disorder. The first reported mutation causing this familial form was mapped to the amyloid precursor protein (APP) gene on chromosome 21. This point mutation causes an amino-acid substitution close to the carboxy terminus of the A β (Goate, Chartier-Harlin et al. 1991). Thereafter, other mutations causing the familial form were identified in the highly homologous presenilin 1 (PS1) gene on chromosome 14 (Sherrington, Rogaev et al. 1995) and in the presenilin 2 (PS2) gene on chromosome 1 (Levy-Lahad, Wasco et al. 1995). Other than these three causal genes, no single, functional, risk-associated mutation was identified. At present, 32 APP missense mutations have been identified in 89 AD families and 185 different AD-related mutations in 405 families have been identified in the PS1 gene, while only 13 mutations in the PS2 gene have been identified in 22 families (Alzheimer Disease & Frontotemporal Dementia Mutation Database, <http://www.molgen.vib-ua.be/ADMutations/>). Mutant APP and presenilin (PS) genes either increase the production of A β 42, the major component of amyloid plaque, or alter the ratio between A β 42 and A β 40 without increasing A β 42 levels (Shioi, Georgakopoulos et al. 2007).

The majority of AD cases are sporadic, not considered genetically inherited; although some genes are risk factors. The best known genetic risk factor is the ϵ 4 allele of the apolipoprotein E (APOE) gene on chromosome 19 (Strittmatter, Saunders et al. 1993). To examine the association between APOE genotype and AD, 5930 patients who met the criteria for probable or definite AD, and 8607 controls who did not, were analyzed with respect to APOE genotypes, including: ϵ 2/ ϵ 2, ϵ 2/ ϵ 3, ϵ 2/ ϵ 4, ϵ 3/ ϵ 3, ϵ 3/ ϵ 4, and ϵ 4/ ϵ 4. The APOE ϵ 4 allele is associated with a three-fold increase in risk of the disease in heterozygotes (ϵ 2/ ϵ 4, ϵ 3/ ϵ 4) and a 15-fold increase in risk in homozygotes (ϵ 4/ ϵ 4). Whereas, the APOE ϵ 2 allele lowers the risk of the disease by half in heterozygotes (ϵ 2/ ϵ 3) and homozygotes (ϵ 2/ ϵ 2) (Farrer, Cupples et al. 1997).

The molecular mechanism for this disease-promoting effect of the ϵ 4 allele of APOE is not clear. APOE regulates transport of cholesterol and phospholipids during early and intermediate phases of the reinnervation process. Its role in the CNS is particularly important in relation to the function of the cholinergic system, which relies to a certain extent on the integrity of phospholipid homeostasis in neurons. A suggestion by Poirer is that the APOE ϵ 4 allele directly impacts cholinergic function in AD (1994). On the other hand, expression of APOE ϵ 3 and APOE ϵ 4 in APP(V717F) transgenic APOE(-/-) mice results in fibrillar A β deposits and neuritic plaques by 15 months of age; and, substantially (>10-fold) more fibrillar deposits are observed in APOE ϵ 4 expressing APP (V717F) TG mice (Holtzman, Bales et al. 2000). Analysis of a large cohort of AD patients and control subjects shows that the APOE ϵ 4 allele is strongly associated with reduced CSF levels of A β 42 in both AD and control populations, an effect which

could reflect a fundamental involvement of APOE in A β metabolism (Prince, Zetterberg et al. 2004). In addition to these findings concerning APOE4, high-throughput genomic association studies on large populations have enabled detection of susceptibility factors for late-onset AD. Recently, three novel risk genes have been identified - CLU, CR1 and PICALM1 (Lambert, Heath et al. 2009; Harold, Abraham et al. 2009).

The amyloid cascade hypothesis

The amyloid cascade hypothesis has been central in AD research, and holds that accumulation of A β in the brain is the primary event driving AD pathogenesis. The rest of the disease process, including formation of NTF, is proposed to result from an imbalance between A β production and A β clearance, which leads to neuronal degeneration and dementia (Hardy and Selkoe 2002). In support of this hypothesis are evidence of familial AD mutations being present in genes encoding either the substrate (APP) or the key enzyme for A β generation (PS). Most of these mutations increase A β 42 production, elevating the A β 42/40 ratio. Mutations in the APP gene cluster near the proteolytic cleavage sites in APP and affect the release of A β (De Jonghe, Esselens et al. 2001). Interestingly, people with trisomy 21 (Down's Syndrome), having an extra gene copy of APP, develop A β plaques early in life, and as adults over 40 years all develop neuropathology sufficient to diagnosis AD (Lott and Head 2005). In a small sample study, duplication of the APP locus was identified in five families that caused autosomal-dominant, early-onset AD, with characteristic accumulation of A β (Rovelet-Lecrux, Hannequin et al. 2006). These results support the notion that life-long APP overexpression triggers A β deposition, resulting in AD. Furthermore, results of several studies indicate that genetic variability in A β catabolism and clearance can increase the risk of late-onset AD (Wavrant-DeVrieze, Lambert et al. 1999; Bertram, Blacker et al. 2000; Ertekin-Taner, Graff-Radford et al. 2000; Myers, Holmans et al. 2000; Olson, Goddard et al. 2001).

In summary, the neurodegenerative process is the consequence of an imbalance between A β production and A β clearance.

Amyloid β -peptide (A β)

Soluble A β aggregates into soluble oligomers and larger insoluble fibrils, which are deposited as plaques. During this process, the A β 42 isoform is more prone to polymerize and trigger the misfolding of other A β species (Jarrett, Berger et al. 1993). Initially, fibrillar A β deposited in plaques was assumed to be neurotoxic, but experiments in vitro and in rodents suggest that soluble oligomers of A β could discretely interfere with synaptic mechanisms mediating aspects of learning and memory, including long-term potentiation (Walsh and Selkoe 2004). Results of one study using the APP-Tg mouse (Tg2576) suggest that an oligomer composed of 12 A β peptides (called A β *56) impairs memory independently of plaques or neuronal loss, and may contribute to cognitive deficits associated with AD (Lesne, Koh et al. 2006). However, this finding has been difficult to reproduce. Meanwhile, other oligomer types have been

proposed as the pathogenic species. For instance, A β dimers isolated from human CSF rapidly inhibit hippocampal long-term potentiation in vivo (Klyubin, Betts et al. 2008). Interestingly, A β 43 is more frequent than A β 40 in amyloid plaque cores from AD brains (Welander, Franberg et al. 2009). Moreover, A β 43 shows a higher propensity to polymerize, and is more neurotoxic than A β 42, as shown in knock-in mice containing a pathogenic PS1 R278I mutation that causes overproduction of A β 43 (Saito, Suemoto et al. 2011). In humans, memory impairment correlates strongly with cortical levels of soluble A β species, including oligomers. Memory impairment is caused by local inflammatory changes, neurofibrillary degeneration, and neurotransmitter deficits, events which are a consequence of early A β accumulation (Walsh and Selkoe 2004).

APP processing

The amyloid β -peptide is generated from APP by sequential cleaving, mediated by β -secretase and γ -secretase (Haass 2004). Along two alternative pathways, non-amyloidogenic and amyloidogenic (Figure 1), several different secretases are nominated to cleave APP. Along the non-amyloidogenic pathway, APP is sequentially cleaved by α -secretase and γ -secretase. α -Secretase cleaves APP at the 17th amino acid, inside the A β peptide sequence, releasing a secreted extracellular domain, soluble APP α (sAPP- α), and a C-terminal transmembrane fragment consisting of 83 amino acids (C83). The transmembrane region of C83 is further cleaved by γ -secretase to release a peptide called P3 and the APP intracellular domain (AICD).

In the amyloidogenic pathway, APP is cleaved by β -secretase either at the first or at the 11th residue of the A β peptide sequence, releasing a secreted extracellular domain, soluble APP- β (sAPP- β), and a transmembrane C-terminal fragment consisting of 99 or 89 amino acids (C99 or C89). γ -Secretase further cleaves the transmembrane region of C99, liberating both AICD and A β (mainly A β 40 and A β 42) (Zhang, Ma et al. 2011).

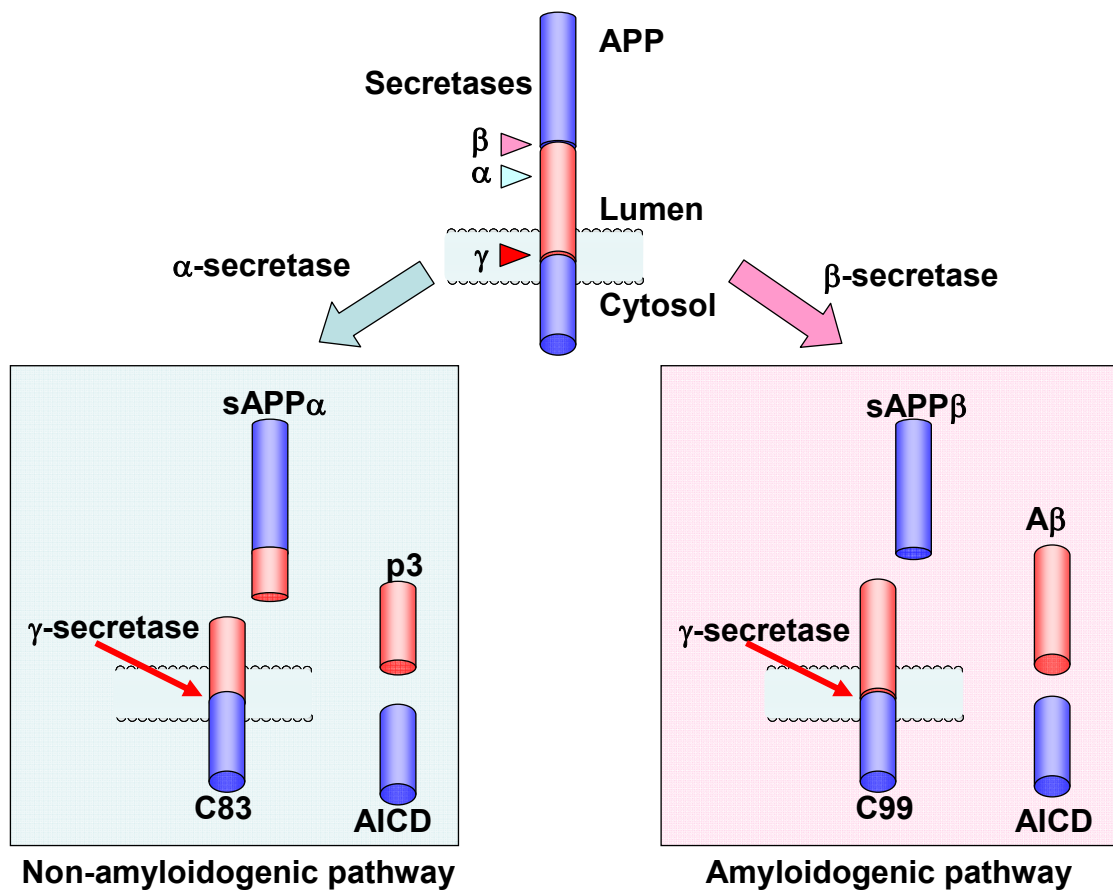


Figure 1. The non-amyloidogenic pathway and the amyloidogenic pathway. APP is undergoing sequential proteolytic processing by α - or β -secretase followed by γ -secretase. The non-amyloidogenic pathway is initiated by α -secretase, which cuts APP within the A β domain and thus excludes A β generation. α -Secretase cleaves APP ectodomain from the membrane, yielding sAPP- α and the membrane-bound C-terminal fragment C83. C83 is cleaved by γ -secretase, which results in the release of AICD and p3. In the amyloidogenic pathway, the ectodomain of APP is cleaved at the N-terminus of the A β region by β -secretase. This initiates amyloidogenic processing of APP by releasing sAPP- β and producing membrane-associated C99 APP-CTF. C99 is further cleaved by γ -secretase to release AICD and A β .

APP

The full-length cDNA of APP was first cloned as a 695-amino-acid protein that was predicted to be a glycosylated integral membrane cell-surface receptor (Kang, Lemaire et al. 1987). APP constitutes a family of different isoforms that are generated by alternative splicing of the 18 exons encoded by the APP gene (Yoshikai, Sasaki et al. 1990). APP695 mRNA lacking exon 7 and 8 is the most abundant APP transcript in the brain. The longest isoform, APP770 (isoforms

are named according to their length), and APP751 were found to be the major primary translation products in peripheral organs (Sandbrink, Masters et al. 1994).

There are two homologues of APP, APP-like proteins 1 and 2 (APLP1 and APLP2). The amino acid sequence of APLP1 is 42% identical and 64% similar to that of APP, while the amino acid sequence of APLP2 is 52% identical and 71% similar to that of APP. APLP1 is only expressed in the brain and is only found in mammals. APLP2 exhibits a pattern of expression very similar to APP in the brain and throughout the body (Wasco, Bupp et al. 1992 ; Wasco, Gurubhagavatula et al. 1993).

APP is a type I integral membrane protein with a large extracellular part, a hydrophobic transmembrane domain, and a short C-terminus, AICD. The extracellular part of APP contains E1 and E2 domains. APLP1 and APLP2 are also both type I integral membrane proteins and share conserved structures with APP, including a large extracellular motif containing the E1 and E2 domains and a short intracellular domain. N-terminal E1 domain is the major interaction interface for dimerization. APP, APLP1, and APLP2 have homo- and hetero-trans-interaction properties resulting in cell–cell adhesion, which specifically depend on E1 domain association (Soba, Eggert et al. 2005).

Genetic studies have shown that APP knock-out (KO) mice are viable. Neurological evaluation showed that the APP-deficient mice exhibited a decreased locomotor activity and forelimb grip strength, indicating a compromised neuronal or muscular function (Zheng, Jiang et al. 1995). Mice with single KOs of APLP1 or APLP2, or double KOs of APP/APLP1 are viable; while APP/APLP2, APLP1/APLP2 double-KO or APP/APLP1/APLP2 triple-KO mice show early postnatal lethality. These studies indicate that APLP2 has a crucial role in the absence of either APP or APLP1 (von Koch, Zheng et al. 1997; Heber, Herms et al. 2000; Herms, Anliker et al. 2004). Both APP/APLP2 double-KO mice and APP/APLP1/APLP2 triple-KO mice showed abnormal development in the peripheral and central nervous systems (Wang, Yang et al. 2005; Yang, Gong et al. 2005; Wang, Yang et al. 2007; Wang, Wang et al. 2009). These genetic studies suggest the critical involvement of these proteins during development.

In addition to these genetic studies, other studies showed APP to promote neurite outgrowth, cell adhesion, and cell proliferation in primary neurons (Annaert and De Strooper 2002). APP knock-down and overexpression studies in rodents showed that a normal APP level is an important factor for proper migration of neuronal precursors into the cortical plate during the development (Young-Pearse, Bai et al. 2007). These reports suggest that APP is involved in neurobiological functions.

α -Secretase

In the non-amyloidogenic pathway, α -secretase cleaves APP after the 16th amino acid inside the A β peptide sequence, resulting in the release of sAPP- α and C83. Members of a family of proteins named A disintegrin and metalloprotease (ADAM), including ADAM-10, ADAM-17 and ADAM-9, have been reported to have an α -secretase-like activity (Buxbaum, Liu et al. 1998;

Koike, Tomioka et al. 1999; Lammich, Kojro et al. 1999). Type I integral membrane proteins, such as ADAMs, belong to the zinc protease super family. ADAM10 may be responsible for constitutive α -cleavage activity, while ADAM9 and ADAM17 are more likely involved in the regulated α -cleavage of APP rather than in constitutive α -cleavage (Zhang, Ma et al. 2011).

β -Secretase

A β generation is initiated by β -cleavage at the ectodomain of APP, which releases sAPP- β and C99. The β -secretase, β -site APP cleaving enzyme 1 (BACE1), was identified and characterized by several groups (Sinha, Anderson et al. 1999; Vassar, Bennett et al. 1999; Yan, Bienkowski et al. 1999). BACE1 is a 501 amino acid type I transmembrane aspartyl protease with its active site on the luminal side of the membrane. BACE shows activity at low pH and the enzyme is predominantly localized in acidic intracellular compartments (e.g., endosomes, trans-Golgi; with pH around 6), its active site in the lumen of the vesicles. The highest expression levels of BACE are found in neurons from brain tissue (Vassar, Kovacs et al. 2009). Brain and primary cortical cultures from BACE1 KO mice showed no detectable β -secretase activity. Knock out of the BACE1 gene prevents A β generation and abolishes A β pathology in the mice expressing the Swedish mutation of human APP. These data indicate that BACE1 has the primary β -secretase activity in brain. A homologue of BACE1 exists also, β -site APP cleaving enzyme 2 (BACE2) (Acquati, Accarino et al. 2000; Hussain, Powell et al. 2000; Xin, Stephans et al. 2000). The amino acid sequence of BACE2 is 45% identical and 71% similar to that of BACE1. BACE2 is also mainly localized in post-Golgi membranes (Ehehalt, Michel et al. 2002). However, the physiological BACE1 activity is higher than BACE2 activity in the CNS, and BACE1 is enriched in neurons of the CNS while BACE2 is mostly expressed in glial cells (Laird, Cai et al. 2005). In addition, BACE2 cleaves more efficiently at a different site within A β (Farzan, Schnitzler et al. 2000). Studies of BACE1 KO mice also demonstrate that lower A β levels are beneficial for the reduction of memory impairments, which indicates that BACE1 could be a therapeutic target for AD (Cai, Wang et al. 2001; Luo, Bolon et al. 2001; Roberds, Anderson et al. 2001; Ohno, Sametsky et al. 2004). However, BACE1 KO mice show alterations in hippocampal synaptic plasticity as well as in performance on tests of cognition and emotion (Laird, Cai et al. 2005). Moreover, BACE1 KO mice have also been found to exhibit hypomyelination and altered neurological behavior, such as reduced grip strength (Hu, Hicks et al. 2006). This phenotype might be caused by physiological functions of other BACE1 substrates, such as the voltage-gated sodium channel β 2 subunit and neuregulin 1 (Willem, Garratt et al. 2006; Gersbacher, Kim et al.). Further studies are needed to clarify potential mechanism-based side effects that may occur with BACE1 inhibitors as a therapeutic strategy for AD.

γ -Secretase (Presenilin, nicastrin, Aph-1, Pen-2)

Both α -secretase and β -secretase generate short APP C-terminal fragments that are further processed by γ -secretase. γ -Secretase is an aspartyl protease

which catalyzes the cleavage of the transmembrane region of short APP C terminal fragments (Wolfe, Xia et al. 1999). Four proteins, PS, nicastrin (Nct), Aph-1 and Pen-2, are sufficient to produce an active γ -secretase complex, which is necessary for the intramembranous proteolysis of proteins such as Notch and APP (Haass 2004).

-PS-

PS1 and PS2 were identified and cloned by two groups and they reported that mutations in the PSs are pathogenic in Familial AD (FAD) (Levy-Lahad, Wasco et al. 1995; Sherrington, Rogaev et al. 1995). The PSs are transmembrane proteins with nine transmembrane domains, the C-terminus fragments of which locate to the lumen/extracellular space (Laudon, Hansson et al. 2005) (Figure 2). During the final assembly of γ -secretase, the PSs are cleaved at the cytoplasmic loop between the sixth and seventh transmembrane regions to generate N-terminal fragments of approximately 28 kDa and a 17 kDa C-terminal fragment (Thinakaran, Borchelt et al. 1996). Two transmembrane aspartyl residues of PS1 have been shown to be critical for endoproteolysis and γ -secretase activity (Wolfe, Xia et al. 1999; Figueroa, Morris et al. 2002; Ahn, Shelton et al. 2010). Transition-state analogue inhibitors designed to interact with the active site of γ -secretase bind directly and specifically to heterodimeric forms of PS fragments, a finding that suggests that N- and C-terminal PS fragments interact with each other and compose the active site of γ -secretase (Esler, Kimberly et al. 2000; Li, Xu et al. 2000). Analysis of transgenic mice over-expressing PSs with FAD mutations showed that overexpression of mutant PSs increases the A β 42/A β 40 ratio. Consequently, PS mutations probably cause AD through a gain of the function that increases the amount of A β 42 (43) in the brain (Duff, Eckman et al. 1996; Qian, Jiang et al. 1998).

-Nicastrin-

Nicastrin was identified as a protein that forms high molecular weight complexes with PS1 or PS2 (Yu, Nishimura et al. 2000). Nicastrin is a type I transmembrane glycoprotein with a large ectodomain (Figure 2), which includes an aminopeptidase/transferrin receptor superfamily domain (Fagan, Swindells et al. 2001). Nicastrin physically interacts with APP and Notch through a glutamate residue at position 333 in the Nct ectodomain (Shah, Lee et al. 2005; Dries, Shah et al. 2009). This mutation differentially affects A β production and Notch protein processing, in vitro and in vivo (Chavez-Gutierrez, Tolia et al. 2008). Furthermore, two other mutants, C213S and C230S, differentially affect APP and Notch processing in vitro. These data demonstrate that single residues in a γ -secretase component, other than presenilin, are able to differentially affect APP and Notch processing (Pamren, Wanngren et al. 2011). Furthermore these data indicate that Nct could present substrates to γ -secretase and, moreover, be involved in substrate selection. With such characteristics, Nct could act as a receptor of candidate substrates.

-PEN-2 and Aph-1-

By using a knockdown approach in *Drosophila*, PEN-2 and Aph-1 were identified as factors required for the activity and accumulation of γ -secretase (Francis, McGrath et al. 2002). APH-1 has seven-transmembrane domains with the N-terminus and even-numbered loops facing the lumen, while the C-terminus and odd-numbered loops reside in the cytosol (Fortna, Crystal et al. 2004)(Figure 2). PEN-2 spans the membrane twice, with the N- and C-termini facing the lumen (Crystal, Morais et al. 2003)(Figure 2). Aph-1 is involved in the early stages of γ -secretase assembly through the stabilization and glycosylation of Nct, and by scaffolding Nct to the immature γ -secretase complex (Lee, Shah et al. 2002; LaVoie, Fraering et al. 2003; Fortna, Crystal et al. 2004). Knockdown of PEN2 abolishes the endoproteolysis of PS1, whereas, overexpression of PEN-2 promotes the production of PS1 fragments. This indicates that PEN-2 is involved in proteolytic cleavage of PS1. Furthermore, overexpression of APH-1 facilitates PEN-2-mediated PS1 proteolysis. These data indicate a direct role for PEN-2 in proteolytic cleavage of PS1 and in the regulatory function of APH-1 in biogenesis of the γ -secretase complex (Luo, Wang et al. 2003).

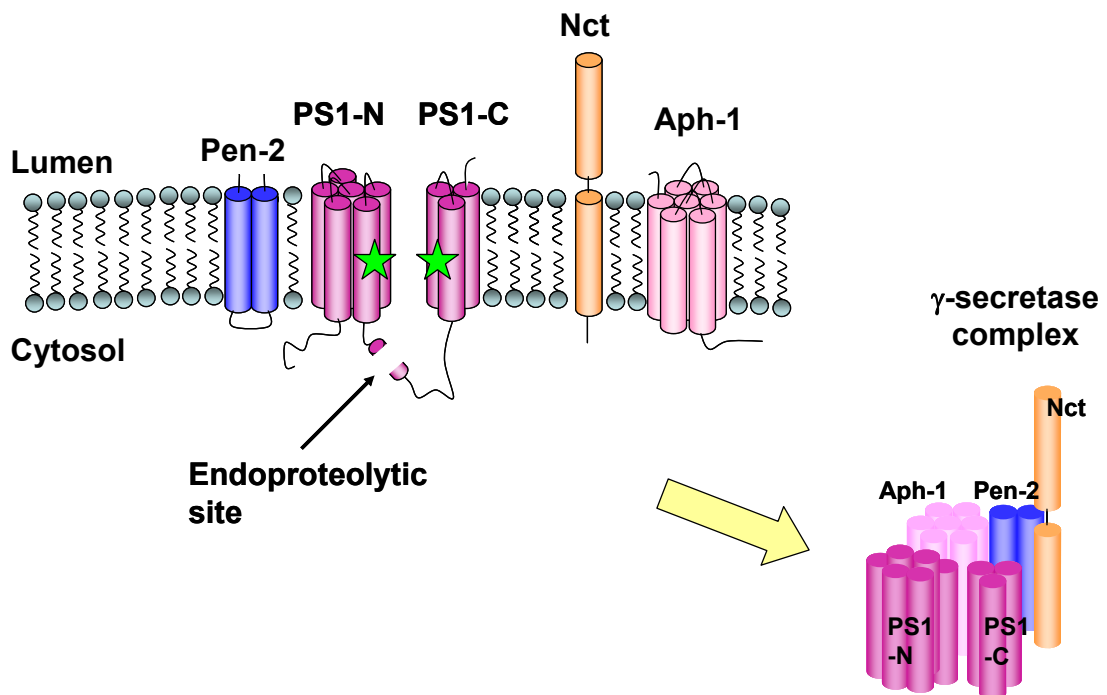


Figure 2. Illustration of γ -secretase.

The four components of the functional γ -secretase complex are shown. The endoproteolytic site is indicated by arrow. The stars indicate the active site, Asp 257 and Asp385.

Assembly of the γ -secretase complex

The γ -secretase complex is assembled in sequential steps. First, immature Nct interacts with Aph-1 to form a scaffold for binding of PS and PEN-2; then, PS binds to the Nct-Aph-1 subcomplex (Takasugi, Tomita et al. 2003; Shirotani, Edbauer et al. 2004). Later, Pen-2 binds to this intermediate complex during the formation of the mature protease. PEN-2 is required for endoproteolytic processing of PS and this process activates the γ -secretase complex (LaVoie, Fraering et al. 2003; Takasugi, Tomita et al. 2003; Niimura, Isoo et al. 2005). The loss of Nct expression in the embryonic fibroblast cells (Nct KO cells) results in decreased levels of Aph-1, PEN-2, and PS1 fragments, accompanied by a significant accumulation of full-length PS1 (Zhang, Luo et al. 2005). Knocking down Aph-1, PEN-2, or Nct in cultured *Drosophila* cells also reduces levels of processed PS and reduces γ -secretase cleavage of APP and Notch (Francis, McGrath et al. 2002). Thus, Nct, PS, Aph-1 and PS interact with and affect the stability of each other.

Substrates of γ -secretase

γ -Secretase cleaves more than 80 type I transmembrane proteins within their transmembrane domain. Of those, APP and Notch are the most studied substrates (Haapasalo and Kovacs 2011). γ -Secretase cleaves APP at multiple sites to generate A β peptides of different lengths (Figure 3). The majority of A β peptides are 40 amino acids long (A β 40). However, peptides ranging from 37 to 49 amino acids are produced by γ -secretase. APP apparently undergoes at least three major cleavages, called gamma-, epsilon- and zeta-cleavage, within its transmembrane domain (Weidemann, Eggert et al. 2002; Zhao, Mao et al. 2004; Sato, Tanimura et al. 2005; Zhao, Cui et al. 2005). APP can be sequentially cleaved along two production lines: A β 49-A β 46-A β 43-A β 40-A β 37 and A β 48-A β 45-A β 42-A β 38 (Chavez-Gutierrez, Bammens et al. 2012). A β 49 and A β 48, which are generated by epsilon-cleavage, are further processed by zeta- and gamma-cleavages sequentially (Zhao, Cui et al. 2005; Tomita 2009). In line with this sequential cleavage of APP by γ -secretase, the predominant species of AICD is CTF- γ 50-99, which is generated by processing between Leu-645 and Val-646 of APP(695) (Lu, Rabizadeh et al. 2000; Sastre, Steiner et al. 2001; Yu, Kim et al. 2001; Sato, Dohmae et al. 2003). AICD is detected in control-sample brain tissue as well as in sporadic AD-brain, and it acts as a positive regulator of apoptosis. Thus, overproduction of AICD may add to the toxic effect of A β 42 oligomers and further accelerate pathogenesis (Passer, Pellegrini et al. 2000). Since AICD is quickly degraded after γ -cleavage, the physiological functions of AICD in vivo are difficult to study. More investigation is required to clarify the biochemical mechanisms of AICD.

Another important substrate, Notch, is cleaved by γ -secretase, resulting in the release of Notch intracellular domain (NICD). NICD is translocated to the nucleus where it transactivates a number of genes that are critical to development, including cell differentiation and proliferation (Kopan, Schroeter et al. 1996; Schroeter, Kisslinger et al. 1998). Genetic studies on PS1/PS2, Nct, or Aph1 knockout mice show embryonic lethality. These embryos exhibit abnormal

somite segmentation phenotypes that are similar to phenotypic patterns of embryos lacking Notch (Shen, Bronson et al. 1997; Donoviel, Hadjantonakis et al. 1999; Li, Ma et al. 2003; Ma, Li et al. 2005). These data indicate that γ -secretase is essential for Notch signaling. In a zebrafish system, moreover, blocking of γ -secretase results in severe side effects due to the inhibition of Notch signaling (Geling, Steiner et al. 2002; Ma, Li et al. 2005). In a murine model, inhibition of γ -secretase results in undesirable biological effects, such as lymphopoiesis and intestinal cell differentiation, because of the inhibition of Notch processing (Wong, Manfra et al. 2004).

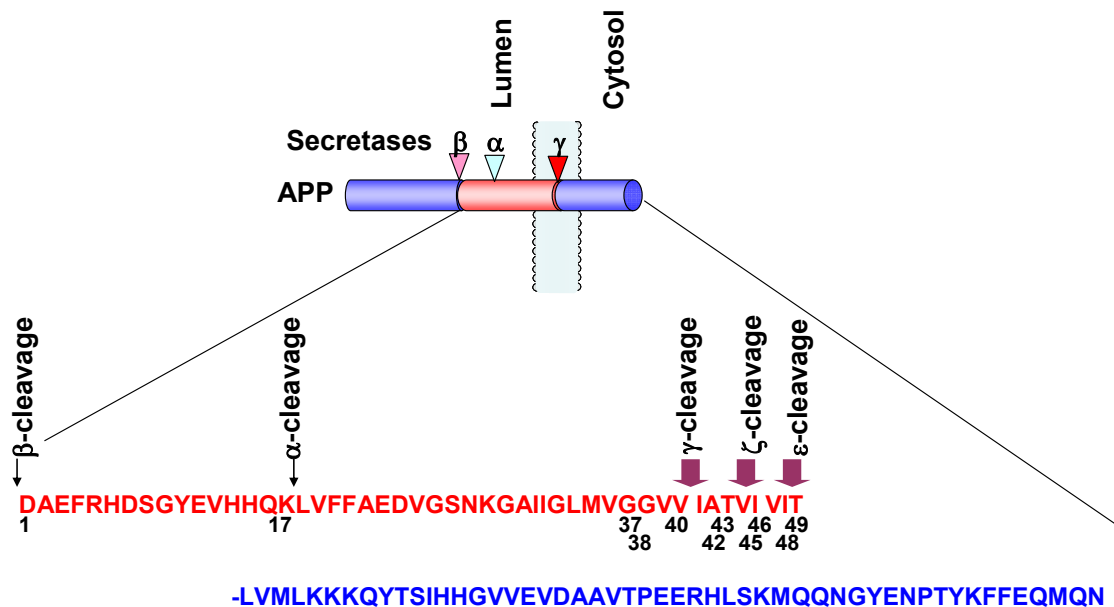


Figure 3. Proteolytic processing of APP

γ -secretase cleaves APP at multiple sites to generate A β peptides of different lengths. Red letters indicate A β peptides ranging from 37 to 49 amino acids that are produced by γ -secretase.

γ -Secretase in DRMs

The cellular membrane is mainly composed of a phospholipid bilayer structure. However, ordering is evident in membrane microdomains (called lipid rafts), which are enriched in cholesterol, sphingolipids, and certain membrane proteins. Lipid rafts are important for the membrane protein trafficking and regulate neurotransmission and receptor trafficking (Simons and Ikonen 1997). Lipid rafts are partly resistant to detergents and can be biochemically isolated by treating cellular membranes with detergents, and then separating by density gradient. The detergent-insoluble lipid-raft fractions float to the top of the gradient and can be collected as detergent-resistant membranes (DRMs). A previous study showed that active γ -secretase is highly enriched in DRMs in human and rat brain (Hur, Welander et al. 2008). Size-exclusion chromatography showed that the DRMs containing γ -secretase elute in the void volume (around 2000 kDa), suggesting that DRMs may contain several copies of γ -secretase complexes, other proteins and lipids. We hypothesized that potential γ -secretase associated

proteins (GSAPs) could interact with γ -secretase in DRMs and affect γ -secretase activity and substrate selection. Thus, that GSAPs localize to the DRM could be of special interest to study.

γ -Secretase in synapses

A loss or alteration of synapse structures has been reported in AD patients and in mouse AD models. Such alteration could be responsible for cognitive deficits long before the absence of neuronal loss. The mechanisms of loss or alteration of synapses in AD are still not fully understood. However, several reports indicate that long-term potentiation, a mechanistic model of synaptic strength and plasticity, can be disrupted by A β ; and that the infusion of A β directly into the brain can rapidly disrupt learned behavior and impair cognitive functions, even without pathognomonic signs of AD. These studies suggest that a toxic effect of A β directly disrupts synaptic signaling (Knobloch and Mansuy 2008). A previous study showed that γ -secretase activity is enriched in crude synaptic membrane and synaptic vesicle fractions (Frykman, Hur et al. 2010), suggesting that the synaptotoxic A β may be produced locally. We hypothesized that potential GSAPs interact with γ -secretase in synapses and affect γ -secretase activity and its substrate selectivity. Thus, studying GSAPs that are localized to synapses could be of special interest.

Developing drugs against AD

At present, drugs used for the treatment of AD only slightly delay the symptomatic progression of the disease and do not affect the main neuropathological hallmarks of the disease. An attractive strategy to develop treatments that modify AD may be inhibiting γ -secretase. However, γ -secretase cleaves a number of other substrates. Moreover, studies using a murine model show that γ -secretase inhibitors alter lymphopoiesis and intestinal cell differentiation by blocking cleavage of one essential substrate, Notch (Wong, Manfra et al. 2004; Evin, Sernee et al. 2006; Wolfe 2008). Furthermore, gastrointestinal and lymphatic system side-effects have been observed in clinical trials of γ -secretase inhibitors, primarily due to the reduced processing of Notch (Panza, Solfrizzi et al. 2009). Such side effects due to the inhibition of physiological function of γ -secretases must be carefully considered (Haass 2004). Further studies on γ -secretase are imperative for development of drugs that selectively reduce A β production. Possible targets for such compounds include proteins that interact with γ -secretase and regulate its activity.

Reported GSAPs

It is possible that proteins, other than the four core components, could interact with γ -secretase and affect its regulatory and selection activity. Examples of GSAPs that regulate A β -production include, for instance, TMP21, CD147, the tetraspanin web and PION (Zhou, Zhou et al. 2005; Chen, Hasegawa et al. 2006; Wakabayashi, Craessaerts et al. 2009; He, Luo et al. 2010).

-TMP21-

TMP21 was immuno-purified from wild-type blastocyst-derived cells expressing PS1 and PS2. TMP21 associates with γ -secretase and modulates APP cleavage, but not Notch cleavage. Knockdown of TMP21 increases A β production while having no effect on Notch cleavage and AICD production (Chen, Hasegawa et al. 2006). TMP21 is a type 1 transmembrane protein and it is a member of the p24 cargo-family, which is involved in secretory protein transport from the endoplasmic reticulum to the Golgi apparatus (Blum, Feick et al. 1996; Barr, Preisinger et al. 2001). TMP21 is expressed in neurons throughout the brain and is co-expressed with γ -secretase subunits in neuronal cells (Vetrivel, Kodam et al. 2008). The expression levels of TMP21 are reduced in AD brains as compared to unaffected controls (Vetrivel, Kodam et al. 2008). The functional domain of TMP21 was examined using a series of TMP21-p24a chimera proteins. It was found that the transmembrane domain of TMP21 binds to γ -secretase and could decrease γ -secretase proteolytic processing. This result was confirmed by a synthetic peptide corresponding to the TMP21 transmembrane helix. This isolated TMP21 transmembrane peptide reduces A β production in a dose-dependent fashion (Pardossi-Piquard, Bohm et al. 2009). However affinity capture experiments using a biotinylated transition-state analogue inhibitor of γ -secretase showed that TMP21 is absent from active γ -secretase complexes (Winkler, Hobson et al. 2009). This observation led to the suggestion that TMP21 could be associated with inactive complexes.

-CD147-

CD147 was identified as an additional γ -secretase complex subunit in a study in which native γ -secretase complexes were purified from HeLa cell membranes. CD147 is a transmembrane glycoprotein belonging to the immunoglobulin family. Knockdown of CD147 by siRNA increases the production of A β without changing the expression level of other γ -secretase components or of APP substrates (Zhou, Zhou et al. 2005). CD147 is expressed in many neuronal and non-neuronal tissues, including hippocampus, pre-frontal cortex, amygdala, thyroid, heart, and placenta (Kanyenda, Verdile et al. 2011). By immunoblotting, CD147 was found to be upregulated in the frontal cortex and thalamus of AD brains compared to control brains. Immunohistochemistry of AD- and control-brain tissue revealed specific upregulation of CD147 in neurons, axons and capillaries of AD frontal cortex and thalamus (Nahalkova, Volkmann et al. 2010). However, affinity capture experiments using a biotinylated transition-state analogue inhibitor of γ -secretase showed that CD147 is absent from active γ -secretase complexes (Winkler, Hobson et al. 2009). As in the case of TMP21, this could mean that CD147 is associated with inactive complexes.

-Tetraspanin web-

Tetraspanins are present in detergent-resistant microdomains in the cell membrane and regulate cell adhesion, cell signalling and proteolysis (Hemler 2008; Charrin, le Naour et al. 2009). By using tandem affinity purification,

tetraspanins (CD81, CD9 and UpK1b) as well as tetraspanin-associated proteins (EWI-2, EWI-F, integrins and CD98) were identified in a complex with PS1 and PS2 in reconstituted PS-deficient fibroblasts (Wakabayashi, Craessaerts et al. 2009). Knockdown of tetraspanins, EWI-F or CD98, inhibited γ -secretase activity and A β release, while their overexpression enhanced A β secretion. γ -Secretase activity was also significantly impaired in cells derived from CD81- and CD9-KO mice (Wakabayashi, Craessaerts et al. 2009).

-Pigeon homologue protein (PION)-

PION selectively increases A β production through a mechanism involving its interactions with both γ -secretase and the carboxy-terminal fragment of APP (APP-CTF). PION does not interact with Notch, nor does it affect its cleavage. PION was identified as a Gleevec (imatinib) target in human embryonic kidney (HEK293) cells (He, Luo et al. 2010). Gleevec is an anticancer drug found to inhibit A β formation without affecting Notch cleavage (Netzer, Dou et al. 2003). Gleevec achieves its A β lowering effect by preventing PION interaction with APP-CTF. Knockdown of PION in a mouse model of AD reduces A β levels and plaque development. Moreover, PION-immunoreactive deposits were found in close association with PS1 and A β in AD brains (Sato, Tabunoki et al. 2012). Aberrant regulation of PION expression could play a key role in acceleration of γ -cleavage of APP-CTF and accumulation of A β in AD brains.

So far, most studies of identification on GSAPs have been performed in cell lines, and little is known about how γ -secretase is regulated in brain. Here, we developed and employed a method based on affinity purification using a γ -secretase inhibitor to isolate the native complex. We searched for GSAPs in microsomal membranes, DRMs and synaptic preparations from brain. Novel GSAPs were identified by liquid chromatography directly coupled to tandem mass spectrometry (LC-MS/MS). Finally, we characterized the function and the localization of these novel GSAPs.

2, Aims of the thesis

AD is a progressive neurodegenerative disorder and the most common cause of dementia. Toxic species formed during the polymerization of A β are involved in AD pathogenesis. Reducing brain A β -levels may be a treatment strategy. However, γ -secretase inhibitors that reduce A β -production cause side effects associated with reduced Notch cleavage. Thus, specific inhibition of A β -production is necessary. The identification and characterization of novel GSAPs could open new possibilities for selective inhibition of A β -production. GSAPs could be novel drug targets for treatment of AD. Identification and characterization of novel GSAPs that selectively affect APP cleavage would increase the understanding of the mechanism of AD. Therefore, the aims of my thesis were the following:

1. To establish suitable pull-down methods for the purification of native γ -secretase from brain materials.
2. To identify novel GSAPs by LC-MS/MS in DRM and investigate the effect of GSAPs on A β production and Notch cleavage.
3. To identify novel GSAPs by LC-MS/MS in synaptic membranes and synaptic vesicles and investigate the effect of GSAPs on A β production and Notch cleavage.
4. To identify novel GSAPs by LC-MS/MS in microsomal fractions and investigate the effect of GSAPs on A β production and Notch cleavage.

3, Materials and methods

Ethical permission (Papers I, II, III, IV)

The animals used in this study were treated according to the Karolinska Institutet, as well as national, guidelines and the study was approved by the Animal Research Ethical Committee of Southern Stockholm (S80-08). Human brain materials were obtained from the Brain Bank at Karolinska Institutet. The study was approved by the regional ethical review board of Stockholm (024-01, 2007/533-32).

Antibodies (Papers I, II, III, IV)

The following antibodies were used: PS1-N-terminal fragment (529591; Calbiochem); PS1-C-terminal fragment (MAB5232; Chemicon); Aph-1aL (PRB-550P; COVANCE); Nct (MAB5556, Chemicon); UD1 raised against the N-terminal residues ERVSNEEKLNL of Pen-2 (a gift from Dr. Jan Näslund, Karolinska Institutet); syntaxin1 (S0664, Sigma); TMP21 (3999, Nordic BioSite); cleaved Notch1 (Val-1744, Cell Signaling Technology); anti-Myc (9E10-biotin, Abcam); voltage-dependent anion channel 1 (VDAC1, sc-8828, Santa Cruz Biotechnology); NADH dehydrogenase [ubiquinone] iron-sulfur protein 7 (NDUFS7, 15728-1-AP, ProteinTech Group); tubulin polymerization-promoting protein (TPPP, AB15346, Millipore); Proton myo-inositol cotransporter (SLC2A13, BMP026, MBL); BDNF/NT-3 growth factors receptor precursor (NTRK2, WH0004915M1, SIGMA). Antibodies directed to NDUFS7 were developed by immunizing rabbits with a peptide from human NDUFS7 (amino acid residues 197-213). Antibodies directed to Probable phospholipid-transporting ATPase IIA (ATPA9A) were developed against a peptide from human ATPA9A (amino acid residues 1-17 and 430-446). Antibodies directed to potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2) were developed against a peptide from human HCN2 (amino acid residues 870-885).

SDS-PAGE and Western blotting (Papers I, II, III, IV)

Samples were boiled in SDS sample buffer and separated by SDS-PAGE. After electrophoresis proteins were transferred to PVDF membranes and probed with specific antibodies. Immune complexes were visualized by SuperSignal enhanced chemiluminescence reagent (Pierce). Hyperfilm ECL (GE Healthcare) was used for exposure, and films were scanned using an AGFA Duoscan. For quantification, the signals of the specific bands were quantified by using a CCD-camera (Fuji, LAS3000). The density of the bands was calculated as a percentage of a standard (input sample) run on each gel. Colloidal gold total protein stain solution (Bio-Rad) was used for the total protein staining on the PVDF membrane.

Synthesis of γ -secretase inhibitor coupled to biotin via a cleavable linker (GCB) (Paper I)

The synthesis of the methyl ester of the L-685,458 acid derivative was described previously (Nadin, Lopez et al. 2001). Overnight, the L-685,458 derivative was reacted with 10 equivalents of diamido-dPEG diamine

(eChemShop) under EDC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) and 1-hydroxybenzotriazole hydrate. The resulting product was treated with 1 equivalent of EZ Link Sulfo-NHS-SS-Biotin (Pierce) overnight. The reaction mixture was purified by silica gel chromatography to give GCB.

γ -Secretase activity assay (Paper I)

Blastocyst-derived ES-cells deficient in PS1 and PS2, but which stably expressed PS1, BD8-PS1 cells, were cultured as previously described (Farmery, Tjernberg et al. 2003). All procedures were carried out at 4 °C. Cells were harvested with cell scraper, pelleted and washed once in ice-cold PBS. Cells were resuspended in 9 volumes of buffer A containing 20 mM Hepes, pH 7.5, 50 mM KCL, 2 mM EGTA and Complete Protease Inhibitor Cocktail (Roche) and sonicated on ice for 30 sec. Cell debris and nuclei were removed by centrifugation at 800 *g* for 10 min. The resulting supernatant was pooled and centrifuged at 100,000 *x g* for 60 min. Membrane proteins were solubilized in buffer H containing 20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EDTA, Complete Protease Inhibitor Cocktail and 1% (w/v) CHAPSO for 30 min at 4 °C. Insoluble debris was removed by centrifugation at 100,000 *x g* for 30 min. The resulting supernatants (solubilized γ -secretase) were diluted with buffer H without CHAPSO to give a final concentration of 0.4% (w/v) CHAPSO. Protein concentration was determined by BCA™-Protein Assay (Pierce). Solubilized γ -secretase (1mg/mL) was incubated in the absence or presence of L-685,458 or GCB for 16 h at 37 °C. The reaction was stopped by adding RIPA (150mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris HCl, pH 8.0) and boiling for 5 min. The samples were centrifuged at 10,000 *x g*, and A β 40 was measured in the supernatants by ELISA (Wako Chemicals). Background, defined as the signal in the presence of 10 μ M L-685,458, was subtracted. IC50 (nM) values were calculated using the GraphPad Prism 4.02 software (GraphPad Software).

Preparation of γ -secretase enriched microsomal membranes (Paper I, IV)

Frozen brains from Sprague-Dawley rats were obtained from ROCKLAND IMMUNOCHEMICALS, INC. (Pennsylvania, USA). Human brain material (40 g of frontal cortex from a non-AD case, 2-20 hr postmortem time) was obtained from Huddinge Brain Bank (Huddinge, Sweden). The tissue was homogenized in buffer A (20 mM Hepes, 50 mM KCL, 2 mM EGTA, pH 7.5) containing a Complete Protease Inhibitor Cocktail (Roche) with 25 strokes at 1500 rpm using a pestle-homogenizer. The brain homogenates were centrifuged at 1,000 *x g* to remove nuclei and cell debris. The post-nuclear supernatants were centrifuged at 10,000 *x g* to remove mitochondria and the resulting supernatants were centrifuged at 100,000 *x g*. The resulting microsomal membrane quantity was resuspended in buffer A supplemented with 20% glycerol, and stored at -80 °C. All centrifugation steps were carried out at 4 °C.

Preparation of DRMs (Paper II)

DRMs were prepared as described previously (Hur, Welander et al. 2008). In brief, to isolate DRMs from brain material, microsomal membrane fraction was resuspended in 600 μ l of buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM

NaCl, 1 mM EDTA, 2.0% CHAPSO, and Complete Protease Inhibitor Cocktail (Roche). The samples were incubated with end-over-end rotation for 20 min at 4 °C. The sample was adjusted to 45% sucrose and placed at the bottom of a 14-ml Ultra-clear centrifuge tube (Beckman Coulter). Then 6.9 ml of 35% sucrose followed by 2.3 ml of 5% sucrose were overlaid. The sample was centrifuged at 100,000 x g for 16 h at 4 °C in a SW40Ti rotor (Beckman Coulter). The fraction at the 5–35% interface was collected using a 5-ml syringe.

Preparation of synaptic membranes and synaptic vesicles (Paper III)

Synaptic membranes and synaptic vesicles were prepared as described previously (Cohen, Blomberg et al. 1977; Frykman, Hur et al. 2010). Briefly, rat brains were homogenized in Buffer AS (0.32 M sucrose, 116 mM NaHCO₃, 1 mM MgCl₂, and 0.5 mM CaCl₂). The P2 (17,300g) pellet was resuspended in Buffer BS (1 mM NaHCO₃, 0.32 M sucrose) and layered on a sucrose gradient in order to purify synaptosomes. The synaptosomes were lysed in 6 mM Tris–HCl pH 8.1 and the lysate was centrifuged at 48,250g to pellet the crude synaptic membranes. The supernatant was further centrifuged at 100,000 x g for 2 h to obtain the synaptic vesicles. For two of the synaptic vesicle experiments, synaptic vesicles were alternatively prepared according to the method of Huttner et al. (Huttner, Schiebler et al. 1983), with direct lysis of the P2 pellet without sucrose gradient purification.

Affinity pulldown of γ -secretase in microsomal membranes, synaptic membranes or synaptic vesicles (Papers I, III, IV)

Microsomal membranes, synaptic membranes or synaptic vesicles were solubilized in buffer A containing Complete Protease Inhibitor Cocktail (Roche) and 1% (w/v) CHAPSO for 30 min at 4 °C. Insoluble debris was removed by centrifugation at 100,000 x g for 30 min. The resulting supernatants (solubilized γ -secretase) were diluted with buffer A without CHAPSO to give a final detergent concentration of 0.5% (w/v) CHAPSO. The samples were incubated with SA-conjugated sepharose beads (GE Healthcare) or SA-conjugated magnetic beads (Invitrogen) to remove endogenous biotinylated proteins for 16 hr at 4 °C. The supernatants were recovered by centrifugation at 1000 g for 5 min and used as starting material (input) for pull-down experiments. The samples were incubated in the presence or absence of 10 μ M L-685,458 as a competing inhibitor for 10 min at room temperature and then incubated with 200nM GCB for 30 min at room temperature. The samples were incubated with SA-beads for 2hr at 4 °C. The resin was washed for 10 minutes 3 times with buffer A with 0.5% CHAPSO at room temperature. The captured γ -secretase complex was eluted from the resin by buffer A containing 100mM DTT and 0.5% CHAPSO, SDS sample buffer, or 0.01% RapiGest (Waters) in 10mM ammonium bicarbonate supplemented with 10mM DTT.

Affinity pulldown of γ -secretase in DRMs (Paper II)

γ -Secretase was captured from DRM fractions using a method modified from the one used in Papers I, II and IV. In brief, CHAPSO was added to DRM fractions to a final concentration of 0.5% (w/v). Samples were pre-cleared with streptavidin-conjugated magnetic beads (Invitrogen) overnight at 4°C. The

magnetic beads were collected by a magnet and the procedure was repeated once. The supernatant was incubated for 30 min at 37 °C in the absence or presence of 50 mM of the γ -secretase inhibitor L-685,458. The samples were incubated further with 200 nM GCB for 1 h at 37 °C, followed by incubation with magnetic beads with end-over-end rotation for 2 h at 4 °C. The magnetic beads were collected by using a magnet. After washing three times with buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5-1.0% CHAPSO, and Complete Protease Inhibitor Cocktail (Roche). The captured γ -secretase complex was eluted from the resin by SDS sample buffer, or 0.01% RapiGest (Waters) in 10mM ammonium bicarbonate supplemented with 10mM DTT.

Tryptic digestion and fractionation of peptides (Papers I, II, III, IV)

The eluted proteins were suspended in trypsin digestion buffer (180 mM ammonium bicarbonate, 2 mM CaCl₂ and 0.3% Rapigest) and digested by trypsin at 37 °C overnight. The digested samples were loaded onto ZipTips C18 (Millipore) after the tips had been equilibrated according to the manufacturer's recommended procedures. The samples were washed with 0.2% formic acid (FA) in water and eluted from the ZipTips C18 with 80% acetonitrile/0.2% FA. The eluted peptides were evaporated to dryness in a vacuum centrifuge. For further fractionation, the eluted peptides were adjusted with 0.1% FA to 45% acetonitrile and loaded onto ZipTips SCX (Millipore) according to the manufacturer's recommendation. The samples were sequentially eluted with 40 mM ammonium formate (pH 3.0) containing 45% acetonitrile, 80 mM ammonium formate (pH 3.0) containing 45% acetonitrile and 5% ammonium hydroxide containing 45% acetonitrile. The eluted peptides were evaporated to dryness in a vacuum centrifuge.

Mass spectrometry (Papers I, II, III, IV)

The digested samples were dissolved in 2% ACN/0.2% FA in water and analyzed on a 6330 Ion Trap LC/MS system (Agilent Technologies). Liquid chromatography was performed on a HPLC chip-system (Agilent Technologies) using a chip with a 150 mm x 75 μ m analytical column and a 160 nL enrichment column, both packed with 5 μ m Zorbax 300SB-C18. Samples were loaded onto the enrichment column, using a mobile phase containing 2% ACN and 0.2% FA in water, at a flow rate of 2 μ l/min and washed for 3 min. Tryptic peptides were eluted into the mass spectrometer using a gradient of increasing mobile phase B at a flow rate of 200 nl/min. The gradient (mobile phase A: H₂O/0.2% FA; mobile phase B: ACN/0.2% FA) was ramped from 3% to 26% B in 92 min, and then from 26% to 36% B in 20 min. As an alternative, the gradient was ramped from 3% to 26% B in 184 min, and then from 26% to 36% B in 40 min. The capillary voltage was set to 1950 V, and the flow rate and the temperature of the dry gas set to 4 l/min and 300 °C, respectively. Spectra were collected over an *m/z* range of 230-1,800, and the five most intense ions were subjected to MS/MS. Precursors were excluded for 30 sec after two MS/MS spectra.

Protein identification by MASCOT Daemon software package (Paper I)

A list of the resulting spectra were generated using the 6300 Series Ion Trap LC/MS Software Version 6.1(Agilent Technologies) with an intensity threshold of 1000. The compound lists were exported as Mascot generic format files (mgf). Protein identification was performed using the MASCOT Daemon software package (Matrix Science), with parameters, including: NCBI nr (database); Homo sapiens or Mammalia (taxonomy); maximum missed cleavage of 1; methionine oxidization (variable modification); monoisotopic mass; unrestricted protein mass, 2.0 Da fragment ion mass tolerance; 0.3 Da MS/MS tolerances; peptide charge state, 2+,3+. Statistically significant peptide data acquired from samples incubated in the presence of competing inhibitor were subtracted from statistically significant peptide data from samples incubated in the absence of competing inhibitor. Finally, peaks for extracted ion chromatogram peptides were compared for the two samples in order to validate the subtraction analysis.

Protein identification by Spectrum Mill MS Proteomics Workbench (Papers I, II, III, IV)

Peak lists were generated from the data obtained from each analysis using the data extractor of the Spectrum Mill MS Proteomics Workbench version A.03.03.078 (Agilent Technologies). The MS and MS/MS data were extracted by limiting the data search to deconvoluted ions observed between 400 and 5000 Da. MS scans with similar precursor mass ($\pm 1.4 m/z$) and retention time within 60 s were merged. Only spectra that contained sequence tag information of three or more residues were submitted for database searching. The resulting extracted data were searched against the Homo Sapiens subset or Human Rodent of SwissProt protein database using the MS/MS search function of Spectrum Mill. Search parameters included a variable modification on methionine residues (oxidized methionine), a 50% minimum matched peak intensity, ± 2.5 Da tolerance on precursor ions and ± 0.3 Da tolerance on product ions, one missed tryptic cleavage, and ESI ion trap scoring parameters as defined by the search algorithm. The initial results were autovalidated using the following parameters for the "protein details" mode: SPI >70% for matches with score >8 for +1, >7 for +2, and >9 for +3; SPI >90% for score >6 on +1. The autovalidation step was done in "peptide" mode using criteria of a score >13 and SPI >70%. In addition, both autovalidation steps required a forward–reverse score >1 for +1 and +2, and >2 for +3 peptides. Results from select additional spectra with lower scores (>10, >70%) were accepted as valid only after manual inspection. The validated peptides were used to identify a set of proteins from which a result file was created (Kapp, Schutz et al. 2005). A second round of searches with unvalidated peptide spectra was performed against the set of proteins in this result file allowing a nonspecific C-terminal or a nonspecific N-terminal. The selected proteins were classified with respect to expression specificity in brain and their functions in brain or neurons according to the General annotation (Subcellular location) of the Swiss-Prot data base.

Cell lines, Cell culture (Papers II, III, IV)

HEK cells overexpressing the human APP 695 isoform (HEK-APP) were kindly provided by Dr. Eirikur Benedikz (Nilsson, Malkiewicz et al. 2006). And HEK cells expressing Notch construct with a deleted ectodomain (HEK-Notch Δ E)

were kindly provided by Dr. Helena Karlström. HEK-APP and HEK-Notch Δ E cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum and 0.5% penicillin-streptomycin (MP Biomedicals). Cells were grown in 5 % CO₂/95% air at 37 °C.

Design and synthesis of siRNA (Papers II, III, IV)

The siRNAs were designed by an algorithm that increases efficiency of the siRNAs while minimizing off-target effects, and all siRNAs passed both vendor and internal quality control. For genes with multiple splice forms, the common sequence was targeted. Additional siRNAs were purchased from Ambion.

RNA Interference and determination of A β levels (Papers II, III, IV)

The transfection procedure for HEK-APP cells was scaled for 96-well (initial experiments) or 24-well plates. Cells were transfected with siRNA oligomers by using the Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's protocol. After three days of transfection, culture medium was replaced with Opti-MEM I reduced-serum medium (Invitrogen). Conditioned medium from siRNA-transfected HEK-APP cells was harvested after 24-hour incubation and analyzed with respect to A β ₄₀ and A β ₄₂ levels by a commercial sandwich ELISA (Wako Chemicals), and compared to the A β levels in cells treated with only Lipofectamine. The knock down efficacy was examined by real time-PCR. The cell viability was analyzed by the WST8 assay (MBL) or alamar blue reagent (Biosource Europe) according to the manufacturer's protocol. The cell viability was calculated by dividing the WST8 or Alamar blue data for the test samples with that of the Lipofectamine control, and the A β levels were adjusted to cell viability.

RNA Interference and determination of NICD level (Papers II, III, IV)

The transfection procedure for HEK-Notch Δ E cells was scaled for 24-well plates, and cells were transfected with siRNA oligomers by using the Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's protocol. After three days of transfection, culture media were replaced with Opti-MEM I reduced-serum medium (Invitrogen). The siRNA transfected HEK-Notch Δ E cells were lysed in RIPA buffer (0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 50 mM Tris-HCl, 150 mM NaCl, pH 8.0), an equal volume of Benzonase solution (25U/ml Benzonase, 50 mM Tris-HCl, 4 mM MgSO₄, pH 8.0) was added and the samples were incubated for 30 min. SDS sample buffer containing 50 mM DTT was added and the samples were subjected to SDS-PAGE, transferred to PVDF membranes, probed with an antibody against the neo-epitope of the γ -secretase cleavage site of Notch-1 and anti-Myc (9E10)-biotin to detect Notch Δ E levels as a internal control.

Real-time PCR (Papers II, III, IV)

After harvesting the conditioned media and determining the cell survival, the siRNA-transfected cells were washed with cold PBS, lysed with lysis solution (Taqman Gene Expression Cells-to-CT kit, Ambion), and reverse transcribed according to the manufacturer's instructions. Gene expression levels were

measured by real-time PCR (7500 Fast Real-time PCR system, Applied Biosystems).

Statistical analysis (Paper III)

Statistical analyses were performed using Independent Sample T-test to test if the mean values were significantly different from 100% control.

Overexpression of VDAC1 and TPPP (Papers II, III)

HEK293 APP695 cells were transiently transfected with a pCMV6-VDAC1 and pCMV6-TPPP TrueORF Gold vector (OriGene) by using TransIT LT-1 transfection agent (Mirus Bio). Cells were grown in 5% CO₂, 95% air at 37 °C. 24 h after transfection, the medium was exchanged to Opti-MEM. After a further 24 h, the medium was analyzed for A β levels as described above, and the cell viability assay (Alamar Blue) was performed. The A β levels were normalized to cell viability data as described above.

Overexpression of HCN2 and SLC2A13 (Paper IV)

HepG2 cells were cultured in RPMI medium 1640 (GIBCO) supplemented with inactivated 10% fetal bovine serum (MP Biomedicals), 0.5% penicillin-streptomycin (MP Biomedicals), 1% MEM Non-Essential Amino Acids (GIBCO) and 1 mM Na-pyruvate (GIBCO) at 37°C in a humidified atmosphere of 5% CO₂ in air. The cells were seeded at 10,000 cells-per-well on 384-well plates. The next day, the BacMam viruses containing C99 (BacMam-C99) were diluted to 20 MOI and SLC2A13 (BacMam-SLC2A13) or HCN2 (BacMam-HCN2) were diluted to 25, 50 and 100 MOI with 0.4% BSA in PBS. BacMam-C99 and BacMam-SLC2A13 or BacMam-HCN2 were added to the cells and incubated for 24 h at 37 °C. Conditioned medium from BacMam-transduced HepG2 cells was harvested after 24 hour incubation and analyzed with respect to A β ₄₀ levels by a commercial A β ₄₀ homogenous time resolved fluorescence (HTRF) assay. After sampling of the culture supernatants for HTRF assay, the cell viability was analyzed by the WST8 assay (MBL) according to the manufacturer's protocol. The relative cell viability was calculated by dividing the WST8 data for the test samples with that of the control BacMam transduced HepG2 cells.

Preparation of recombinant BacMam viruses (Paper IV)

DNA sequences of the C99 GenBank (accession number Y00264) and HCN2 (accession number AF065164) or SLC2A13 (accession number BC047507) were synthesized and cloned into pFASTBac (Invitrogen). Recombinant viruses were constructed by using these transfer vector pFASTBac, and generated by using a Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the manufacturer's instructions. The virus was purified and concentrated by Power prep HP Plasmid Purification System (MARLUGEN Biosciences). Virus titer was determined by a plaque assay on Sf9 cells.

Homogenous time resolved fluorescence (HTRF) assay (Paper IV)

Secreted A β ₁₋₄₀ in the culture supernatants was measured by using the HTRF assay kit (Cisbio Bioassays). The mixture of anti- A β ₁₋₄₀-Cryptate and anti-A β ₁₋₄₀-XL665 reagents was added to 384-well assay plate (Greiner) and then

culture supernatant was applied to the plate, and the reaction mixture was left at 4 °C overnight to reach equilibrium binding. The reaction mixture was read on the EmVision micro plate reader (PerkinElmer).

Immunoprecipitation (Papers III, IV)

For immunoprecipitation, microsomal fractions, synaptic membranes or synaptic vesicles were prepared from rat brain as described above and solubilized in Buffer A containing 0.5% CHAPSO. The samples were preabsorbed with protein A-Dynabeads (Invitrogen) prior to addition of antibody. The GSAP antibody or IgG control from non-immunized rabbit or mouse (Sigma) was incubated with the samples over night at 4 °C. ProteinA-dynabeads were added and the sample was further incubated for 2 h. Subsequently, the beads were washed 3 times with 0.5% CHAPSO in Buffer A prior to elution with SDS sample buffer containing 50 mM DTT. Western blot analysis was performed as described above.

Proximity Ligation Assay (Paper III)

The in situ Proximity Ligation Assay (PLA) using Duolink reagents (Olink Bioscience) was performed according to the manufacturer's instructions (Soderberg, Leuchowius et al. 2008). Briefly, primary murine hippocampal neurons (E16.5) were cultured in Neurobasal medium supplemented with B-27 (Invitrogen) for 14 days in poly-D-lysine (Sigma Aldrich) coated glass bottom microwell dishes (MatTek Corporation). Cells were fixed for 10 min in 4% paraformaldehyde and subsequently treated with 70% ice-cold methanol for 30 s (only for PS1-TPPP experiment), followed by washing in DPBS (Invitrogen) and permeabilization in 0.1% Triton X-100 in DPBS for 5 min at room temperature. After blocking with blocking solution supplied in the kit, cells were probed with PS1 (1:100, MAB5232) and TPPP (1:1000, AB15346) antibodies over night at 4 °C or with PS1 and NDUFS7 antibodies at 37 °C, 1 h. After washing, cells were incubated with anti-mouse PLUS and anti-rabbit MINUS probes, followed by ligation and amplification reactions using the Duolink II Detection Reagents, Far Red. Cells were then stained with Alexa fluor 488 conjugated phalloidin (Invitrogen), mounted with mounting medium containing DAPI (Olink Bioscience) and examined using an inverted laser scanning microscope (LSM 510 META, Zeiss) with the plan-Neofluar 40x/1.3 oil immersion objective.

Immunocytochemistry (Paper III)

Murine hippocampal neurons were cultured, fixed and incubated with primary PS1 and TPPP or NDUFS7 antibodies as described for PLA above. Cells were then washed with DPBS and incubated with 4 µg/ml of secondary Alexa fluor 488 goat anti-mouse IgG and Alexa fluor 555 goat anti-rabbit IgG (Invitrogen) for 1 h at room temperature. After washing with DPBS, cells were mounted with Prolong Gold Antifade reagent with DAPI (Invitrogen) and analyzed using an inverted laser scanning microscope (LSM 510 META, Zeiss, Germany) with the plan-Apochromat 63x/1.4 oil immersion objective.

Immunohistochemistry (Paper III)

Immunohistochemistry was performed on tissue from frontal cortex and hippocampus from 3 AD and 3 age-matched control cases. Brain tissue blocks were fixed in buffered 4% formaldehyde and embedded in paraffin. 7 µm-thick sections were mounted onto Superfrost plus-glass (Menzel). The mounted sections were baked at 37 °C over night, dewaxed, hydrated and autoclaved for 30 min in sodium-citrate buffer pH 6. The sections were blocked for non-specific sites with Dako-protein block (X0909) for 30 min. Primary antibodies against TPPP (AB15346, Millipore) or NDUFS7 (in-house) were incubated at 4°C over night. After this, sections were incubated with biotinylated anti-goat or anti-rabbit IgG antibody (Vector laboratories) in TBS, for 30 min, followed by incubation in ABC-Elite HRP (Vector laboratories) for 1 hour. Reactions were visualized by developing the sections in DAB (Sigma). Between different steps the sections were thoroughly washed in TBS (Tris-buffered-saline, pH 7,6). Finally the sections were dehydrated and mounted in DPX (BDH Prolabo). All sections were treated simultaneously under the same conditions. For control staining the primary antibody was omitted. Sections were counterstained with haematoxylin.

Electron microscopy (Paper II)

DRM fractions were mixed with 2% CHAPSO in PBS and centrifuged at 100,000 x g for 1 h at 4 °C in an SW40 rotor (Beckman Coulter). The bottom fraction was centrifuged once again at 100, 000 x g for 1 h in a TLA55 rotor (Beckman Coulter). The DRM pellets were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose and 3 mM CaCl₂ (pH 7.4) at 4 °C overnight, rinsed in 0.1 M phosphate buffer (pH 7.4) followed by postfixation in 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 2 h, dehydrated in ethanol followed by acetone, and embedded in LX-112 (Ladd, Burlington, VT). Sections were contrasted with uranyl acetate followed by lead citrate and examined in a Leo 906 transmission electron microscope at 80 kV (Leo, Oberkochen). Digital images were obtained using a Morada digital camera (Olympus Soft Imaging System).

4, Results and discussion

Four proteins PS, Nct, Aph-1 and Pen-2 are necessary and sufficient to produce an active γ -secretase complex (Haass 2004). It is possible that other associated proteins affect γ -secretase activity. There are examples of GSAPs, for instance, TMP21, CD147, proteins in the tetraspanin web and PION, which regulate A β production (Zhou, Zhou et al. 2005 ; Chen, Hasegawa et al. 2006; Wakabayashi, Craessaerts et al. 2009; He, Luo et al.). So far, most studies on GSAPs have been performed in cell culture, and little is known about how γ -secretase is regulated in brain. Here, we established an affinity purification method using γ -secretase inhibitors to isolate the native complex from brain material. Novel GSAPs were identified from microsomes, DRMs and synaptic fractions of brain material by using LC-MS/MS. Finally we characterized the function and the localization of these GSAPs.

Affinity pull-down of γ -secretase and GSAPs from brain (Paper I)

We used brain material from rat as well as the frontal cortex from postmortem human brain of non-Alzheimer cases. Since we needed active γ -secretase, brain samples with short postmortem time (less than 22 hours) were selected. In order to enrich the γ -secretase complex before capturing, we used the microsomal fraction as starting material. We used centrifugation to remove nuclei and, in the next step, mitochondria. In the final pellet (microsomes), γ -secretase was enriched approximately 20-fold (Franberg, Welander et al. 2007).

Highly purified native γ -secretase is required to identify GSAPs. Therefore, we designed and synthesized a compound to be used for efficient and selective pull-down of γ -secretase. We chose L-685,458 as an inhibitor part of the compound. L-685,458 has strong γ -secretase inhibitory potency, and interacts with the active site as a transition-state analogue of an aspartyl protease (Shearman, Behr et al. 2000). This γ -secretase inhibitor was coupled to a long hydrophilic linker, which was attached to a biotin group via a disulphide bond (GCB).

The microsomal fraction was solubilized in the detergent CHAPSO (0.5%) and incubated with GCB in the presence or absence of non-biotinylated competing inhibitor. Native γ -secretase complexes that interacted with GCB were captured by streptavidin beads (SA beads). Then, captured γ -secretase complexes were eluted by a reducing reagent (DTT) from the SA beads. To assess the efficacy and the selectivity of the approaches, the capturing of known γ -secretase components was estimated by Western blotting and found to be within the range of 2-20%. The specificity was confirmed by competition using L-685,458. Importantly, elution with reducing reagent (DTT) clearly reduced the background signal compared to elution with SDS sample buffer. The purity and pull-down efficacy of γ -secretase complex was found to be sufficient for LC-MS/MS analysis.

For optimal protein identification, effective digestion of membrane proteins, high resolution separation of peptides, and high sensitivity of MS/MS measurement were conducted. Before analysis by LC-MS/MS, the purified γ -secretase complexes were digested by the protease trypsin. For effective

digestion of membrane proteins, we used 0.01% RapiGest, which enhances solubility of membrane proteins during the digestion. In order to increase the resolution, we pre-fractionated the samples by strong-cation exchange chromatography and used a long LC gradient. For high sensitivity MS/MS analysis, we employed a 6330 Ion Trap LC/MS fitted with a HPLC-chip system (Agilent Technologies). Proteins were identified by a peak-fitting software (Spectrum Mill) using peptide fragment data and a protein data base (SwissProt). The association was evaluated by quantitative analysis of the identified proteins in both the non-competed samples and the competed samples, using the Spectrum Mill software. As a result, all the known γ -secretase complex components were identified by mass spectrometry. Interestingly, TMP21 and syntaxin 1, which were previously reported to be GSAPs in cell lines, were found to associate with γ -secretase also in the brain. The association of TMP21 and syntaxin 1 with γ -secretase was verified by Western blotting.

TMP21, a transmembrane protein involved in trafficking, has previously been reported to co-purify with γ -secretase in preparations from cell lines. In that study an antibody directed to PS1 was used for immunoprecipitation followed by separation by gel electrophoresis and identification by mass spectrometry (Chen, Hasegawa et al. 2006). The synaptic protein syntaxin1 was previously found to be a PS-associated protein using yeast two-hybrid system (Smith, Anderson et al. 2000), but it has not been reported whether it interacts with free PS or with PS within the γ -secretase complex. Here, we show that syntaxin1 is, indeed, part of an active γ -secretase complex. This is of particular interest since synaptic degeneration is an early pathological hallmark in AD, and synaptic activity has been shown to increase A β production (Kamenetz, Tomita et al. 2003).

Previous studies have identified the N-terminus of PS1-C-terminal fragment (PS1-CTF) (Shirotani, Takahashi et al. 1997). However, the C-terminus of PS1-N-terminal fragment (PS1-NTF) has not been reported. Thus, we subjected the MS/MS spectra to a Spectrum Mill search investigating any residue that was allowed in the C-terminus (not only lysine or arginine which terminate the peptides after tryptic cleavage) in order to search for the endogenous C-terminus of PS1-N-terminal fragment. One of the identified PS1 fragments (MLVETAQERNETLFPALIYSST) ended at residue 291, which is threonine. It is likely that the C-terminus of this peptide corresponds to the natural cleavage site of full length PS1. This notion is in line with previous data from N-terminal sequencing of PS-CTF from HEK293 cells (Podlisny, Citron et al. 1997). Thus, we conclude that there is no additional processing by carboxy- or amino-peptidases of the PS fragments after the initial cleavage.

In summary, we have shown that TMP21 and syntaxin1 are associated to γ -secretase in brain and suggest that the present method is suitable for the unbiased identification of GSAPs in brain.

Identification and investigation of novel GSAPs in DRMs (Paper II)

Previous studies using cultured cells or brain tissue have shown that APP, β -secretase, and γ -secretase localize to DRMs (Lee, Liyanage et al. 1998; Wahrle, Das et al. 2002; Eehalt, Keller et al. 2003; Abad-Rodriguez, Ledesma et al.

2004; Urano, Hayashi et al. 2005; Vetrivel, Cheng et al. 2005; Hattori, Asai et al. 2006; Hur, Welander et al. 2008). In particular active γ -secretase is highly enriched in DRMs in human and rat brain (Hur, Welander et al. 2008). Thus, we attempt to find GSAPs that could interact with γ -secretase in DRMs and affect γ -secretase activity and substrate selectivity. Here, we affinity-purified γ -secretase from DRMs and studied their effect on A β production and Notch processing by using siRNA knockdown.

DRMs were isolated by treating rat microsomal fractions with detergents, followed by density gradient centrifugation (Hur, Welander et al. 2008). We confirmed the preparation of DRMs using electron microscopy, which revealed a mixture of mostly vesicular material around 100–200 nm in diameter in a DRM-like structure. Hence, several hundred protein molecules could be present in one vesicle. We needed to modify the GCB pull-down method, which was established in Paper I, since the protocol for detergent soluble γ -secretase fraction is difficult to directly apply on detergent-insoluble fractions.

DRM fractions were pre-cleared with streptavidin-conjugated magnetic beads. The samples were incubated in the absence or presence of the competing inhibitor L-685,458, followed by incubation with GCB. To get an efficient competition, we used the condition of 5 times higher concentration of the competing inhibitor, L-685,458 (50 μ M), than the condition described in Paper I. Using this condition the competition was complete. Since DRMs can contain several copies of γ -secretase complexes in one piece of DRM, high concentration of competing inhibitor could be required for the complete competition. Then, streptavidin-conjugated magnetic beads were added, collected by a magnet after incubation, and repeatedly washed. The DRM preparations gave a higher background than the microsomal preparations. Thus we washed the beads while increasing the CHAPSO concentration (0.5% to 1%), since DRM fractions are stable in more highly concentrated detergent. This washing condition clearly reduced background. After washing, bound components were eluted with a reducing buffer.

The efficiency of the purification and elution procedures was evaluated by using colloidal gold staining and Western blotting. Western blot analysis showed that Nct and PS1-NTF were captured by GCB in the absence of L-685,458. Previously reported GSAPs, TMP21 and syntaxin1, were also specifically captured by GCB in DRMs. To our knowledge, this is the first time GSAPs that are associated with brain DRMs have been presented.

The samples were digested with trypsin. RapiGest was used in the digestion buffer to enhance the solubility of membrane proteins. The tryptic peptides were concentrated and desalted by reversed-phase extraction using a reversed-phase chromatography. The samples were injected into the LC-MS/MS system, eluted by an acetonitrile gradient, and MS and MS/MS spectra were collected. Data from three independent experiments were analyzed and compared by using the Spectrum Mill software. The intensity ratio was then calculated by dividing the spectra mean intensity of the specific binding proteins (found in the absence of a competing inhibitor) by the intensity of the nonspecific binding proteins (found in the presence of a competing inhibitor). We could not identify 4 γ -secretase components from this experiment. A possible explanation is that many other

membrane associated proteins in DRM interfere with the collection of MS/MS spectra of γ -secretase components.

Six proteins were uniquely found in all three independent experiments. Sixteen proteins were uniquely found in two and not at all in one of three experiments. We used the spectra mean intensity ratio to select additional candidates. Based on this method, we selected 5 other proteins. In summary, we have identified 27 potential GSAPs in DRMs. Most of these are trafficking proteins, transporters or channel proteins.

Among the identified proteins, two are the previously reported GSAPs, TMP21 and syntaxin1. The comparison between three samples of non-competed samples and three samples of the competed samples, using the Spectrum Mill software, also showed that TMP21 and syntaxin1 were only identified in the absence of γ -secretase-competing inhibitor samples. Thus, these two GSAPs could interact with detergent-soluble (result of Paper I) as well as DRM-associated active γ -secretase. One of the candidate, voltage-dependent anion channel 1 (VDAC1) was also identified as a potential GSAP in a study using tandem affinity purification method from cell lines. (Wakabayashi, Craessaerts et al. 2009). In our study, we found VDAC1 a significant hit, with a high score only in the absence of γ -secretase-competing inhibitor, as results showed from three independent MS experiments. Although we have taken a novel approach for purification of GSAPs, some of the candidates we identify have also been found in other studies, supporting our notion that the GSAPs we find are relevant. For instance, 14 of the proteins that we identified were found also in the study by Wakabayashi, *et al.* (Wakabayashi, Craessaerts et al. 2009). A clear advantage of using DRMs prepared from brain, compared to cell lines, is that all the relevant proteins are expressed.

To investigate the effect of GSAPs on A β production and Notch cleavage, we employed a knockdown approach. First, we optimized the siRNA knockdown condition. We compared several cell lines - Human Embryonic Kidney 293 cells (HEK293) and human neuroblastoma cells (SH-SY5Y and BE(2)-C), and several different transfection reagents, to obtain the highest knockdown efficacy. We assessed an optimal knockdown condition using HEK-293 cells with Lipofectamine RNAiMAX as the transfection reagent.

Because the brain is most important organ investigated for AD pathology, we selected candidates that are highly expressed in brain. The mRNA levels and tissue expression levels of each candidate in the different organs were checked against public databases, BioGPS and UniProt. Since HEK293 cells are not of neuron-derived cell lines, we confirmed expression levels of candidate proteins in HEK293 cells using Affymetrix and Nimblegen, and candidate proteins that were not expressed in HEK293 cells were excluded. Finally we selected 11 candidates for the knockdown experiment.

To evaluate the effect of GSAPs on A β production, we chose to work with HEK293 cells overexpressing human APP (HEK-APP) (Nilsson, Malkiewicz et al. 2006). Secreted A β at levels from HEK-APP cells were sufficient to quantify by ELISA. We chose siRNA directed to PS1 as a positive control. Treatment with siRNA directed to PS1 reduced A β secretion in a dose-dependent manner. Thirty-five siRNAs directed to 11 candidates were transfected into HEK-APP cells. The knockdown efficiency of each gene at the mRNA level was measured

by RT-PCR, and cell viability was measured by Alamar Blue assay. The siRNAs that gave more than 70% knockdown of gene expression and showed more than 70% cell viability were selected for further studies. Based on this criteria, we selected the candidates, sortilin1 (SORT1), synaptophysin (SYP), contactin1 (CNTN1), VDAC1, syntaxin12 (STX12), cytochrome c oxidase subunit IV isoform 1 (COX4I1), and contactin-associated protein 1 (CNTNAP1) for more detailed studies. The knockdown at the protein level was also confirmed by Western blot analysis.

Next, the effect of siRNA-mediated knockdown of the selected candidates on A β production in culture media was measured by ELISA. The A β 40 and A β 42 levels of each sample were adjusted to cell viability data and compared with Lipofectamine control. Knockdown of SORT1, CNTN1, STX12, and COX4I1 did not show any major reduction in A β levels (less than 30%). For SYP, A β 42 and A β 40 levels were reduced by 44 and 11% at 6 pmol of siRNA, respectively. Knockdown of VDAC1 and CNTNAP1 decreased both A β 40 and 42 levels down to 70% in a siRNA dose-dependent manner. The reduction in A β levels was even greater than for the control, PS1. In summary, VDAC1 and CNTNAP1 appear to regulate A β production by interacting with γ -secretase. CNTNAP1 has not previously been reported to be a GSAP or to affect A β production, while VDAC1 was reported to associate with γ -secretase but no functional data were presented (Wakabayashi, Craessaerts et al. 2009).

To evaluate the effect of GSAPs on Notch processing, we chose to work with HEK-Notch Δ E cells, which express an immediate substrate for γ -secretase. The production of the cleaved fragment, NICD, was analyzed by Western blotting. NICD was detected by an antibody specific for cleaved Notch1 and the intensity of the NICD band was quantified by using a CCD camera. The relative levels of NICD were normalized by cell viability. We chose siRNA directed to PS1 as a positive control of experiment. Treatment with siRNA directed to PS1 decreased NICD levels in a dose-dependent manner. Knockdown of SORT1, STX12, and COX4I1 did not change the NICD levels. Knockdown of SYP, CNTN1, or CNTNAP1 affected Notch cleavage to a similar degree as PS1 did, around 55%. VDAC1 reduced NICD production to a lower degree, 38% at the highest dose (6 pmol) of siRNA. Especially for VDAC1 and CNTNAP1, the effect on A β (52–60% and 69–78% reduction, respectively) was higher than the effect on Notch processing (26 and 41%, respectively). We concluded that VDAC1 and CNTNAP1 affect A β and NICD levels differently.

Finally, we investigated the effect of overexpression of VDAC1 on A β production. HEK-APP cells were transiently transfected with VDAC1-FLAG or empty vector. Although the expression of VDAC1 was confirmed by Western blot analysis, the A β levels did not differ from mock-transfected control. The lack of effect upon overexpression of proteins has been reported previously, for instance for TMP21, which upon silencing reduced A β levels (Chen, Hasegawa et al. 2006). Possibly the endogenous levels could be sufficiently high for maximal interaction with γ -secretase.

Voltage-dependent anion channel protein is a major component of the outer mitochondrial membrane where it regulates fluxes of ions and metabolites. To find this protein to be a GSAP would be unexpected. However, γ -secretase is found also in mitochondria, and accumulation of A β in the mitochondria is

common in AD (Hansson, Frykman et al. 2004; Pavlov, Hansson Petersen et al. 2009). There are several reports indicating that VDAC could be involved in AD. For instance, VDAC accumulates in amyloid plaques in APP/PS1 transgenic mice (Ferrer 2009), and nitrated VDAC1 has been shown to be increased in AD (Sultana, Poon et al. 2006). Furthermore, as shown by immunoprecipitation, the plasma membrane VDAC/caveolin-1 (a marker protein for a lipid raft structure called caveolae) complex is highly expressed in AD human brains compared with healthy controls (Ramirez, Gonzalez et al. 2009).

CNTNAP1 is a transmembrane protein that forms a *cis*-interaction with the glycosylphosphatidylinositol-anchored protein, contactin (Peles, Nativ et al. 1997). These proteins form a complex in the paranodal regions, where glial cells form junctions with axons in myelinated fibers (including the nodes of Ranvier) (Rios, Melendez-Vasquez et al. 2000). CNTNAP1 regulates the transport and processing of contactin (Gollan, Salomon et al. 2003), a protein previously shown to interact with APP and possibly Notch (Bai, Markham et al. 2008; Perreau, Orchard et al.). The CSF and plasma levels of CNTNAP1 are altered in AD individuals, which could indicate potentially association of CNTNAP1 to AD.

In conclusion, we have identified a number of possible GSAP candidates in DRMs by using the biotinylated γ -secretase inhibitor, GCB, for affinity purification followed by LC-MS/MS analysis. VDAC1 and CNTNAP1 associate with γ -secretase in DRM and affect APP processing. These proteins had a less pronounced effect on Notch processing. We suggest that these proteins could be the drug targets for A β lowering therapy in AD.

Identification and investigation of novel GSAPs in synaptic membranes and synaptic vesicles (Paper III)

A loss or alteration of synapse structures has been reported in AD. Such alteration could be responsible for cognitive deficits, and A β may disrupt synaptic signaling. A previous study showed that γ -secretase activity is enriched in crude synaptic membrane and synaptic vesicle fractions (Frykman, Hur et al. 2010). This result suggests that the synaptotoxic A β can be produced locally in synapses. We hypothesized that potential GSAPs could interact with γ -secretase in synapses and affect γ -secretase activity and substrate selectivity. GSAPs at the synapses could be putative drug targets for AD. In Paper III, we identified and investigated novel GSAPs from synaptic membranes and synaptic vesicles prepared from rat brain.

Synaptic membranes (LP1), as well as synaptic vesicles (SV), were prepared from fresh rat brain, since frozen postmortem human brain is difficult to apply to synaptic preparation. In brief, rat brain was homogenized and centrifuged, the resulting pellet resuspended and layered on a sucrose gradient in order to purify synaptosomes. The synaptosomes were lysed and the resulting lysate was centrifuged at 48,250 x g to obtain the pellet (LP1). The supernatant was further centrifuged at 100,000 x g for 2 h to obtain the pellet (SV).

Our GCB method, developed in Paper I, was applied to isolate the γ -secretase complex from synaptic membranes (LP1) and from synaptic vesicles (SV), since using the method γ -secretase in synapses was being solubilized by the

detergent. In brief, LP1 and SV fractions were solubilized in the detergent CHAPSO and incubated with GCB in the presence or absence of competing inhibitor. Native γ -secretase complexes in synapses that interact with GCB were captured by SA beads. Then captured γ -secretase complex was eluted by a reducing reagent (DTT) from the SA beads. To assess the efficacy and the selectivity of pull-down, the purified samples were analyzed by Western blotting for detection of known γ -secretase components. PS1-CTF as well as Nct was successfully pulled-down in LP1 and SV. The signal was fully competed in samples that were pre-incubated with L685,458. Thus, we confirmed that this method applied on synapses is specific.

The purified proteins were digested with trypsin supplement with RapiGest, which enhances solubility of membrane proteins during the digestion, and subjected to analysis by LC-MS/MS. Proteins were identified by Spectrum Mill using peptide fragment data and a protein data base (SwissProt). Samples and negative controls (competed samples) from three different preparations of LP1 as well as four different preparations of SV were analyzed. PS1 and Nct were identified with high scores (41 and 34, respectively) in the LP1, and PS1 was identified with high score (37) in one of the SV samples. In addition, Aph1b was identified in one of the SM samples with significant score (12). No known γ -secretase components were identified in the samples competed with L685,458. In total, 344 proteins were identified in LP1 and 244 proteins were identified in SV.

To select novel GSAPs, the criteria were set to the unique identification of the candidate protein in the sample without inclusion of competing inhibitor in at least two out of three experiments for LP1, or in at least two out of four experiments for SV. We also used the spectra mean intensity ratio to select additional candidates. Based on this method, we added one more candidate. In total, two novel potential GSAPs from LP1 and three novel potential GSAPs from SV were selected. One of these GSAPs, contactin-1, was also identified as a GSAP in a DRM fraction described in Paper II. This finding strengthens the identity of contactin-1 as a GSAP. Other GSAPs we identified from synapses were not found in DRM preparations, indicating that these proteins are synapse-specific GSAPs.

To evaluate the effect of the selected GSAPs on A β production and Notch processing, we planned siRNA knockdown experiments on primary neurons, since primary neurons are the ideal model to evaluate the effect of synapse-specific GSAPs. But the transfection efficacy using transfection reagents was very low on primary neurons. Thus, we decided to use HEK293 cells for knockdown experiments, since knockdown efficacy on HEK293 cells are high and the knockdown condition was optimized in Paper II. We confirmed expression of synaptic GSAPs in HEK293 cells using RT-PCR because HEK293 cells are not derived from a neuron cell line.

To evaluate the effect of GSAPs on A β production, we chose to work with HEK293 cells overexpressing human APP (HEK-APP) (Nilsson, Malkiewicz et al. 2006). We chose siRNA directed to PS1 as a positive control of experiment. Treatment with siRNA directed to PS1 reduced A β secretion in a dose-dependent manner. The knockdown efficiency of each gene at the mRNA level was measured by RT-PCR, and cell viability was measured by Alamar Blue

assay. The siRNAs that gave more than 60% knockdown of gene expression, and showed more than 70% cell viability, were selected for further study. The knockdown of tubulin polymerization-promoting protein (TPPP) significantly increased A β 40 and A β 42 levels. In contrast to TPPP, The knockdown of NADH dehydrogenase [ubiquinone] iron–sulfur protein 7 (NDUFS7) significantly decreased A β 40 and A β 42 levels. We also investigated the effect of overexpression of TPPP on A β production. HEK-APP cells were transiently transfected with pCMV6-VDAC1 or empty vector. However, the A β levels did not differ from mock-transfected control. The levels of TPPP might already be high enough for its maximum effect.

To evaluate the effect of GSAPs on Notch processing, we chose to work with HEK-Notch Δ E cells, which express an immediate substrate for γ -secretase. The production of the cleaved fragment NICD was analyzed by Western blotting. NICD was detected by an antibody specific for cleaved Notch1 and the intensity of the NICD band was quantified by using a CCD camera. The relative levels of NICD were normalized by cell viability. We chose siRNA directed to PS1 as a positive control of the experiment. Treatment with siRNA directed to PS1 decreased NICD levels in a dose-dependent manner. siRNAs against TPPP or NDUFS7 were transfected into HEK-Notch Δ E. We found no significant effect on NICD production for siRNAs directed to TPPP or NDUFS7. Thus, knockdown of TPPP and NDUFS7 have differential effects on APP and Notch processing.

Next, we conducted further biochemical confirmation that γ -secretase interacted with TPPP or NDUFS7 using two different methods, co-immunoprecipitation and Proximity Ligation Assay (PLA).

Immunoprecipitation using antibodies directed to the TPPP or NDUFS7 was performed in SV or LP1 from rat brain. We confirmed that core γ -secretase components, Nct and PS1-CTF, co-immunoprecipitated with NDUFS7 and TPPP. We also tested the interactions in human brain microsomal fractions. The interactions between TPPP and NDUFS7 with γ -secretase were also confirmed by co-immunoprecipitation. These results showed that a small proportion (around 1%) of the γ -secretase components PS1 and Nct were co-precipitated with TPPP and NDUFS7. Thus, all of the γ -secretase complexes do not interact with these GSAPs. One possibility is that the TPPP and NDUFS7 are transiently associated with γ -secretase and regulate its activity.

PLA is an in situ method to directly detect protein-protein interactions with high specificity and sensitivity. In brief, two primary antibodies for different proteins recognize the target antigen of interest. Secondary antibodies, which are labeled with DNA probes, bind to the primary antibodies. If the DNA probes are in close proximity, the DNA strands can interact and be ligated by enzymatic reaction. Ligated DNA is amplified using a polymerase reaction. The amplified products are detected by fluorescently labeled oligonucleotide probes with high sensitivity by microscope (Soderberg, Leuchowius et al. 2008). We performed PLA in primary murine hippocampal neurons using antibodies directed to TPPP or NDUFS7, and the γ -secretase component PS1. Addition of both PS1 and TPPP, and PS1 and NDUFS7 antibodies showed a high number of signals. We confirmed that PS1, indeed, interacts with these two proteins in primary murine hippocampal neurons.

To investigate the subcellular localization of γ -secretase, TPPP and NDUFS7 and their co-localization, we performed immunocytochemistry on hippocampal neurons using PS1 and TPPP, or PS1 and NDUFS7 antibodies. The merged pictures showed partial co-localization of PS1 and TPPP or NDUFS7. These data indicate some portion of TPPP and NDUFS7 could be associated with γ -secretase in hippocampal neurons.

The relationship between GSAPs and AD pathogenesis is important qualification for the drug target strategy. We performed immunohistochemical analysis of sections of hippocampus and frontal cortex from 3 AD and 3 control cases. In AD hippocampus, but not in control, we detected strong TPPP staining of inclusions and fiber structures, particularly in the multipolar neurons of cornu ammonis. In the frontal cortex, the neuronal TPPP staining was weak. Glial staining was intense in both the grey and white matter. No major differences in the TPPP staining of oligodendrocytes or astrocytes were observed between AD and control cases. In contrast to TPPP, the most intense staining of NDUFS7 was observed in pyramidal neurons, both in hippocampal CA regions and in layer III of the frontal cortex. No major differences were observed in NDUFS7 staining between AD and control cases. We observed different staining of TPPP in hippocampus between AD and control cases, which suggests to us that TPPP might be involved in AD pathogenesis.

Treatment of the cells with TPPP siRNA significantly increased A β levels. This result suggests that TPPP might inhibit APP processing. TPPP is an unstructured protein that is predominantly expressed in oligodendrocytes where it promotes tubulin polymerization and oligodendrocyte differentiation (Ovadi and Orosz 2009). The function of TPPP resembles that of tau, a microtubule stabilizing protein that forms tangles in AD brain; and, like Tau, phosphorylation of TPPP inhibits its function (Hlavanda, Klement et al. 2007). Consistent with our detection of TPPP in SV, TPPP has previously been found in pure SV preparations (Takamori, Holt et al. 2006). TPPP is involved in actin and/or tubulin polymerization. Cytoskeleton reformation is important for neurite outgrowth and synaptic plasticity and is impaired in AD. How γ -secretase relates to this impairment remains to be determined, but it is possible that the potential GSAPs affect the trafficking of γ -secretase along the axons. In this study, we have only studied the effect of GSAPs on APP and Notch processing, but it is also possible that γ -secretase affects the function of the GSAPs.

In contrast to TPPP, siRNA directed to NDUFS7 resulted in a decrease in A β levels. NDUFS7 is a core component of complex I of the mitochondrial oxidative phosphorylation system. NDUFS7 could be purified from synaptic mitochondria. In addition, we have previously detected γ -secretase in mitochondria (Hansson, Frykman et al. 2004) and, thus, it is possible that mitochondrial γ -secretase interacts with NDUFS7. Another possibility is that NDUFS7 has an alternative subcellular localization at the synaptic membrane. Indeed, several proteins that previously were believed to be present only in the mitochondria, including VDAC and Hsp60, have recently been detected at the plasma membrane (Cicconi, Delpino et al. 2004; Lawen, Ly et al. 2005). Furthermore, a majority of the genes involved in oxidative phosphorylation, including NDUFS7, were found to be downregulated in brain regions affected by AD (Liang, Reiman et al. 2008). Since our siRNA data show that decreased NDUFS7 expression results in

decreased A β levels, one could speculate that the high expression of NDUFS7 in the pyramidal neurons in hippocampus and cortical layer III would result in high A β levels in these neurons. This could help explain why these neurons are particularly vulnerable to cell death in AD (Braak and Braak 1991; Troncoso, Sukhov et al. 1996).

In conclusion, TPPP and NDUFS7 are associated with γ -secretase in synapses and they alter A β levels without affecting Notch cleavage. Interaction of TPPP and NDUFS7 with γ -secretase was verified by using co-immunoprecipitation and a Proximity Ligation Assay. We suggest that these proteins could be attractive therapeutic drug targets for lowering A β in AD.

Identification and investigation of novel GSAPs in microsomes (Paper IV)

We have identified novel GSAPs from several subcellular fractions in brain in Papers II and III. In those experiments, the amount of starting material was limited, since preparation protocols of DRMs, synaptic vesicles and synaptic membranes require density gradient centrifugation. In Paper IV, we conduct a large scale affinity purification of the γ -secretase complex from microsomal fractions in human and rat brain. The protocol for preparation of microsomal fractions is rather simple in comparison that used for DRM, synaptic vesicle and synaptic membrane preparations, and it is possible to increase the quantity of starting materials. We used LC-MS/MS to identify novel GSAPs, and siRNA-mediated gene knockdown to study their affects on A β production and Notch processing.

We employed the purification and protein identification method as established in Paper 1. We used 40 g of human or rat brain as a starting material for large scale experiment. In total, 174,000 MS/MS spectra were collected from four pulldown samples from human brain (two with and two without competing inhibitor), and 104,000 MS/MS spectra were collected from four pulldown samples from rat brain (two with and two without competing inhibitor). MS/MS spectra were analyzed and compared by using the Spectrum Mill software and the SwissProt human or rodent database. We identified 766 proteins from human brain samples, and 31 proteins were uniquely identified in non-competed (-L-685,458) samples. We also identified 760 proteins from rat brain samples, and 38 of these were uniquely identified in non-competed (-L-685,458) samples. PS1 and Nct were identified with high (more than 20) scores in human and rat brain microsomes. Pen2 and Aph-1B were identified with low but significant scores (14.5 and 12.5 respectively) from the human brain microsomal fraction. No known γ -secretase components were identified in the samples that competed with L685,458. Furthermore, we performed a semi-quantitative analysis. We used the spectra mean intensity ratio to select additional candidates. The intensity ratio was calculated by dividing the spectra mean intensity of the specific binding proteins (found in the absence of a competing inhibitor) by the nonspecific binding proteins (found in the presence of a competing inhibitor). Since the brain is the most important organ for AD pathology, we selected proteins which are highly expressed in brain. The tissue expression profiles of each candidate corresponding with each different organ were checked against

the public database SwissProt. In total, we selected 21 proteins for further functional analysis.

To investigate the effect of the GSAPs on A β production, we knocked down their expression by siRNAs. For each of the 21 candidates, two siRNAs were synthesized and transfected into HEK-APP cells. The siRNAs that gave more than 70% knockdown of gene expression, and showed more than 70% cell viability, were evaluated with respect to their effect on A β 40 and A β 42 production as quantified by a sandwich ELISA. All data were normalized to cell viability and the A β levels were compared to the levels in cells treated with Lipofectamine. siRNA directed to PS1 was used as a positive control. The positive control reduced A β 40 and A β 42 production in a dose-dependent manner, as expected. Many of the siRNAs reduced the A β 40 and A β 42 production, though to a lesser degree than did siRNAs directed to PS1. Phosphatidylinositol phosphatase SAC1 and Band 4.1-like protein 3 increased A β production. These results suggest that they could associate to γ -secretase and suppress A β production. The ratio of A β 40/ A β 42 ratio was unchanged. One intention of this study was to identify GSAPs as potential drug targets, and since it generally is easier to break an interaction than to induce one, we focused on the siRNAs that reduced A β production. Hence, we selected Probable phospholipid-transporting ATPase IIA (ATP9A), BDNF/NT-3 growth factors receptor precursor (NTRK2), Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2), DnaJ homolog subfamily A member 2 (DNAJA2) and Proton myo-inositol cotransporter (SLC2A13) for further studies.

There are the potential risks with such a siRNA experiment, e.g. off target effects. To minimize the risk of false positives, we synthesized additional siRNAs and performed the knockdown experiments at four different concentrations of siRNA. Since the reduction of the A β 42 and A β 40 levels were similar in the previous experiments, we decided to evaluate only A β 42 in the experiments described below. The positive controls, siRNA directed to PS1 and Nct, showed a dose-dependent decrease of A β production. Silencing of ATP9A showed minor reduction in A β levels (less than 20%). Knockdown of NTRK2, HCN2, DNAJA2 and SLC2A13 decreased the A β levels 30-80% in a siRNA dose-dependent manner.

We also tested the effect of the GSAPs on Notch cleavage. siRNAs against the GSAPs were transfected into HEK-Notch Δ E cells. The production of the NICD was analyzed by Western blotting. The signals were normalized to the signal of total Notch Δ E level. The positive controls, siRNA directed to PS1 or Nct, showed a dose-dependent decrease of Notch Δ E production, as expected. Silencing of ATP9A showed minor reduction in Notch Δ E production (less than 20%). Upon silencing of NTRK2 or DNAJA2, the NICD production was, similar to the A β production, decreased by 30-80% in a siRNA dose-dependent manner. Thus, these GSAPs seem to affect both APP and Notch processing in a similar manner. In contrast, when silencing the expression of HCN2 or SLC2A13, NICD production was not affected, suggesting that these GSAPs have a selective effect on APP processing. One possible mechanism is that HCN2 and SLC2A13 bind to the sites of substrate recognition, but further studies are needed to clarify the mechanism of substrate selection. Especially for SLC2A13, the effect of the three different siRNAs on A β (38%, 47% and 60% reduction, respectively) was

greater than their effect on Notch processing (89%, 101% and 105%, respectively).

Since one siRNA for HCN2, and three siRNAs for SLC2A13, affected APP processing but not Notch processing, we further examined the effect of overexpression of HCN2 and SLC2A13 on A β production in HepG2 cells. HepG2 is a perpetual cell line, derived from liver tissue, which shows a low expression of HCN2 and SLC2A13. HepG2 cells were co-infected with BacMam-C99 and BacMam-GSAP. Secreted A β 40 in the culture supernatants was measured by homogeneous time-resolved fluorescence assay kit. Interestingly, overexpression of SLC2A13 increased A β 40 generation, while overexpression of HCN2 did not affect A β 40 generation. This result indicates that SLC2A13 enhances A β production by associating with γ -secretase. On the other hand, overexpression of HCN2 had no effect on A β production. Possibly, the endogenous expression of HCN2 in Hep-G2 cells is sufficient for mediating its effect on γ -secretase.

Since treatment by siRNA directed to ATP9A, NTRK2, HCN2 and SLC2A13 clearly influenced the A β levels, we verified the interaction between these GSAPs and γ -secretase by immunoprecipitation by using antibodies directed to selected proteins. Western blot analysis confirmed that the core γ -secretase components, Nct and PS1-CTF, co-immunoprecipitated with ATP9A, HCN2 or SLC2A13; and PS1-CTF co-immunoprecipitated with NTRK2. We confirmed that four proteins that affect A β production, indeed, associate with γ -secretase by co-immunoprecipitation. These result showed that a small proportion (around 1%) of the γ -secretase complexes were co-precipitated with the selected GSAPs. Thus, all of the γ -secretase complexes do not interact with these GSAPs. One possibility is that these GSAPs are transiently associated with γ -secretase and regulate its activity. We have identified around 70 potential GSAPs which were uniquely identified in non-competing samples by large scale LC-MS/MS experiment. Some of the uniquely identified proteins might be false positive, but all of the selected proteins (ATP9A, HCN2, SLC2A13 and NTRK2) showed association with γ -secretase by co-immunoprecipitation. Thus, we suggest that the number of false positive GSAPs is low.

HCN2 is reported as a hyperpolarization-activated ion channel exhibiting weak selectivity for potassium over sodium ions (Biel, Wahl-Schott et al. 2009). HCN2 is reported to directly interact with X11 β via its MI domain. X11s is the adaptor protein and mainly composed of three domains; MI (Munc-18 interacting region), PTB (phosphotyrosine binding domain) and PDZ (PSD-95/discs large/ZO-1 domain)(Kimura, Kitano et al. 2004). X11 β and X11 α interact also with PS1 via their PDZ Domains (Lau, McLoughlin et al. 2000). Here we show that the core γ -secretase components Nct and PS1-CTF can be co-immunoprecipitated with HCN2. It is possible that HCN2 associates with active γ -secretase through X11s. On the other hand, the PBT domain of X11s binds to intracellular domain of APP (Miller, McLoughlin et al. 2006). X11 α impairs gamma- but not beta-cleavage of APP (King, Cherian et al. 2004). Association of HCN2 to X11s might regulate substrate selectivity of γ -secretase.

There are no previous reports that suggest association between SLC2A13 and γ -secretase. SLC2A13 is a multi-transmembrane protein predominantly expressed in the brain, which facilitates uptake of myo-inositol into neurons and

astrocytes (Uldry, Ibberson et al. 2001). Myo-inositol is the precursor of phosphatidylinositol, the derivatives of which function as second messengers, regulating important processes such as synaptic exo- and endocytosis, polymerization of actin filaments and regulation of ion channels. The intracellular inositol levels are, therefore, of great importance. Interestingly, Down's syndrome patients have increased inositol levels in the brain. Furthermore, SLC2A13 can also transport scyllo-inositol, a stereoisomer of inositol that inhibits aggregation of A β , and which currently is in clinical trials as a potential therapeutic agent for AD. In this study we examined the effect of GSAPs on APP and Notch processing. However, it is also possible that γ -secretase affects the function of the GSAPs. One could speculate that γ -secretase associates with SLC2A13 and affects intracellular inositol levels, and thereby indirectly regulate synaptic functions.

In summary SLC2A13 and HCN were identified as novel GSAPs that are highly expressed in the brain, and which regulate A β production without affecting Notch cleavage; and, thus, could be attractive drug targets for A β lowering therapy in AD.

In Papers II, III, IV, we have identified 6 GSAPs VDAC1, CNTNAP1, TPPP, NDUFS7, SLC2A13 and HCN2, which associate with γ -secretase and affect APP processing. These proteins had a less pronounced effect on Notch processing.

Since inhibition of Notch signaling has been shown to cause side effects in clinical trials with γ -secretase inhibitors, we suggest that inhibiting their interactions with γ -secretase could be a way to specifically decrease A β levels in AD.

5, Conclusions

One therapeutic strategy for the treatment of Alzheimer disease is to reduce γ -secretase-mediated A β -production. However, γ -secretase inhibitors that reduce A β production cause side effects associated with reduced Notch cleavage. Therefore, more finely targeted inhibition of A β -production is needed.

In the studies included in this thesis, the goal was to identify and characterize novel GSAPs that selectively affect APP cleavage in brain. For identification of GSAP, we used different membrane preparations: microsomal membranes, DRMs, synaptic vesicles and synaptic membranes. Since the brain is the most important organ for AD pathology, we investigated potential GSAPs that are highly expressed in brain.

The major findings of Papers I-IV are summarized below

- 1, We established an affinity purification method using a γ -secretase inhibitor based probe to isolate the native complex from brain material, and tandem mass spectrometry to identify novel GSAPs from brain.
- 2, Reported GSAP, TMP21 and the PS-associated protein, syntaxin1 interact with detergent-soluble as well as DRM-associated active γ -secretase in brain. Interaction of TMP21 and syntaxin1 with γ -secretase was confirmed by Western blotting.
- 3, We identified 27 novel potential GSAPs in DRMs. From these, VDAC1 and CNTNAP1 associated with γ -secretase in DRM and affected APP processing. These proteins had a less pronounced effect on Notch processing.
- 4, We identified two novel, potential GSAPs from synaptic membranes and three novel, potential GSAPs from synaptic vesicle. From these, TPPP and NDUFS7 associated with γ -secretases in synapses and altered the A β levels without affecting Notch cleavage. Interaction of TPPP and NDUFS7 with γ -secretase was verified by using co-immunoprecipitation and a Proximity Ligation Assay.
- 5, We identified around 70 potential GSAPs from microsomal fractions by large scale experiment. From these, SLC2A13 and HCN2 were identified as novel GSAPs that regulate A β production without affecting Notch cleavage. Overexpression of SLC2A13 increased A β generation. Interaction of SLC2A13 and HCN2 with γ -secretase was verified by using co-immunoprecipitation.

We suggest that these proteins could be attractive AD therapeutic drug targets aimed at lowering A β .

6, Future perspectives

Interfering with GSAPs that specifically regulate A β generation is a strategy for the treatment of AD that could lead to disease modifying drugs, yet which are free from side effects. In this thesis, we have identified novel GSAPs that regulate A β production without affecting Notch cleavage. Further characterization will be required for these GSAPs.

- 1, Since we evaluate the effect of GSAPs on A β production by overexpression and knockdown using cell lines, a next step is to know their function in cultured neuron or in vivo. An ideal approach would be to investigate the effect in neurons using knockdown experimentation on primary neurons. For this purpose, shRNA-expressing lentivirus vector will be used for the knockdown experiment since transduction of lentivirus vector to primary neuron is highly efficient compared to lipid based transfection of siRNA. Another possibility is that GSAP transgenic and KO mouse will be developed and A β processing will be analyzed using these mouse models.
- 2, The GSAPs will be further evaluated with respect to their subcellular localization. In Paper III, we showed the subcellular localization of γ -secretase, TPPP and NDUFS7 and their co-localization using immunocytochemistry on hippocampal neurons. This method will be employed for other GSAPs which were identified in Papers II and IV.
- 3, In Paper III, we showed interaction of TPPP and NDUFS7 with PS1 using PLA in primary murine hippocampal neurons. To verify the interaction of other GSAPs in neuron, PLA method will be employed for other GSAPs that were identified in Papers II and IV.
- 4, These GSAPs will be subjected to detailed studies aimed at elucidating how they interact with γ -secretase, and how this interaction can be hindered. For this purpose, we will identify interaction domains of GSAPs by using truncation studies. The information on interaction domains will help to establish screening system for searching interaction breakers. For example, detection of interaction between interaction domains of GSAPs and γ -secretase using fluorescently labeled peptide could be performed. For this method, several techniques are useful, as shown below.
 - ✓ Proximity Ligation Assay (PLA)
 - ✓ Fluorescence correlation spectroscopy (FCS)
 - ✓ Fluorescence resonance energy transfer (FRET)
 - ✓ Fluorescence lifetime imaging (FLIM)Such further experimentation will be used for the design and construction of an interaction-breaking drug screen.

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