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The Alzheimer's disease related γ-secretase complex: localization and novel interacting proteins

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ABSTRACT

Alzheimer's disease (AD) is caused by synaptic and neuronal loss in the brain that eventually results in cognitive decline. Characteristic hallmarks of AD are senile plaques containing the amyloid β-peptide (Aβ) and neurofibrillary tangles containing hyperphosphorylated tau protein. Aβ is produced from the amyloid precursor protein (APP) by sequential proteolytic cleavages by β-secretase and γ-secretase, and the polymerization of Aβ into amyloid plaques is thought to be the main pathogenic event in AD. Since γ-secretase mediates the final cleavage that liberates Aβ from APP, γ-secretase has been widely studied as a potential drug target for the treatment of AD. γ-Secretase is a transmembrane protease complex containing presenilin, nicastrin, Aph-1, and Pen-2, which are sufficient for γ-secretase activity. γ-Secretase has more than 60 substrates including APP and Notch. Inhibitors of γ-secretase caused side effects in clinical trials, probably due to the involvement of γ-secretase in Notch signaling. Therefore, more specific regulation or modulation of γ-secretase activity is needed. In the last years, γ-secretase associated proteins (GSAPs) such as CD147, TMP21, and proteins in the tetraspanin web family have been reported to regulate Aβ production. In this thesis, we have characterized γ-secretase in different subcellular localizations, and identified and characterized novel GSAPs in mammalian brain.

Studies in cell lines have shown that γ-secretase is partially localized to lipid rafts, microdomains enriched in cholesterol and sphingolipids, which can be prepared biochemically as detergent resistant membranes (DRMs). In **Paper I**, we show that DRMs from human brain were enriched in γ-secretase. γ-Secretase activity was also high, as accessed by Aβ and AICD production levels. The DRM fraction was subjected to size exclusion chromatography, and all of the γ-secretase components and a lipid raft marker were found in the void volume (> 2000 kDa). The size of the DRMs indicates that they contain other proteins and lipids. In **Paper II**, the distribution of active γ-secretase in different subcellular compartments in brain was investigated. Previously, active γ-secretase has been localized to the Golgi apparatus, endosomes, and plasma membranes in cell studies. Here, we showed that highly active γ-secretase was present in endosomes, synaptic vesicles, and synaptic/plasma membranes in rat brain. The localization of active γ-secretase in synapses and endosomes was also confirmed by fluorescent labeling with an active site inhibitor using confocal microscopy. In **Paper III**, we developed an efficient way to purify γ-secretase and GSAPs. Microsomal membranes from brain were incubated with a γ-secretase inhibitor with cleavable biotin group (GCB). After affinity purification, bound proteins were subjected to LC-MS/MS analysis. All of the known γ-secretase components were identified, and TMP21 and the PS associated protein syntaxin1 were identified as GSAPs. In total, over 90 potential GSAPs were identified. In **Paper IV**, we used GCB affinity purification and identified novel GSAPs in DRMs. By siRNA mediated gene knockdown that a subset of the GSAPs affected Aβ production; voltage-dependent anion channel 1, syntaxin12, and cytochrome C oxidase subunit IV isoform 1.

In summary, we conclude that the active γ-secretase complex is localized to lipid rafts, endosomes, plasma membranes, and at synapses. We identified novel GSAPs in

human brain and in DRMs purified from rat brain. We suggest that the interactions between these proteins and γ-secretase could be potential drug targets to modulate Aβ production in AD.

LIST OF PUBLICATIONS

I. **Hur JY**, Welander H, Behbahani H, Aoki M, Frånberg J, Winblad B, Frykman S and Tjernberg LO.
Active γ-secretase is localized to detergent-resistant membranes in human brain.
The FEBS Journal (2008) 275, 1174-1187.

II. Frykman S, **Hur JY**, Frånberg J, Aoki M, Winblad B, Nahalkova J, Behbahani H and Tjernberg LO.
Synaptic and endosomal localization of active γ-secretase in rat brain.
PLoS One (2010) 5(1):e8948.

III. Teranishi Y, **Hur JY**, Welander H, Frånberg J, Aoki M, Winblad B, Frykman S and Tjernberg LO.
Affinity pulldown of γ-secretase and associated proteins from human and rat brain.
Journal of Cellular and Molecular Medicine (2009) Epub
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IV. **Hur JY**, Teranishi Y, Kihara T, Goto N, Hashimoto M, Hosia W, Winblad B, Frykman S and Tjernberg LO.
Identification of novel γ-secretase associated proteins in detergent resistant membranes from brain.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AICD	APP intracellular domain
Aph-1	Anterior pharynx defective-1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid β-peptide
BACE	β-site APP cleaving enzyme
CTF	C-terminal fragment
DRMs	Detergent resistant membranes
ELISA	Enzyme-linked immunosorbent assay
EndoH	Endo-β-*N*-acetylglucosaminidase
ER	Endoplasmic reticulum
FAD	Familial AD
GCB	γ-Secretase inhibitor with cleavable biotin group
GSAPs	γ-Secretase associated proteins
I-CliPs	Intramembrane-cleaving proteases
KO	Knock-out
LC-MS/MS	Liquid chromatography coupled online to tandem mass spectrometry
NTF	N-terminal fragment
Pen-2	Presenilin enhancer-2
PNGase F	Peptide-*N*-glycosidase F
PS	Presenilin
SAD	Sporadic AD
SCX	Strong cation exchange
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
siRNA	Small interfering RNA
TGN	*trans*-Golgi network
TLC	Thin layer chromatography

1 INTRODUCTION

Why are we doing research on Alzheimer's disease? The answer in common for all is to better understand the mechanisms that cause the Alzheimer's disease. This knowledge could bring us one step closer to possible treatments for Alzheimer's disease in the near future. In this section, what we have learned so far about Alzheimer's disease is introduced.

1.1 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common form of dementia and is caused by the loss of synapses and neurons in the brain (Selkoe, 1999). AD affects 34 million people worldwide and the estimated costs were $422 billion US dollars for 2009 (Wimo et al., 2010). The main risk factor for developing AD is age and the risk is doubled every 5 years after 65 years of age (Querfurth and LaFerla, 2010). The first clinical report on an AD patient was described by Dr. Alois Alzheimer in 1907. According to his report, the 51-year-old patient showed that she was jealous of her husband, developed hallucinations and a rapid loss of memory, and was disoriented in time and space. Autopsy of her brain showed general cortical atrophy and depositions in the cortex (Alzheimer, 1907). These pathological hallmarks of AD are now known as senile plaques, consisting of amyloid β-peptide (Aβ), and neurofibrillary tangles containing the hyperphosphorylated tau protein in neurons (St George-Hyslop, 2000, Perl, 2010). However, plaques and tangles can be found in the brain of the elderly people as a result of normal aging (Alzheimer et al., 1995). The physiological role of Aβ is not well known. It was suggested that Aβ normally has a role in a negative feedback system to prevent neuronal hyperactivity (Kamenetz et al., 2003). Tau is a soluble protein that binds to tubulin and stabilizes microtubules, which are important for the cell structure. Due to hyperphosphorylated tau, microtubules become destabilized and the hyperphophorylated tau self-aggregates, making paired helical filament structures (Querfurth and LaFerla, 2010, St George-Hyslop, 2000). The abnormal accumulation of these proteins in brain leads to the loss of synapses, causes death of neurons and structural changes in the neocortex, the entorhinal area, hippocampus, amygdala, and other regions of brain, and eventually causes symptoms such as memory impairment, cognitive deficits, and behavioural and emotional disturbances (Selkoe, 2001).

1.1.1 Genetics of AD

There are two types of AD, sporadic Alzheimer's disease (SAD) and familial Alzheimer's disease (FAD).

1.1.1.1 Sporadic Alzheimer's disease (SAD)

SAD generally has a late onset (> 65 years of age) and is responsible for over 95% of AD cases. SAD is probably caused by a complex mixture of multiple genetic and environmental factors (Bettens et al., 2010). One major risk factor known for SAD is the ε4 variant of the apolipoprotein E gene (*APOE,* on chromosome 19). APOE is a glycoprotein. The function is to carry lipids and cholesterol, and to maintain lipid and cholesterol homeostasis (Martins et al., 2009). *APOE* has three alleles, *APOE2* (two cysteines at position 112 and at position 158), *APOE3* (cysteine and arginine,

respectively), and *APOE4* (arginine in both positions) (Mahley, 1988). The *APOE ε3* allele is the most common allele and it occurs in 40-90% of the population. The *APOE ε2* allele is known to have a protective effect against AD. On the other hand, the *APOE ε4* allele is a risk factor for AD (St George-Hyslop, 2000). Other possible susceptibility genes are also found; the clusterin gene (*CLU*, aka apolipoprotein J gene, *APOJ)*, *CR1* (the receptor gene for the complement C3b protein), *PICALM1* (encoding for the phosphatidylinositol-binding clathrin assembly protein), *SORL1* (the sortilin-related receptor), and others (www.alzgene.org).

1.1.1.2 Familial Alzheimer's disease (FAD)

FAD is genetically inherited in an autosomal dominant fashion and has an early onset (< 65 years of age) of the disease (Bettens et al., 2010). Even though FAD is only responsible for 1-2% of AD cases (Saunders, 2001), studies on FAD can provide insight into AD. There are mutations in three known genes causing FAD: the amyloid precursor protein gene (*APP*, on chromosome 21), the presenilin1 gene (*PSEN1*, on chromosome 14), and the presenilin2 gene (*PSEN2*, on chromosome 1). Currently, 23 missense mutations have been reported in *APP* (of < 0.1% of AD patients), 178 mutations in *PSEN1*, and 14 mutations in *PSEN2* (AD Mutation Database, http://www.molgen.ua.ac.be/ADMutation). An increased dosage of *APP* also enhances the severity of AD. Duplicated APP is linked to early onset AD with severe cerebral amyloid angiopathy (Bettens et al., 2010). Down syndrome patients with trisomy 21 (three copies of *APP*) show features of AD over the age of 35 (Tyrrell et al., 2001) and Aβ was found in the plaque core of aged Down syndrome patients (Masters et al., 1985). In most of the cases, *APP* mutations increase the ratio of Aβ42/Aβ40 or total Aβ production. The mutations in *PSEN* are missense mutations, insertions, or deletions, and they are located in the transmembrane regions or in the hydrophilic loops in the cytosol. *PSEN* mutations result in increased ratio of Aβ42/Aβ40 (Bettens et al., 2010).

1.1.1.3 Risk or protective factors for AD

There have been many studies on possible risk or protective factors for SAD apart from APOE. However, these factors are probably dependent on other genetic and environmental factors. Possible risk factors such as strong association factors (stroke, high blood pressure in mid-life, obesity), moderate factors (depression, diabetes, excessive alcohol consumption, high cholesterol levels in mid-life), and mild factors (chronic stress, head trauma, low vitamin B_{12} levels, smoking) have been reported. Possible protective factors for AD include strong association factors (education, physical activity such as walking), moderate factors (leisure activities), and mild factors (moderate alcohol consumption, challenging occupation, eating fish (omega-3), eating fruits/vegetables (anti-oxidants)). Since the majority of AD is not caused by the genetic mutations, these risk or protective factors should be considered for the development of drugs for curing AD (Fotuhi et al., 2009).

1.1.2 Amyloid cascade hypothesis

According to the amyloid cascade hypothesis, which is currently dominating in the AD field, the accumulation of Aβ in the brain is the primary cause of AD. Chronic imbalance between production and clearance rate of Aβ may lead to increased Aβ42 levels, followed by Aβ oligomerization, fibril formation and accumulation in plaques.

Both Aβ oligomers and plaques damage neurons by astrocytic activation, oxidative injury, and altered kinase/phosphatase activities, followed by formation of neurofibrillary tangles. Therefore, therapeutics aimed at lowering Aβ levels could be of clinical use for the treatment of AD (Hardy and Selkoe, 2002). However, there are concerns about the amyloid cascade hypothesis. For example, the number of plaques is not well correlated with the severity of disease (Hardy and Selkoe, 2002). Recent studies have shown that soluble Aβ correlates better with the severity of disease. Microinjection with soluble Aβ into rats caused inhibition of long-term potentiation (LTP) in the hippocampus (Walsh et al., 2002). It was also reported that dimers and trimers of Aβ induced decreased LTP in hippocampal brain slices (Klyubin et al., 2008, Walsh et al., 2005). Another concern is that neurofibrillary tangles were shown much earlier than plaques in the extensive human postmortem brain studies by Braak and Braak (Braak and Braak, 1991). However, Down syndrome patients show amyloid deposition earlier than tangles (Lemere et al., 1996). Another strong evidence for Aβ being an initial trigger of the AD process is that frontotemporal dementia with parkinsonism harboring mutations in tau gene only shows the tangle formations but not the depositions of Aβ. When human APP and human tau transgenic mice were crossed, enhanced tangle formations were shown compared to human tau transgenic mice. Thus, tangle formation can probably be triggered by Aβ (Hardy and Selkoe, 2002) but this is still on debate.

1.1.3 APP processing

The amyloid plaques in the brain of AD patients consist of fibrils formed by Aβ. Aβ is produced from the amyloid precursor protein (APP) by sequential proteolytic cleavages of β-secretase (β-site amyloid precursor protein-cleaving enzyme, BACE) and γ-secretase.

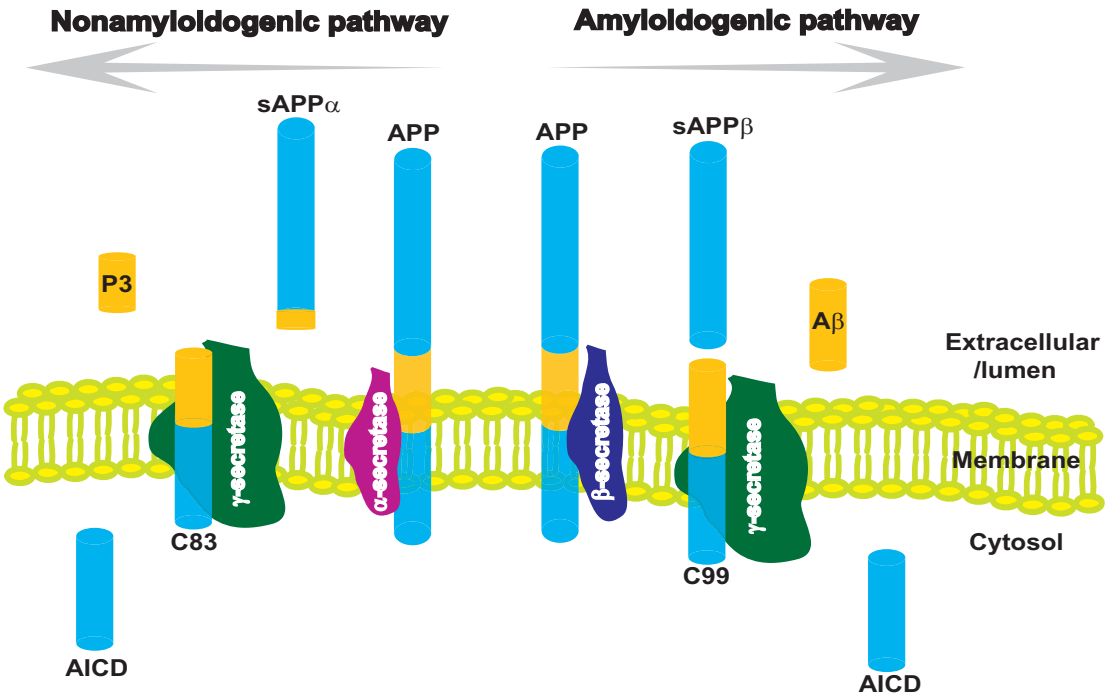

Figure 1. APP processing.

APP and the secretases are transmembrane proteins and the APP processing occurs in the membrane environment. Firstly, BACE cleaves APP extracellularly, and produces soluble APP (sAPPβ) and a membrane bound C-terminal fragment (CTF, C99). C99 is further cleaved in the transmembrane region by γ-secretase resulting in the release of APP intracellular domain (AICD) and Aβ (Selkoe, 2001). This is called amyloidogenic pathway. Two forms of the amyloidogenic peptide are produced, Aβ40 and Aβ42. Aβ42 is more prone to aggregate and more toxic than Aβ40 although Aβ40 is the more abundant one (Iwatsubo et al., 1994, Jarrett et al., 1993, Naslund et al., 2000). In the nonamyloidogenic pathway, APP is cleaved by α-secretase within the Aβ domain, resulting in the release of sAPPα into the extracellular space. Membrane bound C83 is further processed by γ-secretase, and the presumably non-toxic P3 and AICD are produced. P3 is a short hydrophobic protein with the size of 3 kDa and includes Aβ17-40 and Aβ17-42 (De Strooper et al., 2010).

It has been commonly referred as the γ-secretase cleavage to release Aβ40 or Aβ42, and AICD. However, the γ-secretase cleavage site can be separated into γ-, ζ-, and ε-cleavage sites (Xu, 2009). The γ-site ends at Aβ40 or Aβ42 and AICD starts at Aβ49 or Aβ50. This discrepancy with missing amino acid residues led to the new identification of the ε-cleavage site at Aβ49 (Gu et al., 2001, Sastre et al., 2001, Weidemann et al., 2002, Yu et al., 2001). The question whether the γ- and ε-cleavages occur sequentially or independently from each other was answered by another new identification of the ζ-cleavage site at Aβ46 (Qi-Takahara et al., 2005, Zhao et al., 2004). Overall, APP-CTFs formed by the BACE1 cleavage are processed by ε-cleavage resulting in Aβ49 and AICD. Aβ49 is further cleaved at the ζ-site to Aβ46. Aβ46 is mainly processed at Aβ43 by the γ-cleavage and Aβ43 is further processed to Aβ40. Aβ46 can be alternatively cleaved at Aβ42 by the γ-cleavage (Xu, 2009).

1.1.3.1 APP protein

What are the physiological functions of these proteins involved in the APP processing? APP is a type I transmembrane glycoprotein containing a large N-terminal ectodomain, a transmembrane domain of hydrophobic amino acid residues, and a relatively short C-terminal cytoplasmic domain. APP695 is the neuronal isoform and there are other isoforms, APP751 and APP770, expressed in non-neuronal cells (Vetrivel and Thinakaran, 2006). There are two other proteins similar to APP, amyloid precursor-like protein 1 (APLP1) and APLP2 (Wolfe and Guenette, 2007). The APLPs are not involved in AD and no FAD mutations have been found in *APLP*.

APP is highly expressed in fetal tissues (brain, kidney, heart, and spleen), and in the adult (frontal cortex) (www.uniprot.org). APP knock-out (KO) mice are viable, but double (APP/APLP2, APLP1/APLP2) or triple (APP/APLP1/APLP2) KO mice are lethal (Wolfe and Guenette, 2007). Together with KO studies from *C. elegans* and *Drosophila*, these studies suggested that APP is involved in neuronal cell adhesion, cell signaling, protease inhibition, and development (Li et al., 2009). The function of APP was also reported to be a cell-surface receptor (Kang et al., 1987) although its ligand has not been found. Other suggested functions of APP are to stimulate neurite outgrowth (Hung et al., 1992) and to regulate stem cells (Kwak et al., 2006). sAPP proteins released by the cleavage of α-secretase seem to be

neuroprotective/neurotrophic (Turner et al., 2003) and may have a role as a signalling molecule (Gakhar-Koppole et al., 2008). On the other hand, sAPPβ was shown to trigger neuronal death (Nikolaev et al., 2009). Upon a cleavage of APP-CTFs (C89 and C99) by γ-secretase, AICD is released to cytoplasm of cells. AICD forms a complex with FE65 (nuclear adaptor protein) and TIP60 (histone aceyltransferase) and further regulates gene transcription in nucleus (Baek et al., 2002, Cao and Sudhof, 2001).

1.1.3.2 α- and β-Secretases

α-Secretase is a transmembrane protease that belongs to the ADAM (a disintegrin and metalloprotease) family including ADAM9, ADAM10, ADAM17/TACE (tumor necrosis factor-α converting enzyme), ADAM19, MDC-9, and the aspartyl protease BACE2 (Allinson et al., 2003, De Strooper et al., 2010). Several studies indicated that increased α-secretase activity decreases β-secretase cleavage, and in turn lowers Aβ production. However, these findings were not reproducible, and importantly the exact mechanism of which ADAM contributes as α-secretase in APP processing in the human brain is not resolved fully (De Strooper et al., 2010).

β-Secretase (β-site amyloid precursor protein cleaving enzyme, BACE, Asp2, memapsin 2) is a type I transmembrane aspartic protease. BACE was initially reported by five different independent groups using different methods (Hussain et al., 1999, Lin et al., 2000, Sinha et al., 1999, Vassar et al., 1999, Yan et al., 1999). BACE has two isoforms, BACE1 and BACE2, and they share 64% similarity in amino acid sequences. BACE1 is highly expressed in neurons while BACE2 is expressed at low levels in neurons. Therefore, BACE1 is considered to be the real player in APP processing.

BACE1 is a site-specific protease cleaving APP at Asp 1 or Glu 11 (Aβ numbering). One FAD mutation, the Swedish mutation in APP (APPswe) replaces the amino acids KM with NL at positions 670/671, and this enhances the β-secretase cleavage 10 - 100 times. BACE is synthesized in the endoplasmic reticulum (ER) as a zymogen and maturates through post-translational modifications (e.g. glycosylation and acetylation in the ER, the addition of complex carbohydrates and removal of the BACE1 prodomain in Golgi) on its way to the cell surface. BACE activity is highest at low pH, and mature BACE is mainly localized to endosomes and the *trans*-Golgi network (TGN) (Vassar et al., 2009).

BACE seems to play a role in sodium current regulation, synaptic transmission, myelination, and schizophrenia. An initial study showed that BACE1 KO mice were viable and had a normal phenotype. Crossing BACE KO mice with Tg2576 (APPswe transgenic) mice demonstrated that BACE KO indeed reduced Aβ levels (Luo et al., 2001) and rescued memory deficits (Ohno et al., 2004). Therefore, BACE1 was suggested to be a potentially side-effect free therapeutic target for AD. However, in a more recent study, BACE KO mice were found to have hypomyelination in peripheral nerves due to the accumulation of unprocessed neuregulin 1 (Willem et al., 2006).

1.1.3.3 Aβ

The physiological role of Aβ is yet not clear. The length of Aβ found in CSF or brain varies from 38 to 43 amino acids. Aβ42 is more prone to aggregate and more toxic than Aβ40 even though the ratio of production for Aβ42 and Aβ40 is around one to nine.

Aβ42 is the major component of amyloid plaques (Iwatsubo et al., 1994, Roher et al., 1993a, Roher et al., 1993b), and Aβ43 was recently reported to exist in amyloid depositions of human AD brain (Welander et al., 2009). Normally, under physiological conditions, there is a balance between production and degradation of Aβ. Aβ monomers can bind to each other and form oligomers and eventually fibrils (Irvine et al., 2008).

The number of plaques is not well correlated with the severity of AD (Hardy and Selkoe, 2002) although it was shown that dendrites passing through fibrillar Aβ deposits were damaged (Tsai et al., 2004). More recent evidences have pointed out that Aβ oligomers are the toxic species and play an important role in the pathogenesis of AD (Caughey and Lansbury, 2003, Haass and Selkoe, 2007, LaFerla et al., 2007, Klein et al., 2001, Mucke et al., 2000). Another study reported that Aβ dimers are the smallest synaptotoxic species (Shankar et al., 2008). In summary, the amount of soluble Aβ oligomers correlates well with the severity of the disease.

1.1.4 Prevention and treatment for AD

The current treatment for AD is to use acetylcholinesterase inhibitors and *N*-methyl-D-aspartate receptor antagonist, memantine for the symptomatic improvement of AD and quality of life (De Strooper et al., 2010). However, there is still no cure for AD.

Based on the pathology and features of AD, there are many possible treatment strategies. In order to reduce Aβ production, secretases can be targeted either by increasing α-secretase cleavage, or by inhibiting β- or γ-secretase activity. To suppress Aβ oligomerization by polymerization inhibitors or enhance Aβ clearance by Aβ degrading enzymes (insulin degrading enzyme, neprilysin) can be used. Active immunization with Aβ to clear plaques and passive immunization with anti-Aβ antibodies to redistribute Aβ levels from brain to peripheral body (peripheral sink model) are also proposed. However, a phase II study in the clinical trial with active vaccination with aggregated Aβ42 peptide reported a severe side effect, meningoencephalitis in patients and the trial was stopped (Orgogozo et al., 2003). There is a strategy of anti-inflammation such as using non-steroidal anti-inflammatory drugs (NSAIDs) and of cholesterol-lowering drugs such as statins to restore cholesterol homeostasis. At last, the metal chelator could be tried since Aβ aggregation depends on the metal ions (Jakob-Roetne and Jacobsen, 2009).

In this thesis, we focus on the characterization of γ-secretase and to identify novel γ-secretase associated proteins (GSAPs) that affect APP processing. Prior to that, γ-secretase is introduced below.

1.2 γ-SECRETASE

γ-Secretase is a transmembrane protein complex, containing presenilin (PS), nicastrin, anterior pharynx defective-1 (Aph-1), and presenilin enhancer-2 (Pen-2). γ-Secretase belongs to a new class of proteases, intramembrane-cleaving proteases (I-CliPs) (Wolfe and Kopan, 2004) and processes substrates in the lipid bilayer of membranes, which is an unusual cleavage.

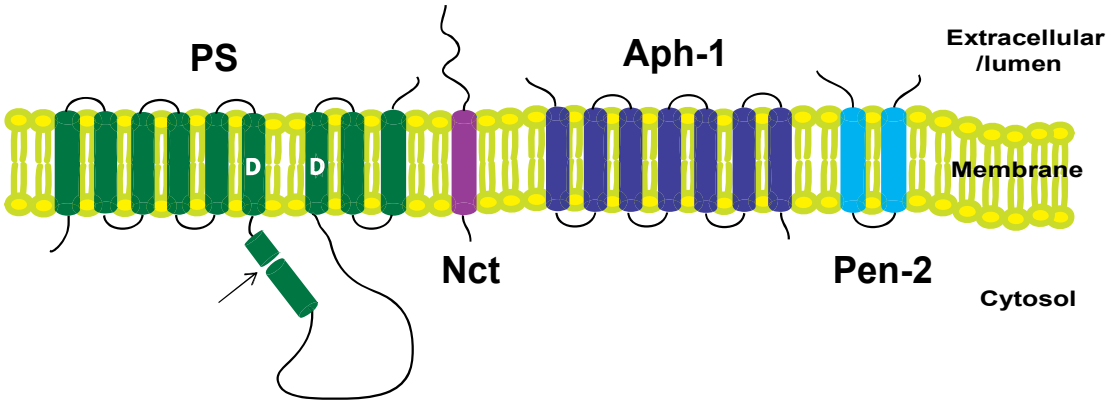

Figure 2. The γ-secretase complex. The two catalytic aspartyl residues in PS1 are indicated by 'D'. PS1 undergoes endoproteolysis and becomes PS1-NTF/PS1-CTF heterodimer (indicated by arrow).

1.2.1 PS, nicastrin, Aph-1, and Pen-2

Compared to BACE, γ-secretase is not strictly site-specific and yields 38 to 43 amino acids long Aβ peptides. There have been evidences to confirm PS as a core of γ-secretase activity. First of all, there are currently 178 known *PSEN*-harboring FAD mutations causing increased Aβ42/40 ratio. Secondly, KO of *PSEN1* decreased γ-secretase cleavage of APP and reduced Aβ production (De Strooper et al., 1998). Thirdly, γ-secretase inhibitors include moieties which can be found in aspartyl protease inhibitors (Wolfe et al., 1999). Altogether, γ-secretase is considered to be an aspartyl protease due to the presence of essential aspartyl residues at positions 257 and 385 within transmembrane domains 6 and 7 of PS (both PS1 and PS2) that are thought to constitute the active site of the protease (Kimberly and Wolfe, 2003, Thinakaran et al., 1996). The mature form of PS is cleaved endoproteolytically between the sixth and the seventh transmembrane domains (between residues N292 and V293) into a N-terminal and a C-terminal fragment (NTF and CTF). The NTF-CTF heterodimer forms the catalytic site of γ-secretase (De Strooper et al., 1998, Herreman et al., 2000, Thinakaran et al., 1996, Zhang et al., 2000). Transition state analogue γ-secretase inhibitors that bind to PS1-NTF and PS1-CTF also supports this notion (Esler et al., 2000, Li et al., 2000).

The topology of PS has been controversial but now the topology with nine transmembrane spanning domains has been accepted (Laudon et al., 2005). PS has two homologues in mammalian, PS1 and PS2 and they share 67% of sequence similarity (Levy-Lahad et al., 1995). The physiological function of PS has been studied in KO mice. PS1 KO mice are lethal, showing Notch signaling deficiency, while the phenotype of PS2 KO mice is normal. Double KO of PS1 and PS2 is embryonic lethal, showing a severe Notch deficiency (Donoviel et al., 1999, Herreman et al., 1999). Apart from its role in γ-secretase complex, PS interacts with many other proteins (Chen and Schubert, 2002), such as syntaxin1 (Smith et al., 2000). In addition, the sarco ER Ca^{2+}-ATPase (SERCA) pumps are regulated by PS (Green et al., 2008).

Whether PS alone works as γ-secretase was initially unclear. Soon the discovery of other partners in γ-secretase was made. Nicastrin was discovered by its association with

PS after immunoaffinity purification using anti-PS antibody (Yu et al., 2000). Two additional cofactors, Aph-1 and Pen-2 were discovered by a genetic screening in *C. elegans* (Francis et al., 2002, Goutte et al., 2002). Nicastrin is a single-pass transmembrane protein with large extracellular domain. Immature nicastrin is ~110 kDa and the apparent molecular weight is increased to ~130 kDa after *N*-glycosylation in the Golgi/TGN compartments. This mature form of nicastrin is associated with the active γ-secretase complex (Arawaka et al., 2002, Edbauer et al., 2002, Kimberly et al., 2002, Leem et al., 2002).

The nicastrin KO mice showed a Notch phenotype with embryonic lethality (Nguyen et al., 2006). Aph-1 is required for cell surface localization of nicastrin (Goutte et al., 2002) and Pen-2 is required for both the expression of PS and the maturation of nicastrin (Steiner et al., 2002). No strong genetic association between Pen-2 and AD has been found (Sala Frigerio et al., 2005). A KO study in zebra fish showed that Pen-2 is important for neuronal cell survival and protects cells from apoptosis (Campbell et al., 2006).

Whether these four subunits of γ-secretase are needed for its activity was investigated. γ-Secretase activity was reconstituted in *Saccaromyces cerevisiae,* which lacks endogenous γ-secretase activity, by co-expressing PS, nicastrin, Aph-1, and Pen-2. Thus, these four proteins appear to be necessary and sufficient for γ-secretase activity (Edbauer et al., 2003). This was also shown in *Drosophila* and mammalian cells (Edbauer et al., 2003, Hayashi et al., 2004, Kimberly et al., 2003, Takasugi et al., 2003, Zhang et al., 2005). Co-expression of all four components also increased the PS heterodimeric form, full glycosylation of nicastrin and γ-secretase activity in mammalian cells (Kimberly et al., 2003). In a postmortem human brain study, it was shown that human brain-derived γ-secretase is present as a high molecular weight protein complex containing PS, nicastrin, Aph-1, and Pen-2, and that these are associated with γ-secretase activity. The activity of complex was inhibited by the specific γ-secretase inhibitor, L-685,458 suggesting that this γ-secretase complex isolated from human brain is functional (Farmery et al., 2003).

1.2.2 The γ-secretase complex assembly

How these γ-secretase components are assembled into a complex has been extensively studied (Fraering et al., 2004, LaVoie et al., 2003). The assembly of the complex is initiated in the ER (Capell et al., 2005) where Aph-1 and nicastrin interact, followed by the binding of PS. Thereafter, Pen-2 binds to the complex and facilitates the endoproteolysis of PS into an amino-terminal fragment (PS1-NTF) and a carboxy-terminal fragment (PS1-CTF), resulting in an active γ-secretase complex (Takasugi et al., 2003). Rer1 was identified as Pen-2 binding protein and play a role in γ-secretase complex assembly (Kaether et al., 2007).

In a γ-secretase activity study using a novel biotinylated affinity ligand, it was confirmed that the PS heterodimers and mature nicastrin exist in the active enzyme complex (Beher et al., 2003).

1.2.3 Stoichiometry of γ-secretase

PS has two homologs, PS1 and PS2. Aph-1 has two homologues Aph-1a and Aph-1b in human, and one additional homologue, Aph-1c, in rodents. Aph-1a has two alternatively spliced forms, Aph-1aL (long form) and Aph-1aS (short form). In total, γ-secretase can form six different complexes in human (Hebert et al., 2004).

The size of the γ-secretase complex is under debate. The molecular weight of the four components is PS1-NTF (~30 kDa), PS1-CTF (~20 kDa), fully glycosylated nicastrin (~130 kDa), Aph-1 (~30 kDa), and Pen-2 (~12 kDa). The molecular weight of the γ-secretase complex is calculated to be around 220 kDat at a stoichiometry of 1:1:1:1 (PS:glycosylated nicastrin:Aph-1:Pen-2). Different methods have been used for preparation and analysis of the complex, resulting in observed molecular weights in the range of 200-2000 kDa (Edbauer et al., 2003, Farmery et al., 2003, Kimberly et al., 2003). The lowest reported molecular weight of the complex is 200-250 kDa, corresponding to a monomeric complex (Kimberly et al., 2003). A molecular weight of ~440 kDa was reported (Edbauer et al., 2003), suggesting a possible stoichiometry of 2:2:2:2. When membranes were prepared from postmortem human brain, the γ-secretase components eluted in a fraction corresponding to a molecular mass of > 1000 kDa (Farmery et al., 2003). The most recent study reported the stoichiometry of active γ-secretase complexes as 1:1:1:1 (Sato et al., 2007). Differences in the molecular weight of the γ-secretase complex might indicate the possibilities of additional proteins, either novel core components or proteins binding transiently. Indeed, the molecular weight of the γ-secretase complex with a new GSAP, TMP21, was reported to be around 660 kDa (Chen et al., 2006).

1.2.4 γ-Secretase structure

The catalytic residues of I-CliPs are located within transmembrane regions, and they hydrolyze the peptide bonds of its substrates in the transmembrane regions (Fraering, 2007). The I-CliP family can be categorized into aspartyl proteases (including γ-secretase and signal peptide peptidase), metalloproteases (site-2 protease, Eep), and serine proteases (Rhomboid, AarA) (Kopan and Ilagan, 2004). To obtain the structure of these transmembrane proteins has been a challenge. Recently, atomic structure of the GlpG rhomboid protease has been reported (Ben-Shem et al., 2007, Wang et al., 2006, Wu et al., 2006). It has been even more challenging for γ-secretase structure studies due to its many subunits and transmembrane domains (PS, nicastrin, Aph-1, and Pen-2 having 9+1+7+2).

Electron microscopy study on the 3D structure of γ-secretase revealed that there is a low density interior chamber and two pores (apical and basal pores) which allows for water molecule entry into the structure. Those pores for water molecules could explain this unusual intramembrane cleavage (peptide bond hydrolysis) by γ-secretase. It was also suggested that Aβ and AICD could be released through two pores into outer spaces (extracellular and cytosolic spaces, respectively) (Lazarov et al., 2006).

How APP interacts with γ-secretase in membranes was investigated, and the substrate docking site was found at the PS-NTF/CTF interface (Kornilova et al., 2005). APP can enter, either in whole or in part, between PS-NTF and PS-CTF to access the internal

active site (Wolfe, 2009). Nicastrin was suggested to be responsible for substrate recognition and binding (Shah et al., 2005), but this function is still under debate.

1.2.5 Trafficking and subcellular localization

In order to understand the sites for Aβ production in cells, it has been studied how APP, BACE1, and γ-secretase are trafficked and processed through subcellular compartments.

Aβ is found in the TGN (Greenfield et al., 1999) and in endosomes (Small and Gandy, 2006). The subcellular localization of Aβ in brain tissue has been found to be mainly endosomal as well (Cataldo et al., 2004, Takahashi et al., 2002). Nascent APP is trafficked through the ER to the plasma membrane in cells. APP undergoes numerous post-translational modifications including *N*- and *O*-glycosylation, ectodomain and cytoplasmic phosphorylation, and tyrosine sulfation. Only 10% of APP reaches the plasma membrane by the secretory pathway while the rest of APP is found in the Golgi and TGN. APP at the cell surface is internalized due to its YENPTY internalization motif in the C-terminus. APP is recycled to the plasma membrane or undergoes a degradation process in the lysosome (the recycling pathway) (Thinakaran and Koo, 2008). APP is believed to be cleaved by α-secretase at the cell surface (Sisodia, 1992) while BACE1 cleavage occurs mostly in the late Golgi/TGN and in endosomes (Koo and Squazzo, 1994).

The localization of γ-secretase has been extensively studied. γ-Secretase components have been found in many subcellular compartments such as the ER (Zhang et al., 1998), ER-Golgi intermediate compartment, Golgi, TGN, endosomes (Lah and Levey, 2000) and at the plasma membrane (Chyung et al., 2005). Interestingly, PS was found in synaptic compartments (Beher et al., 1999, Efthimiopoulos et al., 1998, Lah et al., 1997, Ribaut-Barassin et al., 2003). In addition, all four γ-secretase components were found in phagosomes (Jutras et al., 2005). PS1, nicastrin, and APP were localized in the outer membranes of lysosomes (Pasternak et al., 2003). Due to its loose cleavage site specificity, it has been suggested that γ-secretase could act as a membrane proteasome and active γ-secretase is enriched in lysosomal membranes (Kopan and Ilagan, 2004).

Importantly, the sites for γ-secretase activity have been investigated. A biotinylated active site probe labeled γ-secretase in the plasma membrane of cells (Chun et al., 2004, Chyung et al., 2005). Additionally, a small fraction of active γ-secretase was found in mitochondria (Hansson et al., 2004).

1.2.6 Lipid rafts

The lipid membrane environment can affect the activity of proteins. Since γ-secretase is a transmembrane bound protein, different detergents have been used to extract and study the complex. However, γ-secretase can also be studied in a membrane environment, preserving some of its natural interactions with lipids. Cholesterol and sphingolipids are the major lipid constituent of ordered microdomains in cell membranes. These microdomains are called lipid rafts and are considered to be dynamic platforms for cell signalling, membrane protein sorting and transport (Simons and Ikonen, 1997). Lipid rafts can be studied by treatment of membranes with

detergents such as Triton X-100 at 4 ºC. The insoluble parts of the lipid membranes can be isolated by centrifugation, which are called detergent resistant membranes (DRMs).

Several findings suggest that the trafficking and processing of APP are regulated in lipid rafts (Ehehalt et al., 2003, Urano et al., 2005, Vetrivel et al., 2005, Hattori et al., 2006, Wahrle et al., 2002). APP, BACE and γ-secretase have been shown to localize to lipid rafts. Ehehalt *et al.* reported that APP and BACE residing in separate DRMs can merge in endosomes, where amyloidogenic processing takes place. Urano *et al.* found active γ-secretase in DRMs and Vetrivel *et al.* reported that γ-secretase was active in DRMs from post-Golgi compartments and endosomes. The reconstitution study of γ-secretase with different lipid mixtures showed that a lipid raft like condition gave the highest γ-secretase activity (Osenkowski et al., 2008). A recent high-throughput functional genomics screen identified the orphan G protein-coupled receptor 3 (GPR3) as a modulator of Aβ. GPR3 appears to promote complex assembly of γ-secretase, resulting in increased trafficking of the γ-secretase components and the mature γ-secretase complex to the cell surface and increased localization in DRMs, which eventually leads to an increase in Aβ generation (Thathiah et al., 2009). Therefore, it has been suggested that specific inhibition of γ-secretase in certain organelles or microdomains could be an attractive approach (Cheng et al., 2007, Rajendran et al., 2010, Osenkowski et al., 2008) and an interesting study with a membrane anchored version of β-secretase transition state inhibitor reduced indeed the enzyme activity (Rajendran et al., 2008).

1.2.7 Other γ-secretase substrates

γ-Secretase has more than 60 substrates besides APP, all of them being type 1 transmembrane proteins. They are for instance APLP1 and APLP2 (unknown physiological role), proteins involved in cell adhesion (N-cadherin, E-cadherin, CD44), CSF1 receptor (protein tyrosine kinase), deleted in colorectal cancer (DCC, Netrin-1 receptor), ErbB4 (growth-factor-dependent receptor tyrosine kinase), low-density lipoprotein (LDL) receptor-related protein (LRP, endocytic receptor), Nectin-1α (adherens-junction formation), Notch 1-4 (signalling receptor), Delta and Jagged (Notch ligand), p75 (Neurotrophin co-receptor), and syndecan-3 (cell-surface proteoglycan co-receptor) (Kopan and Ilagan, 2004). γ-Secretase cleavage does not seem to depend on the specific sequence of the substrate. It rather depends on ectodomain shedding (Struhl and Adachi, 2000). In many cases, the intracellular domains (ICDs) released upon γ-secretase cleavage are involved in regulation of gene transcription (Kopan and Ilagan, 2004).

One of the γ-secretase substrates, Notch undergoes similar processing as APP. As APP is cleaved either by α- or β- secretase and the resulting APP-CTFs are cleaved by γ-secretase, Notch undergoes ectodomain shedding by metalloprotease at the S2 site, is further cleaved by γ-secretase at the S3 site, and releases the Notch intracellular domain (NICD) (Kopan and Ilagan, 2004). However, Notch cleavage is stimulated by ligand binding, while it is still unknown whether APP requires a ligand to be activated. Importantly, Notch is mainly cleaved by γ-secretase at the cell surface while APP is processed by intracellular γ-secretase (Tarassishin et al., 2004).

The multitude of γ-secretase substrates has made the development of clinically useful inhibitors difficult. For instance, gastrointestinal side effects related to decreased notch-signalling has been observed (Siemers et al., 2006).

1.3 γ-SECRETASE ASSOCIATED PROTEINS (GSAPS)

Although PS, nicastrin, Aph-1, and Pen-2 are necessary for γ-secretase activity (Edbauer et al., 2003), it is plausible that other GSAPs could have a regulatory role in substrate specificity. In addition, the reported size of the γ-secretase complex varies between 200-2000 kDa (Edbauer et al., 2003, Farmery et al., 2003, Kimberly et al., 2003) indicating that there is a possibility of unknown components to be present in the γ-secretase complex. These GSAPs could be new targets to modulate γ-secretase activity in Alzheimer's disease. Some potential GSAPs have been discovered in the last years and they are discussed below.

1.3.1 CD147

CD147 (also known as basigin, EMMPRIN) was identified as a regulatory subunit of γ-secretase. HeLa cell membranes were solubilized by using FOS-CHOLINE-12 detergent (n-Dodecylphosphocholine, phospholipid analogs with a phosphocholine headgroup) and underwent multipurification steps such as Q-Sepharose HP column, a NaCl step gradient, a lentil lectin column, and a Superdex 200 molecular size exclusion column. The eluting peaks around 250-300 kDa from the Superdex 200 column were pooled and analyzed by SDS-PAGE. The Coomassie blue stained gel was in-gel digested by trypsin, the four known γ-secretase components were identified, and the unidentified 50 kDa band was found to be CD147 from amino acid sequencing. CD147 is a transmembrane glycoprotein with two Ig-like domains (Zhou et al., 2005). CD147 is ubiquitously expressed in various cells and tissues (Muramatsu and Miyauchi, 2003) and is suggested to be involved in many different biological functions such as neural-glial cell interaction, reproduction, neural function, inflammation, protein trafficking, tumor invasion (Zhou et al., 2005). The deletion of CD147 in mice was resulted in severe defects in nervous system development, spatial learning deficits, and working memory deficits (Naruhashi et al., 1997).

Co-immunoprecipitation with anti-PS1-CTF and nicastrin antibodies indicated that CD147 is present in the γ-secretase complex. Suppression of CD147 expression by siRNA resulted in dosage-dependent increased levels of Aβ40 and Aβ42 without changing the expression levels of the other γ-secretase components or APP substrates (APP, sAPPα, sAPPβ). In other words, the presence of CD147 in the γ-secretase complex down-regulates Aβ production. Overexpression of CD147 in CHO-APP695 cells showed that there was no increase in Aβ40 and Aβ42 production, and in the expression levels of PS1, nicastrin, and APP (Zhou et al., 2005).

However, it was later questioned whether CD147 is associated with the γ-secretase complex, and γ-secretase inhibitor pulldown using Merck C did not co-purify CD147 (Winkler et al., 2009).

1.3.2 TMP21

TMP21 (also known as p23) was isolated by the immunoprecipitation with anti-PS antibody in CHAPSO-solubilized membranes from wild-type blastocyst-derived cells expressing PS1 and PS2, and identified by mass spectrometry together with PS1, nicastrin, Aph-1, and Pen-2 (Chen et al., 2006).

TMP21 is a type I transmembrane protein (Blum et al., 1996) and the gene is located at chromosome 14 where PS1 is also located (Sherrington et al., 1995, Trower et al., 1996). It is a member of the p24 cargo-protein family (Blum et al., 1996) and is involved in protein transport and quality control in the ER and Golgi (Jenne et al., 2002). The subcellular localization of TMP21 is at the plasma membrane (Blum et al., 1996) and it is ubiquitously expressed in tissues (www.uniprot.org).

When TMP21 was suppressed by siRNA, Aβ production (both Aβ40 and Aβ42) was increased while the expression levels of γ-secretase and APP were not changed. It was suggested that TMP21 is a component of the γ-secretase complex but it is not necessarily needed for the complex assembly. When p24a was suppressed by siRNA, TMP21 levels were decreased. Suppressing p24a and TMP21 showed about the same levels of Aβ as silencing only TMP21. This was explained by the possibility that TMP21 might have two pools. The major pool joins the p23 cargo family without affecting Aβ production, and the minor makes a complex with PS1. Since TMP21 siRNA only affects Aβ production (γ-cleavage site) but not the AICD or NICD production (ε-cleavage site), TMP21 in the minor pool is thought to regulate only γ-cleavage (Chen et al., 2006).

It is still controversial whether TMP21 is truly a GSAP. For instance, it has been suggested that the reduced Aβ production was due to altered APP trafficking by TMP21 in another study (Vetrivel et al., 2007). No association of TMP21 with active γ-secretase was shown by pulldown experiment using the γ-secretase inhibitor, Merck C (Winkler et al., 2009).

1.3.3 Proteins in the tetraspanin web

Recently, several proteins in the tetraspanin web were identified as GSAPs. PS1 and PS2 deficient mouse embryonic fibroblasts were stably transfected with tagged PS1 and PS2. γ-Secretase was purified by antibody and beads from CHAPSO or CHAPS solubilized membranes. Coomassie blue-stained bands were in-gel digested with trypsin after SDS-PAGE gel running and analyzed by mass spectrometry. PS1, PS2, nicastrin, Aph-1a, and Pen-2 were identified together with previously known PS-interacting proteins, TMP21, β-catenin, γ-catenin, and Rab-11. δ-Catenin, N-cadherin, ApoER2, and FKBP8 were also identified. Functions of these proteins support the subcellular trafficking function of PS and γ-secretase. In total, 59 proteins were identified in the samples from PS1 and PS2 transfected cells, and 46 proteins were found in common. This study focused on proteins (EWI-F, CD81, CD98hc, CD9) in the tetraspanin web. Members of this family form lipid raft-like microdomains (Wakabayashi et al., 2009) and have a role in intracellular and intercellular processes, cell fusion, cell proliferation, adhesion, and migration (Levy and Shoham, 2005). EWI-F makes a primary interaction with CD81, followed by a secondary interaction

with β1-integrin. It is suggested that the tetraspanin web makes a tertiary interaction with the γ-secretase complex. Suppressing *CD81*, *EWI-F* or *CD98hc* by siRNA decreased Aβ production whereas there were no changes on the expression levels of the γ-secretase components and APP. CD81, CD9, and EWI-F are in the active γ-secretase complex as shown by Aβ production in co-immunoprecipitates (Wakabayashi et al., 2009).

2 AIMS OF THE THESIS

According to the current dominant hypothesis in the Alzheimer field, Aβ plays a role in inducing toxicity to neurons in the brain. Since γ-secretase cleaves APP C-terminal fragments to produce Aβ, this enzyme complex has an important role in AD. In this thesis, our goal was to characterize γ-secretase in mammalian brain, with respect to the localization of its activity and ultimately to identify and characterize possible novel γ-secretase associated proteins in brain.

The specific aims of this thesis were:

1. To study active γ-secretase complexes associated with detergent resistant membranes (Paper I)

2. To study the distribution of γ-secretase and γ-secretase activity in different subcellular compartments (Paper II)

3. To identify novel γ-secretase associated proteins in human brain (Paper III)

4. To identify and characterize novel γ-secretase associated proteins in detergent resistant membranes (Paper IV)

3 MATERIALS AND METHODS

The materials and methods used in paper I – IV were briefly summarized in the table below and described in this section. For more information in details, please refer to the each paper indicated. In general, our studies were aimed at studying γ-secretase using brain materials.

Table 1. Materials and methods used in Paper I – IV

	Materials	Fractionations	Methods
I	1. SH-SY5Y cells 2. Rat brains 3. Postmortem human brains	1. Membrane preparation 2. Preparation of DRMs	1. Deglycosylation 2. Co-immunoprecipitation 3. Size exclusion chromatography 4. γ-Secretase activity assay 5. Western blotting 6. Postmortem time study 7. Immunohistochemistry
II	1. Rat brains	1. Iodixanol gradient fractions 2. Preparation of synaptic vesicles and membranes	1. γ-Secretase activity assay 2. Western blotting 3. Electron microscopy 4. Immunohistochemistry
III	1. BD8-PS1 cells 2. Rat brains 3. Postmortem human brains	1. Membrane preparation	1. γ-Secretase activity assay 2. Western blotting 3. Affinity purification with GCB 4. Mass spectrometry 5. Protein identification by MASCOT and SpectrumMill
IV	1. HEK-293 APP695 cells 2. Rat brains	1. Membrane preparation 2. Preparation of DRMs	1. Electron microscopy 2. Thin layer chromatography 3. Affinity purification with GCB 4. Mass spectrometry 5. Protein identification by SpectrumMill 6. RNA interference 7. Real-time PCR 8. Sandwich ELISA 9. Western blotting

3.1 MATERIALS

Cell culture (Paper I, III, IV)

Human neuroblastoma cells (SH-SY5Y, in Paper I), blastocyst-derived embryonic stem cells deficient in PS1 and PS2 stably expressing PS1 (BD8-PS1 cells, in Paper III), and human embryonic kidney 293 cells stably expressing APP695 (HEK-293 APP695 cells, in Paper IV) (Nilsson et al., 2006) were cultured.

Animals (Paper I, II, III, IV)
Male Sprague-Dawley rats (Paper I-IV) were obtained and ethical permit was approved by the Animal Trial Committee of Southern Stockholm. Pregnant C57BL/6 mice were used in Paper II. In Paper IV, Sprague-Dawley rat brains (8 to 12 weeks old) were also purchased from Rockland Immunochemicals and stored at -70 °C before use.

Human brain material (Paper I, III)
The cortex of a non-Alzheimer case (postmortem time 22 hr Paper I) and the frontal cortex of a non-AD case (postmortem time 20 hr, Paper III) from postmortem human brain were obtained from Huddinge Brain Bank, Sweden and stored at -70 °C before use. Ethical permit was granted by the Regional Ethical Review Board in Stockholm.

Postmortem brain material (Paper I)
Since Paper I and III were aimed at characterizing γ-secretase in postmortem human brain, we validated the effect of postmortem time on γ-secretase activity. In order to simulate a slow cooling curve as in the condition of postmortem human brains before autopsy, the rats were killed and kept at room temperature for 2 hours. The head was removed, kept in a box filled with 37 °C water, and the box was kept at 4 °C (Spokes and Koch, 1978), and postmortem brains were collected after 6, 12, 24, or 48 hours. To obtain membranes from postmortem time 0 hr, the rat was sacrificed and the brain was immediately removed and homogenized. Membrane fractions were prepared and AICD was measured by western blotting.

3.2 FRACTIONATIONS

Membrane preparation (Paper I, III, IV)
Membranes were prepared as described previously (Farmery et al., 2003) with some modifications. To prepare membranes, the brain pieces were homogenized in lysis buffer, buffer A (20 mM Hepes (pH 7.5), 50 mM KCl, 2 mM EGTA, and Complete™ protease inhibitor mixture (which inhibits a broad spectrum of serine, cysteine and metalloproteases)). All the procedures were carried out on ice. The samples were centrifuged at 1,000 × g for 10 min to remove nuclei and poorly homogenized material. The post-nuclear supernatant (PNS) was centrifuged at 10,000 × g for 30 min in order to remove mitochondria. The final supernatant was then centrifuged at 100,000 × g for 1 hour to yield the final pellet (P3). All centrifugation steps were carried out at 4 ºC. In Paper I, P3 was freshly prepared for the following DRM preparation. In Paper III and IV, the resulting microsomal pellets were resuspended in buffer A supplemented with 20% glycerol, and stored at -80 ºC before use. In Paper III, cells were sonicated in buffer A. Cell debris and nuclei were removed by centrifugation at 800 × g for 10 min and the resulting supernatant was centrifuged at 100,000 × g for 60 min.

Preparation of DRMs (Paper I, IV)
DRMs were prepared as described previously (Urano et al., 2005) with some modifications. To isolate DRMs from brain material or cells, P3 or the cell pellet, respectively, was resuspended in buffer R (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 2% CHAPSO or 1% Triton X-100, and Complete™ protease inhibitor mixture). 1% Triton X-100 was compared to 2% CHAPSO in Paper I and 2% CHAPSO was found to be superior than 1% Triton X-100 for the localization of

γ-secretase in DRMs. Therefore, 2% CHAPSO was used for the rest of the studies in Paper I and IV. After the incubation with 2% CHAPSO, the sample was adjusted to 45% sucrose and placed at the bottom of a 14 ml tube. Then, 6.9 ml of 35% sucrose followed by 2.3 ml of 5% sucrose was overlaid. The sample was centrifuged at 100,000 × g for 16 hours at 4 °C. Six fractions were collected from the top of the tube. In Paper I, PD-10 desalting columns were used according to the manufacturer's instructions in order to remove sucrose from the six fractions. A buffer H (20 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM EDTA, and Complete™ protease inhibitor mixture) was diluted 7 times and used to equilibrate the columns. The samples were applied, eluted and concentrated to 1 × buffer (7 times) using a vacuum centrifuge. The protein concentration was analyzed by the BCA protein assay. In Paper IV, DRM fractions were diluted with PBS and centrifuged at 100,000 × g for 1 hour two times. The resulting pellets were quickly freezed using liquid nitrogen.

Preparation of iodixanol gradient fractions (Paper II)

Rat brains were homogenized in Buffer I (130 mM KCl, 25 mM Tris-HCl (pH 7.4), 1 mM EGTA, and Complete™ protease inhibitor mixture). The homogenates were centrifuged at 1,000 × g for 10 min to remove nuclei and at 10,000 × g for 15 min to remove mitochondria and synaptosomes. The 10,000 × g supernatant was layered on an iodixanol gradient consisting of 1 ml each of 30, 25, 20, 15, 12.5, 10, 7.5, 5 and 2.5% (w/v) iodixanol in Buffer I and centrifuged at 126,000 × g for 40 min. 1 ml fractions were collected from the bottom of the tube, diluted 4 times with Buffer I and centrifuged at 126,000 × g for 40 min. The pellets were resuspended in Buffer H (150 mM NaCl, 20 mM Hepes-KOH (pH 7.0), 5 mM EDTA and Complete™ protease inhibitor mixture) with 0.4% CHAPSO.

Preparation of synaptic vesicles and membranes (Paper II)

Synaptic vesicles and membranes were prepared as described previously (Cohen et al., 1977). Rat brains were homogenized in Buffer A (0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$). The P2 (17,300 × g) pellet was resuspended in Buffer B (1 mM $NaHCO_3$, 0.32 M sucrose) and layered on a sucrose gradient to purify synaptosomes. The synaptosomes were lysed in 6 mM Tris-HCl (pH 8.1) and the lysate was centrifuged at 48,250 × g to separate membranes (LP1) from synaptic vesicles. Synaptic membranes were further purified from LP1 on a second sucrose gradient to remove synaptic mitochondria, whereas the synaptic vesicles were pelleted at 100,000 × g for 2 hours. The 17,300 × g supernatant was centrifuged at 100,000 × g for 1 hour to obtain a reference pellet (P3). In addition, highly pure synaptic vesicles were kindly provided by Dr. Matthew Holt, Goettingen, Germany. The vesicles were prepared including controlled pored glass chromatography (Huttner et al., 1983). Protein concentrations were determined by the BCA protein assay.

3.3 CHARACTERIZATION OF γ-SECRETASE

Deglycosylation (Paper I)

The glycosylation status of nicastrin was analyzed as described previously (Farmery et al., 2003). To analyze the glycosylation status of nicastrin in the DRM fractions and non-DRM fractions, the samples were denatured by heating for 10 min at 100 °C in the presence of 0.5% (v/v) SDS and 1.0% (v/v) β-mercaptoethanol, cooled on ice

and adjusted to 50 mM sodium citrate, pH 5.5. For endoglycosidase H (Endo-β-*N*-acetylglucosaminidase, Endo H) treatment, 100 milliunits of Endo H was added. For *N*-glycosidase F (peptide-*N*-glycosidase F, PNGase F) treatment, NP-40 was added to a final concentration of 1.0% followed by the addition with 15.4 milliunits of PNGase F. The samples were incubated overnight at 37 °C and analyzed by SDS-PAGE.

Co-immunoprecipitation (Paper I)

To investigate the γ-secretase complex formation in DRMs, DRMs or crude microsomes were immunoprecipitated by anti-nicastrin antibody. Rat membranes (P3) were resuspended in 600 µl of immunoprecipitation buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 2.0% CHAPSO, and Complete™ protease inhibitor mixture). All fractions were pre-cleared with 1:1 ratio of protein A/G Sepharose, followed by the incubation with anti-nicastrin or control rabbit IgG overnight at 4 °C. Protein A/G Sepharose was added for 1 hour at 37 °C. After washing three times with immunoprecipitation buffer, the beads were eluted in SDS-PAGE sample buffer and subjected to SDS-PAGE.

Size exclusion chromatography (Paper I)

To estimate the approximate size of DRMs, the DRM fraction was injected onto a superose 6HR column, using a buffer (20 mM Hepes, pH 7.0, 150 mM KCl, 2 mM EGTA, Complete™ protease inhibitor mixture) and 0.25% CHAPSO or 2% CHAPSO as mobile phase at a flow rate of 0.5 ml/min. Fractions were collected from 10 to 50 min and analyzed by SDS-PAGE.

γ-Secretase activity assay (Paper I, II, III)

Samples were incubated for 16 hours at 37 °C in the absence or presence of the γ-secretase inhibitor L-685,458 or GCB (Paper I, II, III). In some cases, C99-FLAG solubilized in 2,2,2-trifluoroethanol (TFE) was added to the samples (Paper I, II). The production of AICD was analyzed by SDS-PAGE. For Aβ levels, the reaction was stopped by adding RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) and boiling for 5 min. Aβ40 levels were measured by commercial sandwich ELISA (enzyme-linked immunosorbent assay) according to the manufacturer's instructions. The Aβ40 production in the different synaptic fractions was compared with the production in homogenates using unpaired t-test (Paper II).

SDS-PAGE and western blotting (Paper I, II, III, IV)

Proteins were loaded onto SDS-PAGE gels, electrophoresed, and transferred to nitrocellulose or PVDF membranes, and the proteins of interest were detected by specific antibodies.

Thin layer chromatography (Paper IV)

Cholesterol and sphingomylein in DRM fractions were separated on thin layer chromatography plate (TLC plate) together with the known amount of synthetic cholesterol and sphingomyelin. The TLC plate was developed by bromothymol blue and air-dried. The concentration of cholesterol and sphingomyelin in DRMs were quantified using CCD camera.

3.4 MICROSCOPY

Electron microscopy (Paper II, IV)
In Paper IV, DRM fractions were mixed with phosphate buffered saline (PBS) and centrifuged at 100,000 × g for 1 hour. The bottom fraction was centrifuged once again at 100,000 × g for 1 hour. The DRM pellets (Paper IV) and the synaptic vesicle and membrane fractions (Paper II) were fixed in 2% glutaraldehyde and examined in a Leo 906 transmission electron microscope at 80 kV. Digital images were taken by using a Morada digital camera.

Labeling of rat brain sections with a biotinylated γ-secretase inhibitor (Paper II)
Primary cortical neuron cultures were established from cortices dissected from E17 mice (C57BL/6) (Behbahani et al., 2005). The rat brain sections from frontal cortex or mouse primary neurons were fixed in 4% formaldehyde and permeabilized with 0.2% Triton X-100. After blocking, the tissues and cells were pre-incubated with 50 µM L-685,458, followed by incubation with 500 nM GCB. Later, the samples were incubated with streptavidin-Alexa 488, followed by the incubation with primary and secondary antibodies.

Immunohistochemistry and confocal microscopy (Paper I, II)
The cryopreserved brain sections from frontal cortex of postmortem human brain tissue (a non-AD case) were used. Lipid rafts were labelled with the fluorescence conjugated cholera toxin subunit B, which binds to GM1 ganglioside receptor. PS1, nicastrin, APP, and nucleus were also labelled (Paper I). All samples were visualized by an inverted Laser Scanning Microscope (Paper I, II).

3.5 PROTEOMICS

Affinity purification of γ-secretase (Paper III, IV)
γ-Secretase inhibitor with cleavable biotin group (GCB) was synthesized. γ-Secretase was captured by GCB using membranes (Paper III) or DRMs (Paper IV). In brief, samples were pre-cleared with streptavidin-conjugated magnetic beads. Pre-cleared samples were incubated in the absence or presence of the γ-secretase inhibitor L-685,458, followed by the incubation with GCB. Note that different concentrations of L-685,458 and GCB were used in Paper III and IV. The magnetic beads were added and the collected beads were washed to remove non-specific binding. The captured γ-secretase complex was eluted by 100 mM DTT in SDS sample buffer for western blotting. For the mass spectrometry analysis, the samples were eluted with buffer containing 10 mM Dithiothreitol (DTT), 0.01% Rapigest and 10 mM Ambic.

Tryptic digestion and nanoscale liquid chromatography – tandem mass spectrometry (LC-MS/MS) (Paper III, IV)
The samples were digested by trypsin at 37 °C overnight. The tryptic peptides were concentrated by Zip Tip® C_{18} after equilibration according to the manufacturer's instructions. The peptides were washed twice with 10 µl of 0.1-0-2% formic acid followed by elution with 80% acetonitrile/0.1-0.2% formic acid (Paper III, IV). For further fractionation in Paper III, the eluted peptides were adjusted with 0.1% FA to 45% acetonitrile and loaded onto strong cation exchange (SCX) Zip Tip® 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 eluate was dried using a vacuum centrifuge and dissolved in formic acid before the mass spectrometry analysis. The samples were analyzed on a 6330 Ion Trap LC/MS system.

Protein identification by MASCOT Daemon software package (Paper III)
A list of the resulting spectra was generated using the 6300 Series Ion Trap LC/MS Software Version 6.1 with an intensity threshold of 1000. The compound lists were exported as Mascot generic format files (mgf). Protein identification was performed by the MASCOT Daemon software package.

Protein identification by SpectrumMill (Paper III, IV)
Spectra of peaks were obtained from all experiments using the data extractor of the SpectrumMill Proteomics Workbench version A.03.03.078. Proteins were searched in the SwissProt protein database based on MS/MS spectra.

Candidate protein selection (Paper IV)
The data were obtained from the SpectrumMill software. The mean intensity of peptides was normalized to the total intensity of each sample in order to minimize the risk of different efficiency of sample elution from beads. The ratio was calculated by that the value of –L-685,458 was divided by the one of +L-685,458. The minimum score for proteins was 10. To be selected as candidate proteins, minimum two unique peptides per protein were required.

3.6 FUNCTIONAL STUDIES OF CANDIDATE PROTEINS

RNA interference (Paper IV)
The pre-designed small interfering RNA (siRNA) oligos targeting candidate proteins were transfected to HEK-293 APP695 cells by using Lipofectamine^{TM} RNAiMAX. After two days, media were replaced with the fresh one. Conditioned media were harvested after 24-hour incubation and measured for Aβ40 and Aβ42 levels by sandwich ELISA. The Alamar Blue assay was used to check cell viability. Alamar Blue (resazurin, non-fluorescent dye) is soluble and stable in cell medium, cell-permeable, and non-toxic to cells. It is reduced to resorufin (fluorescent dye) by living cells and this indicates cell viability. Even though Alamar Blue has drawbacks like underestimatation or overestimation of cell viability (O'brien J and Pognan F, vol 267), it was superior to WST8 (measuring activity of mitochondrial dehydrogenase) for reproducibility of data in our hands (data not shown).

Real-time PCR (RT-PCR) (Paper IV)
After measuring cell viability, cells were lysed and reverse transcripted according to the manufacturer's instructions. Gene expression levels were measured by real-time PCR.

4 RESULTS AND DISCUSSION

The general goals of the studies included in this thesis were to characterize γ-secretase and to find γ-secretase associated proteins in mammalian brain. By studying its localization and activity, and additional components or modulators, we could better understand γ-secretase and use it as a drug target for curing Alzheimer's disease. Therefore, we investigated the hot spots, where γ-secretase activity takes action, such as microdomains of cell membranes, different organelles in cells, and synapses in brain (Paper I and II). Eventually, we pursued to identify γ-secretase associated proteins by capturing active γ-secretase with a γ-secretase inhibitor designed for affinity purification (Paper III and IV). Since two different pools of starting materials, detergent soluble and detergent insoluble γ-secretase were used (Paper III and IV, respectively), common proteins in these studies could strengthen the significance of identification of proteins.

4.1 ACTIVE γ-SECRETASE IN DRMS (PAPER I)

In many studies on γ-secretase, detergents are used to extract membrane proteins (soluble γ-secretase). However, some studies in cell lines and mouse brain showed that γ-secretase is partially localized to lipid rafts, which are detergent resistant membrane microdomains (DRMs) enriched in cholesterol and sphingolipids. In other words, γ-secretase is tightly bound in lipid rafts and it is still insoluble after treatment with high concentration of detergent. Even though there was an indication of association of γ-secretase in DRMs previously, the activity of γ-secretase localized to DRMs has not been shown. In Paper I, we studied γ-secretase in DRMs from brain, and compared the properties of γ-secretase from human and rat brain.

DRMs were originally prepared by using 1% Triton X-100, and this is still the most commonly used detergent. Previously, it was shown that CHAPSO preserved the γ-secretase activity better than other detergents tested (Franberg et al., 2007). Since we aimed to purify DRMs with preserved γ-secretase activity from brain, we tested 1% Triton X-100 and CHAPSO at different concentrations and investigated the co-fractionation of γ-secretase with lipid raft markers, flotillin-1, caveolin-1 or ganglioside GM1. We found that 2% CHAPSO purified brain DRMs better than 1% Triton X-100, and we also showed that it was more difficult to isolate DRMs from brain than from cell lines. This could be explained by the fact that the brain is more complex and heterogeneous, containing many different kinds of cells (neurons, astrocytes, microglia, and others), and is rich in lipids compared to homogenous cell cultures. Thus, the choice of detergent for preparing DRMs depends on the starting material and the proteins to be studied.

Membrane fractions from human or rat brain were treated with 2% CHAPSO, centrifuged in a stepped sucrose gradient, and the DRMs were collected from the interface between 5% and 35% sucrose. As a result, DRMs enriched in the γ-secretase components (PS1, nicastrin, Aph-1aL, Pen-2) were obtained, while only around 10% of the total protein was found in this fraction. In addition, APP-CTFs were found mainly outside of DRMs. Previous studies showed that APP, BACE, and γ-secretase were associated with lipid rafts, but the degree of localization differs between studies

(Abad-Rodriguez et al., 2004, Ehehalt et al., 2003, Hattori et al., 2006, Lee et al., 1998, Urano et al., 2005, Wahrle et al., 2002, Vetrivel et al., 2005). In order to determine the glycosylation status of nicastrin, the collected fractions were treated with Endo H or PNGase F. Endo H treatment deglycosylated nicastrin and reduced its molecular weight from 125 kDa to 100 kDa indicating the presence of high-mannose oligosaccharides. By the treatment of PNGase F, which removes complex oligosaccharides, the molecular weight of nicastrin was reduced to 80 kDa. Thus, nicastrin was found in its mature, highly glycosylated form in all fractions. Furthermore, the γ-secretase complex was intact as shown by co-immunoprecipitation using anti-nicastrin antibody. Endogenous γ-secretase activity in DRMs was estimated by measuring AICD generation and Aβ production. The fractions were incubated at 37 °C for 16 hours with or without the γ-secretase inhibitor, L-685,458, and the AICD levels were detected by western blot analysis. The AICD production was clearly highest in the DRM fraction. When the fractions were incubated after the addition of exogenous substrate for γ-secretase, C99-FLAG, Aβ production was measured by sandwich ELISA and it was inhibited by L-685,458. Thus, mature and active γ-secretase complexes are localized to DRMs.

Prior to DRM preparations from postmortem human brain, the effect of postmortem time on γ-secretase activity was tested. The rats were sacrificed, heads were kept at conditions which simulated a slow cooling curve (as in the case of the human brain) (Spokes and Koch, 1978), and the brains were analyzed for γ-secretase activity at different postmortem hours. Human brain cortex with 22 hours of postmortem time was used in this study, and γ-secretase should still be active but with reduced activity, according to these results. As expected, DRMs from human brain also showed higher γ-secretase activity than other fractions.

For further chacterization, the DRM fraction was subjected to size exclusion chromatography (SEC) using a Superose 6 SEC column, and fractions were collected and analyzed by western blottiing. All of the γ-secretase components, APP, and flotillin-1 was found in a high molecular weight fraction, > 2000 kDa. This indicates the size of DRMs, and they are likely to contain γ-secretase complexes, many other proteins, lipids, and CHAPSO. In contrast to detergent soluble γ-secretase, DRM associated γ-secretase eluted in a high molecular weight fraction even when using a mobile phase containing 2% CHAPSO. Finally, confocal microscopy showed that PS1, nicastrin, and APP co-localized with the lipid raft marker, GM1 in human brain sections.

We conclude that γ-secretase is localized to DRMs corresponding to lipid rafts in postmortem human brain, and that γ-secretase is highly active in DRMs.

4.2 ACTIVE γ-SECRETASE AT SYNAPSES AND IN ENDOSOMES (PAPER II)

Here, we investigated the subcellular localization of γ-secretase using rat brain. Previously, the localization of γ-secretase has been extensively studied using cell culture. The γ-secretase complex is assembled in the ER and mature γ-secretase is found in the late secretory and/or endosomal pathway (Baulac et al., 2003, Capell et al.,

2005, Kaether et al., 2006, Siman and Velji, 2003, Vetrivel et al., 2004). Many studies have used cell lines that in some cases overexpressed the protein of interest. Since overexpression of certain proteins might affect protein expression in general and the assembly steps in different cellular compartments, these results should be interpreted with caution. However, subcellular localization studies on active γ-secretase in brain tissue has not been performed before.

In Paper II, we studied active γ-secretase in different subcellular compartments prepared by using an iodixanol gradient. We also investigated the localization of γ-secretase and its activity in synaptic vesicles and synaptic membrane preparations. For the iodixanol gradient, rat brains were homogenized and the mitochondria/synaptosome fractions were removed by centrifugation. The resulting 10, 000 x g supernatant was layered on a discontinous 2.5 to 30% iodixanol gradient and centrifugated at 126, 000 x g for 40 min. Fractions were collected from the bottom of the tube and analyzed for γ-secretase components and subcellular markers by western blotting. Fractions 5-7 (15 - 7.5% iodixanol) containnig N-Cadherin (plasma membrane marker) and syntaxin13 (endosome marker) correlated with enrichment of the γ-secretase complex components (PS1-CTF, PS2-CTF, nicastrin, Aph-1aL, Pen-2). Aβ production was measured by incubating the iodixanol gradient fractions at 37 °C for 16 hours in the absence or presence of the γ-secretase inhibitor, L-685,458. Aβ40 levels were detected by sandwich ELISA. Fractions 5-7 were highly enriched in endogenous Aβ40 production as well as in Aβ40 production from an exogenous substrate, C99. Fractions 5-7 indeed contained highly active γ-secretase. Together, these results indicate that highly active γ-secretase is present at endosomes and plasma membrane.

Secondly, crude synaptic membranes (SM) and synaptic vesicles (SV) were prepared by sucrose gradient centrifugation (Cohen et al., 1977) in order to investigate the presence of γ-secretase at synpases. AD is caused by loss of synapses followed by neuronal death, and therefore it is interesting to investigate γ-secretase at synapses. Previously, only PS has been found in synaptic compartments (Beher et al., 1999, Efthimiopoulos et al., 1998, Lah et al., 1997, Ribaut-Barassin et al., 2003). Prepared SM and SV fractions showed the expected structures by electron microscopy and were indeed enriched in organelle maker proteins (PSD-95/N-Cadherin and synaptophysin, respectively) by western blotting. Even though no clear enrichment of γ-secretase complex components in SM and SV fractions was observed, Aβ40 production was high in both fractions. AICD production was also enriched in SM fractions. Interestingly, incubation of a SV fraction did not show any AICD production. To check the stability of AICD in the SV fraction, a SV fraction was incubated with synthetic AICD. Interestingly, no AICD could be detected after incubation. Thus, SV might contain AICD degrading activity. γ-Secretase components were also detected in highly pure SV fractions, which were prepared by controlled pored glass chromatography. Finally, as shown by confocal microscopy, active γ-secretase labelled by a biotinylated γ-secretase inhibitor co-stained with synapses and endosomes. In summary, SM and SV contain highly active γ-secretase.

In conclusion, using brain material, we found high γ-secretase activity in a fraction enriched in endosomes and plasma membrane as well as in synaptic membranes and synaptic vesicles.

4.3 AFFINITY PURIFICATION OF γ-SECRETASE AND GSAPS (PAPER III)

Four proteins appear to be sufficient for γ-secretase activity, but it is possible that other proteins could have a regulatory role. For instance, a recent study showed that TMP21, a protein involved in protein transport and quality control in the ER and Golgi, interacted with γ-secretase and decreased Aβ production (Chen et al., 2006). CD147 was also recently identified to have a regulatory role for γ-secretase activity (Zhou et al., 2005). Furthermore, γ-secretase has several different substrates, and it is possible that unknown proteins interact with γ-secretase and regulate processing of these substrates. In clinical studies, inhibition of γ-secretase showed side effects in patients such as gastrointestinal bleeding, probably due to inhibition of notch processing (Siemers et al., 2006). Therefore, it has been a great interest to find novel γ-secretase associated proteins which could be modulated in such a way that Aβ production is reduced without disturbing the processing of other substrates by γ-secretase. In order to find novel GSAPs in human and rat brain, an efficient way to affinity purify γ-secretase using a γ-secretase inhibitor coupled to biotin by a cleavable linker (GCB) was developed in Paper III.

In this study, we used CHAPSO-soluble γ-secretase. Previously, we have tried affinify purification of γ-secretase using another inhibitor, but it was not efficient for reproducible pulldown probably due to a too short linker (data not shown). Then GCB was designed by coupling L-685,458 to a biotin moiety with a cleavable site (disulfied bond) via long hydrophilic linker (around 70 Å). The use of biotin enabled us to capture the complex with streptavidin beads. GCB has a longer linker than the previous inhibitor we tested, and the importance of the linker was also confirmed by another group (Placanica et al., 2009). To validate how effectively this GCB compund binds to γ-secretase, compared to the well-known L-685,458, the half maximal inhibitory concentration (IC50) was measured by quantifying Aβ production. CHAPSO-solubilized membrane fractions from BD8-PS1 cells were incubated in the absence or presence of L-685,458 or GCB at different concentrations (1, 10, 100, and 1000 nM). The result showed that GCB had an IC50 value at around 13 nM, which was similar to L-685,458 (IC50=10 nM). This indicates that GCB can be used for pulldown of γ-secretase.

The conditions for the affinity purification method were optimized. PS1-NTF, PS2-CTF, and nicastrin were quantified by western blot analysis after pulldown with GCB at different concentrations. The recovery of γ-secretase reached a plateau at around 300 nM of GCB. Therefore, we decided to use GCB at 200 nM for pulldown. For the analysis by liquid chromatography – tandem mass spectrometry (LC-MS/MS), the condition for elution of γ-secretase from the magnetic streptavidin beads was set to 0.01% Rapigest supplemented with 10 mM DTT. This mild elution condition gave a good recovery of PS1, nicastrin, Aph-1, and Pen-2 and also low background levels, which is important for the LC-MS/MS analysis. Different numbers of washing times after affinity purification were also checked and three washes before elution was found suitable.

The eluted proteins which were prepared from rat brain were digested by trypsin, which cleaves after arginine or lysine, and the resulting peptides were analyzed by LC-MS/MS. The MS/MS spectra were searched against the NCBI sequence data bank, using the Mascot software. This approach allows an unbiased identification of proteins. The sample that had been incubated in the presence of the competing γ-secretase inhibitor, L-685,458, before the incubation with GCB, served as control. Nicastrin and PS were readily detected only in the absence of the competing inhibitor, with highly significant scores of 315 and 131, respectively. Proteins identified with a score higher than 40 in the Mascot search are considered to be significant hits. The same sequences that identified PS1 and nicastrin in this study were also found in another study (Wakabayashi et al., 2009). Interestingly, we could also detect TMP21 (score=416) and syntaxin1 (score=119), both by mass spectrometry and western blotting. Previously, TMP21 was reported as a GSAP and reduced Aβ production (Chen et al., 2006) but the specific γ-secretase inhibitor, Merck C, did not co-purify TMP21 (Winkler et al). Nevertheless, TMP21 was clearly associated with active γ-secretase in our experiments. As shown by GCB pulldown, only a minor amount (less than 2%) of TMP21 was associated with γ-secretase, and this could explain this discrepancy. Syntaxin1 was previously reported to be a PS1-associtated protein (Smith et al., 2000). We found that less than 1% of syntaxin1 was associated with active γ-secretase. Our finding shows that TMP21 and syntaxin1 are novel GSAPs in brain. In summary, 91 proteins including the known γ-secretase components (PS1 and nicastrin), TMP21, and syntaxin1 were identified specifically in rat brain. Around 80% of identified proteins had origins of membrane proteins according to UniProt database. Currently, identified proteins are being validated in terms of their effect on Aβ production.

The GCB pulldown method was also applied to human brain samples. Western blotting showed that the pulldown of the four known γ-secretase components was specific, but the recovery was lower than from rat brain samples. The affinity purified samples were trypsinized, pre-fractionated by strong cation exchange (SCX) Zip Tips, analysed by LC-MS/MS, and compared by using the SpectrumMill software. The SpectrumMill software is convenient to compare vast amount data from several samples side by side. Resulting identified proteins with significant scores (> 12, equivalent to a significant score of 40 by Mascot) included the four γ-secretase components, PS1 (score=51.1), nicastrin (37), Aph-1b (13), and Pen-2 (15). Fifty other proteins were also identified, and many of these are currently under investigation with respect to their effect on Aβ production. Examples of GSAPs are transmembrane 9 superfamily member 2 precursor, succinate dehydrogenase flavoprotein subunit, zinc finger protein 532, ribosome-binding protein 1, fatty acid synthase, and melanoma inhibitory activity protein 3 precursor.

In addition, the C-terminus of the PS1-N-terminal fragment was identified from MS/MS spectra using the SpectrumMill software. Previously, the N-terminus of PS1-C-terminal fragment was reported (Shirotani et al., 1997) but the C-terminus of the PS1-N-terminal fragment has not been reported. In this search, one non-tryptic cleavage site was allowed. One PS1 fragment (MLVETAQERNETLFPALIYSST) ending at residue 291 (threonine) was identified and it is most likely the natural cleavage site of full length PS1. The previous data with N-terminal sequencing of

PS-CTF from HEK293 cells are in line with our finding (Podlisny et al., 1997). This indicates that there is no additional processing after the initial cleavage on PS1.

4.4 IDENTIFICATION OF NOVEL GSAPS IN DRMS (PAPER IV)

Since we have shown that γ-secretase is enriched in DRMs (Paper I) and GCB pulldown was an efficient way to purify functional γ-secretase (Paper III), we isolated DRMs from brain enriched in γ-secretase and identified novel components from this fraction by using GCB pulldown, followed by LC-MS/MS analysis.

We have shown by western blotting that DRMs from brain contained the γ-secretase components (PS1, nicastrin, Aph-1aL, Pen-2), small amounts of BACE, full length APP and APP-CTFs (Paper I). SEC showed that DRMs eluted in the void volume (around 2000 kDa), which is more than the sum of molecular weights of the four γ-secretase components (around 220 kDa at a stoichiometry of 1:1:1:1). Protein determination by the BCA protein assay and lipid analysis by thin layer chromatography (TLC) indicated that one DRM fraction approximately contained 40 µg of protein, 3 µg of cholesterol, 0.4 µg of sphingomyelin, and also CHAPSO (data not shown). These data from SEC and TLC suggest that DRMs may contain several copies of γ-secretase complexes, other proteins, lipids and CHAPSO. Therefore, it might be possible to find novel GSAPs in DRMs.

Firstly, DRMs were prepared from rat brain by sucrose gradient centrifugation. Electron microscopy showed that DRMs contained vesicle-like structures around 230 nm in diameter. This means that several hundreds of protein molecules could be present in one vesicle (Takamori et al., 2006). To find out what protiens that were present, the DRMs were analyzed by LC-MS/MS. The DRMs contain high amounts of lipids which interfere with LC-MS/MS analysis. Therefore, the DRMs were treated in two different ways, either by lipid extraction (Folch method) or protein precipitation (aceton precipitation) before the protein fraction was digested and subjected to mass spectrometry. In total, 212 proteins were identified by the Folch method and 242 proteins by aceton precipitation. 173 proteins were found in common between the two different extraction methods. Even though we could detect the known γ-secretase components, PS, nicastrin, Aph-1, and Pen-2 by western blotting, they were not identified by mass spectrometry. This was probably due to the relatively low percentage of γ-secretase present in DRMs compared to other proteins in DRMs. However, some previously reported GSAPs, syntaxin1 and CD147 were identified with significant scores (SpectrumMill score 52 and 15, respectively). DRMs also contained many of the known lipid raft markers including flotillin-1, flotillin-2, and many other proteins (Adam et al., 2008, Martosella et al., 2006, Raimondo et al., 2005). Even though our membrane preparation prior to a DRM preparation included a 10,000 × g centrifugation step in order to remove mitochondria, there were some mitochondrial proteins identified, such as ATP synthase subunits α and β, and dihydrolipoyl dehydrogenase. These might come from contamination of mitochondria in our samples. Another possibility is that those proteins might exist also in other organelles.

After characterization of the starting material, the DRM associated γ-secretase was incubated with GCB in the absence or presence of an excess amount of the competing

inhibitor, L-685,458, followed by pulldown using streptavidin-conjugated magnetic beads as in Paper III. However, the non-specific binding of proteins in the control sample (+L-685,458) was higher than in our previous study on soluble γ-secretase. Therefore, the protocol was optimized for DRMs. The concentration of L-685,458 was increased from 10 μM to 50 μM, and 1% CHAPSO instead of 0.5% CHAPSO was used for washing the beads, in order to reduce non-specific binding to control sample. Western blotting showed that PS1-NTF, nicastrin and TMP21 were specifically captured by GCB in DRMs. Compared to our previous results based on CHAPSO-soluble γ-secretase, TMP21 was clearly more enriched, and to a larger extent associated with γ-secretase, in DRMs. This might indicate that TMP21 regulates γ-secretase activity especially in lipid rafts. However, this needs to be shown experimentally in the future.

For analysis by LC-MS/MS, the samples were eluted with Rapigest and DTT after removing non-specific binding proteins by washing. The eluate was trypsinized, concentrated by using a Zip Tip, and injected into the LC-MS/MS system. The sequential elution by sample buffer after Rapigest elution showed that most of proteins were efficiently eluted by Rapigest and the background levels were low, as shown by Colloidal Gold staining. Western blotting also showed that the elution of PS1-NTF and nicastrin was efficient. However, there is still PS1-NTF left in the +L-685,458 sample indicating the different efficiency of elution between –L685,458 and +L-685,458 samples. We took this different efficiency in elution between samples into account when the data with identified proteins were calculated.

The eluate was trypsinized and injected into the LC-MS/MS system. The data from three samples of –L,685,458 and three from +L-685,458 (three independent experiments) were compared by using the SpectumMill software. Some proteins were either found only in –L-685,458, only in +L-685,458, or in both samples. The spectra mean intensity of each identified peptide was normalized to the total intensity of all peptides identified in the sample. The intensity ratio was then calculated by dividing the spectra mean intensity of the non-specific binding proteins (found in the presence of a competing inhibitor) by the one of the specific binding proteins (found in the absence of a competing inhibitor). We set our protein identification criteria to be strict enough to select candidate proteins for the further functional studies. Firstly, it should have a minimum protein score 10 in order to be significant identification, (equivalent to a significant score at around 40 in the Mascot search). Secondly, each protein should have minimum of two unique peptides identified. After the first criteria, we could detect total 194 proteins including GSAPs, TMP21 and syntaxin1, lipid raft marker proteins, and other possible γ-secretase associated proteins. Thus, TMP21 and syntaxin1 may interact with detergent soluble as well as DRM associated active γ-secretase. As in the proteome of DRMs, none of the four γ-secretase components were identified. The reason could be that it is more difficult to identify γ-secretase in DRM samples since the pulldown efficiency with GCB is lower than in the case of CHAPSO-soluble γ-secretase. We can also speculate that GCB might capture a complex mixture of proteins and/or lipids from DRMs and that the relative percentage of γ-secretase in this mixture might be low. Moreover, the higher concentraion needed for competition may indiciate that several γ-secretase complexes reside in the same DRM.

For functional studies, we chose a group of proteins identified only in the absence of L-685,458 at least two out of three experiments. Six proteins were identified in all three experiments and 14 proteins in two out of three experiments. In addition, two extra proteins were selected; solute carrier family 6, member 17 was found two times uniquely in the absence of a competing inhibitor and one time with higher scores in -L-685,458. Sortilin1 (SORT1) was found with less significant scores, and it was selected since it is similar to sortilin-related receptor (SORL1), which showed a genetic association with AD in a previous study (Rogaeva et al., 2007). In summary, 22 possible GSAPs were selected from DRMs. These proteins are trafficking proteins, transporters or channel proteins, mitochondria related proteins, a signaling protein, and a chaperone.

From an AD perspective, the effect of the potential GSAPs on Aβ production is highly interesting. Hence, we measured Aβ production by sandwich ELISA after silencing candidate genes by siRNA transfection in HEK-293 APP695 cells. Firstly, the expression patterns of mRNA and proteins in organs including brain were checked by the public database (BioGPS and UniProt), and proteins which are expressed in brain were selected. The gene expression patterns in HEK-293 cells were checked by Affymetrix and Nimblegen, and genes which are not expressed in HEK cells were excluded. As a result, the number of proteins to study were narrowed down to eight candidates: voltage-dependent anion channel 1 (VDAC1), guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β-1 (GNB1), excitatory amino acid transporter 1 (SLC1A3), sodium- and chloride-dependent GABA transporter 3 (SLC6A11), cytochrome C oxidase subunit IV isoform 1 (COX4l1), syntaxin12 (STX12), solute carrier family 6, member 17 (SLC6A17), and sortilin1 (SORT1). Twenty-six small interfering RNAs (siRNAs) directed to the eight candidate genes were transfected into HEK-293 APP695 cells. Gene knock-down efficiencies were evaluated by real-time PCR (RT-PCR) and cell viability was measured by the Alamar Blue assay. We set more than 70% gene knock-down levels and more than 70% cell viability as criteria for the siRNAs to be further studied. siRNAs to *SLC1A3*, *SLC6A11*, and *SLC6A17* genes gave less than 70% of gene knock-down and they were excluded. siRNAs directed to *GNB1* was removed due to their toxic effects. However, we can not exclude the possibility that these are still interesting candidates as GSAPs since we only tested limited numbers of siRNAs for those genes. In the next step, Aβ40 and 42 levels were measured three times by sandwich Aβ ELISA after transfection with the remaining siRNAs. Aβ levels of each siRNA were divided by cell viability data. Sortilin1 did not give any significant Aβ lowering effect by silencing *SORT1*. For *COX4l1* and *STX12*, different siRNAs for the same gene gave slightly different effects on Aβ reduction and it was a moderate effect. The data also varied between the three experiments. VDAC1 was the only one which constantly gave more than 30% Aβ reduction in an siRNA dose dependent manner throughout all three experiments. None of these have previously been reported to associate with γ-secretase and regulate Aβ production.

Syntaxin12 (syntaxin12 in rat and syntaxin13 in human) is a SNARE protein involved in fusing/budding of early/recycling endosomes. Endocytosis has been shown to play an important role in the trafficking of APP, BACE, and γ-secretase in cells. Interestingly, individual lipid rafts containing BACE or APP have been suggested to

fuse into one unit during endocytosis, enabling APP to be processed (Ehehalt et al., 2003). Another study found that the γ-secretase in lipid rafts co-fractionated with syntaxin13 (as a marker for endosomes) (Vetrivel et al., 2004) and we have seen the presence of active γ-secretase in endosomes (Paper II). The moderate Aβ reduction upon knockdown of syntaxin12 suggests that suppressing syntaxin12 might have affected the γ-secretase trafficking process in endosomes. It should be verified experimentally whether endosomal lipid rafts were affected by siRNA transfection with *STX12*.

Cytochrome C oxidase is a mitochondrial protein and component of the respiratory chain. Previously, γ-secretase has been found in mitochondria (Hansson et al., 2004), and recently it was shown that cytochrome C oxidase deficiency reduces the amyloid burden in transgenic mice overexpressing Aβ (Fukui et al., 2007). This finding is in line with our data, showing knockdown on *COX4l1* reduced Aβ production in HEK-293 APP695 cells.

Voltage-dependent anion channel protein (VDAC*)* is a major component of the outer mitochondrial membrane and is also present at the neuronal plasma membrane (pl-VDAC) (Ferrer, 2009, Ramirez et al., 2009). VDAC accumulates in amyloid plaques in APP/PS1 transgenic mice (Ferrer, 2009), and nitrated VDAC1 has been shown to be increased in AD (Sultana et al., 2006). In the human brain, pl-VDAC forms a complex with estrogen receptor alpha (ERα) and caveolin-1, which is a marker protein for a lipid raft structure called caveolae. As shown by immunoprecipitation, the pl-VDAC/caveolin-1 complex is highly expressed in AD brains compared to healthy controls (Ramirez et al., 2009). This might indicate that VDAC1 is indeed associated with DRMs, where γ-secretase is located.

By using a cell-system, overexpressing tagged PS that enables tandem affinity purification, De Strooper and co-workers identified proteins in the tetraspanin web (EWI-F, CD81, CD98hc) and CD9 as novel GSAPs, and confirmed the association of TMP21 (Wakabayashi et al., 2009). Two of our candidates, voltage-dependent anion channel 1 and guanine nucleotide-binding protein G, were also among the proteins identified as potential GSAPs in that study. Thus, although we have taken a novel approach for purification of GSAPs, and purified them from brain, some of the candidates we find have been found in other studies, supporting our notion that the GSAPs we find are relevant.

In summary, we identified several possible novel GSAPs in DRMs, and three of these, voltage-dependent anion channel 1, cytochrome C oxidase subunit IV isoform 1, and syntaxin12, affect γ-secretase activity.

5 CONCLUSIONS

In this thesis, the goal was to characterize γ-secretase, especially with respect to its localization and to identify novel GSAPs in mammalian brain. Since AD is a disease of the brain, we studied γ-secretase using human and rat brain. The major findings of Paper I – IV are listed below.

1. γ-Secretase is enriched in DRMs prepared from brain. γ-Secretase in DRMs is highly functional and can cleave APP substrates to release Aβ and AICD.

2. The estimated size of DRMs containing γ-secretase is > 2000 kDa. The DRMs contain γ-secretase components, many other proteins, and lipids.

3. Active γ-secretase is localized to endosomes, plasma/synaptic membranes, and synaptic vesicles prepared from rat brain.

4. An efficient affinity purification method, using a γ-secretase inhibitor coupled to a cleavable biotin group (GCB), was developed. After affinity purification followed by mass spectrometry, all of the known γ-secretase components were identified. Furthermore, previously reported GSAP, TMP21, was confirmed to associate with active γ-secretase. The PS associated protein syntaxin1 was also identified as a GSAP in brain.

5. By using GCB, we identified several potential GSAPs in the soluble microsomal fraction. A subset of these is being investigated with respect to their effect on Aβ production.

6. We identified novel GSAPs in DRMs from rat brain. Three novel GSAPs were shown to affect Aβ production: voltage-dependent anion channel 1, syntaxin12, and cytochrome c oxidase subunit IV isoform 1.

In summary, we suggest that γ-secretase activity and substrate selection can be regulated by its microdomain environment, its subcellular localization, and by γ-secretase associated proteins. This knowledge may be helpful for the development of drugs for curing AD by selective inhibition of APP processing by γ-secretase.

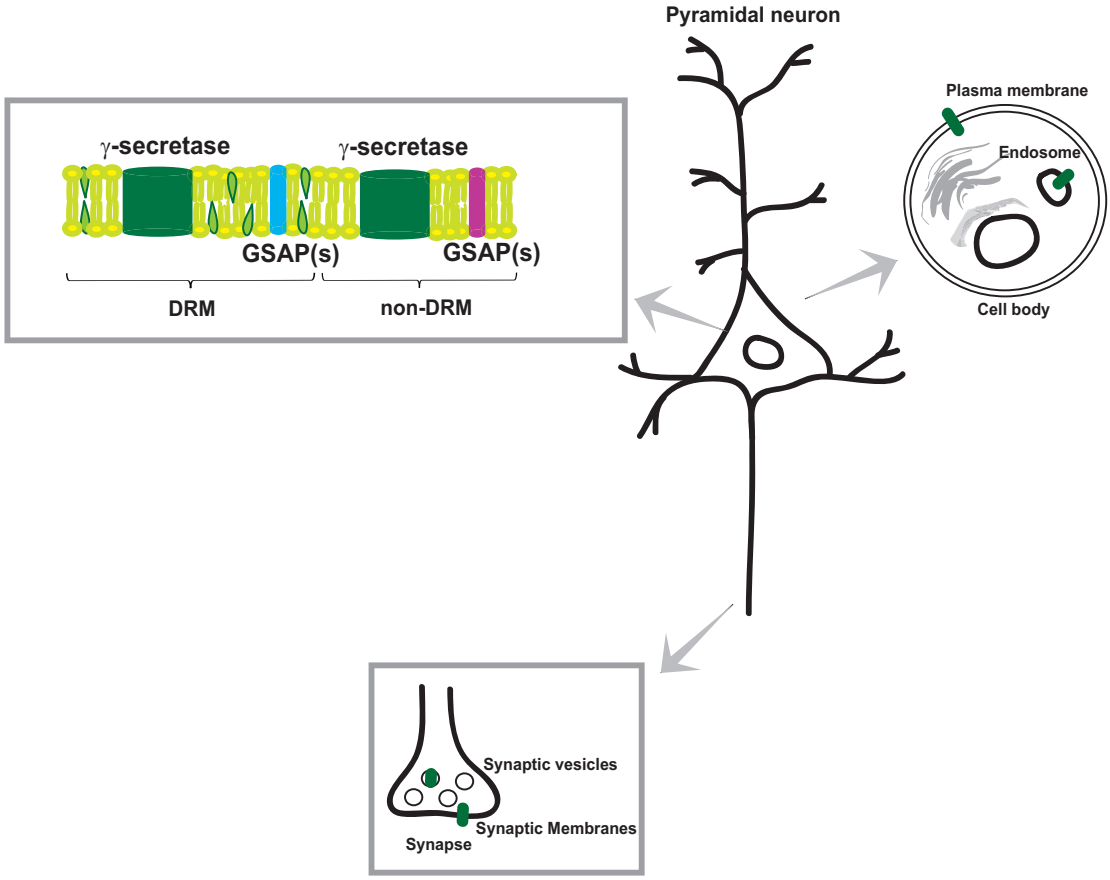

Figure 3. Schematic drawing of the major findings of Paper I-IV.

6 FUTURE PERSPECTIVES

In this thesis, we characterized γ-secretase in respect to the localization of its activity in brain, and identified novel GSAPs.

In Paper III and IV, candidates for novel γ-associated proteins were discovered after affinity pulldown of γ-secretase using the γ-secretase inhibitor, GCB. For the candidates found in Paper III, studies on their effect on Aβ production is currently ongoing. Three novel GSAP candidates found in DRMs, voltage-dependent anion channel 1, syntaxin12, and cytochrome C oxidase subunit IV isoform 1 will be further investigated for their role in Notch processing. Similar to measuring Aβ production after siRNA transfection in cell lines, levels of Notch intracellular domain (NICD) can be measured. Proteins with different effects on APP and Notch processing could be potential targets for drugs aimed at lowering Aβ-production. γ-Secretase processes more than 60 substrates and it is important to know whether inhibiting a specific GSAP affect other signaling pathways of relevant γ-secretase substrates. Furthermore, the GSAP candidates we have identified but not further investigated could be tested for Aβ lowering effects using different cell systems. For the general interest, the localization of the novel GSAPs in lipid rafts and subcellular compartments can be studied by using the methods described in Paper I and II. Their interaction with known γ-secretase components could be studied by co-immunoprecipitation (Farmery et al., 2003) and affinity purification with GCB (Paper III), and the complex assembly step between our novel GSAPs and γ-secretase by 2D PAGE (Fraering et al., 2004, LaVoie et al., 2003).

Currently, it is not known how the candidates from Paper III and IV could modulate γ-secretase activity. Many of the identified proteins are membrane proteins having a role in trafficking, as for instance, TMP21. Based on many studies, including Paper I and II, we have gained more insights about how and where APP is processed by β- and γ-secretases in cells and membranes, and endosomes are suggested as active sites for APP processing. Since syntaxin12 seems to be important for endocytosis and voltage-dependent anion channel 1 is present in lipid rafts, the trafficking process of γ-secretase into endosomal lipid rafts can be evaluated further to decrease Aβ production.

The final goal of these studies is to contribute more information on the mode of action for γ-secretase, and thereby help us to validate γ-secretase as a potential drug target for treating Alzheimer's disease.

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