Thesis for doctoral degree (Ph.D.)
2009

Alzheimer Disease:

Studies on Aβ and γ-secretase in human brain

Hedvig Welander

Karolinska Institutet

Thesis for doctoral degree (Ph.D.) 2009

Alzheimer disease: Studies on Aβ and γ-secretase in human brain

Hedvig Welander

Karolinska Institutet

From The Department of Neurobiology, Care Sciences and Society (NVS)
KI-Alzheimer Disease Research Center
Karolinska Institutet, Stockholm, Sweden

Alzheimer disease: Studies on Aβ and γ-secretase in human brain

Hedvig Welander

Karolinska Institutet

Stockholm 2009

Cover picture: A Congo stained plaque core, reproduced with permission from the publisher.

Published by Karolinska Institutet. Printed by Larserics Digital Print AB.

ISBN 978-91-7409-727-6

Droppen urholkar stenen inte genom sin tyngd
utan genom att falla ofta

ABSTRACT

Alzheimer disease (AD) is a devastating neurodegenerative disorder and the most prevalent form of dementia. One hallmark of the disease is the extracellular deposition of amyloid β-peptide (Aβ) into senile plaques in the brain. Biochemical and genetic studies reveal Aβ as a key player in AD pathogenesis. The most common forms of Aβ are 40 (Aβ40) or 42 (Aβ42) residues long. Aβ40 is produced at higher levels than Aβ42; while Aβ42 is more hydrophobic, prone to aggregate, and form the toxic species. Thus, the length of the hydrophobic C-terminus of Aβ is very important for oligomerization and neurotoxicity. Aβ is generated through sequential processing of the amyloid β- precursor protein (APP) by the enzymes β- and γ-secretases. The γ-secretase cleavage is performed by a transmembrane protein complex containing presenilin (PS), nicastrin (Nct), anterior pharynx defective-1 (Aph-1), presenilin enhancer-2 (Pen-2), and possibly other components. The biological understanding of γ-secretase remains elusive, as does the mechanism by which Aβ causes neurodegeneration in AD.

The work presented in this thesis has focused on studies of γ-secretase activity and localization in mammalian brain, as well as on identification and quantification of Aβ species that are deposited in AD brains. In **paper I**, a detailed analysis was performed to see how active γ-secretase is best prepared from brain material, and in what subcellular fraction the activity is highest. The γ-secretase activity was highly affected by detergents; and the fraction containing endosomes, endoplasmic reticulum, Golgi and synaptic vesicles revealed the highest activity. It was possible to measure Aβ production under the optimized conditions. In **paper II**, active γ-secretase was further studied in detergent resistant membranes (DRMs). Active γ-secretase was localized to DRMs in human and rat brain. The size of DRMs containing active γ-secretase, and possibly other proteins and lipids, was estimated to be > 2000 kDa. Furthermore, it was possible to measure Aβ production in DRMs. In **paper III** and **paper IV** the product of amyloidogenic γ-secretase cleavage was studied and a detailed investigation performed on Aβ species deposited in AD brains. A method was established for quantification of C-terminal Aβ species in purified plaque cores and in total amyloid preparations from sporadic and familial AD brains. It was found that a longer Aβ species, i.e. Aβ43, was more frequent than Aβ40. Immunohistochemistry that was performed supported these findings. In **paper IV**, Aβ species were quantified in six different brain regions obtained from two mutation carriers having the I143T *PSEN1* mutation, reported here in Sweden for the first time. As in **paper III**, Aβ43 was much more frequent than Aβ40.

In conclusion, we have determined the optimal conditions for studies of active γ-secretase in brain and have showed that this active enzyme complex is localized to lipid rafts in human and rat brain. Further, we have found a longer Aβ species, Aβ43, to be more frequent than Aβ40 in amyloid depositions in AD brains. This species polymerizes rapidly, and we suggest that Aβ43 may be of importance in AD etiology.

LIST OF PUBLICATIONS

I. Jenny Frånberg, **Hedvig Welander**, Mikio Aoki, Bengt Winblad, Lars O Tjernberg and Susanne Frykman
Rat brain γ-secretase activity is highly influenced by detergents
Biochemistry (2007) 46, 7647-7654

II. Ji-Yeun Hur, **Hedvig Welander**, Homira Behbahani, Mikio Aoki, Jenny Frånberg, Bengt Winblad, Susanne Frykman and Lars O Tjernberg
Active γ-secretase is localized to detergent-resistant membranes in human brain
The FEBS Journal (2008) 275, 1174-1187

III. **Hedvig Welander**, Jenny Frånberg, Caroline Graff, Erik Sundström, Bengt Winblad and Lars O Tjernberg
Aβ43 is more frequent than Aβ40 in amyloid plaque cores from Alzheimer disease brains
Journal of Neurochemistry (2009) 110, 697-706

IV. *Lina Keller, ***Hedvig Welander**, Huei-Hsin Chiang, Lars O Tjernberg, Inger Nennesmo, Åsa K Wallin and Caroline Graff
The PSEN1 I143T mutation in a Swedish Alzheimer family: Clinical report and quantification of Aβ variants in different brain regions
Manuscript

*** These authors contributed equally**

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid β-peptide
AD	Alzheimer disease
ADDLs	Aβ-derived diffusible ligands
AICD	APP intracellular domain
ApoE	Apolipoprotein E
Aph-1	Anterior pharynx defective-1
APP	Amyloid β- precursor protein
APLP	APP-like protein
APPSwe	Swedish APP mutation (K670N/M671L)
BACE	β-site APP cleaving enzyme
CAA	Cerebral amyloid angiopathy
CNBr	Cyanogen bromide
CMC	Critical micelle concentration
CSF	Cerebrospinal fluid
CTF	C-terminal fragment
DRMs	Detergent resistant membranes
DS	Down syndrome
ELISA	Enzymed-linked immunosorbent assay
EOAD	Early onset AD
FA	Formic acid
FAD	Familial AD
HPLC	High performance liquid chromatography
IP	Immunoprecipitation
LC-MS/MS	Liquid chromatography combined with tandem mass spectrometry
MS	Mass spectrometry
Nct	Nicastrin
NFT	Neurofibrillary tangles
NICD	Notch intracellular domain
Pen-2	Presenilin enhancer-2
PS	Presenilin
PS1 and PS2	Presenilin 1 and 2
RIP	Regulated intramembrane proteolysis
SAD	Sporadic AD
SDS	Sodium dodecyl sulfate
SP	Senile plaque
TFE	Trifluoro ethanol
TBS	Tris-buffered saline
Tht	Thioflavin T
WB	Western blotting

INTRODUCTION

ALZHEIMER DISEASE

Alzheimer disease (AD), recognized as the most common form of dementia, is a progressive neurodegenerative disorder characterized by memory dysfunction and cognitive impairment. AD was originally described by the German physician, Alois Alzheimer, who in 1906 found remarkable pathological changes in the post-mortem brain from his demented patient Auguste D (Alzheimer, 1907). Age is the major risk factor for AD as the number of cases increases exponentially with advancing age. The estimated prevalence is around 1 % for people over the age of 65, and 20 % for individuals over 85 years of age (Lobo et al., 2000). Due to a global increase in life expectancy AD is a growing problem that will bring even greater cost for society and more immense suffering for the victims and their families. The duration of the disease is 5-15 years and the cause of death is often a secondary illness such as pneumonia. There is no cure for AD at present and the current pharmacological treatments are only symptomatic.

Neuropathology of Alzheimer disease

Macroscopically, AD is characterized by cortical atrophy with enlargement of ventricles and widening of sulci. The most severly affected brain areas include temporal and parietal lobes, and parts of the frontal cortex and the cingulate gyrus. Two major neuropathological hallmarks in AD observed at the microscopic level are extracellular senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs) (Figure 1). Other observations associated with AD pathology include loss of synapses and neurons, activated microglia and astrocytes, dystrophic neuritis, and cerebral amyloid angiopathy (CAA). SPs are extracellular deposits mainly composed of fibrils formed by the β-peptide (Aβ) whereas NFTs are composed of hyperphosphorylated tau protein.

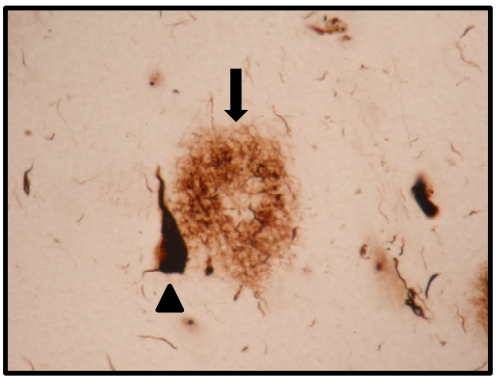

Figure 1. The main hallmarks in AD. An extracellular senile plaque (arrow) and intracellular neurofibrillary tangles (arrow head). Courtesy of Dr. Nenad Bogdanovic.

Plaques are divided into two major categories based on their morphology - neuritic plaques and diffuse plaques. The neuritic plaques stain positive for Congo red, which is a β-sheet specific dye for amyloid structures. The definition of amyloid has varied to some extent over the years; but amyloid is now defined as an extracellular deposit of protein fibrils having a typical organization that features characteristic properties observed after staining with Congo red (Westermark et al., 2005). However, the term amyloid has wider usage as it may refer also to intracellular depositions in disorders termed amyloidoses (Westermark et al., 2005) seen for example, in Creutzfeldt-Jakob disease, Parkinson disease and Lewy body dementia.

Neuritic plaques (sometimes called mature plaques or SPs) are associated with typical AD pathology, such as dystrophic neurites, activated microglia and reactive astrocytes. The plaque cores are mainly composed of Aβ42, which is the species of particular importance in early plaque formation (Iwatsubo et al., 1994). The diffuse plaques (also referred to as preamyloid plaques) are Congo-negative Aβ42 deposits (Gowing et al., 1994) that are detectable using immunohistochemical methods. Diffuse plaques have been found in healthy, aged humans with normal intellectual status, leading to the hypothesis that the diffuse plaques represent precursor lesions to the neuritic plaques and might not be toxic. This hypothesis is supported by studies of transgenic mice that develop diffuse deposits before neuritic plaques and by studies of patients with Down's syndrome (DS). Individuals with DS develop AD relatively early in life and commonly display diffuse deposits already in their teenage years; but they do not show neuritic plaques until decades later (Lemere et al., 1996). Another type of plaque are the so called cotton wool plaques, which are bigger than both the neuritic and diffuse plaques. They consist mainly of Aβ42, are not congophilic, and have most often been observed in familial AD (FAD) (Shepherd et al., 2009).

While Aβ42 is the main species in the plaque types described above, Aβ40 is the most common peptide in CAA (Suzuki et al., 1994).

Genetics and risk factors of AD

AD is a complex disorder that results most likely from a combination of environmental and genetic factors. Most AD patients have no family history of the disease and are classified as sporadic AD cases (SAD). However, a few percent of all AD cases are inherited and are classified as familial forms (Saunders, 2001) with mutations in genes

encoding the amyloid precursor protein (APP), presenilin 1 (PS1) or presenilin 2 (PS2) (Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995). These mutations associated with familial AD (FAD) are typically linked to early onset (before 65 years of age). A nearly complete penetrance and an increased Aβ42/40 ratio are observed.

In the 1960s it was observed that patients with DS, who carried an extra copy of chromosome 21, and consequently an extra copy of the *APP* gene, later developed AD brain pathology (Olson and Shaw, 1969). In 1984, Glenner and Wong published two papers reporting on their discoveries of the amino acid sequence of Aβ through purification of amyloid from CAA in AD brains and DS brains (Glenner and Wong, 1984a; Glenner and Wong, 1984b). One year later, Masters and co-workers isolated Aβ from plaque cores in AD and DS brains (Masters et al., 1985). Taken together, these findings eventually lead to the discovery that Aβ is derived from APP and in 1987 the *APP* gene was mapped to chromosome 21 (Kang et al., 1987). *APP* was later the first gene to be linked to FAD (Goate et al., 1991).

All pathogenic *APP* mutations linked to FAD reported to date are located within the Aβ peptide region or in the close proximity of the protease cleavage sites. They thereby influence either APP processing and Aβ production, or the production of Aβ species that have increased inclination to assemble into neurotoxic fibrils. To date, 32 mutations in the *APP* gene are known (see the AD mutation database for an updated list: http://www.molgen.ua.ac.be/ADMutations/). The Swedish mutation (*APP*Swe) (Mullan et al., 1992), which immediately precedes the beginning of the Aβ sequence, is a double point mutation that causes substitution of two adjacent amino acids, i.e. LysMet to AsnLeu. The *APP*Swe mutation causes an increased production of total Aβ (Citron et al., 1992; Citron et al., 1994; Scheuner et al., 1996). Another example of an *APP* mutation is the Arctic mutation, in which a glutamic acid in the Aβ sequence is substituted by glycine at position 22. This mutation gives rise to reduced extracellular Aβ levels, both in culture media from transfected cells and in plasma from mutation carriers; but appears to give rise to production of soluble aggregation intermediates, so called protofibrils, that aggregate rapidly (Kamino et al., 1992; Nilsberth et al., 2001). The London mutation is located C-terminally of the Aβ region and causes an amino acid substitution from valine to isoleucine (Goate et al., 1991). This mutation gives rise to an increased Aβ42/40 ratio. Other examples of *APP* mutations are the Flemish (Hendriks et al., 1992) and Dutch (van Broeckhoven et al., 1990; Levy et al., 1990)

mutations both located close to the α-secretase cleavage site. These mutations give rise to severe amyloid angiopathy and/or presenile dementia and hereditary cerebral hemorrhage with amyloidosis, Dutch Type.

Mutations in the *PS1* gene located on chromosome 14 and in the *PS2* gene located on chromosome 2 are also linked to FAD. To date, 177 pathogenic *PSEN1* mutations and 14 pathogenic *PSEN2* mutations are known (see the AD mutation database for an updated list: http://www.molgen.ua.ac.be/ADMutations/). The mutations located in the presenilin genes are suggested to result in an increased ratio of Aβ42/40 (Scheuner et al., 1996).

Besides advanced age, the next most important risk factor for AD is polymorphism in the apolipoprotein E (*APOE*) gene. In humans, the *APOE* gene has three alleles: ε2, ε3 and ε4. Carriers of the ε4 allele have an increased risk of developing AD and the ε4 allele is over represented, both in late onset FAD and in SAD cases. *APOE* ε4 seems to act in a dose dependent manner; carriers of one allele of ε4 have a three-fold risk for having AD; while two alleles of ε4 increase the risk 15 times (Corder et al., 1993). The mechanism by which the ε4 allele elevates the risk of developing late onset AD is not known. However, studies show a higher Aβ burden in the brains from AD patients carrying the *APOE* ε4 allele, suggesting that ApoE binds to Aβ. However, the *APOE* ε4 allele is not required for the development of AD, and many other unidentified disease genes probably exist. A large number of gene candidates have been suggested, for example *CLU* (also known as *APOJ*) and *PICALM* (Harold et al., 2009), but so far no association between these and AD has been verified.

There are several other suggested risk factors for AD in addition to advanced age and *APOE* ε4. Head trauma, female gender, cardiovascular disease, high cholesterol diet, hypertension, lack of social interactions and low education are some likely risk factors (Mayeux R., 2003).

MOLECULAR MECHANISMS OF AD

The Aβ precursor protein, APP

APP was cloned by Kang and co-workers in 1987 (Kang et al., 1987) and is a type 1 integral membrane spanning protein that is ubiquitously expressed throughout the body

(Dyrks et al., 1988). It has a large extracellular N-terminal domain, a transmembrane domain, and a short cytosolic C-terminal domain. APP belongs to a protein family that also comprises two homologues in mammals, including APP like proteins (APLP) 1 and 2 respectively. APP exists in three major isoforms of varying lengths - 695, 751 and 770 amino acids residues long, and each variant contains the Aβ domain. No other APP family member contains the Aβ sequence. The longer isoforms contain a Kunitz protease inhibitor domain (Kido et al., 1990). The APP695 isoform is primarily expressed in neurons (Weidemann et al., 1989). The biological function of APP still remains elusive; although several functions have been proposed, which implicate APP in neuronal protection and neural outgrowth (Milward et al., 1992; Olsson et al., 2004), as a cell adhesion molecule (Beher et al., 1996; Breen et al., 1991), and in axonal transport (Kamal et al., 2001). APP is transported along the secretory pathway, to reach the cell surface and undergoes several post-translational modifications, such as phosphorylation, N- and O-linked glycosylations and sulfation (Lyckman et al., 1998; Olstersdorf et al., 1990; Weidemann et al., 1989).

APP processing and generation of the Aβ peptide

In 1984, the Aβ peptide was isolated by Glenner and Wong (Glenner and Wong, 1984a; Glenner and Wong, 1984b). On the basis of further study, it was concluded that Aβ is the main component of senile plaques in brains from patients with AD and DS (Masters et al., 1985). The Aβ peptide is a cleavage product of APP, and during its intracellular transport, and when reaching the cell membrane, APP can be cleaved by different enzymes termed α-, β- and γ-secretases.

The cleavage of APP can be divided into either a non-amyloidogenic pathway or an amyloidogenic pathway (Figure 2). In the non-amyloidogenic pathway, APP is cleaved by α-secretase in the location between amino acids 16 and 17 within the Aβ region. This produces a soluble, secreted αAPP and an 83 amino acid C-terminal fragment, i. e. C83. This fragment, C83, is retained in the membrane, and is subsequently cleaved by γ-secretase, which produces a fragment called p3 and the APP intracellular C-terminal domain (AICD) (Kojro et al., 2005). Importantly, since cleavage by α-secretase occurs within the Aβ region, formation of Aβ is precluded. There are several candidates in the ADAM (a disintegrin and metalloprotease) family suggested to possess α-secretase activity, ADAM9, 10 and 17 (Koike et al., 1999; Lammich et al., 1999; Buxbaum et al., 1998).

The amyloidogenic pathway, on the other hand, leads to Aβ generation. The initial cleavage in this pathway is mediated by β-secretase (BACE) (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999) generating soluble βAPP that is secreted, and a 99 amino acid C-terminal fragment (C99). Subsequent cleavage of C99 by γ-secretase liberates Aβ as well as AICD. APP can also undergo cleavage by β-secretase at the so called β' site which is located after amino acid 10 yet within the Aβ region, resulting in C89 which is further processed by γ-secretase to generate a short Aβ variant (Aβ11-x) and AICD.

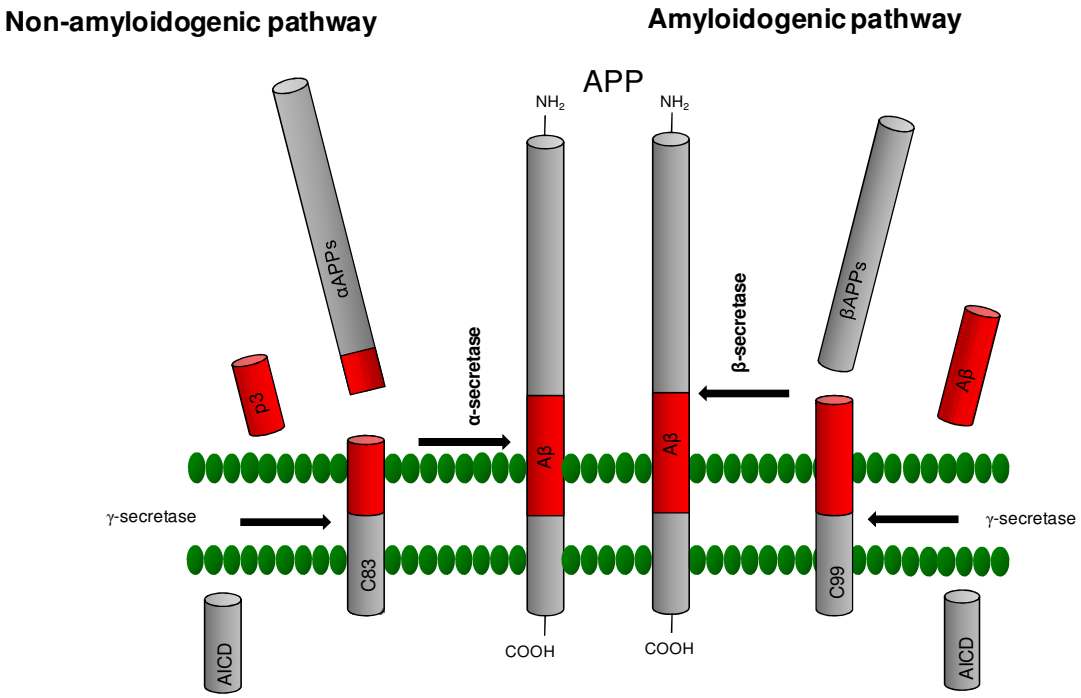

Figure 2. APP processing in the non-amylodogenic (left) and the amyloidogenic (right) pathways. APP is cleaved by α-secretase in the non-amyloidogenic pathway resulting in the C83 peptide which undergoes cleavage by γ-secretase producing the p3 peptide, AICD and the soluble sAPPα fragment. In the amyloidogenic pathway APP is cleaved by β-secretase resulting in the C99 peptide. Subsequent cleavage by γ-secretase results in the production of Aβ and AICD.

The γ-secretase complex

γ-Secretase is an enzyme complex located in the membrane and contains at least four subunits (Figure 3): Presenilin 1 or presenilin 2 (PS1 or PS2), nicastrin (Nct), anterior pharynx defective-1 (Aph-1) and presenilin enhancer-2 (Pen-2) (Edbauer et al., 2003). Endoproteolytical cleavage of PS between the sixth and seventh transmembrane domains gives rise to the N- and C-terminal fragments of PS. These two fragments form a heterodimer that is the active version of PS (Fraering et al., 2004). Knockouts of both PS1 and PS2 completely inhibit all γ-secretase activity (Herreman et al., 2000),

demonstrating that PSs are required for γ-secretase activity. Two aspartic residues (Asp257 and Asp385) within transmembrane domain 6 and transmembrane domain 7 of PS constitute the catalytic site (Wolfe et al., 1999). The second complex member identified is Nct, a type 1 transmembrane glycoprotein with a large extracellular ectodomain and a short cytoplasmic tail (Yu et al., 2000; Shah et al., 2005). Nct is suggested to be involved in stability of the γ-secretase complex, and may function as a substrate receptor (Shah et al., 2005). Aph-1 exists as two homologues in humans (Aph-1a and Aph-1b) and as three homologues in rodents (Aph-1a, Aph-1b and Aph-1c). Aph-1 has a topology of seven transmembrane domains, with the N-terminus facing the lumen and the C-terminus facing the cytosol (Fortna et al., 2004). Aph-1 seems to play a role as an initial scaffold in γ-complex assembly; while Pen-2 is required for endoproteolytic processing of PS (Takasugi et al., 2003).

The activity of γ-secretase can be reconstituted by co-expression of PS, Nct, Aph-1, and Pen-2 in *Saccaromyces cerevisiae*, which lack endogenous γ-secretase activity. These four proteins appear to be necessary and sufficient for γ-secretase activity (Edbauer et al., 2003). However, it is possible that other proteins have a regulatory role. For instance, TMP21 interacts with γ-secretase and decreases the production of Aβ (Chen et al., 2006). Also, CD147, another protein, that is reported to be component of γ-secretase, regulates the production of Aβ and exclusion of CD147 from the γ-secretase complex increases the Aβ production (Zhou et al., 2005).

Inhibition of γ-secretase as a drug target is complicated, because there are around fifty type 1 transmembrane proteins, which in addition to APP, are cleaved by γ-secretase. Among these are the Notch receptors (Notch 1-4), the Erb-B4 receptor and E-cadherin (Suh and Checler, 2002). In all substrates, the γ-cleavage appears to take place in the membrane and releases a luminal/extracellular and a cytosolic fragment. This process is called “regulated intramembrane proteolysis” (RIP) (Ebinu and Yankner, 2002) and requires two cleavage events. The first occurs outside the membrane, often in response to ligand binding, which triggers a second intramembrane cleavage that releases a cytoplasmic fragment. In some cases, the cytoplasmic fragment translocates to the nucleus and activates gene expression. The cell surface receptor Notch-1 for instance, is activated by ligands such as Delta or Serrate/Jagged. Notch-1 is cleaved at the membrane resulting in the release of an intracellular domain of Notch (NICD). The γ-secretase-mediated Notch-1 signalling plays an important role in the regulation of

developmental cells survival in many organ systems (Jarriault et al., 1995), and dysregulation in Notch signalling results in developmental defects (Shen et al., 1997).

The stoichiometry and the size of the γ-secretase complex are currently not known. The molecular weight of the complex has been estimated in the range of 250-2000 kDa depending on the experimental set-ups and techniques used (Edbauer et al., 2003; Kimberly et al., 2003; Farmery et al., 2003). This discrepancy is not sufficiently explained by limitations in the techniques: Modulator proteins interacting with the γ-secretase complex, and/or multimeric subunits or lipids are present and may account for the difference. Recent work by Sato and co-workers has suggested a 1:1:1:1 stoichiometry of the four γ-components (Sato et al., 2007). This is in line with a recent study in which the mass of the purified γ-secretase complex has by electron microscopy been estimated to 230 kDa at a 12 Å resolution (Osenkowski et al., 2009).

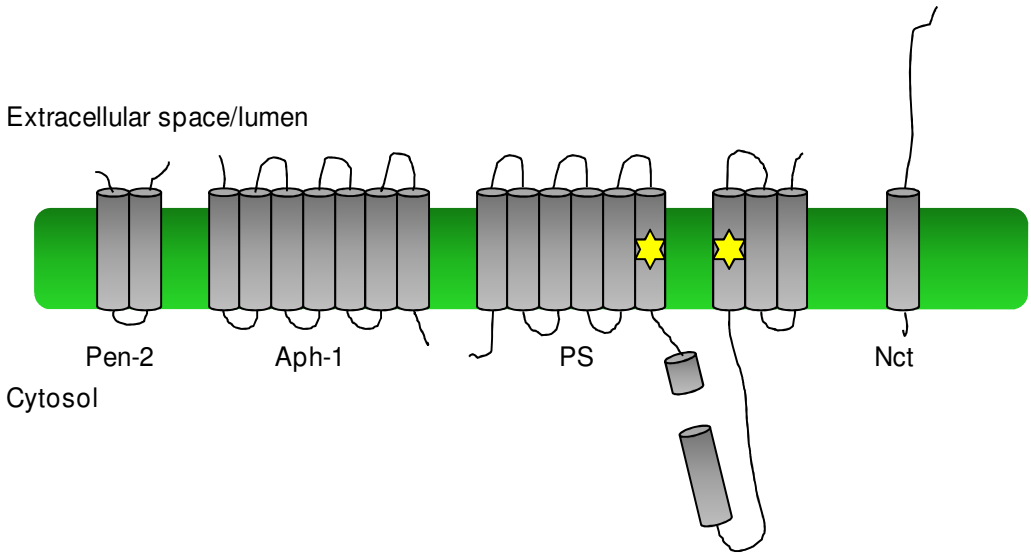

Figure 3. Schematic drawing of the γ-secretase complex. The stars indicate the active sites in PS, Asp257 and Asp385.

Lipid rafts and AD

Several studies indicate that cholesterol is an important factor in the pathogenesis of AD. High cholesterol levels increase Aβ production and deposition. ApoE is involved in cholesterol transport, and the ε4 allele of *APOE* has been identified as an important risk factor for AD (Strittmatter et al., 1993). Cholesterol and sphingolipids are the main constituents of lipid rafts, which are small platforms, i.e. microdomains, in the cell membranes. These microdomains are more ordered and tightly packed than the surrounding bilayer. Lipid rafts are considered to be dynamic platforms for cell

signalling, membrane protein sorting, and transport (Simons and Ihonen, 1997). The size of lipid rafts is suggested to be in the range of 5-100 nm and they are highly dynamic (life-times less than 10 ms) (Prior et al., 2003; Sharma et al., 2004; Eggeling et al., 2009), and they are, therefore, difficult to study in cells. The lipid membranes can be treated with detergents such as Triton X-100 at 4 ºC, resulting in partial dissolution of the membranes. The insoluble parts of the lipid membranes, called detergent resistant membranes (DRMs), can be isolated by centrifugation and are thought to at least partly reflect the composition of the lipid rafts. Certain proteins are concentrated to lipid rafts. Several findings suggest that the trafficking and processing of APP partly depends on lipid rafts. Several research groups report that APP, BACE and γ-secretase localize to lipid rafts. However, these studies show different results as to the degree of localization (Ehehalt et al., 2003; Hattori et al., 2006; Hur et al., 2008; Urano et al., 2005; Vetrievel et al., 2005; Wahrle et al., 2002). Possible explanations for the different results include choice of cell line, overexpression of the proteins of interest, and different detergents used for preparation of DRMs.

The amyloid cascade hypothesis

It has been almost 20 years since Hardy and Higgins first proposed the *amyloid cascade hypothesis*, which since then has been the predominant hypothesis in the field. This hypothesis states that the amyloid deposition of Aβ in the brain is the primary influence driving AD pathogenesis as manifested in the formation of NFTs, inflammatory processes, synaptic deficits, and several toxic events that ultimately give rise to dementia and death (Hardy and Higgins, 1992). Initially, it was believed that the fibrillar insoluble depositions of Aβ in plaques were the cause of the disease. However, the focus has turned towards soluble Aβ oligomers, which are now considered the most toxic species and the amyloid cascade hypothesis has consequently been updated (Hardy and Selkoe, 2002) (Figure 4).

Several findings using genetic and biochemical data, as well as animal models, constitute evidence that Aβ has a causative role in AD. These findings include: Aβ oligomers are toxic and the levels of oligomers are increased in AD brains, and that synthetic Aβ peptides are also toxic to neurons and synapses (Selkoe, 2002). In addition, inherited mutations in the *APP* gene give rise to an elevated total Aβ production, or an increased Aβ42/40 ratio, and cause early onset AD. Inherited mutations within the *PS1* or *PS1* genes result in an increased Aβ42/40 ratio and very

early and aggressive forms of early onset AD. Studies also show that transgenic mice expressing both mutant tau and APP show an accelerated tau pathology while the amyloid plaque deposition is unchanged (Jada et al., 2001). Also, DS patients develop AD because they carry an extra copy of chromosome 21 where the *APP* gene is located (Olson and Shaw, 1969). Finally, *APOE* ε4 allele is a major risk factor for developing late onset AD, and brains from patients carrying one or two copies of the ε4 allele have a severe Aβ burden (Corder et al., 1993).

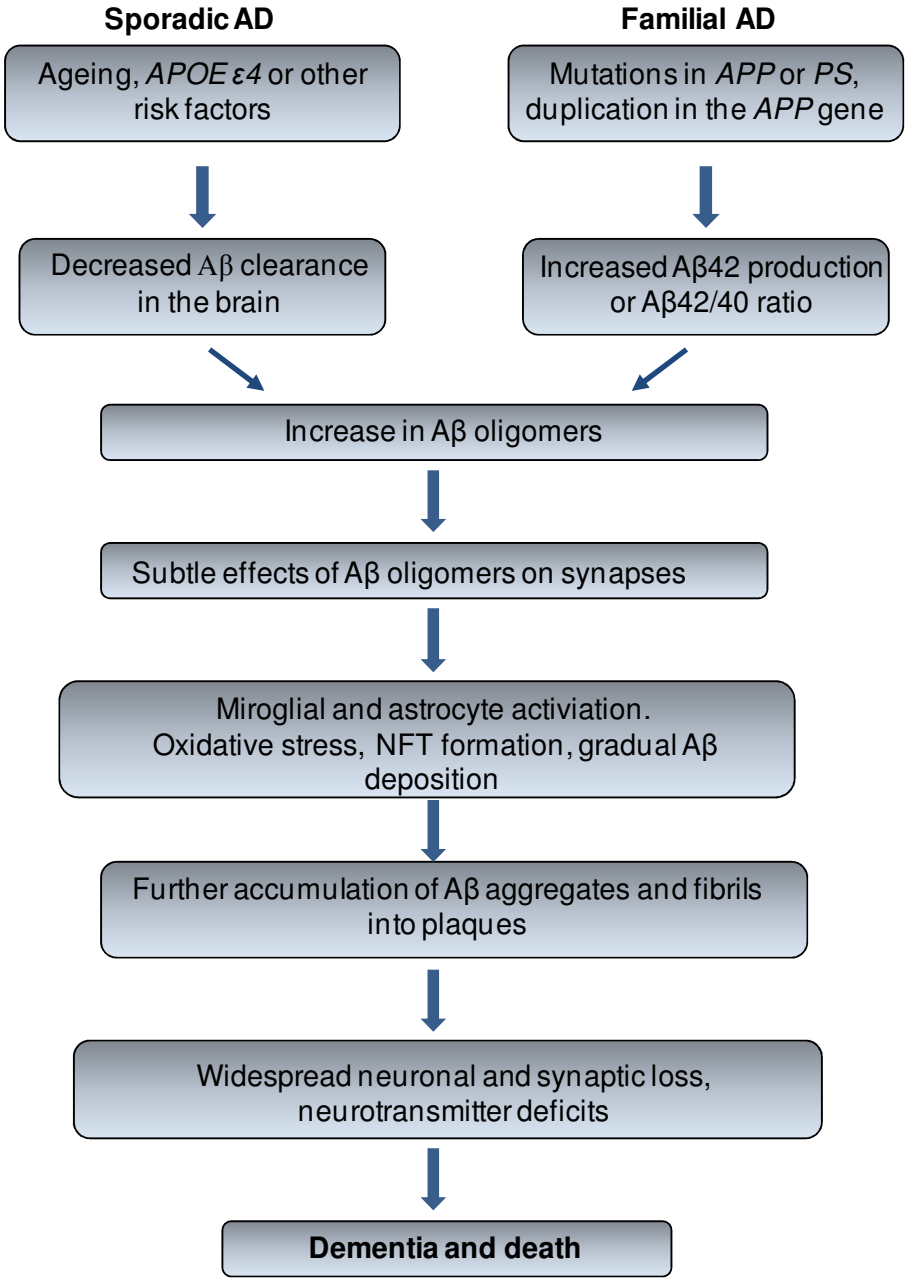

Figure 4. According to the amyloid cascade hypothesis, the central event in AD pathogenesis is the accumulation of Aβ which leads to a cascade of pathogenic processes, such as: NFT formation, oxidative stress, cognitive disturbances and eventually dementia and death.

Aβ aggregation

Extracellular senile plaques and vascular amyloid are primarily composed of Aβ fibrils. Aβ is prone to aggregation and the fibrillogenesis is a nucleation dependent phenomenon. Such a process is dependent on a seed and has three characteristic features (Jarrett and Lansbury, 1993): A critical concentration below which no fibrils can form, a lag phase that is the time required before a seed is formed (above the critical concentration), and a growth phase. The lag time is strongly dependent on the concentration; but once a seed is added, the polymerization starts instantly. Aβ40 is the major species in plasma and cerebrospinal fluid (CSF), whereas Aβ42 is more hydrophobic and prone to aggregation. Aβ42 is the species that appears important for early plaque formation (Iwatsubo et al., 1994). Furthermore, studies show that the length of the C-terminus of Aβ is important for the rate of amyloid formation. Small amounts of insoluble peptides with the critical C-terminus resulted in immediate aggregation of soluble model peptides. Aβ42 forms fibrils at lower concentrations than Aβ40 and trace amounts of Aβ1-42 and Aβ1-43 could seed the formation of amyloid plaques *in vivo* (Jarrett et al., 1993).

The concentration of Aβ in plasma and CSF in humans is in the picomolar to low nanomolar range while the concentration required for Aβ polymerization *in vitro* is in the μM range (Vanderstichele et al., 2000). It is plausible that Aβ polymerization in brain is dependent on several different factors, such as: Higher local concentrations of Aβ in certain cell compartments (Hu et al., 2009), metal ion concentrations (Maynard et al., 2005; Religa et al., 2006), pH, or that Aβ binding proteins enable polymerization through seeding at much lower concentrations *in vivo* (McLaurin et al., 2000). Studies have reported that seeding might also be possible *in vivo*. Microinjections of AD brain tissue homogenate into primate brains induced cerebral amyloid (Baker et al., 1993). Similar results were obtained from microinjections of AD brain tissue extracts into APP transgenic mice (Meyer-Luehmann et al., 2006). Under normal conditions Aβ peptides can be cleared from the brain by different enzymes, insulin-degrading enzyme and neprilysin being the major ones (Miners et al., 2008).

Amyloid fibrils stain with Congo red and show a green/red birefringence when examined under polarized light, indicating an ordered structure. Aβ fibrils isolated from brain tissue show very similar properties to those formed *in vitro*; and therefore *in vitro* systems serve as good models for studies of Aβ fibrillization. The fibrils have a

characteristic cross-β sheet structure with the peptide strands running perpendicular to the fibril axis (Pauling et al., 1951). This is a common structure for all amyloid fibrils. The distance between two β-sheets perpendicular to the fibril axis has by X-ray diffraction pattern been shown to be between 10-11 Å, and the distance between the hydrogen bonded β-strands has been shown to be 4.7-4.8 Å (Kirschner et al., 1986; Malinchik et al., 1998).

The Aβ monomer is a 4 kDa unfolded peptide with a hydrophobic C-terminal starting at residue 29, and corresponds to half of the transmembrane part of APP. The N-terminal part of the peptide is hydrophilic, thus the Aβ sequence has an amphipathic character. Aβ fibril formation is a multistep process generating various transient intermediates with different properties. Several intermediate Aβ species have been isolated during the aggregation of monomers into fibrils. The polymerization process is not completely understood. Important to bear in mind is that some of the intermediate species have only been detected *in vitro* and, consequently, details regarding the *in vivo* polymerization are uncertain. Several different oligomeric species occurring during Aβ fibrillization process *in vitro* have been identified, including: Dimers (Walsh et al., 2002; Shankar et al., 2008), Aβ-derived diffusible ligands (ADDLs) (Gong et al., 2003; Lambert et al., 1998), a 56-kDa Aβ assembly called Aβ*56 (Lesne et al., 2006), globulomers (Barghorn et al., 2005; Gellerman et al., 2008), and protofibrils (Harper et al., 1997; Walsh et al., 1997) (Figure 5). These range from 8 kDa to over 100 kDa and are all soluble - the definition embracing all species of Aβ that remain in aqueous solution after high-force centrifugation.

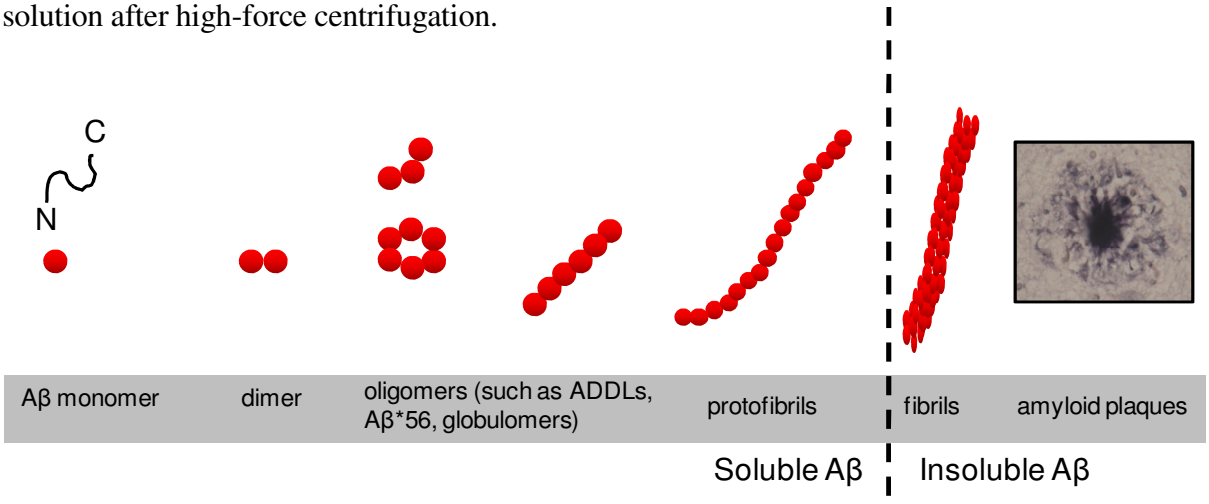

Figure 5. Aβ aggregation process from monomer to insoluble fibrils and amyloid plaque. Many oligomeric species have been detected *in vitro*, some of them are schematically illustrated here. Photo, courtesy of Dr. Nenad Bogdanovic.

Toxicity of Aβ

Aβ is present in CSF and in the brain of healthy as well as demented subjects throughout life, and is also produced by cells under normal metabolism. Thus, the presence of Aβ by itself does not lead to neuronal injury or eventual dementia. Instead neurodegeneration follows the aggregation process of Aβ. Initially, it appeared that fibrillar insoluble depositions of Aβ in plaques were the primary toxic species, a condition resulting from its accumulation in senile plaques. However, as described earlier in the introduction, *the amyloid cascade hypothesis* has changed the focus from plaques to soluble oligomeric forms of Aβ now considered the most toxic pathogens in AD. One reason for this shift was a weak correlation between AD severity and amyloid plaque burden (Terry et al., 1991).

As described above, many soluble oligomeric species have been isolated *in vitro* by several different research groups searching for the most toxic species. Nevertheless, it is important to take into consideration that most studies of soluble Aβ levels have employed methods that cannot reveal the aggregation status of the species identified. However, recent studies have reported antibodies specific to oligomeric forms of Aβ (Englund et al., 2007; Georganopoulou et al., 2005). One must also take into account the intrinsic amyloidogenic properties of Aβ that cause technical difficulties when studying the peptide. For example, Aβ oligomerizes quickly in solution, a process that is highly dependent on Aβ concentration, pH, and temperature. Furthermore, it is common with a batch-to-batch variation when using synthetic Aβ. The variation could be due to the presence of oligomeric seeds, that cause difficulty in reproducing results.

Several groups have with different approaches studied the toxicity of Aβ. For instance, Lesne et al. (2006) present data that demonstrate cognitive decline in transgenic AD mice being associated with Aβ*56. Furthermore, isolated Aβ*56 extracted from transgenic mice brains was injected into rat brain, which resulted in decreased performance in a spatial memory test. Fibrillar Aβ injected into the cerebral cortex of aged rhesus monkeys produce plaque cores similar to those observed in humans (Geula et al., 1998). Shankar and colleagues recently demonstrated that dimers isolated from AD brains result in inhibited long-term potentiation and enhanced long-term depression in normal rodent hippocampus (Shankar et al., 2008). Protofibrils have been demonstrated to alter synaptic physiology and cell death (Hartley et al., 1999) and many of the described soluble oligomeric species have been shown to cause

neurotoxicity in cell cultures (Lambert et al., 1998; Dahlgren et al. 2002; Barghorn et al., 2005).

Various mechanisms have been described that offer to explain how Aβ induced toxicity is mediated, including: Oxidative stress (Markesbery et al., 1999), Aβ pore formation in membranes and resulting changes in cell membrane permeability (Lashuel et al., 2002; Allen et al., 1997; Arispe et al., 1993), induction of hyperphosphorylation of tau (Busciglio et al., 1995), binding to ApoE (Gylys et al., 2003), and induction of mitochondrial dysfunction (Bubber et al., 2005).

Long Aβ peptide species

The major Aβ species produced are Aβ40 and Aβ42. Aβ40 is predominant in CSF and plasma. The longer form, Aβ42, is more prone to form toxic oligomers and is the species initially deposited in the brain in senile plaques (Iwastubo et al., 1994). The FAD mutations in *PSEN1*, *PSEN2* and *APP* cause an increased production of Aβ42 (an increased Aβ42/40 ratio or a total increased Aβ production) (Selkoe, 2001). Importantly, the longer Aβ variant is more neurotoxic than the shorter species (Jarrett et al., 1993; Liao et al., 2007). In a recent study, Jan and co-workers demonstrated that the relative concentration of Aβ42 is a critical factor for the rate of amyloidogenesis (Jan et al., 2008). Furthermore, *in vitro* experiments from Jarrett and colleagues demonstrate that low concentrations of the longer C-terminal variants, Aβ42 and Aβ43, have a seeding effect on soluble Aβ peptides, which could be of great importance for Aβ fibrillization and plaque formation *in vivo* (Jarret et al., 1993). Longer and more hydrophobic forms other than Aβ42, polymerize faster and at lower concentrations than the more abundant shorter variants, and could thus seed polymerization of Aβ into amyloid *in vivo*.

Studies from cell lines and transgenic mice clearly show that Aβ species longer than Aβ42 are formed. Qi-Takahara and co-workers identified longer variants, such as: Aβ43, Aβ45, Aβ46 and Aβ48, all obtained in cell lysates from cells expressing *APP* and *PS1* mutations, in a system using urea gels (Qi-Takahara et al., 2005). These variants were also detected in APP-transgenic mouse brain homogenates. A similar approach from Yagishita and colleagues also revealed longer Aβ forms in lysates from several cell lines (Yagishita et al., 2006). Longer forms of Aβ have also been studied in transgenic mice by other groups using different techniques, such as high performance

liquid chromatography (HPLC), mass spectrometry (MS), or detection in gelsystems (Esh et al., 2005; Van Vickle et al., 2007; Shimojo et al., 2008). Important to consider is that these cell lines and transgenic animals over-express Aβ and thus do not reflect the situation in human AD brain. A few studies of longer Aβ species in human brain from SAD and FAD cases, detected longer Aβ species such as Aβ43 (Miravalle et al., 2005; Mori et al., 1992; Van Vickle et al., 2008; Roher et al., 2004) but these studies were not quantitative. However, a widely used technique enabling measurement of both soluble and insoluble Aβ (Aβ40 and Aβ42) employs enzyme-linked immunosorbent assay (ELISA), facilitating quantitative studies in plasma, CSF and cell lysate. When employing ELISA for Aβ measurements it is crucial to consider that the presence of oligomers could interfere with the analysis and cause underestimation of Aβ due to epitope masking (Englund et al., 2009). MS is a technique that allows identification and characterization of peptides and proteins. It is frequently used for example, in combination with immunoprecipitation (IP) when analyzing Aβ in CSF (Portelius et al., 2007). However, research on longer C-terminal Aβ species is lacking. Furthermore, the role of longer Aβ in the AD pathogenesis is not known.

AIMS OF THE STUDY

γ-Secretase cleavage of APP leads to formation of the toxic Aβ peptide, and the deposition of Aβ into amyloid plaques, is one key event in the pathogenesis of AD and one of the hallmarks of the disease. The general aim of this study was to investigate γ-secretase activity and localization in different compartments in mammalian brain, and to identify and quantify the Aβ species that are deposited in AD brains.

The specific aims of this thesis were:

Paper I: To determine the optimal conditions for further studies of active γ-secretase in brain.

Paper II: To explore if active γ-secretase is localized to detergent resistant membranes in rat and human brain.

Paper III: To investigate if longer Aβ species are deposited in AD brains, to develop a method for quantification of the identified species and perform a quantititative study of Aβ species in SAD and FAD brains.

Paper IV: To use the conditions for quantifications of Aβ species established in paper III in order to identify, compare and quantify Aβ species in different brain regions from Swedish *PSEN1* I143T mutation carriers.

METHODOLOGICAL CONSIDERATIONS

Several different methods and techniques were used for this thesis and detailed descriptions are found in papers I-IV. Some of the techniques are summarized and commented on in this chapter, which focuses on less conventional techniques.

DETERGENTS

Since γ-secretase is a transmembrane protein complex, detergents are often used for purification prior to studies of the γ-secretase complex. To study active γ-secretase, it is crucial to choose a detergent that preserves the γ-secretase activity. Although earlier studies have used a variety of detergents, authors have not agreed on the choice of optimal detergent for studies of γ-secretase (Gu et al., 2001; McLendon et al., 2000; Pinnix et al., 2001; Li et al., 2000). Eight detergents were included in our study (paper I): CHAPS (zwitterionic), CHAPSO (zwitterionic), DDM (non-ionic), Triton X-100 (non-ionic), Lubrol (non-ionic), Brij-35 (non-ionic), Tween-20 (non-ionic), and a relatively new detergent called PreserveX, which is a mixture of polymeric amphiphiles. We studied the effect of each detergent at 0.25% or 1% (w/v) on AICD production.

γ-SECRETASE ACTIVITY ASSAY

The production of AICD endogenously produced was assayed by incubation of the membrane fractions in buffer S (20 mM Hepes, pH 7.0, 150 mM NaCl, 5 mM EDTA) containing Complete™ protease inhibitor mixture, with or without detergent for 16 hours at 37 °C for the indicated time. The protein concentration was kept at 1 mg/ml in the experiments in which detergents were present. The reactions were halted by cooling the samples on ice. Thereafter AICD in the supernatant (or in the total sample for experiments including detergents), and APP C-terminal fragements (CTFs) in the membrane pellet, were analysed by western blotting (WB) (paper I).

In paper II the production of AICD was investigated by incubation of the samples for 16 hours at 37 °C in the absence or presence of the γ-secretase inhibitor L-685.458.

ELISA

ELISA was used to study the levels of Aβ40 and Aβ42. In paper I, *de novo* generation of Aβ40 was measured after 16 h of incubation of the 100,000 × g pellet, enriched in

endosomes, endoplasmic reticulum, Golgi, and synaptic vesicles. In paper II the DRM fraction was incubated for 16 h in the presence of 20 ng of the exogenous substrate C99-FLAG. In paper IV secreted Aβ40 and Aβ42 in cell medium as well as cellular Aβ40 and Aβ42 from fibroblasts were measured. A buffer containing 0.1% sodium dodecyl sulfate (SDS) was added to the samples before all the measurements described above to prevent Aβ aggregation. Still, one cannot exclude that some aggregates did form, hiding epitopes from the detection antibody and thereby giving incorrect values.

PREPARATION OF DRMs FROM BRAIN

To isolate DRMs, the 100.000 × g pellet was re-suspended in 600 µl of a buffer with 2.0 % CHAPSO supplemented with Complete™ protease inhibitor mixture. The samples were incubated with end-over-end rotation for 20 min at 4 °C. The samples were then adjusted to 45% sucrose. Then, 6.9 ml of 35% sucrose followed by 2.3 ml of 5% sucrose was overlaid onto the 45% sample fraction. The samples were centrifuged at 100,000 × g for 16 h at 4 °C. Six fractions were collected from the top of the tube using a 5 ml syringe. Initially, different CHAPSO concentrations were tried in the gradient; but 2% CHAPSO gave the best separation between the fractions.

BRAIN MATERIAL

We have focused on rat brain and human brain in our studies, but in some cases cell lines have been used. All studies are approved by the human ethical committee in Stockholm and the animal ethical committee of South Stockholm. Numerous studies on AD employ cell systems that over express the protein of interest. These systems could serve as good models for studying certain mechanisms in a controlled environment; but it is important to keep in mind that these models are simplified, and consequently it is hard to project the results and information to AD in humans.

PLAQUE-CORE PREPARATION

In brief, plaque cores from frozen human brain were prepared by homogenization of 0.5-1 g of tissue in 4 mL Tris-buffered saline (TBS)/g of tissue, pH 7.4, supplemented with Complete™ protease inhibitor mixture using a mechanical pestle-homogeniser. The homogenate was centrifuged at 4,000 × g for 2 min at 4 °C and the resulting pellet was washed in TBS and stained overnight with Congo red (50% saturated in TBS). The pellet was filtered through a 100 µm nylon mesh to remove poorly dissolved material, adjusted to 46% sucrose, and centrifuged at 16,000 × g for 1h at 4

°C, followed by one more centrifugation of the pellet at 16,000 × g for 5 min at 4 °C and dilution in 1% SDS in TBS. After filtration through a 60 µm nylon mesh and centrifugation at 16,000 × g for 5 min at room temperature (24 °C), the pellet was washed three times in TBS buffer and centrifuged at 16,000 × g for 5 min at room temperature (24 °C). The final pellet was stored at –20 °C (Figure 6). To estimate the yield of the method, Congo red-stained plaque cores in preparations from four brains were counted at each step of the purification procedure. Thirty µL of both supernatant and diluted pellets from each step in the purification protocol were collected throughout the procedure and viewed under polarized light. The total number of plaque cores was calculated with respect to the total volume of the sample in each step. We found that the total recovery varied between 9-20% and this has been taken into account in the calculations.

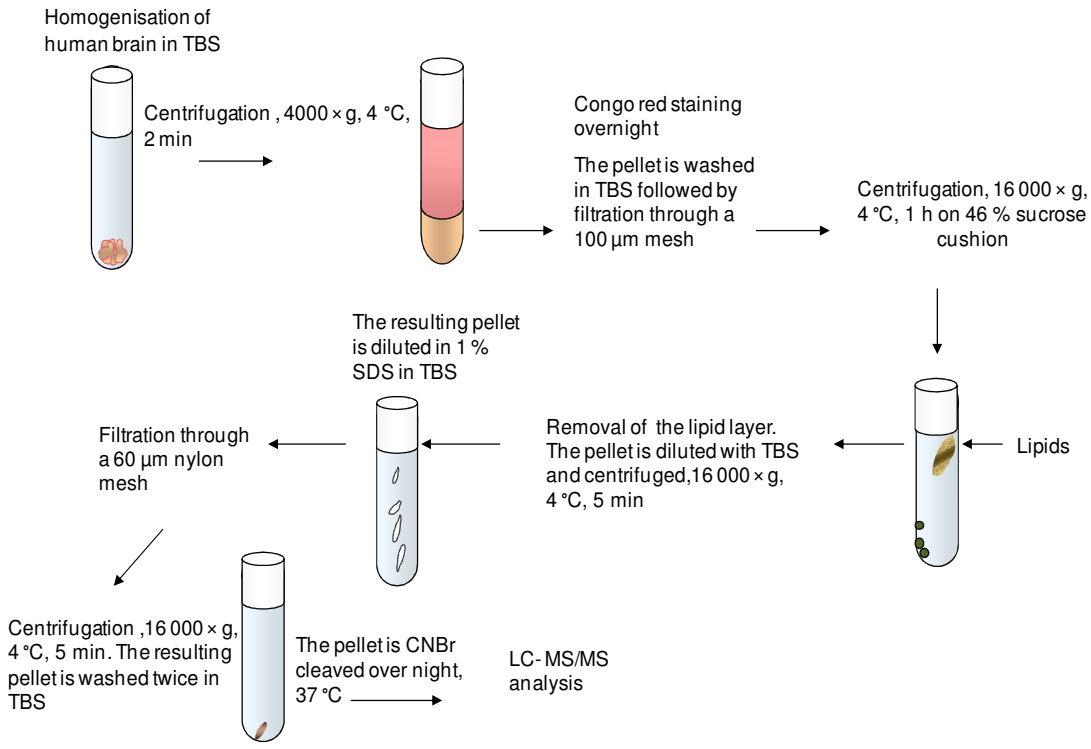

Figure 6. The plaque core preparation procedure. Welander et al. (2009), J Neurochem 110, 697-706.

LC-MS/MS AND Aβ QUANTIFICATIONS

MS is a very powerful technique that allows identification and characterization of peptides and proteins. The principle of MS is that the molecules in the sample are ionized in the ion source and then separated according to their mass-to-charge ratio (m/z) and finally detected by a detector. There are several different ion sources, mass

analysers and detectors available on the market. The equipment and method we have used in our laboratory is liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI) (Fenn et al., 1998) coupled to an ion trap. In positive ESI mode, positive ions from the liquid containing the analyte are generated from the solution with the help of a strong electric field. Positive charges in the solution are then separated and an aerosol spray of positively charged droplets is generated. The solvent is evaporated and the droplet shrinks in size. When the surface tension of the droplet is exceeded by the charge repulsion the droplet breakes apart and ions are formed in atmospheric pressure. The ions then pass through an orifice into the high vacuum of the mass analyser.

We have used synthetic Aβ peptides for quantifications of C-terminal Aβ peptides in samples prepared from purified plaque cores and total amyloid preparations. The synthetic Aβ peptides - Aβ36-40, Aβ36-42, Aβ36-43, Aβ36-44, Aβ36-45 and Aβ36-46 - were used as standards for the quantifications. The peptides were dissolved in 80% formic acid (FA) and diluted in 0.1% FA. A standard curve was created by mixing Aβ36-40, Aβ36-42 and Aβ36-43 at a ratio of 20:20:1, and injecting 1 µL of 500, 200, 50 and 20 fmoles of Aβ36-40 and Aβ36-42, and 25, 10, 2.5 and 1 fmoles of Aβ36-43, in the same solution. Injections were performed from lower to higher concentrations with blank injections of 0.1% FA between each standard injection. Initially, we found that around 20 times more Aβ36-42 was detected as compared to Aβ36-43 in plaque cores from human brain. Standard concentrations were then chosen to reflect the situation in brain samples. The monoisotopic masses for the Aβ fragments of interest were plotted as extracted ion chromatograms, and quantification was performed based on peak areas. The peak areas were then used in the equations from the standard curves in order to calculate the concentrations expressed as nmol Aβ/g of wet brain tissue.

The detection limits of the standard peptides were approximately 10 fmoles for Aβ36-40, 2.5 fmoles for Aβ36-42, 1 fmole for Aβ36-43; and significantly higher for the longer and more hydrophobic peptides, i.e. 50 fmoles for Aβ36-44 and Aβ36-45, and 500 moles for Aβ36-46.

Sample preparation

The samples were dissolved over night at 37 °C in 80% FA supplemented with 4 mg cyanogen bromide (CNBr) per sample in a shaker at 600 rpm. Prior to LC-MS/MS-

analysis the samples were concentrated and desalted using C18 ZipTips, dried in a vacuum centrifuge, and finally dissolved in 10 µL of 0.1% FA. One µL of the samples was injected (Figure 7). We chose CNBr to generate shorter and less hydrophobic C-terminal Aβ fragments compared to the fragments generated by the more commonly used trypsin. CNBr cleaves C-terminally of methionine (Aβ35), thereby generating Aβ36-x fragments (Figure 8). Trypsin is widely used for peptide digestion prior to MS analysis, but trypsin cleavage would yield fragments that are not well suited for analysis by LC-MS/MS due to their high hydrophobicity. Furthermore, plaques are very difficult to dissolve and around 80% FA is required. Since the CNBr cleavage reaction requires concentrated FA, the dissolution of samples and CNBr reaction could be combined.

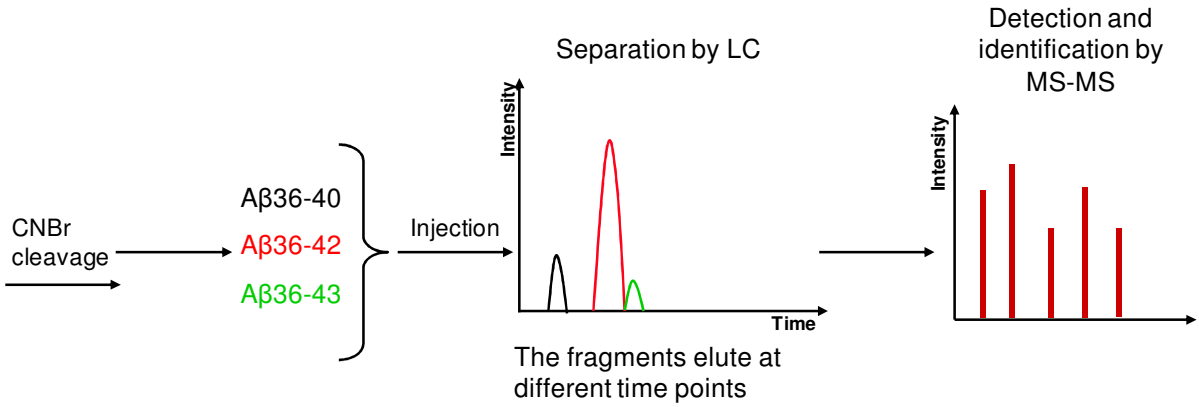

Figure 7. A schematic presentation of the sample analysis procedure using LC-MS/MS.

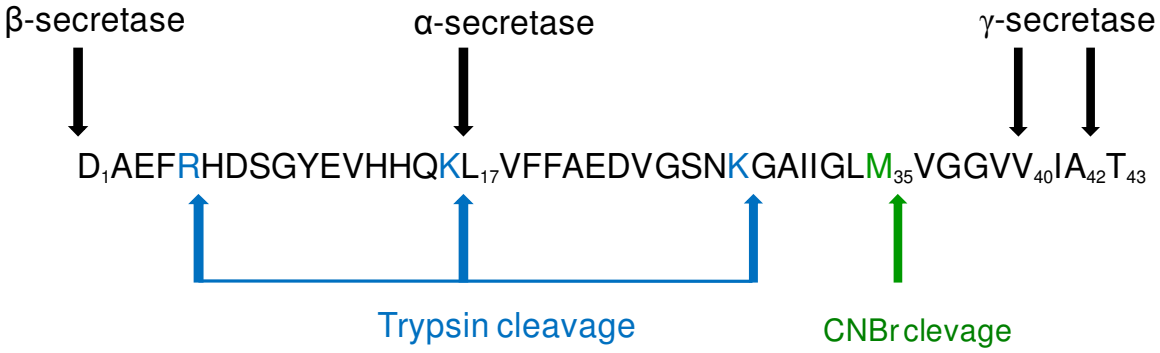

Figure 8. The Aβ sequence indicating the CNBr and trypsin cleavage sites. The α-, β- and γ-cleavage sites are indicated with black arrows.

IMMUNOHISTOCHEMISTRY USING AN Aβ43 SPECIFIC ANTIBODY

There are only a few Aβ43 antibodies available on the market to date. We used a relatively new one, not tested for immunohistochemistry before. The specificity of the antibody was tested by a dot blot procedure. Two µL of a solution containing 1 µg/µL of synthetic Aβ1-40, Aβ1-42 or Aβ1-43 was placed on a nitrocellulose membrane. The membrane was then dried and blocked in 5% bovine serum albumin in TBS supplemented with 0.1% Tween-20, and incubated with Aβ1-43 antibody (1:1000) for 1 h. Next, the membrane was washed 3 times in TBS supplemented with 0.1% Tween-20 and incubated in secondary antibody anti-mouse (1:2000) for 30 minutes. Finally, the membrane was washed as above, followed by one wash in TBS. The antibody was highly reactive to Aβ1-43 but not to Aβ1-40 and Aβ1-42. Furthermore, the specificity of the antibody was tested by immunostaining of brain sections from two non-AD cases, resulting in no immunoreactivity.

RESULTS AND DISCUSSION

In this part of the thesis the results from papers I-IV are summarized and discussed. All figures are found in their respective papers, along with detailed descriptions of results.

RAT BRAIN γ-SECRETASE ACTIVITY IS HIGHLY INFLUENCED BY DETERGENTS

As mentioned in the introduction, γ-secretase is a crucial enzyme for the generation of the toxic Aβ which is central for the development of AD. Since γ-secretase is a transmembrane protein complex, detergents have been frequently used for solubilization and purification of the γ-complex. However, to our knowledge, no thorough investigation of the effect of detergents on γ-secretase activity has been reported. Moreover, most of the knowledge about γ-secretase to date is obtained from models where transfected cell lines are used. These systems over-express for example γ-secretase components, or APP, and do not fully reflect the situation in brain. Furthermore, these systems lack complexity and do not express all protein present in nerve cells. An extensive and detailed investigation was performed on rat brain to show how detergents influence γ-secretase activity. Thus, this study determined the optimal conditions for studying an active protein complex. To measure the activity, an activity assay was used to detect endogenous production of AICD. It appears to be an advantage to quantify the endogenous AICD production compared to other studies, where often an exogenous substrate is used in the artificial systems.

The rat brain was first fractionated in three centrifugation steps, 1000 g, 10,000 × g and 100,000 × g, and resulting pellets were compared to determine which was enriched in γ-secretase components. The comparison showed that the 100,000 × g pellet (P3) was enriched in Nct, PS1, Pen-2 and the γ-secretase substrate APP CTFs, as well as in endosomes, synaptic vesicles, Golgi and ER. Equal amounts of protein from the three pellets were incubated for 16 h at 37 °C and centrifuged for 100,000 × g, where after AICD formation was measured by WB. The highest relative activity (AICD/µg protein) was found in the P3 pellet, in concordance with the enrichment of γ-secretase components in this fraction, and thus the conclusion was made that this fraction served as a good source of active γ-secretase. Consequently, this fraction was used in the following studies. This is in agreement with a study from Gu and co-workers (Gu et al.,

2001). However, one study showed higher activity in the 10,000 × g pellet (Pinnix et al., 2001), possibly due to differences in detergents, centrifugation times, buffer composition and homogenization procedures. Next pH dependence of γ-secretase activity was studied and found to be in concordance with earlier studies, reporting the optimum to be around pH 7 (McLendon et al., 2000; Pinnix et al., 2001; Li et al., 2000). An earlier study of AICD production in crude membrane preparations from cells showed that AICD is rapidly degraded (Edbauer et al., 2002). The P3 fraction was incubated for different time points between 0 and 16 h and a time-dependent generation of AICD fragment was observed with the highest levels occurring at 16 h. The endogenous substrate levels were sufficient for generation of detectable levels of AICD fragment; and APP CTFs were present even after 16 h. This was advantageous compared to other systems using cells, where AICD is quickly degraded by soluble proteases (Edbauer et al., 2002). In this respect, rat brain is also a good option instead of human brain, since the post-mortem time has a negative effect on γ-secretase activity (Hur et al., 2008).

The investigation turned to examine the influence of seven detergents on γ-secretase activity, including: CHAPS, CHAPSO, DDM, Triton X-100, Lubrol, Brij-35, Tween-20 and PreserveX; at either 0.25% or 1%. All detergents except PreserveX reduced γ-secretase activity at 1%. This low activity could be due to dissociation of the complex, changes in lipid environment, or dissociation of substrate and complex; or combinations of these. It is interesting to note that 1% Tween-20 or Brij-35 showed only a small reduction in activity, in concordance with a previous study (Gu et al., 2001). However, most protein and lipids were shown to be resistant to 1% Tween-20 (Schuck et al., 2003) which is a week detergent. Perhaps the environment for γ-secretase is changed only to a minor degree at this concentration.

To investigate the ability of the detergents to solubilize the γ-secretase complex, turbidity measurements were performed, which demonstrated no changes in turbidity in 1% PreserveX, Tween-20, or Brij-35; and all of the γ-secretase components were present in the 100,000 × g pellet after solubilization of the samples in the presence of these detergents. Therefore, even though γ-secretase was active at 1% PreserveX, Tween-20, or Brij-35, these detergents are not suitable for solubilizing the complex. The activity was barely detectable in 1% CHAPSO or CHAPS, and completely exterminated in DDM, Lubrol and in 1% Triton X-100. At a concentration of 0.25%,

PreserveX, CHAPS, and Brij-35 showed similar activity as did samples without detergents. CHAPSO showed an even more increased activity at this concentration and hence was selected as the detergent at which the optimal CHAPSO concentration was established in order to preserve γ-secretase activity. The optimal CHAPSO concentration was 0.4%. This concentration is just below the critical micelle (CMC) concentration for CHAPSO, which is 0.5%. It seems plausible that γ-secretase is dependent on lipid environment for its activity, since γ-secretase is found in DRM that are enriched in cholesterol and sphingolipids (Wahrle et al., 2002; Vetrievel et al., 2005; Hur et al., 2008), and cholesterol and sphingolipids seem to have a positive influence on γ-secretase activity (Wahrle et al., 2002; Sawamura et al., 2004). This positive effect on γ-secretase activity could depend on the ability of CHAPSO to form mixed micelles with membrane lipids just below the CMC (Womack et al., 1983).

Assays were also conducted to learn whether Aβ was produced at the optimal conditions established for AICD production, made possible by using a sensitive Aβ40 ELISA. It was possible to measure Aβ in our system and its production was inhibited by addition of the γ-secretase inhibitor L-685.458.

Next, γ-secretase activity at different CHAPSO concentrations was observed to determine whether activity stemmed from the soluble or insoluble fraction of the samples. This study concluded that most of the activity was detected in the supernatant at CHAPSO concentrations over CMC, while the soluble activity was low at concentrations below CMC. The γ-components were only partly soluble (around 50%) at 1% CHAPSO; and therefore we hypothesized that the low activity in the pellet could be caused by low substrate levels. To investigate this, an exogenous substrate, i.e. C99-FLAG, was added, at 10 ng per sample. Activity was then observed in the pellets at CHAPSO concentrations around CMC, showing that γ-secretase is partly insoluble at these CHAPSO concentrations. Interestingly, it was also noted that the treatment of the exogenous substrate was important for the effect on AICD production. Pre-treatment with trifluoroethanol (TFE) had a favorable effect on the production of AICD, probably because TFE diminishes aggregation and keeps the transmembrane region in a native α-helical conformation. However, the concentration of exogenous substrate was much higher than the endogenous concentrations required for production of AICD. Finally, it was concluded that 1% CHAPSO solubilized a substantial amount of the γ-secretase components, and the low activity at 1% could be fully restored by diluting the sample

to 0.4% CHAPSO. Hence, solubilizing the sample in 1% CHAPSO and then diluting the sample to 0.4% was a good procedure for obtaining soluble and active γ-secretase.

ACTIVE γ-SECRETASE IS LOCALIZED TO DETERGENT-RESISTANT MEMBRANES IN HUMAN BRAIN

Studies have shown that the lipid membrane environment is important for γ-secretase activity; and that γ-secretase is partly localized to DRMs (Wahrle et al., 2002; Vetrievel et al., 2005). DRMs are ordered micro domains in the cell membranes, enriched in sphingolipids and cholesterol. Cholesterol appears to have an important role in AD pathogenesis and APP processing, on the basis that high cholesterol levels in mid-life correlate with the risk of developing AD later in life; and Aβ production is increased at high cholesterol levels (Cordy et al., 2006). Previous studies of DRMs and γ-secretase are based on findings from mouse brain and cultured cells, which suggest that γ-secretase, BACE, and APP are partly localized to DRMs. As mentioned in the introduction of this thesis, the degree of localization of the γ-secretase components differs between the studies, possibly due to the particular detergent that is used for the preparation of DRMs, and whether the cell systems over-express the protein of interest. Since many of the studies are conducted from transfected cell lines, further studies in brain would contribute to the understanding of γ-secretase localization and APP processing in brain.

Previously, no study regarding the association of γ-secretase with DRMs in humans has been reported. Also, to our knowledge, no report on γ-secretase activity in DRMs obtained from mammalian brain has been described. Therefore, this study included human brain samples to examine co-localization of active γ-secretase with DRMs. Also, this study intended to compare the distribution and activity of γ-secretase in DRMs in human and rat brain, and therefore preparations from rat brain were included in the study. To explore the association of γ-secretase complex with lipid rafts a protocol was used in which membranes were dissolved in detergent, placed in a sucrose gradient, and centrifuged. The DRMs were then localized to a fraction between 5% and 35% sucrose allowing them to be isolated. CHAPSO was selected since in **paper I** it was concluded that it best preserves γ-secretase activity. In another study 2% CHAPSO was used when preparing DRMs from SH-SY5Y neuroblastoma cells containing active γ-secretase (Urano et al., 2005). We found that a CHAPSO concentration of 0.4% resulted in a poor separation between soluble components and DRMs, but the

separation was improved using 2% CHAPSO. This study also found that separation in SH-SY5Y neuroblastoma cells was more distinct than in brain samples, perhaps due to greater heterogeneity of the brain samples and also due to the presence of myelin. For comparison, a DRM preparation was performed using Triton X-100, which is a detergent frequently used in DRM preparation protocols. Interestingly, no PS1-NTF was detected in the DRM fraction using this detergent. Thus it was concluded that 2% CHAPSO was appropriate for DRMs preparation. Also noted was that the 5-35% interface was enriched in all four known γ-complex components and a lipid raft marker as shown by WB along with antibodies directed to γ-complex components, including: BACE, APP, APP-CTFs; and raft and non-raft markers. These results suggested that the majority of γ-secretase in human brain was localized to lipid rafts, which is in concordance with earlier reports on γ-secretase association from mouse brain (Urano et al., 2005; Vetrievel et al., 2004; Vetrievel et al., 2005). The localization of PS1 and Nct to lipid rafts was further supported by immunofluoresence confocal microscopy on human brain sections. Interestingly, BACE, full-length APP, and APP-CTFs were found to be located outside the DRM fraction. This experiment was repeated using rat brain, and obtained results were consistent with human brain DRMs preparation.

Previous studies have also shown that < 25% of BACE is associated with lipid rafts (Ehehalt et al., 2003; Vetrievel et al., 2005; Hattori et al., 2006; Abad-Rodriguez et al., 2004). Interestingly, Ehehalt and co-workers have suggested that raft association is essential for the activity of BACE. Hence, one way of decreasing the levels of Aβ could be to lower the amount of raft-associated BACE. Furthermore, results from our study show that most of the APP is localized outside the DRM fraction. The low levels of BACE and APP in DRMs might indicate that the initial cleavage step in the amyloidogenic pathway occurs outside the rafts. Earlier studies have reported a raft association of APP between zero and 20% (Ehehalt et al., 2003; Vetrievel et al., 2005; Hattori et al., 2006; Abad-Rodriguez et al., 2004). Despite the low levels of APP CTFs in the current study, AICD was detected in preparations from both human and rat brain. To our knowledge, this is the first study to show γ-secretase activity in DRMs in mammalian brain, and the processing of an endogenous substrate in DRMs.

We wished to measure endogenous production of Aβ in the DRMs, but it was not possible. When adding the exogenous substrate C99-FLAG, treated in the same way as in **paper I**, detection of Aβ production was feasible and was inhibited when the γ-

secretase inhibitor L-685.458 was added. Next, we wished to further purify the DRMs containing the γ-secretase complex by size exclusion chromatography (SEC). Fractions were collected and analysed by WB with antibodies directed to γ-complex components. When using 0.25% CHAPSO in the mobile phase, the γ-secretase components, APP, and a raft marker eluted in the high molecular fraction, > 2000 kDa. Earlier studies obtained varying results regarding the size of the γ-secretase complex. Nevertheless, the complex is estimated to have molecular weights between 200 and 2000 kDa (Edbauer et al., 2003; Farmery et al., 2003; Kimberly et al., 2003; Gu et al., 2004; Evin et al., 2005). We suggest that the estimated molecular weight obtained by SEC images the size of the DRMs rather than the γ-secretase complex. When using a CHAPSO concentration of 2% in the mobile phase instead of 0.25%, the γ-secretase complex still eluted in the void volume, indicating a complex stably associated with DRMs. In support of this notion, co-immunoprecipitation of PS1, Aph-1aL and Pen-2 was possible in 2% CHAPSO. Furthermore, γ-secretase was observed still active in DRMs from a human brain sample with post-mortem time of 22 h.

An investigated was made of the effect of different postmortem times on γ-secretase activity in rat brain. A conclusion was made that γ-secretase activity, as measured by AICD production, decreased rapidly after short postmortem times, but was still detectable through all time points studied (0-48 h), indicating that studies of active γ-secretase in human brain with longer postmortem times would be possible.

Aβ43 IS MORE FREQUENT THAN Aβ40 IN AMYLOID PLAQUE CORES FROM ALZHEIMER DISEASE BRAINS

In **paper III** the study addressed one of the pathological hallmarks in AD - the amyloid plaque, which result from the γ-secretase dependent production of Aβ. As described in the introduction, studies have shown that Aβ42 is the main constituent in plaques. This study aimed to investigate whether we could identify other, longer (>Aβ42) Aβ species and determine the concentration of all C-terminal variants of Aβ. Aβ42 has a higher aggregation propensity than Aβ40 and is of particular importance in early plaque formation (Iwatsubo et al., 2004; Jarrett et al., 1993). Species longer than Aβ42 are even more hydrophobic and could possibly seed plaque formation *in vivo*. As mentioned in the introduction, studies have shown that it is possible to detect longer Aβ species, but there has been no thorough study of longer C-terminal variants from plaque

cores. One early experiment from our laboratory revealed that not only Aβ42 and Aβ40 were present in plaque cores, but also a longer species, Aβ43.
Initially, a procedure was set up to obtain purified plaque cores. The most conventional method when preparing amyloidogenic material is SDS preparations. The pellet from these preparations reflects the total insoluble material, which includes not only plaque cores, but also amyloid from vessels and diffuse deposits. Through a series of centrifugation steps including sucrose fractionation and filtration steps, plaque core pellets were obtained which served as starting material for the study. We also prepared SDS insoluble material.

To generate shorter and less hydrophobic peptides, as compared to those generated by trypsin cleavage, CNBr was used to cleave the samples. Conditions were optimized for detection of Aβ species and we started with studies of the synthetic standard peptides. It was necessary to use a low concentration of acetonitrile (ACN) in the loading buffer (1%) as well as at the start of the gradient (2%) in order to trap the short hydrophilic Aβ36-40 fragment. Aβ36-42 and Aβ36-43 had similar retention times and so the gradient was then optimized with respect to separation of these. A gradient from 2% to 14% ACN in 20 min was observed to give good separation between the closely eluted species. The detection limits of the standard peptides were also investigated. The detection limit was higher for Aβ36-40 when compared to Aβ36-42 and Aβ36-43. This was probably due to the short Aβ36-40 fragment not being as easily trapped on the enrichment column. This is an integral part of the chip column and therefore not possible to bypass. It was interesting to note that the detection limit for the longer and more hydrophobic peptides increased significantly compared to the shorter ones. Therefore, it appeared possible that longer peptides than Aβ36-43 were present at low levels in the samples, but which had escaped detection in our system. The monoisotopic masses for the Aβ fragments of interest were plotted as extracted ion chromatograms, and quantification was performed based on peak area. The peak areas were used in the equations from the standard curves in order to calculate the concentrations expressed as nmol Aβ/g of wet brain tissue. The conditions for LC-MS/MS analysis were also used in paper **paper IV**.

C-terminal Aβ variants in plaque cores were analysed along with SDS preparations (SDS-insoluble material from brain homogenates) from six SAD and two *APP*Swe cases. Samples were prepared from the occipital cortex, which was chosen since it is

known to be a plaque core rich region (Arnold et al., 1991). Interestingly, we found a longer species ending at residue 43 in all cases. This species was clearly more frequent than Aβx-40, which was detectable only in the two *APP*Swe cases and in one SAD case. Interestingly, this SAD case was the only *APOE* ε4/4 carrier. As expected, Aβx-42 was present and readily detected in all samples analysed. Further, frontal cortex was included from SAD cases, *APP*Swe cases, and one I143T *PSEN1* case, in the study in order to compare Aβ variants in a region with a lower plaque load (Arnold et al., 1991). In frontal cortex, Aβx-43 was detectable in seven out of ten brains, Aβx-42 was present in all brains and Aβx-40 was only found in the *APP*Swe cases. In line with earlier studies, showing that the frontal cortex is a less plaque core rich region, the Aβ concentrations were in general lower in this area compared to the occipital cortex (Arnold et al., 1991). No variants longer than Aβx-43 were detected in any of the brains. In agreement with earlier studies, we conclude that Aβ42 is the major species in amyloid plaques in AD brain (Iwatsubo et al., 1994).

In order to investigate whether longer Aβ species could be found in diffuse deposits and in the walls of cerebral vessels, as well as to study the relation between different variants, Aβ was studied in the insoluble material from SDS preparations obtained from occipital cortex of four SAD, two *APP*Swe, and from the frontal cortex of the I143T *PSEN1*. Aβx-42 and Aβx-43 were readily detected in all cases and Aβx-40 was found in all but one SAD case, in all *APP*Swe cases, but not in the I143T *PSEN1*. The higher abundance of Aβ40 in the SDS preparations is probably because of CAA, which to a great extent consists of Aβ40 (Gravina et al., 1995; Iwatsubo et al., 1994).

Since only C-terminal fragments were analysed in our study one could speculate that these fragments could have been derived from the P3 peptide (Aβ17-42). A study of diffuse plaques in SAD identified Aβ17-42 as well as other truncated Aβ1-42 peptides (Gowing et al., 1994). To investigate whether the C-terminal fragments found in this study could have been derived from P3, the chromatograms were analysed to see if the N-terminal peptide that would have been produced by CNBr cleaved P3, Aβ17-35, was present in our samples. A peak was no found in the extracted chromatograms with m/z corresponding to the mass of this fragment, and therefore the conclusion was made that the majority of the C-terminal fragments originate from β-cleaved APP.

Next, we studied the aggregation propensity of Aβ1-40, Aβ1-42, Aβ1-43 and compared these with the long and hydrophobic Aβ1-46 peptide using a thioflavin T (Tht) assay. Presumably, Aβ1-46 has a high propensity to polymerize but this has not, to our knowledge, been shown. The peptides used in the Tht assay were dissolved in hexafluoroisopropanol (HFIP) and fluorescence was measured after different time points. We concluded that, as expected, the fluorescence was lowest for Aβ1-40 and highest for Aβ1-46. The aggregation properties for Aβ1-42 and Aβ1-43 were similar and this suggested that the Tht results reflect the hydrophobic properties of the peptides investigated.

As a complement to the results obtained from the LC-MS/MS analysis, the identified Aβ species was studied by immunohistochemistry. Occipital cortex was examined in two SAD cases, one with low levels of Aβx-40, another with high levels (the *APOE* ε4/4 carrier), and one *APP*Swe case. The immunohistochemical analysis revealed that Aβx-42 staining was intense and enabled several plaques to be visible. Aβx-43 was found in plaques but the intensity of the staining was lower compared to Aβx-42 staining. The Aβx-40 staining was more variable. The *APOE* ε4/4 carrier showed mostly vascular staining, but some plaques were also visible. The other SAD cases showed only weak Aβx-40 staining while the *APP*Swe case displayed strong, mostly vascular staining.

THE *PSEN1* I143T MUTATION IN A SWEDISH ALZHEIMER FAMILY: CLINICAL REPORT AND QUANTIFICATION OF Aβ IN DIFFERENT BRAIN REGIONS

Methods developed for plaque core preparations and quantifications of C-terminal Aβ species in **paper III** were applied also in **paper IV**. The aim was to compare and quantify Aβ variants in different brain regions. In this study, the opportunity was taken to include six brain regions- frontal, occipital, parietal and temporal cortex; as well as anterior hippocampus and cerebellum, all obtained from two mutation carriers of a I143T *PSEN1* mutation, which causes a very severe and aggressive dementia. The clinical and neuropathological characteristics of this three generational family with a I143T *PSEN1* mutation were reported. This study provided the first identification in Sweden of this mutation, which had been previously reported in other countries.

The mutation present in this family manifests in a very severe form of familial EOAD. The reported mean age of onset is 34 years of age and the mean age of death is about 41 years of age (http://www.molgen.ua.ac.be/ADMutations). Clinical investigation revealed characteristics of cognitive impairments typical seen in AD, such as: Memory impairment, disorientation, dysphasia and dysphraxia. Other clinical features identified included coordination problems associated with myoklonic jerks, a history of falling, and epileptic seizures, which are symptoms similar to a Belgian family with the same mutation described as early as 1940 (Martin et al., 1991; Cruts et al., 1995; van Bogaert et al., 1940). In later stages of the disease hallucinations, aggressiveness, and paranoid illusions appear. The youngest generation was treated using cholinesterase inhibitors, with little or no effect on rapid progression of the disease. Brains from three of the family members were subject for autopsy. In brief, autopsied brains of three of the family members showed atrophy consistent with Alzheimer pathology. Also the cerebellum, which is normally relatively spared in AD, was severely affected by amyloid in cases III:1 and III:2.

A quantitative study was performed on the C-terminal Aβ fragments in plaque cores as well as SDS insoluble material from the six brain regions from two mutation carriers in the EOAD family earlier discussed, case III:2 and III:2. In plaque cores, Aβx-42 was readily detected in all regions in both cases. Interestingly, Aβx-40 was not at all detected in any of the regions. We found the longer species, which was identified in **paper III**, Aβx-43, in all regions in both cases except for the cerebellum. The highest concentrations of both Aβx-42 and Aβx-43 were found in occipital cortex, which is consistent with the results in **paper III**. The lowest concentrations of Aβx-42 were found in cerebellum, where, Aβx-43 was not at all detected. The Aβx-42 concentrations were similar in frontal cortex and anterior hippocampus, but higher in temporal and parietal cortex. No significant difference in Aβx-43 concentration was observed between these regions. Aβ was also analysed in SDS insoluble material from brain homogenates from the six brain regions investigated. Aβx-42 was readily detected in all regions in both case samples. As for the results from plaque cores samples, the lowest concentrations of Aβx-42 were found in cerebellum and the highest concentrations were found in occipital cortex. Aβx-43 was readily detected in all regions from both cases. Interestingly, Aβx-40 was detected only in occipital cortex and cerebellum of case III:2. In accordance with earlier studies, the conclusion

was made that Aβx-42 is the most prevalent species in amyloid plaque cores in AD brain. Interestingly, there were no detectable levels of Aβx-40 in plaque cores.

Immunohistochemical analysis was also performed on consecutive sections of the brain regions. Sections from the six regions of the two cases were stained with antibodies specific for the C-terminus of Aβx-40, Aβx-42, or Aβx-43. The immunohistochemical analysis revealed that Aβx-40 was mainly localized to the cerebral vessels, while Aβx-42 and Aβx-43 were mainly present in plaques. An intense staining of the Aβx-43 antibody was achieved and a high background was observed. This antibody is the same kind (not same batch) as used in **paper III**, although results varied, and in **paper III**, the staining was much weaker. Note that the immunohistochemical experiments were performed in different laboratories using different protocols. In this study, **paper IV**, the staining was visualized in a Bond™ Polymer Refine Detection system enhancing the immune response.

In a complementary study, Aβ production was analysed in cultured fibroblasts from the *PSEN1* I143T mutation carrier, three healthy controls, and one *APP*Swe mutation carrier; using a sensitive ELISA. The sample from the *APP*Swe mutation carrier clearly showed a higher concentration of secreted Aβ40 and Aβ42 when compared to all other samples. In samples where secreted Aβ was measured in the presence of a γ-secretase inhibitor the concentrations were distinctly reduced. The cellular Aβ40 concentration did not differ significantly between the samples. Also, the cellular Aβ42 concentrations were similar between the samples, but lower than cellular Aβ40 levels. Interestingly, the cellular Aβ levels were not inhibited by the γ-secretase inhibitor. One could speculate that this is due to an inability of the inhibitor to enter the cell; or that these cellular Aβ levels were already present from time zero. The highest ratio of Aβ42/40 was observed for the I143T mutation carrier for secreted Aβ, which was consistent with earlier studies (Borchelt et al., 1996; Citron et al., 1997).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

AD is a complex disorder and its underlying pathophysiological mechanisms are under intense research by many groups all over the world. Studies of the genetics and the pathology of AD, as well as data from transgenic mice and cell lines suggest a central role for Aβ in AD pathogenesis. Despite thorough studies it remains unclear how Aβ causes the neurodegeneration in AD.

In this thesis two main parts can be distinguished. The first part (**paper I** and **paper II**) focused on the γ-secretase complex, the activity of which gives rise to the generation of Aβ, eventually deposited as amyloid plaques in AD brains. The second part (**paper III** and **paper IV**), focused on the product of γ-secretase cleavage and the analyses of Aβ peptides in amyloid deposits.

In **paper I** a detailed investigation determined how γ-secretase activity is best preserved. Since γ-secretase is a transmembrane protein complex, detergents were used to extract the complex from the membrane. It was crucial to ensure during the procedures that the γ-secretase complex remained active and stable, particularly since γ-secretase associated proteins are under intense investigation, as for example by our group (Teranishi et al., 2009). Most knowledge about γ-secretase today comes from systems using exogenous substrate and cell lines over-expressing the proteins of interest. These conditions do not reflect the situation in brain. One may conclude from our study that the rat brain serves as a good substitute for the human brain. The highest γ-secretase activity was found in the fraction enriched in endosomes, ER, Golgi, and synaptic vesicles; as well as in γ-secretase components. The γ-secretase activity was highly affected by detergents, including CHAPSO, which resulted in the highest AICD production. CHAPSO, at a concentration of 1% proved good for solubilizing the complex, and subsequent dilution to 0.4% CHAPSO restored the activity. Furthermore, γ-secretase activity was highly dependent on pH and incubation time. Aβ was produced, and could be measured by a sensitive ELISA. The conditions established in **paper I** were again used in **paper II**, for studying active γ-secretase and investigating the association of γ-secretase and lipid rafts in DRMs in rat and human brain. The findings demonstrated that active γ-secretase was localized to lipid rafts in human as

well as in rat brain. The size of the DRMs containing active γ-secretase was estimated by SEC to be > 2000 kDa, indicating the presence of other proteins and lipids. Aβ production in DRMs could be measured, but it was necessary to add an exogenous substrate. Importantly, it was concluded that the majority of BACE was located outside the raft. Interestingly, an earlier study had concluded that lipid raft association of BACE is necessary for activity (Ehehalt et al., 2003). This might have therapeutic potential since decreasing raft-associated BACE could result in decreased levels of Aβ. Furthermore, our observation indicated that the amyloidogenic pathway may be initiated outside the rafts. Further studies are needed to clarify this issue.

In **paper III** and **paper IV** the focus turned to the product of γ-secretase cleavage and deposited Aβ species in AD brains. This study addressed the following questions: Are Aβ variants longer than Aβ42 present in plaque cores from AD brains? What are the levels of these variants and how might these be important for disease? Could Aβ polymerization *in vivo* be seeded by long Aβ variants? No extensive study on longer Aβ species is reported although longer variants are detected in cell lines, transgenic animals, and a few FAD and SAD cases as earlier described in this thesis. A method was developed in **paper III** to purify plaque cores. We also established a method for quantification of C-terminal Aβ species, and performed an extensive quantitative study, comparing two brain regions of SAD and FAD cases. Indeed, a longer variant was found ending at Thr43, Aβ43, which was much more frequent than Aβ40. Interestingly, in SAD cases Aβ40 was found in plaque cores only from one case, which carried the *APOE* ε4/4 genotype. As expected, Aβ42 was the predominant species found in plaque cores as well as in the total amyloid preparations. Meanwhile, around 5% of the peptides in the samples were Aβ43. In general, the concentrations of the three detected species were higher in occipital cortex compared to frontal cortex. In **paper IV** the optimized conditions for quantifications from **paper III** were used to quantify and compare C-terminal Aβ species in two mutation carriers with an I143T *PSEN1* mutation, reported now in Sweden for the first time. Six brain regions were included. Aβ43 was much more frequent than Aβ40, which was not detected at all in plaque cores, and the highest levels were found in occipital cortex. Since Aβ43 was present in most AD cases, one might suggest that Aβ43 is important in AD pathogenesis. Further *in vivo* and *in vitro* studies that focus on Aβ43 are warranted.

An important future study is to examine whether longer Aβ species are present also in oligomers in the human brain. Oligomer specific antibodies are available, for example 8F5 binding to Aβ globulomers or M93 binding to ADDLs (Barghorn et al., 2005; Lambert et al., 2001). To first investigate the role of Aβ43 in oligomers, ELISA system would highly facilitate these investigations, both for studies of Aβ43 in soluble fractions from brain and also in CSF. To date, no Aβ43 ELISA is available on the market and only a few Aβ43 antibodies are available at present. An initial step for future studies would therefore be to develop an Aβ43 ELISA system. Furthermore, if Aβ43 is present in oligomeric Aβ species, vaccination using C-terminal Aβ43 antibodies might serve as a future target approach.

CSF can be used for diagnostics in neurodegenerative diseases since it is in direct contact with the brain via the interstitial fluid. Moreover, CSF, since it fills intracranial cavities may to some extent reflecting the metabolic processes in the brain. Several studies have shown that Aβ1-42 levels in CSF as well as the Aβ42/40 ratio are significantly decreased in AD patients compared to controls (Blennow, 2005). This finding has made Aβ42 levels in CSF a candidate biomarker for AD. It is hypothesized that the lower levels of Aβ42 reflect the peptide composition of accumulated Aβ in the brain. A summary of 6 previous studies has shown that the mean sensitivity to differentiate AD from controls was 89% when using CSF Aβ42 levels as a diagnostic biomarker for AD (Blennow, 2005). Although these results appear promising, it would be interesting also to study the presence of Aβ43 in CSF and the ratio of Aβ43/40. Doing so would add more information about the significance of Aβ43 in AD etiology, and enable the examination of Aβ43 as a potential, future diagnostic marker for the disease, alone or in combination with other candidate biomarkers.

ACKNOWLEDGEMENTS

I would especially like to thank the following people who in different ways have supported and encouraged me during the years:

My main supervisor **Lars Tjernberg**. First of all I want to thank you for taking me on as a PhD student and for trusting me with interesting ideas and projects, and especially for letting me struggle with mass spectrometry. ☺ I think you never give any easy answers, but I have learned a lot during these years. Your sense of humor makes things feel easier. Working with you has been everything but boring and I can't understand how quickly these years have passed!

My co-supervisor **Erik Sundström**, for your excellent knowledge in the neuroscience field and for good advice in different matters. Also for taking your time to answer questions when you have your little green "welcome" sign at your office door!

My co-supervisor **Bengt Winblad**, for giving me the opportunity to work in the department. Thank you for encouragement and for creating a very professional research atmosphere!

Jenny Frånberg. First of all, thank you for your outstanding support when I first started here, showing me everything in the lab and teaching me new things. Your friendship has meant so much during these years and now, and actually, I want to say that after have known you for just a few weeks it felt like I had known you for all my life! Without our everlasting talks about absolutely everything, many fun moments, laughs, coffees and many cakes, this would not have been half as fun! **Ji-Yeun Hur**. Thank you for welcoming me when I first started at Kaspac, your friendship means a lot to me, for introducing me to your nice Korean food (especially kimchi), for your concern, you being so social and cheer things up and for many nice talks!

Previous post doc **Lisa Stone**, for your friendship, mental support and comforting emails (now from the other side of the world), your fabulous sense of humor, help with practical matters and last but not least for teaching me so much English slang and nasty words!! ☺ I'm looking forward to seeing you soon in NY and visiting you in Liverpool was great fun!

Masakazu (Masa) Hashimoto, for your kindness, wonderful sense of humor, nice and fun moments, laughs, your concern and supportive attitude and for teaching me your special ELISA! Former co-worker **Yasuhiro (Yasu) Teranishi**, for being so kind, all the nice talks we had about many things and your sense of humor and for many laughs.

Waltteri Hosia, for your nice attitude, humor and for being kind. **Susanne Frykman**, for your enthusiasm and energy.

Birgitta (Bitti) Wiehager for being so kind and for nice talks and company in the lab. **Homira Behbahani** for always taking your time to listen, being supportive and for nice company and many talks in the lab.

Caroline Graff, for nice collaboration, for your positive and very encouraging attitude, and for never being too tired or stressed for interesting discussions and for taking your time. **Lina Rosvall**, for such a nice collaboration– it has been pleasant to work with you, for many nice talks and your concern. **Huei-Hsin Chiang** for being so kind and for nice sightseeing together in Chicago (also during huge thunder storm!). Collaborators **Inger Nennesmo**, for good collaboration and for your great enthusiasm and **Åsa K Wallin**.

Inga Volkmann, for all your help with immunohistochemistry, for your enthusiasm and good mood. Also, thank you for providing me with nice honey from your bee farm. **Nenad Bogdanovic**, for giving excellent lectures and for providing me with the nice plaque and tangle pictures for this thesis.

All other nice people at **Kaspac** and **KI-ADRC**, both present and former: **Anna Sillén** for nice talks, **Annica Rönnbäck** for being so positive and for taking your time, **Hanna Sagelius**, **Jesper Brohede** for your positive attitude, **Maria Ankarcrona**, **Shunwei Zhu**, **Natsuko Goto** and **Takahiro (Taka) Kihara** for being so nice new members of our γ-group, **Pavel Pavlov**, **Balbir Kaur**, **Jenny Björkström**, **Hiroyoshi (Hiro) Kakuyama**, **Mikio Aoki** and **Akira Yamazaki** for nice company in our group. **Gunilla Johansson**, for excellent help with many practical matters. **Kristina Sinegube-Lundh**, for being so nice.
A special thanks to **Lotta Forsell** and **Lena Lilius** for nice talks and laughs, your nice attitude and for making this a special place to be!

PhD-students for nice lunches and coffee breaks, everyone of you has contributed to a nice atmosphere: **Raffaella Crinelli** for nice discussions and your comforting and nice attitude and for being glamorous, **Erik Hjort** for your sense of humor, **Anna Sandebring** for being so nice, **Louise Hedskog** for your kindness and fair attitude, **Annelie Svensson** for nice coffee and cake moments and for your positive and calm attitude. **Eric Westman**, **Johanna Wanngren**, **Per-Henrik (PH) Andersson**, **Anna Lilja**, **Alina Codita** and **Linn Wicklund** for nice lunches. **Xing-Mei Zhang** for bringing us to China town in Chicago, **Michael Schöll** for your kind attitude, **Tamanna Mustafiz** for being so nice and **Andreas Frick** for nice talks during lunches. Post docs, **Monica Perez-Manso**, **Laura Mateos** and **Francisco (Patxi) Gil-Bea**.

Lena (Hullan) Holmberg and **Ewa-Britt Samuelsson** for nice talks during lunches.

Senior scientists **Marianne Schultzberg** for your positive attitude and sharing many lunches with us PhD-students in the lunch room, **Tatjana Nilsson** for being so kind and for nice talks about Denmark among other things, **Ronnie Folkesson**, **Elisabeth Åkesson**, **Helena Karlström** for being so positive and for arranging the PhD student seminars, **Angel Cedazo-Minguez**, **Jin-Jing Pei** and **Eirikur Benedikz**.

Så till alla utanför NVS som på så många sätt stöttat mig, jag vill speciellt tacka:

Karin Husman på institutionen för medicin i Solna. Utan Ditt stöd under hösten 2003 hade jag inte fortsatt på den här banan! Tack! Jag är väldigt glad att jag får möjlighet att skriva detta nu.
Caroline (KU), för snart 30 års vänskap! Tack för så mycket roligt, säkert fyrsiffrigt antal telefonsamtal, pluggande tillsammans och stöd under åren! Ett stort tack också till Din familj för att jag alltid har känt mig så välkommen hos er!
Sophie, för allt roligt från gymnasiet och framåt och för knäppa konsertbesök! Tack också för att Du visat sådant intresse för det jag gör och orkat lyssna på allt som jag oroar mig för... Tack också till **Joakim** och **Susanne** (**Sanna) S** för omtanke och trevliga och roliga middagar och fester!
Lina. För vänskap sedan lågstadiet. För att Du är så positiv, kreativ och klarar hur mycket som helst på en gång!
Magnus. För vänskap, roliga konsertbesök och Roskildefestivalen, och allt stöd under KTH-tiden!
Jenny-Li, för att man när som helst kan ringa Dig även om vi inte ses ofta längre, för allting roligt och jobbigt vi upplevt genom åren, för allt man kan fråga Dig till råds om.
Roonak, för vänskap, att vi klarade den tråkiga datalogikursen tillsammans och för att jag genom Dig fått träffa så många trevliga människor.
Åsa, **Kanchana** och **Sanna** för allt roligt vi hittat på under och efter KTH-tiden.

Min familj:
Det känns omöjligt att här med ord beskriva hur mycket ni betyder för mig och vilket stöd ni är, men jag vill ändå säga någonting till er:

Pappa. Tack för allt stöd, uppmuntran och omtanke genom åren och inte minst det stora intresse Du alltid visat för allt jag gör! Tack också för att Du introducerade mig för löpning för 25 år sedan, utan alla löprundor hade jag definitivt inte alltid haft ”huvudet upp och fötterna ned”, tack för allt trevligt genom åren och för alla minnen vi delar av farmor, farfar och Misty.

Mamma och Göran för allt ert stöd, enorma omtanke och allting roligt genom åren och nu! Mamma också för telefonsamtal dagligen ”på väg hem” som gör att vägen känns kortare och Göran för att på olika sätt visa sådan omtanke, ge kloka råd och att aldrig stressa upp sig i onödan! **Anette**. För Din omtanke, uppmuntran och allt roligt vi haft och har när vi ses!

Resten av min **danska** och **svenska familj**!
Hela Carls familj!

Min **Carl**. För allt enastående tålamod, stöd och uppmuntran, för att Du är den Du är. För att allting känns roligare med Dig och att vardagen aldrig är tråkig. När jag tänker på Dig blir jag varm i hjärtat, och jag är glad och lycklig varje stund för att jag har träffat just ***Dig***!

****

I am most grateful to different sources that have supported this thesis. I would like to express my sincere gratefulness to: Bernard Osher Foundation, Dainippon Sumitomo Pharma, Demensförbundet, Gamla Tjänarinnor, Gun och Bertil Stohnes Stiftelse and Socialstyrelsen.

REFERENCES

Abad-Rodriguez L, Ledesma MD, Craessaerts K et al. (2004) Neuronal membrane cholesterol loss enhances amyloid peptide generation, J Cell Biol 167, 953-960.

Allen DD, Galdzicki Z, Brining SK et al. (1997) Beta-amyloid induces increase in choline flux across PC12 cell membranes, Neurosci Lett 234, 71-73.

Alzheimer A. (1907) Über eine eigenartige Erkrankung der Hirnrinde, Allgemeine Zeitschrift für Psychiatrie und Psychisch-Gerictliche Medizin 64, 146-148.

Arispe N, Rojas E, Pollard HB. (1993) Alzheimer disease amyloid β protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminium, Proc Natl Acad Sci USA 90, 567-571.

Arnold SE, Hyman BT, Flory J, et al. (1991) The topographical and neuroanatomical distribution of neurofibrillary tangles and neuritic plaques in the cerebral cortex of patients with Alzheimer's disease, Cereb Cortex 1, 103-116.

Baker HF, Ridley RM, Duchen LW et al. (1993) Evidence for the experimental transmission of cerebral β-amyloidosis to primates, Int J Exp Pathol 74, 441-454.

Barghorn S, Nimmrich V, Striebinger A et al. (2005) Globular amyloid β-peptide oligomer – a homogenous and stable neuropathological protein in Alzheimer's disease, J Neurochem 95, 834-847.

Beher D, Hesse L, Masters CL et al. (1996) Regulation of amyloid protein precursor (APP) binding to collagen and mapping of the binding sites on APP and collagen type I, J Biol Chem 271, 1613-1620.

Blennow K. (2005) CSF biomarkers for Alzheimer's disease: use in early diagnosis and evaluation of drug treatment, Expert Rev Mol Diagn 5, 661-672.

Borchelt DR, Thinakaran G, Eckman CB et al. (1996) Familial Alzheimer's disease-linked presenilin 1variants elevate Aβ1-42/1-40 ratio in vitro and in vivo, Neuron 17, 1005-1013.

Breen KC, Bruce M, Anderton BH. (1991) β amyloid precursor protein mediates neuronal cell-cell and cell-surface adhesion, J Neurosci Res 28, 90-100.

Bubber P, Haroutunian V, Fisch G et al. (2005) Mitochondrial abnormalities in Alzheimer brain: mechanistic implications, Ann Neurol 57, 695-703.

Busciglio J, Lorenzo A, Yeh J et al. (1995) β-amyloid fibrils induce tau phosphorylation and loss of microtubule binding, Neuron 14, 879-888.

Buxbaum JD, Liu KN, Luo Y et al. (1998) Evidence that tumor necrosis factor α converting enzyme is involved in regulated α-secretase cleavage of the Alzheimer amyloid protein precursor, J Biol Chem 273, 27765-27767.

Chen F, Hasegawa H, Schmitt-Ulms G et al. (2006) TMP21 is a presenilin complex component that modulates γ-secretase but not ε-secretase activity, Nature 440, 1208-1212.

Citron M, Oltersdorf T, Haass C et al. (1992) Mutation of the β-amyloid precursor protein in familial Alzheimer's disease increases β-protein production, Nature 360, 672-674.

Citron M, Vigo-Pelfrey C, Teplow DB et al. (1994) Excessive production of amyloid β-protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation, Proc Natl Acad Sci USA 91, 11993-11997.

Citron M, Westaway D, Xia W et al. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β-protein in both transfected cells and transgenic mice, Nat Med 3, 67-72.

Corder EH, Saunders AM, Strittmatter WJ et al. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families, Science 261, 921-923.

Cordy JM, Hooper NM, Turner AJ. (2006) The involvement of lipid rafts in Alzheimer's disease, Mol Membr Biol 23, 111-122.

Cruts M, Backhovens H, Wang SY et al. (1995) Molecular genetic analysis of familial early-onset Alzheimer's disease linked to chromosome 14q24.3, Hum Mol Genet 4, 2363-2371.

Dahlgren KN, Manelli AM, Stine WB et al. (2002) Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability, J Biol Chem 277, 32046-32053.

Dyrks T, Weidemann A, Multhaup G et al. (1988) Identification, transmembrane orientation and biogenesis of the amyloid A4 precursor Alzheimer's disease, EMBO J 7, 949-957.

Ebinu JO, Yankner BU. (2002) A RIP tide in neuronal signal transduction, Neuron 34, 499-502.

Edbauer D, Willem M, Lammich S et al. (2002) Insulin-degrading enzyme rapidly removes the β-amyloid precursor protein intracellular domain (AICD), J Biol Chem 277, 13389-13393.

Edbauer D, Winkler E, Regula JT et al. (2003) Reconstruction of γ-secretase activity, Nat Cell Biol 5, 486-488.

Eggeling C, Ringemann C, Medda R et al. (2009) Direct observation of the nanoscale dynamics of membrane lipids in a living cell, Nature 457, 1159-1162.

Ehehalt R, Keller P, Haass C et al. (2003) Amyloidogenic processing of the Alzheimer β-amyloid precursor protein depends on lipid rafts, J Cell Biol 160, 113-123.

Englund H, Sehlin D, Johansson AS et al. (2007) Sensitive ELISA detection of amyloid-β protofibrils in biological samples, J Neurochem 103, 334-345.

Englund H, Degerman Gunnarsson M, Brundin RM et al. (2009) Oligomerization partially explains the lowering of Aβ42 in Alzheimer's disease cerebrospinal fluid, Neurodegener Dis 6, 139-147.

Esh C, Patton L, Kalback W et al. (2005) Altered APP processing in PDAPP (Val717 – Phe) transgenic mice yields extenden-length Aβ peptides, Biochemistry 44, 13807-13819.

Evin G, Canterford LD, Hoke DE et al. (2005) Transition-state analogue γ-secretase inhibitors stabilize a 900 kDa presenilin/nicastrin complex, Biochemistry 44, 4332-4341.

Farmery MR, Tjernberg LO, Pursglove SE et al. (2003) Partial purification and characterization of γ-secretase from post-mortem human brain, J Biol Chem 278, 24277-24282.

Fenn JB, Mann M, Meng CK et al. (1989) Electrospray ionization for mass spectrometry of large biomolecules, Science 246, 64-71.

Fortna RR, Crystal AS, Morais VA et al. (2004) Membrane topology and nicastrin-enhanced endoproteolysis of aph-1, a component of the γ-secretase complex, J Biol Chem 279, 3685-3693.

Fraering PC, Ye W, Strub JM et al. (2004) Purification and characterization of the human γ-secretase complex, Biochemistry 43, 9774-9789.

Gellerman GP, Byrnes H, Striebinger A et al. (2008) Aβ-globulomers are formed independently of the fibril pathway, Neurobiol Dis 30, 212-220.

Georganopoulou DG, Chang L, Nam JM et al. (2005) Nanoparticle-based detection in cerebral spinal fluid of a soluble pathogenic biomarker for Alzheimer's disease, Proc Natl Acad Sci USA 102, 2273-2276.

Geula C, Wu C, Saroff D et al. (1998) Aging renders the brain vulnerable to amyloid beta-protein neurotoxicity, Nat Med 4, 827-831.

Glenner GG, Wong CW. (1984a) Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein, Biochem Biophys Res Commun 120, 885-890.

Glenner GG, Wong CW. (1984b) Alzheimer's disease and Down's syndrome: Sharing of a unique cerebrovascular amyloid fibril protein, Biochem Biophys Res Commun 122, 1131-1135.

Goate A, Chartier-Harlin MC, Mullan M et al. (1991) Segregation of the missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease, Nature 349, 704-706.

Gong Y, Chang L, Viola KL et al. (2003) Alzheimer's disease affected brain: presence of oligomeric Aβ ligands (ADDLs) suggests a molecular basis for reversible memory loss, Proc Natl Acad Sci USA 100, 10417-10422.

Gowing E, Roher AE, Woods AS et al. (1994) Chemical characterization of Aβ 17-42 peptide, a component of diffuse amyloid deposits of Alzheimer disease, J Biol Chem 269, 10987-10990.

Gravina SA, Ho L, Eckman CB et al. (1995) Amyloid β protein (Aβ) in Alzheimer's disease brains, J Biol Chem 270, 7013-7016.

Gu Y, Misonou H, Sato T et al. (2001) Distinct intramembrane cleavage of the β-amyloid precursor protein family resembling γ-secretase-like cleavage of notch, J Biol Chem 276, 35235-35238.

Gu Y, Sanjo N, Chen F et al. (2004) The presenilin proteins are components of multiple membrane-bound complexes that have different biological activities, J Biol Chem 279, 31329-31336.

Gylys KH, Fein JA, Tan AM et al. (2003) Apolipoprotein E enhances uptake of soluble but not aggregated amyloid-β protein into synaptic terminals, J Neurochem 84, 1442-1451.

Hardy JA, Higgins GA. (1992) Alzheimer's disease: the amyloid cascade hypothesis, Science 256, 184-185.

Hardy J, Selkoe DJ. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics, Science 297, 353-356.

Harold D, Abraham R, Hollingworth P et al. (2009) Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease, Nat Genet 41, 1088-1093.

Harper JD, Wong SS, Lieber CM et al. (1997) Observation of metastable Aβ amyloid protofibrils by atomic force microscopy, Chem Biol 4, 119-125.

Hartley DM, Walsh DM, Ye CP et al. (1999) Protofibrillar intermediates of amyloid β-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons, J Neurosci 19, 8876-8884.

Hattori C, Asai M, Onishi H et al. (2006) BACE1 interacts with lipid raft proteins, J Neurosci Res 84, 912-917.

Hendriks L, van Duijn CM, Cras P et al. (1992) Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the β-amyloid precursor protein gene, Nat Genet 1, 218-221.

Herreman A, Serneels L, Annaert W et al. (2000) Total inactivation of γ-secretase activity in presenilin-deficient embryonic stem cells, Nat Cell Biol 2, 461-462.

Hu X, Crick SL, Bu G et al. (2009) Amyloid seeds formed by cellular uptake, concentration, and aggregation of the amyloid-β peptide, Proc Natl Acad Sci USA, Epub ahead of print.

Hur JY, Welander H, Behbahani H et al. (2008) Active γ-secretase is localized to detergent-resistant membranes in human brain, FEBS J 275, 1174-1187.

Hussain I, Powell D, Howlett DR et al. (1999) Identification of a novel aspartic protease (Asp 2) as β-secretase, Moll Cell Neurosci 14, 419-427.

Iwatsubo T, Odaka A, Suzuki N et al. (1994) Visualization of Aβ42(43) and Aβ40 in senile plaques with end-specific Aβ monoclonals: evidence that an initiallydeposited species is Aβ42(43), Neuron 13, 45-53.

Jada L, Dickson DW, Lin WL et al. (2001) Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP, Science 293, 1487-1490.

Jan A, Gokce O, Luthi-Carter R et al. (2008) The ratio of monomeric to aggregated forms of Aβ40 and Aβ42 is an important determinant of amyloid-β aggregation, fibrillogenesis, and toxicity, J Biol Chem 283, 28176-28189.

Jarrett JT, Berger EP, Lansbury PT Jr. (1993) The carboxy terminus of the β amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease, Biochemistry 32, 4693-4697.

Jarrett JT, Lansbury PT Jr. (1993) Seeding "one-dimensional crystallization"of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie?, Cell 73,1055-1058.

Jarriault S, Brou C, Logeat F et al. (1995) Signalling downstream of activated mammalian Notch, Nature 377, 355-358.

Kamal A, Almenar-Queralt A, LeBlanch JF et al. (2001) Kinesin-mediated axonal transport of a membrane compartment containing β-secretase and presenilin-1 requires APP, Nature 414, 643-648.

Kamino K, Orr HT, Payami H et al. (1992) Linkage and mutational amalysis of familial Alzheimer disease kindreds for the APP gene region, Am J Hum Genet 51, 998-1014.

Kang J, Lemaire HG, Unterbeck A et al. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor, Nature 325, 733-736.

Kido H, Fukutomi A, Schilling J et al. (1990) Protease-specificity of kunitz inhibitor domain of Alzheimer's disease amyloid protein precursor, Biochem Biophys Res Commun 167, 716-721.

Kimberly WT, LaVoie MJ, Ostaszewski BL et al. (2003) γ-secretase is a membrane protein complex comprised of presenilin, nicastrin, aph-1, and pen-2, Proc Natl Acad Sci USA 100, 6382-6387.

Kirschner DA, Abraham C, Selkoe DJ. (1986) X-ray diffraction from intraneuronal paired helical filaments and extraneuronal amyloid fibers in Alzheimer disease indicates cross-β conformation, Proc Natl Acad Sci USA 83, 503-507.

Koike H, Tomioka S, Sorimachi H et al. (1999) Membrane-anchored metalloprotease MDC9 has an α-secretase activity responsible for processing the amyloid precursor protein, Biochem J 343, pt 2, 371–375.

Kojro, E, Fahrenholz, F. (2005) The non-amyloidogenic pathway: structure and function of α-secretases, Subcell Biochem 38, 105-127.

Lambert MP, Barlow AK, Chromy BA et al. (1998) Diffusable, nonfibrillar ligands derived from Aβ1-42 are potent central nervous system neurotoxins, Proc Natl Acad Sci USA 95, 6448-6453.

Lambert MP, Viola KL, Chromy BA et al. (2001) Vaccination with soluble Aβ oligomers generates toxicity-neutralizing antibodies, J Neurochem 79, 595-605.

Lammich S, Kojro E, Postina R et al. (1999) Constitutive and regulated α-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease, Proc Natl Acad Sci USA 96, 3922-3927.

Lashuel HA, Hartley DM, Petre BM et al. (2002) Neurodegenerative disease: Amyloid pores from pathogenic mutations, Nature 418, 291.

Levy E, Carman MD, Fernandez-Madrid IJ et al. (1990) Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, dutch type, Science 248, 1124-1126.

Levy-Lahad E, Wasco W, Poorkaj P et al. (1995) Candidate gene for the chromosome 1 familial Alzheimer's disease locus, Science 269, 973-977.

Lemere CA, Blusztajn JK, Yamaguchi H et al. (1996) Sequence of deposition of heterogenous amyloid β-peptides and APO E in Down syndrome: Implications for initial events in amyloid plaque formation, Neurobiol Dis 3, 16-32.

Lesne S, Koh MT, Kotilinek L et al. (2006) A specific amyloid-β protein assembly in the brain impairs memory, Nature 440, 352-357.

Li YM, Lai MT, Xu M et al. (2000) Presenilin 1 is linked with γ-secretase activity in the detergent solubilized state, Proc Natl Acad Sci USA 97, 6138-6143.

Liao MQ, Tzeng YJ, Chang LY et al. (2007) The correlation between neurotoxicity, aggregative ability and secondary structure studied by sequence truncated Aβ peptides, FEBS Letters 581, 1161-1165.

Lobo A, Launer LJ, Fratiglioni L et al. (2000) Prevalence of dementia and major subtypes in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group, Neurology 54, S4-9.

Lyckman AW, Confaloni AM, Thinakaran G et al. (1998) Post-translational processing and turnover kinetics of presynaptically targeted amyloid precursor superfamily proteins in the central nervous system, J Biol Chem 273, 11100-11106.

Malinchik SB, Inouye H, Szumowski KE et al (1998) Structural analysis of Alzheimer's β(1-40) amyloid: protofilament assembly of tubular fibrils, Biophys J 74, 537-545.

Markesbery WR, Carney JM. (1999) Oxidative stress in Alzheimer's disease, Brain Pathol 9, 133-146.

Martin JJ, Gheuens J, Bruyland M et al. (1991) Early-onset Alzheimer's disease in 2 large Belgian families, Neurol 41, 62-68.

Masters CL, Simms G, Weinman NA et al. (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome, Proc Natl Acad Sci USA 82, 4245-4249.

Mayeux R. (2003) Epidemiology of neurodegeneration, Annu Rev Neurosci 26, 81-104.

Maynard CJ, Bush AI, Masters C et al. (2005) Metals and amyloid-β in Alzheimer's disease, Int J Path 86, 147-159.

McLaurin J, Yang DS, Yip CM et al. (2000) Modulating factors in amyloid-β fibril formation, J Struct Biol 130, 259-270.

McLendon C, Xin TP, Ziani-Cherif C et al. (2000) Cell-free assays for γ-secretase activity, FASEB J 14, 2383-2386.

Meyer-Luehmann M, Coomaraswamy J, Bolmont T et al. (2006) Exogenous induction of cerebral β-amyloidogenesis is governed by agent and host, Science 313, 1781-1784.

Milward EA, Papadopoulos R, Fuller SJ et al. (1992) The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth, Neuron 9, 129-137.

Miners JS, Baig S, Palmer J et al. (2008) Aβ-degrading enzymes in Alzheimer's disease, Brain Pathol 18, 240-252.

Miravalle L, Calero M, Takao M et al. (2005) Amino-terminally truncated Aβ peptide species are the main component of cotton wool plaques, Biochemistry 44, 10810-10821.

Mori H, Takio K, Ogawara M et al. (1992) Mass spectrometry of purified amyloid β protein in Alzheimer's disease, J Biol Chem 267, 17082-17086.

Mullan M, Crawford F, Axelman K et al. (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of β-amyloid, Nat Genet 1, 345-347.

Nilsberth C, Westlind-Danielsson A, Eckman CB et al. (2001) The 'arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Aβ protofibril formation, Nat Neurosci 4, 887-893.

Olson MI, Shaw CM. (1969) Presenile dementia and Alzheimer's disease in mongolism, Brain 92, 147-156.

Olsson A, Csajbok L, Ost M et al. (2004) Marked increase of β-amyloid(1-42) and amyloid precursor protein in ventricular cerebrospinal fluid after severe traumatic head injury, J Neurol 251, 870-876.

Oltersdorf T, Ward PJ, Henriksson T et al. (1990) The Alzheimer amyloid precursor protein. Identification of a stable intermediate in the biosynthetic/dagradative pathway, J Biol Chem 265, 4492-4497.

Osenkowski P, Li H, Ye W et al. (2009) Cryoelectron microscopy structure of purified γ-secretase at 12 Å resolution, J Mol Biol 385, 642-652.

Pauling L, Corey RB, Branson HR. (1951) The structure of proteins: two hydrogen-bonded helical configurations of the polypeptide chain, Proc Natl Acad Sci USA 37, 205-211.

Pinnix I, Musunuru U, Tun H et al. (2001) A novel γ-secretase assay based on detection of the putative c-terminal fragment- γ of amyloid β protein precursor, J Biol Chem 276, 481-487.

Portelius H, Tran A, Andreasson U et al. (2007) Characterization of amyloid β peptides in cerebrospinal fluid by an automated immunoprecipitation procedure followed by mass spectrometry, J proteome Res 6, 4433-4439.

Prior IA, Muncke C, Parton RG et al. (2003) Direct visualization of ras proteins in spatially distinct cell surface microdomains, J Cell Biol 160, 165-170.

Qi-Takahara Y, Morishima-Kawashima M, Tanimura Y et al. (2005) Longer forms of amyloid β protein: implications for the mechanism of intramembrane cleavage by γ-secretase, J Neurosci 25, 436-445.

Religa D, Strozyk D, Cherny RA et al. (2006) Elevated cortical zinc in Alzheimer disease, Neurology 67, 69-75.

Roher AE, Kokjohn TY, Esh C et al. (2004) The human amyloid-β precursor protein$_{770}$ mutation V717F generates peptides longer than amyloid- β-(40-42) and flocculent amyloid aggregates, J Biol Chem 279, 5829-5836.

Sato T, Diehl TS, Narayanan S et al. (2007) Active γ-secretase complexes contain only one of each component, J Biol Chem 282, 33985-33993.

Saunders AM. (2001) Gene identification in Alzheimer's disease, Pharmacogenomics 2, 239-249.

Sawamura N, Ko M, Yu W et al. (2004) Modulation of amyloid precursor protein cleavage by cellular sphingolipids, J Biol Chem 279, 11984-11991.

Selkoe DJ. (2001) Alzheimer's disease: genes, proteins, and theraphy, Physiol Rev 81, 741-766.

Selkoe D. (2002) Alzheimer's disease is a synapthic failure, Science 298, 789-791.

Scheuner D, Eckman C, Jensen M et al. (1996) Secreted amyloid β-protein simliar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease, Nat Med 2, 864-870.

Schuck S, Honsho M, Ekroos K et al. (2003) Resistance of cell membranes to different detergents, Proc Natl Acad Sci USA 100, 5795-5800.

Shah S, Lee SF, Tabuchi K et al. (2005) Nicastrin functions as a γ-secretase-substrate receptor, Cell 122, 435-447.

Shankar GM, Mehta TH, Garcia-Munoz A et al. (2008) Amyloid-β protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory, Nat Med 14, 837-842.

Sharma P, Varma R, Sarasij RC et al. (2004) Nanoscale organization of multiple GPI-anchored proteins in living cell membranes, Cell 116, 577-589.

Shen J, Bronson RT, Chen DF et al. (1997) Skeletal and CNS defects in presenilin-1-deficient mice, Cell 89, 629-639.

Shepherd C, McCann H, Halliday GM. (2009) Variations in the neuropathology of familial Alzheimer's disease, Acta Neuropathol 118, 37-52.

Sherrington R, Rogaev EI, Liang Y et al. (1995) Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease, Nature 375, 754-760.

Shimojo M, Sahara N, Mizoroko T et al. (2008) Enzymatic characteristics of I213T mutant presenilin-1/γ-secretase in cell models and knock-in mouse brains: FAD linked mutation impairs γ-site cleavage of APP-CTFβ, J Biol Chem 283, 16488-16496.

Simons K, Ikonen E. (1997) Functional rafts in cell membranes, Nature 387, 569-572.

Sinha S, Anderson JP, Barbour R et al. (1999) Purification and cloning of amyloid precursor protein β-secretase from human brain, Nature 402, 537-540.

Strittmatter WJ, Saunders AM, Schmechel D et al. (1993) Apolipoprotein E: high-avidity binding to β-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease, Proc Natl Acad Sci USA 90, 1977-1981.

Suh YH, Checler F. (2002) Amyloid precursor protein, presenilins, and α-synuclein: molecular pathogenesis and pharmacological applications in Alzheimer disease, Pharmacol Rev 54, 469-525.

Suzuki N, Iwatsubo T, Odaka A et al. (1994) High tissue content of soluble beta 1-40 is linked to cerebral amyloid angiopathy, Am J Pathol 145, 452-460.

Takasugi N, Tomita T, Hayashi I et al. (2003) The role of presenilin cofactors in the γ-secretase complex, Nature 422, 438-441.

Teranishi Y, Hur JY, Welander H et al. (2009) Affinity pulldown of γ-secretase and associated proteins from human and rat brain, J Cell Mol Med, Epub ahead of print.

Terry RD, Masliah E, Salmon PD et al. (1991) Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment, Ann Neurol 30, 575-580.

Urano Y, Hayashi I, Isoo N et al. (2005) Association of active γ-secretase complex with lipid rafts, J Lipid Res 46, 904-912.

Van Bogaert L, Maere M, de Smedt E, (1940) Sur les formes familiales précoces de la maladie d'Alzheimer, Mschr Psychiat Neurol 102, 249-301.

Van Broeckhoven CV, Haan J, Bakker E et al. (1990) Amyloid β protein precursor gene and hereditary cerebral hemorrhage with amyloidosis (dutch), Science 248, 1120-1122.

Van Vickle GD, Esh CL, Kalback WM et al. (2007) TgCRND8 amyloid precursor protein transgenic mice exhibit an altered γ-secretase processing and an aggressive, additive amyloid pathology subject to immunotherapeutic modulation, Biochemistry 46, 10317-10327.

Van Vickle GD, Esh CL, Kokjohn TA et al. (2008) Presenilin-1 280Glu-Ala mutation alters c-terminal APP processing yielding longer Aβ peptides: implications for Alzheimer's disease, Mol Med 14, 184-194.

Vanderstichele H, Van Kerschaver E, Hesse C et al. (2000) Standardization of measurements of β-amyloid (1-42) in cerebrospinal fluid and plasma, Amyloid 7, 245-258.

Vassar R, Bennett BD, Babu-Khan S et al. (1999) β-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE, Science 286, 735-741.

Vetrivel KS, Cheng H, Lin W et al. (2004) Association of γ-secretase with lipid rafts in post-golgi and endosome membranes, J Biol Chem 279, 44945-44954.

Vetrivel KS, Cheng H, Kim SH et al. (2005) Spatial segregation of γ-secretase and substrates in distinct membrane domains, J Biol Chem 280, 25892-25900.

Wahrle S, Das P, Nyborg A et al. (2002) Cholesterol-dependent γ-secretase acticity in buoyant cholesterol-rich membrane microdomains, Neurobiol Dis 9, 11-23.

Walsh DM, Lomakin A, Benedek GB et al. (1997) Amyloid β-protein fibrillogenesis . Detection of a protofibrillar intermediate, J Biol Chem 272, 22364-22372.

Walsh DM, Klyubin I, Fadeeva JV et al. (2002) Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo, Nature 416, 535-539.

Weidemann A, König G, Bunke D et al. (1989) Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein, Cell 57, 115-126.

Westermark P, Benson MD, Buxbaum JN et al. (2005) Amyloid: Toward terminology clarification. Report from the Nomencalture Committee of the International Society of Amyloidosis, Amyloid 12, 1-4.

Wolfe MS, Weiming X, Ostaszewski BL et al. (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase acticity, Nature 398, 513-517.

Womack MD, Kendall DA, MacDonald RC. (1983) Detergent effects on enzyme activity and solubilization of lipid bilayer membranes, Biochim Biophys Acta 733, 210-215.

Yagishita S, Morishima-Kawashima M, Tanimura Y et al. (2006) DAPT-induced intracellular accumulations of longer amyloid β-proteins: further implications for the mechanism of intramembrane cleavage by γ-secretase, Biochemistry 45, 3952-3960.

Yan R, Bienkowski MJ, Shuck ME et al. (1999) Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity, Nature 402, 533-537.
Yu G, Nishimura M, Arawaka S et al. (2000) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and βAPP processing, Nature 407, 48-54.

Zhou S, Zhou H, Walian PJ et al. (2005) CD147 is a regulatory subunit of the γ-secretase complex in Alzheimer's disease amyloid β-peptide production, Proc Natl Acad Sci USA 102, 7499-7504.