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Studies on γ-secretase activity and products

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ABSTRACT

γ-Secretase is a transmembrane aspartyl protease involved in processing of a multitude of functionally diverse substrates within their transmembrane domains. γ-Secretase cleavage of the amyloid precursor protein (APP) generates the APP intracellular domain (AICD) and the amyloid β-peptide (Aβ). Aβ is a key player in the pathogenesis of Alzheimer disease (AD) and the main constituent of senile plaques, one of the hallmarks found in the brain of AD patients. Various lengths of Aβ peptides are produced but the most common forms are 40 or 42 residues long (Aβ40 or Aβ42). γ-Secretase cleavage of Notch results in the release of the Notch intracellular domain (NICD) which is known to activate transcription. Notch signaling is important for cell differentiation during embryonic development as well as in adulthood. In analogy with APP and Notch, γ-secretase cleavage of other substrates also generates secreted peptides and intracellular domains (ICDs). γ-Secretase is a protein complex consisting of presenilin (PS), nicastrin, anterior pharynx defective-1 and presenilin enhancer-2, where PS is suggested to harbor the active site.

The work of this thesis has focused on studies of γ-secretase activity and its products in AD brain and model systems such as cell lines and rat brain. In **paper I**, we examined the effect of subcellular localization, pH and detergents, on γ-secretase activity, i.e. AICD production, in rat brain membranes. A fraction containing Golgi, endoplasmic reticulum, endosomes and synaptic vesicles demonstrated the highest AICD production and the optimal pH was found to be around 7.0. In addition, the γ-secretase activity was highly affected by detergents and CHAPSO at a concentration of 0.4% was found to enhance the activity. In **paper II**, the γ-secretase dependent production of Aβ40 as well as of ICDs from APP, Notch1, N-cadherin, ephrinB and p75-neurotrophin receptor was found to be down-regulated in adult compared to embryonic rat brain membranes. Processing of all of the substrates was observed in embryonic rat brain membranes while only APP and Notch1 processing was detected in the adult rat brain membranes. In **paper III**, the large hydrophilic loop of PS1 was examined with respect to its effect on APP and Notch processing. Deletion of the PS1 loop resulted in a striking decrease in production of Aβ38, Aβ39, Aβ40, whereas production of Aβ42 was affected to a lesser extent, and the production of AICD and NICD was not impaired. In particular, the most C-terminal amino acids of the loop were important for the differential effect on APP processing. In **paper IV**, C-terminal Aβ variants deposited in sporadic and familial AD brains were identified and quantified. Both Aβ deposited in plaque cores and in total amyloid preparations was examined. Apart from Aβ40 and Aβ42, a longer Aβ species, Aβ43, was detected. Aβ43 was detected more frequently than Aβ40, especially in plaque cores. Taken together, we have optimized conditions for studies of γ-secretase processing of APP in rat brain, demonstrated an age-dependent production of ICDs from several substrates in rat brain and showed that the most C-terminal part of the PS1 loop influences the Aβ profile. Finally, a longer Aβ variant, Aβ43 was frequently detected in amyloid depositions in AD brains.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their roman numerals.

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. **Jenny Frånberg**, Helena Karlström, Bengt Winblad, Lars O. Tjernberg and Susanne Frykman
γ-Secretase dependent production of intracellular domains is reduced in adult compared to embryonic rat brain membranes
Manuscript

III. Johanna Wanngren, **Jenny Frånberg**, Annelie I. Svensson, Hanna Laudon, Bengt Winblad, Frank Liu, Jan Näslund, Johan Lundkvist and Helena Karlström
The large hydrophilic loop of presenilin 1 is important for regulating γ-secretase complex assembly and for dictating the Aβ profile without affecting Notch processing
Journal of Biological Chemistry, Epub 2010 jan 27, doi:10.1074

IV. 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

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid β-peptide
AD	Alzheimer Disease
ADAM	A disintegrin and metalloproteinase
AICD	APP intracellular domain
ApoE	Apolipoprotein E
Aph-1	Anterior pharynx defective-1
APLP	APP-like protein
APP	Amyloid precursor protein
APPSwe	Swedish APP mutation (K670N/M671L)
BACE	β-site APP cleaving enzyme
CAA	Cerebral amyloid angiopathy
CMC	Critical micelle concentration
CNBr	Cyanogen bromide
CSF	Cerebrospinal fluid
CTF	C-terminal fragment
DS	Down's syndrome
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FA	Formic acid
FAD	Familial AD
HPLC	High performance liquid chromatography
ICD	Intracellular domain
IDE	Insulin-degrading enzyme
LC-MS/MS	Liquid chromatography combined with tandem mass spectrometry
MS	Mass spectrometry
NICD	Notch intracellular domain
NTF	N-terminal fragment
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
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
ThT	Thioflavin T

INTRODUCTION

γ-Secretase is one of the most extensively investigated intramembrane proteases, primarily due to its critical role in the neuropathogenesis of Alzheimer disease. Despite these research efforts, many basic features of the γ-secretase complex remain to be answered.

ALZHEIMER DISEASE

Alzheimer disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia in the elderly, accounting for 50-70% of all cases [1]. The prevalence of dementia increases exponentially with age, from below 1% in individuals of 60-64 years of age up to 24-33% in people over age 85. In 2001, around 24 million people worldwide had dementia and the number is expected to double every 20 years as the population ages [2]. The clinical features of AD includes slow progressive impairment of episodic memory and orientation, language deficits and apraxia as well as impairments in attention and executive functions such as decision-making and processing of information. During the course of the disease, behavioral symptoms usually occur at some point, such as depression, agitation and apathy, which negatively affect the cognitive and functional status further. The patient history, together with clinical, neurological and psychiatric examination, provides the basis for establishing the clinical diagnosis of AD. Laboratory tests and neuroimaging are valuable tools to exclude other causes of dementia, such as deficiencies in thyroid function, brain tumors and cerebral infarcts. However, AD can only be definitively diagnosed by postmortem neuropathologic assessment. In addition to the impact on the lives of AD patients and caregivers, AD is of major public health concern and efficient pharmacological treatment is needed. Even though of symptomatic benefit, neither of the available treatments today stops the progression of the disease.

Neuropathology

Once the clinical symptoms occur and AD is diagnosed, the process of neurodegeneration is estimated to have proceeded for 20-30 years [3]. Cortical atrophy and concomitant enlargement of ventricles and sulci, particularly in the frontal, temporal and parietal lobes, are characteristic changes in AD. The occipital lobe and the sensory and motor regions of cortex are relatively spared. The first

neurodegenerative changes are observed in hippocampus and entorhinal cortex and measurement of hippocampal atrophy can discriminate between AD and non-affected elderly people [4]. Atrophy of these regions is, however, also present in other dementias and is not specific for AD. The atrophy in AD is due to decreased number of synapses, degenerated neurites and neuronal loss [5]. The cholinergic neurons in the basal forebrain appear to be particularly affected [6, 7].

At a microscopic level, AD is characterized by intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein [8, 9] and extracellular plaques composed of fibrils of polymerized amyloid β-peptide (Aβ) [10], the two diagnostic brain lesions initially described by Alois Alzheimer in 1906 [11]. Tau is the major microtuble-associated protein in the axon. Phosphorylation of tau regulates the levels of free and microtuble-bound tau and thereby the morphology of the neuron and the axonal transport. In AD, tau is abnormally hyperphosphorylated as a result of an imbalance in kinase and phosphatase activities. This hyperphosphorylation disrupts the binding of tau to microtubules. In addition, the hyperphosphorylation stimulates the self-assembly of tau, which finally forms paired helical filaments that in turn assemble into neurofibrillary tangles. Plaques can be subdivided into neuritic or diffuse plaques. The core of the neuritic plaques are mostly composed of Aβ42, an Aβ species ending at amino acid 42 [12], and can be visualized by staining with Congo red, a dye which binds to amyloid fibrils. The amyloid fibrils show a green/red birefringence when stained with Congo red and viewed under polarized light, which indicates an ordered structure. Neuritic plaques are surrounded by structurally abnormal neurites, activated microglia and reactive astrocytes, changes typically found in AD. In constrast, the majority of the diffuse plaques lack the associated dystrophic neurites and glia. Even though the amorphous diffuse plaques also contain Aβ42 [13] they are non-birefringent when stained with Congo red. Instead, the diffuse plaques can be detected with immunohistochemical staining using antibodies recognizing Aβ. The diffuse plaques are not only found in limbic and association cortices, where often large numbers of the neuritic plaques are found, but also in regions which do not generally display the typical AD pathology, such as cerebellum, striatum and thalamus. In addition, healthy, non-demented, aged individuals show only diffuse plaques in brain regions where AD affected individuals show both diffuse and neuritic plaques. Together these findings, suggest that diffuse plaques are precursors to neuritic plaques. This hypothesis is

supported in particular by studies of patients with Down's syndrome (DS). Individuals with DS carry an extra copy of chromosome 21, where the APP gene is located, and consequently they have been shown to develop AD with an early onset [14]. Studies showed that diffuse Aβ deposits occurred commonly in twenty to thirty year-old patients with DS, whereas few or no neuritic plaques were observed at this age [15-17]. Apart from plaques, Aβ is also deposited in the walls of blood vessels within the cerebral cortex, leading to the development of cerebral amyloid angiopathy (CAA), another common pathological observation in AD [18]. In fact, Aβ was first isolated and sequenced from meningeal vessels in AD cases and DS cases, one year prior to the isolation from plaques [19, 20]. The predominant Aβ species deposited in the vessels, is Aβ40, as judged by immunoreactivity, [21, 22].

Genetics and risk factors of AD

AD exists as sporadic or familial forms. Familial Alzheimer disease (FAD) is a monogenic disease in which the mutations are inherited in an autosomal dominant way, causing an almost complete penetrance. The familial form of the disease, however, is rare compared to the sporadic form (SAD), accounting for only a few percent of the total AD cases. The onset of disease in most FAD cases occurs before 65 years of age (early onset) while in sporadic AD the onset occurs usually after 65 years of age (late onset). So far, mutations in three genes, the amyloid precursor protein (*APP)* gene on chromosome 21, the presenilin 1 (*PSEN1*) gene on chromosome 14 and the presenilin 2 (*PSEN2*) gene on chromosome 1 have been shown to cause FAD [23-25]. To date, 32 mutations in *APP*, 178 mutations in *PSEN1* and 14 mutations in *PSEN2* have been reported (http://www.molgen.ua.ac.be/ADMutations/). All three proteins encoded by these genes are linked to the production of Aβ. APP is the precursor protein of Aβ and presenilin 1 and 2 are integral parts of γ-secretase, the enzyme responsible for generating Aβ from APP [26]. In addition to the genes involved in FAD, several putative susceptibility genes for AD have been reported. However, the only well established genetic risk factor for AD is the apolipoprotein E (*APOE*) genotype. There are three allelic variants of the *APOE* gene: ε3, ε2 and ε4, encoding the corresponding isoforms. The ε4 allele is associated with an increased risk of AD in a dose-related manner, as well as an earlier onset of the disease [27, 28]. On the contrary, ε2, the least common allele, is suggested to be protective [29]. The apoE isoforms are suggested to

mainly influence the risk of developing AD by differentially affecting the aggregation and clearance of Aβ [30].

Besides age and genetics, which are the most important risk factors for AD, epidemiological studies suggest that other factors such as traumatic brain injury, female gender, low physical and social activity, hypertension and high serum cholesterol levels at midlife are associated with AD [1]. Even though further work is needed to elucidate the impact of some of these factors and their possible role in pathogenesis, it is promising that there could be ways to reduce the risk of AD by for example maintaining an active lifestyle and control vascular risk factors.

γ-SECRETASE

The γ-secretase complex

γ-Secretase is a multi-protein complex dependent primarily on four transmembrane proteins for its function: presenilin (PS), nicastrin, anterior pharynx defective-1 (Aph-1) and presenilin enhancer-2 (Pen-2) (Figure 1).

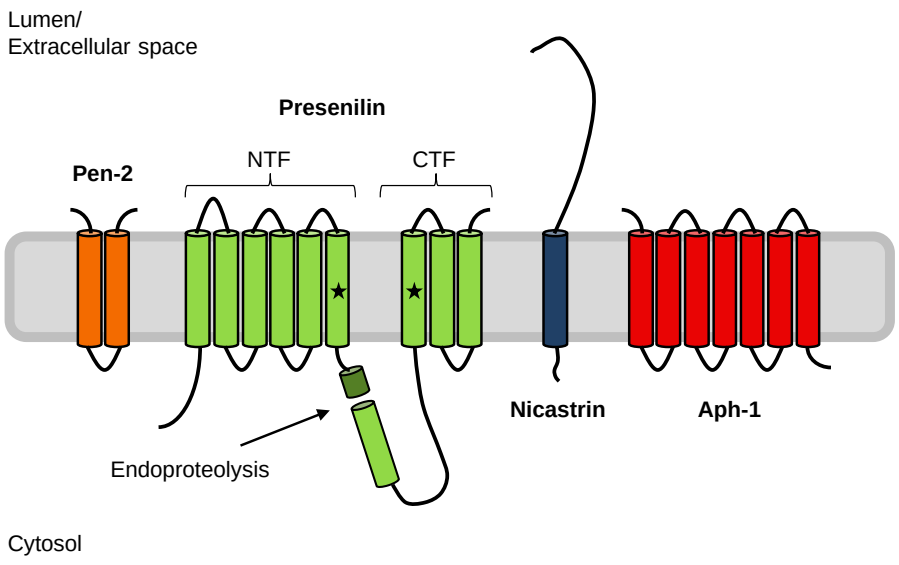

Figure 1. The components of the γ-secretase complex. The stars indicate the catalytic aspartates.

Studies in mammalian cells, demonstrated that overexpression of PS, nicastrin, Aph-1 and Pen-2 enhanced γ-secretase activity and that they physically interacted in a high molecular weight complex [31]. Multistep purification of γ-secretase from cell lines followed by masspectrometry analysis also revealed these four proteins [32]. Further, γ-

secretase activity has been reconstituted in *Saccharomyces cerevisiae,* which lacks endogenous γ-secretase components and activity, by co-expression of the four components [33]. Furthermore, the γ-secretase subunits as well as assembled complexes are ubiquitously expressed in adult mouse tissues as determined by western blot analysis [34].

Presenilin

The genes encoding PS were discovered in 1995 through genetic linkage analysis of families with FAD. PS exists as two homologues, PS1 and PS2, which share an overall amino acid sequence identity of 67% but as high as 95% in certain transmembrane regions, suggesting that the major functions of PS are associated to these hydrophobic domains. PS has nine transmembrane domains with the N-terminus located in the cytoplasm and the C-terminus in the lumen [35]. PS is endoproteolytically cleaved, within the large cytosolic loop between transmembrane domains six and seven, into N- and C-terminal fragments (NTF and CTF) [36] probably by autoproteolysis. Together these fragments form a stable heterodimer which constitutes the active form of PS. Each fragment donates one aspartate residue to the active site of the γ-secretase complex [37, 38]. Alanine mutations of Asp257 and Asp385 in PS1 and the analogues Asp263 and Asp366 in PS2 within transmembrane domains six and seven abolish Aβ production and prevent PS endoproteolysis. In addition, transition-state inhibitors of γ-secretase, designed to bind to the active site of an aspartyl protease, directly bind to PS-NTF and PS-CTF [39-42]. Mutation of the Asp residues abrogates inhibitor binding [43]. Therefore, γ-secretase is considered to be an aspartyl protease and PS the catalytic core of the complex. The large hydrophilic loop between transmembrane domain six and seven of PS is one of the most divergent regions between PS1 and PS2. β-Catenin has been shown to bind to the hydrophilic loop of PS1 but not of PS2 [44, 45]. Apart from endoproteolysis, this nonconserved region is also subjected to cleavage by caspases [46] and several FAD mutations are confined to the loop. However, when comparing non-loop FAD mutations of PS1 and PS2 with or without the hydrophilic loop domain, removal of the loop did not affect Aβ production [44]. Consistent with the idea of the loop being dispensable for γ-secretase activity, introduction of a PS1 molecule lacking the loop is able to rescue the lethal phenotype of PS1 deficiency [47]. Nevertheless, a regulatory role of the PS1 loop on γ-secretase activity has been proposed by Deng et al. [48]. They generated PS1 knock-in mice containing a deletion

of exon 10, encoding most of the hydrophilic loop including the β-catenin binding site, which showed a reduced Aβ40 formation and exacerbated plaque pathology [48].

Nicastrin

Nicastrin was isolated by affinity chromatography via its interaction with PS [49]. Nicastrin is a type I transmembrane glycoprotein with a large extracellular domain containing several glycosylation sites. However, glycosylation of nicastrin is not critical for either complex assembly or activity [50]. Maturation of nicastrin, including glycosylation, and its trafficking through the early secretory pathway, is dependent on the presence of PS [51, 52]. PS is also required for the conformational alteration of the nicastrin ectodomain during complex assembly [53]. In contrast to glycosylation, the conformational changes of the nicastrin ectodomain as well as its conserved DYIGS motif are essential for γ-secretase activity. Nicastrin has been suggested to be important for the stability of the γ-secretase complex [54] and to be involved in initial substrate recognition [55]. The glutamic residue (Glu333) in the nicastrin ectodomain is proposed to interact with the N-terminus of γ-secretase substrates and Glu333 mutated nicastrin has been shown to reduce APP processing compared to wild-type nicastrin [56]. This residue has, however, also been shown to affect complex assembly rather than activity [57].

Aph-1 and Pen-2

Through genetic screens in *Caenorhabditis elegans,* searching for genes interacting with the PS genes, Aph-1 and Pen-2 were identified [58, 59]. Aph-1 has seven transmembrane domains with the N-terminus facing the lumen and the C-terminus oriented into the cytoplasm. Two homologues of Aph-1 exist in human, Aph-1b and Aph-1a (56% amino acid sequence identity), which in turn can be alternatively spliced (Aph-1aS and Aph-1aL). Mice have an additional third isoform, Aph-1c. Aph-1 is the most stable component of the complex and it has been suggested to play a role in complex assembly, as a scaffold for the complex [60]. Finally, Pen-2 is a small hairpin protein composed of two transmembrane domains with its loop located in the cytosol and the N- and C-terminus facing the lumen [61]. The C-terminus and the first transmembrane domain of Pen-2 are vital for endoproteolysis of the PS holoprotein and stabilization of the subsequently produced PS fragments [62, 63].

Assembly, localization and stoichiometry of the γ-secretase complex

The γ-secretase complex assembly is suggested to be initiated in the early secretory pathway by Aph-1 and nicastrin forming a subcomplex [64-66], followed by the sequential incorporation of the PS holoprotein and Pen-2 [67-69]. Following incorporation of Pen-2 the PS holoprotein is cleaved into NTF and CTF [68]. How the assembly is regulated is not fully known, but Rer1p, a membrane receptor operating in Golgi retrieval, has been shown to be involved in the regulation by interacting with unassembled nicastrin and Pen-2 [70, 71]. Rer1p recycles nicastrin back to the endoplasmic reticulum (ER) where it can bind to Aph-1 and continue the formation of a mature complex [70]. In addition, phospholipase D1, a phospholipid-modifying enzyme, was demonstrated to bind to PS1 and thereby disrupting the association of the γ-secretase subunits and inhibiting the catalytic activity of γ-secretase [72]. On the contrary, over-expression of the G-protein coupled receptor 3 (GPR3) was shown to facilitate the assembly of the γ-secretase components into mature complexes [73]. When the assembly is completed, the γ-secretase complex is trafficked to its functional sites, where the substrates are encountered.

Assembled, mature and active γ-secretase complexes have been reported to reside in numerous subcellular compartments including the Golgi/trans-Golgi network [52, 74, 75], ER [76], endosomes [74, 77, 78], lysosomes [79], mitochondria [80], the synapse [78] and plasma membrane [77, 81]. However, how much each of these subcellular compartments contributes to the cellular γ-secretase activity needs to be further evaluated. Moreover, the subcellular compartment where substrate and γ-secretase co-localize could vary between different substrates.

Another unresolved question regarding γ-secretase concerns the stoichiometry and size of the complex. Depending on the experimental techniques and conditions used, such as the use of different detergents, the molecular mass of the complex has been estimated in a range of 250-2000 kDa [31, 39, 45, 82-84]. However, the sum of the molecular masses of the four components is approximately 220 kDa in a 1:1:1:1 stoichiometry. Sato et al. recently demonstrated that active γ-secretase complexes isolated by immunoprecipitation indeed contain one of each subunit per complex [85]. Consistent with this 1:1:1:1 stoichiometry, the size of purified γ-secretase was shown to be 230 kDa as determined by scanning transmission electron microscopy [86]. The

observation of larger complexes could be explained by multimeric subunits or complexes or additional regulatory components. Indeed, two proteins CD147, a transmembrane glycoprotein, and TMP21, a protein involved in protein transport and quality control in the ER and Golgi, have been reported as putative γ-secretase interacting proteins that negatively modulate the production of Aβ [87, 88]. The synaptic protein syntaxin 1 and a number of other proteins have also recently been shown to associate with an active γ-secretase complex isolated from rat brain [89]. In addition, several proteins have been shown to associate with active γ-secretase in preparations from γ-secretase reconstituted PS deficient fibroblasts, including members of the tetraspanin web [90].

APP PROCESSING

One of the most extensively studied γ-secretase substrates, APP, was cloned in 1987 [91] and belongs to a conserved gene family which also includes the homologous APP like proteins 1 and 2 (APLP1 and APLP2). APP is a type I integral membrane glycoprotein, with a single membrane-spanning domain, a large N-terminal region located in the lumen and a shorter C-terminal region located in the cytosol [92]. There are three isoforms of APP; 695, 751 and 770 amino acids in length. APP is expressed throughout the body, and APP695 is the predominant form found in neurons [93]. During its trafficking from ER to the plasma membrane, APP is posttranslationally modified by N- and O-glycosylations, phosphorylations and sulfations. APP is subsequently internalized from the cell surface, and trafficked through the endocytic and recycling compartments and back to the cell surface or degraded. Proteolytic processing of APP proceeds along two different pathways, the amyloidogenic pathway, which generates Aβ, or the non-amyloidogenic pathway (Figure 2). The amyloidogenic processing of APP is initiated through the cleavage by β-secretase, identified as β-site APP-cleaving enzyme 1 (BACE1) [94-98], leading to the release of the N-terminal ectodomain, sAPPβ, and the production of a 99 amino acid long membrane-anchored carboxy terminal fragment (C99). C99 is subsequently cleaved by γ-secretase to generate Aβ [26]. BACE1 can also cleave APP at the β' site, within the Aβ sequence, resulting in C89 and, following γ-secretase cleavage, Aβ11-40/42 [99]. A related protein, BACE2, is also known to exert β-secretase activity [96]. However, it is expressed at very low levels in brain [100]. In the non-amyloidogenic pathway, APP is sequentially processed by α-secretase and γ-secretase [101]. α-Secretase cleavage

results in the secretion of the N-terminal ectodomain, sAPPα, and generates an 83 amino acid long membrane-bound fragment (C83). Several α-secretase candidates exist, which are all members of the ADAM (a disintegrin and metalloproteinase) family, ADAM 9, ADAM 10 and ADAM 17 (also known as TACE, tumor necrosis factor-α converting enzyme) [102-105]. Since the α-secretase cleavage occurs within the Aβ sequence, Aβ formation is precluded [106], and instead, γ-secretase cleavage of C83 generates a shorter peptide, p3, which biological role is not established. In addition to the Aβ and p3 generating sites (γ-sites) in the middle of the transmembrane domain, γ-secretase cleaves C99 and C83 at the ε-site, close to the membrane-cytoplasm boundary, generating the APP intracellular domain (AICD) [107, 108]. Moreover, γ-secretase has been described to cleave APP at an intermediate position, between the γ- and ε-sites, which is called the ζ-site [109].

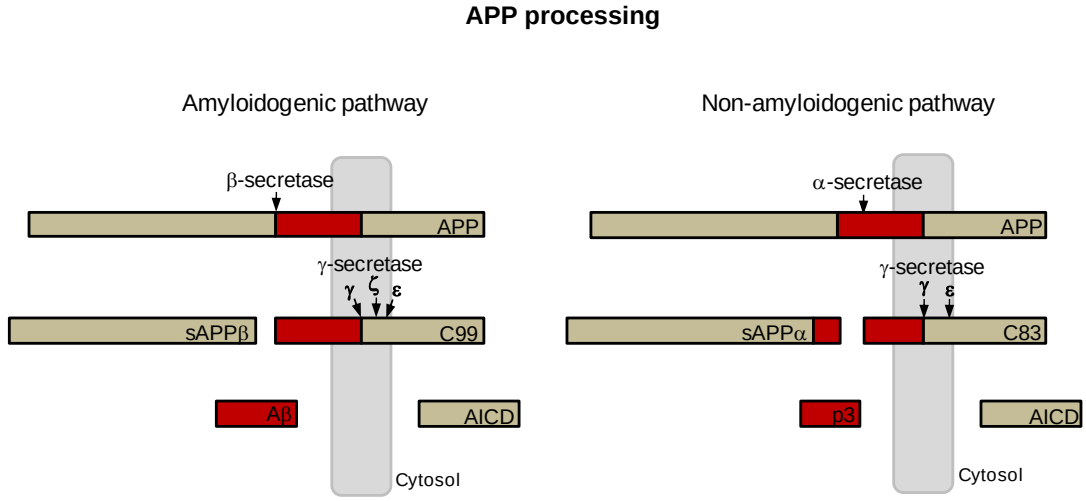

Figure 2. Schematic illustration of APP processing through the amyloidogenic and non-amyloidogenic pathways.

The biological function of APP is still not fully clear. In mice, APP deficiency displays several phenotypes, including reduced body and brain size, hypersensitivity to seizures, impaired spatial learning and long-term potentiation [110]. These deficits can be rescued by the introduction of sAPPα, indicating that the ectodomain of APP is responsible for most of the functions of APP [111]. sAPPα has also been shown to have neuroprotective properties when added to cortical or hippocampal cell cultures. In contrast, the sAPPβ fragment has been demonstrated to suppress neuronal activity and trigger neuronal death [112, 113]. While single knockouts of the APP, APLP1 or APLP2 genes in mice are viable, APP-APLP2 and APLP1-APLP2 double knockouts as

well as APP-APLP1-APLP2 triple knockouts result in perinatal lethality [114, 115]. These findings demonstrate an important role of the APP family in development and suggest a functional redundancy of the family members. In agreement, a recent study using RNA interference, has demonstrated a critical role for APP in neuronal migration during development [116]. In addition, APP has been proposed to serve as an adhesion molecule [117] and a cell-surface receptor [91]. A role of APP in signaling is supported by the observation that AICD can activate transcription through the adaptor proteins Fe65 and Tip60 [118-120]. Several putative target genes of AICD have been suggested, including p53, neprilysin, APP and GSK-3β [121]. The role of AICD in regulating transcription is, however, rather controversial since the protein levels of APP, GSK-3β and neprilysin have been shown to be unaffected in fibroblasts deficient of PS [122]. Further, AICD has been shown to be rapidly degraded by insulin-degrading enzyme (IDE) both *in vitro* and *in vivo* [123, 124] resulting in low levels of AICD. Therefore, the use of systems overexpressing AICD or APP for assessing the effect of AICD on transcription, has also been criticized.

C-terminal Aβ variants

Sequential cleavage of APP by β- and γ-secretase generates Aβ of various lengths, the majority being 40- or 42 amino acids long (Aβ40 and Aβ42 respectively). Aβ40 is the most commonly produced of the two, while the less abundant Aβ42 is more hydrophobic and fibrillogenic and the species particularly implicated in plaque formation [12]. In addition to Aβ40 and Aβ42, less abundant and shorter peptides such as Aβ37, Aβ38 and Aβ39 have been identified in cell medium by several groups [125-127]. γ-Secretase cleavage at the ζ-site has been shown to generate a longer Aβ species ending at residue 46 [109]. Further, Aβ peptides longer than Aβ42, including Aβ43, Aβ45, Aβ46, Aβ48 and Aβ49 have also been detected in cell lysates and in homogenates of APP-transgenic mouse brains by combining immunoprecipitation and a sodium dodecyl sulfate (SDS)/urea gel system [127-129]. Additional studies in transgenic mice, carrying either an APP or PS1 mutation, as well as a few non-quantitative studies in human brain from SAD and FAD cases, also reported on longer Aβ species [130-134]. The different Aβ species are proposed to be produced by γ-secretase cleavage of APP CTFs in a stepwise manner starting at the ε-site and then cleaving roughly every third residue via the ζ-site to the γ-site [127, 135]. Importantly, Aβ40 and Aβ42 are suggested to be generated through independent production lines.

As the peptides become shorter approaching the γ-site they are more likely to be released whereas the longer forms of Aβ are more likely to be retained within the membrane. However, the precise temporal order and mechanism of this multiple processing is still unclear and the model does not explain the production of Aβ38. Aβ is rapidly cleared from the brain and one way to regulate the Aβ levels are through Aβ-degrading enzymes [136]. For example neprilysin and IDE have been shown to degrade Aβ *in vivo* [124, 137].

Polymerization of Aβ

The heterogeneity of the C-terminus of Aβ peptides has implications for aggregation properties. Aβ fibril formation is suggested to be a nucleation-dependent mechanism. Such a mechanism is dependent on the formation of a seed and is characterized by a critical concentration below which polymerization will not occur, a lag time, which refers to the time it takes for a seed to form, above the critical concentration, and a growth phase [138]. The lag time is dependent on protein concentration, but the addition of a seed results in instant polymerization. Studies on synthetic Aβ peptides have demonstrated that peptides ending at amino acids 39 or 40 have a relatively long lag time, while peptides ending at amino acids 42 or 43 polymerize rapidly [139, 140]. In addition, the critical concentration is lower for Aβ1-42 compared to Aβ1-40. Thus, the length of the C-terminus strongly affects the propensity to aggregate, due to the addition of hydrophobic residues in the C-terminus of the longer variants. Polymerization of Aβ peptides with long lag times, Aβ39 and Aβ40, has been shown to be seeded by co-incubation with low concentrations of Aβ42 or Aβ43 [140]. The Aβ fibrils, which are the major component in plaques, are ordered structures composed of repeating units of β-sheets aligned perpendicular to the fibre axis, and due to the high β-sheet content they bind dyes such as Congo red and thioflavin T (ThT) [141, 142].

Several intermediate oligomeric assemblies of Aβ have been described, including dimers and trimers [143, 144], a dodecamer ($A\beta^{*}56$) [145], Aβ-derived diffusible ligands (ADDLs) [146], annular structures [147, 148], globulomers [149] and protofibrils [150].

The amyloid cascade hypothesis

In 1984, Glenner and Wong extracted Aβ from deposits in blood vessels from AD brains and DS brains and provided a partial sequence [19, 20]. A year later, Aβ was identified as the main component of neuritic plaques in AD brain [10]. Shortly thereafter, the APP gene was cloned and shown to localize to chromosome 21 [91]. These findings, together with the earlier recognition that individuals with DS, carrying an extra copy of chromosome 21, develop amyloid plaques and neurofibrillary tangles [14], resulted in the formulation of the amyloid cascade hypothesis [151]. This hypothesis states that Aβ accumulation in the brain is a primary event in AD pathogenesis, initiating a cascade of events, including: Alterations of synaptic function, microglial and astrocytic activation, hyperphosphorylation and oligomerization of tau, synaptic spine loss and dystrophic neurites, oxidative stress, synaptic dysfunction and ultimately progressive neuronal loss and dementia. The amyloid cascade hypothesis has since its proposal in 1992 been the prevailing hypothesis in AD research. However, in recent years, it has become apparent that soluble oligomers of Aβ rather than insoluble Aβ fibrils are the key mediators in the early stages of the disease process [152-154]. Soluble Aβ, including oligomers, correlate better with the degree of synaptic loss and cognitive decline than plaques do [155-157]. Soluble Aβ, is defined as Aβ that is soluble in aqueous buffer and remains in solution after high speed centrifugation. Oligomers of Aβ produced by cultured cells and ADDLs of synthetic Aβ, have been shown to inhibit hippocampal long-term potentiation, an electrophysiological correlate of learning and memory [146, 158, 159]. Importantly, antibodies raised against Aβ prevented the oligomer induced inhibition of LTP. Further, cell-derived oligomers have been demonstrated to interfere with memory function in rats [160]. In addition, Aβ dimers and trimers, but not monomers, secreted from cells have been shown to trigger loss of hippocampal spines and synapses through a pathway that requires NMDA receptors [161]. In accordance, Aβ dimers isolated from cerebral cortex of individuals with AD were shown to reduce spine density and also to impair synaptic plasticity in rodent hippocampus and to disrupt memory function in rats [162]. Strong support for the role of Aβ, particularly Aβ42, in AD pathogenesis comes from the observation that AD-causing mutations in the genes encoding APP, PS1 and PS2 either increase total Aβ levels, the Aβ42/Aβ40 ratio or enhance the oligomerization of Aβ [163]. The FAD-linked mutations on the *APP* gene are either located adjacent to β- or γ-secretase cleavage sites or within the Aβ region [164]. For example, the Swedish APP mutation

(K670N/ M671L) [165], which immediately precedes the β-secretase cleaving site, increase the production of total Aβ [166, 167]. The AD-associated PS mutations are mostly found within the transmembrane regions of PS and they have been shown to increase the Aβ42/Aβ40 ratio [167-169]. Aβ is generated from cells during normal metabolism and is also present in cerebrospinal fluid (CSF) of normal humans [26, 170, 171]. However, in the FAD cases, an increase in total Aβ or in the relative levels of Aβ42, seem sufficient to cause early onset AD. The relative amount of Aβ42 is of interest since this C-terminally longer variant is far more prone to oligomerize and to form fibrils than the more abundantly produced Aβ40. Interestingly, mice expressing high levels of Aβ40 did not show plaque pathology [172]. In fact, Aβ40 has been suggested to impede Aβ42 deposition [173].

γ-SECRETASE IN REGULATED INTRAMEMBRANE PROTEOLYSIS

As exemplified by the processing of APP, γ-secretase cleaves its substrates within the hydrophobic environment of the membrane. This intramembraneous proteolytic event is an example of a highly conserved process, called regulated intramembrane proteolysis (RIP) [174]. Three classes of proteases are currently known to possess the ability to cleave peptide bonds within the lipid bilayer, aspartyl proteases (represented by γ-secretase and Signal peptide peptidase), serine proteases (represented by the Rhomboids) and metalloproteases (represented by the Site-2 protease) [175]. RIP has emerged as a novel mechanism in cell signaling and involves in most cases sequential cleavage events of type I or type II transmembrane proteins. The first cleavage, referred to as ectodomain shedding, takes place within the ectodomain, close to the membrane, and results in the release of a large soluble extracellular fragment and a membrane-associated CTF. Subsequently, in a second cleavage event, the CTF is cleaved within its transmembrane region, releasing the intracellular domain (ICD) which mediates diverse effects at other intracellular locations, e.g. as a transcription factor. The ectodomain shedding can be regulated by different stimuli such as ligand-binding, be dependent upon subcellular localization or be constitutive. The proteases catalyzing the event include members of the ADAM family, BACE1, BACE2 and matrix metalloproteases.

The Notch receptors (Notch 1-4) are other well characterized substrates of γ-secretase, and Notch1 signaling is an established example of a pathway mediated by RIP [176].

During maturation, the Notch receptor is cleaved at the S1 site by furin-like convertases within the secretory pathway forming a heterodimer that is targeted to the plasma membrane (Figure 3). Activation of the Notch heterodimer, by binding of its ligands Delta or Jagged, presented by neighboring cells, induces cleavage by ADAM metalloproteases. This cleavage occurs at the extracellular S2 site and results in a membrane-bound truncated form of Notch, which is further processed by γ-secretase at sites S3 and S4. The S3 cleavage of Notch corresponds to the ε-cleavage in APP and results in the liberation of the Notch intracellular domain (NICD) which enters the nucleus and regulates transcription [177-180]. Cleavage at the S4 site, equivalent to the γ-site in APP, releases the Nβ peptide [181].

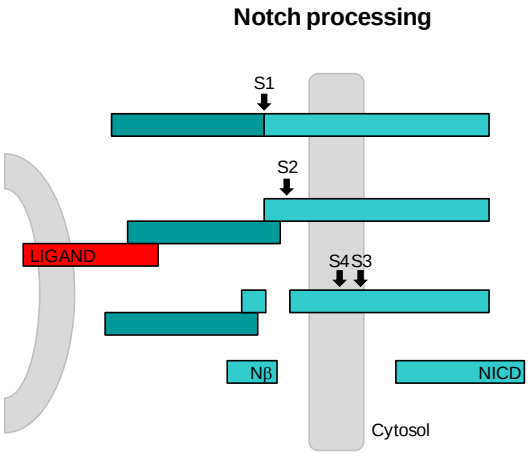

Figure 3. Schematic illustration of Notch processing. Notch is initially cleaved at the S1 site in the secretory pathway by furin-like convertases. The resulting Notch extracellular domain and the membrane-bound domain form a heterodimer which is transported to the plasma membrane. Upon ligand-binding the heterodimer is further cleaved by ADAMs at the S2 site. Finally, γ-secretase cleaves at the S4 and S3 sites releasing Nβ and NICD respectively.

The Notch1 signaling pathway is vital during development, controlling events such as cell fate decision, apoptosis and cell renewal, and consequently Notch1 deficiency causes an embryonic-lethal phenotype associated with severe developmental deficits [182, 183]. Notch1 continues to be expressed during adulthood and plays important roles in self-renewing organs as well as in cancer.

In addition to APP and Notch, γ-secretase cleaves more than 60 other substrates [184] within their transmembrane domains, including: EphrinB, a ligand for receptor tyrosine kinase Eph receptors and involved in neuritogenesis and angiogenesis [185]; ErbB4, a receptor of various growth factors [186]; CD44, E-cadherin and N-cadherin which are cell adhesion molecules [187-189]; SorLA and Sortilin which are sorting receptors responsible for intracellular sorting and trafficking of various cargo proteins [190]; interleukin-1 receptors, which take part in the immune response [191, 192]; and p75-neurotrophin receptor (p75-NTR), a neurotrophin receptor involved in axonal transport

and neuronal survival [193, 194]. In analogy to NICD, γ-secretase released intracellular domains from other substrates have also been shown to mediate signal transduction/transcriptional activation. For example, the intracellular domain of ErbB4 has been shown to be part of a complex that translocates to nucleus and represses expression of astrocytic genes [195]. In addition, N-cadherin ICD has been shown to bind and promote degradation of the transcription factor CREB binding protein [188]. Even though RIP was initially defined in 1999 as the release of membrane-bound transcription factors, the concept has since then been ascribed broader functions, such as signaling to other cells (Rhomboids). For some substrates, the γ-secretase mediated intramembrane proteolysis also seem to mediate non-nuclear signaling functions such as formation/disassembly of receptor complexes (p75-NTR) or src activation (EphrinB). No consensus sequence motif within the γ-secretase substrates has been identified. However, the substrates are, with few exceptions, type I transmembrane proteins that have undergone ectodomain shedding resulting in a short ectodomain, preferentially less than 50 amino acids [196]. The continuously increasing number of γ-secretase substrates and the poor sequence specificity of γ-secretase, have led to the suggestion that γ-secretase functions as the proteasome of the membrane, removing the transmembrane domains generated following shedding, when the full-length proteins have fulfilled their purpose [197]. In order for the intramembrane hydrolysis of the substrates to occur, water needs to reach the active site. Recently, the structure of purified γ-secretase was determined by cryo-electron microscopy (EM) at a resolution of 12 Å [86]. The cryo-EM structure reveals three low-density regions which can potentially be water-accessible cavities. Further, a vertically oriented groove in the membrane surface was observed, which is likely to be an initial substrate docking site [198]. The transmembrane part of the substrate is thought to initially interact with a docking site on PS, before being sequestered into the active site. In support of this model, active site directed transition state γ-secretase inhibitors were shown not to affect the interaction of PS with APP CTFs [199, 200].

REGULATION OF γ-SECRETASE ACTIVITY

Even though there seem to be only a few prerequisites for the substrates to be cleaved by γ-secretase, different variants of the γ-secretase complex can differ in activity and substrate specificity. Since neither PS1 and PS2 or the different Aph-1 isoforms (including splice variants) co-exist in the same γ-secretase complex, at least six distinct

complexes with different subunit composition in human could be inferred that have different properties and functions [34, 201-203]. The PS1 complexes display higher Aβ production than the PS2 complexes and the PS1- and PS2-mediated activities exhibit different sensitivity to γ-secretase inhibitors [204]. In addition, PS1 deficient mice exhibit perinatal lethality and marked inhibition of γ-secretase activity, whereas the PS2-deficient mice are viable and develop a mild phenotype with preserved γ-secretase activity [205-207]. Aph-1a is the Aph-1 isoform implicated in γ-secretase activity during development [208] while, Aph-1b is proposed to be engaged in γ-secretase processing of neuregulin-1 [209]. Further, inactivation of the three *Aph-1* genes in mice also displays differences in phenotype and γ-secretase activity [202]. As mentioned earlier, γ-secretase activity could also be regulated by interacting proteins, such as TMP21 and CD147, which both have been shown to decrease Aβ production upon interaction. However, recently it was shown that CD147 stimulated extracellular degradation of Aβ rather than regulating γ-secretase activity [210]. Interestingly, TMP21 inhibit γ-secretase cleavage at the γ-site while the cleavage at the ε-site remains unaffected, which suggests that these two cleavages can be regulated independently. Further, γ-secretase inhibitor binding density has been shown to be higher in postnatal rat brains compared to adult, implying that γ-secretase could be developmentally regulated [211]. Moreover, substrate selectivity could be spatially regulated by compartmentalization of γ-secretase and its substrates (in lipid rafts/non-raft domains) [74, 212, 213]. For example, γ-secretase has been suggested to reside in non-raft membranes together with several substrates during development, but being transferred to lipid rafts, containing a limited number of substrates including APP, in adult brain [214]. Recently, expression of the lipid raft located G protein-coupled receptor, GPR3, has been suggested to target γ-secretase to lipid rafts, and to cause an increased Aβ production both *in vitro* and *in vivo* [73]. Further supporting the importance of lipids in proteolysis, phosphatidylcholine and sphingomyelin have been shown to increase γ-secretase activity without changing cleavage specificity within APP [32].

Since γ-secretase is the enzyme generating the Aβ42 peptide, which plays a central role in AD pathogenesis, modulation or inhibition of γ-secretase can be a therapeutic strategy. However, γ-secretase cleaves several vital substrates besides APP and also the products of APP processing are suggested to have important functions. The side effects

observed following administration of γ-secretase inhibitors, such as gastrointestinal toxicity and interference with B- and T-lymphocyte maturation, seem mainly to be attributed to impaired Notch processing and signaling [215, 216]. Therefore, understanding the mechanisms of how γ-secretase activity is regulated is valuable in the design of γ-secretase modulators or inhibitors that selectively reduce the levels of Aβ42. Certain nonsteroidal anti-inflammatory drugs selectively decrease Aβ42 production without affecting AICD or NICD production [217] by binding to APP [218]. Other small molecule inhibitors that selectively inhibits Aβ42 production, by binding to an allosteric site in the γ-secretase complex, has been shown to cause a conformational change within the active site of γ-secretase [219, 220].

AIMS

γ-Secretase is an enzyme complex that cleaves a multitude of type I transmembrane proteins, of which the Notch receptor and APP are the most extensively studied. Processing of Notch is vital for cell differentiation and proliferation processes during development as well as in adulthood, while cleavage of APP generates Aβ, suggested to initiate a cascade of toxic events ultimately leading to AD. Polymerization and deposition of Aβ into plaques is one of the cardinal features in AD pathology. Thus, inhibiting the production of Aβ, especially Aβ42, without affecting processing of the other γ-secretase substrates is a possible therapeutic approach. Development of such selective inhibitors requires additional knowledge about the mechanisms of γ-secretase activity. The general aim of this thesis was to study γ-secretase activity and products in AD brain and model systems such as cell lines and rat brain.

The specific aims of this thesis were:

- To characterize the optimal conditions for γ-secretase dependent production of AICD in membranes prepared from rat brain, including: Subcellular fraction, pH and effect of detergents on activity and solubilization of γ-secretase components (Paper I).

- To study γ-secretase processing of APP, Notch1, N-cadherin, ephrinB and p75-NTR in membranes prepared from embryonic, adult and old rat brain (Paper II).

- To investigate the role of the large hydrophilic loop of PS1, the catalytic component of γ-secretase, in γ-secretase complex formation and processing of Notch and APP (Paper III).

- To identify and quantify the Aβ species that are deposited in plaque cores and other SDS-insoluble aggregates in SAD and FAD brains (Paper IV).

METHODOLOGY

In this section some of the materials and methods used in this thesis have been summarized. A detailed description of all the different procedures can be found in papers I-IV.

MATERIALS

cDNA constructs, cell culture and transfection (Paper II and III)

To study the function of the large hydrophilic loop of PS1 (Paper III), several different cDNA constructs were created: Full-length wildtype (wt) PS1 (PS1wt), PS1 NTFwt, PS1 CTFwt, PS1Δexon 10 and the PS1 CTF N-terminally truncated constructs, CTFcasp, CTF start 355, CTF start 365, CTF start 375 and CTF start 375 D385A. The DNA sequence of all constructs was verified by sequencing. The different cDNA constructs were transiently transfected into blastocyst-derived embryonic stem cells devoid of both PS1 and PS2 (BD8 cells) [221] or BD8 cells stably expressing either APP (BD8:APP) or PS1 NTFwt (BD8:NTF) using Lipofectamine2000. In paper II BD8 cells and blastocyst-derived embryonic stem cells lacking one allele of PS1 and both alleles of PS2 (BD3 cells), were used [221]. All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol and non-essential amino acids.

Brain material (Study I, II and IV)

In paper I, brains from young Sprague-Dawley rats were used and in paper II brains from embryonic (E17), young (2-3 month-old) or old (16-18 month-old) Sprague-Dawley rats were used. Frontal and occipital cortex from SAD and FAD cases were obtained from Huddinge brain bank for studies in paper IV. The FAD cases included four *APP*Swe mutations (KM670/671NL) and one *PSEN1* Ile143Thr mutation. All studies using rat or human brain were approved by the animal ethical committee of southern Stockholm or the human ethical committee in Stockholm, respectively.

Membrane preparations from rat brain and cells (Paper I-III)

Rat brains and cells were homogenized using a mechanical pestle-homogenizer in a buffer supplemented with protease inhibitor cocktail. In paper I, the homogenate was centrifuged at 1000 × g for 10 min and the post-nuclear supernatant was further

fractionated by sequential centrifugation at 10 000 × g for 30 min and at 100 000 × g for 1 h. The three membrane fractions were collected. In paper II and III, the homogenate was centrifuged at 1000 × g for 10 min and the post-nuclear supernatant was subsequently centrifuged at 100 000 × g for 1 h to obtain a crude 100 000 × g membrane pellet. All centrifugations steps were carried out at 4 °C.

Plaque core and SDS preparations from human-postmortem brain (Paper IV)

For plaque core preparations, the human-postmortem brain tissue was homogenized in Tris-buffered saline (TBS), pH 7.4, supplemented with protease inhibitor mixture (4 ml buffer/g of tissue) using a mechanical pestle-homogenizer. The homogenate was centrifuged at 4000 × g for 2 min at 4 °C and the obtained pellet was washed in TBS and stained overnight with Congo red. The pellet was washed in TBS and filtered through a 100 µm nylon mesh, adjusted to 46% sucrose and centrifuged at 16 000 × g for 1 h at 4 °C. The pellet was suspended in TBS and centrifuged again at 16 000 × g for 5 min at 4 °C. After removing the supernatant the pellet was suspended in 1% SDS in TBS, filtered through a 60 µm nylon mesh and centrifuged at 16 000 × g for 5 min at 24 °C. The pellet was washed twice in TBS and stored at –20 °C. For the SDS preparations from human-postmortem brain the homogenization was performed as for the plaque preparation but the homogenate was centrifuged at 50 000 × g for 1 h at 24 °C. The resulting pellet was re-homogenized in TBS containing 1% SDS and centrifuged at 50 000 × g for 1 h. This procedure was performed three more times. The pellet was then washed five times in TBS and stored at –20 °C.

ANALYSIS OF γ-SECRETASE COMPLEXES

Affinity pulldown (Paper II and III)

To capture active γ-secretase complexes, membranes prepared from embryonic and adult rat brain (Paper II) or BD8 cells stably expressing PS1wt, PS1Δexon 10 or the empty vector pcDNA5 (Paper III) were subjected to affinity pulldown. The membrane preparations were resuspended in buffer H (20 mM Hepes pH 7.0, 150 mM NaCl, 5 mM EDTA and protease inhibitor cocktail) containing 0.5% CHAPSO, and biotinylated proteins were removed by incubation with strepatvidin magnetic beads. Subsequently, the samples were incubated with 200 nM of an affinity probe based on L-685,458, a transition state inhibitor of γ-secretase, coupled to biotin by a cleavable

linker (GCB) [89]. As a negative control, 10 μM L-685,458, was added to the sample prior to incubation with GCB. The active γ-secretase complexes were captured by magnetic streptavidin beads, washed, eluted, separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blot using antibodies directed to the γ-secretase components.

Co-immunoprecipitation (Paper III)

Transiently transfected BD8 cells were harvested and lysed by sonication in co-immunoprecipitation buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% CHAPSO and protease inhibitor cocktail). The lysates were centrifuged at 100 000 × g for 20 min and the supernatant was pre-cleared with protein A and protein G sepharose. Samples were incubated with antibodies recognizing nicastrin or Aph-1aL. Purified rabbit IgG was used as a negative control. Following addition of protein A and protein G sepharose, the captured proteins were washed, eluted, separated by SDS-PAGE and analyzed by western blot using antibodies raised against PS1-NTF or Pen-2. In contrast to affinity pulldown described above, co-immunoprecipitation will detect not only interactions in active complexes but also inactive mature complexes and subcomplexes.

IN VITRO γ-SECRETASE ACTIVITY ASSAY (PAPER I AND II)

In vitro γ-secretase activity assay was used to study the endogenous production of intracellular domains and Aβ40 from rat brain membrane preparations as well as AICD-FLAG following addition of the exogenous APP CTF substrate, C99-FLAG. The membrane preparations were resuspended in buffer S (20 mM Hepes, pH 7.0, 150 mM KCl, 2 mM EDTA, 2 mM EGTA) or buffer H (20 mM Hepes, pH 7.0, 150 mM NaCl, 5 mM EDTA). Both buffers were supplemented with protease inhibitor mixture and buffer H was prepared with or without detergent. In paper II, only buffer H was used. The samples were incubated for the indicated time, mostly 16 hours, at 37 °C in the absence or presence of 1 μM of the γ-secretase inhibitor L-685,458. In the case of Aβ40 production in study II, buffer H was supplemented with 5 mM 1,10-phenanthroline and 10 μM thiorphan, two inhibitors of Aβ degrading enzymes.

DETECTION AND QUANTIFICATION OF Aβ

Enzyme-linked immunosorbent assay (ELISA) (Paper I and II)

Commercial sandwich ELISA (Wako chemicals, Osaka, Japan) was used according to the instructions of the manufacturer to measure Aβ40 generated in *the vitro* γ-secretase activity assay from rat membrane preparations. RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) was added to the samples prior to analysis. The capturing antibody was directed against amino acids 11-28 of Aβ and the detection antibody against the C-terminal of Aβ40.

Sandwich immunoassay (Paper III)

To quantify Aβ peptides secreted into conditioned medium sandwich immunoassay were performed using Meso Scale Discovery reagents and equipment. Stable BD8:APP cells were transfected with either PS1wt or PS1Δexon 10 or a combination of NTFwt and truncated PS CTF. All cells were transfected with CMV-β-gal and GFP. Empty pcDNA5 vector was added to ensure equal amounts of DNA in the different cells. Next, the cells were incubated with DMSO or 1 μM L-685,458 for 36 h and the conditioned medium was analyzed. Conditioned medium and Aβ peptide standards were added to Multi-Array plates, pre-coated with either C-terminal antibodies recognizing specifically Aβ38, Aβ40 and Aβ42 (3-Plex) or 6E10. Ruthenylated 6E10 (SULFO-TAG^{TM}) detection antibody was added to the samples in the plate coated with Aβ38, Aβ40 and Aβ42. Ruthenylated Aβ42 (SULFO-TAG^{TM}) detection antibody or primary anti-Aβ40 and anti-Aβ39 antibodies was added to the samples on the plate pre-coated with 6E10. All the samples were incubated overnight. Next a secondary ruthenylated anti-rabbit (SULFO-TAG^{TM}) antibody was added to the plates with primary anti-Aβ40 and anti-Aβ39 antibodies. Total Aβ was detected by incubating samples overnight on plates pre-coated with 6E10 followed by incubation with ruthenylated 4G8 (SULFO-TAG^{TM}) antibody. For detection, light emitted at 620 nm was measured following electrochemical stimulation. The secreted Aβ peptide levels were adjusted for differences in transfection efficacy according to β-galactosidase activity and for protein expression of PS1-NTF with western blot using an anti-PS1 antibody.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Paper IV)

LC-MS/MS was used to detect and quantify C-terminal Aβ variants in plaque cores and SDS preparations. The plaque cores and SDS preparations were dissolved overnight at 37 °C in 80% formic acid (FA) with 4 mg of cyanogen bromide (CNBr) per sample. CNBr cleavage generates shorter and less hydrophobic C-terminal Aβ fragments compared to the more commonly used trypsin. The samples were concentrated, desalted, dried and finally dissolved in 0.1% FA prior to LC-MS/MS. Since CNBr cleaves after methionine (Aβ35), synthetic Aβ peptides Aβ36-40, Aβ36-42, Aβ36-43, Aβ36-44, Aβ36-45 and Aβ36-46 were used as standards for quantification. The standards were also dissolved in 80% FA and diluted in 0.1%. A standard curve was created by mixing Aβ36-40, Aβ36-42 and Aβ36-43 at different concentrations (at a 20:20:1 ratio). One µl of samples and standards was injected onto an HPLC-system coupled to an electrospray ionization (ESI)-ion trap mass spectrometer. The different C-terminal Aβ species in the samples were identified by MS/MS and quantified based on the respective peak area in extracted ion chromatograms.

Immunohistochemistry (Paper IV)

The different Aβ variants found in AD brains were also studied with immunohistochemistry on consecutive sections from occipital cortex from two SAD cases and one *APP*Swe case. Antibodies specific for the C-terminus of Aβ40, Aβ42 and Aβ43 were used. The anti-Aβ43 antibody had not been tested for immunohistochemistry and thus the specificity was examined by dot blot procedure using standard Aβ peptides 1-40, 1-42 and 1-43. The antibody was specific for Aβ1-43 and showed no immunoreactivity in non-AD cases.

DETECTION OF INTRACELLULAR DOMAINS

SDS-PAGE and western blot (Paper I and II)

The intracellular domains generated from rat brain membrane preparations during the activity assay were separated by SDS-PAGE and analyzed by western blot using C-terminal antibodies for the different substrates. For semi-quantitative measurements of AICD production in paper II, a two-point calibration was made by loading dilutions of a 50-amino acid AICD peptide on the same gel as the samples. Quantification was performed using a CCD-camera.

Luciferase reporter gene assay (Paper III)

AICD and NICD formation in BD8 cells transiently transfected with either PS1wt or PS1Δexon 10 or a combination of NTFwt and truncated CTFs was measured by luciferase reporter gene assay as previously described [222]. In addition to the PS constructs, MH100, CMV-β-gal, GFP and C99-GVP or NotchΔE-GVP was included in the transfection mixtures. NotchΔE is a truncated form of the Notch1 receptor which resembles S2-cleaved Notch. γ-Secretase cleavage of C99-GVP and NotchΔE-GVP liberates the intracellular domains of APP and Notch1 with the incorporated GVP domain, which translocate to the nucleus and activate transcription the luciferase gene. The luciferase activity was normalized for transfection efficacy according to β-galactosidase activity and for protein expression of PS1-NTF with western blot using an anti-PS1 antibody.

RESULTS AND DISCUSSION

In this section, the results from papers I-IV are summarized and discussed. Figures and detailed description of the results are found within the respective paper.

γ-SECRETASE ACTIVITY (PAPER I-III)

γ-Secretase cleavage of APP occurs at multiple sites and generates AICD and the secreted peptides p3 and Aβ, where the Aβ peptide is implicated in the development of Alzheimer disease. Apart from APP, γ-secretase cleaves more than 60 substrates with different functions in a similar manner. Selective inhibition of Aβ production without affecting processing of other substrates is considered a possible therapeutic approach. Thus, assays for studying γ-secretase and basic knowledge on how γ-secretase activity is regulated could contribute to the development of γ-secretase inhibitors and modulators.

Rat brain γ-secretase activity is highly influenced by detergents (Paper I)

In paper I and II, γ-secretase activity was studied in rat brain with a focus on production of intracellular domains. Most of the data on γ-secretase is derived from studies in cell lines which often over-express γ-secretase components or APP. These systems do not fully reflect the situation in brain and its complexity. Since γ-secretase is a transmembrane protein complex, detergents are frequently used to solubilize and purify the complex to facilitate the studies thereof. To our knowledge, no extensive comparison of the effect of detergents on γ-secretase activity in rat brain has been reported. Hence, in paper I, the optimal conditions for γ-secretase activity in rat brain membranes were established, including influence of a range of different detergents. Activity was measured by detection of endogenously produced AICD by western blot.

As a first step, rat brain was homogenized and fractionated by three sequential centrifugations, 1000 × g, 10 000 × g and 100 000 × g. The generated pellets were analyzed for γ-secretase components, APP CTFs and subcellular markers by western blot. The 100 000 × g pellet was enriched in nicastrin, PS1, Pen-2 and APP CTFs as well as in endosomes, ER, Golgi and synaptic vesicles. In accordance with the enrichment of γ-secretase components and APP CTFs, the highest relative activity

(AICD/µg protein) was found in the 100 000 × g pellet. Thus, the 100 000 × g fraction was used in the subsequent studies. This fraction has also been shown to be highly active by Gu and collaborators [223] whereas Pinnix et al. showed higher activity in the 10 000 × g pellet [224]. The discrepancies could be due to different buffer compositions, homogenization procedures and centrifugation times. Further, the influence of pH on γ-secretase activity was examined and γ-secretase was found to have an optimum around pH 7, which is in line with previous studies [39, 224, 225]. We also observed an increase in AICD formation with time and the highest levels were detected after 16 h. However, the APP CTF levels did not decline significantly. Importantly, no significant degradation of AICD was observed in this *in vitro* system, which is an advantage compared to studies in cells where AICD is rapidly degraded [123].

Using the above established conditions, the effect of eight detergents at 0.25% or 1% (w/v) on γ-secretase activity was examined. The detergents were of varying type and structurally different and included: CHAPS, CHAPSO, DDM, Triton X-100, Lubrol, Brij-35, Tween-20 and PreserveX. With the exception of PreserveX, all detergents reduced AICD formation at a concentration of 1% compared to no detergent. Brij-35 and Tween-20 showed the least negative effect. The reduced activity could be due to conformational changes or dissociation of the complex, changes in lipid environment or a combination of these. For example, 1% DDM has been shown to dissociate endogenous γ-secretase complex in membranes prepared from HeLa cells [69]. For CHAPSO, the low activity is probably not due to dissociation of γ-secretase since all components can be co-immunoprecipitated in 1% CHAPSO [39]. At a concentration of 0.25%, PreserveX, CHAPS and Brij-35 did not substantially decrease the AICD levels, and interestingly, CHAPSO even increased the activity at this concentration.

Next, the ability of the detergents to solubilize γ-secretase components at 0.25% and 1% was studied. PreserveX, Brij-35 and Tween-20 were unable to solubilize the components even at a concentration of 1%. Thus, despite the fact that γ-secretase was active in these detergents they are not suitable for solubilization of the complex. The γ-secretase components were poorly solubilized in 0.25% CHAPSO, but around 50% of the components were solubilized in 1% CHAPSO.

Since the highest activity was detected in 0.25% CHAPSO, and γ-secretase, even though not active, was solubilized in 1% CHAPSO, the next step was to pinpoint the CHAPSO concentration at which γ-secretase was both active and soluble. The optimal CHAPSO concentration for AICD production was demonstrated to be 0.4%, just below the critical micelle concentration (CMC) of CHAPSO which is 0.5%. Aβ40 production was also detected in 0.4% CHAPSO. It is likely that the lipid environment is important for γ-secretase activity and the positive effect could be due to the ability of CHAPSO to form mixed micelles with lipid membranes below the CMC [226]. However, when the soluble activity at different CHAPSO concentrations was examined (that is the activity remaining in solution after centrifugation at 100 000 × g for 1 h) it was low at concentrations below the CMC. In contrast, in 1% CHAPSO, most of the γ-secretase activity was solubilized as assessed by cleavage of both endogenous and exogenous substrate and the low activity in 1% could be restored by diluting the sample to 0.4%.

γ-Secretase dependent production of intracellular domains is reduced in adult compared to embryonic rat brain membranes (Paper II)

In paper II, we turned to investigating whether γ-secretase processing of other substrates besides APP could be detected in membrane preparations from rat brain, and if the processing and substrate specificity was age-dependent. Cleavage of five γ-secretase substrates with important functions in the developing and adult nervous system: APP, Notch1, N-cadherin, ephrinB and p75-NTR, were investigated. Activity assay was performed on membrane preparations from embryonic, 2-3 month-old and 16-18 month-old rat brain and the respective intracellular domain and substrate levels (CTFs) were detected by western blot and Aβ40 by ELISA. Even though 0.4% CHAPSO was found to be the optimal detergent for AICD production in paper I, initial experiments showed that NICD production was substantially decreased under this condition. Thus, the activity assay in paper II was performed without detergent. In addition, it was demonstrated in paper I that plasma membrane was partially lost in the 10 000 × g centrifugation step during membrane preparation. Since the above mentioned substrates reside at the plasma membrane, this centrifugation step was removed in paper II.

Intracellular domains from N-cadherin, ephrinB and p75-NTR were detected in embryonic rat brain membranes but not in the adult rat brain. Production of AICD and

NICD was detected in both embryonic and adult rat brain membranes but to a higher extent in the embryonic rat brain membranes. Noticeably, production of AICD and NICD in membrane preparations from BD3 cells ($PS1^{+/-}$, $PS2^{+/+}$) can also be detected without addition of exogenous APP and Notch substrates. The higher ICD production in embryonic rat brain membranes could be due to the higher levels of substrate CTFs in membranes prepared from embryonic rat brain compared to adult. However, there was only a 20% decrease in APP CTF levels in the adult membranes compared to embryonic membranes while there was an approximately three-fold decrease in AICD production. This finding, together with the observation in paper I that the APP CTFs were present even after long incubation times, suggests either that the amount of γ-secretase was a limiting factor or that the processing was regulated. The reduced AICD formation despite the presence of substrate could be explained by spatial separation of γ-secretase and APP CTFs. We have previously demonstrated, that in adult rat brain membranes, γ-secretase is located to lipid rafts, whereas APP CTFs as well as full-length APP are located outside lipid rafts [213]. However, production of AICD-FLAG, following addition of an exogenous APP based substrate, i.e. C99-FLAG, was also decreased in the adult rat brain compared to embryonic rat brain, indicating that the limiting factor is not the substrate concentration per se. Another explanation for the decrease in AICD formation could be degradation since AICD has been shown to be degraded by IDE [123] and therefore, ETDA was added to the assay buffer to block IDE. In this case, no significant degradation was observed of either AICD or NICD. In addition to the production of ICDs, the production of Aβ40 was also higher in embryonic rat brain membranes compared to adult rat brain membranes. The generally higher γ-secretase processing observed in membranes from embryonic rat brain is in line with the reported higher levels of γ-secretase inhibitor binding in postnatal rat brain compared to adult rat brain [211].

As mentioned above, lower levels of γ-secretase could account for the decreased activity in the adult rat brain membranes. Thus, the levels of γ-secretase components in membranes prepared from embryonic and adult rat brain were compared. Apart from PS2-CTF, the γ-secretase components were present to a higher degree in embryonic rat brain membranes. The increase in PS2-CTF levels in the adult brain might be of importance since an increase in PS2 containing γ-secretase complexes have been associated with an increased Aβ42/40 ratio [227]. To study whether the total amount of

γ-secretase components reflected their actual incorporation into active complexes, affinity pulldown with GCB was used. The affinity probe GCB, based on L-685,458, is efficient in capturing active γ-secretase complexes and blocks Aβ production with a similar IC50 as L-685,458 [89]. Indeed, higher levels of captured active γ-secretase complexes were also found in embryonic rat brain. However, the increase in PS2-CTF levels in the adult brain was not reflected in increased amount of PS2-CTF in active complexes.

To further investigate the effect of age on γ-secretase cleavage of the different substrates; γ-secretase processing was compared in 2-3 month-old (young) and 16-18 month-old (old) rat brain membranes. The production of AICD, NICD and Aβ40 as well as the levels of APP CTFs and S2 cleaved Notch1, was similar in young and old rat brain membranes. In contrast to this study, Placanica and co-workers demonstrated a sharp decrease in Notch processing as well as nicastrin and PS1 levels in aged mice [228]. The divergent results could be explained by the different ways to study NICD processing (using a recombinant substrate or endogenous Notch), age or species differences. Further, neither AICD-FLAG production following addition of C99-FLAG, nor the levels of γ-secretase components differed substantially between the two age groups. No ICD production from ephrinB, N-cadherin or p75-NTR was detected.

The large hydrophilic loop of presenilin 1 is important for regulating γ-secretase complex assembly and dictating the Aβ profile without affecting Notch processing (Paper III)

As the catalytic core of the complex, PS is the target of many γ-secretase modulators and inhibitors. Side-effects observed following inhibition of γ-secretase seem mainly to be due to interference with Notch signaling. In accordance, only APP and Notch processing was observed in the adult brain of the five γ-secretase substrates examined in paper II. Thus, paper III focused on investigating γ-secretase processing of APP and Notch from a molecular point of view, by studying the importance of the large hydrophilic loop of PS1, located between transmembrane domain six and seven. To address this question, BD8 cells devoid of PS1 and PS2 were transfected with a construct encoding human PS1 lacking most of the loop region (amino acids 320-374), called PS1Δexon 10. Since the endoproteolytic site (Met292) was present in PS1Δexon 10, the endoproteolytic event could occur but it was 10-fold decreased compared to

PS1wt. This indicates the importance of the large hydrophilic loop for the rate of endoproteolysis. Even though the endoproteolysis was impaired for PS1Δexon 10, the NTFs generated were shown by co-immunoprecipitation to interact with nicastrin, Aph-1 and Pen-2. Further, the PS1Δexon molecule was demonstrated to bind to GCB, a transition state analogue inhibitor of γ-secretase. Taken together, this suggests that PS1Δexon 10 assemble into an active γ-secretase complex. We also studied whether the removal of the loop changed the topology of PS, in which the C-terminus is believed to be facing the lumen. By assessing the glycosylation pattern following introduction of a glycosylation acceptor site in the C-terminus, the C-terminus of PS1Δexon 10 was found to have the correct, luminal orientation. The decreased rate of endoproteolysis, and therefore lower levels of NTF in cells transfected with PS1Δexon 10, could be explained by less stable NTF fragments and/or γ-secretase complexes. However, for NTFs that was not the case; since the NTFs generated from PS1Δexon 10 were as stable as the ones generated from PS1wt.

Next, we investigated the functional impact of PS1Δexon 10 on production of AICD and NICD by luciferase reporter gene assay and of secreted Aβ by sandwich immunoassay. PS1Δexon 10 was found not to reduce the AICD or NICD production compared to PS1wt. In the case of AICD, this is in contrast to data from Deng et al., which indirectly demonstrated a decreased AICD formation by showing an accumulation of APP-CTFs in PS1Δexon 10 knock-in mice [48]. On the contrary, the total Aβ production was strikingly reduced in cells with PS1Δexon 10 compared to PS1wt and interestingly, the production of Aβ42 was not impaired to the same degree as the production of Aβ38, Aβ39 and Aβ40. Thus, an increase in the Aβ42/Aβ40 ratio was observed for PS1Δexon 10 compared to PS1wt, which is a feature associated with FAD-linked PS1 mutations [229]. One could speculate that the increase in Aβ42/Aβ40 ratio could be due to a conformational change in the active site of PS1Δexon 10, since we observed a tendency of decreased binding to a transition state inhibitor compared to PS1wt. In fact, genetic and pharmacological manipulations known to increase the Aβ42/Aβ40 ratio, have been demonstrated to cause a conformational change in the PS1 active site [220]. Interestingly, a similar Aβ profile and total secreted Aβ levels was observed with PS1Δexon 10 as with co-expression of NTFwt and CTF start 375, which also lacks exon 10. This indicates that the effect on Aβ production pattern is not

dependent on endoproteolysis. Taken together, this study suggests that the large hydrophilic loop of PS1 differentially affects the cleavage at the ε/S3-site and γ-site.

To further investigate which part of exon 10 that is responsible for the changes described above, the loop region was subjected to partial deletions. Partial deletions from the N-terminal end of the loop towards the C-terminus resulted in a gradual decrease in production of all Aβ species as more of the loop was deleted, while AICD production was unperturbed. Interestingly, removal of the last C-terminal ten amino acids of the loop (comparing CTF start 365 and CTF start 375) caused a further decrease in Aβ38, Aβ39 and Aβ40 but not in Aβ42, suggesting that indeed these amino acids of the loop are responsible for the differential effect on production of Aβ42 versus the shorter Aβ peptides.

γ-SECRETASE PRODUCTS (PAPER IV)

One of the neuropathological hallmarks of AD is the extracellular plaques composed of Aβ, which is a product of the enzymatic action of γ-secretase on APP. The AD neuropathology also includes deposition of Aβ in cerebral blood vessels (CAA). The major Aβ peptide found in plaques is Aβ42, while CAA predominantly is associated with Aβ40. Aβ42 is more hydrophobic than Aβ40 and has a higher propensity to aggregate [140]. Aβ peptides longer than Aβ42 is even more hydrophobic and it is possible that such variants could seed the aggregation of Aβ into fibrils that forms plaques. However, no extensive quantitative study regarding Aβ C-terminal variants longer than Aβ42 in human AD brains has been reported.

Aβ43 is more frequent than Aβ40 in amyloid plaque cores from Alzheimer disease brains (Paper IV)

In paper IV the aim was to identify and quantify Aβ species present in plaque core preparations and SDS-insoluble fractions from cortex from SAD and FAD cases. The samples were cleaved with CNBr to generate C-terminal Aβ fragments (Aβ36-x) and injected onto an HPLC-system coupled to an ESI-ion trap mass spectrometer. For quantification of the identified Aβ fragments, the corresponding synthetic Aβ peptides were used as standards.

Plaque cores were prepared from occipital and frontal cortex to compare Aβ variants in regions with different plaque load. Occipital cortex is known to be plaque core rich while frontal cortex shows lower plaque load (Arnold et al 1991). In the study of plaque cores from occipital cortex, six SAD cases and two *APP*Swe cases were included. Three different C-terminal variants were found in the plaque cores: Aβ40, Aβ42 and Aβ43. Interestingly, the longer fragment, Aβ43, as well as Aβ42 were detected in all cases. In contrast, Aβ40 was detected in the two *APP*Swe cases and in one SAD case, which was the only *APOE*ε4/4 carrier. As expected from previous studies, Aβ42 was the species present at highest quantities. Plaque cores from frontal cortex were included from five SAD cases, four *APP*Swe cases and one I143T *PSEN1* case. As in the occipital cortex, Aβ42 was present in all brains. However, Aβ40 was only detected in the *APP*Swe cases. Interestingly, Aβ43 was also frequently detected in the frontal cortex (in seven out of ten brains). In accordance with the observation that the occipital cortex has a higher plaque load compared to the frontal cortex, the Aβ levels were higher in the plaque core preparations from occipital cortex of SAD cases. Further, the propensity of the Aβ peptides 1-40, 1-42, 1-43 and 1-46 to aggregate was studied by ThT binding assay. As reflected by the hydrophobicity of the peptides, ThT binding was lowest for Aβ1-40, similar between Aβ1-42 and Aβ1-43 and highest for Aβ1-46. The lower tendency of Aβ40 to polymerize is in agreement with its rare occurrence in plaque cores from SAD cases. However, in *APP*Swe cases, the total Aβ production is increased and accordingly the concentration of Aβ40 could be high enough to induce polymerization and subsequent plaque deposition.

To investigate whether longer Aβ species could be found in cerebral blood vessels or diffuse deposits, SDS preparations were prepared from occipital cortex of four SAD cases and two *APP*Swe cases as well as from frontal cortex of the I143T *PSEN1* case. Aβ42 and Aβ43 were detected in the SDS-insoluble fractions from all brains included. In contrast to plaque cores, Aβ40 was more frequently detected in the SDS-insoluble fractions from SAD cases, and at higher levels. Further, Aβ40 was found in the two *APP*Swe cases but not in the I143T *PSEN1* case. The higher abundance of Aβ40 in SDS preparations possibly reflects the Aβ deposition in cerebral vessels, which is associated with Aβ40 [12]. In accordance, immunohistochemical staining of sections

from the occipital cortex showed that Aβ40 was found mainly in the cerebral vessels whereas Aβ42 and Aβ43 were localized to plaques.

No Aβ variants longer than Aβ43 were detected in any of the brains. However, the detection limit for the standard peptides increased concomitantly with the length of the C-terminal; 1 fmole for Aβ36-43, 50 fmoles for Aβ36-44 and Aβ36-45, and 500 fmoles for Aβ36-46. Thus, we cannot exclude that longer variants are not present because of limitations in our system. Since this study only focused on analyzing C-terminal Aβ variants (Aβ36-x) it could be possible that the identified fragments originated from p3 (Aβ17-42). CNBr cleavage of p3 would have generated an N-terminal peptide Aβ17-35. However, no peaks were found in the extracted ion chromatograms with m/z that corresponded to this fragment. Thus, the majority of C-terminal fragments identified are likely to be derived from Aβ and not from p3.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The work of this thesis has focused on γ-secretase processing and factors that influence its activity (**papers I-III**) and on one important γ-secretase product, the Aβ peptide (**paper IV)**. Over the past decades, since the discovery of the Aβ peptide, biochemical and genetic studies have implicated a central role for Aβ in the pathogenesis of AD. Further, there has been an immense increase in the knowledge about γ-secretase. At least four proteins make up the high molecular weight γ-secretase complex which has not only been found to cleave APP at multiple sites but also a multitude of functionally diverse substrates. Nevertheless, the exact mechanism of how Aβ causes neurodegeneration in AD and how γ-secretase processing is regulated remains elusive.

In **paper I** a detailed investigation determined the optimal conditions for γ-secretase activity in membranes prepared from rat brain by measuring AICD formation. Since γ-secretase is a transmembrane protein complex, detergents are frequently used for its purification and solubilization. In this study, AICD production was found to be highly affected by different detergents, which emphasizes the importance of the choice of detergent as well as its concentration in studies of γ-secretase. Solubilization in 1% CHAPSO, and dilution to 0.4% was demonstrated to be a suitable procedure for obtaining a soluble and active γ-secretase. These results could be of value for further studies where the activity and stability of the γ-secretase complex needs to be preserved, such as investigations of γ-secretase interacting proteins using active site directed inhibitors or co-immunoprecipitation. Further, the highest γ-secretase activity was found in a membrane fraction enriched in Golgi, ER, endosomes and synaptic vesicles as well as γ-secretase components and APP CTFs. Besides detergents, AICD production was also dependent on pH and increased in a time dependent manner. Under the established conditions the APP CTF levels were sufficient not only to monitor the production of AICD but also of Aβ40.

As mentioned earlier, apart from APP, several other γ-secretase substrates have been revealed. Thus, in **paper II** we expanded our studies on γ-secretase activity in rat brain membranes to include APP and four other important neuronal substrates: Notch1, N-

cadherin, ephrinB and p75-NTR. In addition, processing of the substrates was compared between embryonic, young and aged rat brain to study the temporal regulation of γ-secretase. γ-Secretase dependent production of ICDs from these substrates, as well as Aβ40 production, was concluded to be down-regulated in the adult compared to embryonic rat brain membranes. Interestingly, the production of Aβ40 did not increase in aged compared to young rat brain membranes. This may indicate that the elevated levels of Aβ in AD brains are due to other mechanisms than an age-dependent increase in γ-secretase activity. ICDs generated from all substrates were observed in the embryonic rat brain. The ability to detect γ-secretase processing of APP as well as several other substrates in embryonic rat brain, provides an additional approach in which the effect and specificity of γ-secretase inhibitors could be examined. Further, only processing of Notch1 and APP was detected in the adult and aged rat brain, and to a lesser extent. The persistent cleavage of Notch1 during adulthood is in line with the side-effects observed following γ-secretase inhibition. These side-effects, including gastrointestinal toxicity appear mainly to be due to impaired Notch signaling. Thus, for the evaluation of selective γ-secretase inhibitors in this brain membrane based assay, the development of a quantitative method for NICD detection, such as ELISA, would be valuable.

In **paper III**, a molecular investigation of the large hydrophilic loop in PS1, demonstrated its specific importance for regulation of Aβ production. Deletion of the loop region encoded by exon 10 caused a drastic decrease in formation of the shorter Aβ peptides (Aβ38, Aβ39 and Aβ40) and the total secreted Aβ, whereas the Aβ42 production was only partially decreased. In particular the last ten C-terminal amino acids of the loop were found to be important for this differential effect. In accordance with this study, studies on FAD-linked PS mutations and artificial mutations suggest that genetic modifications cause a more severe loss of Aβ variants shorter than Aβ42. However, further studies are needed to fully elucidate the mechanism behind the effect of the large hydrophilic loop of PS. Importantly, the production of NICD and AICD was intact following removal of the loop. Thus, the differential effect at the ε/S3-site and γ-site of the large hydrophilic loop of PS1 observed in this study may be of importance in the design of selective γ-secretase inhibitors.

In **paper IV**, an extensive study was performed in order to detect and quantify the C-terminal Aβ species that are deposited in occipital and frontal cortex of SAD and FAD cases. As expected from studies by other research groups, Aβ42 was the major Aβ species detected in plaque cores as well as in total amyloid preparations. Aβ40 was with one exception only found in plaque core preparations from *APP*Swe cases, although in the total amyloid preparations it was also found in SAD cases. Interestingly, an Aβ species ending at Thr43, Aβ43, was identified in plaque cores and SDS preparations from occipital as well as frontal cortex. Aβ43 was clearly more frequent than Aβ40 and accounted for approximately 5% of the Aβ peptides in the samples. The presence of Aβ43 in most AD cases suggests that this species could be of importance for AD pathogenesis, but further studies to clarify its significance are needed. Since Aβ43 is more hydrophobic than the C-terminally shorter variants, it is possible that Aβ43 could seed Aβ polymerization *in vivo*. The focus of future studies could be to investigate whether oligomeric Aβ43 species are found *in vivo* or whether Aβ43 is present in CSF and if the levels change in AD patients compared to non-demented individuals. Depending on the results, Aβ43 could be the target for vaccination trials or used as a diagnostic marker.

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