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DIFFERENT TYPES OF γ-SECRETASE COMPLEXES AND THEIR EFFECT ON SUBSTRATE PROCESSING

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To my family

"Life is what happens to you when you're busy making other plans"

John Lennon

ABSTRACT

The γ-secretase complex is a transmembrane aspartyl protease that generates the Alzheimer disease (AD) related amyloid β-peptide (Aβ) from the amyloid precursor protein (APP). The γ-secretase complex cleaves APP at two different sites (γ- and ε-sites) generating Aβ-peptides and the APP intracellular domain (AICD). The Aβ-peptide can vary in length, where the most common lengths are of 40 or 42 residues (Aβ40 and Aβ42). The longer Aβ42 peptide is more hydrophobic and prone to aggregate into toxic oligomers. These oligomers will eventually form the extracellular plaques, which are one of the hallmarks found in the brain of AD patients. The γ-secretase complex processes many other substrates besides APP. One important substrate is the Notch receptor that is crucial for critical signaling and cell fate decisions. The failure of γ-secretase inhibitors used in clinical trials can partly be explained by the large number of substrates. Most of these inhibitors give severe side effects related to the impairment of the Notch signaling pathway. Therefore, it is essential to identify strategies to affect the APP processing without disturbing the processing of other substrates. The γ-secretase complex is composed of four components; Presenilin (PS), Nicastrin (Nct), Anterior pharynx defective-1 (Aph-1), and Presenilin enhancer-2 (Pen-2). Both PS and Aph-1 exist as two homologues (PS1/PS2 and Aph-1a/Aph1b) and the Aph-1a homologue can also undergo alternative splicing generating a short (Aph-1aS) or a long (Aph-1aL) isoform. Thus, the different homologues and splice variants can generate up to six distinct γ-secretase complexes with possible diverse functions. In addition, the γ-secretase complex can also undergo caspase cleavage, which may change the properties of the complex. Inhibiting or modulating certain types of γ-secretase complexes could be one way to avoid severe side effects. The general aim of this thesis is therefore to achieve a more detailed understanding of the different γ-secretase complexes and their components, with respect to their properties and substrate selectivity.

In **Paper I**, we reported that single residues in a γ-secretase component besides presenilin, such as Nicastrin, affected the processing of γ-secretase substrates differently. In **Paper II**, we examined how γ-secretase processing of APP and Notch was affected by the caspase cleavage of PS1. We found that caspase-cleaved γ-secretase complexes still could process APP and Notch, but with an increased intracellular Aβ42/Aβ40 ratio. In **Paper III**, we investigated whether PS1 and PS2 show different substrate specificity by analyzing the processing of the γ-secretase substrates APP, Notch, N-cadherin, and ephrinB. We found that while the PS1 depletion affected the cleavage of all substrates, the effect of PS2 deficiency was minor. In the final study, **Paper IV**, we found that whereas γ-secretase complexes containing either Aph-1a or Aph-1b processed APP and Notch to the same extent, they showed different preference of forming complexes with the PS proteins. Aph-1a favored PS1-containing complexes, while Aph-1b rather was incorporated into PS2-containing complexes. All together, these findings support the existence of different active γ-secretase complexes and their possible diverse effects on substrate processing.

LIST OF PUBLICATIONS

This thesis is based on the papers below. Two of the papers are published under the previous last name Svensson.

I. **Pamrén A**, Wanngren J, Tjernberg LO, Winblad B, Bhat R, Naslund J, Karlstrom H.
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II. Hedskog L, Petersen CA**, Svensson AI**, Welander H, Tjernberg LO, Karlström H, Ankarcrona M.
γ-secretase complexes containing caspase-cleaved presenilin-1 increase intracellular Aβ42/Aβ40 ratio.
J Cell Mol Med. 2011 Oct; 15(10):2150-63. doi: 10.1111/j.1582-4934.2010.01208.x

III. Frånberg J*, **Svensson AI***, Winblad B, Karlström H, Frykman S.
Minor contribution of presenilin 2 for γ-secretase activity in mouse embryonic fibroblasts and adult mouse brain.
Biochem Biophys Res Commun. 2011 Jan 7; 404(1):564-8. Epub 2010 Dec 10.

IV. **Pamrén A**, Hedskog L, Berling A, Ankarcrona M, Karlström H
Investigation of regions in Aph-1 isoforms for differences in substrate processing
Manuscript

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LIST OF ABBREVIATIONS

Aβ	Amyloid β
AD	Alzheimer Disease
ADAM	a disintegrin and metalloproteinase
AICD	APP intracellular domain
Aph-1	Anterior pharynx defective-1
ApoE/APOE	apolipoprotein E
APLP	amyloid precursor-like protein
APP	Amyloid precursor protein
BACE	β-site APP cleaving enzyme-1
C83	C-terminal fragment of 83 residues (generated from APP)
C99	C-terminal fragment of 99 residues (generated from APP)
caspCTF	caspase-cleaved CTF
cDNA	complementary DNA
CTF	C-terminal fragment
DAP	DYIGS and peptidase homologous region
EphB	ephrinB receptor
ELISA	Enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FAD	Familial AD
GSAP	γ-secretase associated proteins
GWAS	Genome-wide association studies
ICD	intracellular domain
iCLIPs	intramembrane-cleaving proteases
KPI	Kunitz protease inhibitor
LRP	Low-density lipoprotein receptor-related protein
MEF	mouse embryonic fibroblasts
MSD	Meso scale discovery technology
Nct	Nicastrin
NFT	neurofibrillary tangles
NICD	Notch intracellular domain
NTF	N-terminal fragment
p3	3 kDa peptide (generated from APP)
Pen-2/PSENEN	Presenilin enhancer-2
PS/PSEN	Presenilin
RAGE	receptor for advanced glycosylation end products
SAD	sporadic AD
sAPPα/sAPPβ	soluble APP fragment produced by α- or β-cleavage
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
TMD	transmembrane domain
Wt	wild type

1 INTRODUCTION

1.1 ALZHEIMER DISEASE

Alzheimer disease (AD) is the most common type of dementia affecting the elderly population. In the report ”Dementia: a public health priority”, published in 2012 by the World Health Organization (WHO) together with Alzheimer's Disease International (ADI), the estimated number of people with dementia worldwide in 2010 was nearly 36 million. This number is believed to increase to 115 million people in 2050, unless better treatments are developed. In Sweden alone, approximately 25 000 patients are diagnosed each year and there are currently about 160 000 Swedish residents with dementia (Wimo A, 2007). Of all cases, about 60-80% are believed to suffer from AD.

Early signs of the disease are memory deficits, disorientation, and challenges with performing activities of daily living. As the disease progresses, the patient's mood and personality may change, becoming more anxious and suspicious. The patient begins to have sleep disturbances and difficulties recognizing close relatives and friends. In later stages, more severe symptoms are developed, such as speaking difficulties, loss of appetite and failure to control normal body functions. At this stage, the patient is completely dependent on caregivers. In the end stage of the disease, the patient is extra vulnerable to infections such as pneumonia and this is generally the cause of death.

The disease is not only devastating for the patient but also for family and friends. In addition, the disease is also a large cost for the society. Considering the increase in life expectancy, the number of people developing AD will increase and thereby also the cost. Despite the large research efforts in the field during the last decades, there is still no cure for AD. The available drugs can only alleviate the symptoms, not stop the underlying progression of the disease. Thus, AD is a major health problem and will be an even larger issue in the future without effective treatments (WHO, 2012).

1.1.1 Neuropathology

The first documented case of AD was described in a publication from 1907 by the German psychiatrist and neuropathologist Alois Alzheimer. He described atrophy of the brain and the prevalence of intracellular neurofibrillary tangles (NFTs) and extracellular plaques (Alzheimer et al., 1995). The cortical atrophy is due to synaptic and neuronal loss, especially in regions such as the temporal and parietal lobes, along with areas of the frontal cortex and the cingulate gyrus (Terry et al., 1991). In addition, a chronic inflammation response is observed in AD, manifested by the increased number of activated microglia and astrocytes (Shepherd et al., 2009).

The NFTs in the AD brain are composed of hyperphosphorylated tau. Tau is a microtubule-associated protein that binds to microtubules to reduce their dynamic instability. Tau is normally phosphorylated at several sites, but becomes hyperphosphorylated and starts to

self-assemble into NFTs in AD. The phosphorylation of tau reduces its capacity to bind and stabilize microtubuli (Goedert, 1993). The breakdown of the microtubule network causes an impaired axonal transport, which leads to synapse loss and retrograde degeneration (Iqbal et al., 2005). The NFT pathology begins in the entorhinal cortex and spreads through the hippocampus and amygdala to the neocortex (Braak and Braak, 1991).

The extracellular plaques are composed of aggregated β-amyloid (Aβ) peptides and exhibit a spectrum of morphology from so called neuritic plaques to diffuse plaques based on their characteristics. It is believed that diffuse plaques are first developed and may later mature into neuritic plaques. This is partly based on the fact that deceased Down's syndrome patients show diffuse plaque morphology before fully developing AD (Giaccone et al., 1989; Rumble et al., 1989). The regional distribution of plaques differs from the tangle distribution mentioned above. The plaques are first visible in neocortex and spread further to allocortical brain regions, diencephalic nuclei, striatum, several brainstem nuclei, and finally to the cerebellum and additional brainstem nuclei (Thal et al., 2002).

1.1.2 Genetics and risk factors

AD is a multi-factorial disease likely influenced by both environmental and genetic factors. The disease can be subdivided into two major forms depending on the age of onset: early-onset (before 65 years of age) and late-onset (after 65 years of age). The most common form is late-onset where most cases are considered to be sporadic (SAD), i.e. there is no obvious family history or mutation that can explain the cause of the disease. Some of the early-onset cases display a family history of the disease and a few of these are found to be caused by autosomal dominant mutations in the genes *APP* (located on chromosome 21), *PSEN1 (*located on chromosome 14), or *PSEN2* (located on chromosome 1) (Lambert and Amouyel, 2011). Mutations in these genes are often referred to as familial AD (FAD) mutations in the literature. The genes encode the amyloid precursor protein (APP), the presenilin 1 (PS1) protein, and the presenilin 2 (PS2) protein and are all linked to the generation of Aβ (Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995). APP is the precursor protein of Aβ (Haass et al., 1992b), and PS1 and PS2 are components of the γ-secretase complex that finalizes the last cleavage step of APP to generate Aβ (Wolfe et al., 1999).

Currently, 33 pathogenic mutations in the *APP* gene, 185 pathogenic mutations in the *PSEN1* gene, and 13 pathogenic mutations in the *PSEN2* gene have been reported (AD mutation database: http://www.molgen.ua.ac.be/ADMutations). The pathogenic *PSEN1* and *PSEN2* mutations cause an increase in the Aβ42/Aβ40 ratio either by decreasing the production of the shorter less toxic Aβ40 peptide or enhancing the production of the longer more toxic Aβ42 peptide (Bentahir et al., 2006; Citron et al., 1997; Kretner et al., 2011; Kumar-Singh et al., 2006; Scheuner et al., 1996). *APP* mutations cause an increase in the Aβ42/Aβ40 ratio, elevate total Aβ levels, or enhance the oligomerization of Aβ (Mullan et al., 1992; Nilsberth et al., 2001; Suzuki et al., 1994). Some of these reported mutations are not point mutations but rather duplications of the *APP* gene (Rovelet-Lecrux et al., 2006; Sleegers et al., 2006; Thonberg et al., 2011) causing an overexpression of APP, which results in increased Aβ production. The harmful effect of the APP duplication can also be observed in patients with

Down's syndrome that have an extra copy of chromosome 21 where the *APP* gene is located. These individuals often develop AD after the age of 35 (Olson and Shaw, 1969; Tyrrell et al., 2001). Thus, both changes in the Aβ42/Aβ40 ratio and increased levels of Aβ seem to be important for the disease.

A promoter deletion and mutations in the *PSENEN* gene, encoding the γ-secretase component Pen-2, have also been identified in AD and mild cognitive impaired (MCI) patients in Italy. However, the pathogenic role of these mutations in AD is unclear since no segregation analyses have been performed and the mutations show no effect on APP processing (Albani et al., 2007; Andreoli et al., 2011; Sala Frigerio et al., 2005).

A major genetic risk factor is the presence of one or two ε4 alleles of the gene *APOE* located on chromosome 19. This gene encodes for apolipoprotein E (ApoE), a protein involved in lipoprotein metabolism and cholesterol homeostasis by transporting lipids and cholesterol between cells (Corder et al., 1993; Martins et al., 2009; Rebeck et al., 1993). Genetic studies have revealed three different allelic variants; ε2 (with cysteines at position 112 and 158), ε3 (with a cysteine at position 112 and an arginine at position 158), and ε4 (where both cysteines have been replaced with arginines) (Mahley, 1988). The most common allele in humans is the ε3 allele, while the least common allele is ε2. Carriers of the *APOE* ε4 allele have an increased risk of developing AD and an earlier onset of the disease (Corder et al., 1993; Strittmatter et al., 1993). In contrast, the ε2 allele has been proposed to be protective (Corder et al., 1994). The ApoE proteins may influence the risk of developing AD by differentially affecting the aggregation and clearance of Aβ (Kim et al., 2009). Other possible susceptibility genes have also been suggested to contribute to a higher risk of developing AD. These genes have been identified by genome-wide association studies (GWAS) and include; *BIN1 (*encoding the Myc box-dependent-interacting protein 1), *CLU* (encoding the clustrin protein), *ABCA7* (encoding the ATP-binding cassette sub-family A member 7), *CR1* (encoding the complement receptor 1), and *PICALM1* (encoding the phosphatidylinositol-binding clathrin assembly protein) (www.alzgene.org).

Epidemiological studies have also identified several possible risk or protective factors. While the major risk factor is age, other possible risk factors associated with the disease are: brain injuries, high blood pressure and high cholesterol levels during mid-life, female gender, diabetes, obesity, depression, chronic stress, smoking, low physical and social activity, low vitamin B12 levels, and excessive alcohol consumption. There are also some suggested protective factors such as education, physical activity, challenging occupation, and intake of food rich in omega-3 and antioxidants (Fotuhi et al., 2009; Mayeux and Stern, 2012; Qiu et al., 2009). The mechanisms by which these risk and protective factors contribute to, or protect from, developing the disease are not fully understood.

1.1.3 Apoptosis and caspase activation in AD

Apoptosis is a cell death mechanism reported to cause the synaptic and neuronal loss in AD (Cicconi et al., 2007; Stadelmann et al., 1999). Apoptosis is an energy-dependent, non-inflammatory process, where the cells break down and vanish with minimal effects on the surrounding tissue. Cells dying by apoptosis undergo several morphological and biochemical changes. Upon apoptotic signaling, cells round up and blebs are formed on the plasma membrane. This is followed by chromatin condensation, DNA fragmentation, exposure of phosphatidylserine on the cell surface, and the formation of apoptotic bodies. The apoptotic bodies are then subjected to phagocytosis by macrophages or neighboring cells (Bredesen et al., 2006; Mirkes, 2002).

Apoptotic signaling is often divided into two main pathways: an extrinsic pathway that is initiated by the activation of death receptors located at the surface of the cell, and an intrinsic pathway that is mediated via the mitochondria (Figure 1) (Bredesen et al., 2006). Both these pathways involve activation of cysteine-aspartic proteases, called caspases, that cleave their substrates after an aspartate residue. Caspases reside in the cells as procaspases and target a broad spectrum of cellular proteins after being activated (Alnemri et al., 1996).

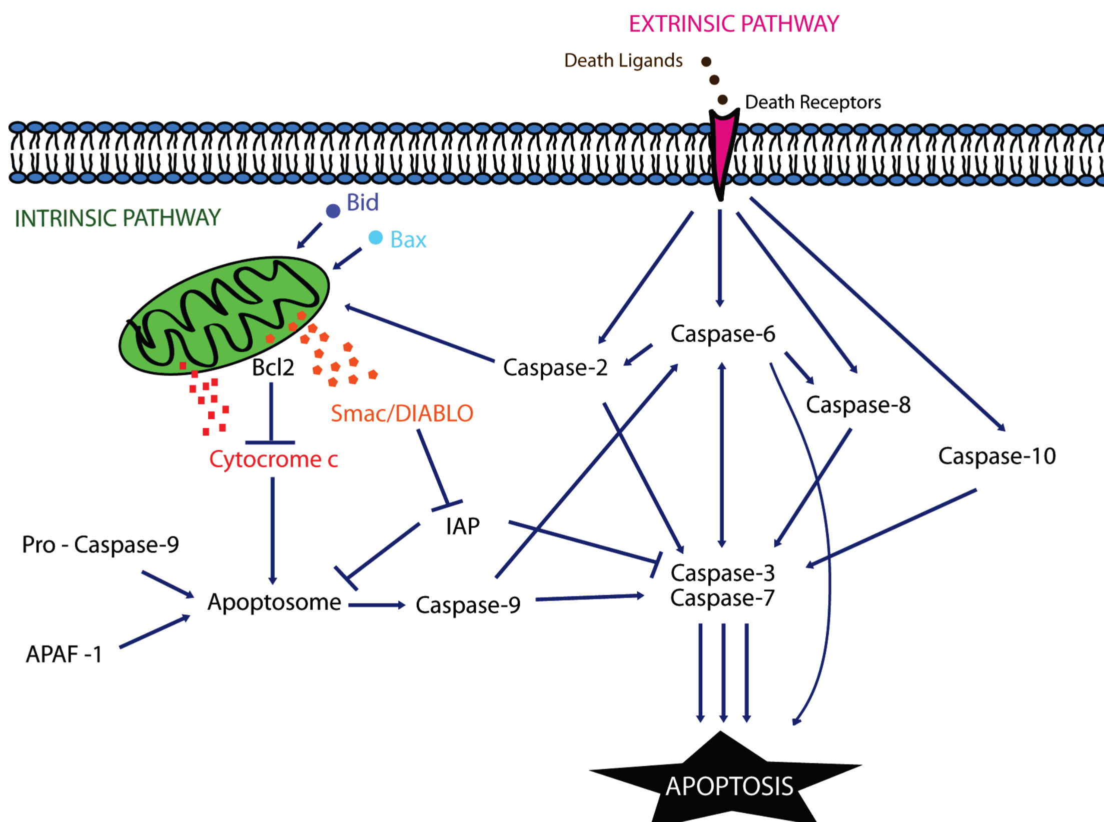

Figure 1. The intrinsic and extrinsic pathways in apoptosis. The extrinsic pathway is initiated by the activation of death receptors located at the surface of the cell, while the intrinsic pathway begins with an imbalance between Bcl-2 proteins (e.g. Bid and Bax) and is mediated via the mitochondria. These pathways are regulated by inhibitors of the apoptosis protein (IAP) family (Deveraux et al., 1998) and other apoptotic mediators such as Smac/DIABLO (Verhagen et al., 2000).

The extrinsic pathway is initiated by activation of death receptors at the cell surface, which activates initiator caspases (-2,-8,-9, and -10) to further activate the effector caspases (-3,-6, and -7). However, the classification of caspases into “initators” or “effectors” has been re-evaluated the last years since some caspases, including caspase-3 and caspase-6, appear to have both initiator and effector properties (Uribe et al., 2012). The intrinsic pathway begins with an imbalance between members of the Bcl-2 family. If the imbalance shifts towards the pro-apoptotic members of the Bcl-2 family, the permeability of the mitochondrial outer membrane increases. This leads to a release of pro-apoptotic factors such as cytochrome C into the cytosol. Together, cytochrome C, Apaf-1, and procaspase-9 form the apoptosome, which activates caspase-9. Caspase-9 subsequently activates caspases-3, -6 and -7. Thus, the extrinsic and intrinsic signaling pathways converge and the effector caspases-3, -6, and -7 will in both cases cleave cellular proteins, eventually leading to the controlled demise of the cell (Bredesen et al., 2006; Xu and Shi, 2007).

Several studies suggest that caspase activation is linked to the synaptic loss and neuronal death seen in the AD brain and that the activation occurs before the appearance of plaque and tangles (de Calignon et al., 2010; Hyman, 2011). Caspase activation has also been associated with an increase in β-secretase cleavage (see section “APP processing” for more details about the β-secretase cleavage) (Tesco et al., 2007) and elevated Aβ production (Cicconi et al., 2007; Galli et al., 1998; LeBlanc, 1995; Xie et al., 2007).

Caspase-3 and caspase-6 are especially activated in AD brains and were earlier believed to be activated only during apoptosis. Recently however, it has been reported that they are also involved in neuronal and synaptic plasticity without causing cell death (Gilman and Mattson, 2002; Jo et al., 2011; Uribe et al., 2012). Caspase-6 may play a key role in the development of AD since it has been found to be activated both in SAD and FAD. Its activation increases the formation of Aβ and is therefore suggested to be an early event in AD (Albrecht et al., 2009). In addition, caspase-6 is also linked to axonal degeneration, which contributes to the neuronal loss in AD (Uribe et al., 2012). It has been suggested that a N-terminal APP fragment activates caspase-6 via the death receptor 6 (DR6) (Nikolaev et al., 2009). Caspase-6 further cleaves caspase-2, -3, or -8 (Nikolaev et al., 2009; Uribe et al., 2012), as well as other substrates, such as cytoskeleton-associated proteins (Kawahara et al., 1998; Klaiman et al., 2008), and the AD related proteins tau and APP (de Calignon et al., 2010; LeBlanc et al., 1999). The cleavage of tau by caspase-3 or -6 generates a truncated form of tau that triggers tangle formation and induce mitochondrial dysfunction (de Calignon et al., 2010; Guo et al., 2004; Lee and Shea, 2012; Quintanilla et al., 2009). APP can be cleaved by caspase -3, -6, -8, or -9 (Gervais et al., 1999; LeBlanc et al., 1999; Lu et al., 2003; Pellegrini et al., 1999), generating the peptides Jcasp and C31 that have been suggested to be potentially cytotoxic (Lu et al., 2000; Nikolaev et al., 2009; Park et al., 2009). However, when this caspase site was removed in a transgenic mouse model of AD, the mice still showed neuronal and behavior impairments (Harris et al., 2010).

Besides APP and tau, caspases also cleave the γ-secretase components PS1 (Kim et al., 1997; Loetscher et al., 1997) and PS2 (Loetscher et al., 1997; Vito et al., 1997). Caspase-cleaved PS1 and PS2 appear to sensitize cells to apoptotic stimuli, suggesting that the caspase-cleaved PS C-terminal fragments (CTF) may serve as pro-apoptotic effectors (Kim et al., 1997). While PS2 is preferably cleaved by caspase-3 (Cai et al., 2006; Vito et al., 1997), several caspases have been reported to cleave PS1. The caspase cleavage site $ENDD_{333}$ in PS1 is cleaved in decreasing

order by caspase-8, -6, and -11, and the $AQRD_{345}$-site by caspase-3, -7, and -1. Both these cleavage sites are located in PS1 CTF, distal to the endoproteolytic site but still within the hydrophilic loop (van de Craen et al., 1999). Cleavage of the caspase site $AQRD_{345}$ gives rise to a truncated form of CTF (caspCTF) that starts at the serine residue 346 (see figure 2A in **Paper II**). This caspCTF displaces wild type (wt) CTF in the γ-secretase complex during apoptosis with remained activity (Hansson et al., 2006). In summary, increasing evidence points toward a central role of apoptotic signaling and caspase activation in AD.

1.2 THE AMYLOID PRECURSOR PROTEIN

The amyloid precursor protein (APP) is a type I transmembrane protein containing a large N-terminal ectodomain with high affinity to bind copper and zinc (Bush et al., 1993; Hesse et al., 1994), one transmembrane domain, and a small C-terminal part facing the cytosol (Kang et al., 1987). APP is subjected to several post-translational modifications such as N- and O-glycosylation, tyrosine sulfation, and phosphorylation (Gandy et al., 1988; Weidemann et al., 1989) during its way through the secretory pathway to the plasma membrane. However, only a small fraction reaches the plasma membrane. The majority of APP is instead localized to the Golgi apparatus. If APP is not ectodomain-shedded at the plasma membrane, it will be internalized into the endosomes where it can be i) ectodomain-shedded, ii) recycled back to the cell surface or iii) degraded in lysosomes (Haass et al., 2012; Haass et al., 1992a).

APP belongs to a small protein family including the amyloid precursor-like proteins 1 and 2 (APLP1 and APLP2) (Slunt et al., 1994; Wasco et al., 1992). The APP family share conserved regions within the ectodomain and the cytosolic C-terminal, but APLP1 and APLP2 lack the Aβ domain (Sprecher et al., 1993). However, they still undergo the same type of processing as APP, generating Aβ-like fragments and intracellular domains (ICD) (Eggert et al., 2004). APP and APLP2 are ubiquitously expressed in most tissues, while APLP1 is restricted to the nervous system (Lorent et al., 1995; Slunt et al., 1994).

To investigate the function of the APP family members, different knock-out combinations have been generated in mice. APP knock-out mice show reduced grip strength, reactive gliosis, reduced body (and brain) weight, as well as impairments in behaviour and LTP (Dawson et al., 1999; Zheng et al., 1995). When APLP1 is absent in mice, the mice are viable and fertile, but like the APP deficient mice, show reduction in body weight (Heber et al., 2000). Surprisingly, mice lacking APLP2 show no apparent abnormalities (von Koch et al., 1997). When analysing the APP/APLP1, APP/APLP2, APLP1/APLP2 double, and the APP/APLP1/APLP2 triple knocked-out mice, only the APP/APLP1 double knocked-out mice were viable, the other displayed early postnatal lethality (Heber et al., 2000; Wang et al., 2005; von Koch et al., 1997). All together, this suggests that the APP family members serve partially redundant functions and play an essential role in normal brain development and early postnatal survival (Heber et al., 2000; Herms et al., 2004).

APP exists in three isoforms containing 695, 751, or 770 residues (Kang et al., 1987; Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). All isoforms are ubiquitously expressed throughout the body but APP695 is the most abundant form in the brain and is mainly

produced by neurons (Weidemann et al., 1989). All isoforms contain the Aβ peptide domain but the longer isoforms, 751 and 770, also express a Kunitz protease inhibitor (KPI) domain in their ectodomains. The KPI domain has lately been shown to interfere with certain types of dimerizations (Isbert et al., 2012). APP and the other APP family members can dimerize into homo- or hetero-dimers via one of their GxxxG domains in the transmembrane region (Munter et al., 2007), or by cis- and trans-dimerization via their E1 domains in the ectodomains, promoting cell-cell adhesion (Soba et al., 2005).

The physiological function of APP is not fully understood and is difficult to address since APP processing generates various products that all can have specific functions. In addition, members of the APP family have some overlapping functions making the identification of its function more difficult. However, several functions of APP have been suggested, such as the involvement in: neuronal migration during development (Young-Pearse et al., 2007), neuroprotection and stimulation of neurite outgrowth (Perez et al., 1997; Small et al., 1994), synaptogenesis (Moya et al., 1994; Priller et al., 2006), regulation of stem cells (Kwak et al., 2006), axonal transport (Kamal et al., 2001), cell adhesion (Beher et al., 1996; Breen et al., 1991), maintaining copper homeostasis in the cell (Barnham et al., 2003; Treiber et al., 2004), and function as a cell surface receptor (Kang et al., 1987).

1.2.1 APP processing

APP is processed through two major pathways; the amyloidogenic pathway (associated to AD) or the non-amyloidogenic pathway (Figure 2).

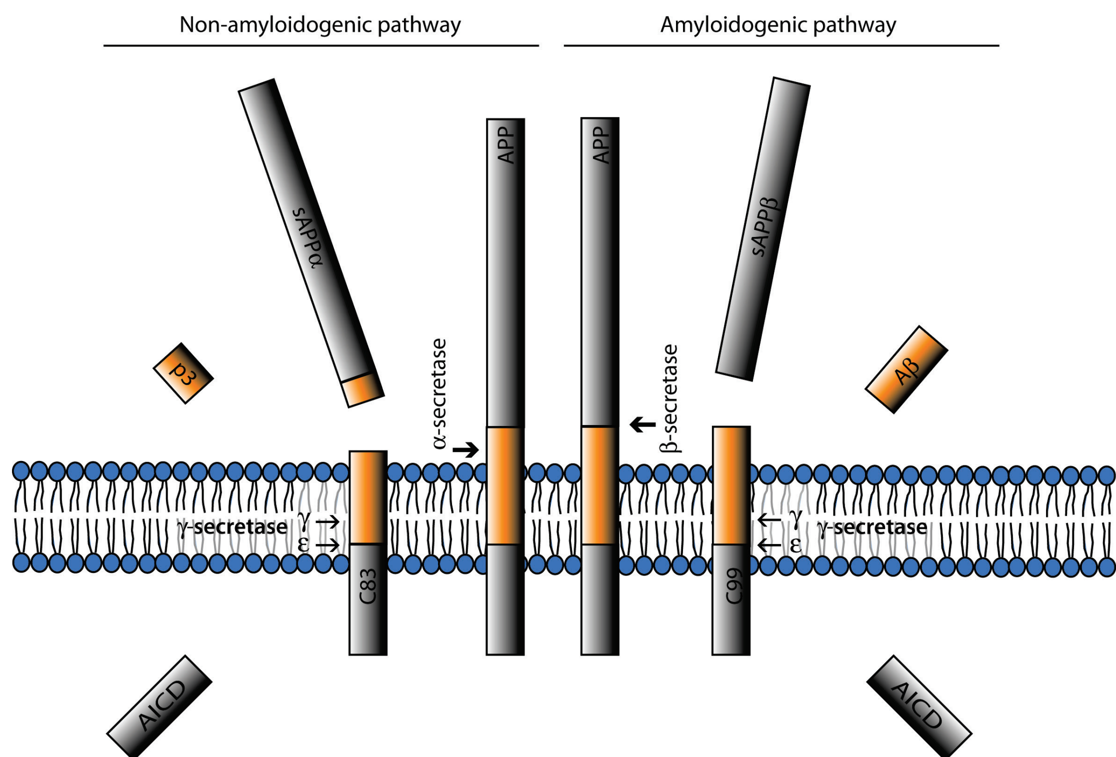

Figure 2. APP processing. APP is processed through the non-amyloidogenic pathway at the plasma membrane where α-secretase mostly is located and active (Sisodia, 1992). The amyloidogenic pathway is suggested to occur in intracellular compartments (including the trans-Golgi network, endosomes, and lysosomes) with acidic pH that makes BACE1 highly active (Vassar, 2004).

The amyloidogenic pathway appears to be the favored pathway in neurons, while the non-amyloidogenic pathway is predominantly found in all other cell types (Vetrivel and Thinakaran, 2006). In the non-amyloidogenic pathway, APP is first processed by α-secretase followed by the γ-secretase complex. α-Secretase is identified as a metalloprotease belonging to the "a disintegrin and metalloproteinase" (ADAM) family. Several ADAM family members have been suggested to be α-secretases, especially ADAM9, ADAM10, and ADAM17 (Koike et al., 1999; Lammich et al., 1999; Slack et al., 2001). However, ADAM10 is considered to be responsible for the α-secretase cleavage occurring in primary neurons (Kuhn et al., 2010). α-Secretase cleaves APP within the Aβ peptide sequence (between aa 16-17), thereby preventing Aβ production. The cleavage releases the neuroprotective soluble APPα fragment, sAPPα (Esch et al., 1990; Gakhar-Koppole et al., 2008; Ring et al., 2007; Sisodia, 1992; Taylor et al., 2008) from the remaining membrane-bound fragment of 83 residues (C83). C83 is then processed further by the γ-secretase complex to release both a 3 kDa peptide (p3) and the APP intracellular domain (AICD) from the membrane (Gu et al., 2001; Haass et al., 1993; Sastre et al., 2001).

In the amyloidogenic pathway, APP is first processed by an enzyme called β-secretase, followed by the γ-secretase complex. The β-site APP cleaving enzyme-1 (BACE1) has long been considered to be the main candidate for β-secretase (Vassar, 2004; Vassar et al., 1999). β-secretase cleavage releases a soluble fragment, sAPPβ, from the membrane-retained fragment of 99 residues (C99). Unlike the sAPPα formed by the non-amyloidogenic pathway, sAPPβ is believed to be involved in suppressed neuronal activity and triggering neuronal death (Furukawa et al., 1996; Nikolaev et al., 2009). C99 is subsequently cleaved by the γ-secretase complex to release the AD-related Aβ peptide and AICD from the membrane (Gu et al., 2001; Haass et al., 1993; Sastre et al., 2001).

The processing of C99 by the γ-secretase complex occurs at multiple sites. First, γ-secretase cleaves at the ε-site to release AICD into the cytosol, followed by a stepwise cleavage of the remaining membrane-bound fragments to generate Aβ peptides of different lengths (Kakuda et al., 2006). The step-wise processing occurs with a distance of 3-4 residues, corresponding to one complete turn of the α-helix structure (Qi-Takahara et al., 2005). Based on this information, the cleavage at the ε-site in APP has been proposed to occur at two possible sites (either at position 48-49 or 49-50 in C99) (Gu et al., 2001) followed by the step-wise process generating either the Aβ49→Aβ46→Aβ43→Aβ40 or the Aβ48→Aβ45→Aβ42→Aβ38 product lines (Funamoto et al., 2004; Takami et al., 2009).

1.2.2 The Amyloid β-peptide

Aβ is not only produced in the AD brain but also in the brain of healthy people (Seubert et al., 1992; Vigo-Pelfrey et al., 1993). Although the physiological function of Aβ is unknown, the balance between the production and degradation of Aβ seems to be of importance. Normally, Aβ is rapidly cleared from the brain by different Aβ degrading enzymes such as Neprilysin and the insulin degrading enzyme (Farris et al., 2003; Iwata et al., 2000). Aβ can also be taken up by endocytosis in the synapse and become phagocytosed by microglia and astrocytes (Querfurth and LaFerla, 2010). In addition, Aβ can be transported in and out of the brain by the receptor for advanced glycation end products (RAGE) (Deane et al., 2003) and the low-

density lipoprotein receptor-related protein 1 (LRP1) (Shibata et al., 2000), respectively. Interestingly, the LRP-1 expression is reduced (Deane et al., 2008; Hashimoto et al., 2012; Kang et al., 2000; Shibata et al., 2000) and the RAGE expression is enhanced (Carnevale et al., 2012; Deane et al., 2003; Lue et al., 2001; Yan et al., 1996) in AD patients and AD animal models, promoting an increased level of Aβ in the brain.

Due to the stepwise cleavage process mediated by the γ-secretase complex, Aβ peptides can vary in length. While the Aβ40 peptide is the major form produced (Beher et al., 2002; Wang et al., 1996), the longer Aβ42 peptide is more hydrophobic and thereby more prone to aggregate and form plaques (Iwatsubo et al., 1994). In fact, Aβ42 forms fibrils very rapidly and at lower concentration than Aβ40 (Jarrett et al., 1993). The longer variant Aβ43 has also been found in amyloid deposits of AD brains (Welander et al., 2009), and like the Aβ42 peptide, has a seeding effect of other soluble Aβ peptides for the formation of plaques (Saito et al., 2011). Longer peptides have also been identified but they are believed to stay within the membrane due to their hydrophobic properties (Qi-Takahara et al., 2005; Yagishita et al., 2006; Zhao et al., 2005). The amount of produced Aβ42 in relationship to Aβ40 has shown to be important for the pathogenesis of the disease and only a minor increase in the Aβ42/Aβ40 ratio is enough to enhance oligomer formation (Jan et al., 2008; Kuperstein et al., 2010). In addition, most of the FAD mutations in APP and the PS homologues do not increase the total amount of Aβ but rather the Aβ42/Aβ40 ratio. This strongly indicates that the ratio is highly relevant for AD pathogenesis.

Monomeric Aβ can aggregate into fibrils and sequentially form amyloid plaques by a complex multi-step procedure involving different oligomeric intermediates such as dimers and trimers (Podlisny et al., 1995; Shankar et al., 2008; Walsh et al., 2000), Aβ-derived diffusible ligands (ADDLs) (Gong et al., 2003; Lambert et al., 1998), a 56-kDa Aβ assembly called dodecamer (Aβ*56) (Lesne et al., 2006), globulomers (Barghorn et al., 2005; Gellermann et al., 2008), and protofibrils (Harper et al., 1997; Walsh et al., 1997). The relevance of these intermediates *in vivo* is uncertain but it is considered that soluble oligomers are the most neurotoxic pathogens in AD (Haass and Selkoe, 2007; Hardy, 2006). This is supported by studies confirming that soluble oligomers correlate better to the degree of cognitive decline and synaptic loss than the plaques (DaRocha-Souto et al., 2011; McLean et al., 1999; Naslund et al., 2000). Although the plaques are found extracellularly, accumulated Aβ is also found intracellularly in compartments such as lysosomes and endosomes (Aoki et al., 2008; Gouras et al., 2010; Hashimoto et al., 2012; Hu et al., 2009). The combination of the low pH and the high concentration of Aβ in these compartments increase the risk for spontaneous aggregation of Aβ. These aggregates may be secreted and seed the extracellular plaque formation.

1.2.3 The Amyloid cascade hypothesis

The predominant and most supported hypothesis for what causes AD is the amyloid cascade hypothesis. This hypothesis was initially formulated 20 years ago but has been updated in year 2002. It states that the major cause of the events observed in AD is the accumulation of Aβ, which leads to a cascade of pathogenic events such as: the formation of NTFs, activation of microglia and astrocytes, oxidative stress, and neuronal and synaptic loss that subsequently causes neurotransmitter deficits. This leads to dementia and finally death (Hardy and Selkoe, 2002; Hardy and Higgins, 1992).

The hypothesis is supported by different findings. First, Aβ is found as the main component of the neuritic plaques (Glenner and Wong, 1984; Masters et al., 1985) and the intermediate Aβ oligomers, that are toxic to synapses and neurons, are found in elevated levels in AD brains (Selkoe, 2002). Second, inherited mutations in the *APP* and *PSEN* genes give rise to an increased Aβ42/Aβ40 ratio, an elevated total Aβ production, or an enhancement of the oligomerization of Aβ, causing early onset FAD (Nilsberth et al., 2001; St George-Hyslop, 2000). Third, Down's syndrome patients show plaque pathology and develop AD due to their extra copy of chromosome 21, where APP is located (Olson and Shaw, 1969). Fourth, the APOE ε4 allele is a major risk factor for AD, and patients carrying this allele show a severe Aβ burden (Corder et al., 1993). Fifth, many GWAS have identified genetic variants that are associated to Aβ related mechanisms (Reitz, 2012). Sixth, imaging techniques and chemical biomarkers that indicate specific changes occurring *in vivo*, demonstrate that Aβ begins to accumulate before tau-mediated neuronal injuries and clinical symptoms can be detected (Jack et al., 2010). Even though many findings support the amyloid cascade hypothesis, the connection between Aβ accumulation and the following events in the cascade, still remains to be explained.

1.3 THE γ-SECRETASE COMPLEX

The γ-secretase complex is a large multimeric aspartylic protease responsible for cleaving various type I transmembrane proteins such as APP, Notch, N-cadherin, and ephrinB. The γ-secretase complex consists of the four components presenilin (PS), Nicastrin (Nct), anterior pharynx defective-1 (Aph-1) and presenilin enhancer-2 (Pen-2), which all are required for the complex to become active (Francis et al., 2002; Goutte et al., 2002; Wolfe et al., 1999; Yu et al., 2000)(Figure 3). Both PS and Aph-1 exist as two homologues (PS1/PS2 and Aph-1a/Aph1b) and in addition to that, the Aph-1a homologue can undergo alternative splicing generating a short (Aph-1aS) or a long (Aph-1aL) isoform. Thus, there can be up to six compositions of the γ-secretase complex (Shirotani et al., 2004a).

The assembly of the components takes place in the endoplasmic reticulum (ER) (Capell et al., 2005). First, Aph-1 and the immature form of Nct form a subcomplex to which PS binds, followed by Pen-2 (Capell et al., 2005; Gu et al., 2003; Hu and Fortini, 2003; LaVoie et al., 2003; Shirotani et al., 2004b; Takasugi et al., 2003). The complex will thereafter undergo endoproteolysis, cleaving the PS protein into N- and C-terminal fragments (NTF and CTF) (Thinakaran et al., 1996). The association of Pen-2 with the other components is also suggested to trigger a conformational change of Nct, causing an immediate release of the

complex from ER to the Golgi apparatus (Capell et al., 2005). The γ-secretase complex will there be subjected to several post-translational modifications such as the glycosylation and sialylation of Nct and the S-palmitoylation of both Aph-1 and Nct (Cheng et al., 2009; Herreman et al., 2003; Yu et al., 2000), leading to the fully mature form of Nct. The process from complete assembly in the ER to a mature form of the γ-secretase complex in the Golgi apparatus is believed to occur very rapidly. The reason for this is that the activation of γ-secretase is believed to occur in connection with the endoproteolysis of PS. In addition, it is mainly the mature form of Nct that interacts with the endoproteolysed fragments of PS as observed by immunoprecipitation studies (Capell et al., 2005; Edbauer et al., 2002; Herreman et al., 2003; Kimberly et al., 2002).

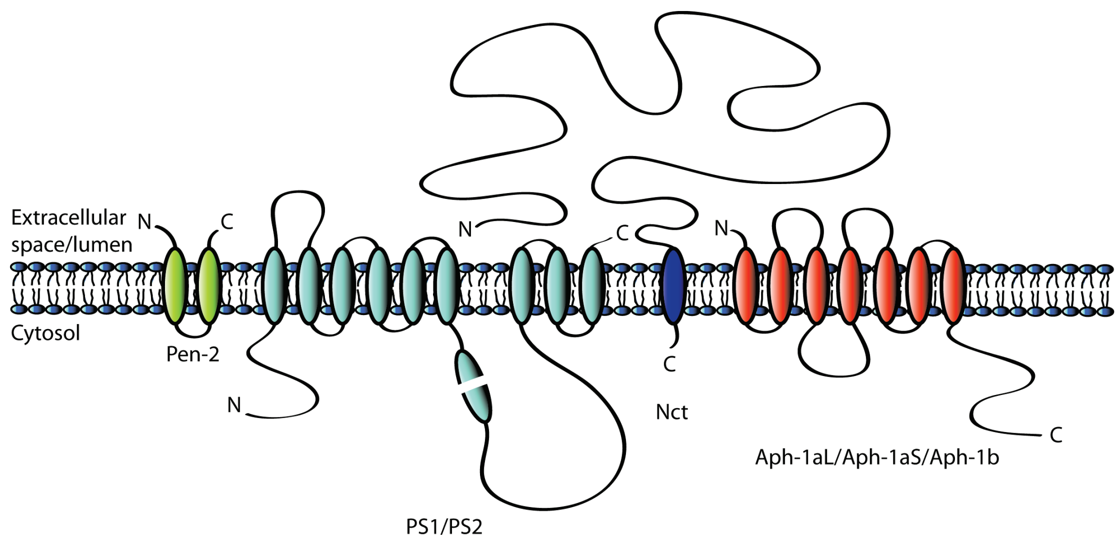

Figure 3. The γ-secretase complex. The γ-secretase complexes are composed of the components presenilin (PS1 or PS2), nicastrin (Nct), Aph-1 (Aph-1aL, Aph-1aS, or Aph-1b), and Pen-2, generating up to six different types of complexes.

Several other proteins have been suggested to associate with the γ-secretase complex such as CD147, TMP21, syntaxin 1, the tetraspanin web proteins, the tubulin polymerization promoting protein (TPPP), the NADH dehydrogenase ubiquinone iron-sulfur protein 7 (NDUFS7), the voltage-dependent anion channel 1 (VDAC1), the contactin-associated protein 1 (CNTNAP1), and Erlin-2 (Chen et al., 2006; Frykman et al., 2012; Hur et al., 2012; Teranishi et al., 2010; Teranishi et al., 2012; Wakabayashi et al., 2009; Zhou et al., 2005). However, these γ-secretase associated proteins (GSAPs) are believed to be of importance for modulating the γ-secretase complex in aspect of location and/or substrate specificity rather than being additional components. The co-expression of PS, Nct, Aph-1, and Pen-2 in *Saccaromyces cerevisiae*, that lacks endogenous γ-secretase components and activity, clearly indicates that these components are sufficient for γ-secretase activity. (Edbauer et al., 2003). Additional reconstitution studies using cells from different insect and mammalian species support this conclusion (Hayashi et al., 2004; Kimberly et al., 2003; Takasugi et al., 2003; Zhang et al., 2005).

The γ-secretase complex cleaves its substrates within the membrane by a process referred to as regulated intramembrane proteolysis (RIP) (Brown et al., 2000). Membrane-embeded proteases that have this property belong to the family of intramembrane-cleaving proteases (iCLIPs). The proteins within the iCLIP family are aspartyl proteases (e.g. the γ-secretase complex and Signal peptide peptidase), serine proteases (e.g. Romboids), or metalloproteases (e.g. the Site-2 protease) (Beel and Sanders, 2008). In most cases, the ectodomain of the substrates needs to be shedded by another protease before the members of the iCLIPs can cleave. Compared to other iCLIP proteins, the γ-secretase complex is quite unique due to its size and its many subunits.

The stoichiometry of the γ-secretase complex is most likely 1:1:1:1 (Fraering et al., 2004; Kimberly et al., 2003; Osenkowski et al., 2009; Sato et al., 2007), which corresponds to a molecular mass of approximately 220-230 kDa. Due to the large size and high hydrophobicity, containing 19 transmembrane domains (TMD) in total, structure analyses of the γ-secretase complex have been difficult to perform. However, x-ray crystallographic analyses of other ICLiPs have been accomplished, indicating that the active sites reside in water cavities within the lipid bilayer (Feng et al., 2007; Wang et al., 2006; Wu et al., 2006). The three-dimensional models of the γ-secretase complex that have been created so far, have been analyzed by electron microscopy (Lazarov et al., 2006; Ogura et al., 2006; Osenkowski et al., 2009; Renzi et al., 2011). The two most recent three-dimensional structures display a resolution of 12 and 18 Ångström, which is too low for detailed analysis but give an insight into the overall structure (Osenkowski et al., 2009; Renzi et al., 2011). These structure models strongly support the 1:1:1:1 stoichiometry and display, like the other iCLIPs, water-accessible cavities that enables the enzyme to process its substrates.

1.3.1 The γ-secretase components

1.3.1.1 Presenilin

Two PS homologues, PS1 and PS2, exist in humans. PS1 is slightly larger than PS2 with its 467 residues compared to the 448 residues of PS2 (Levy-Lahad et al., 1995; Sherrington et al., 1995). The PS proteins contain nine TMDs and have their N-terminals facing the cytosol while the C-terminals are facing the lumenal/extracellular space (Figure 4) (Henricson et al., 2005; Laudon et al., 2005; Oh and Turner, 2005; Spasic et al., 2006). Both proteins are subjected to endoproteolysis within their large cytosolic loop between TMD 6 and 7, generating a C- and N-terminal fragment (CTF and NTF). These fragments constitute the active conformation of PS by forming a stable heterodimer (Thinakaran et al., 1996; Wolfe et al., 1999).

The endoproteolysis of PS is believed to be generated by the γ-secretase complex itself and to be processed in a step-wise manner, like the cleavage of its substrates such as APP. Since a major cleavage site is detected in PS1 after residue 298, and a minor site after residue 292 (Podlisny et al., 1997), endoproteolysis may be initiated between residue 292 and 293 and then proceed via a cleavage at residues 295/296 to finally generate the last cleavage after residue 298 (Fukumori et al., 2010). Similar observations have also been made for PS2 (Jacobsen et al., 1999; Shirotani et al., 1997).

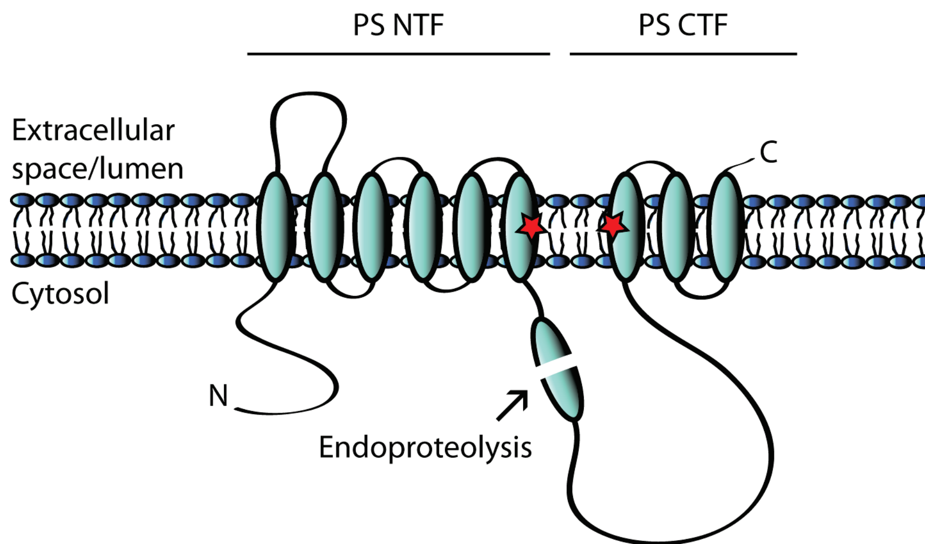

Figure 4. Presenilin (PS). PS is endoproteolysed into C- and N-terminal fragments (CTF and NTF). The aspartylic residues responsible for the catalytic activity are shown by red stars.

The aspartate residues, which are responsible for the catalytic activity of the enzyme, are located in the TMD6 of PS CTF (PS1 D257 and PS2 D263) and in TMD7 of PS NTF (PS1 D385 and PS2 D366). If these aspartates are mutated to alanines by site-directed mutagenesis, no endoproteolysis will occur and the complex stays inactive (Kimberly et al., 2000; Wolfe et al., 1999). This is also supported by the fact that transisition-state inhibitors, designed to bind to the active site, binds directly to both CTF and NTF (Berezovska et al., 2000; Esler et al., 2000; Li et al., 2000; Seiffert et al., 2000). This binding is abolished if the aspartates are mutated (Berezovska et al., 2000; Wrigley et al., 2004). Recent studies have suggested that in addition to TMD6 and 7 where the aspartylic acids reside, the PAL motif located in connection to TMD9, together with other residues of TMD1 and 9, are also involved in forming the water accessible catalytic pore of the enzyme (Sato et al., 2006; Sato et al., 2008; Takagi et al., 2010; Tolia et al., 2006; Tolia et al., 2008; Wang et al., 2006).

PS1 and PS2 are suggested to play distinct roles since PS1 knock-out mice are embryonically lethal (De Strooper et al., 1998) while PS2 knock-out mice are viable (Herreman et al., 1999). Supporting this theory, PS1 or PS2 containing γ-secretase complexes show differences in APP processing and sensitivity to some γ-secretase inhibitors (Lai et al., 2003; Zhao et al., 2008; Borgegard et al., in press). It has been speculated that PS2 is responsible for the residual Aβ secretion seen in PS1 deficient neurons. However, endogenous PS2 fails to rescue the lethal phenotype of PS1 deficient mice. Hence, PS1 and PS2 seem to have only partial and not completely overlapping functions (Herreman et al., 1999).

PS1 and PS2 share approximately 63-67% identity at the protein level (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Levy-Lahad et al., 1995; Rogaev et al., 1995). In certain TMDs, the identity can be as high as 95% suggesting that these regions are of importance for the function of PS. By comparing the protein sequence of PS1 and PS2, the largest diversity is found in the hydrophilic loop between TMD6 and 7 (Rogaev et al., 1995), where they only

share 16 % homology at protein level, when performing alignments (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The PS2 loop is smaller (84 residues compared to 110 residues in PS1), but other properties also differ concerning this region. For example, β-catenin binds to the PS1 loop but not to PS2 (Saura et al., 2000). The lethal phenotype of PS1 deficient mice could be rescued by introducing a PS1 molecule lacking the loop, suggesting that the loop is dispensable for γ-secretase activity (Xia et al., 2002). In addition, different truncations of PS1 CTF indicate that the first proteolytic step at the ε-site does not require the loop (Wanngren et al., 2010). However, the loop seems to be of importance for the enzyme to continue its stepwise cleavage pattern to generate the shorter Aβ peptides Aβ38, Aβ39, and Aβ40 (Deng et al., 2006; Wanngren et al., 2010). This demonstrates that the loop is important for normal processing at the γ-site. Interestingly, the loop region can also be cleaved by caspases and still be part of an active complex (Hansson et al., 2006; Kim et al., 1997). This suggests that caspase activation might modulate the Aβ-profile during apoptosis by its effect on PS CTF.

1.3.1.2 Nicastrin

Nct is the largest component in the γ-secretase complex with its 709 residues. It is a type I membrane protein where almost the entire protein is facing the lumenal/extracellular space (Figure 3) (Yu et al., 2000). While the short cytosolic tail seems to have no important function, domains like the TMD and the juxtamembrane region have been shown to be important for complex assembly (Capell et al., 2003; Morais et al., 2003; Walker et al., 2006). Nct is highly glycosylated in its mature form and is also subjected to other post-translational modifications such as S-palmitoylation and sialylation (Cheng et al., 2009; Yu et al., 2000). The complex assembly and activity is dependent on the conformational change of Nct, which occurs during its maturation (Shirotani et al., 2003). The glycosylation itself is although not required for the conformational change (Herreman et al., 2003).

The large ectodomain of Nct adopts a highly thermostable secondary structure and contains regions resembling several other proteins (Fluhrer et al., 2011). One region in the ectodomain shows similarity to the superfamily of aminopeptidases and the transferrin receptors (Fagan et al., 2001). Since Nct lacks catalytic activity, the structure similarities suggest that Nct could function as some kind of receptor. In fact, there is an ongoing debate whether Nct acts as a substrate receptor or if it rather stabilizes the γ-secretase complex. Nct has been reported to interact with APP- and Notch-derived γ-secretase substrates by a region between residues 312-340 in the DAP domain (DYIGS *and peptidase* homologous region). Specifically, glutamate 333 (E333) in Nct was shown to interact with the substrates (Dries et al., 2009; Shah et al., 2005). This proposed substrate receptor-like role for Nct has been challenged, as both *in vivo* and *in vitro* studies suggest that the maturation and assembly of the γ-secretase complex, rather than its activity, were affected by mutating this glutamate residue (Chavez-Gutierrez et al., 2008). Another argument against the substrate receptor role is that the signal peptide peptidase SPPL2b, another aspartyl protease belonging to the iCLIPs family, possesses intrinsic enzyme activity without additional co-factors and shows similar substrate requirements as γ-secretase, although it processes type II membrane proteins instead of type I membrane proteins (Martins et al., 2009). In addition, two other studies suggest that Nct is dispensable

for γ-secretase activity and instead has a stabilizing function (Futai et al., 2009; Zhao et al., 2010). Recently however, a tetratricopeptide repeat (TPR) domain was characterized downstream of the DAP domain. This domain is commonly involved in peptide recognition. Mutagenesis analyses within this domain resulted in the loss of γ-secretase activity but it did not affect the assembly, suggesting that the TPR-like domain may play an important role in substrate binding (Zhang et al., 2012). Thus, it remains unclear whether Nct is involved in substrate selectivity or has a more general role in the stabilization and maturation of the γ-secretase complex.

1.3.1.3 Anterior pharynx defective-1

Aph-1 exists as two homologues in humans (Aph-1a and Aph-1b) (Francis et al., 2002) and as three homologues in rodents (Aph-1a, Aph-1b, and Aph-1c) (Ma et al., 2005; Serneels et al., 2005). The additional Aph-1c gene found in rodents is probably caused by a gene duplication of the Aph-1b gene. Human Aph-1a and Aph-1b show 56-59% sequence identity and contain seven TMDs (Figure 3) (Francis et al., 2002; http://blast.ncbi.nlm.nih.gov/Blast.cgi). While Aph-1b consists of 257 residues, Aph-1a can contain either 265 or 247 residues since it can be alternatively spliced generating two isoforms, Aph-1aL and Aph-1aS (where L stands for longer and S for shorter) (Lee et al., 2002). Topology studies have shown that the N-terminus and the loops 2, 4, and 6 are located in the lumenal/extracellular space, while the C-terminus together with the loops 1, 3, and 5 are situated in the cytosolic space (Fortna et al., 2004).

All three different forms of Aph-1 (Aph-1aL, Aph-1aS, and Aph-1b) have been shown to be part of active γ-secretase complexes but cannot be found in the same complex, giving the possibility of six different types of γ-secretase complexes when taking the two PS homologues into account (Shirotani et al., 2004a). The down-regulation of one Aph-1 homologue does not affect the other, which suggests that the Aph-1 homologues lack compensatory properties (Saito and Araki, 2005). Moreover, Aph-1a knock-out mice die during embryogenesis while Aph-1b and Aph1c null mice are viable (Ma et al., 2005). Aph-1b-containing γ-secretase complexes have shown to produce longer Aβ peptides than Aph-1a-containing γ-secretase complexes. In addition, Aph-1bc knock-out mice shows a significant reduction of Aβ production without causing any severe side effects, implicating that Aph-1bc do not have a large impact on the Notch signaling pathway (Serneels et al., 2005; Serneels et al., 2009). These findings lead to the conclusion that Notch processing is more dependent on Aph-1a, while Aph-1b seems more involved in APP processing (Ma et al., 2005; Serneels et al., 2009).

All human Aph-1 variants contain a tandem GxxxG sequence in TMD4 that is essential for the assembly of the γ-secretase complex (Araki et al., 2006; Lee et al., 2004; Niimura et al., 2005), especially for the interaction with PS that is believed to occur with TMD7 of the PS CTF (Araki et al., 2006; Steiner et al., 2008). Aph-1 is shown to have a stabilizing effect on PS and immature Nct (Araki et al., 2006; Luo et al., 2003). However, the interaction with Nct is probably not affected by the GxxxG-motif (Araki et al., 2006; Fortna et al., 2004; Niimura et al., 2005), but rather occurs downstream of TMD4 (Chiang et al., 2012; Fortna et al., 2004). Interestingly, two histidines (H171 and H197) located in TMD5 and TMD6, are important for γ-secretase assembly and activity (Pardossi-Piquard et al., 2009). These histidine residues have

also been suggested to participate in the binding with both full-length substrates and their CTFs after ectodomain-shedding (Chen et al., 2010). This suggests that Aph-1, like Nct, may have a role in the initial association of the γ-secretase complex with its substrates (Chen et al., 2010; Dries et al., 2009; Shah et al., 2005). A recent publication suggests that Aph-1 together with Nct as a subcomplex may contribute to this role (Mao et al., 2012). In addition, Aph-1 has been shown to have sequence motifs resembling Type II CAAX proteases, which raises the possibility that Aph-1 may possess proteolytic activity. However, since no protease activity of Aph-1 has been shown experimentally, it is more likely that Aph-1 lost the protease activity during evolution and now has another function within the γ-secretase complex (Pei et al., 2011).

1.3.1.4 Presenilin enhancer-2

Pen-2 is a small hairpin protein consisting of 101 residues and is composed of two TMDs connected by a cytosolic loop, where the N- and C-terminus are facing the lumenal/extracellular space (Figure 3) (Crystal et al., 2003; Francis et al., 2002). Pen-2 is the component that finalizes the assembly of the complex and thereby initiates the endoproteolysis of PS. While the N-terminus is important for the interactions with PS (Crystal et al., 2003), the C-terminus is suggested to stabilize the generated PS NTF and CTF fragments (Prokop et al., 2005; Prokop et al., 2004). The first TMD of Pen-2 has been suggested to be important for endoproteolysis (Kim and Sisodia, 2005). However, contradicting results have recently been presented, suggesting that Pen-2 is dispensable for endoproteolysis. They found that PS became endoproteolysed in Pen-2 knock-down cells but that the endoproteolysed fragments were rapidly degraded by the proteasome (Mao et al., 2012). Hence, Pen-2 seems to be more important for the stabilization of the endoproteolytic fragments rather than the endoproteolysis itself.

1.3.2 γ-Secretase substrates

The list of γ-secretase substrates is continually growing and to date, more than 90 substrates have been identified. Most of the substrates are type I membrane proteins and have become ectodomain-shedded by another protease before being processed by the γ-secretase complex, although not all ectodomain-shedded type I membrane proteins are γ-secretase substrates (Haapasalo and Kovacs, 2011; Lleo, 2008). In addition to the ectodomain shedding, the juxtamembrane domain, the TMD, and the cytoplasmic domain of the substrates are of importance for correct processing (Hemming et al., 2008; Ren et al., 2007; Struhl and Adachi, 2000; Zhang et al., 2002). Hence, there is no specific sequence motif for the cleavage of the γ-secretase complex. The many substrates in combination with the lack of a clear sequence motif suggest that the γ-secretase complex may have a general degrading function to remove TMDs when the full-length proteins have fulfilled their purpose (Kopan and Ilagan, 2004). However, some substrates display a functional role also after γ-secretase processing, such as Notch, N-cadherin, and ephrinB.

Substrate processing by the γ-secretase complex results in the release of intracellular domains (ICD) that in some cases are translocated into the nucleus and are involved in signal

transduction (e.g. the ICD of Notch) (Figure 5). Other ICDs function as cytosolic signaling molecules (e.g. the ICD of ephrinB), while some may not have a function. The function of the APP derived AICD product is not established, partly because AICD is rapidly degraded and therefore difficult to study. In addition, more than 20 proteins are suggested to interact with AICD (Muller et al., 2008). AICD could therefore have different functions depending on which protein it interacts with. To make it even more challenging, AICD can also become phoshorylated at three sites which might affect its function (Oishi et al., 1997). AICD has been suggested to be translocated into the nucleus and regulate gene expression together with the proteins Fe65 and Tip60 (Cao and Sudhof, 2001; Nakaya and Suzuki, 2006). However, it is possible that AICD itself is not translocated into the nucleus, but instead induces a conformational change in Fe65 necessary for its translocation into the nucleus (Cao and Sudhof, 2004). There is also conflicting data suggesting that neither AICD nor APP is required for this signaling event (Giliberto et al., 2008; Hass and Yankner, 2005). Thus, it is still not clear if AICD i) translocates into the nucleus and regulates gene transcription like the ICD of Notch, ii) regulates gene transcription without being translocated into the nucleus like the ICD of N-cadherin, or iii) function as a cytosolic signaling molecule like the ICD of EphrinB.

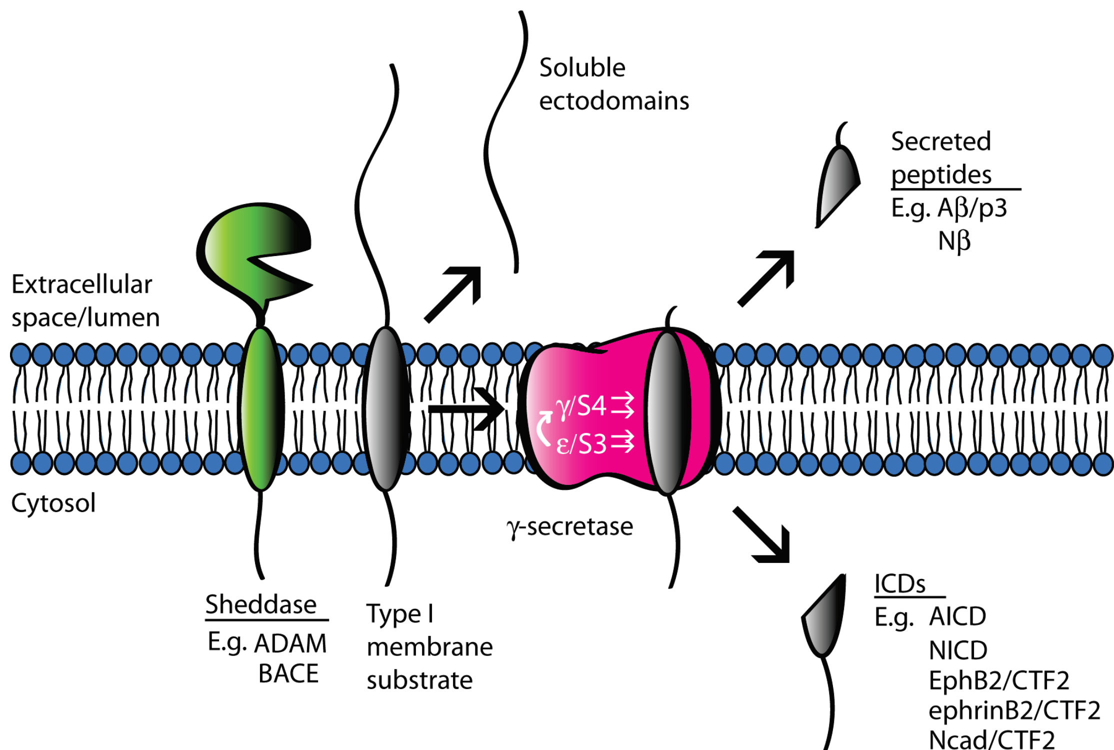

Figure 5. Substrate processing by the γ-secretase complex. Many ectodomain shedded type I membrane proteins are substrates for the γ-secretase complex. The cleavage by the γ-secretase complex generates ICDs where many act as signaling molecules or translocate into the nucleus and regulate gene transcription. Some of the substrates also release Aβ-like peptides, such as Notch, APLP1, APLP2, CD44, and the interleukin-1 receptor II (Eggert et al., 2004; Kuhn et al., 2007; Lammich et al., 2002; Okochi et al., 2002; Yanagida et al., 2009).

1.3.2.1 Notch

One of the most investigated γ-secretase substrates beside APP is the Notch receptor. Four Notch receptors exist (Notch 1-4) and they are important for critical signaling and cell fate decisions during both embryogenesis and adulthood (Lleo and Saura, 2011). The Notch 1 receptor continues to be expressed in the adult brain and is initially cleaved at the S1-site by furin-like convertases forming a heterodimer (Blaumueller et al., 1997; Logeat et al., 1998). The heterodimer localizes to the plasma membrane where it becomes activated by the binding of its ligands Jagged or Delta (Nye and Kopan, 1995). The activation will induce ectodomain shedding of Notch at the S2-site by the protease ADAM10, generating a membrane-bound fragment called NotchΔE or NEXT (van Tetering et al., 2009). The fragment is then processed at the S3-site, analogous to the ε-site in APP, by the γ-secretase complex (De Strooper et al., 1999; Schroeter et al., 1998). This event releases the Notch intracellular domain (NICD) (Schroeter et al., 1998), that translocates into the nucleus and regulates transcription of genes such as the hairy and enhancer of split (HES) family. These genes are involved in the inhibition of neuronal differentiation by acting as negative regulators of other pro-neuronal genes. They are also critical for the development of many tissues and organs (Jarriault et al., 1995). The γ-secretase complex will also cleave at another site in Notch called the S4-site, which corresponds to the γ-site in APP, and releases an Aβ-like peptide called Nβ (Okochi et al., 2002). The function of Nβ is still unknown.

1.3.2.2 N-cadherin and ephrinB

Neural (N)-cadherin is a cell adhesion receptor highly expressed in neurons and is involved in important functions such as adhesion, axonal growth, synaptogenesis, and long-term potentiation (lléo and Saura, 2011). N-cadherin is ectodomain-shedded by ADAM10 and further processed by the γ-secretase complex to release an ICD referred as Ncad/CTF2 in the literature. The ICD will bind to the CREB-binding protein CBP in the cytosol and thereby block its translocation into the nucleus and promote its degradation (Junghans et al., 2005). Hence, the ICD of N-cadherin downregulates CBP/CREB-dependent transcription without being translocated into the nucleus.

Both the tyrosine kinase EphrinB (EphB) receptor and its ligands ephrinB1-2 are substrates for the γ-secretase complex. The interaction between the ligand ephrinB of one cell with a EphB receptor of an adjacent cell, trigger a bidirectional signaling that is crucial for the development of the nervous system. In the adult brain, the activation will regulate synaptic plasticity and long-term potentiation (Georgakopoulos et al., 2006).

The activation of the EphB2 receptor can either occur via a ligand, that will induce endocytosis and ectodomain shedding, or by calcium influx that promote ADAM protease cleavage. Nevertheless, the ectodomain-shedded CTF will be further processed by the γ-secretase complex to release the ICD, referred to as EphB2/CTF2 in the literature, into the cytosol. This ICD promotes phosphorylation and surface expression of NMDA receptors (Barthet et al., 2012).

The ligand ephrinB will first be ectodomain-shedded after the binding of the EphB-receptor, and subsequently processed by the γ-secretase complex to generate an ICD referred to as ephrinB2/CTF2. The ICD activates Src kinases and promotes the phosphorylation of its full length protein ephrinB2 (Georgakopoulos et al., 2006). Hence, neither the ICD of the EphB2 receptor nor its ligand ephrinB translocate into the nucleus but are instead important cytosolic signaling molecules.

2 AIMS OF THE THESIS

The γ-secretase complex cleaves over 90 substrates, of which APP and the Notch receptor are the most extensively studied. Several inhibitors of the γ-secretase complex have failed in clinical trials due to severe side effects such as skin cancer, gastrointestinal tract disorders, and immunosuppression (Imbimbo and Giardina, 2011). All these side effects are connected to an impairment of the Notch signaling pathway. Hence, it is of importance to find ways of inhibiting APP processing without disturbing other vital processes such as the Notch signaling pathway. For this challenge, a larger understanding of the γ-secretase complex is crucial. The different homologues and splice variants of the γ-secretase components can generate up to six distinct γ-secretase complexes with possible diverse functions. In addition, the γ-secretase complex can also undergo caspase cleavage, which may change the properties of the complex. Inhibiting or modulating only one type of γ-secretase complexes might avoid severe side effects. The general aim of this thesis is therefore to achieve a deeper understanding of the different γ-secretase complexes and their components, with respect to their properties and substrate processing.

The specific aims of the thesis were:

Paper I: To examine the role of the Nct ectodomain for APP and Notch processing.

Paper II: To investigate how the γ-secretase complex and its activity is affected by caspase cleavage.

Paper III: To study the different PS homologues and their activity on the neuronal substrates APP, Notch, ephrinB, and N-cadherin.

Paper IV: To explore if low homology regions within the Aph-1 proteins affect APP and Notch processing.

3 METHODOLOGICAL CONSIDERATIONS

Several methods have been used in the papers for this thesis. In this section, comments concerning some of the materials and methods will be presented and discussed. Detailed descriptions of the procedures can be found in **Paper I-IV**.

3.1 CONSIDERATIONS WHEN CHOOSING MATERIAL AND MODEL SYSTEMS

There is unfortunately no such thing as the perfect model system when studying AD. Human brain samples are in theory the best material to reflect the disease as humans are the only species that develops AD. Human brain material is essential for studying which brain areas that are affected by the disease and its neuropathological characteristics. It is also a good model to examine how different proteins are expressed and affected in the AD brain. However, brain material is collected post-mortem and therefore it is difficult to diverse processes occurring before and after death, particularly if the post-mortem time is long. The availability of human brain material is limited and considering that the disease occurs at such late stage of life, other life factors may create a large individual difference between brains, demanding a large subset for comparison. Another disadvantage with brain material is that it cannot reflect changes occurring throughout the disease progression. This is especially relevant to AD, which is probably caused by multiple pathogenic processes and progresses over a long period of time.

To be able to examine the progress and the various stages of the disease, different animal models are more suitable considering that they can be sacrificed at different time points. However, no animal model can reflect all the neuropathological hallmarks of the disease but rather mimic different aspects of it. It is therefore of great importance to find the most relevant animal model when analyzing a particular aspect. Other advantages with animal models are less individual differences and short post-mortem time. However, it can be difficult to extrapolate the data from animal studies to humans. If the purpose with a study is to investigate molecular processes within a cell, immortalized cell lines can instead be suitable models.

Cell lines make it possible to study a mechanism in a more simple and controlled environment. Different proteins can be silenced, knocked-out, or overexpressed. Less ethical considerations are required and the amount of material is unlimited due to their immortality. Cell lines lack complexity and can be cultured under the same conditions, which generate less individual differences compared to human and animal samples. The drawback with the lack of complexity is that it is even more difficult than animal studies to extrapolate the data to the human disease.

3.1.1 Cell lines

In this thesis, molecular interactions within the γ-secretase complex and its properties have been analyzed. When performing these types of experiments, cell line based models have been considered to be sufficient with the benefit that they do not require sacrificed animals. The cell lines used were Nct knock-out mouse embryonic fibroblast (Nct-/-MEF) cells in **Paper I**, blastocyst-derived embryonic stem cells lacking PS1 and PS2 (BD8), and neuroblastoma (SH-SY5Y) cells in **Paper II**, wt and different PS knock-out mouse embryonic fibroblast (PS-/-, PS1-/-, and PS2-/-MEF) cells in **Paper III**, and Aph-1 knock-out mouse embryonic fibroblast (Aph1abc -/-MEF) cells in **Paper IV**. These immortal cell lines are valuable tools to study molecular processes in the cell.

When knock-out studies have been carried out in animals, Nct, PS1, Aph-1a, or Pen-2 deficient mice have died during embryogenesis due to phenotypes resembling Notch signaling impairment. During the last decades, conditional knock-out mice have been developed. These express all proteins during embryogenesis and the desired protein can be silenced during a later stage. However, it is essential to take into account that the effects of the knock-outs do not necessary describe the function of the lacking protein, rather the impact it has on the surrounding environment when it is absent. To investigate the function of each γ-secretase component, cell lines that are knocked-out of the protein of interest is very convenient. In this thesis, several modifications of the different components have been transfected into knock-out cell lines (**Paper I, II, and IV**) to compare these modifications with the wt protein. For this purpose, cell lines are a suitable method.

Different cDNA constructs have been both transiently and stably transfected depending on which type of experiment that has been performed. Both transfection methods have their benefits and drawbacks. The transient transfection is a fast method where the protein is expressed already within 24 hours, but the cDNA begins to degrade already after 48 hours since the cDNA is not inserted into the cell genome. In addition, each experiment is based on a new transient transfection, leading to different expression levels each time. For this reason, it is important to normalize with transfection efficiency. This can be achieved by co-expressing a CMV-β-gal vector and measure β-galactosidase activity in the samples. The benefit with stable expression of proteins is that the protein is inserted into the genome of the cell and therefore expressed during a long time. This generates reproducible protein levels. However, depending on its location, it can interfere with the expression of other genes. When comparing different stable cell lines with each other, one important aspect is to select clones with similar expression levels. This must also be controlled over time since a stable cell clone can decrease its expression although treated with medium containing antibiotics. The stable overexpression of proteins can also expose the cells to stress. While many experiments can be made with transient transfections, some methods require stable overexpressing of proteins to amplify the signal from background noise.

3.1.2 Mouse material

To extrapolate the findings in cell lines to mice, PS2 knock-out mice as well as wt mice have been studied (**Paper III**). For cell localization studies, hippocampal primary cultures from wt mouse embryos have been used (**Paper IV**). The mouse brain material and the primary mouse hippocampal neurons have been approved by the Animal Trial Committee of Southern Stockholm (S30-09, and S165-11).

3.1.3 cDNA constructs

Many different cDNA constructs have been generated and used throughout this thesis project. The constructs include single or multiple amino acid replacements in Nct (**Paper I**) and Aph-1 (**Paper II**), as well as the PS1 wt and truncation of PS1wt (caspCTF) together with the substrate C99 (**Paper I**). These were all generated with wt cDNA as a template using the Quikchange Multi Site-directed mutagenesis (**Paper I**), the QuikChange Site-Directed Mutagenesis (**Paper IV**), or by combining PCR with cloning using restriction enzymes (**Paper IV**).

For **Paper I**, a Flp-In cell line was generated in Nct deficient cells. The Flp-in system is based on a Flp recombination target site cell vector (pFRT/lacZeo) that is transfected into the cells. After selection with the antibiotic Zeocin, clones were picked and the complete genome was analyzed by southern blotting to identify the amount of FRT sites that have been integrated. The clone with the minimum amount of integration sites was chosen for further use. When transfecting different Nct constructs situated in a pcDNA5FRT/TO vector, these vectors will be integrated into the FRT sites, making sure that the constructs will be integrated at the same location and expressed in equal amounts in all cell lines. The drawback with the Flp-In system is that it is time consuming and not always gives equal amounts of protein expression. All cDNA constructs were sequenced using the BigDye® Terminator Version 3.1 Cycle Sequencing kit (Applied Biosystems) for verification.

3.2 ANALYSIS OF γ-SECRETASE COMPLEXES AND THEIR COMPONENTS

3.2.1 Protein detection by Western immunoblotting and immunohistochemistry

Most of the methods used in this thesis include detection by immunoblotting using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). The samples were first treated with sample buffer (containing SDS and β-mercaptoethanol), sometimes followed by a heat step, to denature the proteins and make them negatively charged. The samples were then loaded on the gel based on protein concentration or transfection efficiency. The proteins were separated in the gel according to size and transferred to a nitrocellulose or a PVDF membrane for immunoblotting. By probing proteins of interest with specific antibodies, the proteins can be detected with chemoluminescence by either X-ray film or CCD camera. When quantification was required, images taken by the CCD camera was preferred because its software easily detects possible saturation of the chemoluminescent signal. Protein

expression levels can also be detected qualitatively by immunohistochemistry (**Paper IV**). This method visualizes regional differences of the protein expression within the cell.

3.2.2 Cycloheximide and Staurosporine treatment

Mutations (**Paper I**) and truncation of proteins (**Paper II**) can affect the stability and the half time of the proteins. In these studies, we investigated this by treating cells with cycloheximide, which blocks the translation of mRNA into protein and thereby inhibits protein synthesis. The degradation of the altered proteins was then compared to the corresponding wt proteins. To investigate events occurring during cell apoptosis, such as chromatin condensation (**Paper II**), cells were treated with staurosporine (STS). STS inhibits different protein kinases and activates caspases and is therefore often used to induce apoptosis.

3.2.3 Co-immunoprecipitation and affinity pulldown/GCB

Co-immunoprecipitation is a way to analyze protein-protein interactions between γ-secretase components (**Paper I, II, IV**). This method can reveal if the lack of activity for mutants are due to an impairment of protein interactions. For example, in **Paper I**, the C4 mutant lacked γ-secretase activity because it could no longer interact with PS and Pen-2. Although the co-immunoprecipitation method displays protein-protein interactions, the detected proteins can not only be parts of active complexes but also be localized in subcomplexes or inactive complexes.

One way to detect proteins that are only situated within active complexes is to use an affinity pulldown assay called GCB (**Paper I-IV**). This method resembles the co-immunoprecipitation method but requires an additional preparation step to amplify the material by generating membrane fractions. These samples are incubated with the γ-secretase transient analogue (inhibitor) L-685,458 coupled to a long hydrophilic linker with a cleavable biotin group (GCB) and then pulled down with streptavidin beads.

3.3 MONITORING γ-SECRETASE ACTIVITY

3.3.1 Detection of Aβ

In this thesis, Aβ production was analyzed by Meso Scale Discovery (MSD) technology (**Paper I**), Enzyme-linked immunosorbent assay (ELISA) (**Paper II-IV**) and mass spectrometry (**Paper II**). Both ELISA and MSD are based on protein standard curves to estimate the concentration of the analyte and uses antibodies to quantify molecules in solution. The drawback with the use of antibodies for Aβ quantification is that Aβ easily forms aggregates that can hide epitopes from the detection antibody and thereby give incorrect results. Although ELISA and MSD are both based on antibodies, they have different detection systems. The ELISA can be measured by absorbance or fluorescence, while the MSD technology is based on electrochemiluminescence. Some of the MSD's advantages are its minimal background signal

and the high signal-to-noise ratios. In addition, MSD MULTI-SPOT plates can simultaneously measure several molecules in the same well under the same conditions. This also decreases the sample volume required. While the MSD technology has many advantages, the major drawback is that it requires an expensive instrument for the read-out. ELISA is in comparison an uncomplicated well-established assay that only requires an ordinary absorbance or fluorescent plate-reader.

3.3.2 Detection of intracellular domains

Intracellular domains (ICDs) have been investigated using a Luciferase-Reporter Gene Assay (**Paper I, II, and IV**) and a cell membrane based activity assay (**Paper II and III**). The Luciferase-Reporter Gene Assay monitors AICD or NICD formation from a modified ectodomain-shedded APP (C99) or Notch (NotchΔE) constructs containing an incorporated Gal4/VP16 (GVP) domain. When γ-secretase cleaves these modified substrates, the ICDs are translocated into the nucleus where the GVP region binds to the UAS promotor upstream of the luciferase-reporter gene and thereby activates the transcription of the Luciferase enzyme. By adding the luciferase substrate Luciferin and ATP, the activity is then measured by luminescence. This method is very sensitive, shows a high specificity, and makes it possible to study APP and Notch processing under same conditions in similar setups. The method however, requires several cDNA constructs to be transfected and requires normalization against transfection efficiency. The many cDNA constructs may have an impact on the cells. Therefore the samples are always compared to the activity generated by the wt protein undergoing the same procedure and the results imply the changes between them. To control the results from this method, SDS-PAGE was performed on the same cell lysates based on the transfection efficiency (**Paper I and II**).

For the detection of endogenous ICDs, a cell membrane based activity assay was used (**Paper II and III**). This method also enables the detection of ICDs from other γ-substrates such as N-cadherin and ephrinB. Since endogenous levels were monitored, we isolated membrane fractions in order to increase the starting material. These are then solubilized and incubated in 37°C for 14-16 hours with or without the inhibitor L685,458, followed by detection of the ICDs with immunoblotting. This method better reflects the physiological process within the cells, but the quantification is not as sensitive as the luciferase-reporter gene assay and more material is required.

4 RESULTS AND DISCUSSION

In this section, the main findings from **Paper I-IV** are summarized and discussed. Figures and more details concerning the results are found within respective paper.

4.1 MUTATIONS IN NICASTRIN DIFFERENTIALLY AFFECT APP AND NOTCH PROCESSING

Most research about the γ-secretase complexes has been focused on the PS component. This is partly because it was the first component to be discovered, but mostly because it contains the catalytic site of the enzyme. In addition, all FAD mutations linked to the γ-secretase complex are located within PS, which highlights its importance for the complex. However, PS still requires the other components to form an active complex (Edbauer et al., 2003) and the functions of these components are less clear.

The largest component in the complex is Nct. In contrast to the other components, the main part of Nct is located in the lumenal/extracellular space. An alignment between human, mouse, *Drosophila*, and *C. elegans* revealed a conserved DYIGS motif and four evenly spaced cysteines located in the ectodomain of Nct (Yu et al., 2000). The DYIGS motif was shown to be included in the larger DAP domain that resembles domains found in the superfamily of aminopeptidases and transferrin receptors (Fagan et al., 2001). Nct, like the transferring receptors, lacks residues required for the catalytic activity of the aminopeptidases. Despite this, structure similarities imply that the DAP domain of Nct can bind peptides or proteins. In fact, a direct interaction between the glutamate residue 333 (E333) located within the DAP domain and ectodomain shedded substrates has been found, suggesting Nct to act as a substrate receptor docking the substrates into the catalytic site of PS (Dries et al., 2009; Shah et al., 2005). However, this receptor-like role of Nct has been challenged by a study suggesting that E333 is instead important for the maturation and the assembly of the complex.

To further explore the role of Nct, we mutated the four evenly spaced conserved cysteine residues to serines, C195S (C1), C213S (C2), C230S (C3), and C248S (C4), and monitored their effect on APP and Notch processing (**Paper I**). We, like others, found that C4 lacked activity for both substrates due to impaired complex assembly (Shirotani et al., 2004b). Hence, the cysteine at position 248 in Nct is required for essential interactions within the complex or for proper protein folding. We also found that C2 and C3 differently affected APP and Notch processing at the ε/S3-site by reducing the level of AICD while maintaining the level of produced NICD. When we investigated the processing at the γ-site by measuring extracellular Aβ40 levels, a similar trend as for the processing at the ε-site was observed. Based on the hypothesis of the step-wise processing by the γ-secretase complex that generates the Aβ49→Aβ46→Aβ43→Aβ40 or the Aβ48→Aβ45→Aβ42→Aβ38 product lines, we therefore suggest that the mutations have a decreased production of Aβ due to interference at the ε-site. However, since Aβ42 levels were too low for detection, we cannot fully clarify the exact mechanism behind these mutations.

Another feature we found was that C2 and C3 generated less of the mature form of Nct compared to wt Nct. This might imply that the processing of APP, in comparison to Notch, is more dependent on the mature form of Nct. In addition, C3 formed very few active complexes. Despite this, the low level of active complexes was sufficient to cleave Notch but not APP. One possible explanation to this result might be that C3 changes the conformation of Nct in a way so that also immature Nct can integrate into active complexes. These complexes might then favor Notch processing or become localized to subcellular compartments where Notch is primary processed. To conclude, this is to our knowledge the first study showing that single residues in a γ-secretase component except PS, differently affect APP and Notch processing. These results suggest that Nct is not just a stabilizing factor. However, it is still unclear whether Nct is directly involved in substrate recognition or if it has an indirect role such as gating the substrates into the catalytic site.

4.2 CASPASE CLEAVAGE OF PS1 INCREASES THE INTRACELLULAR Aβ42/Aβ40 RATIO

Caspase activation is linked to the synaptic and neuronal loss observed in the AD brain and seems to occur before the appearance of plaques and tangles (de Calignon et al., 2010; Hyman, 2011; Uribe et al., 2012). Caspase activation has also been associated with an increased Aβ production (Albrecht et al., 2009; Cicconi et al., 2007; Galli et al., 1998; LeBlanc, 1995; Xie et al., 2007) but the mechanism behind this is not fully understood.

Caspases cleave the γ-secretase components PS1 (Kim et al., 1997; Loetscher et al., 1997) within the hydrophilic loop generating a truncated form of CTF (caspCTF) (van de Craen et al., 1999). The PS1 caspCTF has been shown to displace PS1 wtCTF in the γ-secretase complex during apoptosis with remained activity (Hansson et al., 2006). In **Paper II**, we wanted to investigate how these caspase-cleaved γ-secretase complexes affected the processing of APP and Notch. γ-secretase complexes containing PS1 caspCTF were indeed active and produced AICD and NICD levels comparable to the complexes containing PS1 wtCTF. In addition, we identified a shift at the γ-site that resulted in an increased intracellular Aβ42/Aβ40 ratio. Therefore, we suggest that γ-secretase complexes containing PS1 caspCTF contribute to the increased Aβ42 production during apoptosis. The interest of intracellular Aβ and its possible biological function has grown during the last years. Several publications report observations of intraneuronal Aβ accumulation, especially of the Aβ42 species, in animal models and brains of AD and Down's syndrome patients (Gouras et al., 2010; Hashimoto et al., 2010). These observations suggest that intraneuronal Aβ accumulation is an early event in AD that precedes both NFTs and plaque formation.

4.3 THE PROCESSING OF THE APP, NOTCH, N-CADHERIN, AND EPHRINB, ARE MORE DEPENDENT ON PS1 THAN PS2

PS1 and PS2 are suggested to play distinct roles since PS1 knock-out mice die during embryogenesis (De Strooper et al., 1998), while PS2 knock-out mice are viable (Herreman et al., 1999). In addition, PS1- or PS2-containing γ-secretase complexes also show differences in APP processing and sensitivity to some γ-secretase inhibitors (Lai et al., 2003; Zhao et al., 2008; Borgegard et al, in press). To investigate whether PS1 and PS2 show differences in substrate specificity, we used PS1 and PS2 deficient cells and compared the catalytic activity of PS1 and PS2 on the neuronal substrates APP, Notch, N-cadherin, and ephrinB (**Paper III**). We observed that while PS1-containing γ-secretase complexes could process all substrates, PS2-containing complexes could only process APP and to a very low extent also Notch. This shows a clear difference in substrate specificity between PS1 and PS2. The lack of N-cadherin processing by PS2-containing complexes can be explained by the fact that the Catenin protein p120 (p120ctn) bridges cadherins to PS1-CTF by binding to the sequence 330-360 of PS1. This sequence is not present in PS2 (Kouchi et al., 2009) and N-cadherin might therefore only be processed by PS1-containing complexes. The ligand ephrinB was, like N-Cadherin, also shown to only be processed by PS1-containing complexes. If the processing of ephrinB occurs through a similar mechanism as for N-cadherin is, however, unknown.

In this study, we also observed that less PS2 was found in active γ-secretase complexes. However, this was found in fibroblast cells (MEFs), and it is possible that different cell types can contain various amounts of PS1- and PS2-containing complexes. For instance, it has been reported that most of the γ-secretase complexes in microglia contain PS2 (Jayadev et al., 2010). Another factor that can regulate the presence of different PS-containing complexes is age. When comparing active PS1- and PS2-containing γ-secretase complexes in embryonic vs. adult rat brain, the level of PS2-containing complexes was much higher than PS1-containing complexes in adult rat brain (Franberg et al., 2010). The low amount of active PS2-containing γ-secretase complexes during development in combination with the low ability to process Notch can therefore be the reason why PS1 deficient mice exhibit a perinatal lethality. Although, it is possible that the lethality in the PS1 deficient mice is due to impaired processing of substrates only processed by PS1-containing complexes, it is more likely that the lethality is caused by impaired Notch signaling since the mice resembles the phenotype of Notch deficient mice.

Of the four neuronal substrates we investigated, only APP and to a minor extent Notch were processed in PS1 deficient cells. The difference in processing efficacy between the PS proteins appears to be less pronounced for APP than for the other substrates. This suggests that PS2 complexes have a preference for APP as a substrate, at least in fibroblasts. The experiments performed on brain tissue from the PS2 deficient mice indicated that the ability for PS1 to process the substrates in the brain were the same as in the fibroblasts. However, due to the perinatal lethality of PS1 deficient mice, we can only speculate that PS2 shows the same preference in the brain as in the fibroblast. If the amount of active PS2-containing complexes in the human brain increases with age, as shown in rat brains (Franberg et al., 2010), inhibiting PS2-containing complexes could be sufficient to lower Aβ production without generating severe side effects. However, a PS2-sparing γ-secretase inhibitor has recently been found to

be well tolerated in wt mice, suggesting that the inhibition of PS1-containing complexes can also be a therapeutic strategy (Borgegard et al, in press).

4.4 THE APH-1 PROTEINS SHOW DIFFERENT PREFERENCE OF FORMING EITHER PS1- OR PS2-CONTAINING COMPLEXES

Similar to the PS proteins, the different forms of Aph-1 can all be incorporated into active γ-secretase complexes and are not found within the same complex. In addition, Aph-1a knock-out mice die during embryogenesis due to impaired Notch signaling, while Aph-1bc knock-out mice are viable and show a significant reduction of Aβ production (Ma et al., 2005; Serneels et al., 2009). These findings indicate that complexes containing different forms of Aph-1 can have distinct substrate specificity. The Aph-1 proteins also seem to differently affect the processing of APP at the ε- and γ-site. While the AICD production is similar between the proteins, Aph-1b-containing γ-secretase complexes have shown to produce longer Aβ peptides than Aph-1a-containing γ-secretase complexes (Serneels et al., 2009).

An alignment between the protein sequences of Aph-1a and Aph-1b identified the loops and the C-terminus as the regions with lowest homology (**Paper IV**). By generating chimeras, where these regions have been replaced in Aph-1aL with the corresponding residues of Aph-1b, our aim was to investigate if these regions contributed to changes in substrate processing. We could not observe any difference in AICD and NICD production, which was expected based on previous findings (Serneels et al., 2009). However, we suspected to see a difference in the extracellular Aβ42/Aβ40 ratio, since Aph-1b-containing complexes have been shown to produce longer Aβ-peptides (Serneels et al., 2009). Although, Aph-1b displayed a slightly higher Aβ42/Aβ40 ratio, the increase was not significant. The previous finding of longer Aβ-peptides was identified using microsomes. This will include both intracellular and extracellular production of Aβ. Therefore, it is possible that the difference observed by the earlier study reflected the intracellular Aβ production. Unfortunately, in our setup we could not monitor the intracellular Aβ values, so in order to test this hypothesis, the conditions need to be further optimized.

When we investigated the expression of the Aph-1 proteins and their ability to form active complexes, we observed different expression patterns and preferences for interactions with PS1 and PS2. Aph-1a rather formed PS1-containing complexes, while Aph-1b preferred PS2-containing complexes. When comparing chimeras with the wt proteins, the replacement of loop 2 in Aph-1a did not change the preference for PS1-containing complexes. The replacement of loop 6 in Aph-1a resulted in a reduction of active complexes and equal amounts of PS1- and PS2-containing complexes. The replacement of the C-terminal in Aph-1a resembled Aph-1a wt, but since the C-terminal chimera displayed a lower level of Aph-1 expression, additional experiments with a higher expression of the chimera are required to better understand its properties. Nevertheless, the finding of different preferences for PS-containing complexes supports the suggested existence of various γ-secretase complexes and their ability to exhibit different effects on substrate processing.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

During the last decades, a major effort has been devoted to understand the biological mechanisms behind AD and to find a cure for the disease. The treatments available today can only alleviate the symptoms for patients with AD, not cure them or slow down the disease progression. To date, there are two types of symptomatic drugs on the market; cholinesterase inhibitors and the N-methyl-D-aspartic acid (NMDA)-receptor antagonist Memantine. Cholinesterase inhibitors are used for treating mild to moderate AD while Memantine is prescripted to treat moderate to severe AD. These treatments interfere with the cholinergic and glutaminergic neurotransmitter systems in the brain; systems that are especially vulnerable and disrupted in AD (Lukiw, 2012; Molinuevo et al., 2005).

Research groups all over the world are trying to find new medical drugs that via various strategies can treat AD. The advantage with the current research field is that it covers numerous of different treatment strategies, which enhance the chance of developing better treatments in a near future. Strategies that are focused on targeting the Aβ production include the elimination of Aβ aggregations and improving the clearance of Aβ by for example vaccination methods. In addition, there is also a large focus on reducing or modulating the Aβ production by the use of BACE-1 inhibitors or modulators and inhibitors of the γ-secretase complex (Reitz, 2012).

In order to find a drug that can influence the generation of Aβ, it is beneficial to understand as much as possible about the mechanism underlying Aβ production. Since the many substrates of the γ-secretase complex prohibit the development of a drug that inhibits the complex and thereby the production of all its substrates, we need to increase our knowledge about the γ-secretase complex. The work of this thesis has therefore been to explore the properties of the γ-secretase components by focusing on their intrinsic function and substrate selectivity. The long-lasting aim beyond this thesis is to find ways of inhibiting or modulating APP processing via the γ-secretase complex without affecting other important signaling pathways.

The many different compositions of the γ-secretase complex open up the possibility of inhibiting a certain type of the complex without causing severe side effects. For this purpose, it is important to characterize the different γ-secretase complexes and investigate their effect on several substrates. It has been suggested that Aph-1b-containing γ-secretase complexes could be good targets for reducing the production of the longer and more toxic Aβ peptides. Although, we could not detect a significant difference in the secreted Aβ42/Aβ40 ratio between the Aph-1a- and Aph-1b-containing complexes, it is possible that the intracellular Aβ42/Aβ40 ratio or the proportion of Aph-1a- and Aph-1b-containing complexes within the brain can be relevant. This highlights the importance of investigating the proportion of different γ-secretase complexes in the human brain, especially at an old age.

We should also not rule out PS1- or PS2-containing complexes as targets for limiting the Aβ production. Although PS2 only had a limited contribution to the γ-secretase activity in MEFs and adult mouse brain, PS2-containing complexes seemed to process fewer substrates compared to PS1-containing complexes and would thereby generate less side effects if they

were inhibited. On the other hand, wt mice treated with a PS2-sparing γ-secretase inhibitor reduced the Aβ production without exhibiting any Notch-related toxicity (Borgegard et al, in press). Hence, PS1-containing complexes can also be promising targets since most reported side effects from clinical trials using γ-secretase inhibitors have been Notch-related.

Besides targeting distinct γ-secretase complexes, inhibiting GSAPs might be an effective target. It is interesting that different GSAPs, like p120ctn (mentioned in "Results and Discussion"), can link the substrate to the γ-secretase complex by binding to a certain part of the complex. These binding sequences within the γ-secretase complex are probably located in the loops of the components, since the loops are the sequences that differs the most between Aph-1 and PS isoforms. This might also imply that Nct with its large ectodomain might affect the recognition and recruitment of several substrates.

Last but not least, there is a large potential of γ-secretase modulators that change the complex cleavage properties at the γ-site but not at the ε-site. Since many of the signal molecule substrates seem to be more dependent on the ICD formation rather than generating Aβ-like peptides, this might become a future treatment for AD.

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