Studies on the γ-Secretase Complex and Processing of the Alzheimer's Disease-associated Amyloid Precursor Protein

Anna Bergman

Karolinska Institutet

Neurotec

Stockholm 2004

ABSTRACT

Alzheimer's disease (AD) is a devastating neurodegenerative disorder that causes the most common form of dementia. Pathological lesions, such as plaques consisting of the amyloid β-peptide (Aβ), are found in the brains of AD patients. Aβ is produced by sequential cleavages of the amyloid precursor protein (APP) by β- and γ-secretase. Concomitant with γ-cleavage, another cleavage, termed ε-cleavage, occurs seven to nine residues C-terminal of the γ-site, releasing the APP intracellular domain (AICD). The γ- and ε-cleavages critically require the γ-secretase complex consisting of four well-conserved proteins, namely presenilin (PS), nicastrin, Aph-1 and Pen-2. Interestingly, this processing occurs within the anhydrous environment of the lipid membrane bilayer where PS is proposed to provide the catalytic core of the complex, acting as an aspartyl protease.

The work presented in this thesis describes the intramembrane processing of APP and how disease-causing familial AD mutations in the APP protein affect ε-cleavage, and thereby generation of AICD. Also, the intricate biogenesis and assembly of the γ-secretase complex was investigated by detailed studies of the Pen-2 and PS1 proteins.

In addition to cleavage of APP, PS is required for the processing of a number of type I membrane proteins, such as the Notch receptors. γ-Secretase provides a potential therapeutic target for AD. However, inhibition of γ-secretase can possibly lead to unwanted side effects due to impaired signaling of other PS substrates. In **paper I**, a novel γ-secretase reporter assay was developed. The assay specifically and quantitatively records total γ-cleavage occurring in intact cells, enabling detailed studies of the intramembrane processing of APP. In addition, the reporter assay can be used for screening compound libraries for drugs that differentially affect γ-secretase processing of APP and other PS substrates such as Notch. Generation of AICD was characterized in **paper II** by using the reporter assay developed in paper I. Formation of AICD was found to occur in a compartment downstream of the endoplasmic reticulum (ER) in the secretory pathway, thus overlapping with the reported site of production of Aβ. Furthermore, familial AD mutations showed unchanged levels of AICD generation. Thus, the disease-causing consequences of these APP mutations are unlikely to be mediated by the amount of AICD fragment produced.

Understanding of the γ-secretase complex is essential for unraveling the pathogenic mechanism(s) leading to AD. Pen-2, the smallest protein in the γ-secretase complex, was studied in depth in **paper III**. Presence or absence of PS had a great impact on cellular levels and distribution of Pen-2. In PS null cells, Pen-2 was destabilized and restricted to the ER, compared with cells expressing PS1, where Pen-2 levels were stable and the Pen-2 protein was trafficked further in the secretory pathway. Destabilization of Pen-2 in PS null cells was mediated by ubiquitylation and proteasomal degradation. In the absence of PS, the Pen-2 protein appeared to be retrotranslocated out of the ER into the cytosol prior to ubiquitylation and degradation. These observations suggest an ER-associated proteasomal degradation pathway mediating regulation of protein levels and trafficking of Pen-2, and possibly other components, not incorporated into the γ-secretase complex. Analysis of the γ-secretase complex was continued in **paper IV** and focused on the C-terminal domain of PS1. Truncations of seven to seventeen residues in the PS1 C terminus resulted in PS1 molecules deficient in supporting γ-secretase activity, with accompanying impairment in γ-secretase complex formation and endoproteolysis of the PS1 molecule. However, intramolecular PS1 heterodimer formation was shown to occur for C-terminally truncated molecules that were unable to associate with nicastrin and Aph-1. On a PS null background the C-terminal fragment of PS1 was by itself able to interact with Aph-1 and nicastrin, thus suggesting an additional function for Aph-1 and nicastrin apart from stabilizing the full-length PS1 molecule.

Although the main components of the γ-secretase complex are known, the molecular mechanisms underlying the inter-regulation, assembly and actual stochiometry of the complex are less well understood. The studies presented here provide insights into intramembrane processing of APP and detailed information about the γ-secretase complex components Pen-2 and PS1, thereby providing a framework for future studies of the intricate regulation of the γ-secretase complex and its proteolytic function in terms of APP processing.

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications, which are referred to in the text by their roman numerals.

I A sensitive and quantitative assay for measuring cleavage of presenilin substrates

Helena Karlström, **Anna Bergman**, Urban Lendahl, Jan Näslund and Johan Lundkvist

The Journal of Biological Chemistry (2002) **277**, 6763-6766

II APP intracellular domain formation and unaltered signaling in the presence of familial Alzheimer's disease mutations

Anna Bergman, Dorota Religa, Helena Karlström, Hanna Laudon, Bengt Winblad, Lars Lannfelt, Johan Lundkvist and Jan Näslund

Experimental Cell Research (2003) **287**, 1-9

III Pen-2 is sequestered in the endoplasmic reticulum and subjected to ubiquitylation and proteasome-mediated degradation in the absence of presenilin

Anna Bergman*, Emil Hansson*, Sharon E. Pursglove, Mark R. Farmery, Lars Lannfelt, Urban Lendahl, Johan Lundkvist and Jan Näslund

* These authors contributed equally

The Journal of Biological Chemistry (January 14, 2004)
10.1074/jbc.M313999200

IV The extreme C terminus of presenilin 1 is essential for γ-secretase complex assembly and activity

Anna Bergman, Hanna Laudon, Bengt Winblad, Johan Lundkvist and Jan Näslund

Manuscript

TABLE OF CONTENTS

ABBREVIATIONS

Aβ	amyloid β-peptide
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease
AICD	APP intracellular domain
Aph-1	Anterior pharynx defective-1
APLP	APP like protein
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BACE	β-site APP cleaving enzyme
CERAD	Consortium to establish a registry of Alzheimer's disease
CTF	C-terminal fragment
DSM IV	Diagnostic and statistical manual of mental disorders fourth edition
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
GVP	Gal4 VP16
HES	Hairy/Enhancer of split
ICD-10	International classification of disease, 10^{th} revision
KPI	Kunitz-type protease inhibitor
NICD	Notch intracellular domain
NINCDS-ADRDA	National institute of neurological and communicative disorders and stroke and Alzheimer's disease and related disorders association
NTF	N-terminal fragment
Pen-2	Presenilin enhancer-2
PS	Presenilin
RIP	Regulated intramembrane processing
TACE	Tumor necrosis factor-α converting enzyme
TMD	Transmembrane domain

INTRODUCTION

Background

Alzheimer's disease (AD) is the most common form of dementia, affecting approximately 5% of the population over the age of 65 years in Europe (Lobo et al., 2000). The Bavarian neuropathologist and psychiatrist Alois Alzheimer first described this neurodegenerative disease in a publication in 1907 (Alzheimer, 1907). Working at a clinic in Munich, he encountered and described the case of a woman who suffered from a rapidly progressing dementia and died at the age of 51. In the histopathological examination of the diseased woman's brain Alois Alzheimer described thick fibrils and foci with special dye characteristics in the cerebral cortex. These structures are the two neuropathological hallmarks of AD: plaques composed of the amyloid β-peptide (Aβ) and neurofibrillary tangles consisting of the microtubule-binding protein tau.

Symptoms

Short-term memory impairment, disorientation, aphasia, and a general cognitive decline are common symptoms early in disease development. The symptoms reflect, to some extent, the brain regions that are affected in the disease (Haroutunian et al., 1998). As the disease progresses, spatial and motor abilities are affected and the patient becomes bedridden and completely dependent of the caretaker. The disease lasts 5-15 years, and the cause of death is generally a secondary illness such as pneumonia, or other infections.

Diagnosis

Clinical diagnosis

To clinically diagnose AD several investigations are performed such as neuropsychological tests, physical examination, evaluation of the patient's medical history and tests to exclude other diseases. Diagnostic criteria have been developed to help make the clinical diagnosis. The National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) have developed a set of criteria for diagnosis. The diagnosis is divided into possible, probable and definite AD according to these criteria (McKhann et al., 1984). In probable AD the patient has a disease history typical of AD, specific findings in mental and physical examinations, and other types of dementia have been excluded. Possible AD is diagnosed when there are atypical features in the clinical course, or when another brain or systemic disorder is present, but not considered to be the cause of dementia. Definitive AD can only be used as diagnosis after a post mortem examination has been performed. In the examination specific neuropathological criteria have to be confirmed together with a clinical diagnosis of the disease (Khachaturian, 1985; Mirra et al., 1991). The ICD-10 (International Classification of Disease, 10th revision) (WHO, 1992) and DSM IV (Diagnostic and Statistical Manual of Mental Disorders fourth edition) (American Psychiatric Association, 1994) are other sets of internationally accepted criteria for clinical diagnosis of AD.

The accuracy of clinical diagnosis has been much improved over recent years by technical achievements that enable visualization of the brain, such as computed tomography, magnetic resonance imaging, positron emission tomography and single photon emission computed tomography. In nine out of ten cases, the correct diagnosis of AD can be made at specialist centers, when comparing clinical diagnosis with post mortem examination (Klatka et al., 1996).

Neuropathological diagnosis

Brains from AD patients are atrophic with accompanying widening of sulci, smaller gyri and larger ventricles. The first brain regions to be affected in the disease are the hippocampus and entorhinal cortex. As the disease progresses more regions are affected including the temporal and parietal lobes (Braak and Braak, 1991). At the microscopic level, extracellular amyloid plaques and intracellular neurofibrillary tangle lesions are found. Mature amyloid plaques, so-called neuritic plaques, consist of Aβ, with its longer 42-residue form in the core and with Aβ40 and Aβ42 surrounding the central part of the plaque (Iwatsubo et al., 1994). Plaques can be stained by the histological amyloid dyes Congo Red and Thioflavin S or T. Surrounding the fibrillar amyloid are activated microglia and reactive astrocytes, which cause an inflammatory reaction around the plaques by releasing complement factors and cytokines (McGeer and McGeer, 2001). Dystrophic neurites are present close to the plaques, indicating that a neurodegenerative process is taking place. The neurofibrillary tangles occur within neurons and consist of paired helical filaments of hyperphosphorylated tau that aggregate within the cytoplasm. Tangle formation is not exclusive for AD, but is also a common feature in Parkinson's disease, frontotemporal lobe dementia, and other dementias (Lee et al., 2001).

There are a number of different sets of criteria for the neuropathological establishment of AD such as the Consortium to Establish a Registry of Alzheimer's Disease (CERAD) criteria, Tierny criteria and Khachaturian's criteria (Mirra et al., 1991; Tierney et al., 1988; Khachaturian, 1985). These criteria include the presence of amyloid plaques in specific regions of the brain. For the Tierny criteria, an accompanying distribution of neurofibrillary tangles is required. Fulfillment of the neuropathological criteria alone is not sufficient for the diagnosis of AD. There has to be a clinical diagnosis supporting the neuropathological findings and ruling out other diseases or dementias.

Epidemiology and risk factors

The prevalence of AD is about 1-2% at the age of 65, but it can vary depending on the population (Fratiglioni et al., 2000). With increasing age the prevalence rises, and after 65 years of age it doubles in every five years (Jorm et al., 1987). AD is the most common form of dementia and accounts for around 50% of all dementia cases (Lobo et al., 2000).

Many risk factors for developing AD have been proposed. They have often been controversial and a matter of debate, but old age, a family history of dementia, hypertension, head trauma, and female gender, are considered well-established risk factors (Munoz and Feldman, 2000).

Genetics in AD

Genetics has provided powerful insights into the underlying mechanisms of AD. Through genetics, genes and proteins involved in the disease have been identified. This knowledge has lead to an increased understanding about the molecular events that give rise to Aβ release and pathogenesis of the disease.

A subset of AD cases is inherited in an autosomal dominant pattern indicating linkage to specific genes. As early as the 1960's there was an indication that chromosome 21 plays an important role in AD. Olson and co-workers made the interesting observation that patients suffering from Down's syndrome, which is caused by trisomy of chromosome 21, displayed AD-like symptoms and pathology at an early age (Olson and Shaw, 1969). The next set of information making AD researchers even more interested in chromosome 21 was the identification of a partial amino acid sequence of Aβ in protein purification from a Down's syndrome brain (Glenner and Wong, 1984). Using this sequence, the gene encoding the amyloid precursor protein (APP), located on chromosome 21, was cloned (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987).

Familial AD

The literature is rich in reports describing mutations causing familial AD, with approximately 150 disease-causing mutations identified as of today (http://molgen-www.uia.ac.be/AD Mutations/). Individuals suffering from familial AD often develop dementia at 40-60 years of age, and display an aggressive disease progression. All AD-causing mutations discovered so far are concentrated to three genes, which all are directly involved in the generation of Aβ and are described below. The first identified missense mutation causing familial AD was localized to the APP gene (Goate et al., 1991), from which Aβ is excised. Subsequent studies have increased the number of identified AD-causing mutations in the APP gene, and presently thirteen mutations have been reported.

The vast majority of all known familial AD mutations have been found in the gene encoding presenilin (PS) 1, accounting for over 130 mutations. PS1 was identified by genetic linkage to chromosome 14 for an autosomal dominant form of hereditary early-onset AD (Schellenberg et al., 1992; Sherrington et al., 1995). The name of the gene is taken from its role in familial *pre senile* dementia. The identification of many mutations in PS1 suggested that the protein plays a central role in the pathological events leading to AD, and its function will be discussed in detail in the section *Presenilin protein function*. A protein homologous to PS1, namely PS2, was found on chromosome 1 by homology searches of databases for amino acid sequences with high similarity to PS1 (Levy-Lahad et al., 1995). In contrast to PS1, very few mutations leading to AD have been found in the PS2 gene. Families reported with mutations in the PS2 gene have a later age of onset than PS1 mutation carriers, ranging from mid-forties to late seventies (Renbaum and Levy-Lahad, 1998). The later onset of the disease could be a possible explanation for the low number of mutations identified in PS2 compared to PS1. The clinical and neuropathological manifestation of PS1 and PS2 mutations is typical AD, with rare reports of spastic paraparesis, epileptic seizures and cerebral amyloid angiopathy for a subset of the cases (Kwok et al., 1997; Houlden et al., 2000; Renbaum and Levy-Lahad, 1998). A few mutations in PS1 present a pathological phenotype with so-called cotton-wool plaques that are large and lack a distinct core (Houlden et al., 2000), compared to neuritic plaques that are smaller and have a dense core.

Genetic risk factors

In 1993 Apolipoprotein E (ApoE) was identified as a susceptibility gene for AD (Corder et al., 1993). ApoE plays a central role in lipoprotein metabolism and cholesterol homeostasis. ApoE has three different isoforms, E2, E3 and E4 encoded by the three alleles ε2, ε3 and ε4, with the ε4 allele being a risk factor for AD. The effect for carriers of the ε4 allele is an increased probability for getting the disease, but it is not a prerequisite for developing the disease. Studies from knockout and transgenic mice suggest that ApoE, and in particular the E4 isoform, promotes Aβ deposition and aggregation (Bales et al., 1997; Holtzman et al., 2000). Several other genes have been reported to show association to AD, for example α-2 macroglobulin, α1-antichymotrypsin, angiotensin converting enzyme and the very low-density lipoprotein receptor (Blacker et al., 1998; Hinds et al., 1994; Alvarez et al., 1999; Okuizumi et al., 1995). However, the ApoE allele ε4 is the most widely reported genetic influence for AD, and it shows the strongest correlation to the disease among the susceptibility genes reported today (Rocchi et al., 2003).

Amyloid cascade hypothesis

The amyloid cascade hypothesis is currently the most favored model explaining the pathogenic events causing AD (Fig. 1). The hypothesis was first presented in 1991 (Selkoe, 1991; Hardy and Higgins, 1992), and over time it has been subjected to some modifications. However, the basic mechanisms described by the hypothesis remain the same, and give a reasonable sequence of events that could explain the development of dementia in AD. The amyloid cascade hypothesis states that the deposition of Aβ is causative for the disease, and that tangles, inflammatory response, cell loss, vascular damage and dementia follow as a direct result of this deposition. The hypothesis is supported by several findings. First, individuals with Down's syndrome are carriers of an additional copy of the APP gene and therefore generate more Aβ. Early in life, Down's syndrome patients develop AD-like dementia and neuropathology (Olson and Shaw, 1969). Second, mutations causing hereditary AD in the APP or PS genes increase the production of the longer and more amyloidogenic Aβ42 form of the Aβ peptide (St George-Hyslop, 2000). Third, the levels of deposited Aβ correlate with cognitive decline and severity of the disease in AD patients as well as in transgenic animals (Näslund et al., 2000; Hsiao et al., 1996; Gordon et al., 2001). Fourth, the Aβ peptide *per se* is neurotoxic (Yankner et al., 1990). Fifth, transgenic mice expressing familial AD mutant APP and that are knocked out for β-secretase, an enzyme directly involved in Aβ formation, show rescue of memory impairments found in the parental strain with an intact β-secretase gene (Ohno et al., 2004). This indicates that overexpression of APP alone does not cause memory deficits, and that the production of Aβ and/or APP C-terminal fragments are required for the manifestation of reduced memory function in the mice. Sixth, tangle formation seem intimately linked to Aβ since tau deposition is increased in transgenic mice expressing human mutant tau together with mutant APP, as compared to mice expressing mutant tau alone (Lewis et al., 2001). Moreover, injection of fibrillar Aβ42 enhances the tangle formation in the tau transgenic mice (Götz et al., 2001). Recently, attention has been focused on in which form (fibrils, protofibrils, oligomers or monomers) the amyloid species has its most deleterious effects. Several reports point to the oligomeric and protofibrillar state as being the most toxic form of Aβ (Lambert et al., 1998; Walsh et al., 2002; Dahlgren et al., 2002).

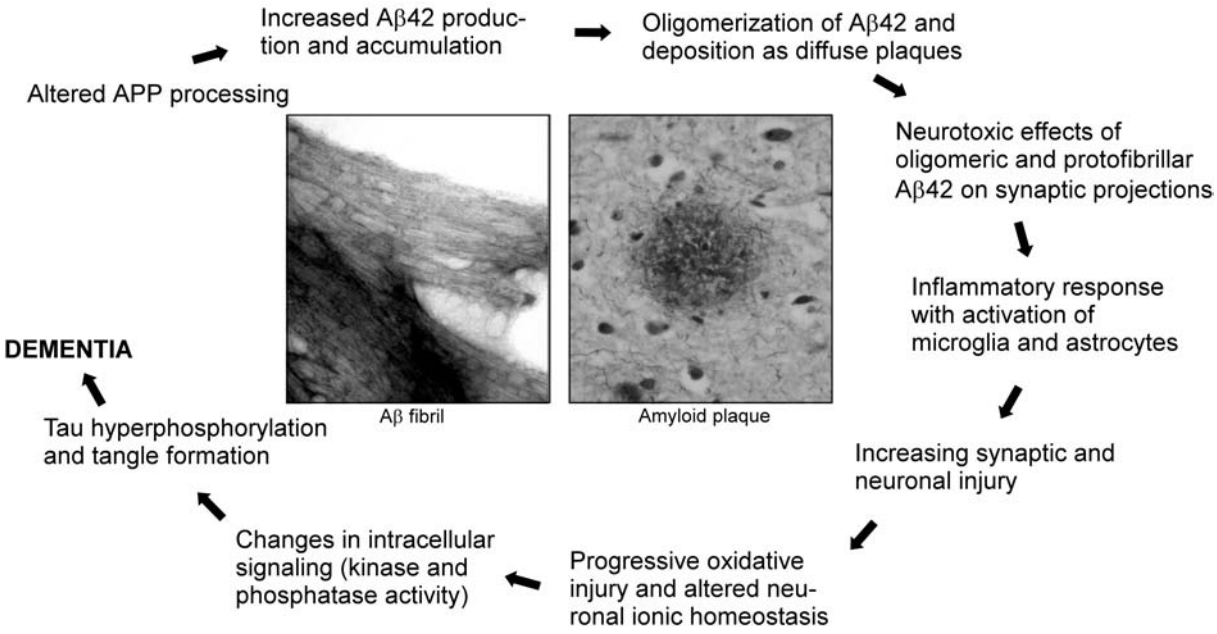

Figure 1. Outline of the amyloid cascade hypothesis, as suggested by Hardy and Selkoe 2002 (Hardy and Selkoe, 2002). The electron micrograph shows Aβ fibrils. A plaque composed of aggregated Aβ fibrils is depicted in the histological picture to the right. Dr. Johan Thyberg and Dr. Nenad Bogdanovic are acknowledged for the micrographs.

Even if the amyloid cascade hypothesis is convincing, there are theories implying that the role of tangles and/or inflammatory response is not fully explained by the amyloid cascade hypothesis (Lee, 2001; McGeer and McGeer, 1998). One argument against the amyloid cascade hypothesis concerns transgenic mice that overexpress APP or PS1 mutations. These mice only develop plaque pathology while no neurofibrillary tangles are formed (Janus et al., 2000). However, a promising triple transgenic mouse model was recently developed. The mice overexpress mutant APP and mutant tau on a PS1 mutant knock-in background (Oddo et al., 2003). Plaque depositions precede tau pathology in these mice, and with age the mice display synaptic dysfunction. Finally, the absence of plaque pathology for patients with mutations in tau, causing frontotemporal lobe dementia, and the presence of both plaques and tangles for patients with mutations in APP, causing AD, suggests that amyloid toxicity precedes tau in development of AD.

APP cell biology

APP is an evolutionary conserved glycoprotein with ubiquitous expression throughout the body. The type I transmembrane APP protein has a large extracellular N-terminal domain, and a short intracellular C-terminal tail protruding into the cytoplasm (Fig. 2) (Dyrks et al., 1988). Alternative splicing of exons 7, 8 and 15 generates eight different isoforms of APP, ranging from 695 to 770 residues in length (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al.,

1988). The main APP isoform expressed in neurons is the shortest variant, APP695, which lacks exons 7 and 8 (Haass et al., 1991; Rohan de Silva et al., 1997). Non-neuronal cells, such as microglia and astrocytes, express APP lacking exon 15 (APP751 and 770). These longer isoforms of APP contain a Kunitz-type protease inhibitor (KPI) domain encoded by exon 7 (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). The KPI domain inhibits serine proteases, and can thus protect the protein from degradation.

APP is produced in the endoplasmic reticulum (ER), and trafficked through the secretory pathway to the plasma membrane. Maturation of APP occurs within the Golgi apparatus by N- and O-linked glycosylation and tyrosine-sulfation (Fig. 2) (Weidemann et al., 1989). Another post-translational modification of the APP molecule is phosphorylation in the intracellular tail and ectodomain of the protein (Fig. 2) (Gandy et al., 1988; Oltersdorf et al., 1990; Hung and Selkoe, 1994). The internalization signal sequence YENP found in the C terminus of APP can mediate internalization from the plasma membrane (Perez et al., 1999), but the trafficking of APP is not yet fully understood, in part due to the fact that the functions of APP still remain largely unknown.

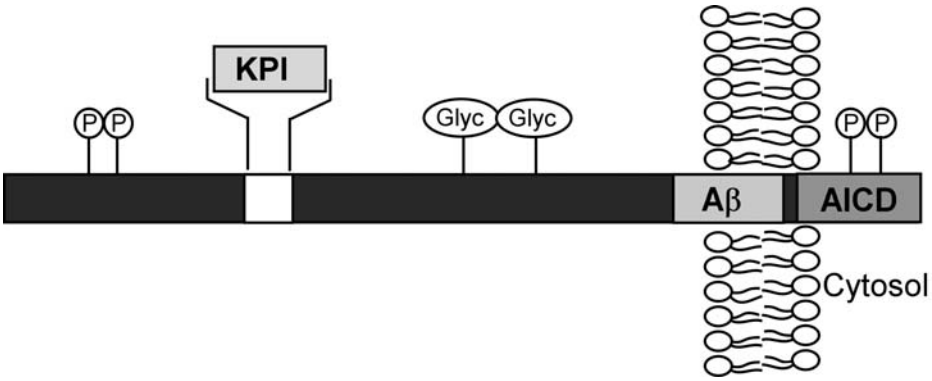

Figure 2. Outline of the APP protein with the alternatively spliced KPI domain indicated. Glycosylation in the extracellular domain and phosphorylation of intracellular and extracellular domains are shown (Glyc and P, respectively). Aβ and the APP intacellular domain (AICD) are depicted in the C-terminal part of the protein.

APP was originally suggested to be a receptor (Kang et al., 1987), but no ligand has been identified. Another suggested function for APP has been as a mediator of interaction with kinesin in microtubule-associated vesicle transport (Kamal et al., 2001). APP has also been proposed to function in cell adhesion (Schubert et al., 1989). Recently, the APP intracellular domain (AICD) was detected in the nucleus and found to interact with the nuclear adaptor protein Fe65 and the histone acetyltransferase Tip60 (Cao and Südhof, 2001). AICD has together with these two proteins been reported to activate transcription of a Gal4-reporter gene, suggesting a gene regulatory role for APP. In addition, AICD has been shown to regulate phosphoinositide-mediated calcium signaling (Leissring et al., 2002). The multitude of functions reported for APP suggests that the protein indeed is involved in numerous cellular processes. This could indicate that APP conveys some general effect and/or several specific functions. In many cases, the generation of a knockout mouse provides useful information about the function of the protein that has been ablated. The APP knockout mice were viable with a normal phenotype, suggesting that APP is not an essential gene. A closer

examination of these animals reveals a slight decrease in weight and reduced locomotor activity (Zheng et al., 1995). Most likely, the APP-like proteins (APLP) 1 and 2, which are highly homologous to APP, can compensate for at least some of the functions of APP in these mice. Similar to APP knockout mice, neither deficiency in APLP1 or APLP2 causes a distinct phenotype. Interestingly, however APP/APLP2 and APLP1/APLP2 double knockout mice die early after birth, while the APP/APLP1 deficient mice survive and appear normal (Heber et al., 2000). The rather complex set of data emerging from the knockout mouse studies shows that the APP and APLP protein family mediate some vital function, and that a partial functional replacement between the proteins probably can occur. However, the specific functions of these three proteins remain to be elucidated.

APP processing

A central feature of APP biology is the proteolysis of the molecule. Upon synthesis, the APP molecule is proteolytically processed by so-called secretases. Hydrolysis close to the lipid bilayer releasing the luminal N-terminal domain of APP is performed by α- or β-secretase. These processing events produce the N-terminal fragments sAPPα or sAPPβ, and the C-terminal fragments C83 or C99 (Fig. 3). Subsequently, the C-terminal fragments are substrates for γ-secretase, generating the p3 peptide and Aβ, respectively. Intriguingly, the physiological function or effects of the generated fragments are, in parallel with intact APP, largely unclear.

α-Secretase

The α-cleavage is performed by tumor necrosis factor-α converting enzyme (TACE) (Buxbaum et al., 1998), which belongs to the ADAM (a disintegrin and metalloprotease) family of metalloproteases. Other members of the ADAMs have been shown to support α-cleavage, such as MDC-9 and ADAM-10 (Koike et al., 1999; Lammich et al., 1999). TACE has a broad substrate specificity, cleaving a number of type I proteins such as tumor necrosis factor-α, p75 TNF receptor, L-selectin, transforming growth factor-α and Notch (Black et al., 1997; Moss et al., 1997; Peschon et al., 1998; Brou et al., 2000; Mumm et al., 2000).

Processing at the α-secretase site is considered non-amyloidogenic since it cleaves within the Aβ sequence and thus obliterates the formation of intact amyloidogenic Aβ (Fig. 3). Furthermore, α-secretase cleaves within the KLVFF-motif of APP, which has been identified as an important sequence for aggregation of the Aβ peptide (Tjernberg et al., 1996).

α-Secretase activity occurs in two variants, one constitutive variant with a basal level of α-secretase cleavage, and one inducible variant. Protein kinase C signaling can stimulate α-secretase (Hung et al., 1993). Thus, protein kinase C activation, through activation of signaling pathways or phorbol ester stimulation, can preclude Aβ formation by increasing the amount of APP cleaved in the so-called non-amyloidogenic pathway.

β-Secretase

Cleavage at the Aβ N terminus is executed by β-site APP cleaving enzyme (BACE) (Fig. 3) (Lin et al., 2000; Sinha et al., 1999; Yan et al., 1999; Vassar et al., 1999; Hussain et al., 1999). Two homologs of BACE have been identified. BACE1 is mainly expressed in brain and pancreas (Vassar et al., 1999), while BACE2 has a low expression in brain and probably little

effect on processing of APP (Bennett et al., 2000). BACE1 is an aspartyl protease with one transmembrane domain (TMD), and belongs to the pepsin protease family. The active site resides in the N-terminal domain of the protein and contains two catalytic aspartate residues (Hong et al., 2000). BACE1 is expressed as a pro-enzyme, and trafficked through the Golgi where it is glycosylated and the pro-peptide is cleaved off (Capell et al., 2000b). Surprisingly, the release of the pro-peptide is not necessary for BACE1 to be able to cleave APP (Creemers et al., 2001). Subcellular localization of BACE1 is mainly within Golgi and endosomes (Vassar et al., 1999). Furthermore, APP is not the only protein hydrolyzed by BACE1, other substrates identified are sialyl-transferase ST6Gal, P-selectin glycoprotein ligand-1, APLP1 and APLP2 (Kitazume et al., 2001; Lichtenthaler et al., 2003; Li and Sudhof, 2003).

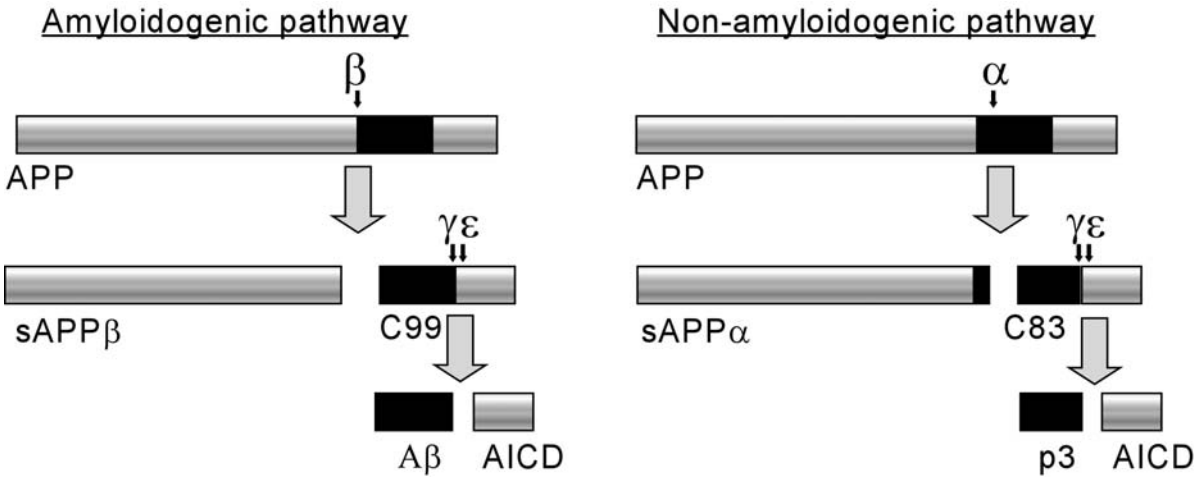

Figure 3. Processing of APP. The amyloidogenic pathway is outlined in the left panel where APP is cleaved by β-secretase to generate sAPPβ and the C-terminal fragment C99. In the right panel the non-amyloidogenic pathway is shown where a cleavage performed by α-secretase generates sAPPα and the C-terminal fragment C83. The C-terminal fragments from both pathways are processed by γ-secretase generating Aβ (amyloidogenic pathway) and p3 (non-amyloidogenic pathway). Processing at the ε-site generates AICD from both pathways.

γ-Secretase

The final catalytic step in Aβ formation from APP processing is performed by γ-secretase. The C-terminal fragments generated from α- and β-secretase cleavage, C83 and C99, respectively, are cleaved within their TMDs to produce the p3 peptide or Aβ (Fig. 3). γ-Secretase processing shows low sequence specificity and generates Aβ peptides differing in length, with Aβ40 being the most abundant product and the longer Aβ42 variant being generated to a lesser extent (Lichtenthaler et al., 1999; Wang et al., 1996). In parallel with Aβ-generating γ-secretase processing, another cleavage event, designated ε-cleavage, has been shown to occur after position 49 in Aβ (Sastre et al., 2001; Yu et al., 2001; Weidemann et al., 2002). Both γ- and ε-processing critically require the presence of PS proteins, which will be discussed further in the *Presenilin* section (Herreman et al., 2000; Zhang et al., 2000; Sastre et al., 2001). The C-terminal peptide fragment resulting from ε-cleavage, the AICD (Fig. 3), is rapidly turned over, but can be stabilized by interaction with Fe65 (Kimberly et al., 2001). Production of Aβ has been suggested to occur in several subcellular compartments, including the trans Golgi network and the plasma membrane (Hartmann, 1999).

APP mutations

It is striking that mutations in APP that cause familial AD are located close to the secretase cleavage sites in the molecule (Fig. 4). There is a cluster of mutations C-terminal to the Aβ42 cleavage site. Disease-causing mutations at this position all change the preference of the site in which APP is processed from Aβ40 to Aβ42 and thereby increase the Aβ42 to Aβ40 ratio (Hutton et al., 1998; Goate, 1998). Aβ42 is more prone to aggregate than Aβ40 (Jarrett et al., 1993), and is the major Aβ species found in the core of amyloid plaques (Iwatsubo et al., 1994).

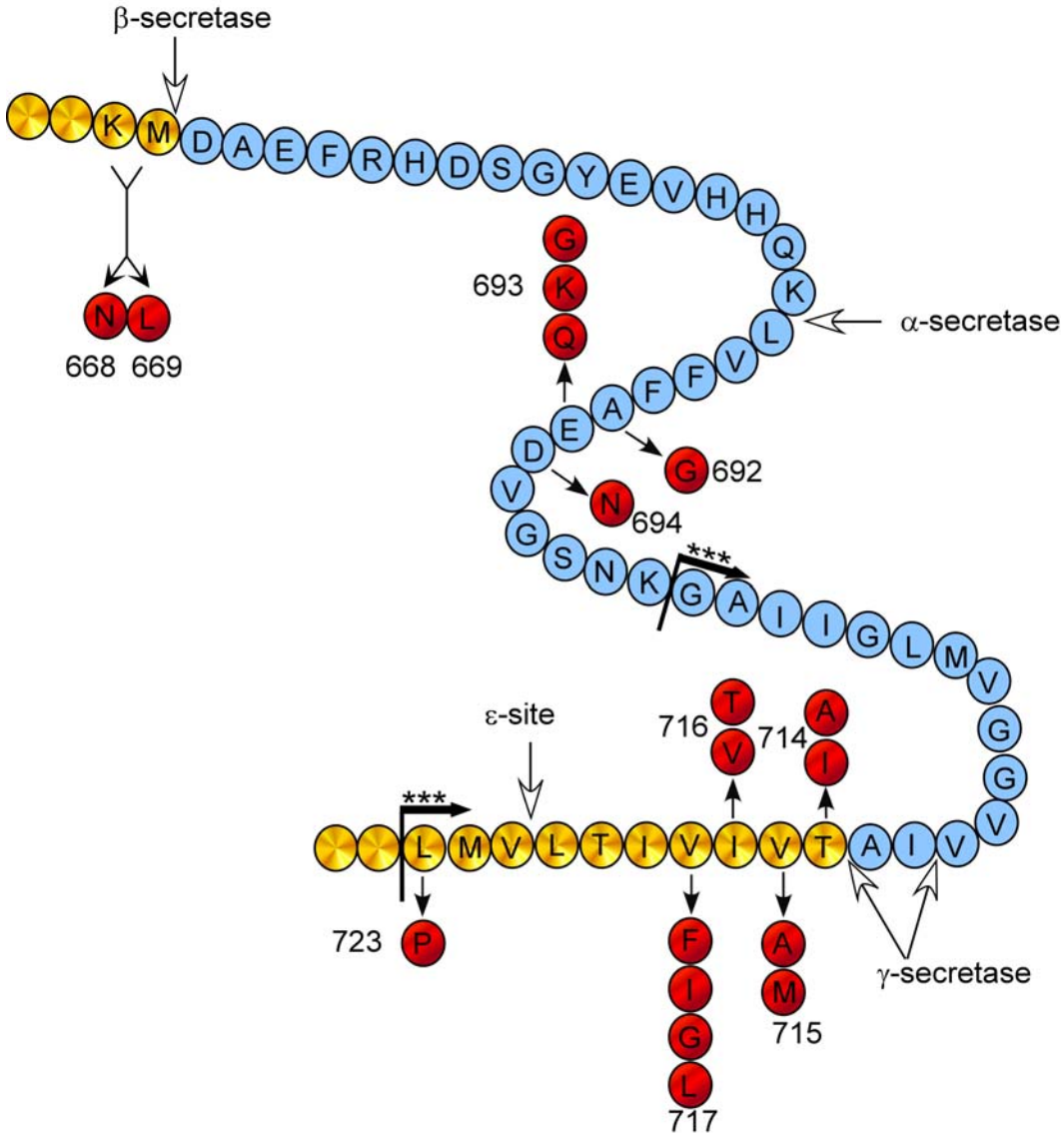

Figure 4. A part of APP containing the Aβ sequence (in blue), and a few residues N- and C-terminal to the Aβ sequence (in yellow) are outlined. Pathogenic mutations (in red) and the different cleavage sites (indicated by arrows) are shown. Filled arrows marked with *** indicate the proposed membrane-spanning domain. Numbering refers to the APP 770 isoform.

Distal to these mutations, a double mutation has been found in a Swedish family causing hereditary AD (Mullan et al., 1992). The mutation is located just N-terminal of the Aβ N terminus, and it results in increased production of total Aβ, including Aβ42 (Fig. 4).

Within the middle of the Aβ sequence is a cluster of mutations that show different pathogenic features, such as cerebral amyloid angiopathy, cerebral hemorrhages, white matter changes, and dementia (Fig. 4) (Revesz et al., 2002). The Arctic mutation is located at this middle region of Aβ exchanging a glutamic acid for a glycine (E693G), and causes AD and white matter changes (Nilsberth et al., 2001). Interestingly, this mutation changes the fibrillogenic characteristics of the Aβ peptide by stabilizing protofibrils – a kinetic intermediate in the Aβ aggregation process.

Presenilin

The homologous PS1 and PS2 proteins are conserved multipass transmembrane proteins. From studies of *Caenorhabditis elegans* it was found that the PS homolog Sel-12 was an essential mediator of Notch signaling in development (Levitan et al., 1996). Mice deficient in PS1 die late embryonically or at birth, indicating the developmental importance of PS1 also in mammalian systems (Shen et al., 1997; Wong et al., 1997). In contrast to the lethal phenotype of PS1 ablation, PS2 knockout mice were viable and fertile (Herreman et al., 1999). This suggests that a partial redundancy can occur between the two PS proteins, where it is likely that PS1 can functionally substitute for PS2, whereas PS2 is not able to replace PS1. Double PS1 and PS2 knockout mice die at embryonic day 9.5 with a phenotype resembling the phenotype for Notch 1 knockout mice, further emphasizing the role of PS in development (Donoviel et al., 1999; Herreman et al., 1999). The involvement of PS proteins in AD was first suggested by the genetic findings of multiple mutations in the PS1 gene causing familial AD. Importantly, neuronal cells from PS1 knockout mice secrete dramatically reduced amounts of Aβ, indicating a gain of function mechanism for disease-causing PS1 mutations (De Strooper et al., 1998). The requirement of PS for Aβ generation was corroborated by the analysis of blastocyst-derived stem cells from PS null mice, which were found to produce no Aβ (Herreman et al., 2000; Zhang et al., 2000). PS1 and PS2 have been suggested to be the enzymatically active components of a high-molecular weight γ-secretase complex, acting as an aspartyl protease with its catalytic site within the membrane bilayer. Mutagenesis of either one or both aspartates in TMD six and seven (Fig. 5) results in PS molecules deficient in supporting Aβ generation (Wolfe et al., 1999).

Presenilin cell biology

The orientation of PS within the membrane has been a matter of debate. Currently, the most favored topological model for PS suggests an eight transmembrane topology with the N and C termini located in the cytoplasm (Fig. 5) (Li and Greenwald, 1996), while other studies predict a seven transmembrane topology (Nakai et al., 1999). Both topological models predict a cytoplasmic loop in which PS undergoes endoproteolysis, generating a stable N- and C-terminal fragment (NTF and CTF) heterodimer (Fig. 5) (Thinakaran et al., 1996; Capell et al., 1998). Overexpression of PS in cell culture systems does not lead to increased Aβ formation, instead overexpressed PS accumulates as full-length protein, suggesting that endoproteolysis of full-length PS into the presumably active heterodimer is highly regulated (Thinakaran et al., 1997).

Full-length PS1 protein is mainly found in the ER, whereas the NTF and CTF are trafficked further in the secretory pathway (Zhang et al., 1998). PS endoproteolysis has been proposed to

be an autocatalytic process requiring the aspartyl residues in TMD six and seven (Wolfe et al., 1999), or to be performed by an unknown protease, presenilinase (Campbell et al., 2003). Endoproteolysis of PS into the NTF-CTF heterodimer has been suggested to be required for the PS molecule to support Aβ generation. However, the naturally occurring mutation causing familial AD, PS1Δexon9, which lacks exon 9 and hence the site for endoproteolysis, is still active with respect to γ-secretase activity. An artificial point mutation, PS1M292D, also shows resistance to endoproteolysis, yet can still generate Aβ (Steiner et al., 1999). Recently, it was suggested that PS1 can exist as an oligomer consisting of two NTF-CTF heterodimers, though the *in vivo* validity of this model has not yet been shown (Schroeter et al., 2003).

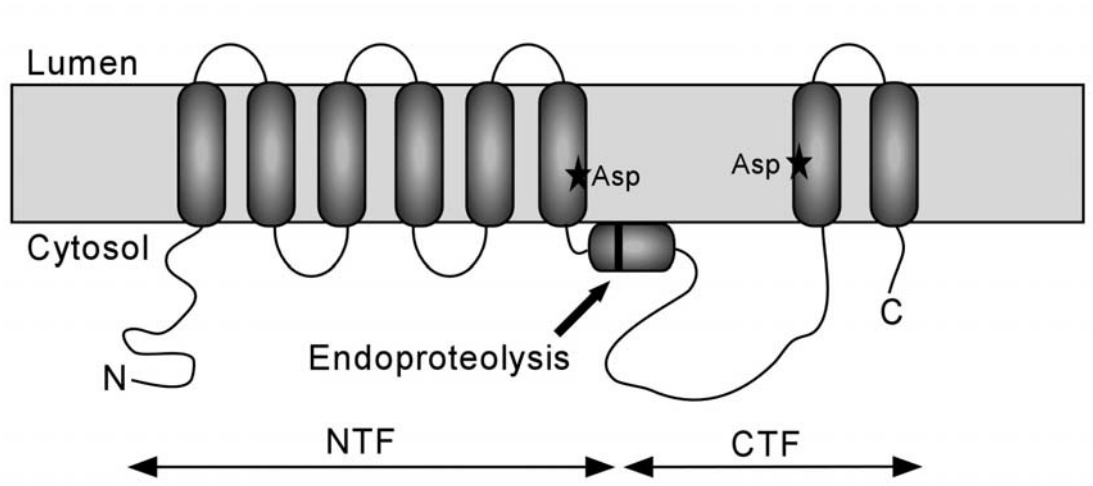

Figure 5. Cartoon of PS1 with eight membrane-spanning domains. The endoproteolysis site for generation of NTF and CTF is shown. The conserved aspartyl residues in TMD six and seven that are critical for γ-secretase activity are indicated by★.

Presenilin protein function

The function of PS as the catalytic component in γ-secretase processing has been a matter of intense debate with the main arguments against it being: (*i*) the unusual structure of PS with regards to it being a protease, and (*ii*) the so-called spatial paradox, suggesting that γ-secretase activity and PS are localized in different intracellular compartments. These arguments have now, at least partly, been resolved. PS ability to function as a protease is supported by the identification of a new class of transmembrane aspartyl proteases, the protein family of signal peptide peptidases. The signal peptide peptidases are homologous to PS and are catalytically active within the lipid membrane bilayer (Weihofen et al., 2002). Further evidence for PS being an aspartyl protease is the labeling of PS1 NTF and CTF by γ-secretase transition state analogs (Esler et al., 2000; Li et al., 2000), the conserved aspartyl protease motif identified in the molecule (Steiner et al., 2000), and the critically required aspartate residues in TMD six and seven (Wolfe et al., 1999). The spatial paradox emphasizes that PS mainly is located to the ER while γ-secretase processing occurs in a compartment downstream of the ER (Cupers et al., 2001; Checler, 2001). However, PS1 has recently been found at the plasma membrane (Kaether et al., 2002; Berezovska et al., 2003), which is one proposed site for γ-secretase

activity. It is noteworthy that the interaction between PS and APP, as estimated by fluorescence lifetime imaging microscopy, reaches its highest intensity at this localization (Berezovska et al., 2003).

Other proposed functions for PS are involvement in the folding of proteins in the ER by affecting the unfolded protein response (Katayama et al., 1999). Also, PS regulates calcium homeostasis within the cell (Guo et al., 1996) and familial AD PS1 and PS2 mutations alter intracellular Ca^{2+}-signaling and make cells more susceptible to apoptosis (Leissring et al., 2000; Herms et al., 2003; Popescu and Ankarcrona, 2000). PS interacts with a number of different proteins, such as glycogen synthase kinase-3β and β-catenin, and through these interactions PS may have other functions (Van Gassen et al., 2000).

Presenilin mutations

A large number of mutations have been identified in the gene encoding PS1, whereas few mutations have been found in the PS2 gene. The mutations cause familial AD with an early age of onset. There are cases displaying symptoms as early as in their late teens (Moehlmann et al., 2002), but more commonly the mutations cause disease in the fourth or fifth decade of life in affected individuals (Hutton and Hardy, 1997). At the cellular level, all mutations in PS1 result in an increase of the Aβ42 to Aβ40 ratio, thus producing more of the longer and more hydrophobic Aβ42 variant (Hutton and Hardy, 1997). It is interesting to note that despite the stereotype effect of PS1 mutations on Aβ production, disease-causing PS1 mutations are distributed over all hydrophobic and membrane spanning regions of the protein. This feature indicates that the conserved hydrophobic regions are important with regards to Aβ production. However, the molecular mechanisms of how PS1 mutations *de facto* alter Aβ generation is presently not fully understood.

Notch processing

Notch is a protein with important signaling functions in development. Despite obvious lack of homologous domains or sequences between Notch and APP, the proteins share several common features in the way they are processed. Notch is first proteolyzed by a furin-like convertase in the Golgi at the so-called S1-site in the molecule. The two resulting protein fragments associate into a heterodimer and are trafficked to the plasma membrane (Blaumueller et al., 1997). Ligand activation of Notch occurs at the cell surface and induces a second cleavage, S2. Processing at the S2-site releases the Notch extracellular domain, and is performed by the same enzyme that mediates α-secretase cleavage of APP, namely TACE (Brou et al., 2000; Mumm et al., 2000). Subsequent to S2-cleavage, the molecule undergoes PS-dependent intramembrane cleavage at the S3-site generating the Notch intracellular domain (NICD) (Schroeter et al., 1998; De Strooper et al., 1999). NICD translocates into the nucleus where it activates transcription of the cell-fate determining HES (Hairy/Enhancer of split) genes, thus initiating a non-neuronal development of the cell (Jarriault et al., 1995; de la Pompa et al., 1997). The S3-cleavage is similar to the ε-cleavage event occurring in APP. Both the S3- and ε-cleavage occur close to the cytosolic interface of the membrane bilayer, and both cleavages critically require PS (Schroeter et al., 1998; Sastre et al., 2001). APP and Notch processing show further parallel features since Notch was recently found to undergo dual intramembrane cleavage, releasing a p3-like peptide from a site, S4, equivalent to the γ-secretase site in APP (Okochi et al., 2002).

Regulated intramembrane processing

The term regulated intramembrane processing (RIP) is used for a process where enzymes cleave their substrates within their TMDs (Brown et al., 2000). In addition to PS, there are three other groups of RIP enzymes: two of the groups process type II transmembrane proteins, site-2 protease and signal peptide peptidases, while PS and rhomboid recognize type I transmembrane proteins as substrates (Urban and Freeman, 2002).

APP and Notch were the first proteins found to be cleaved by PS-dependent RIP. The list of PS substrates is rapidly growing and today includes over 20 proteins. Among them are the APLP proteins, the Notch ligands Delta and Jagged, CD44, ErbB-4, Syndecan 3, E-cadherin and N-cadherin (Scheinfeld et al., 2002; Ikeuchi and Sisodia, 2003; Okamoto et al., 2001; Ni et al., 2003; Schulz et al., 2003; Marambaud et al., 2002; Marambaud et al., 2003). The proteins require shedding of their ectodomain to become accessible for PS-mediated RIP. Subsequently, intramembrane cleavage releases the cytoplasmic domains of the substrates, which then translocate to sites where they have their signaling action, for example in the nucleus. The intracellular domains of Notch, Jagged, APP, APLP1 and APLP2 have been found to translocate into the nucleus (Schroeter et al., 1998; LaVoie and Selkoe, 2003; Cao and Südhof, 2001; Walsh et al., 2003). RIP has been suggested to constitute a signaling mechanism conserved from bacterial to mammalian cells. As of today, no function has been ascribed to the short hydrophobic peptide fragment resulting after ectodomain shedding and intracellular domain liberation of RIP substrates. Speculatively, these "by-products" from RIP processing generally undergo degradation. However, when Aβ is produced, its fibrillogenic and persistent characteristics inhibit its clearance, hence causing aggregation and deposition of Aβ. An intriguing feature of RIP is the dual intramembrane cleavage reported for APP, Notch, CD44, and the APLP proteins (Sastre et al., 2001; Okochi et al., 2002; Eggert et al., 2004). Is this an adaptation to varying membrane thickness common for a wide range of RIP substrates, or is it an effect of certain molecules having extremely hydrophobic membrane spanning domains that require additional processing to enable release of their cytosolic fragment? Future studies are required to shed light on these matters.

γ-Secretase complex

Intense research efforts over recent years have provided much new information about γ-secretase. Not only is PS critical, but also three other γ-secretase complex components, nicastrin, Anterior pharynx defective-1 (Aph-1), and Presenilin enhancer-2 (Pen-2), are required for γ-cleavage to occur (Chung and Struhl, 2001; Goutte et al., 2002; Francis et al., 2002). The proteins assemble together with PS into a multi-component high molecular weight γ-secretase complex and are described in the following paragraphs.

Nicastrin

Nicastrin is a 709-residue type I transmembrane glycoprotein with a large ectodomain and a short cytoplasmic tail (Fig. 6). A stretch of conserved residues, DYIGS, has been identified in the extracellular domain of the protein (Yu et al., 2000). This motif is required for nicastrin to support Aβ generation and its deletion abrogates Aβ production, while point mutations within this site cause an increased secretion of Aβ42. The function of nicastrin is unknown, but it has been suggested that nicastrin brings the substrate and γ-secretase together (Yu et al., 2000; Hu

et al., 2002). Ablation of nicastrin leads to abolished Aβ production, accumulation of APP and perturb internalization of APP (Li et al., 2003). The nicastrin knockout mice die by embryonic day 10.5, whereas heterozygot nicastrin$^{+/-}$ mice are viable without obvious clinical phenotype. This notion is of particular interest considering the marked reduction of secreted Aβ from nicastrin$^{+/-}$ fibroblasts.

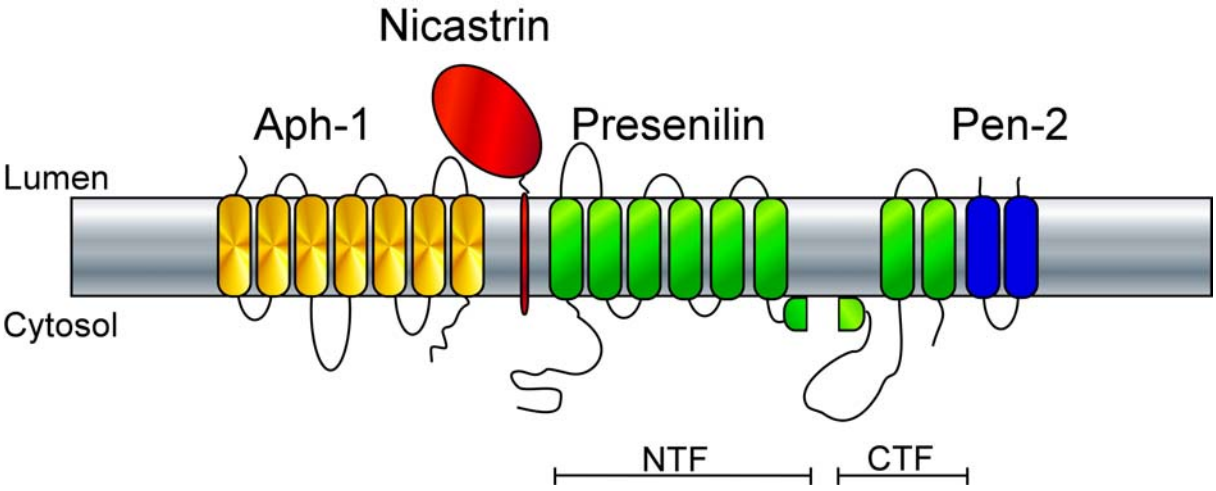

Figure 6. Members of the γ-secretase complex are illustrated: Aph-1, a seven TMD protein (in yellow), the type I glycoprotein nicastrin (in red), the PS1 heterodimer consisting of NTF and CTF (in green) and Pen-2 (in blue). Association of all four of the proteins into a high-molecular weight γ-secretase complex is required for γ-secretase activity.

Aph-1 and Pen-2

Recently, two new proteins required for γ-cleavage were identified (Goutte et al., 2002; Francis et al., 2002) (Fig. 6). The Aph-1 and Pen-2 proteins were isolated in genetic *Caenorhabditis elegans* screens for phenotypes resembling PS homolog deficiency. The seven TMD Aph-1 protein has two mammalian homologs, Aph-1a and Aph-1b (Goutte et al., 2002). Aph-1a also exists in two major splice variants, depicted L and S for the long and short variants (Francis et al., 2002; Lee et al., 2002). The topology of Aph-1 resembles that of seven TMD receptors with the N terminus in the lumen, while the C terminus resides in the cytoplasm (Fig. 6) (Fortna et al., 2004). The molecule undergoes endoproteolysis and the stable C-terminal part of Aph-1 is incorporated into the γ-secretase complex (Fortna et al., 2004).

Pen-2 has two predicted TMDs and one human homolog has been found (Francis et al., 2002). The Pen-2 molecule adopts an inverted hairpin structure with the N and C termini facing the ER lumen (Fig. 6) (Crystal et al., 2003; paper III, this study). A Pen-2 knockout mouse has been developed which displays a phenotype similar to Notch 1-deficient mice; the embryos die before birth at embryonic day 9-10 (Li et al., 2002a).

Regulation and function within the γ-secretase complex

The proteins in the γ-secretase complex are conserved in sequence and in function, as shown by the ability of mammalian homologs of the γ-secretase complex components to rescue, or partially rescue, *Caenorhabditis elegans* deficient phenotypes of corresponding proteins (Levitan et al., 1996; Levitan et al., 2001; Francis et al., 2002). Within the γ-secretase complex the components regulate each other in an intricate, and not yet fully understood manner (De Strooper, 2003).

Nicastrin requires PS1 to become glycosylated and fully mature (Leem et al., 2002; Yang et al., 2002; Edbauer et al., 2002; Tomita et al., 2002), however the maturation status of nicastrin does not seem to affect γ-secretase activity *per se* (Herreman et al., 2003). Reciprocally, PS requires nicastrin for its trafficking to the cell surface (Edbauer et al., 2002). Moreover, in nicastrin null cells PS1 NTF and CTF are undetectable, indicating a dramatic destabilization of PS1 fragments in the absence of nicastrin (Li et al., 2003). The knowledge of the functional impact of the two most novel γ-secretase components, Aph-1 and Pen-2, is just emerging. Pen-2 has been reported to be essential for the endoproteolytic cleavage of PS1 (Luo et al., 2003a). In the absence of PS proteins, Pen-2 is rapidly degraded (Steiner et al., 2002) while the stability of Aph-1 is not affected in an appreciable way (Edbauer et al., 2002). Aph-1, alone or together with nicastrin, has been proposed to stabilize the PS1 full-length protein (Hu and Fortini, 2003; Luo et al., 2003a). A subcomplex containing Aph-1 and nicastrin has been shown to form rapidly in the ER (LaVoie et al., 2003; Hu and Fortini, 2003; Fortna et al., 2004). In addition, by using detergent dissociation of γ-secretase, other subcomplexes have been identified composed of (*i*) nicastrin, Aph-1 and PS1 CTF, (*ii*) Pen-2 and PS1 NTF, and (*iii*) the PS1 NTF-CTF heterodimer (Fraering et al., 2004). However, the exact functions for each of the components and subcomplexes remain largely unclear due to the difficulties in analyzing the proteins individually in the absence of the other γ-secretase components.

Overexpression of either one of the four γ-secretase complex components does not lead to an increased Aβ generation while overexpressing all four proteins facilitates γ-processing of APP (Takasugi et al., 2003; Edbauer et al., 2003; Kimberly et al., 2003b). Under these conditions PS1 processing into stable heterodimers is increased, and more fully glycosylated nicastrin protein is produced. Moreover, overexpression of all four components in yeast cells, that cannot endogenously process APP, restores γ-secretase processing and Aβ/AICD formation (Edbauer et al., 2003). The γ-secretase complex has been purified from human brain, indicating an association also *in vivo* of the four γ-secretase complex components (Farmery et al., 2003). The specific details of how the γ-secretase complex assembles, associates with its substrates, and performs the actual hydrolysis are still unclear. Possibly, *in vitro* reconstitution of γ-secretase activity from purified components could bring insight into these matters.

Ubiquitylation and the proteasome

Protein levels can be regulated by the two major degradation systems present in cells, proteasomal and lysosomal degradation. Covalent attachment of the well-conserved 76-residue ubiquitin protein can target proteins for degradation by the proteasome (Hershko and Ciechanover, 1998). The addition of ubiquitin can also regulate such diverse mechanisms as DNA repair, cell cycle control, vesicle transport, antigen presentation, signal-transduction pathways and transcription (Yew, 2001; Aguilar and Wendland, 2003; Weissman, 2001).

Ubiquitylation of target proteins is an ATP-dependent process performed by three enzymes (Pickart, 2001). The first step is activation of ubiquitin by a ubiquitin-activating enzyme, E1. The activated ubiquitin is next transferred to a ubiquitin-conjugating enzyme, E2. Finally, the ubiquitin moiety is linked to a lysine residue in the target protein by the E3 ubiquitin-ligase enzyme, which possesses the substrate-recognition function in this enzymatic cascade that mediates ubiquitylation. Degradation by the proteasome is mediated by poly-ubiquitylation, which may require a chain elongation factor (E4) (Koegl et al., 1999). The 2.5 MDa 26S proteasome resides in the cytoplasm. At one or both ends of the proteasome there is a gating subunit that only allows poly-ubiquitylated proteins to enter into the catalytic sites within the proteasome (Voges et al., 1999). The ubiquitylation pathway is very well conserved with only three residues differing between yeast and mammalian ubiquitin. It is a complex and diverse system indicating an intricate regulation to fine-tune cellular protein levels and activity in ways not yet fully understood.

Interestingly, proteasomal degradation has been implicated for the PS proteins. PS2 has been shown to be poly-ubiquitylated and degraded by the proteasome (Kim et al., 1997). The levels of full-length PS1 or NTF and CTF that are not incorporated into stable complexes are tightly regulated by the proteasome. In contrast, NTF and CTF incorporated into heterodimers are stable and resistant to proteasomal degradation (Steiner et al., 1998). The enzyme facilitating ubiquitylation of PS1 was identified by Jinhe Li and colleagues to be Sel-10, which also participates in the ubiquitylation of Notch (Li et al., 2002b; Öberg et al., 2001). Furthermore, findings of ubiquitin conjugated to tau protein in neurofibrillary tangles in brains from AD patients, and ubiquitin-like immunoreactivity in amyloid plaques, suggests a role for ubiquitylation in AD (Checler et al., 2000).

Treatment of AD – today and in the future

Despite intense research efforts, no causal treatment of AD is clinically available at present. The symptomatic drugs used today are acetylcholine esterase inhibitors and a glutamate NMDA-receptor antagonist (Scarpini et al., 2003). Acetylcholine esterase inhibitors increase neurotransmission by decreasing the rate of degradation of acetylcholine in the synaptic cleft, resulting in more functional neurotransmitter. The glutamate non-competitive NMDA-receptor antagonist inactivates ionotropic receptors of the NMDA type and thereby decreases intracellular Ca^{2+}-levels.

The enzymes generating the pathological Aβ peptide, β- and γ-secretase, are potential therapeutic targets. However, the multitude of substrates makes γ-secretase a difficult target. The γ-secretase inhibitor DAPT (Dovey et al., 2001) was used in an *in vivo* study in zebra fish. The treated animals displayed an impaired developmental phenotype similar to Notch deficiency (Geling et al., 2002). Inhibition of γ-secretase will not only affect Aβ generation, but also influence the processing and down-stream effects of all PS-dependent RIP substrates. Hypothetically, a partial inhibition of γ-secretase may lead to a beneficial lowering of Aβ without having deleterious effects on other PS-dependent RIP-substrates, however this remains to be investigated.

β-Secretase, BACE1, provides an attractive drug target since the phenotype of BACE1 knockout mice appears normal and no Aβ is generated (Cai et al., 2001; Luo et al., 2001; Luo et al., 2003b). The crystal structure of the active site has been solved and provides useful

information in the search to find BACE1-specific inhibitors (Hong et al., 2000). However, the search can be challenging since the active site cleft is wide and it may be difficult to target using small molecules with high specificity.

The clinical trial attracting most interest in recent years is the immunization studies performed by Wyeth/Elan. Results from the pre-clinical studies were very promising, showing reduced plaque pathology and complete clearance of plaques in transgenic mice immunized with the Aβ42 peptide (Schenk et al., 1999). Trials on humans was initiated in 2001 and stopped in January 2002 when rare cases of encephalitis were discovered. In the only case report available, a partial clearance of Aβ was seen in the cortex (Nicoll et al., 2003). A complete analysis of the study is not yet available, it awaits decoding and compilation. The severe adverse effects of the present immunization protocol preclude its use as a therapy for AD. Despite the setbacks, vaccination is still intensely investigated. Different parts of the Aβ peptide may be used as the immunogen (Sigurdsson et al., 2001). Alternatively, passive immunization can be performed where no immunogen is administrated. Instead, exogenously produced Aβ antibodies are supplied (Bard et al., 2000).

Treatment of other disorders has shown beneficial effects for the incidence of AD for two different sets of drugs: cholesterol lowering compounds and nonsteroidal anti-inflammatory drugs (Wolozin et al., 2000; Jick et al., 2000; in t' Veld et al., 2001). The molecular mechanisms of cholesterol lowering drugs with respect to AD are not well known, though in cell culture studies depletion of cholesterol reduces Aβ formation (Simons et al., 1998). In contrast, high-cholesterol diets increase Aβ pathology in animals (Sparks et al., 2000; Refolo et al., 2000). A subset of nonsteroidal anti-inflammatory drugs directly affect Aβ-generation by shifting the preferred C-terminal cleavage site from residue 42 to residue 38, thus decreasing the amount of longer and more amyloidogenic Aβ42 variant (Weggen et al., 2001).

There have been numerous attempts in the quest to find an AD therapy that could improve cognition and reduce neurodegeneration, but the results have so far been disappointing. Still, several promising approaches are now under investigation, and the increased knowledge about the pathogenic mechanisms underlying AD provides researchers of today with powerful tools in the endeavor to develop an efficient therapy.

AIMS OF THE STUDY

The devastating neurodegenerative process observed in AD is believed to be initiated by the pathological effects of the Aβ peptide. Both the Aβ peptide and the AICD fragment are generated by the γ-secretase complex. The work presented in this thesis aimed to characterize the intramembrane cleavage of APP by investigating AICD generation, and to reach an increased understanding of the regulation and assembly of the γ-secretase complex.

The specific aims in each of the studies were:

Paper I — To develop a cell-based reporter assay for the transmembrane cleavages of APP and Notch.

Paper II — To investigate AICD generation from wild-type APP and APP with familial AD mutations. In addition, we wanted to determine the intracellular site for production of AICD.

Paper III — To describe the basic cell-biological properties of the γ-secretase complex component Pen-2 by determining the topology, interacting proteins, stability and degradation pathway of the Pen-2 protein.

Paper IV — To elucidate the importance and impact of the absolute C terminus of PS1 for APP processing and for the interaction of PS1 with other γ-secretase complex components.

RESULTS AND DISCUSSION

I. Development of a sensitive and quantitative assay for measuring cleavage of presenilin substrates

The general method to assess γ-secretase cleavage *in vitro* is by measuring Aβ concentration in cell culture media by ELISA (enzyme-linked immunosorbent assay). Alternatively, γ-secretase activity can be estimated by preparing membrane microsomes and analyzing the amount of AICD generated and Aβ produced from exogenously provided substrate, for example C100-Flag (Kimberly et al., 2003a). We wanted to develop a reporter system with features not present in the available assays to measure γ-secretase activity. Most ELISA assays measure secreted Aβ, whereas intracellular pools of Aβ are not accessible for analysis. In addition, Aβ peptides are known to aggregate, which can cause masking of the antibody epitopes, precluding a measurement of the total amount of Aβ. The *in vitro* microsomal assay is performed by purifying membranes from cells, which could possibly lead to only a subset of the cellular γ-secretase activity being purified. Disruption of cells prior to activity analysis could also lead to artificial effects not seen in measurements from whole cells. Hence, we wanted to develop an assay where whole cells were analyzed and total γ-cleavage was recorded. Importantly, γ-secretase cleaves both APP and Notch, therefore it was desirable to be able to measure γ-cleavage of both substrates.

In paper I, a cell-based γ-secretase assay was developed, where processing of the TMD of APP or Notch was recorded. The reporter-assay utilizes Gal4-VP16-driven transcription of luciferase as read-out for intramembrane cleavage. The γ-secretase substrates were modified by incorporation of a Gal4 DNA binding domain and a transactivating VP16 domain, together abbreviated GVP. The GVP-domain was inserted into the intracellular part of the protein, between the membrane-spanning region and the cytoplasmic tail (Fig. 7A). The reporter molecules consisted of ectodomain-shedded Notch 1 or APP, Notch ΔE-GVP and C99-GVP, respectively. The S2- and β-secretase cleaved variants of the proteins were chosen since we wanted our reporter molecules to be direct γ-secretase substrates. In the case for Notch, it was also important to circumvent the need for ligand activation of the molecule. Upon γ-cleavage, the C-terminal part of the proteins, NICD-GVP and AICD-GVP, respectively, were released from the membrane, and translocated to the nucleus where transcription of the luciferase reporter gene was activated. The luciferase activity was subsequently quantified by luminescence.

In PS null cells, no γ-secretase activity was observed in the absence of PS1 and PS2. However, γ-cleavage was robustly restored by exogenously expressing PS1 or PS2 together with the reporter constructs (Fig. 7B). The sensitivity of the reporter-system was shown by transfecting increasing amounts of cDNA encoding PS1 together with the reporter constructs in PS-deficient cells. Using low levels of PS1 cDNA (1-10 ng/transfection), luciferase activity was observed, whereas PS1 protein detection by immunoblotting was not evident until 50 ng cDNA/transfection was used. In cells with all four γ-secretase complex proteins present, overexpression of either one of the γ-secretase components is not sufficient for increasing γ-secretase activity (Edbauer et al., 2003; Kimberly et al., 2003b; Takasugi et al., 2003). Instead, all four γ-secretase complex proteins have to be exogenously expressed to augment γ-secretase activity. In this study, when PS-null cells were transfected with low levels of cDNA encoding PS1, the PS1 protein probably constituted the limiting factor for γ-secretase activity.

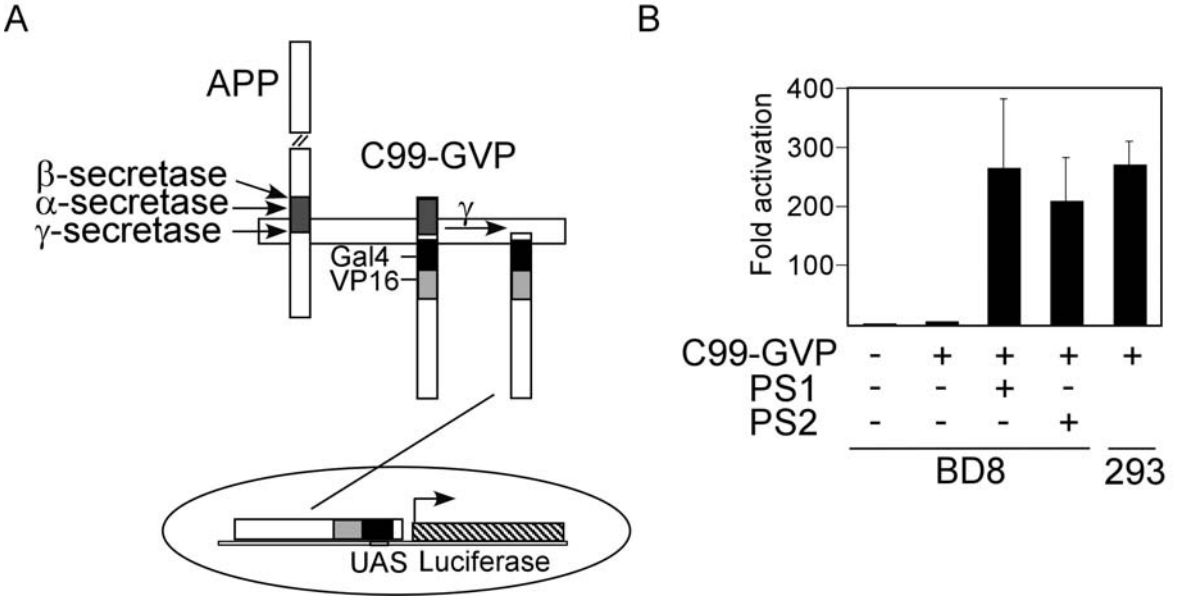

Figure 7. **A**. Illustration of the luciferase reporter system. The reporter molecule C99 with a Gal4 and a VP16 domain inserted after the TMD of the molecule is shown. Upon intramembrane cleavage, the intracellular domain is released and activates transcription of luciferase via the upstream activating sequence (UAS). **B**. Luciferase activity from PS null cells transfected with reporter molecules with or without PS1 or PS2. The bar to the right shows luciferase activity from reporter molecules in HEK293 cells expressing endogenous PS.

However, with increasing amounts of PS1 being expressed it is likely that PS1 would become in excess compared with the other γ-secretase complex components. Due to the limiting amount of either nicastrin, Aph-1 or Pen-2, γ-secretase activity could not increase correspondingly when higher levels of PS1 cDNA were used for the transfections.

The assay was sensitive to the specific γ-secretase inhibitors MW167 and L-685,458, indicating that a true γ-cleavage was recorded in HEK293 cells. In addition, IC_{50} values for the inhibitors were determined to 50 μM and 200 nM, respectively, in good agreement with earlier published studies (Berezovska et al., 2000; Shearman et al., 2000). Furthermore, the assay recapitulated the phenotype for PS1 aspartyl mutants in terms of C99-GVP processing, with no rescue of γ-activity for either one of the three mutants in PS null cells. Regarding processing of Notch ΔE-GVP, γ-cleavage could not be rescued by PS1 with mutations D257A or D257A/D385A, while D385A had a minor rescuing effect. The lack of γ-secretase processing of C99-GVP for PS1 aspartyl mutants is in agreement with other studies (Wolfe et al., 1999; Nyabi et al., 2003). In contrast, the PS1 D385A mutant displayed low levels of luciferase activity for the Notch ΔE-GVP substrate though it has previously been reported to be inactive. We have in subsequent experiments, using different experimental paradigms, been unable to consistently repeat this small rescuing effect of PS1 D385A. Still, not all reports have considered the aspartyl mutants inactive. In a study performed by Capell and co-workers, Aβ generation was detected from cells transfected with PS1 D257A (Capell et al., 2000a). In addition, Kim et al reported Aβ production from PS1 D257A and the double mutant PS1 D257A/D385A (Kim et al., 2001). This could, at least partially, be explained by the use of cells expressing endogenous PS.

In conclusion, a new, sensitive and quantitative assay for measuring γ-secretase cleavage has been developed for the γ-secretase substrates APP and Notch. The method has several advantages. First, it was specific since the GVP domain was inserted 13-15 residues from the proposed γ-secretase cleavage site, thus minimizing recording of unspecific cleavage. Second, it was sensitive since VP16 is a strong transactivator of the upstream activating sequence promoter used. Third, it has the advantage, with respect to APP processing, that it detects the total γ-cleavage including the cleavage generating Aβ within the cell, in contrast to other methods that rely on antibody detection of Aβ secreted into the media of cultured cells.

The need for a causal therapeutical intervention in AD is urgent, and one obvious drug target is γ-secretase. However, the broad substrate specificity of the enzyme could cause unwanted effects when inhibiting γ-cleavage. A γ-secretase inhibitor would decrease Aβ generation, but also alter signaling from other proteins processed by γ-secretase. This assay provides a means to screen compounds in the search for drugs that could potently reduce Aβ generation while γ-secretase processing of other substrates, for instance Notch, remain largely unaltered.

II. APP intracellular domain formation and unaltered signaling in the presence of familial Alzheimer's disease mutations

In a report by Sastre and colleagues, the ε-cleavage site of APP and the generation of AICD were described (Sastre et al., 2001). This report and others made us realize that the cleavage assay used by us, and described in paper I, recorded AICD generation. All isolated C-terminal APP species reported so far correspond to peptide fragments starting at the ε-site (Sastre et al., 2001; Weidemann et al., 2002), and no intermediates between Aβ and AICD have been found.

Benefiting from the reporter assay developed in paper I, we wanted to describe some aspects of AICD formation that previously have been thoroughly investigated for Aβ generation. Two features were analyzed, namely subcellular localization for AICD production and the impact of familial AD mutations in APP on AICD generation.

To investigate the subcellular localization for AICD generation, cells transfected with C99-GVP were subjected to pharmacological treatment. Monensin, which inhibits transport of vesicles from the Golgi apparatus to the plasma membrane, and brefeldin A, a fungal metabolite that redistributes the Golgi apparatus into the ER were used. Both compounds decreased AICD generation (Fig. 8A,B). In addition, AICD formation for C99-GVP with an ER retrieval signal (two lysine residues were introduced into the C terminus of the molecule) was severely compromised (Fig. 8C). Thus, using pharmacological treatment and a compartment-specific mutant, it was shown that AICD generation was predominantly occurring downstream of the ER in the secretory pathway.

APP TMD mutants associated with familial AD and artificial TMD mutants, were tested for their effect on AICD generation. In cell culture systems it is known that these mutations lead to an increase in secreted Aβ42 (Selkoe, 1999). The mutants analyzed were found not to differ from the wild-type molecule in terms of AICD generation (Fig. 8D). Mutant C99-GVP and wild-type molecules had the same inhibitory profile for AICD generation using the γ-secretase inhibitor L-685,458. To ensure that the hybrid reporter molecules did not behave differently due to the insertion of the GVP-domain, Aβ ELISA analysis was performed.

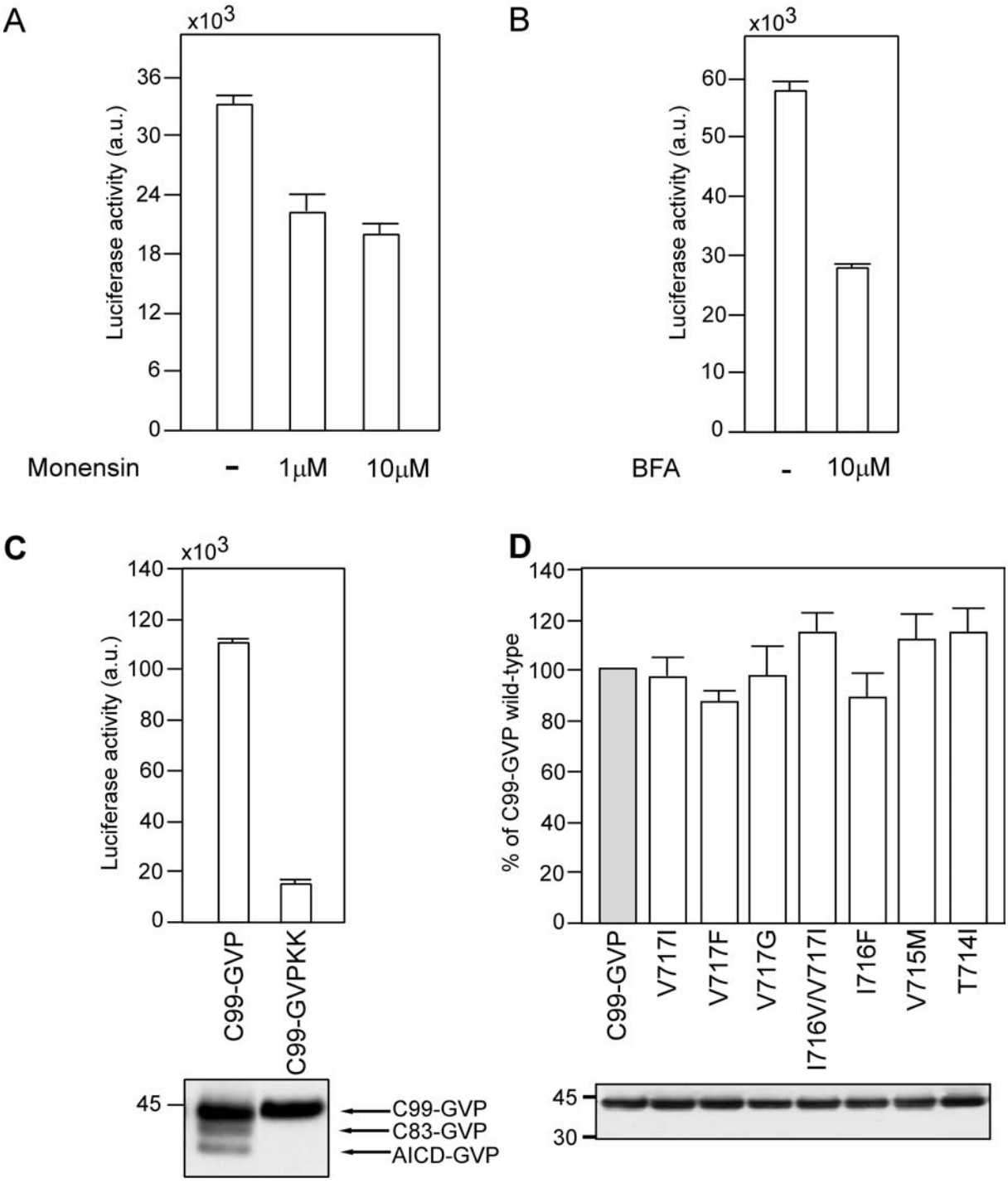

Figure 8. **A**. AICD signaling in HEK293 cells stably expressing reporter constructs for the luciferase assay after treatment with monensin (1 and 10 μM). **B**. AICD signaling in HEK293 cells stably expressing reporter constructs for the luciferase assay after treatment with brefeldin A (BFA; 10 μM). **C**. CHOPro5 cells transiently expressing C99-GVP wild-type and with an ER-retention motif (KK) were analyzed for AICD formation. In the lower panel an immunoblot is shown. Note the disappearance of AICD-GVP and C83-GVP for the ER-retained molecule. **D**. AICD signaling from C99-GVP wild-type and C99-GVP containing TMD mutations in CHOPro5 cells transiently transfected with reporter constructs for the luciferase assay. Immunoblotting is shown in the lower panel to ascertain equal expression of the constructs.

Mutant hybrid constructs showed increased secretion of Aβ42 and decreased secretion of Aβ40 compared to the wild-type C99-GVP construct. Consequently, the introduction of the GVP domain did not affect the expected behavior of the C99-GVP molecules.

AICD generation, like Aβ formation, was found to be critically dependent on PS proteins and could be inhibited by a well-characterized γ-secretase inhibitor (Shearman et al., 2000). Further, the study showed that AICD formation, again like Aβ generation, mainly occurs in compartments downstream of the ER. Both the pharmacological treatment and the ER retention motif used here, have shown diminished Aβ generation in previous studies (Peraus et al., 1997; Soriano et al., 1999; Maltese et al., 2001).

Importantly, TMD mutations known to increase Aβ42 secretion in cell culture systems, did not have an altered AICD generation compared to the wild-type molecule, indicating that total ε-processing is not noticeably affected by the TMD mutations. However, in a recent report by Sato and co-workers, cells expressing APP with familial AD mutations V717F or V717G showed a slight shift in specificity of AICD fragment formation. The mutant APP molecules mainly generated AICD starting at leucine-49 compared with wild-type APP, which mainly produced AICD starting at valine-50, as assessed by mass spectrometry analysis (Sato et al., 2003). Notably, the peak for fragments starting at residue 52 was increased for both APP molecules bearing familial AD mutations, further indicating that cleavage site specificity for AICD formation can be shifted in mutant APP. This shift in specificity strengthens the resemblance between Aβ and AICD generation, in that the site preference of both cleavages is influenced by TMD mutations. The reporter assay used here is, by its design, not able to distinguish between AICD fragments differing in length, instead it detects the total amount of AICD released. The unchanged level of AICD generation from mutations in APP found in this study was supported by the report from Chen and colleagues that observed maintained AICD production for two APP TMD mutations (Chen et al., 2002). In contrast to APP mutations, disease-causing mutations in PS1 have been shown to decrease the amount of AICD fragments formed (Chen et al., 2002; Moehlmann et al., 2002). The findings of unaltered AICD formation, but with a shift in specificity for APP TMD mutations agree with the idea of γ-secretase being one single enzyme activity performing a dual intramembrane cleavage with low sequence specificity. To conclude, the unchanged levels of AICD formation found for familial AD mutations imply that the pathological effect of the mutations are mediated via other pathways than the amount of AICD produced. These other pathways are likely to involve Aβ production, fibrillogenesis and deposition, as proposed by the amyloid cascade hypothesis (Selkoe, 1991; Hardy and Higgins, 1992).

III. Pen-2 is sequestered in the endoplasmic reticulum and subjected to ubiquitylation and proteasome-mediated degradation in the absence of presenilin

The two most recently identified members of the γ-secretase complex are the Aph-1 and Pen-2 proteins (Goutte et al., 2002; Francis et al., 2002). At the time of identification of the proteins, their functions were unknown, except that they were critically required for γ-secretase activity. The study presented in paper III aimed to describe the basic cell biological properties of the Pen-2 protein.

Firstly, Pen-2 was cloned from a cDNA library and an antibody (UD-1) towards the N-terminal part of the protein was developed. Pen-2 was found to interact with PS1 and nicastrin

by co-immunoprecipitations using a mild detergent, CHAPS, confirming the interaction reported by other groups (Luo et al., 2003a; Steiner et al., 2002). Interestingly, the N-terminal Pen-2 antibody was not able to immunoprecipitate endogenous Pen-2 protein under conditions preserving association between the γ-secretase complex components. Successful pull-down of endogenous Pen-2 by UD-1 required stronger detergents. This could indicate that the N terminus of Pen-2 might be embedded within the complex, thereby masking the epitope of the antibody. Notably, another Pen-2 antibody raised against a similar epitope showed the same immunoprecipitation characteristics (Steiner et al., 2002), suggesting that the lack of immunoprecipitation of endogenous Pen-2 under mild detergent conditions was a shared characteristic of two independent antibodies.

Subsequently, the intracellular distribution of Pen-2 was studied. The subcellular localization of Pen-2 was affected by the presence or absence of PS. In cells lacking PS, Pen-2 showed a restricted distribution to the ER, whereas in cells expressing PS1, Pen-2 was further trafficked in the secretory pathway. Trafficking of other γ-secretase complex components has been shown to be inter-related within the γ-secretase complex. In the case of nicastrin and PS1, nicastrin requires PS1 to become fully glycosylated and trafficked to the cell surface (Goutte et al., 2002; Leem et al., 2002). Similarly, PS1 requires nicastrin to reach the plasma membrane (Edbauer et al., 2002).

We observed a destabilization of the Pen-2 protein in cells devoid of PS compared to PS1 expressing cells, this being in agreement with previous studies (Steiner et al., 2002; Luo et al., 2003a). Using a range of proteasomal, lysosomal and calpain inhibitors it was shown that the destabilization of Pen-2 in PS null cells was mediated by the proteasome. Proteasomal degradation is mainly achieved by ubiquitylation, where ubiquitin moieties are conjugated to lysine residues in the target protein that will be degraded by the proteasome. Pen-2 was found to be ubiquitylated when PS1 was present in limiting amounts by transiently expressing Pen-2 in 293T cells (Fig. 9A). For further investigations of the ubiquitylation of Pen-2, it was important to determine the orientation (luminal vs. cytosolic) of the lysine residues in the protein. Hence, it was essential to establish the topology of Pen-2 within the membrane. To this end, glycosylation acceptor sites consisting of Asn-Ser-Thr tripeptides were introduced in the loop domain, and in the N- and C-terminal domain, respectively, of the Pen-2 protein. In addition, a proteinase K protection assay was performed to confirm the topological findings for Pen-2. The protein adopted an inverted hairpin structure with the N and C termini in the lumen, and a cytosolic loop between the two membrane spanning domains. Our topological findings for Pen-2 are consistent with the topology reported by Crystal and colleagues (Crystal et al., 2003). The Pen-2 molecule contains three lysine residues, and according to the topology only one of these resides in the cytosol. Mutagenesis of the cytosolic lysine into an arginine (Pen-2-K54R) was performed. In PS null cells, the lysine mutant Pen-2 molecule had an increased stability compared to the wild-type protein (Fig. 9B). However, Pen-2-K54R was still ubiquitylated (Fig. 9C), which implied that the molecule was retrotranslocated out of the ER prior to ubiquitylation and degradation. A number of ER-resident proteins, luminal as well as transmembrane, have been shown to be regulated by so-called endoplasmic reticulum associated degradation (ERAD) performed by the proteasome (Hampton, 2002). The translocon has been proposed to be involved in retrotranslocation of ERAD substrates. This retrotranslocation enable proteins to exit out of the ER into the cytosol, where they would become accessible to ubiquitylation by cytosolic E3-ligases. Pen-2 immunoreactivity was

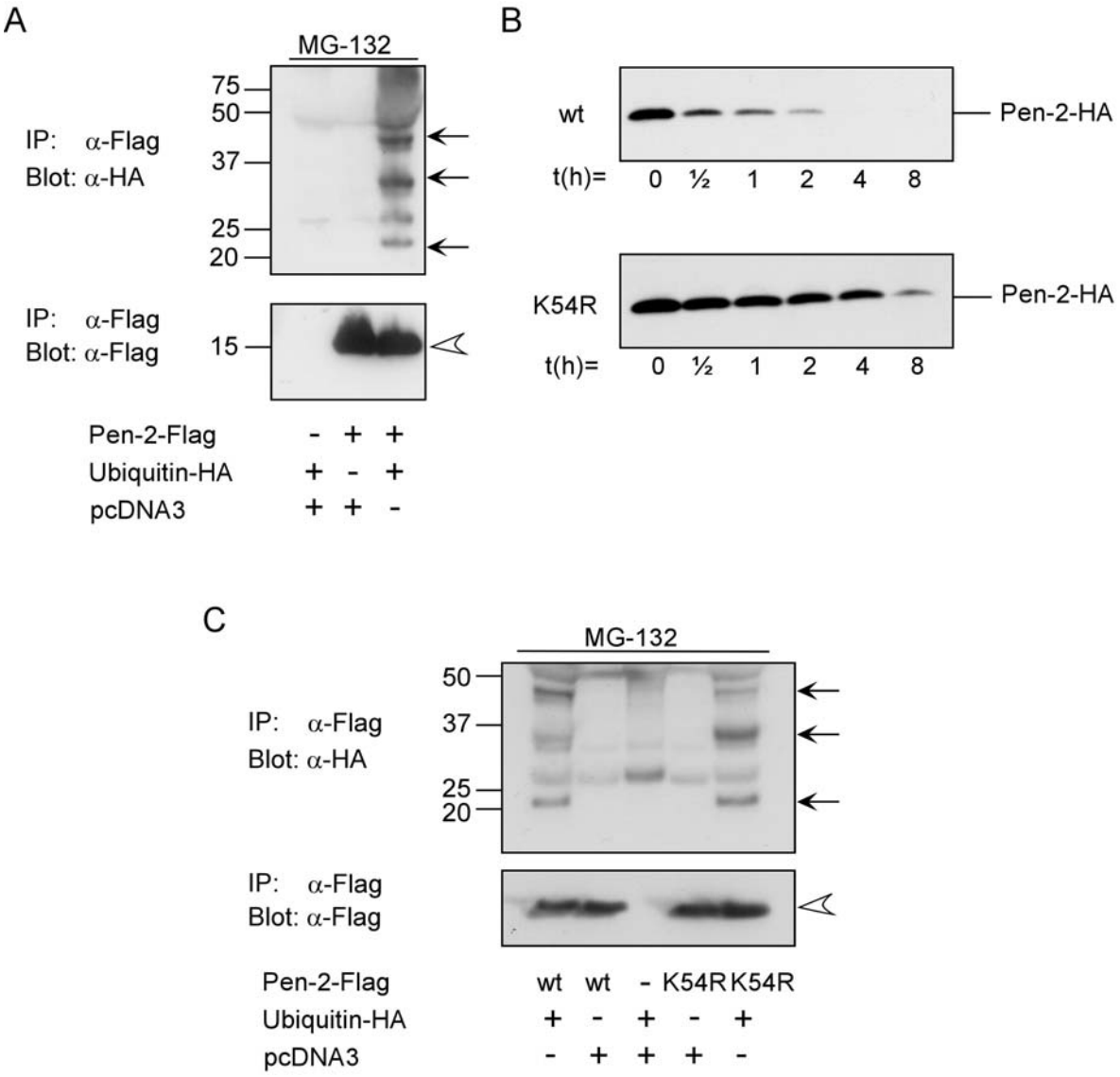

Figure 9. **A**. 293T cells were co-transfected with constructs encoding Flag-tagged Pen-2 and HA-tagged ubiquitin and then cultured in the presence of MG-132 (5 μM). Lysates were immunoprecipitated using anti-Flag M2 antibodies followed by Western blot analysis. **B**. PS null cells were transfected with wild-type (wt) and K54R mutant Pen-2-HA. The day after transfection, cells were exposed to cycloheximide (50 μg/ml) for the indicated time-points. The expression levels of the Pen-2HA constructs were analyzed by Western blot. **C**. 293T cells were co-transfected with DNA constructs encoding ubiquitin-HA and either Flag-Pen-2 or Flag-Pen-2-K54R and then exposed to MG-132 (5 μM). Lysates were immunoprecipitated using anti-Flag M2 antibodies followed by Western blot analysis. Pen-2 is indicated by an arrowhead and ubiquitylated Pen-2 species are indicated by arrows.

found in the cytosol of cells treated with proteasome inhibitor, thus suggesting that Pen-2 is retrotranslocated out of the ER prior to the addition of ubiquitin moieties. Taken together, this set of data suggested that Pen-2 molecules that are not incorporated into γ-secretase complexes could be degraded by ERAD.

Regulation of the abundance of the different γ-secretase complex components appears tightly linked. In addition to reduced levels of Pen-2, absence of PS has been shown to destabilize nicastrin (Leem et al., 2002; Edbauer et al., 2002). The degradation pathway mediating decreased levels of nicastrin has not yet been identified. Down regulation of protein levels using siRNA of either nicastrin, Aph-1 or Pen-2 was reported to result in decreased amounts of PS (Francis et al., 2002). Interestingly, PS1 and PS2 have been shown to be ubiquitylated (Li et al., 2002b; Kim et al., 1997), and the level of full-length PS1 to be regulated by the proteasome (Steiner et al., 1998). This could indicate that ERAD-mediated degradation is involved in regulating cellular levels of other γ-secretase complex components in addition to Pen-2. Hypothetically, ERAD could constitute the common cellular mechanism that removes unincorporated γ-secretase complex components. However, more studies are needed before detailed conclusions regarding the degradation pathways for nicastrin, Aph-1 and PS can be made.

IV. The extreme C terminus of presenilin 1 is required for γ-secretase complex assembly and activity

The C terminus of PS1 has been shown to be important for γ-secretase cleavage of APP (Tomita et al., 1999; Tomita et al., 2001). In order to further investigate the most C-terminal domain of PS1, point mutations and truncations were introduced in the PS1 C terminus. Modified PS1 molecules were analyzed in PS-deficient cells by the γ-secretase assay described in paper I. The single point mutations analyzed did not affect γ-secretase activity or endoproteolysis. However, PS1 proteins with truncations ranging from three to seventeen residues in length showed decreased γ-secretase activity, with molecules containing the largest deletions exhibiting the most severely impaired function (Fig. 10A, upper panel). To further elucidate the impact of the truncations, the ability of C-terminally deleted PS1 proteins to become incorporated into γ-secretase complexes was investigated by co-immunoprecipitations. PS1 molecules with C-terminal deletions larger than four residues were found to have a reduced ability to incorporate mature γ-secretase complexes.

Furthermore, the truncations caused reduced endoproteolysis (Fig. 10A, lower panel). To elucidate whether the impairment in activity and complex formation was due to reduced endoproteolysis, or if the reduced functionality could be directly attributed to the truncations, NTF was co-expressed with C-terminally truncated CTF. With this experimental setup it was possible to circumvent the endoproteolysis step of PS1 (Laudon et al., 2004). The reduction observed in γ-secretase activity and γ-secretase complex formation for the truncated PS1 molecules was found to not be an effect of reduced endoproteolysis *per se*. NTF together with C-terminally truncated CTF displayed the same pattern of severely impaired γ-secretase activity and complex formation as observed for the truncated full-length protein. While the interaction of PS1 with nicastrin and Aph-1 was perturbed by the deletions, heterodimer

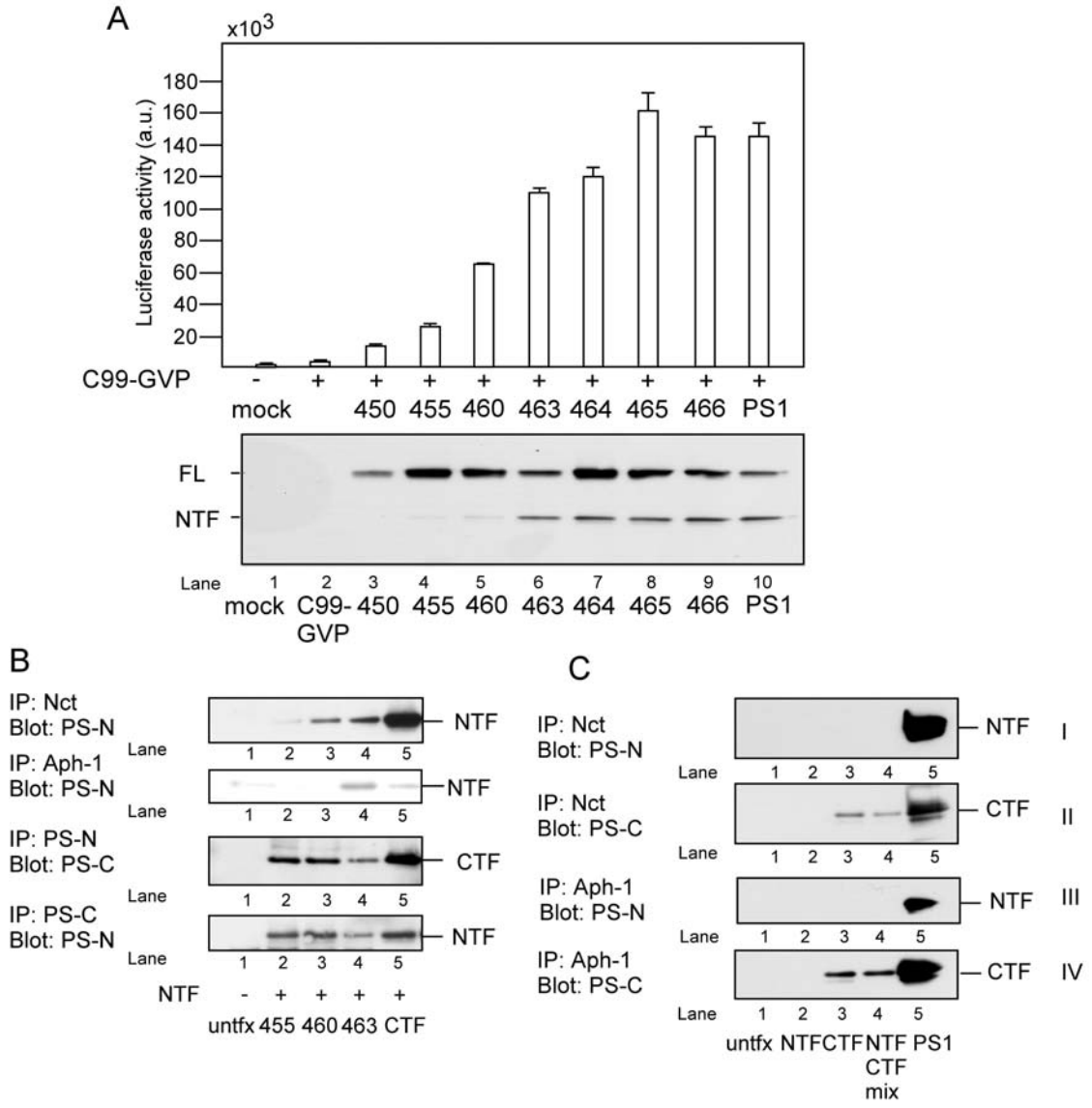

Figure 10. **A**. Luciferase activity was monitored to measure AICD generation from the C99-GVP reporter molecule in PS-deficient BD8 cells transfected with PS1 wild-type or PS1 having C-terminal deletions lacking one (466), two (465), three (464), four (463), seven (460), twelve (455) or seventeen (450) residues, respectively. In the lower panel an immunoblot of the cell lysates from the luciferase measurements is shown. Note the decrease in endoproteolysis for PS1 with the larger deletions. **B**. Co-immunoprecipitation with indicated antibodies of BD8 cell lysates co-transfected with wild-type NTF and wild-type CTF or CTF with C-terminal truncations of four, seven or twelve residues, respectively. Interaction of NTF and CTF with nicastrin (Nct) or Aph-1 was decreased for C-terminally truncated CTF molecules compared to CTF wild-type (the two upper panels). Note that the interaction between NTF and CTF was intact even when the CTF was C-terminally truncated (the two lower panels). **C**. Cell lysates from PS nulls stably transfected with PS1 wild-type, NTF or CTF were immunoprecipitated with anti-Nct or anti-Aph-1 antibodies. NTF associated with Nct in PS1 wild-type expressing cells, but not in cells expressing NTF alone, or in mixed cell lysates from NTF and CTF cells (panel I). CTF associated with Nct in cells expressing PS1 wild-type or CTF, as well as in NTF mixed with CTF lysates (panel II). NTF co-precipitated with Aph-1 in PS1 wild-type expressing cells, but not in cells expressing NTF alone, or in cell lysates mixed from NTF and CTF cells (panel III). CTF co-purified with Aph-1 in lysates from cells expressing PS1 wild-type, and CTF, and in mixed NTF/CTF lysates (panel IV).

formation between the PS1 endoproteolytic products, NTF and CTF, was maintained (Fig. 10B). For further investigations of the C terminus of PS1, co-immunoprecipitations were performed from cells expressing PS1 NTF or CTF on a PS null background. Unexpectedly, nicastrin and Aph-1 interacted with the CTF alone (Fig. 10C). Supposedly, the C-terminal part of PS1 could promote a direct interaction with nicastrin and Aph-1, and this contact could be lost when the PS1 molecule was truncated.

Taken together, we found that the most C-terminal part of PS1 was required for intact γ-secretase activity and complex formation. Earlier studies of point and deletion mutations in the C-terminal domains of PS1 and PS2 molecules have shown an effect on Aβ secretion by increasing the Aβ42 to Aβ40 ratio (Tomita et al., 1999). These studies were performed in cells expressing endogenous PS. Replacement of endogenous PS by transfection can be challenging, and the PS molecules already present in the cells can hide effects from the exogenously expressed PS. Our findings, in terms of reduced endoproteolysis, agree with the report from Tomita and co-workers regarding truncated PS1 molecules. In contrast, a point mutation, I467R, was impaired in endoproteolysis in the Tomita et al. study, whereas we found it fully functional and undergoing endoproteolysis to the same extent as wild-type PS1. Our data showed that truncations of the PS1 C terminus severely impaired γ-secretase function. This effect was most likely a result from the lack of the C-terminal amino acids, and not from the reduction in endoproteolysis that the truncations induced. This conclusion was made since NTF together with C-terminally truncated CTF showed the same reduction in γ-secretase activity and complex formation as the truncated full-length protein. It is noteworthy that even though the association between PS1 and other γ-secretase complex components was lost for the truncated constructs, the interaction between NTF and CTF remained intact, suggesting that the intramolecular association is mediated by different parts of the PS1 molecule than those critically required for association with nicastrin and Aph-1. By detergent dissociation of the high-molecular weight γ-secretase complex Fraering and colleagues have been able to detect four subcomplexes (Fraering et al., 2004). One of the subcomplexes contained NTF and CTF, and another subcomplex consisted of CTF, nicastrin and Aph-1. Our data are in agreement with this report, and could provide the additional information that the presence of full-length PS1 or NTF is not a prerequisite for interaction between CTF, nicastrin and Aph-1. In studies by others, an Aph-1-nicastrin subcomplex has been proposed (LaVoie et al., 2003; Hu and Fortini, 2003; Fraering et al., 2004). The function of this subcomplex, or of Aph-1 alone, has been suggested to stabilize the full-length PS1 protein. However, the interaction detected here, between the endoproteolytic product CTF, nicastrin and Aph-1 could indicate that there are additional functions of the subcomplex components also after endoproteolysis of full-length PS1. This unusual enzyme activity has proved very challenging to understand since each component requires the presence of the other components so as to be functional. Future studies are needed to understand how PS1 interacts with the other γ-secretase components during folding and assembly to form an active γ-secretase complex.

CONCLUSIONS AND FUTURE PERSPECTIVES

Aβ is undoubtedly a key player in the pathophysiological events causing AD. Our understanding of the generation of Aβ has been greatly increased by the identification, cloning and characterization of the enzymes involved in processing of APP. Research regarding the generation of the Aβ C terminus is currently focused on PS, a central molecule in the multiprotein γ-secretase complex. PS has been found to process not only APP, but a wide range of type I membrane proteins. PS is thus an important enzyme that regulates a variety of cellular functions, such as development via Notch signaling and cell adhesion via E-cadherin (Levitan and Greenwald, 1995; Marambaud et al., 2003).

Development of a cell-based reporter assay for γ-secretase substrates provides a good tool for studying intramembrane cleavage. The assay described in paper I includes several attractive features as it is sensitive, quantitative, and records the total γ-secretase processing of reporter molecules within whole cells. An additional advantage with the reporter system is that γ-cleavage is monitored from the reporter molecules *per se*, and not from endogenous γ-secretase substrates. Thereby it enables studies of effects on intramembrane processing from specific mutations or alterations introduced into the reporter molecules, in the absence of effects attributable to endogenous proteins. In the search for an efficient Aβ-lowering drug, the reporter assay could potentially be used to screen for compounds differentially affecting the processing of APP and other γ-secretase substrates.

The identification of AICD generation from the ε-site in APP, a cleavage site C-terminal to the Aβ-generating site, raised a number of questions pertaining to this newly described processing event. In paper II, we addressed two aspects of AICD formation. Intracellularly, AICD generation was observed in a compartment downstream of the ER, thereby overlapping with sites reported for production of Aβ. Furthermore, APP reporter molecules harboring mutations that cause familial AD were found to generate AICD to the same extent as wild-type molecules. Hence, we concluded that the pathogenic mechanism of the mutations is not acting via altered AICD formation. It is intriguing that AD causing mutations in APP and PS1 can have a differential effect on AICD generation (Chen et al., 2002; Moehlmann et al., 2002). Hypothetically, the mutations in APP might slightly shift the positioning of the γ- and ε-cleavages sites within the membrane, thereby altering the length but not the amount of generated fragments. In contrast, mutations in PS1, might inflict a conformational change within the molecule. This could affect the PS active site, changing both efficiency and specificity in substrate cleavage. Alternatively, the mutations could cause an altered trafficking of the molecules to compartments where cleavage normally does not occur. Different intracellular compartments have varying membrane thickness, which could affect the positioning of intramembrane hydrolysis. It seems likely that the membrane lipid milieu affects γ-cleavage since cholesterol depletion reduces Aβ generation (Simons et al., 1998), and thus an altered trafficking would have an impact on the production of Aβ. These are presently speculations that are not easily addressed, and the underlaying questions need to be approached with more research.

It is now widely believed that PS is the enzymatically active component of the γ-secretase complex and that the other γ-secretase complex components regulate and provide functions required for PS to become an efficient aspartyl protease. However, the precise function of the γ-secretase complex components and the intricate regulation within the complex is largely

unsolved due to difficulties in analyzing each protein separately in the absence of the other components. In paper III we studied the Pen-2 protein in cells with and without PS. The subcellular distribution of Pen-2 was restricted to ER and Pen-2 was destabilized in the absence of PS, in accordance with data presented for the other γ-secretase complex components (De Strooper, 2003). PS and nicastrin have been shown to inter-regulate each other's cellular distribution, and both proteins are dependent on Aph-1 for stability and maturation. The destabilization observed for Pen-2 in PS null cells was mediated by ubiquitylation and proteasomal degradation. It would be interesting to investigate the degradation pathway for the other γ-secretase complex components to determine if there is a common cellular mechanism regulating the level of unincorporated γ-secretase proteins. Pen-2 has been suggested to be required for endoproteolysis of PS, however no motif with proteolytic function has so far been found in the molecule (Luo et al., 2003a). It would be interesting to search the Pen-2 molecule for functional domains, and to identify the part of the molecule that mediates interaction with the γ-secretase complex and is required for sustaining endoproteolysis of PS. The tight regulation of the γ-secretase complex makes studies of the individual components very challenging. Hence, these proposed studies would benefit from being performed in Pen-2 null cells. Such cells could potentially be obtained from a Pen-2 knockout mouse, for example the one developed by Jinhe Li and co-workers (Li et al., 2002a). Alternatively, endogenous Pen-2 could be down regulated by siRNA.

Intense research efforts have much improved our knowledge about PS1. Several domains important for γ-secretase function have been identified in the PS1 molecule. In paper IV, we identify the most C-terminal domain of PS1 to be critical for γ-secretase activity and complex assembly. Wild-type CTF alone could associate with Aph-1 and nicastrin, whereas C-terminal deletions of full-length PS1 abrogated the interaction with nicastrin and Aph-1. This led us to speculate that nicastrin and/or Aph-1 could be physically interacting with the most C-terminal residues in PS1. However, we were not able, using the present experimental setup, to identify which amino acids mediate the association between the proteins. In addition, with the high number of hydrophobic domains in the molecules, it is likely that important interactions are occurring within the membrane lipid bilayer. It would be very interesting to directly identify which residues are participating in the association between the different complex components. Experimentally this could potentially be achieved by performing cross-linking studies.

Major progress has been made by identifying the γ-secretase components. However, we are only just beginning to understand the complexity of this intriguing enzymatic activity. Further studies are needed to obtain detailed information about the different components, in order to acquire a more complete depiction of the events underlying generation of Aβ. In particular, it would be beneficial to distinguish possible APP specific features in the γ-secretase complex. This type of knowledge would increase the chances of inhibiting Aβ production, while minimizing the adverse side effects from perturbed signaling of other γ-secretase substrates. Our understanding of the molecules involved in the disease is rapidly growing, thus the prospect of developing a causal treatment for AD is steadily increasing. Hopefully, in the years to come, success will be attained in this important endeavor.

MATERIAL AND METHODS

DNA constructs and mutagenesis

DNA constructs used in papers I-IV were encoding the following proteins PS1, NTF, CTF, C99-GVP, Notch ΔE-GVP, luciferase, β-galactosidase, ubiquitin, and Pen-2. Mutagenesis was performed to generate point and deletion mutations in DNA constructs for PS1, CTF, C99-GVP and Pen-2. The PCR-based QuickChange site-directed mutagenesis protocol (Stratagene) was applied using complementary primers encoding the desired mutation. All constructs were verified by sequencing using either DYEnamic terminators (Amersham) or BigDye (Applied Biosystems) sequencing kits. For generation of PS1, NTF and CTF stable cell lines, the desired constructs were cloned into the pCAGiRESpuro vector with puromycin resistance.

Cell culture and transfections

PS-deficient cells (BD8 cells) derived from blastocysts from $PS1^{-/-}PS2^{-/-}$ mice were a kind gift from Dorit Donoviel (Donoviel et al., 1999). Culturing of BD8 cells was performed in DMEM supplemented with 10% fetal calf serum, 2.4 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, and non-essential amino acids. HEK293 and CHOPro5 cells were used for studies where cells expressing endogenous PS were suitable for the experimental set-up. HEK293 and CHOPro5 cells were cultured in DMEM and α-MEM, respectively, supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

Transfections were performed with Lipofectamine Plus (Life Technologies) according to the manufacturer's recommendations. Cells from transient transfections were analyzed one or two days after the transfection. Stably expressing BD8 cells were generated by selection with 1 μg/ml of puromycin. Single clones were analyzed with Western blot for expression, and expanded.

Luciferase assay

In paper I the luciferase assay was developed, and in papers II and IV this assay was used to measure AICD generation from the C99-GVP hybrid reporter molecule. The luciferase-based reporter assay was performed in 24-well tissue culture plates. Cells were plated at a suitable density the day before transfection. Cells were transfected with 100 ng MH100, 50 ng CMV-βgal, and 100 ng of C99-GVP or Notch ΔE-GVP, per well. Vectors encoding full-length PS1 (100 ng) or PS1 fragments (100 ng + 100 ng) were included for transfections of PS-deficient cells. Empty pcDNA3.1 vector (100 ng) was added to adjust for differences in DNA amounts. Within each luciferase reporter experiment, triplicates were performed, i.e. three wells were transfected with each of the DNA constructs analyzed. Cells were harvested 24 h after transfection in 100 μl lysis buffer per well (10 mM Tris, pH 8, 1 mM EDTA, 150 mM NaCl and 0.65% NP40), and luciferase activity was monitored luminometrically after addition of luciferin and ATP (BioThema). The β-galactosidase activity of the cell lysates was determined by measuring absorbance at 405 nm in β-gal buffer (10 mM KCl, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 50 μM β-mercaptoethanol and 8 mM O-nitrophenyl-β-D-galactopyranoside), to equalize for differences in transfection efficiencies.

ELISA

To ensure that the hybrid C99-GVP molecules did not show an altered processing due to the insertion of the GVP domain into the molecule, we performed ELISA in paper II. Cells were transfected with cDNA encoding C99-GVP wild-type or with mutations reported to increase secretion of Aβ42 into cell media. After transfection, cells were grown in OptiMEM media with 5% newborn calf serum. The media was harvested two days after transfection and analyzed by a sandwich ELISA using 6E10 (Senetech) as capture antibody and detection of Aβ40 and Aβ42 was mediated by polyclonal end-specific antibodies.

Immunoprecipitations

Immunoprecipitations were performed in papers III and IV from cells with stable or transient expression of the protein of interest. The cells were grown in 10 cm or 6-well tissue culture dishes. Cells were lysed in immunoprecipitation buffer, and all subsequent incubations were carried out at 4°C. Co-immunoprecipitations were performed in buffer containing CHAPS (paper III) or CHAPSO (paper IV). For direct immunoprecipitations in paper III, either buffers containing Triton X-100 and NP-40 (to precipitate Pen-2 with UD-1 antibody) or with SDS, sodium deoxycholate, and Triton X-100 supplemented with NEM (to precipitate ubiquitylated Pen-2) were used. The immunoprecipitations were prepared by pre-clearing cell lysates with protein A and G sepharose (Amersham) for 30 min. Primary antibody was incubated with the lysates with end-over-end rotation over night (or 1h for precipitation of ubiquitylated Pen-2). Subsequently, a mixture of protein A and G sepharose was added to the samples, and the incubation continued for 1 h. The immunoprecipitates were washed three times in immunoprecipitation buffer and once in PBS prior to Western blot analysis.

Western blot

In papers I-IV immunoblotting was performed. Specific details for the antibodies used are described in each paper. In brief, cell lysates or immunoprecipitates were incubated with Laemmli sample buffer (Sigma). The samples were loaded on precasted 10%, 16%, 10-20% Tris-Tricine gels or 4-12% Bis-Tris gels (Invitrogen), and resolved using electrophoresis. The proteins were transferred to nitrocellulose membrane (Bio-Rad), and incubated with indicated antibodies. The proteins were visualized with SuperSignal West Pico chemiluminescence (Pierce), and exposure on Hyperfilm ECL (Amersham). For indicated experiments in papers III and IV, protein concentration of the cell lysates was determined using BCA protein assay kit (Pierce), and equal amount of protein was loaded from each sample. In paper III, the intensity of immunoreactive bands was determined using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Antibodies

In paper III, the polyclonal antibody UD-1 towards Pen-2 was raised against the N-terminal residues ERVSNEEKLNL of Pen-2. Antibodies used for immunoprecipitations, immunoblotting and immunocytochemical experiments were anti-APP CTF 369 and C1/6.1 (generously provided by Drs. Sam Gandy and Paul Mathews, respectively), anti-calnexin (a kind gift from Dr. Ralf Pettersson), anti-Flag M2 (Sigma), anti-HA 12CA5 (Berkeley Antibodies Inc.), anti-GM130 (BD Transduction labs), anti-nicastrin N1660 (Sigma), anti-PS1 NTF Ab14 and NT1 (generously supplied by Drs. Sam Gandy and Paul Mathews, respectively), anti-PS1 loop MAB5232 and AB5308 (Chemicon), anti-Ubiquitin (Dako) and anti-Aph-1a^{L} H2D (Calbiochem).

Pharmacological treatment

Different pharmacological treatments were used in papers I, II and III. Concentrations used and incubation times are detailed in the papers. Inhibition of γ-secretase was achieved by using the specific inhibitors L-685,458, MW167 and DAPT. In paper II, brefeldin A and monensin, agents disturbing intracellular trafficking, were used. In paper III we wanted to elucidate the degradation pathway for Pen-2 in PS-deficient cells. We used the proteasomal inhibitors lactacystin, MG-132 and ALLN. For lysosomal inhibition chloroquine was used and for calpain inhibition, cells were treated with calpeptin. Further in paper III, stability of wild-type Pen-2 was compared with Pen-2 K54R in PS null cells. Cycloheximide was added to transfected BD8 cells to inhibit protein synthesis. The degradation rate of the two different Pen-2 proteins was assessed in a time-course experiment by Western blotting.

Topological study

The topology of Pen-2 was determined in paper III. Glycosylation acceptor sites consisting of Asn-Ser-Thr tripeptides were introduced by site-directed mutagenesis in the loop, N-, and C-terminal domain of the Pen-2 protein. DNA constructs encoding Pen-2 with glycosylation acceptor sites were transiently expressed in CHOPro5 cells and analyzed by Western blotting. Retarded migration of proteins in Western blot analysis indicated glycosylated species. Deglycosylation was performed to verify that the shift in electrophoretic mobility was truly caused by the addition of carbohydrates. The deglycosylation assay was performed in a buffer consisting of 10 mM Hepes, pH 7.4, 250 mM sucrose, 200 mM sodium citrate, 10 mM KCl, 100 mM NaCl, 5 mM EDTA+EGTA, 1.5 mM $MgCl_2$, 0.3% SDS, 0.6% β-mercaptoethanol and protease inhibitors. Endoglycosidase H (Roche) and peptide N-glycosidase F (Roche) were added to the deglycosylation reactions, and the reactions were incubated at 37°C for 16 hours followed by Western blot analysis.

The proteinase K protection assay was carried out at 4°C. Membranes prepared from CHOPro5 cells transfected with cDNA encoding Pen-2-HA were exposed to proteinase K (100 μg/ml) for 30 min and subsequently analyzed by Western blotting.

Subcellular fractionation

In paper III, subcellular fractionation was performed to analyze the cellular distribution of Pen-2 in PS null cells and cells expressing PS1. Cells grown in 15 cm tissue culture plates to near confluency were used for fractionation experiments. The cells were homogenized in ice-cold homogenization buffer (130 mM KCl, 25 mM NaCl, 1 mM EGTA, 25 mM Tris, pH 7.4) supplemented with protease inhibitors using a Dounce homogenizer. Lysates were cleared by centrifugation at 1,000 x g for 10 minutes. The supernatant was overlayed onto the top of a step gradient consisting of 1 ml each of 30, 25, 20, 15, 12.5, 10, 7.5, and 5% (vol/vol) Iodixanol (OptiPrep reagent, Axis-Shield PoC AS) in homogenization buffer. After a 3 hour centrifugation at 126,000 x g (SW40 rotor, Beckman), 12 fractions were collected from the top of the gradient. Fractions were analyzed by Western blotting.

Immunocytochemistry

To determine the intracellular localization for Pen-2 under different conditions (in paper III) immunocytochemistry was performed. Cells were seeded out on glass slides, and in some experiments transfected with indicated constructs. Cells were either treated with empty vehicle (DMSO) or with different pharmacological agents prior to analysis. The cells were then fixed with 4% formaldehyde in PBS (pH 7.4) at 4°C for 15 min, blocked for 1 h at room temperature in blocking solution (PBS supplemented with 5% BSA, 10% goat serum, and 0.3% Triton X-100), and stained with primary antibodies at 4°C over night. Following extensive washing in PBS, the cells were incubated with Alexa 546- and 488-conjugated goat anti-mouse and goat anti-rabbit antibodies (Molecular Probes) and DAPI in darkness for 40 minutes. After thorough washing in PBS, the cells were mounted in ProLong mounting medium (Molecular Probes). Immunoreactivity was visualized in a Zeiss Axioplan2 microscope and photographed using a Zeiss Axiocam. The subcellular localization of Pen-2 in the presence or absence of PS1 was determined using a BioRad Radiance confocal microscopy unit. Pictures were assembled using PhotoShop (Adobe).

REFERENCES

Aguilar, R. C. and Wendland, B. (2003). Ubiquitin: not just for proteasomes anymore. *Curr Opin Cell Biol* **15**, 184-90.

Alvarez, R., Alvarez, V., Lahoz, C. H., Martinez, C., Pena, J., Sanchez, J. M., Guisasola, L. M., Salas-Puig, J., Moris, G., Vidal, J. A., Ribacoba, R., Menes, B. B., Uria, D. and Coto, E. (1999). Angiotensin converting enzyme and endothelial nitric oxide synthase DNA polymorphisms and late onset Alzheimer's disease. *J Neurol Neurosurg Psychiatry* **67**, 733-6.

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

American Psychiatric Association. (1994). Committee on Nomenclature and Statistics. Dignostical and statistical manual of mental disorders 4th edn. Washington DC., 133-155.

Bales, K. R., Verina, T., Dodel, R. C., Du, Y., Altstiel, L., Bender, M., Hyslop, P., Johnstone, E. M., Little, S. P., Cummins, D. J., Piccardo, P., Ghetti, B. and Paul, S. M. (1997). Lack of apolipoprotein E dramatically reduces amyloid beta-peptide deposition. *Nat Genet* **17**, 263-4.

Bard, F., Cannon, C., Barbour, R., Burke, R. L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Lieberburg, I., Motter, R., Nguyen, M., Soriano, F., Vasquez, N., Weiss, K., Welch, B., Seubert, P., Schenk, D. and Yednock, T. (2000). Peripherally administered antibodies against amyloid β-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. *Nat Med* **6**, 916-9.

Bennett, B. D., Babu-Khan, S., Loeloff, R., Louis, J. C., Curran, E., Citron, M. and Vassar, R. (2000). Expression analysis of BACE2 in brain and peripheral tissues. *J Biol Chem* **275**, 20647-51.

Berezovska, O., Jack, C., McLean, P., Aster, J. C., Hicks, C., Xia, W., Wolfe, M. S., Kimberly, W. T., Weinmaster, G., Selkoe, D. J. and Hyman, B. T. (2000). Aspartate mutations in presenilin and γ-secretase inhibitors both impair notch1 proteolysis and nuclear translocation with relative preservation of notch1 signaling. *J Neurochem* **75**, 583-93.

Berezovska, O., Ramdya, P., Skoch, J., Wolfe, M. S., Bacskai, B. J. and Hyman, B. T. (2003). Amyloid precursor protein associates with a nicastrin-dependent docking site on the presenilin 1-γ-secretase complex in cells demonstrated by fluorescence lifetime imaging. *J Neurosci* **23**, 4560-6.

Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J. and Cerretti, D. P. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. *Nature* **385**, 729-33.

Blacker, D., Wilcox, M. A., Laird, N. M., Rodes, L., Horvath, S. M., Go, R. C., Perry, R., Watson, B., Jr., Bassett, S. S., McInnis, M. G., Albert, M. S., Hyman, B. T. and Tanzi, R. E. (1998). Alpha-2 macroglobulin is genetically associated with Alzheimer disease. *Nat Genet* **19**, 357-60.

Blaumueller, C. M., Qi, H., Zagouras, P. and Artavanis-Tsakonas, S. (1997). Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell* **90**, 281-91.

Braak, H. and Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol (Berl)* **82**, 239-59.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., Cumano, A., Roux, P., Black, R. A. and Israel, A. (2000). A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol Cell* **5**, 207-16.

Brown, M. S., Ye, J., Rawson, R. B. and Goldstein, J. L. (2000). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* **100**, 391-8.

Buxbaum, J. D., Liu, K.-N., Luo, Y., Slack, J. L., Stocking, K. L., Peschon, J. J., Johnson, R. S., Castner, B. J., Cerretti, D. P. and Black, R. A. (1998). Evidence that Tumor necrosis factor α converting enzyme is involved in regulated α-secretase cleavage of the Alzheimer amyloid protein precursor. *J Biol Chem* **273**, 27765-27767.

Cai, H., Wang, Y., McCarthy, D., Wen, H., Borchelt, D. R., Price, D. L. and Wong, P. C. (2001). BACE1 is the major β-secretase for generation of Aβ peptides by neurons. *Nat Neurosci* **4**, 233-4.

Campbell, W. A., Reed, M. L., Strahle, J., Wolfe, M. S. and Xia, W. (2003). Presenilin endoproteolysis mediated by an aspartyl protease activity pharmacologically distinct from γ-secretase. *J Neurochem* **85**, 1563-74.

Cao, X. and Südhof, T. C. (2001). A transcriptively active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science* **293**, 115-120.

Capell, A., Grunberg, J., Pesold, B., Diehlmann, A., Citron, M., Nixon, R., Beyreuther, K., Selkoe, D. J. and Haass, C. (1998). The proteolytic fragments of the Alzheimer's disease-associated presenilin-1 form heterodimers and occur as a 100-150-kDa molecular mass complex. *J Biol Chem* **273**, 3205-11.

Capell, A., Steiner, H., Romig, H., Keck, S., Baader, M., Grim, M. G., Baumeister, R. and Haass, C. (2000a). Presenilin-1 differentially facilitates endoproteolysis of the β-amyloid precursor protein and Notch. *Nat Cell Biol* **2**, 205-11.

Capell, A., Steiner, H., Willem, M., Kaiser, H., Meyer, C., Walter, J., Lammich, S., Multhaup, G. and Haass, C. (2000b). Maturation and pro-peptide cleavage of β-secretase. *J Biol Chem* **275**, 30849-54.

Checler, F., da Costa, C. A., Ancolio, K., Chevallier, N., Lopez-Perez, E. and Marambaud, P. (2000). Role of the proteasome in Alzheimer's disease. *Biochim Biophys Acta* **1502**, 133-8.

Checler, F. (2001). The multiple paradoxes of presenilins. *J Neurochem* **76**, 1621-7.

Chen, F., Gu, Y., Hasegawa, H., Ruan, X., Arawaka, S., Fraser, P., Westaway, D., Mount, H. and St George-Hyslop, P. (2002). Presenilin 1 mutations activate γ_{42}-secretase but reciprocally inhibit ε-secretase cleavage of amyloid precursor protein (APP) and S3-cleavage of notch. *J Biol Chem* **277**, 36521-6.

Chung, H. M. and Struhl, G. (2001). Nicastrin is required for Presenilin-mediated transmembrane cleavage in Drosophila. *Nat Cell Biol* **3**, 1129-32.

Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., Roses, A. D., Haines, J. L. and Pericak-Vance, M. A. (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261**, 921-3.

Creemers, J. W., Ines Dominguez, D., Plets, E., Serneels, L., Taylor, N. A., Multhaup, G., Craessaerts, K., Annaert, W. and De Strooper, B. (2001). Processing of β-secretase by furin and other members of the proprotein convertase family. *J Biol Chem* **276**, 4211-7.

Crystal, A. S., Morais, V. A., Pierson, T. C., Pijak, D. S., Carlin, D., Lee, V. M. and Doms, R. W. (2003). Membrane topology of γ-secretase component PEN-2. *J Biol Chem* **278**, 20117-23.

Cupers, P., Bentahir, M., Craessaerts, K., Orlans, I., Vanderstichele, H., Saftig, P., De Strooper, B. and Annaert, W. (2001). The discrepancy between presenilin subcellular localization and γ-secretase processing of amyloid precursor protein. *J Cell Biol* **154**, 731-40.

Dahlgren, K. N., Manelli, A. M., Stine, W. B., Jr., Baker, L. K., Krafft, G. A. and LaDu, M. J. (2002). Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability. *J Biol Chem* **277**, 32046-53.

de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. and Conlon, R. A. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124**, 1139-48.

De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K. and Van Leuven, F. (1998). Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391**, 387-90.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A. and Kopan, R. (1999). A presenilin-1-dependent γ-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518-22.

De Strooper, B. (2003). Aph-1, Pen-2, and Nicastrin with Presenilin generate an active gamma-Secretase complex. *Neuron* **38**, 9-12.

Donoviel, D. B., Hadjantonakis, A.-K., Ikeda, M., Zheng, H., St George-Hyslop, P. and Bernstein, A. (1999). Mice lacking both presenilin genes exhibit early embryonic patterning defects. *Genes Devel* **13**, 2801-2810.

Dovey, H. F., John, V., Anderson, J. P., Chen, L. Z., de Saint Andrieu, P., Fang, L. Y., Freedman, S. B., Folmer, B., Goldbach, E., Holsztynska, E. J., Hu, K. L., Johnson-Wood, K. L., Kennedy, S. L., Kholodenko, D., Knops, J. E., Latimer, L. H., Lee, M., Liao, Z., Lieberburg, I. M., Motter, R. N., Mutter, L. C., Nietz, J., Quinn, K. P., Sacchi, K. L., Seubert, P. A., Shopp, G. M., Thorsett, E. D., Tung, J. S., Wu, J., Yang, S., Yin, C. T., Schenk, D. B., May, P. C., Altstiel, L. D., Bender, M. H., Boggs, L. N., Britton, T. C., Clemens, J. C., Czilli, D. L., Dieckman-McGinty, D. K., Droste, J. J.,

Fuson, K. S., Gitter, B. D., Hyslop, P. A., Johnstone, E. M., Li, W. Y., Little, S. P., Mabry, T. E., Miller, F. D. and Audia, J. E. (2001). Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J Neurochem* **76**, 173-81.

Dyrks, T., Weidemann, A., Multhaup, G., Salbaum, J. M., Lemaire, H. G., Kang, J., Muller-Hill, B., Masters, C. L. and Beyreuther, K. (1988). Identification, transmembrane orientation and biogenesis of the amyloid A4 precursor of Alzheimer's disease. *EMBO J* **7**, 949-57.

Edbauer, D., Winkler, E., Haass, C. and Steiner, H. (2002). Presenilin and nicastrin regulate each other and determine amyloid β-peptide production via complex formation. *Proc Natl Acad Sci U S A* **99**, 8666-71.

Edbauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H. and Haass, C. (2003). Reconstitution of γ-secretase activity. *Nat Cell Biol* **5**, 486-8.

Eggert, S., Paliga, K., Soba, P., Evin, G., Masters, C. L., Weidemann, A. and Beyreuther, K. (2004). The proteolytic processing of the amyloid precursor protein gene family members APLP-1 and APLP-2 involves α-, β-, γ-, and ε-like cleavages. Modulation of APLP-1 processing by N-glycosylation. *J Biol Chem*, In press 10.1074/jbc.M311601200.

Esler, W. P., Kimberly, W. T., Ostaszewski, B. L., Diehl, T. S., Moore, C. L., Tsai, J. Y., Rahmati, T., Xia, W., Selkoe, D. J. and Wolfe, M. S. (2000). Transition-state analogue inhibitors of γ-secretase bind directly to presenilin-1. *Nat Cell Biol* **2**, 428-34.

Farmery, M. R., Tjernberg, L. O., Pursglove, S. E., Bergman, A., Winblad, B. and Näslund, J. (2003). Partial purification and characterization of γ-secretase from post-mortem human brain. *J Biol Chem* **278**, 24277-84.

Fortna, R. R., Crystal, A. S., Morais, V. A., Pijak, D. S., Lee, V. M. and Doms, R. W. (2004). Membrane topology and nicastrin-enhanced endoproteolysis of APH-1, a component of the γ-secretase complex. *J Biol Chem* **279**, 3685-93.

Fraering, P. C., LaVoie, M. J., Ye, W., Ostaszewski, B. L., Kimberly, W. T., Selkoe, D. J. and Wolfe, M. S. (2004). Detergent-dependent dissociation of active γ-secretase reveals an interaction between Pen-2 and PS1-NTF and offers a model for subunit organization within the complex. *Biochemistry* **43**, 323-33.

Francis, R., McGrath, G., Zhang, J., Ruddy, D. A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M. C., Parks, A. L., Xu, W., Li, J., Gurney, M., Myers, R. L., Himes, C. S., Hiebsch, R., Ruble, C., Nye, J. S. and Curtis, D. (2002). aph-1 and pen-2 are required for Notch pathway signaling, γ-secretase cleavage of βAPP, and presenilin protein accumulation. *Dev Cell* **3**, 85-97.

Fratiglioni, L., Launer, L. J., Andersen, K., Breteler, M. M., Copeland, J. R., Dartigues, J. F., Lobo, A., Martinez-Lage, J., Soininen, H. and Hofman, A. (2000). Incidence of dementia and major subtypes in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. *Neurology* **54**, S10-5.

Gandy, S., Czernik, A. J. and Greengard, P. (1988). Phosphorylation of Alzheimer disease amyloid precursor peptide by protein kinase C and Ca2+/calmodulin-dependent protein kinase II. *Proc Natl Acad Sci U S A* **85**, 6218-21.

Geling, A., Steiner, H., Willem, M., Bally-Cuif, L. and Haass, C. (2002). A γ-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. *EMBO Rep* **3**, 688-94.

Glenner, G. G. and Wong, C. W. (1984). Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. *Biochem Biophys Res Commun* **122**, 1131-5.

Goate, A., Chartier-Harlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L. and et al. (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* **349**, 704-6.

Goate, A. M. (1998). Monogenetic determinants of Alzheimer's disease: APP mutations. *Cell Mol Life Sci* **54**, 897-901.

Goldgaber, D., Lerman, M. I., McBride, O. W., Saffiotti, U. and Gajdusek, D. C. (1987). Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease. *Science* **235**, 877-80.

Gordon, M. N., King, D. L., Diamond, D. M., Jantzen, P. T., Boyett, K. V., Hope, C. E., Hatcher, J. M., DiCarlo, G., Gottschall, W. P., Morgan, D. and Arendash, G. W. (2001). Correlation between cognitive deficits and Aβ deposits in transgenic APP+PS1 mice. *Neurobiol Aging* **22**, 377-85.

Goutte, C., Tsunozaki, M., Hale, V. A. and Priess, J. R. (2002). APH-1 is a multipass membrane protein essential for the Notch signaling pathway in Caenorhabditis elegans embryos. *Proc Natl Acad Sci U S A* **99**, 775-9.

Guo, Q., Furukawa, K., Sopher, B. L., Pham, D. G., Xie, J., Robinson, N., Martin, G. M. and Mattson, M. P. (1996). Alzheimer's PS-1 mutation perturbs calcium homeostasis and sensitizes PC12 cells to death induced by amyloid beta-peptide. *Neuroreport* **8**, 379-83.

Götz, J., Chen, F., van Dorpe, J. and Nitsch, R. M. (2001). Formation of neurofibrillary tangles in P301L tau transgenic mice induced by Abeta 42 fibrils. *Science* **293**, 1491-5.

Haass, C., Hung, A. Y. and Selkoe, D. J. (1991). Processing of β-amyloid precursor protein in microglia and astrocytes favors an internal localization over constitutive secretion. *J Neurosci* **11**, 3783-93.

Hampton, R. Y. (2002). ER-associated degradation in protein quality control and cellular regulation. *Curr Opin Cell Biol* **14**, 476-82.

Hardy, J. and Higgins, G. A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science* **256**, 184-5.

Hardy, J. and Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**, 353-6.

Haroutunian, V., Perl, D. P., Purohit, D. P., Marin, D., Khan, K., Lantz, M., Davis, K. L. and Mohs, R. C. (1998). Regional distribution of neuritic plaques in the nondemented elderly and subjects with very mild Alzheimer disease. *Arch Neurol* **55**, 1185-91.

Hartmann, T. (1999). Intracellular biology of Alzheimer's disease amyloid beta peptide. *Eur Arch Psychiatry Clin Neurosci* **249**, 291-8.

Heber, S., Herms, J., Gajic, V., Hainfellner, J., Aguzzi, A., Rulicke, T., von Kretzschmar, H., von Koch, C., Sisodia, S., Tremml, P., Lipp, H. P., Wolfer, D. P. and Muller, U. (2000). Mice with combined gene knock-outs reveal essential and partially redundant functions of amyloid precursor protein family members. *J Neurosci* **20**, 7951-63.

Herms, J., Schneider, I., Dewachter, I., Caluwaerts, N., Kretzschmar, H. and Van Leuven, F. (2003). Capacitive calcium entry is directly attenuated by mutant presenilin-1, independent of the expression of the amyloid precursor protein. *J Biol Chem* **278**, 2484-9.

Herreman, A., Hartmann, D., Annaert, W., Saftig, P., Craessaerts, K., Serneels, L., Umans, L., Schrijvers, V., Checler, F., Vanderstichele, H., Baekelandt, V., Dressel, R., Cupers, P., Huylebroeck, D., Zwijsen, A., Van Leuven, F. and De Strooper, B. (1999). Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. *Proc Natl Acad Sci U S A* **96**, 11872-7.

Herreman, A., Serneels, L., Annaert, W., Collen, D., Schoonjans, L. and De Strooper, B. (2000). Total inactivation of γ-secretase activity in presenilin-deficient embryonic stem cells. *Nat Cell Biol* **2**, 461-2.

Herreman, A., Van Gassen, G., Bentahir, M., Nyabi, O., Craessaerts, K., Mueller, U., Annaert, W. and De Strooper, B. (2003). γ-Secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation. *J Cell Sci* **116**, 1127-36.

Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem* **67**, 425-79.

Hinds, T. R., Kukull, W. A., Van Belle, G., Schellenberg, G. D., Villacres, E. C. and Larson, E. B. (1994). Relationship between serum α1-antichymotrypsin and Alzheimer's disease. *Neurobiol Aging* **15**, 21-7.

Holtzman, D. M., Bales, K. R., Tenkova, T., Fagan, A. M., Parsadanian, M., Sartorius, L. J., Mackey, B., Olney, J., McKeel, D., Wozniak, D. and Paul, S. M. (2000). Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A* **97**, 2892-7.

Hong, L., Koelsch, G., Lin, X., Wu, S., Terzyan, S., Ghosh, A. K., Zhang, X. C. and Tang, J. (2000). Structure of the protease domain of memapsin 2 (β-secretase) complexed with inhibitor. *Science* **290**, 150-3.

Houlden, H., Baker, M., McGowan, E., Lewis, P., Hutton, M., Crook, R., Wood, N. W., Kumar-Singh, S., Geddes, J., Swash, M., Scaravilli, F., Holton, J. L., Lashley, T., Tomita, T., Hashimoto, T., Verkkoniemi, A., Kalimo, H., Somer, M., Paetau, A., Martin, J. J., Van Broeckhoven, C., Golde, T., Hardy, J., Haltia, M. and Revesz, T. (2000). Variant Alzheimer's disease with spastic paraparesis and cotton wool plaques is caused by PS-1 mutations that lead to exceptionally high amyloid-β concentrations. *Ann Neurol* **48**, 806-8.

Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F. and Cole, G. (1996). Correlative memory deficits, Aβ elevation, and amyloid plaques in transgenic mice. *Science* **274**, 99-102.

Hu, Y., Ye, Y. and Fortini, M. E. (2002). Nicastrin is required for γ-secretase cleavage of the Drosophila Notch receptor. *Dev Cell* **2**, 69-78.

Hu, Y. and Fortini, M. E. (2003). Different cofactor activities in γ-secretase assembly: evidence for a nicastrin-Aph-1 subcomplex. *J Cell Biol* **161**, 685-90.

Hung, A. Y., Haass, C., Nitsch, R. M., Qiu, W. Q., Citron, M., Wurtman, R. J., Growdon, J. H. and Selkoe, D. J. (1993). Activation of protein kinase C inhibits cellular production of the amyloid β-protein. *J Biol Chem* **268**, 22959-62.

Hung, A. Y. and Selkoe, D. J. (1994). Selective ectodomain phosphorylation and regulated cleavage of β-amyloid precursor protein. *EMBO J* **13**, 534-42.

Hussain, I., Powell, D., Howlett, D. R., Tew, D. G., Meek, T. D., Chapman, C., Gloger, I. S., Murphy, K. E., Southan, C. D., Ryan, D. M., Smith, T. S., Simmons, D. L., Walsh, F. S., Dingwall, C. and Christie, G. (1999). Identification of a novel aspartic protease (Asp 2) as β-secretase. *Mol Cell Neurosci* **14**, 419-27.

Hutton, M. and Hardy, J. (1997). The presenilins and Alzheimer's disease. *Hum Mol Genet* **6**, 1639-46.

Hutton, M., Perez-Tur, J. and Hardy, J. (1998). Genetics of Alzheimer's disease. *Essays Biochem* **33**, 117-31.

Ikeuchi, T. and Sisodia, S. S. (2003). The Notch ligands, Delta1 and Jagged2, are substrates for Presenilin-dependent "γ -secretase" cleavage. *J Biol Chem* **278**, 7751-4.

in t' Veld, B. A., Ruitenberg, A., Hofman, A., Launer, L. J., van Duijn, C. M., Stijnen, T., Breteler, M. M. and Stricker, B. H. (2001). Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. *N Engl J Med* **345**, 1515-21.

Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N. and Ihara, Y. (1994). Visualization of Aβ 42(43) and Aβ 40 in senile plaques with end-specific Aβ monoclonals: evidence that an initially deposited species is Aβ 42(43). *Neuron* **13**, 45-53.

Janus, C., Chishti, M. A. and Westaway, D. (2000). Transgenic mouse models of Alzheimer's disease. *Biochim Biophys Acta* **1502**, 63-75.

Jarrett, J. T., Berger, E. P. and Lansbury, P. T., Jr. (1993). The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* **32**, 4693-7.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A. (1995). Signalling downstream of activated mammalian Notch. *Nature* **377**, 355-8.

Jick, H., Zornberg, G. L., Jick, S. S., Seshadri, S. and Drachman, D. A. (2000). Statins and the risk of dementia. *Lancet* **356**, 1627-31.

Jorm, A. F., Korten, A. E. and Henderson, A. S. (1987). The prevalence of dementia: a quantitative integration of the literature. *Acta Psychiatr Scand* **76**, 465-79.

Kaether, C., Lammich, S., Edbauer, D., Ertl, M., Rietdorf, J., Capell, A., Steiner, H. and Haass, C. (2002). Presenilin-1 affects trafficking and processing of βAPP and is targeted in a complex with nicastrin to the plasma membrane. *J Cell Biol* **158**, 551-61.

Kamal, A., Almenar-Queralt, A., LeBlanc, J. F., Roberts, E. A. and Goldstein, L. S. (2001). Kinesin-mediated axonal transport of a membrane compartment containing β-secretase and presenilin-1 requires APP. *Nature* **414**, 643-8.

Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., Multhaup, G., Beyreuther, K. and Muller-Hill, B. (1987). The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325**, 733-6.

Katayama, T., Imaizumi, K., Sato, N., Miyoshi, K., Kudo, T., Hitomi, J., Morihara, T., Yoneda, T., Gomi, F., Mori, Y., Nakano, Y., Takeda, J., Tsuda, T., Itoyama, Y., Murayama, O., Takashima, A., St George-Hyslop, P., Takeda, M. and Tohyama, M. (1999). Presenilin-1 mutations downregulate the signalling pathway of the unfolded-protein response. *Nat Cell Biol* **1**, 479-85.

Khachaturian, Z. S. (1985). Diagnosis of Alzheimer's disease. *Arch Neurol* **42**, 1097-105.

Kim, S. H., Leem, J. Y., Lah, J. J., Slunt, H. H., Levey, A. I., Thinakaran, G. and Sisodia, S. S. (2001). Multiple effects of aspartate mutant presenilin 1 on the processing and trafficking of amyloid precursor protein. *J Biol Chem* **276**, 43343-50.

Kim, T. W., Pettingell, W. H., Hallmark, O. G., Moir, R. D., Wasco, W. and Tanzi, R. E. (1997). Endoproteolytic cleavage and proteasomal degradation of presenilin 2 in transfected cells. *J Biol Chem* **272**, 11006-10.

Kimberly, W. T., Zheng, J. B., Guenette, S. Y. and Selkoe, D. J. (2001). The intracellular domain of the beta-amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in a notch-like manner. *J Biol Chem* **276**, 40288-92.

Kimberly, W. T., Esler, W. P., Ye, W., Ostaszewski, B. L., Gao, J., Diehl, T., Selkoe, D. J. and Wolfe, M. S. (2003a). Notch and the amyloid precursor protein are cleaved by similar γ-secretase(s). *Biochemistry* **42**, 137-44.

Kimberly, W. T., LaVoie, M. J., Ostaszewski, B. L., Ye, W., Wolfe, M. S. and Selkoe, D. J. (2003b). γ-Secretase is a membrane protein complex comprised of presenilin, nicastrin, aph-1, and pen-2. *Proc Natl Acad Sci U S A* **100**, 6382-6387.

Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S. and Ito, H. (1988). Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature* **331**, 530-2.

Kitazume, S., Tachida, Y., Oka, R., Shirotani, K., Saido, T. C. and Hashimoto, Y. (2001). Alzheimer's β-secretase, β-site amyloid precursor protein-cleaving enzyme, is responsible for cleavage secretion of a Golgi-resident sialyltransferase. *Proc Natl Acad Sci U S A* **98**, 13554-9.

Klatka, L. A., Schiffer, R. B., Powers, J. M. and Kazee, A. M. (1996). Incorrect diagnosis of Alzheimer's disease. A clinicopathologic study. *Arch Neurol* **53**, 35-42.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U. and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* **96**, 635-44.

Koike, H., Tomioka, S., Sorimachi, H., Saido, T. C., Maruyama, K., Okuyama, A., Fujisawa-Sehara, A., Ohno, S., Suzuki, K. and Ishiura, S. (1999). Membrane-anchored metalloprotease MDC9 has an α-secretase activity responsible for processing the amyloid precursor protein. *Biochem J* **343**, 371-5.

Kwok, J. B., Taddei, K., Hallupp, M., Fisher, C., Brooks, W. S., Broe, G. A., Hardy, J., Fulham, M. J., Nicholson, G. A., Stell, R., St George Hyslop, P. H., Fraser, P. E., Kakulas, B., Clarnette, R., Relkin, N., Gandy, S. E., Schofield, P. R. and Martins, R. N. (1997). Two novel (M233T and R278T) presenilin-1 mutations in early-onset Alzheimer's disease pedigrees and preliminary evidence for association of presenilin-1 mutations with a novel phenotype. *Neuroreport* **8**, 1537-42.

Lambert, M. P., Barlow, A. K., Chromy, B. A., Edwards, C., Freed, R., Liosatos, M., Morgan, T. E., Rozovsky, I., Trommer, B., Viola, K. L., Wals, P., Zhang, C., Finch, C. E., Krafft, G. A. and Klein, W. L. (1998). Diffusible, nonfibrillar ligands derived from $A\beta_{1-42}$ are potent central nervous system neurotoxins. *Proc Natl Acad Sci U S A* **95**, 6448-53.

Lammich, S., Kojro, E., Postina, R., Gilbert, S., Pfeiffer, R., Jasionowski, M., Haass, C. and Fahrenholz, F. (1999). Constitutive and regulated α-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc Natl Acad Sci U S A* **96**, 3922-7.

Laudon, H., Mathews, P. M., Karlström, H., Bergman, A., Farmery, M. R., Nixon, R. A., Winblad, B., Gandy, S. E., Lendahl, U., Lundkvist, J. and Näslund, J. (2004). Co-expressed presenilin 1 NTF and CTF form functional γ-secretase complexes in cells devoid of full-length protein. *J Neurochem*, In press 10.1046/j.1471-4159.2003.02298.x.

LaVoie, M. J., Fraering, P. C., Ostaszewski, B. L., Ye, W., Kimberly, W. T., Wolfe, M. S. and Selkoe, D. J. (2003). Assembly of the γ-secretase complex involves early formation of an intermediate subcomplex of Aph-1 and nicastrin. *J Biol Chem* **278**, 37213-22.

LaVoie, M. J. and Selkoe, D. J. (2003). The Notch ligands, Jagged and Delta, are sequentially processed by α-secretase and presenilin/γ-secretase and release signaling fragments. *J Biol Chem* **278**, 34427-37.

Lee, S. F., Shah, S., Li, H., Yu, C., Han, W. and Yu, G. (2002). Mammalian APH-1 interacts with presenilin and nicastrin and is required for intramembrane proteolysis of amyloid-β precursor protein and Notch. *J Biol Chem* **277**, 45013-9. .

Lee, V. M. (2001). Tauists and βaptists united--well almost! *Science* **293**, 1446-7.

Lee, V. M., Goedert, M. and Trojanowski, J. Q. (2001). Neurodegenerative tauopathies. *Annu Rev Neurosci* **24**, 1121-59.

Leem, J. Y., Vijayan, S., Han, P., Cai, D., Machura, M., Lopes, K. O., Veselits, M. L., Xu, H. and Thinakaran, G. (2002). Presenilin 1 is required for maturation and cell surface accumulation of nicastrin. *J Biol Chem* **277**, 19236-40.

Leissring, M. A., Akbari, Y., Fanger, C. M., Cahalan, M. D., Mattson, M. P. and LaFerla, F. M. (2000). Capacitative calcium entry deficits and elevated luminal calcium content in mutant presenilin-1 knockin mice. *J Cell Biol* **149**, 793-8.

Leissring, M. A., Murphy, M. P., Mead, T. R., Akbari, Y., Sugarman, M. C., Jannatipour, M., Anliker, B., Muller, U., Saftig, P., De Strooper, B., Wolfe, M. S., Golde, T. E. and LaFerla, F. M. (2002). A physiologic signaling role for the γ-secretase-derived intracellular fragment of APP. *Proc Natl Acad Sci U S A* **99**, 4697-702.

Lewis, J., Dickson, D. W., Lin, W. L., Chisholm, L., Corral, A., Jones, G., Yen, S. H., Sahara, N., Skipper, L., Yager, D., Eckman, C., Hardy, J., Hutton, M. and McGowan, E. (2001). Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science* **293**, 1487-91.

Levitan, D. and Greenwald, I. (1995). Facilitation of lin-12-mediated signalling by sel-12, a Caenorhabditis elegans S182 Alzheimer's disease gene. *Nature* **377**, 351-4.

Levitan, D., Doyle, T. G., Brousseau, D., Lee, M. K., Thinakaran, G., Slunt, H. H., Sisodia, S. S. and Greenwald, I. (1996). Assessment of normal and mutant human presenilin function in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* **93**, 14940-4.

Levitan, D., Yu, G., St George Hyslop, P. and Goutte, C. (2001). APH-2/nicastrin functions in LIN-12/Notch signaling in the Caenorhabditis elegans somatic gonad. *Dev Biol* **240**, 654-61.

Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D. M., Oshima, J., Pettingell, W. H., Yu, C.-e., Jondro, P. D., Schmidt, S. D., Wang, K., Crowley, A. C., Fu, Y.-H., Guenette, S. Y., Galas, D., Nemens, E., Wijsman, E. M., Bird, T. D., Schellenberg, G. D. and Tanzi, R. E. (1995). Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science* **269**, 973-977.

Li, J., Myers, R., Shuang, R., Carter, D., McKinley, D., Hiebsch, R., Ellerbrock, B., Fleck, T., Himes, C., Ruble, C., Carozza, M., Drong, R., Pauley, A., Schellin, K., Seaver, L., Slightom, J. and Nye, J. S. (2002a). Presenilin enhancers PEN-1 and PEN-2 and their roles in Aβ production. *Eigth International Conference on Alzheimer's Disease and Related Disorders*, Poster 777.

Li, J., Pauley, A. M., Myers, R. L., Shuang, R., Brashler, J. R., Yan, R., Buhl, A. E., Ruble, C. and Gurney, M. E. (2002b). SEL-10 interacts with presenilin 1, facilitates its ubiquitination, and alters A-beta peptide production. *J Neurochem* **82**, 1540-8.

Li, Q. and Sudhof, T. C. (2003). Cleavage of APP and APLP's by BACE 1. *J Biol Chem*, In press 10.1074/jbc.M3100001200.

Li, T., Ma, G., Cai, H., Price, D. L. and Wong, P. C. (2003). Nicastrin is required for assembly of presenilin/γ-secretase complexes to mediate Notch signaling and for processing and trafficking of β-amyloid precursor protein in mammals. *J Neurosci* **23**, 3272-7.

Li, X. and Greenwald, I. (1996). Membrane topology of the c. elegans SEL-12 presenilin. *Neuron* **17**, 1015-1021.

Li, Y. M., Xu, M., Lai, M. T., Huang, Q., Castro, J. L., DiMuzio-Mower, J., Harrison, T., Lellis, C., Nadin, A., Neduvelil, J. G., Register, R. B., Sardana, M. K., Shearman, M. S., Smith, A. L., Shi, X. P., Yin, K. C., Shafer, J. A. and Gardell, S. J. (2000). Photoactivated γ-secretase inhibitors directed to the active site covalently label presenilin 1. *Nature* **405**, 689-94.

Lichtenthaler, S. F., Wang, R., Grimm, H., Uljon, S. N., Masters, C. L. and Beyreuther, K. (1999). Mechanism of the cleavage specificity of Alzheimer's disease γ-secretase identified by phenylalanine-scanning mutagenesis of the transmembrane domain of the amyloid precursor protein. *Proc Natl Acad Sci U S A* **96**, 3053-8.

Lichtenthaler, S. F., Dominguez, D. I., Westmeyer, G. G., Reiss, K., Haass, C., Saftig, P., De Strooper, B. and Seed, B. (2003). The cell adhesion protein P-selectin glycoprotein ligand-1 is a substrate for the aspartyl protease BACE1. *J Biol Chem* **278**, 48713-9.

Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A. and Tang, J. (2000). Human aspartic protease memapsin 2 cleaves the β-secretase site of β-amyloid precursor protein. *Proc Natl Acad Sci U S A* **97**, 1456-60.

Lobo, A., Launer, L. J., Fratiglioni, L., Andersen, K., Di Carlo, A., Breteler, M. M., Copeland, J. R., Dartigues, J. F., Jagger, C., Martinez-Lage, J., Soininen, H. and Hofman, A. (2000). Prevalence of dementia and major subtypes in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. *Neurology* **54**, S4-9.

Luo, W. J., Wang, H., Li, H., Kim, B. S., Shah, S., Lee, H. J., Thinakaran, G., Kim, T. W., Yu, G. and Xu, H. (2003a). PEN-2 and APH-1 coordinately regulate proteolytic processing of Presenilin 1. *J Biol Chem* **278**, 7850-7854.

Luo, Y., Bolon, B., Kahn, S., Bennett, B. D., Babu-Khan, S., Denis, P., Fan, W., Kha, H., Zhang, J., Gong, Y., Martin, L., Louis, J. C., Yan, Q., Richards, W. G., Citron, M. and Vassar, R. (2001). Mice deficient in BACE1, the Alzheimer's β-secretase, have normal phenotype and abolished β-amyloid generation. *Nat Neurosci* **4**, 231-2.

Luo, Y., Bolon, B., Damore, M. A., Fitzpatrick, D., Liu, H., Zhang, J., Yan, Q., Vassar, R. and Citron, M. (2003b). BACE1 (β-secretase) knockout mice do not acquire compensatory gene expression changes or develop neural lesions over time. *Neurobiol Dis* **14**, 81-8.

Maltese, W. A., Wilson, S., Tan, Y., Suomensaari, S., Sinha, S., Barbour, R. and McConlogue, L. (2001). Retention of the Alzheimer's amyloid precursor fragment C99 in the endoplasmic reticulum prevents formation of amyloid β-peptide. *J Biol Chem* **276**, 20267-79.

Marambaud, P., Shioi, J., Serban, G., Georgakopoulos, A., Sarner, S., Nagy, V., Baki, L., Wen, P., Efthimiopoulos, S., Shao, Z., Wisniewski, T. and Robakis, N. K. (2002). A presenilin-1/γ-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *EMBO J* **21**, 1948-56.

Marambaud, P., Wen, P. H., Dutt, A., Shioi, J., Takashima, A., Siman, R. and Robakis, N. K. (2003). A CBP binding transcriptional repressor produced by the PS1/ε-cleavage of N-cadherin is inhibited by PS1 FAD mutations. *Cell* **114**, 635-45.

McGeer, E. G. and McGeer, P. L. (1998). The importance of inflammatory mechanisms in Alzheimer disease. *Exp Gerontol* **33**, 371-8.

McGeer, P. L. and McGeer, E. G. (2001). Inflammation, autotoxicity and Alzheimer disease. *Neurobiol Aging* **22**, 799-809.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D. and Stadlan, E. M. (1984). Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* **34**, 939-44.

Mirra, S. S., Heyman, A., McKeel, D., Sumi, S. M., Crain, B. J., Brownlee, L. M., Vogel, F. S., Hughes, J. P., van Belle, G. and Berg, L. (1991). The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* **41**, 479-86.

Moehlmann, T., Winkler, E., Xia, X., Edbauer, D., Murrell, J., Capell, A., Kaether, C., Zheng, H., Ghetti, B., Haass, C. and Steiner, H. (2002). Presenilin-1 mutations of leucine 166 equally affect the generation of the Notch and APP intracellular domains independent of their effect on Abeta 42 production. *Proc Natl Acad Sci U S A* **99**, 8025-30.

Moss, M. L., Jin, S. L., Milla, M. E., Bickett, D. M., Burkhart, W., Carter, H. L., Chen, W. J., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R., Kost, T. A., Lambert, M. H., Leesnitzer, M. A., McCauley, P., McGeehan, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L. K., Schoenen, F., Seaton, T., Su, J. L., Becherer, J. D. and et al. (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. *Nature* **385**, 733-6.

Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B. and Lannfelt, L. (1992). A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. *Nat Genet* **1**, 345-7.

Mumm, J. S., Schroeter, E. H., Saxena, M. T., Griesemer, A., Tian, X., Pan, D. J., Ray, W. J. and Kopan, R. (2000). A ligand-induced extracellular cleavage regulates γ-secretase-like proteolytic activation of Notch1. *Mol Cell* **5**, 197-206.

Munoz, D. G. and Feldman, H. (2000). Causes of Alzheimer's disease. *Cmaj* **162**, 65-72.

Nakai, T., Yamasaki, A., Sakaguchi, M., Kosaka, K., Mihara, K., Amaya, Y. and Miura, S. (1999). Membrane topology of Alzheimer's disease-related presenilin 1. *J Biol Chem* **274**, 23647-23658.

Ni, C. Y., Yuan, H. and Carpenter, G. (2003). Role of the ErbB-4 carboxyl terminus in γ-secretase cleavage. *J Biol Chem* **278**, 4561-5.

Nicoll, J. A., Wilkinson, D., Holmes, C., Steart, P., Markham, H. and Weller, R. O. (2003). Neuropathology of human Alzheimer disease after immunization with amyloid-β peptide: a case report. *Nat Med* **9**, 448-52.

Nilsberth, C., Westlind-Danielsson, A., Eckman, C. B., Condron, M. M., Axelman, K., Forsell, C., Stenh, C., Luthman, J., Teplow, D. B., Younkin, S. G., Näslund, J. and Lannfelt, L. (2001). The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Aβ protofibril formation. *Nat Neurosci* **4**, 887-93.

Nyabi, O., Bentahir, M., Horre, K., Herreman, A., Gottardi-Littell, N., Van Broeckhoven, C., Merchiers, P., Spittaels, K., Annaert, W. and De Strooper, B. (2003). Presenilins mutated at Asp-257 or Asp-385 restore Pen-2 expression and Nicastrin glycosylation but remain catalytically inactive in the absence of wild type Presenilin. *J Biol Chem* **278**, 43430-6.

Näslund, J., Haroutunian, V., Mohs, R., Davis, K. L., Davies, P., Greengard, P. and Buxbaum, J. D. (2000). Correlation between elevated levels of amyloid β-peptide in the brain and cognitive decline. *JAMA* **283**, 1571-7.

Oddo, S., Caccamo, A., Shepherd, J. D., Murphy, M. P., Golde, T. E., Kayed, R., Metherate, R., Mattson, M. P., Akbari, Y. and LaFerla, F. M. (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Aβ and synaptic dysfunction. *Neuron* **39**, 409-21.

Ohno, M., Sametsky, E. A., Younkin, L. H., Oakley, H., Younkin, S. G., Citron, M., Vassar, R. and Disterhoft, J. F. (2004). BACE1 Deficiency Rescues Memory Deficits and Cholinergic Dysfunction in a Mouse Model of Alzheimer's Disease. *Neuron* **41**, 27-33.

Okamoto, I., Kawano, Y., Murakami, D., Sasayama, T., Araki, N., Miki, T., Wong, A. J. and Saya, H. (2001). Proteolytic release of CD44 intracellular domain and its role in the CD44 signaling pathway. *J Cell Biol* **155**, 755-762.

Okochi, M., Steiner, H., Fukumori, A., Tanii, H., Tomita, T., Tanaka, T., Iwatsubo, T., Kudo, T., Takeda, M. and Haass, C. (2002). Presenilins mediate a dual intramembranous γ-secretase cleavage of Notch-1. *EMBO J* **21**, 5408-16.

Okuizumi, K., Onodera, O., Namba, Y., Ikeda, K., Yamamoto, T., Seki, K., Ueki, A., Nanko, S., Tanaka, H., Takahashi, H. and et al. (1995). Genetic association of the very low density lipoprotein (VLDL) receptor gene with sporadic Alzheimer's disease. *Nat Genet* **11**, 207-9.

Olson, M. I. and Shaw, C. M. (1969). Presenile dementia and Alzheimer's disease in mongolism. *Brain* **92**, 147-56.

Oltersdorf, T., Ward, P. J., Henriksson, T., Beattie, E. C., Neve, R., Lieberburg, I. and Fritz, L. C. (1990). The Alzheimer amyloid precursor protein. Identification of a stable intermediate in the biosynthetic/degradative pathway. *J Biol Chem* **265**, 4492-7.

Peraus, G. C., Masters, C. L. and Beyreuther, K. (1997). Late compartments of amyloid precursor protein transport in SY5Y cells are involved in β-amyloid secretion. *J Neurosci* **17**, 7714-24.

Perez, R. G., Soriano, S., Hayes, J. D., Ostaszewski, B., Xia, W., Selkoe, D. J., Chen, X., Stokin, G. B. and Koo, E. H. (1999). Mutagenesis identifies new signals for β-amyloid precursor protein endocytosis, turnover, and the generation of secreted fragments, including Aβ42. *J Biol Chem* **274**, 18851-6.

Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russell, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N., Boyce, R. W., Nelson, N., Kozlosky, C. J., Wolfson, M. F., Rauch, C. T., Cerretti, D. P., Paxton, R. J., March, C. J. and Black, R. A. (1998). An essential role for ectodomain shedding in mammalian development. *Science* **282**, 1281-4.

Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70**, 503-33.

Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I. and Fuller, F. (1988). A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. *Nature* **331**, 525-7.

Popescu, B. O. and Ankarcrona, M. (2000). Neurons bearing presenilins: weapons for defense or suicide? *J Cell Mol Med* **4**, 249-261.

Refolo, L. M., Malester, B., LaFrancois, J., Bryant-Thomas, T., Wang, R., Tint, G. S., Sambamurti, K., Duff, K. and Pappolla, M. A. (2000). Hypercholesterolemia accelerates the Alzheimer's amyloid pathology in a transgenic mouse model. *Neurobiol Dis* **7**, 321-31.

Renbaum, P. and Levy-Lahad, E. (1998). Monogenic determinants of familial Alzheimer's disease: presenilin-2 mutations. *Cell Mol Life Sci* **54**, 910-9.

Revesz, T., Holton, J. L., Lashley, T., Plant, G., Rostagno, A., Ghiso, J. and Frangione, B. (2002). Sporadic and familial cerebral amyloid angiopathies. *Brain Pathol* **12**, 343-57.

Robakis, N. K., Ramakrishna, N., Wolfe, G. and Wisniewski, H. M. (1987). Molecular cloning and characterization of a cDNA encoding the cerebrovascular and the neuritic plaque amyloid peptides. *Proc Natl Acad Sci U S A* **84**, 4190-4.

Rocchi, A., Pellegrini, S., Siciliano, G. and Murri, L. (2003). Causative and susceptibility genes for Alzheimer's disease: a review. *Brain Res Bull* **61**, 1-24.

Rohan de Silva, H. A., Jen, A., Wickenden, C., Jen, L. S., Wilkinson, S. L. and Patel, A. J. (1997). Cell-specific expression of beta-amyloid precursor protein isoform mRNAs and proteins in neurons and astrocytes. *Brain Res Mol Brain Res* **47**, 147-56.

Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multhaup, G., Condron, M. M., Teplow, D. B. and Haass, C. (2001). Presenilin-dependent γ-secretase processing of β-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. *EMBO Rep.* **2**, 835-841.

Sato, T., Dohmae, N., Qi, Y., Kakuda, N., Misonou, H., Mitsumori, R., Maruyama, H., Koo, E. H., Haass, C., Takio, K., Morishima-Kawashima, M., Ishiura, S. and Ihara, Y. (2003). Potential link between amyloid β-protein 42 and C-terminal fragment γ 49-99 of β-amyloid precursor protein. *J Biol Chem* **278**, 24294-301.

Scarpini, E., Scheltens, P. and Feldman, H. (2003). Treatment of Alzheimer's disease: current status and new perspectives. *Lancet Neurol* **2**, 539-47.

Scheinfeld, M. H., Ghersi, E., Laky, K., Fowlkes, B. J. and D'Adamio, L. (2002). Processing of β-amyloid precursor-like protein-1 and -2 by γ-secretase regulates transcription. *J Biol Chem* **277**, 44195-201.

Schellenberg, G. D., Bird, T. D., Wijsman, E. M., Orr, H. T., Anderson, L., Nemens, E., White, J. A., Bonnycastle, L., Weber, J. A., Alonso, M. E., Potter, H., Heston, L. L. and Martin, G. M. (1992). Genetic linkage evidence for a familial Alzheimer's disease locus on chromosome 14. *Science* **258**, 668-671.

Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandevert, C., Walker, S., Wogulis, M., Yednock, T., Games, D. and Seubert, P. (1999). Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* **400**, 173-7.

Schroeter, E. H., Kisslinger, J. A. and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382-6.

Schroeter, E. H., Ilagan, M. X., Brunkan, A. L., Hecimovic, S., Li, Y. M., Xu, M., Lewis, H. D., Saxena, M. T., De Strooper, B., Coonrod, A., Tomita, T., Iwatsubo, T., Moore, C. L., Goate, A., Wolfe, M. S., Shearman, M. and Kopan, R. (2003). A presenilin dimer at the core of the γ-secretase enzyme: insights from parallel analysis of Notch 1 and APP proteolysis. *Proc Natl Acad Sci U S A* **100**, 13075-80.

Schubert, D., Jin, L. W., Saitoh, T. and Cole, G. (1989). The regulation of amyloid β protein precursor secretion and its modulatory role in cell adhesion. *Neuron* **3**, 689-94.

Schulz, J. G., Annaert, W., Vandekerckhove, J., Zimmermann, P., De Strooper, B. and David, G. (2003). Syndecan 3 intramembrane proteolysis is presenilin/γ-secretase-dependent and modulates cytosolic signaling. *J Biol Chem* **278**, 48651-7.

Selkoe, D. J. (1991). The molecular pathology of Alzheimer's disease. *Neuron* **6**, 487-98.

Selkoe, D. J. (1999). Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* **399**, A23-31.

Shearman, M. S., Beher, D., Clarke, E. E., Lewis, H. D., Harrison, T., Hunt, P., Nadin, A., Smith, A. L., Stevenson, G. and Castro, J. L. (2000). L-685,458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid β-protein precursor γ-secretase activity. *Biochemistry* **39**, 8698-704.

Shen, J., Bronson, R. T., Chen, D. F., Xia, W., Selkoe, D. J. and Tonegawa, S. (1997). Skeletal and CNS defects in Presenilin-1-deficient mice. *Cell* **89**, 629-39.

Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K. and et al. (1995). Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* **375**, 754-60.

Sigurdsson, E. M., Scholtzova, H., Mehta, P. D., Frangione, B. and Wisniewski, T. (2001). Immunization with a nontoxic/nonfibrillar amyloid-β homologous peptide reduces Alzheimer's disease-associated pathology in transgenic mice. *Am J Pathol* **159**, 439-47.

Simons, M., Keller, P., De Strooper, B., Beyreuther, K., Dotti, C. G. and Simons, K. (1998). Cholesterol depletion inhibits the generation of β-amyloid in hippocampal neurons. *Proc Natl Acad Sci U S A* **95**, 6460-4.

Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Caccavello, R., Davis, D., Doan, M., Dovey, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensaari, S. M., Wang, S., Walker, D., John, V. and et al. (1999). Purification and cloning of amyloid precursor protein beta-secretase from human brain. *Nature* **402**, 537-40.

Soriano, S., Chyung, A. S., Chen, X., Stokin, G. B., Lee, V. M. and Koo, E. H. (1999). Expression of β-amyloid precursor protein-CD3gamma chimeras to demonstrate the selective generation of amyloid β(1-40) and amyloid β(1-42) peptides within secretory and endocytic compartments. *J Biol Chem* **274**, 32295-300.

Sparks, D. L., Martin, T. A., Gross, D. R. and Hunsaker, J. C., 3rd. (2000). Link between heart disease, cholesterol, and Alzheimer's disease: a review. *Microsc Res Tech* **50**, 287-90.

St George-Hyslop, P. H. (2000). Genetic factors in the genesis of Alzheimer's disease. *Ann N Y Acad Sci* **924**, 1-7.

Steiner, H., Capell, A., Pesold, B., Citron, M., Kloetzel, P. M., Selkoe, D. J., Romig, H., Mendla, K. and Haass, C. (1998). Expression of Alzheimer's disease-associated presenilin-1 is controlled by proteolytic degradation and complex formation. *J Biol Chem* **273**, 32322-31.

Steiner, H., Romig, H., Pesold, B., Philipp, U., Baader, M., Citron, M., Loetscher, H., Jacobsen, H. and Haass, C. (1999). Amyloidogenic function of the Alzheimer's disease-associated presenilin 1 in the absence of endoproteolysis. *Biochemistry* **38**, 14600-5.

Steiner, H., Kostka, M., Romig, H., Basset, G., Pesold, B., Hardy, J., Capell, A., Meyn, L., Grim, M. L., Baumeister, R., Fechteler, K. and Haass, C. (2000). Glycine 384 is required for presenilin-1 function and is conserved in bacterial polytopic aspartyl proteases. *Nat Cell Biol* **2**, 848-51.

Steiner, H., Winkler, E., Edbauer, D., Prokop, S., Basset, G., Yamasaki, A., Kostka, M. and Haass, C. (2002). PEN-2 is an integral component of the γ-secretase complex required for coordinated expression of presenilin and nicastrin. *J Biol Chem* **277**, 39062-5.

Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G. and Iwatsubo, T. (2003). The role of presenilin cofactors in the γ-secretase complex. *Nature* **422**, 438-41.

Tanzi, R. E., Gusella, J. F., Watkins, P. C., Bruns, G. A., St George-Hyslop, P., Van Keuren, M. L., Patterson, D., Pagan, S., Kurnit, D. M. and Neve, R. L. (1987). Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. *Science* **235**, 880-4.

Tanzi, R. E., McClatchey, A. I., Lamperti, E. D., Villa-Komaroff, L., Gusella, J. F. and Neve, R. L. (1988). Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. *Nature* **331**, 528-30.

Thinakaran, G., Borchelt, D. R., Lee, M. K., Slunt, H. H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A. I., Gandy, S. E., Jenkins, N. A.,

Copeland, N. G., Price, D. L. and Sisodia, S. S. (1996). Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. *Neuron* **17**, 181-90.

Thinakaran, G., Harris, C. L., Ratovitski, T., Davenport, F., Slunt, H. H., Price, D. L., Borchelt, D. R. and Sisodia, S. S. (1997). Evidence that levels of Presenilins (PS1 and PS2) are coordinately regulated by competition for limiting cellular factors. *J Biol Chem* **272**, 28415-28422.

Tierney, M. C., Fisher, R. H., Lewis, A. J., Zorzitto, M. L., Snow, W. G., Reid, D. W. and Nieuwstraten, P. (1988). The NINCDS-ADRDA Work Group criteria for the clinical diagnosis of probable Alzheimer's disease: a clinicopathologic study of 57 cases. *Neurology* **38**, 359-64.

Tjernberg, L. O., Näslund, J., Lindqvist, F., Johansson, J., Karlstrom, A. R., Thyberg, J., Terenius, L. and Nordstedt, C. (1996). Arrest of β-amyloid fibril formation by a pentapeptide ligand. *J Biol Chem* **271**, 8545-8.

Tomita, T., Takikawa, R., Koyama, A., Morohashi, Y., Takasugi, N., Saido, T. C., Maruyama, K. and Iwatsubo, T. (1999). C terminus of presenilin is required for overproduction of amyloidogenic Aβ42 through stabilization and endoproteolysis of presenilin. *J Neurosci* **19**, 10627-34.

Tomita, T., Watabiki, T., Takikawa, R., Morohashi, Y., Takasugi, N., Kopan, R., De Strooper, B. and Iwatsubo, T. (2001). The first proline of PALP motif at the C terminus of presenilins is obligatory for stabilization, complex formation, and gamma-secretase activities of presenilins. *J Biol Chem* **276**, 33273-81.

Tomita, T., Katayama, R., Takikawa, R. and Iwatsubo, T. (2002). Complex N-glycosylated form of nicastrin is stabilized and selectively bound to presenilin fragments. *FEBS Lett* **520**, 117-21.

Urban, S. and Freeman, M. (2002). Intramembrane proteolysis controls diverse signalling pathways throughout evolution. *Curr Opin Genet Dev* **12**, 512-8.

Walsh, D. M., Klyubin, I., Fadeeva, J. V., Rowan, M. J. and Selkoe, D. J. (2002). Amyloid-β oligomers: their production, toxicity and therapeutic inhibition. *Biochem Soc Trans* **30**, 552-7.

Walsh, D. M., Fadeeva, J. V., LaVoie, M. J., Paliga, K., Eggert, S., Kimberly, W. T., Wasco, W. and Selkoe, D. J. (2003). γ-Secretase cleavage and binding to FE65 regulate the nuclear translocation of the intracellular C-terminal domain (ICD) of the APP family of proteins. *Biochemistry* **42**, 6664-73.

Van Gassen, G., Annaert, W. and Van Broeckhoven, C. (2000). Binding partners of Alzheimer's disease proteins: are they physiologically relevant? *Neurobiol Dis* **7**, 135-51.

Wang, R., Sweeney, D., Gandy, S. E. and Sisodia, S. S. (1996). The profile of soluble amyloid β protein in cultured cell media. Detection and quantification of amyloid β protein and variants by immunoprecipitation-mass spectrometry. *J Biol Chem* **271**, 31894-902.

Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G. and Citron, M. (1999). β-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735-41.

Weggen, S., Eriksen, J. L., Das, P., Sagi, S. A., Wang, R., Pietrzik, C. U., Findlay, K. A., Smith, T. E., Murphy, M. P., Bulter, T., Kang, D. E., Marquez-Sterling, N., Golde, T. E. and Koo, E. H. (2001). A subset of NSAIDs lower amyloidogenic Aβ42 independently of cyclooxygenase activity. *Nature* **414**, 212-6.

Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J. M., Masters, C. L. and Beyreuther, K. (1989). Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* **57**, 115-26.

Weidemann, A., Eggert, S., Reinhard, F. B. M., Vogel, M., Paliga, K., Baier, G., Masters, C. L., Beyreuther, K. and Evin, G. (2002). A novel ε-cleavage within the transmembrane domain of the Alzheimer amyloid precursor protein demonstrates homology with Notch processing. *Biochemistry* **41**, 2825-2835.

Weihofen, A., Binns, K., Lemberg, M. K., Ashman, K. and Martoglio, B. (2002). Identification of signal peptide peptidase, a presenilin-type aspartic protease. *Science* **296**, 2215-8.

Weissman, A. M. (2001). Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* **2**, 169-78.

WHO. (1992). The ICD classification of mental and behavioural disorders: WHO Geneve.

Voges, D., Zwickl, P. and Baumeister, W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu Rev Biochem* **68**, 1015-68.

Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T. and Selkoe, D. J. (1999). Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase activity. *Nature* **398**, 513-517.

Wolozin, B., Kellman, W., Ruosseau, P., Celesia, G. G. and Siegel, G. (2000). Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. *Arch Neurol* **57**, 1439-43.

Wong, P. C., Zheng, H., Chen, H., Becher, M. W., Sirinathsinghji, D. J., Trumbauer, M. E., Chen, H. Y., Price, D. L., Van der Ploeg, L. H. and Sisodia, S. S. (1997). Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm. *Nature* **387**, 288-92.

Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brashier, J. R., Stratman, N. C., Mathews, W. R., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, L. A., Heinrikson, R. L. and Gurney, M. E. (1999). Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity. *Nature* **402**, 533-7.

Yang, D. S., Tandon, A., Chen, F., Yu, G., Yu, H., Arawaka, S., Hasegawa, H., Duthie, M., Schmidt, S. D., Ramabhadran, T. V., Nixon, R. A., Mathews, P. M., Gandy, S. E., Mount, H. T., St George-Hyslop, P. and Fraser, P. E. (2002). Mature glycosylation and trafficking of nicastrin modulate its binding to presenilins. *J Biol Chem* **277**, 28135-42.

Yankner, B. A., Duffy, L. K. and Kirschner, D. A. (1990). Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science* **250**, 279-82.

Yew, P. R. (2001). Ubiquitin-mediated proteolysis of vertebrate G1- and S-phase regulators. *J Cell Physiol* **187**, 1-10.

Yu, C., Kim, S.-H., Ikeuchi, T., Xu, H., Gasparini, L., Wang, R. and Sisodia, S. S. (2001). Characterization of a presenilin-mediated amyloid precursor carboxyl-terminal fragment γ. *J Biol Chem* **276**, 43756-43760.

Yu, G., Nishimura, M., Arawaka, S., Levitan, D., Zhang, L., Tandon, A., Song, Y. Q., Rogaeva, E., Chen, F., Kawarai, T., Supala, A., Levesque, L., Yu, H., Yang, D. S., Holmes, E., Milman, P., Liang, Y., Zhang, D. M., Xu, D. H., Sato, C., Rogaev, E., Smith, M., Janus, C., Zhang, Y., Aebersold, R., Farrer, L. S., Sorbi, S., Bruni, A., Fraser, P. and St George-Hyslop, P. (2000). Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and βAPP processing. *Nature* **407**, 48-54.

Zhang, J., Kang, D. E., Xia, W., Okochi, M., Mori, H., Selkoe, D. J. and Koo, E. H. (1998). Subcellular distribution and turnover of presenilins in transfected cells. *J Biol Chem* **273**, 12436-42.

Zhang, Z., Nadeau, P., Song, W., Donoviel, D., Yuan, M., Bernstein, A. and Yankner, B. A. (2000). Presenilins are required for γ-secretase cleavage of β-APP and transmembrane cleavage of Notch-1. *Nat Cell Biol* **2**, 463-5.

Zheng, H., Jiang, M., Trumbauer, M. E., Sirinathsinghji, D. J., Hopkins, R., Smith, D. W., Heavens, R. P., Dawson, G. R., Boyce, S., Conner, M. W. and et al. (1995). β-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. *Cell* **81**, 525-31.

Öberg, C., Li, J., Pauley, A., Wolf, E., Gurney, M. and Lendahl, U. (2001). The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. *J Biol Chem* **276**, 35847-53.