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INTRACELLULAR DYNAMICS OF ALZHEIMER DISEASE-RELATED PROTEINS

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One of the greatest discoveries a man makes, one of his great surprises, is to find out he can do what he was afraid he could not do. Henry Ford

Abstract

Alzheimer's disease is a devastating neurodegenerative disorder characterized by accumulation of amyloid-β peptide and formation of amyloid plaques and aggregates of hyperphosphorylated tau protein forming neurofibrillary tangles.

The amyloid-β peptide is generated through sequential processing of the amyloid precursor protein (APP) by β- and γ-secretase. This processing is thought to occur at different locations along the intracellular secretory pathway that APP has to travel prior to reaching the cell surface.

In our studies we have focused on the role of secretory pathway trafficking of APP in its amyloidogenic processing. In particular, we have investigated the significance of retrograde protein transport mediated by coatomer protein 1 in processing of APP. The potential significance of a novel endoplasmic reticulum (ER) chaperone ERdj5, belonging to a group of proteins that help the cell to handle misfolded proteins, in amyloidogenic processing of APP and AD pathogenesis was studied. We have also explored the mechanisms underlying the accumulation of an aberrant form of ubiquitin (that normally serves as a proteasomal degradation signal for intracellular and misfolded proteins) UBB+1 in neurodegenerative disorders, including AD, and its inhibitory effect on proteasomal function.

Studies included in this thesis demonstrate that (i) retrograde transport of proteins between Golgi complex and the endoplasmic reticulum mediated by COPI is required for proper intracellular trafficking of APP and its amyoidogenic processing, (ii) early steps in the secretory pathway occurring in the ER and Golgi are significant for amyloidogenic processing of APP, (iii) ERdj5 binds to immature APP, enhances its processing to amyloid-β peptide, and has altered expression in AD brain, (iv) stability of UBB+1 and its accumulation in cells in AD is due to the shortness of its C-terminal extension, leading us to propose that proteasomal substrates need a certain length in order to efficiently degraded by the proteasome.

The major premise of this thesis was to follow the turnover of proteins in the cell: from synthesis and quality control in the endoplasmic reticulum, trafficking along the secretory pathway to a protein's site of action and finally protein degradation by the proteasome. We have demonstrated that all these pathways are significant for neurodegenerative disorders, such as Alzheimer's disease.

List of publications

This thesis is based on the following papers, which will be referred to in the text in bold by their roman numerals.

I. Alexandra Selivanova, Bengt Winblad, Mark R. Farmery, Nico P. Dantuma, and Maria Ankarcrona. COPI-mediated retrograde transport is required for efficient γ-secretase cleavage of amyloid precursor protein. *Biochem. Biophys. Res. Comm.* (2006) 350:220-226

II. Alexandra Selivanova, Bengt Winblad, Nico P. Dantuma and Mark R. Farmery. Biogenesis and processing of the amyloid precursor protein in the secretory pathway. *Biochem. Biophys. Res. Comm.* (2007) In press

III. Paula M. Cunnea, Alexandra Selivanova, Nenad Bogdanovic, Mark R. Farmery and Giannis Spyrou. ERdj5 interacts with amyloid precursor proteins and is expressed in Alzheimer's disease. *Submitted.*

IV. Lisette G.G.C. Verhoef, Alexandra Selivanova, Daria Krutauz, Michael Glickman and Nico P. Dantuma. Proteasome substrates require a minimum length for efficient processing by proteasome-associated deubiquitylation activity and subsequent degradation. *Submitted.*

Abbreviations

Aβ	amyloid-β peptide
AD	Alzheimer's disease
ADAM	a disentegrin and metalloprotease
AICD	APP intracellular domain
APOE	apolipoprotein E
APP	amyloid precursor protein
sAPP	soluble fragment of APP
Aph-1	Anterior pharynx defective-1
APLP1 and APLP2	APP-line protein 1 and 2
BACE1 and BACE2	β-site APP cleavage enzyme
BiP	immunoglobulin heavy chain binding protein
cdk5	protein kinase-5
COPs	coatomer proteins
DAPT	*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FAD	familial Alzheimer's disease
FTDP-17	frontotemporal dementia with Parkinsonism linked to chromosome 17
GSK-3	glycogen synthase kinase-3
Hsp	heat shock protein
LTP	long-term potentiation
MHC	major histocompatibility complex
NFT's	neurofibrillary tangles
ORP150	150-kDa oxygen-regulated protein
Pen-2	presenilin enhancer-2
TACE	tumor necrosis factor alpha converting enzyme
UBB+1	product of ubiquitin B transcript with +1 frame shift
UCH-L1	ubiquitin C-terminal hydrolase L1
UFD	ubiquitin fusion degradation
UPR	unfolded protein response
UPS	ubiquitin-proteasome system

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INTRODUCTION

Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder currently recognized as the major cause of dementia in the elderly. Approximately 50% of people over 85 years of age may suffer from AD. AD is a progressive disease, implying that early mild symptoms worsen over time. The early signs include memory deficits, loss of orientation in familiar surroundings, and problems with performing routine tasks (daily chores such as shopping). During further development of the disease, personality changes, such as increased anxiety and suspiciousness, manifest themselves. Patients also begin to suffer from sleep disturbances and have trouble recognizing their relatives and friends. Late stages of AD are characterized by more severe symptoms, including loss of speech, loss of appetite, and failure to control normal bodily functions. The patients are thus completely dependent on hospital care. Considering the increase in life expectancy in the industrialized countries, it is currently estimated that in 50 years more than 100 million people worldwide will require constant medical care due to AD symptoms. It is not difficult to realize that this will put an enormous strain on the world's medicine and economy, making Alzheimer research a matter of great public interest (Suh and Checler, 2002).

Development of AD pathology

The major pathological characteristics of AD include severe neuronal loss in the cerebral cortex and the hippocampus, the presence of intra- and extracellular proteinaceous deposits, neurofibrillary tangles (NFTs) and amyloid (or senile) plaques, respectively.

Neurofibrillary tangles

NFTs are aggregates of tau protein, which are abnormally hyperphosphorylated at several sites (Grundke-Iqbal et al., 1986). NFTs are present in a number of neurodegenerative disorders, referred to as taupathies, such as AD, frontotemporal dementia, and progressive supranuclear palsy. Discovery that mutations in the *tau* gene are correlated with the inherited form of frontotemporal dementia (frontotemporal dementia with Parkinsonism linked to chromosome-17, FTDP-17) led

to the notion that malfunction of tau protein can be a causative factor in neurodegeneration (Hutton et al., 1998).

Tau is a microtubule-associated protein, which is primarily expressed in neurons. Tau is thought to play a role in stabilizing microtubules during their assembly (Iqbal et al., 2005). Microtubules regulate the axonal transport of a number of organelles and small molecules and are essential for neuronal activity. Hyperphosphorylation of tau has been suggested to reduce its affinity for microtubules (Biernat et al., 1993; Sengupta et al., 1998), since most tau hyperphosphorylation sites flank tau microtubule-binding domains. Thus, the degree of tau phosphorylation regulates its biological function. Balanced activity of several protein kinases (such as glycogen synthase kinase-3 (GSK-3) and cyclin-dependent protein kinase-5 (cdk5)) and phosphatases (primarily protein phosphatase 2A and protein phosphatase-1) monitors phosphorylation state of tau. Overactivity of these protein kinases and compromised activity of phosphatases lead to decline in neuronal function and are associated with AD (Iqbal et al., 2005). Tau hyperphosphorylation also facilitates its aggregation into insoluble protein deposits, i.e. neurofibrillary tangles, which are detrimental for the cell (Iqbal et al., 2005). Hyperphosphorylated tau appears to sequester normal tau as well as other microtubule-binding proteins, leading to disassembly of microtubules, compromised axonal transport and finally neuronal loss (Alonso et al., 1996; Alonso et al., 1997).

Amyloid plaques and Aβ peptide

Amyloid plaques are mainly composed of misfolded aggregates of amyloid-β (Aβ) peptide (Glenner et al., 1984). Aβ production is due to the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases, and is a part of normal cell function. Under ordinary conditions, Aβ is efficiently cleared from the brain via the action of several enzymes. For instance, mammalian endopeptidase neprilysin has a role in degrading membrane-associated Aβ and is one of the main Aβ degrading enzymes in the brain(Iwata et al., 2001; Ling et al., 2003). Insulin-degrading enzyme is also able to degrade Aβ (Selkoe, 2001). Other factors playing a role in Aβ clearance include: alpha-2-macroglobulin (a glycoprotein that binds numerous extracellular ligands including Aβ), endothelin-converting enzyme, and apolipoprotein E (ApoE), which has been shown to bind Aβ and regulate its clearance

(Holtzman, 2004). The processes described above are not properly balanced in AD, as evidenced by increased production of Aβ peptide in AD brain.

Two major forms of Aβ peptide exist - $A\beta_{1-40}$ (40 amino acids long) and the longer form, $A\beta_{1-42}$. The longer peptide is more hydrophobic and more prone to aggregation. Aβ aggregation is regarded as a crucial event in the development of AD pathology (Suh and Checler, 2002). Usually, $A\beta_{1-42}$ constitutes about 10% of the total pool of secreted Aβ. Studies of mutations in the APP gene have revealed that these mutations modify APP processing, so that $A\beta_{1-42}$ concentration rises, thus increasing the $A\beta_{1-42}$ to $A\beta_{1-40}$ ratio (Selkoe, 2002; Suzuki et al., 1994).

Many studies have confirmed that Aβ peptide is neurotoxic. This can occur via a number of mechanisms, e.g. disruption of calcium homeostasis and neuronal signalling, accumulation of free radicals and induction of inflammatory response pathways via activation of microglia and astrocytes (Suh and Checler, 2002). These processes are thought to lead to progressive neuronal loss and deficiencies in synaptic transmission causing cognitive dysfunction in AD (Haass and Selkoe, 2007).

One argument against the amyloid cascade hypothesis that states that gradual accumulation of Aβ peptide leads to neuronal loss and cognitive decline is represented by post-mortem examinations of older healthy individuals. These studies have revealed substantial amounts of amyloid deposits in the brain, which are mainly diffuse, do not contain amyloid fibrils and are not associated with neuronal pathology normally found in AD (Dickson, 1997). Others have stated that correlation between the amounts of amyloid plaques in the brains of AD patients and cognitive impairment is rather weak (Haass and Selkoe, 2007). However, additional studies show an apparent relationship between the levels of soluble Aβ peptide and cognitive decline (Lue et al., 1999; Naslund et al., 2000).

An important conundrum has thus been which species of Aβ is most toxic – soluble Aβ, Aβ oligomers and fibrils, or the mature amyloid plaques?

APP transgenic mice start to exhibit changes in neuronal signalling long before the formation of amyloid plaques, suggesting that soluble Aβ or Aβ oligomers may mediate neurotoxicity (Hsia et al., 1999). Indeed, a recent study has revealed that a specific soluble Aβ assembly induces memory loss in transgenic mice independent of plaque formation (Lesne et al., 2006). On the other hand, fibrillar deposits of Aβ may also contribute to neurotoxicity as suggested by a study that demonstrated that

dendrites and axons passing near fibrillar amyloid deposits are subject to damage, leading to disruption of neuronal connections (Tsai et al., 2004). Possibly, an interplay between different Aβ species is an important contributor to neuronal injury.

What are then the mechanisms through which Aβ induces cognitive decline and disrupts synaptic transmission? It has long been recognized that repeated stimulation of certain neuronal connections in hippocampus, for example, leads to potentiation of neurotransmitter release and neuronal signalling referred to as long-term potentiation (LTP). LTP is an essential mechanism involved in learning and memory (Angelo et al., 2006). Evidence now exists that Aβ assemblies can inhibit LTP (Walsh et al., 2002). LTP disruption by Aβ may occur via inhibition of a deubiquitinating enzyme UCH-L1 (ubiquitin C-terminal hydrolase L1), which is important for recycling of ubiquitin, a major player in protein degradation (Gong et al., 2006). Recent studies indicate that Aβ depresses synaptic transmission by influencing the trafficking of neuronal receptors mediating excitatory synaptic transmission (Hsieh et al., 2006; Ting et al., 2007).

Importantly, there are indications that Aβ may have a normal physiological function. Aβ appears to participate in a negative feedback loop, as it is secreted from normal neurons in response to synaptic activity and then decreases synaptic transmission (Kamenetz et al., 2003).

Plaques or tangles – what comes first?

There is an ongoing debate in the Alzheimer field concerning the sequence of events underlying AD pathology. Which of the AD lesions arises first and how are they related to one another? It was noted that accumulation of hyperphosphorylated tau is directly associated with dementia (Alafuzoff et al., 1987; Arriagada et al., 1992), prompting many researchers to believe that formation of neurofibrillary tangles is the key event leading to neuronal loss in AD. However, studies using mutant tau transgenic mice injected with $A\beta_{1-42}$ and double tau/APP mutants showed that Aβ may induce the formation of neurofibrillary tangles (Gotz et al., 2001; Lewis et al., 2001). In support of this view, others have shown that cdk5 activity is increased in AD (Patrick et al., 1999) as well as demonstrating that Aβ may induce prolonged activation of cdk5 (Lee et al., 2000). Using a triple transgenic mouse model of AD expressing mutant versions of APP, presenilin and tau, Oddo and colleagues have shown that accumulation of amyloid occurs prior the appearance of neurofibrillary

tangles in the brain (Oddo et al., 2003). This implies that hyperphosphorylation of tau and formation of neurofibrillary tangles occurs downstream of Aβ generation.
On the other hand, tau hyperphosphorylation and neurofibrillary tangle formation occurs in other neurodegenerative disorders that develop in the absence of amyloid plaques. These include FTDP-17, Pick's disease, progressive supranuclear palsy, corticobasal degeneration and argyrophilic grain disease (Williams, 2006). Interestingly, a recent study has demonstrated that a decrease in endogenous tau levels in an AD mouse model can reduce toxic effects of Aβ peptide (Roberson et al., 2007).

Risk factors in AD

Sporadic (non-familiar) AD accounts for most cases of AD-linked dementia. Major risk factor for sporadic AD is age, and even healthy individuals exhibit a small number of amyloid plaques and tangles in the brain as they age (Goedert and Spillantini, 2006). A number of environmental factors may also increase the risk of developing AD. These are, for example, low levels of education, lack of social interactions, history of head trauma, as well as consumption of high-calorie, high-fat and low folate diet. Other risk factors may include stroke, hypertension and diabetes mellitus (Turner, 2006). A recent study concluded that stress may lead to enhancement of AD pathology (Ni et al., 2006). Down's syndrome (trisomy 21) is another risk factor for AD. This is likely due to overexpression of APP and increased Aβ production, since APP is encoded on chromosome 21 (Turner, 2006). Currently, the only identified genetic risk factors for late-onset sporadic AD are polymorphisms in ApoE, but the molecular mechanisms underlying their role in the development of AD pathology are unknown (Goedert and Spillantini, 2006). ApoE is involved in lipid and cholesterol transport in the blood and has also been shown to play a role in metabolism and clearance of Aβ, as mentioned above. Three major ApoE polymorphisms exist, ε2, ε3, and ε4. Inheritance of the ε4 allele increases the risk of AD (Goedert and Spillantini, 2006; Turner, 2006; Vetrivel and Thinakaran, 2006). Genes contributing to inherited, familial form of AD (FAD) with early onset have also been identified. Mutations in the *APP* gene have been found to lead to an increase in Aβ production or increased levels of the more amyloidogenic $A\beta_{1-42}$ peptide (Citron et al., 1992; Suzuki et al., 1994). However, they account for only a small fraction of FAD cases. Most *APP* mutations flank the Aβ region, yet some are found within the Aβ sequence. The latter may sometimes enhance Aβ aggregation and fibril formation

without effecting the levels of Aβ (Wisniewski et al., 1991). Among well-known APP mutations linked to AD is the "Swedish" mutation located close to β-secretase cleavage site and associated with increased levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ in plasma (Mullan et al., 1992). Another pathogenic APP mutation identified a few years ago is the "Arctic" mutation located within the Aβ sequence. It appears to lead to decreased levels of Aβ in the plasma but enhanced fibril formation (Nilsberth et al., 2001). However, the majority of FAD cases are caused by mutations in *presenilin-1* gene, while some *presenilin-2* mutations have also been linked to AD. Presenilin-1 mutations cause the most aggressive form of FAD, where onset of the disease can sometimes occur in individuals around 30 years of age (Vetrivel and Thinakaran, 2006). To date, more than 160 *presenilin-1* mutations have been described (Goedert and Spillantini, 2006). The effect of *presenilin* mutations is to increase the ratio of $A\beta_{1-42}$ to $A\beta_{1-40}$. Some of these mutations appear to be associated with reduced γ-secretase activity (Citron et al., 1992). Recently, Bentahir and co-workers have shown that certain *presenilin* mutations lead to a decrease in $A\beta_{1-40}$ levels while others lead to enhancement of $A\beta_{1-42}$ production (Bentahir et al., 2006). Interestingly, all the *presenilin* mutations tested in this study led to a decrease in ε-cleavage (see below) activity of γ-secretase. Thus, *presenilin* mutations appear to have different effects on the function of γ-secretase (Bentahir et al., 2006).

Amyloid precursor protein

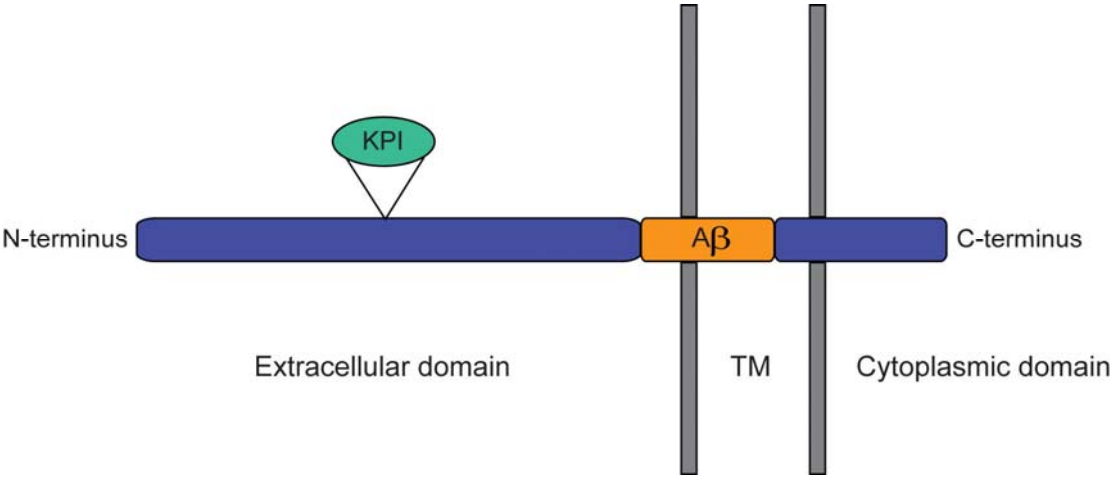

Fig 1. Schematic representation of APP domain structure. KPI, Kunitz protease inhibitor domain. TM, transmembrane domain.

Amyloid precursor protein (APP) is an evolutionarily conserved type I membrane protein. It belongs to a protein family that also includes APP-like proteins 1 and 2 (APLP1 and APLP2), which lack the Aβ sequence.

APP is comprised of a large N-terminal extracellular domain, a transmembrane domain, and a short C-terminal cytoplasmic domain. The N-terminal domain of APP is responsible for protein-protein interactions and growth-factor-like properties of APP and could also be involved in maintaining copper homeostasis in the cell (Reinhard et al., 2005). APP intracellular domain (AICD) appears to mediate most of the interactions of APP with other proteins, primarily through its evolutionary conserved YENPTY sequence (De Strooper and Annaert, 2000).

APP is expressed in most tissues and cell types. APP mRNA is subject to several splicing events leading to generation of several APP isoforms. APP695 (695 amino acids) is the major APP isoform found in neurons, while APP751 and APP771 that contain a Kunitz protease inhibitor domain, are expressed in other cell types (Annaert and De Strooper, 2002).

APP is subject to a number of post-translational modifications: it is N- and O-glycosylated, phosphorylated, and undergoes specific proteolytic cleavage. Different fragments of APP appear mediate a number of important processes in the cell, making APP a multi-functional molecule. It has been proposed to act as cell surface receptor, mediate cell adhesion, regulate neurite outgrowth and synaptic plasticity, have a role in axonal transport (Kamal et al., 2001); though this idea has been subject of some controversy recently (Lazarov et al., 2005); act as signalling molecule, regulate gene transcription and calcium homeostasis, and play a role in cell survival and cell death (Reinhard et al., 2005). Surprisingly, APP knockout mice appear normal, possibly due to the compensational effect of APLPs. However, these mice do exhibit deficiencies in synaptic transmission (Turner et al., 2003). Thus, the precise physiological function of APP is at present unclear.

APP processing

APP is processed through either the amyloidogenic and non-amyloidogenic pathway. This sequential cleavage is mediated by three proteases: α-, β-, and γ-secretases.

In the non-amyloidogenic pathway, APP is cleaved within the Aβ sequence by α-secretase, giving rise to extracellular soluble fragment of APP (sAPPα) and a C-terminal membrane-anchored fragment C83. A number of proteins possessing α-

secretase activity have now been identified. These include tumor necrosis factor alpha converting enzyme (TACE) or ADAM17 (a disentegrin and metalloprotease), as well as ADAM9 and ADAM10.

Amyloidogenic pathway involves cleavage of APP by β-secretase, releasing sAPPβ and giving rise to membrane-tethered C99 fragment. BACE1 and BACE2 (β-site APP cleavage enzyme) are now recognized as β-secretase.

The final cleavage in both non-amyloidogenic and amyloidogenic pathways is mediated by γ-secretase. γ-Secretase is a multiprotein complex comprised of presenilin 1 or 2 (thought to form the active site of the complex), Nicastrin, Aph-1 (anterior pharynx defective-1) and Pen-2 (presenilin enhancer-2) (Annaert and De Strooper, 2002; Ling et al., 2003). It has been suggested that additional proteins may be a part of the complex. Recently, TMP21, a protein involved in protein trafficking and quality control in ER and Golgi, was found to be incorporated into presenilin, nicastrin, Aph-1 and Pen-2 complexes and modulate γ-secretase activity (Chen et al., 2006).

Both APP C-terminal fragments produced by α- and β-cleavages are γ-secretase substrates. γ-Secretase complex is an unusual protease in the sense that it cleaves its plasma membrane-anchored substrates via a process termed regulated intramembrane proteolysis. Cleavage of C83 produces a fragment called p3, which does not possess aggregation properties while cleavage of C99 produces Aβ peptide. γ-Secretase executes an additional cleavage, termed ε-cleavage, which is thought to liberate AICD (Russo et al., 2005; Sastre et al., 2001; Weidemann et al., 2002). The amyloidogenic pathway of APP processing appears to be the preferred pathway in neurons, while the non-amyloidogenic pathway is mainly found in other cell types (Vetrivel and Thinakaran, 2006).

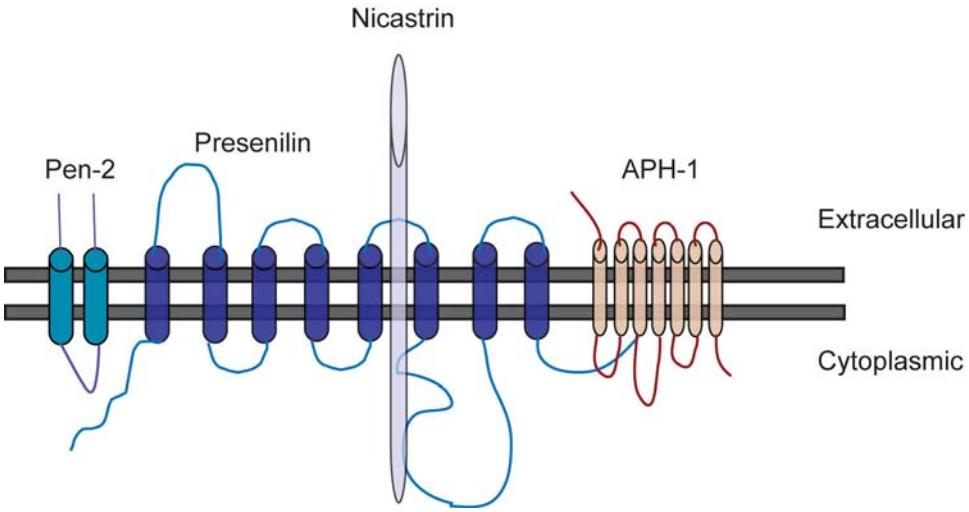

Fig 2. Components of the γ-secretase complex. (Adapted from De Strooper and Woodget 2003.)

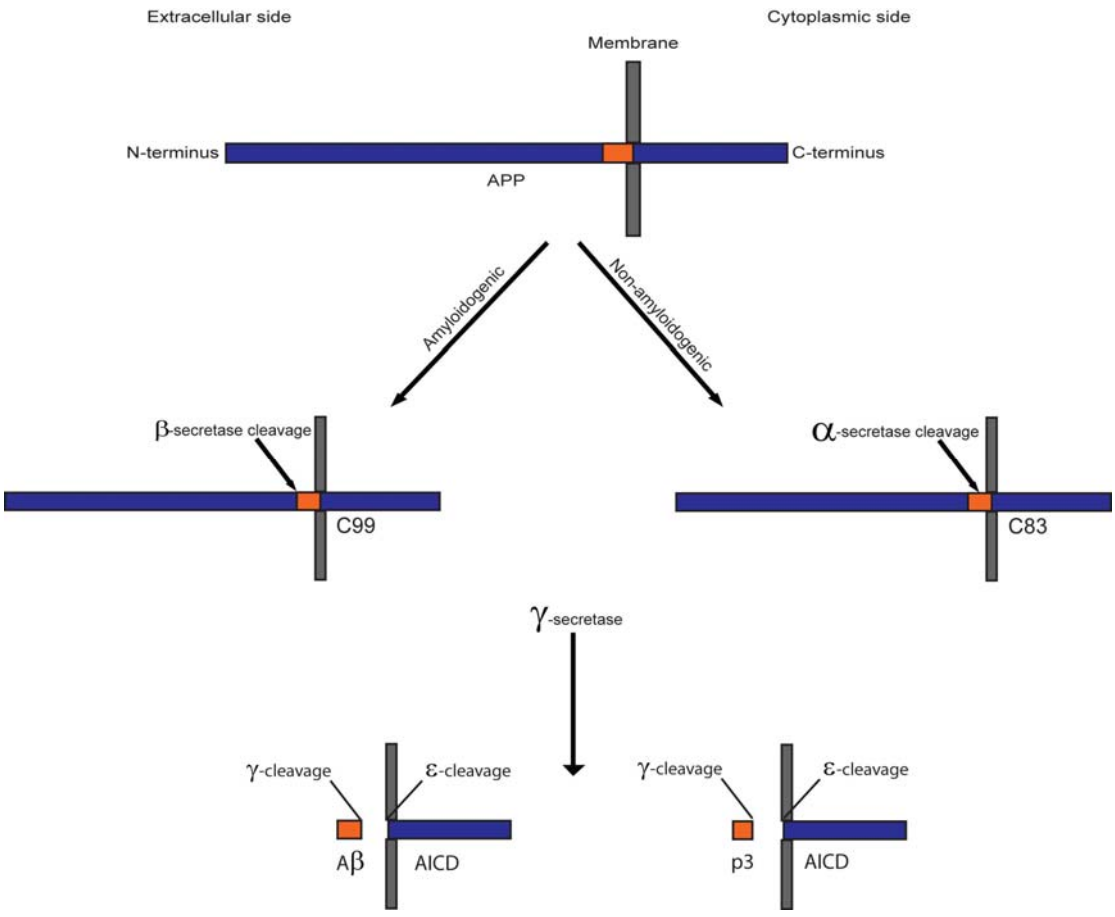

Fig.3 A schematic representation of APP processing pathways.

APP trafficking and Aβ production

Since APP is an transmembrane protein, it needs to travel via the secretory pathway (see below), i.e., to be exported from endoplasmic reticulum and pass through Golgi complex prior to reaching the plasma membrane. APP residence at the cell surface is normally of short duration. Instead, APP is internalised and delivered to endosomes. This is followed by either recycling back to the cell surface or lysosomal degradation. APP processing enzyme activity has been localized to the endosomal lysosomal pathway as well as a number of other organelles in the secretory pathway (Cook et al., 1997; Koo and Squazzo, 1994; Vetrivel and Thinakaran, 2006). Thus, an important challenge of APP biology has been to determine where in the cell APP processing and in particular, the amyloidogenic cleavage occurs.

β-secretase activity has been found in trans-Golgi network and the endoplasmic reticulum (Huse et al., 2002), while BACE and APP interactions have been identified at the cell surface and endosomes (Cordy et al., 2003; Kinoshita et al., 2003).

γ-Secretase components have been localized to endoplasmic reticulum, Golgi, plasma membrane, as well as endosomes (Annaert et al., 1999). APP and components of the γ-secretase complex can also co-localize in lysosomes (Pasternak et al., 2003). In another recent study, nicastrin, presenilin, Aph-1, and Pen-2 were found in mitochondria as an active γ-secretase complex, but its function in this organelle is at present unclear (Hansson et al., 2004).

A number of studies have presented evidence that Aβ can be generated at the cell surface (Chyung and Selkoe, 2003; Kaether et al., 2006), while others have demonstrated that Aβ is produced in the Golgi and other locations along the secretory pathway (Annaert et al., 1999; Jolly-Tornetta and Wolf, 2000). Endosomal pathway has also been shown to have a bearing on Aβ production and AD pathology, since β-secretase cleaved APP was found in endosomal compartments (Mathews et al., 2002). Upregulation of endosomal pathway leads to endosomal morphology similar to that seen in affected neurons in AD as well as increase in β-cleavage and Aβ secretion (Grbovic et al., 2003).

The question whether Aβ can be generated in the endoplasmic reticulum is a matter of debate. However, some studies support this view. $A\beta_{1-40}$ was shown to be generated in the Golgi, while $A\beta_{1-42}$ can be generated in both ER and Golgi (Greenfield et al., 1999; Hartmann et al., 1997; Wilson et al., 2002). There are indications that the site of Aβ generation may be dependent on the cell type (Hartmann et al., 1997). Others have suggested that $A\beta_{1-42}$ production in the ER may be presenilin-independent (Wilson et al., 2002). In additon, it has been shown that retention of APP in the ER leads to an increase in $A\beta_{1-42}$ production (Wild-Bode et al., 1997) and intracellular pool of insoluble Aβ identified in neurons was found to originate in ER and intermediate compartment (Skovronsky et al., 1998).
In summary, factors influencing intracellular trafficking of APP will also regulate its amyloidogenic processing.

Protein life cycle: from synthesis and trafficking to degradation

Quality control in the endoplasmic reticulum – handling misfolded proteins

Secretory and membrane proteins (such as APP) are synthesized on ribosomes of rough endoplasmic reticulum (rough ER). They are co-translationally inserted into the ER through the action of signal recognition particle that recognizes the ER targeting signal in the nascent polypeptide (Martoglio and Dobberstein, 1998) and ER translocon protein complex (Sec61), a channel through which proteins enter the ER (Clemons et al., 2004). Environment in the ER lumen is uniquely specialized for protein folding and maturation, mediating several processes that cannot take place in the cytosol, such as disulfide bond formation and N-linked glycosylation. The majority of proteins that fold in the ER only do so under the oxidizing conditions in the ER lumen (van Anken and Braakman, 2005).
Protein folding in the ER needs to be tightly regulated, ensuring that only properly folded proteins can leave the ER (Ellgaard and Helenius, 2003). Therefore, ER possesses a sophisticated quality control system that monitors protein folding. Two levels of quality control exist in the ER: primary and secondary quality control. Primary quality control is a general system that applies to all proteins. It is based on certain structural features that distinguish properly folded from misfolded proteins,

such as exposed hydrophobic patches, tendency to aggregate, and unpaired cysteine residues. Proteins involved in primary quality control are ER resident chaperones. These include BiP(GRP78) (immunoglobulin heavy chain binding protein), calnexin, calreticulin, protein disulfide isomerase and ERp57. In general, chaperones recognize characteristic features of misfolded proteins and bind them, thereby retaining misfolded proteins in the ER until they achieve their native conformation. Secondary quality control is more selective and regulates the export of specific proteins or protein families out of the ER, usually after they have been properly folded or when late folding intermediates have been formed. These mechanisms are often cell-type specific (Ellgaard and Helenius, 2003; Ellgaard et al., 1999). Examples include dependence of MHC (major histocompatibility complex) class I on its chaperone tapasin for incorporation of antigenic peptides and subsequent export from the ER (Sadasivan et al., 1996).

ER and neurodegeneration

Disturbance in the function of ER quality control causes accumulation of misfolded proteins, leading to a condition known as ER stress. ER stress leads to activation of a number of signalling pathways collectively termed unfolded protein response (UPR). In brief, UPR decreases protein synthesis and promotes degradation of misfolded proteins, as well as enhancing the expression of ER chaperones, such as BiP (GRP78), the major chaperone acting in the UPR pathways. Other ER resident proteins involved in UPR are membrane proteins ATF6, PERK, and IRE1 (Zhang and Kaufman, 2006). BiP is normally bound to these three proteins, keeping them in the inactive state. When the levels of misfolded proteins increase, BiP dissociates. PERK can then lead to decline in protein synthesis by phosphorylating the eukaryotic translation initiation factor, while ATF6 exits the ER and travels to the Golgi, where it is activated by proteolytic cleavage events allowing it to act as a transcription factor that stimulates the synthesis of BiP. IRE1 possesses endonuclease activity and cleaves off a portion of XBP-1 mRNA, which is then transcribed as a functional transcription factor for other genes (e.g. genes coding for ER chaperones) involved in ER stress response. After activation of these pathways, misfolded proteins are translocated out of the ER through the Sec61 channel and degraded by the ubiquitin-proteasome system, via a process termed ER-associated degradation (ERAD) (Meusser et al., 2005). If the

demands on UPR pathways become too large, ER function is severely disturbed, and cell death pathways mediated by ER-resident caspase-12, gadd153 (CHOP), and mitochondria are activated (Paschen and Frandsen, 2001; Paschen and Mengesdorf, 2005; Rao et al., 2004). It is thought that perturbations in the ubiquitin-proteasome system (UPS) responsible for protein degradation can also contribute to ER stress (Ciechanover and Brundin, 2003). Neurodegenerative disorders, including AD, are characterized by accumulation of misfolded proteins. This suggests that ER stress pathways may be involved in AD pathogenesis. Indeed, cells expressing mutant presenilin involved in familiar early-onset AD are more sensitive to ER stress compared to normal cells (Guo et al., 1999a; Guo et al., 1999b). Mutant presenilin 1 also inhibits IRE1, one of the ER sensors that detect the presence of misfolded proteins (Lindholm et al., 2006). One of the chaperone targets of the UPR, BiP/GRP78 has been found to be upregulated in AD brain (Hoozemans et al., 2005). Yet, more studies are needed to completely elucidate the role of ER dysfunction in the development of AD.

APP and chaperones

Several studies indicate that ER resident chaperones involved in quality control interact with APP, regulating its processing and Aβ aggregation. For example, BiP (GRP78) binds APP and reduces the production of $A\beta_{1-40}$ and $A\beta_{1-42}$ (Kudo et al., 2006; Yang et al., 1998). Others have shown that ER chaperones Hsp90 (heat shock protein 90) and Hsp70 can inhibit Aβ aggregation (Evans et al., 2006). Another recent study demonstrated that GRP78 co-chaperones ERdj3 and ERdj4 enhanced the inhibitory effect of GRP78 on Aβ production, while overexpression of calnexin and ORP150 (150-kDa oxygen-regulated protein) also inhibited Aβ generation (Hoshino et al., 2007).

In addition, ER chaperone calreticulin can function as chaperone for APP (Johnson et al., 2001) while both calreticulin and ERp57 were found to bind Aβ peptide in cerebrospinal fluid (Erickson et al., 2005).

Intracellular trafficking

Trafficking of secretory and membrane proteins in eukaryotic cells is a dynamic process mediated by membrane-bound structures. It serves not only as a transport

system but is also important for organelle maintenance, and changes in organelle structure when such alterations are required by cell metabolism.

Export of properly folded proteins from the ER occurs in specialized regions of the ER called “exit sites”. ER exit sites are relatively large dynamic domains devoid of ribosomes (Lippincott-Schwartz et al., 1998). Nascent proteins are recruited to ER exit sites via their sorting signals and are then packaged into vesicles. Vesicle traffic from the ER is mediated by specialized proteins, COPs (coatomer proteins) (Lee et al., 2004). These are involved in vesicle formation, budding, as well as recognition of protein cargo. ER-Golgi traffic is dependent on COPII coated vesicles (Antonny and Schekman, 2001). Upon reaching the Golgi, COPII vesicles fuse with the Golgi membrane, thereby releasing their contents. Vesicle-target membrane recognition is thought to depend on a group of proteins, v- and t-SNARES (vesicle and target), which are present on the surfaces of vesicles and target membranes and bind each other with high specificity (Lippincott-Schwartz et al., 1998).

In the Golgi, proteins may undergo additional post-translational modifications, and can be either retained in the Golgi or transported further to the plasma membrane. However, certain ER resident proteins as well as incompletely folded proteins can sometimes escape the ER. In order to minimize the toxic effect misfolded proteins can have on the cell, and ensure that ER resident proteins exert their function in the ER, retrograde transport pathways exist between Golgi and the ER. Retrograde protein trafficking is mediated by at least two independent pathways. In the conventional pathway, proteins are transported between Golgi and ER in COPI coated vesicles (Lee et al., 2004). An additional retrograde transport pathway that is independent of COPI was identified a few years ago (Storrie et al., 2000). Considering that misfolded proteins that are transported back to the ER may be given another chance to complete their folding and maturation, retrograde protein trafficking between Golgi and ER may play a role in protein maturation. Recent evidence concerning maturation of MHC class I appears to support this view (Paulsson and Wang, 2003; Paulsson and Wang, 2004).

APP and intracellular transport pathways

As mentioned above, intracellular trafficking pattern of APP may be important for cleavage of APP and Aβ production.

This notion is supported by several studies. For instance, presenilin 1 can regulate trafficking and delivery of APP to the cell surface, and presenilin 1 mutants linked to familiar forms of Alzheimer's disease are associated with diminished amounts of APP at the cell surface. This suggests that modulation of APP trafficking by presenilin mutants can influence APP processing (Cai et al., 2003; Leem et al., 2002). A recent study also put forward the idea that precision of γ-secretase cleavage of APP is dependent on the subcellular location of this cleavage (Fukumori et al., 2006).
In addition, presenilins are subject to retrograde COPI-dependent transport while presenilin knockouts and presenilin dominant negative mutants decrease the amounts of C-terminal fragments of APP packaged into COPI vesicles. Thus, intracellular transport of APP and its derivatives can be regulated by presenilins, which is likely to affect amyloidogenic processing of APP (Rechards et al., 2006; Rechards et al., 2003). In addition, a COPI-independent retrograde transport pathway that requires GTPase Rab6 appears to influence the processing of APP (McConlogue et al., 1996).

The ultimate destiny – protein degradation

Degradation of protein complexes and intracellular organelles is mediated by lysosomal pathways. This process is thought to involve autophagy, during which double-membrane-bounded structures known as autophagic vesicles are formed. These vesicles then fuse with lysosomes and their contents are degraded by lysosomal hydrolases. Interestingly, knockout of genes responsible for autophagy leads to neurodegeneration in transgenic mice (Hara et al., 2006), making it an important player in preventing protein misfolding. On the other hand, autophagy-mediated degradation is generally considered to involve low specificity in substrate recognition (Rubinsztein, 2006).
Regulation of levels of via degradation intracellular proteins and destruction of aggregation-prone and misfolded intracellular proteins, on the other hand, is a more tightly regulated process.
UPS is responsible for two-step mechanism that degrades intracellular proteins. Proteins are first targeted for degradation by attachment of ubiquitin molecules, which are then recognized by a protease complex, the 26S proteasome (Reinstein and Ciechanover, 2006).

Ubiquitin is a highly evolutionary conserved protein of 76 amino acids. It is synthesized as a precursor protein, which is then cleaved into several ubiquitin moieties by ubiquitin C-terminal hydrolases (Glickman and Ciechanover, 2002). Ubiquitin conjugation to protein substrate occurs in a number of steps. First, ubiquitin is activated by E1 (ubiquitin-activating enzyme) in an ATP-dependent reaction. Ubiquitin is then transferred from E1 to the substrate through the action of one of the several E2 enzymes (ubiquitin-conjugating enzymes). The substrate itself is specifically bound to an E3 ubiquitin ligase, which is a member of a large protein family of ubiquitin-protein ligases, and catalyses the last step of ubiquitination process. E3 ubiquitin ligases thus confer specificity on UPS.

In general, ubiquitin molecules are attached to an internal lysine in the protein and successive addition of other ubiquitin molecules to internal lysines on previously added ubiquitins synthesizes the polyubiquitin chain (Ciechanover, 2003; Glickman and Ciechanover, 2002).

The polyubiquitin chain is then recognized by the 26S proteasome. This large protease complex is composed of two subcomplexes: 20S core particle responsible for proteolytic activity and the 19S regulatory particle. 20S proteasome is a barrel-shaped structure that is capped by the 19S complex. 19S proteasome has a dual function: it recognizes the proteins destined for degradation and assists in unfolding of the substrate and its insertion into the proteolytic core. Since these processes are energy-dependent, 19S contains several ATPase subunits (Ciechanover, 2003). Prior to degradation by the proteasome, the substrate is deubiquitinated by deubiquitinating enzymes, and ubiquitin molecules can be released and re-used.

Controlled protein degradation mediated by the ubiquitin-proteasome system is responsible for regulating many essential biological processes. These include synaptic plasticity, long-term memory, cell differentiation, regulation of cell cycle and transcription, cellular stress responses, DNA repair, and many others (Ciechanover, 2003).

Ubiquitin-proteasome system and disease

Considering the number of essential mechanisms regulated by the UPS, it was not unexpected to find that dysfunction in the UPS can be detrimental and lead to many disease states. These can be mediated either by a loss of function mechanism, meaning mutation in a UPS enzyme or a target protein leading to impaired

degradation and stabilization of certain proteins, or a gain of function mechanism leading to accelerated degradation of certain protein substrates.
A number of protein misfolding diseases have been associated with impaired proteasome activity. For instance, in Parkinson's disease the levels of 26S proteasomes and proteasome activity are decreased (Rubinsztein, 2006).
Association of pathological lesions with ubiquitin conjugates has been reported for many neurodegenerative disorders, such as Parkinson's disease, Huntington's disease, spinocerebellar ataxia, as well as AD. However, it has been difficult to establish a link between neurodegeneration and UPS. Accumulation of ubiquitin may be due to the activity of protein degradation machinery attempting to degrade aggregates of misfolded proteins. Another possibility is that misfolded proteins themselves have an inhibitory effect on the UPS (Bence et al., 2001; Glickman and Ciechanover, 2002).

Role of ubiquitin-proteasome system in AD pathogenesis

In AD, accumulation of ubiquitin is associated with neurofibrillary tangles and senile plaques (Ciechanover, 2003; Cras et al., 1991). However, the molecular mechanisms that link the ubiquitin-proteasome system to AD are not completely understood. Interestingly, a human homologue of E3 ubiquitin ligase sel-10 can interact with presenilin 1 and assist in its ubiquitination. In addition, co-expression of APP and sel-10 leads to increased Aβ production (Li et al., 2002). In a recent proteomic study of APP-interacting proteins in AD, an association between APP and deubuquitinating enzyme UCHL-1 (also implicated in Parkinson's disease) was reported (Cottrell et al., 2005).
The role of the ubiquitin-proteasome system is further evidenced by findings that turnover of BACE1 as well as components of the γ-secretase complex is mediated by the proteasome (Bergman et al., 2004; Fraser et al., 1998; He et al., 2006; He et al., 2007; Qing et al., 2004). Inhibition of the proteasome also leads to increased levels of Aβ, suggesting that age-related decline in proteasomal function and expression may contribute to AD pathogenesis (Flood et al., 2005; Keller et al., 2000). In addition, even though the idea is still controversial, there is evidence that C99 can be processed by both γ-secretase and the proteasome (Christie et al., 1999; Nunan et al., 2001; Nunan et al., 2003; Skovronsky et al., 2000).
Recent data has shed more light on the link between the UPS and AD, namely, the discovery of transcriptional error that causes a frameshift mutation in the ubiquitin

transcript. This mutation generates an aberrant ubiquitin molecule with C-terminal glycine substituted for tyrosine, followed by a 19 amino acid extension, UBB+1. The UBB+1 transcript is produced at low frequency in normal cells but its levels are low or undetectable. However, UBB+1 has been found to accumulate in affected neurons in AD brains, particularly in patients with late-onset AD, as well as in Huntington's disease brains (de Pril et al., 2004; van Leeuwen et al., 1998). There are indications that UBB+1 could play a role in Aβ-mediated neurotoxicity (Song et al., 2003).
UBB+1 can be polyubiquitinated owing to the presence of lysine residues, but due to glycine to tyrosine substitution deubiquitinating enzymes cannot remove its C-terminal extension and UBB+1 cannot be part of a ubiquitin chain.
Thus, UBB+1 behaves as a ubiquitin fusion degradation (UFD) substrate (Lindsten et al., 2002). UFD substrates possess uncleavable N-terminal ubiquitin that can serve as an acceptor of polyubiquitin chains and can thus be efficiently degraded by the proteasome (Johnson et al., 1995). As mentioned above, UBB+1 levels are generally low in normal cells, as it is degraded by the proteasome due to the presence of UFD signal. Degradation of UBB+1, however, appears to be a more demanding process compared to other proteasome substrates, as evidenced by its accumulation under the conditions of mildly compromised ubiquitin-proteasome system (Menendez-Benito et al., 2005). Indeed, increased levels of UBB+1 induce severe dysfunction of the ubiquitin- proteasome system, leading to cell cycle arrest and apoptosis (De Vrij et al., 2001; Lindsten et al., 2002). Other substrates that resist proteasomal degradation have been described; these include expanded polyglutamine repeats found in certain neurodegenerative disorders (Verhoef et al., 2002), which is a further indication of the involvement of the ubiquitin-proteasome system in neurodegeneration.
This published data suggests that UBB+1, owing to its unusual properties (being a UFD substrate which appears to be a demanding substrate for the proteasome), may be a factor in pathological cascade leading to AD symptoms.

AIMS

The general aims of this study were to investigate processing and trafficking of APP in the secretory pathway and mechanisms of protein degradation related to neurodegenerative disorders.

The specific aims of this thesis were as follows:

- To study the role of COPI-dependent retrograde protein transport in APP processing.
- To study biogenesis and processing of APP in the early secretory pathway using an *in vitro* translation system based on semi-permeabilized cells.
- To investigate a potential role of novel ER chaperone ERdj5 in Alzheimer's disease.
- To investigate molecular mechanisms underlying the resistance to proteasomal degradation of UBB+1, and inhibition of proteasomal activity mediated by UBB+1.

MATERIALS AND METHODS

Tissue culture and transfections

Chinese hamster ovary (CHO) cells and CHO-derived cell line LdlF-2 expressing a temperature-sensitive mutant of COPI ε subunit (kindly provided by Dr. Monty Krieger, MIT, Cambridge, MD) were cultured at permissive (34°C) or restrictive (39°C) temperature in Ham's F-12 medium (Invitrogen) with 5mM glutamine containing 5% fetal bovine serum, 100 μg/ml penicillin, and 100μg/ml streptomycin. CHO and LdlF-2 stable cell lines expressing APPwt were generated by transfection with pcDNA3.1 APPwt 695 and selection with 150μg/ml G418 (Invitrogen) for three weeks. Surviving clones were expanded and APP expression was analysed by Western blot. Transfections were performed using Lipofectamine PLUS reagent (Invitrogen), according to manufacturer's instructions.

Human embryonic kidney cells (HEK 293) endogenously expressing APP or HEK 293 cells overexpressing APPwt were cultured either in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 10 U/ml penicillin, and 100 μg/ml streptomycin or in DMEM containing 10% fetal bovine serum, 10 U/ml penicillin, and 100 μg/ml streptomycin with the addition of 100 μg/ml G418.

Human cervical carcinoma epithelial cells (HeLa) or HeLa cells stably expressing Ub^{G76V}-GFP reporter construct (see below) were cultured in DMEM containing 10% fetal calf serum (Gibco), 10 U/ml penicillin and 10 μg/ml streptomycin. Transient transfections were performed using polyethylenimine (Polysciences) or lipofectamine (Life Technologies), according to manufacturer's instructions.

Western blotting

Cells were lysed, samples separated by SDS-PAGE and blotted onto PVDF membrane. Membrane was blocked with 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.1 % Tween and then incubated with appropriate primary and secondary antibodies.

Immunoprecipitation

Cells were solubilized in either CHAPSO (50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 % CHAPSO (3-[(3-choloamydopropyl)dimethylammonio]-2-hydroxy-1propanesulfonate), protease inhibitor cocktail (Roche)) or RIPA buffer (50mM Tris, pH8, 150mM NaCl, 0.5 % deoxycholic acid, 0.1 % SDS, 1 % NP-40, and protease inhibitor

cocktail (Roche). Lysates were centrifuged to remove insoluble material, and incubated overnight at 4 °C with appropriate antibodies and Protein A/G Sepharose. After several washing steps, sepharose beads were eluted by incubation in Laemmli buffer at 60 °C and samples were analyzed by SDS-PAGE and Western blotting.

Immunostaining

Cells were grown on sterile coverslips, fixed with 2 % paraformaldehyde and 0.2 % glutaraldehyde, and blocked in PBS containing 10% goat serum and 5 % bovine serum albumine (BSA). Cells were then incubated with appropriate primary antibodies diluted in PBS with 10% goat serum and 5 % BSA, and after several washing steps incubated with Alexa Fluor secondary antibodies. Immunostainings were analysed using confocal laser scanning microscope (Carl Zeiss) and software (LSM 5 image browser).

Cell surface biotinylation

Cultured cells were treated with 100 μM chloroquine for 15 h or left untreated. Subsequently, cells were washed with PBS and incubated with 1 mg/ml EZ-link Sulfo-NHS-LC-Biotin (Pierce) in PBS for 30 min at 4 °C. Cells were then lysed in PBS containing 1 % NP-40 and protease inhibitor cocktail and samples centrifuged to remove insoluble material. Lysates were incubated with streptavidin-agarose beads (Pierce) overnight at 4 °C. After several washing steps, streptavidin-agarose beads were eluted by addition of Laemmli buffer and boiling for 5 min. Results were analysed by SDS-PAGE and Western blotting.

γ-Secretase cleavage assay

The γ-secretase reporter assay has been described previously (Karlstrom et al., 2002). Briefly, HEK 293 cells were transfected with 100ng MH100 (Gal4/VP16 (GVP)-fusion regulated luciferase expression vector), and 50ng CMV-β-galactosidase plasmid (described in (Iwatsubo et al., 1994), combined with 100 ng C99-GVP reporter construct (Fig. X) and either 100 ng pIRES-Erdj5 expression vector or en empty pIRES plasmid. CHO and LdlF-2 cells were transfected with 100ng MH100 (Gal4/VP16 (GVP)-fusion regulated luciferase expression vector), 50ng CMV-β-galactosidase plasmid, and with C99-GVP reporter construct or control pCDNA 3 empty plasmid.

Twenty-four hours after transfection, cells were harvested and analysed for reporter gene activity using bioluminescence reader. When appropriate, γ-secretase inhibitors L685,458 (10μM) or *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) (2.5μM) were added to the culture medium during the recovery phase.

Preparation of semi-permeabilized cells

HEK 293 cells were harvested, treated with digitonin to achieve partial digestion of the plasma membrane, and cytosolic components were removed by centrifugation and washing steps. To remove endogenous nucleic acids, semi-permeabilized (SP) cells were treated with micrococcal nuclease (Sigma), which was subsequently inactivated with EGTA. SP cells were aliquoted, frozen in liquid nitrogen and stored at –80 °C until use.

In vitro transcription and translation

Appropriate expression vectors were linearized prior to transcription. Linearized vector DNA was then transcribed using RiboMax (T7) RNA production system kit (Promega), in accordance with manufacturer's instructions.

In vitro translations were performed using Rabbit Reticulocyte Lysate System (Promega). SP cells were reconstituted with rabbit reticulocyte lyzate, and S^{35}-labeled methionine, methionine-free amino acid mix, and appropriate mRNA were added. SP cells were then solubilized and immunoprecipitations performed. Samples were then separated using SDS-PAGE and S^{35}-labeled translation products were analysed using phosphoimager.

Green fluorescent protein-based reporter substrates

In our studies, we have utilized green fluorescent protein (GFP)-based proteasomal reporter substrates, which have been previously successfully used to monitor protein degradation mediated by the ubiquitin-proteasome system (Dantuma et al., 2000). These substrates possess a ubiquitin moiety that cannot be cleaved off by deubiquitinating enzymes coupled to green fluorescent proteins. The uncleavable ubiquitin serves as an acceptor of polyubiquitin chains and these engineered substrates are efficiently cleaved by the proteasome. Thus, under normal conditions, cells expressing the reporters emit very low fluorescence. When the function of the ubiquitin-proteasome system is perturbed, the reporter substrates accumulate and GFP fluorescence can be monitored and readily detected.

Flow cytometry

For flow cytometry analysis, HeLa cells were fixed with cytofix/cytoperm solution (BD Biosciences) and washed with perm/wash solution (BD Biosciences). Staining was performed using the indicated primary antibody and APC-conjugated secondary antibody. Where appropriate, cells were stained with APC-conjugated primary antibody. Flow cytometry was performed with a FACSort flow cytometer (Beckton & Dickinson) and analysed with Cellquest software.

Glycerol gradients

Forty-eight hours after transfection, cells were lysed in proteasome homogenization buffer (PHB) supplemented with protease inhibitor cocktail, N-ethylmaleimide, and 4 mM ATP. 10-40 % glycerol gradient was prepared in PHB buffer and stored overnight at 4 °C to allow linear gradient to form. Total lyzates were added onto the gradient and subjected to ultracentrifugation at 40 000 g for 20 h. Fractions were collected and trichloroacetic acid precipitated with an equal volume of 25% trichloroacetic acid for minimum 30 min on ice. After several centrifugation and washing steps, the samples were air-dried and resuspended in SDS loading buffer.

RESULTS AND DISCUSSION

COPI-mediated retrograde transport is required for efficient γ-secretase cleavage of APP.

APP is able to interact with components of γ-secretase complex at various sites during its trafficking through the secretory pathway to the cell surface.

As mentioned in the introduction, amyloidogenic cleavage of APP can occur at the cell surface as well as in the ER, Golgi, and the endosomes (Annaert et al., 1999; Chyung and Selkoe, 2003; Kaether et al., 2006). To what extent each of these locations contributes to total pool of Aβ is not completely understood.

Secretory and membrane proteins (e.g. APP) are subject to the action of quality control machinery in the ER, which ensures that immature and misfolded proteins do not leave the ER. ER-resident proteins and misfolded nascent polypeptides can sometimes escape the ER and need to be transported back from the Golgi complex to the ER. Currently, two mechanisms are known that mediate this process, a COPI-dependent pathway and a transport pathway that does not require COPI (Lee et al., 2004; Storrie et al., 2000).

As mentioned earlier, presenilin-1 and APP cleavage fragments are found in COPI-coated vesicles, while Rab6-dependent retrograde transport pathway appears to influence APP cleavage (McConlogue et al., 1996; Rechards et al., 2006; Rechards et al., 2003). Thus, both APP and presenilin undergo COPI-dependent retrograde transport, while it is unknown whether this pathway in any respect influences intracellular processing of APP.

In **Paper I** we aimed to study the role of COPI-dependent retrograde transport in processing and trafficking of APP. We took advantage of a Chinese Hamster Ovary (CHO)-derived cell line LdlF-2 expressing a temperature-sensitive mutant of ε subunit of the COPI complex. Restrictive temperature renders COPI protein complex unstable and unable to perform its function in retrograde transport.

We first investigated whether APP interacts with COPI complex in cells, since an interaction between APP and COPI would point strongly towards the conclusion that APP is subject to COPI-dependent retrograde transport. We found by immunoprecipitating APP from HEK293 cells and HEK293 cells overexpressing

APP, that APP interacts with the β subunit of the COPI complex. Notably, when these immunoprecipitation experiments were performed in LdlF-2 cells and control CHO cells under restrictive and non-restrictive temperature, APP-β-COPI interaction was abrogated in LdlF-2 cells, but not in control cells under restrictive temperature. Thus, we concluded that APP is indeed subject to COPI-dependent retrograde transport and its interaction with COPI is dependent on the integrity of the COPI complex.

It has been suggested that retrograde transport is required for maturation and cell surface delivery of transmembrane proteins (Paulsson and Wang, 2004). Therefore, if APP maturation is dependent on retrograde transport, inhibition of COPI pathway would decrease maturation of APP and lead to decreased delivery of mature APP to the cell surface. In order to test this hypothesis, we studied the levels of cell surface APP in LdlF-2 and control CHO cells under restrictive and non-restrictive conditions using immunostaining and cell surface biotinylation. We found that cell surface expression of APP was strongly reduced following inhibition of COPI-dependent retrograde transport, suggesting that COPI-mediated trafficking is of importance for cell surface delivery of APP and can influence its maturation.

Next, we chose to investigate whether APP processing is affected by the absence of a functional COPI complex. Full-length APP and APP C-terminal fragments were detected by immunoblotting in control CHO cells and LdlF-2 cells under restrictive and non-restrictive conditions. After inhibition of COPI-dependent retrograde transport we noted an accumulation of full-length APP, which coincided with a decrease in APP C-terminal fragments. This indicated that dysfunctional COPI-mediated protein transport leads to accumulation of full-length APP and a reduction of APP processing.

Accumulation of full-length APP would imply that both β- and γ-secretase cleavage is impaired during inhibition of COPI pathway. We wished next to focus on γ-secretase cleavage, due to the relevance of this process to AD pathogenesis. Using a well-characterized γ-secretase cleavage reporter assay (Karlstrom et al., 2002), we first found no significant differences in γ-secretase cleavage of the reporter in the control and LdlF-2 cells at the non-restrictive temperature. Notably, at the restrictive temperature, γ-secretase cleavage of the reporter and AICD production was completely blocked in LdlF-2 cells and was not affected by the temperature change in

the control cells. These data show that inhibition of retrograde transport has a significant effect on γ-secretase cleavage of APP.

Paper I is the first study to demonstrate that inhibition of COPI-dependent retrograde transport in cells that express APP induces (i) decreased cell surface expression of APP, (ii) accumulation of full-length APP, (iii) decreased processing of C99 by γ-secretase, (iv) decreased levels of AICD.

Biogenesis of amyloid precursor protein in the early secretory pathway.

As discussed above, there is abundant evidence for the processing of APP and Aβ production occurring at multiple sites along the secretory pathway, cell surface, and the endosomes, even though the production of Aβ in early secretory pathway compartments has been subject to some controversy (Chyung and Selkoe, 2003; Cook et al., 1997; Greenfield et al., 1999; Huse et al., 2002; Kaether et al., 2006).

In **Paper II** we have studied the role of early secretory pathway in the processing of APP. For this purpose we used an *in vitro* translation system that involves SP cells that had been treated with digitonin. In these cells ER and Golgi complex are intact, so proteins can be synthesized in the ER and travel to the Golgi but are unable to reach the cell surface. *In vitro* translation of proteins of interest in SP cells can be used to monitor the behaviour of newly synthesized proteins in the early secretory pathway (Farmery et al., 2000; Plutner et al., 1992; Wilson et al., 2000; Wilson et al., 1995).

We followed the fate of nascent APP by *in vitro* translating APP in the presence of [^{35}S]methionine in SP cells. First we wished to ascertain that APP was targeted to ER in our system. Therefore, we immunoprecipitated Sec61β, a subunit of the translocon complex responsible for insertion of nascent proteins into the ER, from SP cells and then analyzed the immunoprecipitates by autoradiography for [^{35}S]APP. We found that full-length APP was recovered in Sec61β immunoprecipitates and concluded that *in vitro* translated APP was indeed translocated into the ER in SP cells.

Considering the putative significance of the early secretory pathway in APP processing, we wished to investigate whether interaction between APP and the γ-secretase complex can occur in the early secretory pathway compartments. For this purpose, we immuniprecipitated γ-secretase components presenilin 1 and nicastrin

from SP cells after *in vitro* translating APP. Radiolabeled APP could be detected in nicastrin and presenilin 1 immunoprecipitates. These results indicate that APP interacts with γ-secretase early in the secretory pathway. This is in line with previously published data indicating a role for presenilin 1 in regulation of trafficking of certain membrane proteins, including APP, to the cell surface (Cai et al., 2003; Naruse et al., 1998; Uemura et al., 2003). Furthermore, presenilin and APP co-localize in COPI-coated vesicles mediating retrograde transport of proteins from the Golgi to the ER (Rechards et al., 2006), while COPI-mediated retrograde transport is required for efficient processing of APP by γ-secretase (Selivanova et al., 2006). Together these results suggest that early secretory pathway may be significant for amyloidogenic processing of APP.

As functional plasma membrane and endocytosis are absent in our *in vitro* system, we were able to investigate the importance of γ-secretase activity in APP cleavage in early secretory compartments. SP cells were treated with specific γ-secretase inhibitor L685, 458 or left untreated prior to *in vitro* translating APP. In addition to full-length APP, we identified a fragment corresponding in size to the β-secretase APP cleavage product, C99. The identity of full-length APP and C99 was confirmed by immunoprecipitation with antibodies specifically recognizing the N-terminal and central part of Aβ peptide. Detection of Aβ peptide was more problematic in our assay, probably due to release of Aβ from the membrane once it is produced and its subsequent loss in the washing steps after *in vitro* translation.

Notably, pre-treatment of SP cells with γ-secretase inhibitor led to increased levels of both full-length APP and C99. Together, these results suggest that APP can be cleaved by β- and γ-secretase in the early secretory pathway. Due to the fact that our *in vitro* studies were restricted to APP metabolism in the early secretory pathway, it was not possible to evaluate using SP cell system the relative contribution of early secretory pathway to APP processing compared to APP cleavage occurring at the cell surface or endosomes.

Next, we turned our attention to ER resident chaperones. Published data points towards the role of certain ER chaperones in APP processing and Aβ production (Johnson et al., 2001; Yang et al., 1998). Since ER chaperones resist the accumulation of misfolded proteins in the cell, they should be of particular significance for the metabolism of proteins that tend to aggregate. We have chosen to study potential

interactions between ER chaperones calnexin, calreticulin, ERp57 and APP. Interactions of APP with calreticulin have been reported by others (Johnson et al., 2001), but to our knowledge interaction of APP with calnexin has not been accounted for. Immunoprecipitations using calnexin and calreticulin-specific antibodies showed that *in vitro* translated full-length APP could bind to these chaperones. These interactions indicate that APP is subject to quality control in the ER. Precipitations with ERp57-specific antibody revealed that full-length APP interacted with this chaperone as well. In a recent study, it was shown that ERp57, which is normally localized in the ER lumen, is found in a complex with Aβ peptide in cerebrospinal fluid of AD patients (Erickson et al., 2005). Since ERp57 did not interact with C99 in our system, even though C99 contains the Aβ sequence, we hypothesize that interaction between ERp57 and APP in the ER is different from ERp57/Aβ complexes found in cerebrospinal fluid.

The above results indicate that APP interacts with calnexin, calreticulin, and ERp57 prior to its processing by β- and γ-secretase.

In summary, in **Paper II** we show that (i) early events in APP processing and biogenesis can be studied by using SP cells, (ii) full-length APP but not C99 interacts with three quality control chaperones in the ER, (iii) APP can be processed by β- and γ-secretase in the early secretory pathway. This study implies that APP processing can occur soon after synthesis and that biogenesis of APP in the early secretory pathway may be significant for its amyloidogenic processing.

ERdj5 interacts with amyloid precursor protein and has reduced expression in Alzheimer's disease

ER is an essential organelle for protein synthesis, folding, maturation, and trafficking. These processes require stringent quality control mechanisms, ensuring that proteins can efficiently achieve their native conformation and exit the ER only when completely folded. Protein folding and retention of misfolded proteins in the ER are mediated by ER-resident proteins known as chaperones.

Disturbances in protein folding lead to accumulation of misfolded proteins and activation of several signalling pathways collectively known as unfolded protein response (UPR) (Zhang and Kaufman, 2006), leading to upregulation of chaperone

expression and a general downregulation of protein synthesis. If the strain on UPR mechanisms is too great and ER function cannot be rescued, pathways leading to programmed cell death are activated (Lindholm et al., 2006).

Recent findings indicate that certain ER chaperones, such as BiP and calreticulin, have a role in processing and maturation of APP (Johnson et al., 2001; Kudo et al., 2006; Yang et al., 1998).

ERdj5, a recently identified human ER resident protein, was found to contain several domains possessing different functions related to protein folding (Cunnea et al., 2003; Hosoda et al., 2003). In addition, it interacts with BiP and is upregulated following ER stress (Cunnea et al., 2003; Hosoda et al., 2003), implicating ERdj5 as an ER chaperone.

In **Paper III** we investigated a potential role for ERdj5 in AD. In the initial stage of the study, we examined a previously unexplored area of ERdj5 biology, namely, the expression patterns of ERdj5 in AD and age-matched control post-mortem brains by immunohistochemistry. ERdj5 was expressed predominantly in pyramidal neurons in both control and AD brains, with a significantly stronger immunostaining pattern in AD brains. In the few degenerating neurons in normal brains, similar ERdj5 staining pattern was identified. Additionally, morphological changes in the nucleus of degenerating cells expressing high amounts of ERdj5 were noted. Double staining for ERdj5 and phosphorylated tau in AD brain revealed that with increased tau staining ERdj5 staining was decreased, probably due to decrease of the ER volume as the tangle load inside the cell rises. These results indicate that degenerating neurons contain increased amounts of ERdj5.

Certain ER chaperones have been found to have altered expression levels in AD brain (Taguchi et al., 2000). For this reason, we decided to inspect mRNA levels of ERdj5 in AD and control brains by using quantitative real-time PCR analysis of cDNA from AD and control brains. On the whole, the levels of ERdj5 mRNA were decreased in AD as compared to age-matched controls. We have also examined the expression levels of chaperones BiP and calreticulin in AD and control brains, as well as expression levels of CHOP, a mediator of ER stress induced cell death pathway. Our results show that in most cases the expression levels of these proteins were decreased in AD, mimicking the pattern of ERdj5 expression. In some AD cases the levels of calreticulin were comparable with those of age-matched controls. Thus, ERdj5 expression levels are decreased in AD brain, yet an increased number of samples from

different stages of AD and different brain areas would be required to further determine the pattern of ERdj5 expression in AD. Recent work has indicated that ER stress plays a role in AD pathology (Katayama et al., 2004; Lindholm et al., 2006), with UPR induction (i.e. upregulation of ER chaperones such as BiP) first having a neuroprotective effect followed by promotion of cell death after prolonged activation of UPR pathways (Lindholm et al., 2006). This, in turn, may lead to neurodegeneration. What mechanisms mediate this switch from beneficial to harmful UPR is a matter of further investigation.

Considering the role ER resident chaperones may play in the processing of APP and the reported interaction between ERdj5 and BiP (Cunnea et al., 2003; Hosoda et al., 2003), we wondered whether ERdj5 was able to interact with APP. Interestingly, our immunoprecipitation experiments using HEK 293 cells expressing APP, showed that ERdj5 interacted specifically with immature non-glycosylated form of APP. It has been shown that BiP, a co-chaperone of ERdj5 interacts with immature APP and was proposed to influence its subcellular localization during ER stress (Kudo et al., 2006). Our data indicates that ERdj5 can be involved in the early steps of APP metabolism, prior to its full maturation and cleavage by β- and γ-secretase.

The next question we addressed was whether ERdj5 is involved in later stages of APP processing. Therefore, we explored the interaction between APP β-secretase cleavage product C99 and ERdj5. We used an *in vitro* translation system based on semi-permeabilized cells, which serve as a source of functionally intact ER and Golgi complex, to express the C99 fragment and then carry out immunoprecipitation experiments. We found that ERdj5 interacted with C99 in this system. In addition, GST pull-down experiments also demonstrated that ERdj5 could interact with C99. Since ERdj5 is an ER resident protein, its interaction with C99 provides additional evidence for APP processing being able to occur in the early stages of the secretory pathway.

Having found that ERdj5 interacted with APP as well as product of APP cleavage, we wondered whether ERdj5 could in any respect influence the amyloidogenic processing of APP. To address this issue, we used a well-established γ-secretase cleavage reporter assay (Karlstrom et al., 2002; Selivanova et al., 2006). Expression of ERdj5 enhanced γ-secretase cleavage of C99, as evidenced by increased amounts of AICD produced. ERdj5 mutant without a functional KDEL ER retention signal

also increased γ-secretase cleavage, but not to the same extent as wt ER resident ERdj5. These results, together with an interaction between ERdj5 and C99 are indicative of the role of ERdj5 in amyloidogenic processing of APP. Since ERdj5 is also upregulated during ER stress it would be of interest to investigate to what extent increased expression of ERdj5 during ER stress influences amyloidogenic processing and subcellular localization of APP.

In summary, in **Paper III** we have (i) demonstrated for the first time that ERdj5 is expressed in high amounts in degenerating neurons in AD brain, (ii) found that ERdj5 mRNA levels as well as those of ER chaperones BiP and calreticulin and an ER stress response mediator CHOP, are decreased in AD, (iii) demonstrated that ERdj5 interacts with immature APP and C99 and increases γ-secretase activity.

Proteasome substrates require a minimum length for efficient processing by proteasome-associated deubiquitylation activity and subsequent degradation.

Proteasomal degradation of proteins is essential for many cellular processes. Therefore, this process must be subject to tight regulation, i.e., proteasomal substrates need to be specifically targeted for degradation, recruited to the proteasome, unfolded, and finally degraded. This is accomplished via a mechanism involving conjugation of ubiquitin molecules to protein substrates, forming a polyubiquitin chain. Polyubiquitin chains then serve as tags for proteasomal degradation. These can be directly recognized by the 19S regulatory particle of the proteasome. Also, shuttling factors that bind polyubiquitin exist, assisting in the delivery of substrates to the proteasome. Deubiquitination of substrate proteins is required for their efficient degradation and this task is performed by proteasome-associated deubiquitination enzymes. Most proteasomal substrates can be efficiently processed by the proteasome. However, certain proteins are more resistant to proteasomal degradation (Dantuma and Masucci, 2002; Verhoef et al., 2002). Among them is UBB+1, an aberrant transcript of the ubiquitin B gene usually expressed at low levels that encodes a ubiquitin moiety with a C-terminal substitution of glycine to tyrosine together with 19 amino acids long extension. It accumulates in affected neurons in neurodegenerative disorders, including Alzheimer's disease (de Pril et al., 2004; van Leeuwen et al.,

1998) and has also been found to be stabilized and interfere with proteasomal function when it is expressed at high levels (Lindsten et al., 2002).

In **Paper IV,** we aimed to investigate the cause for the stability of UBB+1 and for its inhibition of the ubiquitin-proteasome system.

We first decided to investigate whether the 19 amino acid extension (+1 extension) of UBB+1 conferred stabilizing properties on this protein by fusing the +1 sequence to green fluorescent protein (GFP)-based proteasomal substrates. Flow cytometry and Western blot analysis revealed that +1 extension did not influence the stability of proteasomal reporter substrates, leading us to conclude that +1 sequence does not act as a stabilization signal.

The major difference between UBB+1 and the proteasomal reporter substrates is the limited length of the C-terminal part of UBB+1. Therefore, we wondered whether this characteristic could be the cause of inefficient degradation of UBB+1. The GFP reporter substrate was then truncated, obtaining a proteasomal substrate of the same length as UBB+1, but with a different extension sequence. This construct was found to behave in a manner similar to UBB+1, being stable and did not further accumulate in response to proteasome inhibition. This result suggested that the stability of UBB+1 due to the length of its +1 extension and not to the +1 sequence.

We then hypothesized that an extension of 19 amino acids coupled to a ubiqutin moiety is too short to be efficiently processed by the proteasome. To test this hypothesis, we engineered a series of ubiquitin fusions, with C-terminal domains of different lengths. Our results show that ubiquitin fusion of 20 amino acids was stable and not processed by the proteasome, while fusions of 25 and 30 amino acids accumulated following inhibition of the proteasome. There was a minimal difference in stability between the 25 and 30 amino acids long extension. We conclude that proteasomal substrates require a minimum length in order to be rapidly degraded by the proteasome.

The inhibitory effect of UBB+1 on the ubiquitin-proteasome system may be due to its stability coupled with its ability to be ubiquitinated, making the proteasome try and degrade a ubiquitinated protein that cannot be efficiently processed. To test this idea, we monitored the inhibitory effect of our ubiquitin fusions by transfecting them into cells stably expressing GFP-based proteasomal reporter substrates. Expression of 20 amino acid fusion was associated with increased GFP fluorescence, suggesting that there was indeed an impairment of the ubiquitin-proteasome system. In contrast, in

reporter cells expressing the 25 amino acid construct there was no increase in GFP fluorescence, implying that the ubiquitin-proteasome system was functional in these cells. These results indicate that ubiquitin fusion substrates that are too short to be degraded by the proteasome can lead to impairment of the ubiquitin-proteasome system.

We also investigated whether inefficient degradation of short ubiquitin fusions was due to weak interaction with the proteasome. We performed co-immunoprecipitations and analysed cell lysates by glycerol gradients, finding that both 20 and 25 amino acid fusions interacted with the proteasomal subunits and were found in gradient fractions that contained the proteasome. Therefore, we concluded that the stability of short proteasomal substrates is not due to their weak binding to the proteasome.

Surprisingly, our results also showed that the majority ubiquitin fusions bound to the proteasome lacked polyubiquitin chains. We then tested the migration in glycerol gradients of UBB+1 and two UBB+1 mutants: UBB+1 K29, 48R, with two lysines required for the formation of polyubiquitin chains that serve as degradation tags changed to arginines, and UBB+1 KO which lacked all lysine residues. UBB+1 and the UBB+1 lysine mutants were found to be associated with the proteasome, implying that recruitment of ubiquitin fusions to the proteasome does not require polyubiquitination. This is in agreement with the previously published results showing that certain proteins can associate with the proteasome in ubiquitin-independent manner through an N-terminal ubiquitin-like domain (Buchberger, 2002). It has been shown that proteasome is able to cleave off with low efficacy the N-terminal ubiquitin moiety (Guterman and Glickman, 2004) while our results described above indicate that unmodified ubiquitin fusions can be bound to the proteasome. Therefore, we next wished investigate the role of proteasome-associated deubiquitination activity in degradation of ubiquitin fusion substrates. This was accomplished by *in vitro* methods using purified proteasomes and recombinant His-tagged ubiquitin fusions. We demonstrated that 25 aa fusion was efficiently processed by proteasomal deubiquitination activity while the processing of 20 aa fusion was highly impaired, implying that deubiquitination of ubiquitin fusions requires a minmal length similar to that needed for efficient proteasomal degradation. In addition, in our *in vitro* system we showed that short ubiquitin fusion substrates inhibit the degradation of other proteasomal substrates.

In summary, in **Paper IV** we have identified the reason for stability of UBB+1 and have gained further insight into how proteasome manages its substrates. Our results show that (i) a minimum length is necessary for proteasomal substrates to be efficiently deubiquitinated and degraded by the proteasome, (ii) short ubiquitin fusion substrates can cause a general inhibition of the ubiquitin-proteasome system. Perturbation of the function of the ubiquitin-proteasome system may influence the pathogenesis of many neurodegenerative disorders (Ciechanover and Brundin, 2003) while UBB+1 has been suggested to play an important role in Aβ-induced toxicity (Song et al., 2003). The precise role of UBB+1 in neurodegeneration is still unknown, yet our data suggests that UBB+1 is a problematic substrate for the proteasome due both to the length of its +1 extension and the fact that its ubiquitin moiety cannot be cleaved off, thus releasing it from the proteasome. This would then lead to impaired degradation of other proteasomal substrates and misfolded proteins in the cell, which may, in turn, be detrimental for normal cell function.

Conclusions and Future Perspectives

In **Paper I**, we showed that COPI-mediated retrograde transport between Golgi complex and the ER is significant for subcellular localization of APP and its processing by γ-secretase. This implies that a retrograde transport step mediated by COPI is required for proper intracellular trafficking and proteolytic cleavage of APP. In this study we found that inhibition of COPI-dependent transport led to decreased processing of C99 by γ-secretase. Others have found that presenilin 1 is subject to COPI-mediated retrograde transport (Rechards et al., 2003). A question arises which may be of interest to address: whether this is of significance for presenilin maturation or whether presenilin trafficking by COPI is part of a different regulatory pathway; i.e. presenilin is delivered to the ER and is incorporated into γ-secretase complex. Several studies indicate that APP processing can occur in the ER (Cook et al., 1997; Greenfield et al., 1999; Huse et al., 2002). Thus, interference with COPI-dependent transport may alter the trafficking of an essential component of γ-secretase complex and affect γ-secretase activity. It is tempting to speculate whether γ-secretase complex is properly assembled when COPI-mediated transport is inhibited. On the other hand, altered trafficking of APP in the absence of functional COPI may lead to accumulation of immature APP that "escaped" from the ER in the Golgi. Immature APP may not be cleavage-competent and will not be transported to the cell surface, where γ-secretase cleavage is also known to occur (Chyung and Selkoe, 2003; Kaether et al., 2006). Further studies are needed to investigate these possibilities. Since APP is processed at different locations along the secretory pathway, it is important to identify factors that regulate intracellular trafficking of APP and its processing, thus gaining insight into biological processes underlying AD pathogenesis.

In **Paper II**, we investigated early events in biogenesis and processing of APP in the secretory pathway. Using an *in vitro* translation system that involves semi-permeabilized cells possessing functionally intact ER and Golgi, we have shown that β-secretase cleavage product C99 is produced early in the secretory pathway. In addition, APP interacted with γ-secretase complex components shortly after synthesis

and processing of APP in the early secretory pathway in our *in vitro* system was γ-secretase-dependent. These results suggest that APP can indeed be subject to amyloidogenic cleavage in the early stages of the secretory pathway. The question of to what extent secretory pathway organelles, plasma membrane, and the endosomal pathway contribute to the production of different species of Aβ in different cell types and whether physiological conditions influence these processes has not yet been sufficiently explored.

In **Paper III**, we found that newly characterized ER chaperone ERdj5 is expressed in degenerating neurons in the brains of AD patients and its mRNA levels, along with other ER chaperones, are decreased in AD brain. Notably, we have also shown that ERdj5 interacts preferentially with an immature form of APP as well as with APP. cleavage product C99. ERdj5 was also found to enhance γ-secretase cleavage of C99. These results point towards a role for ERdj5 in the early steps in APP biogenesis in the secretory pathway as well as in processing of APP by γ-secretase. Significantly, ERdj5 partner chaperone BiP has been shown to bind to APP and decrease processing of APP to Aβ (Yang et al., 1998). The question of whether ERdj5 exerts its APP-related function independently or in concert with BiP merits further investigation. In line with addressing this issue, it will be of interest to explore how downregulation of ERdj5 expression by RNAi would effect maturation and processing of APP. ERdj5 has also been shown to be upregulated during ER stress (Cunnea et al., 2003; Hosoda et al., 2003) and there is a lot of evidence that ER stress is involved in the pathogenesis of AD (Katayama et al., 2004; Lindholm et al., 2006). For this reason, it is important to further explore how upregulation of ERdj5 during ER stress influences APP processing. As discussed earlier, during neurodegneration ER stress has initially a neuroprotective effect, as expression levels of ER chaperones are increased, thus helping the cell handle misfolded proteins. On the other hand, prolonged activation of the UPR pathways may lead to cell death and eventually induce neurodegeneration in AD. What molecular mechanisms mediate the change in UPR from being a beneficial to cell death-inducing response is a matter that needs to be further investigated.

In **Paper IV,** we studied UBB+1, an aberrant ubiquitin that accumulates in cells in neurodegenerative disorders, including AD. Our results show that the cause of the unusual stability of UBB+1 is the short length of its C-terminal extension that follows the ubiquitin moiety. Thus, we propose that proteasomal substrates generally require a minimum length in order to be effectively processed by the proteasome. Such reluctant substrates can also lead to a general impairment of the ubiquitin-proteasome system. Our results also show that short proteasomal substrates cannot be properly deubiquitinated by the proteasome-associated deubiquitination activities. This, in turn, means that a short substrate is not released from the proteasome that keeps trying to degrade a difficult substrate. The consequence would be that other proteasomal substrates (such as misfolded proteins) accumulate and finally overwhelm the ubiquitin-proteasome system, as proteasomes that are not occupied with degrading a difficult substrate (e.g. UBB+1) must deal with increasing substrate load.

It is currently unclear what role UBB+1 plays in development of AD. Interestingly, it was found to mediate Aβ toxicity via inhibition of the proteasome (Song et al., 2003) Accumulation of UBB+1 in AD and other neurodegenerative disorders (Van Leeuwen et al., 2000) and its inhibitory effect on the ubiquitin-proteasome system (Lindsten et al., 2002) suggest that the presence of a reluctant proteasomal substrate can have detrimental effects for the cell. There is some evidence that proteasomal function decreases with age (Keller et al., 2000). Since UBB+1 is usually expressed at low levels, the ubiquitin-proteasome system can under normal conditions degrade it (Lindsten et al., 2002). However, during aging, as the proteasomal function decreases, UBB+1 would tend to accumulate. This would present an additional challenge to the already weakened ubiquitin-proteasome system and impede degradation of other misfolded proteins, in the end leading to cell death. Further studies are needed to investigate this issue.

It is apparent that neurodegeneration is caused by a complex interplay of environmental and genetic factors. At the cellular level, multiple pathways intermingle to either contribute to normal cell function or lead to cell death and ultimately disease state. Deciphering this intricate puzzle is one of the goals of medical research. In this thesis, we have chosen to explore neurodegeneration in relation to a protein life cycle. Starting with protein synthesis and quality control in the ER (**paper II** and **III**), we went on to study protein trafficking in the early

secretory pathway (**paper I**), finishing with proteasomal degradation of unwanted or misfolded proteins (**paper IV**). Thus, mechanisms that are essential for normal protein turnover in the cell can play an important role in neurodegeneration.

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References

Alafuzoff, I., Iqbal, K., Friden, H., Adolfsson, R. and Winblad, B. (1987) Histopathological criteria for progressive dementia disorders: clinical-pathological correlation and classification by multivariate data analysis. *Acta Neuropathol (Berl)*, **74**, 209-225.

Alonso, A.C., Grundke-Iqbal, I. and Iqbal, K. (1996) Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. *Nat Med*, **2**, 783-787.

Alonso, A.D., Grundke-Iqbal, I., Barra, H.S. and Iqbal, K. (1997) Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration: sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau. *Proc Natl Acad Sci U S A*, **94**, 298-303.

Angelo, M., Plattner, F. and Giese, K.P. (2006) Cyclin-dependent kinase 5 in synaptic plasticity, learning and memory. *J Neurochem*, **99**, 353-370.

Annaert, W. and De Strooper, B. (2002) A cell biological perspective on Alzheimer's disease. *Annu Rev Cell Dev Biol*, **18**, 25-51.

Annaert, W.G., Levesque, L., Craessaerts, K., Dierinck, I., Snellings, G., Westaway, D., George-Hyslop, P.S., Cordell, B., Fraser, P. and De Strooper, B. (1999) Presenilin 1 controls gamma-secretase processing of amyloid precursor protein in pre-golgi compartments of hippocampal neurons. *J Cell Biol*, **147**, 277-294.

Antonny, B. and Schekman, R. (2001) ER export: public transportation by the COPII coach. *Curr Opin Cell Biol*, **13**, 438-443.

Arriagada, P.V., Marzloff, K. and Hyman, B.T. (1992) Distribution of Alzheimer-type pathologic changes in nondemented elderly individuals matches the pattern in Alzheimer's disease. *Neurology*, **42**, 1681-1688.

Bence, N.F., Sampat, R.M. and Kopito, R.R. (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. *Science*, **292**, 1552-1555.

Bentahir, M., Nyabi, O., Verhamme, J., Tolia, A., Horre, K., Wiltfang, J., Esselmann, H. and De Strooper, B. (2006) Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms. *J Neurochem*, **96**, 732-742.

Bergman, A., Hansson, E.M., Pursglove, S.E., Farmery, M.R., Lannfelt, L., Lendahl, U., Lundkvist, J. and Naslund, J. (2004) Pen-2 is sequestered in the endoplasmic reticulum and subjected to ubiquitylation and proteasome-mediated degradation in the absence of presenilin. *J Biol Chem*, **279**, 16744-16753.

Biernat, J., Gustke, N., Drewes, G., Mandelkow, E.M. and Mandelkow, E. (1993) Phosphorylation of Ser262 strongly reduces binding of tau to microtubules: distinction between PHF-like immunoreactivity and microtubule binding. *Neuron*, **11**, 153-163.

Buchberger, A. (2002) From UBA to UBX: new words in the ubiquitin vocabulary. *Trends Cell Biol*, **12**, 216-221.

Cai, D., Leem, J.Y., Greenfield, J.P., Wang, P., Kim, B.S., Wang, R., Lopes, K.O., Kim, S.H., Zheng, H., Greengard, P., Sisodia, S.S., Thinakaran, G. and Xu, H. (2003) Presenilin-1 regulates intracellular trafficking and cell surface delivery of beta-amyloid precursor protein. *J Biol Chem*, **278**, 3446-3454.

Chen, F., Hasegawa, H., Schmitt-Ulms, G., Kawarai, T., Bohm, C., Katayama, T., Gu, Y., Sanjo, N., Glista, M., Rogaeva, E., Wakutani, Y., Pardossi-Piquard, R.,

Ruan, X., Tandon, A., Checler, F., Marambaud, P., Hansen, K., Westaway, D., St George-Hyslop, P. and Fraser, P. (2006) TMP21 is a presenilin complex component that modulates gamma-secretase but not epsilon-secretase activity. *Nature*, **440**, 1208-1212.

Christie, G., Markwell, R.E., Gray, C.W., Smith, L., Godfrey, F., Mansfield, F., Wadsworth, H., King, R., McLaughlin, M., Cooper, D.G., Ward, R.V., Howlett, D.R., Hartmann, T., Lichtenthaler, S.F., Beyreuther, K., Underwood, J., Gribble, S.K., Cappai, R., Masters, C.L., Tamaoka, A., Gardner, R.L., Rivett, A.J., Karran, E.H. and Allsop, D. (1999) Alzheimer's disease: correlation of the suppression of beta-amyloid peptide secretion from cultured cells with inhibition of the chymotrypsin-like activity of the proteasome. *J Neurochem*, **73**, 195-204.

Chyung, J.H. and Selkoe, D.J. (2003) Inhibition of receptor-mediated endocytosis demonstrates generation of amyloid beta-protein at the cell surface. *J Biol Chem*, **278**, 51035-51043.

Ciechanover, A. (2003) The ubiquitin proteolytic system and pathogenesis of human diseases: a novel platform for mechanism-based drug targeting. *Biochem Soc Trans*, **31**, 474-481.

Ciechanover, A. and Brundin, P. (2003) The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. *Neuron*, **40**, 427-446.

Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I. and Selkoe, D.J. (1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature*, **360**, 672-674.

Clemons, W.M., Jr., Menetret, J.F., Akey, C.W. and Rapoport, T.A. (2004) Structural insight into the protein translocation channel. *Curr Opin Struct Biol*, **14**, 390-396.

Cook, D.G., Forman, M.S., Sung, J.C., Leight, S., Kolson, D.L., Iwatsubo, T., Lee, V.M. and Doms, R.W. (1997) Alzheimer's A beta(1-42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Nat Med*, **3**, 1021-1023.

Cordy, J.M., Hussain, I., Dingwall, C., Hooper, N.M. and Turner, A.J. (2003) Exclusively targeting beta-secretase to lipid rafts by GPI-anchor addition up-regulates beta-site processing of the amyloid precursor protein. *Proc Natl Acad Sci U S A*, **100**, 11735-11740.

Cottrell, B.A., Galvan, V., Banwait, S., Gorostiza, O., Lombardo, C.R., Williams, T., Schilling, B., Peel, A., Gibson, B., Koo, E.H., Link, C.D. and Bredesen, D.E. (2005) A pilot proteomic study of amyloid precursor interactors in Alzheimer's disease. *Ann Neurol*, **58**, 277-289.

Cras, P., Kawai, M., Lowery, D., Gonzalez-DeWhitt, P., Greenberg, B. and Perry, G. (1991) Senile plaque neurites in Alzheimer disease accumulate amyloid precursor protein. *Proc Natl Acad Sci U S A*, **88**, 7552-7556.

Cunnea, P.M., Miranda-Vizuete, A., Bertoli, G., Simmen, T., Damdimopoulos, A.E., Hermann, S., Leinonen, S., Huikko, M.P., Gustafsson, J.A., Sitia, R. and Spyrou, G. (2003) ERdj5, an endoplasmic reticulum (ER)-resident protein containing DnaJ and thioredoxin domains, is expressed in secretory cells or following ER stress. *J Biol Chem*, **278**, 1059-1066.

Dantuma, N.P., Lindsten, K., Glas, R., Jellne, M. and Masucci, M.G. (2000) Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. *Nat Biotechnol*, **18**, 538-543.

Dantuma, N.P. and Masucci, M.G. (2002) Stabilization signals: a novel regulatory mechanism in the ubiquitin/proteasome system. *FEBS Lett*, **529**, 22-26.

de Pril, R., Fischer, D.F., Maat-Schieman, M.L., Hobo, B., de Vos, R.A., Brunt, E.R., Hol, E.M., Roos, R.A. and van Leeuwen, F.W. (2004) Accumulation of aberrant ubiquitin induces aggregate formation and cell death in polyglutamine diseases. *Hum Mol Genet*, **13**, 1803-1813.

De Strooper, B. and Annaert, W. (2000) Proteolytic processing and cell biological functions of the amyloid precursor protein. *J Cell Sci*, **113 (Pt 11)**, 1857-1870.

De Vrij, F.M., Sluijs, J.A., Gregori, L., Fischer, D.F., Hermens, W.T., Goldgaber, D., Verhaagen, J., Van Leeuwen, F.W. and Hol, E.M. (2001) Mutant ubiquitin expressed in Alzheimer's disease causes neuronal death. *Faseb J*, **15**, 2680-2688.

Dickson, D.W. (1997) The pathogenesis of senile plaques. *J Neuropathol Exp Neurol*, **56**, 321-339.

Ellgaard, L. and Helenius, A. (2003) Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol*, **4**, 181-191.

Ellgaard, L., Molinari, M. and Helenius, A. (1999) Setting the standards: quality control in the secretory pathway. *Science*, **286**, 1882-1888.

Erickson, R.R., Dunning, L.M., Olson, D.A., Cohen, S.J., Davis, A.T., Wood, W.G., Kratzke, R.A. and Holtzman, J.L. (2005) In cerebrospinal fluid ER chaperones ERp57 and calreticulin bind beta-amyloid. *Biochem Biophys Res Commun*, **332**, 50-57.

Evans, C.G., Wisen, S. and Gestwicki, J.E. (2006) Heat shock proteins 70 and 90 inhibit early stages of amyloid beta-(1-42) aggregation in vitro. *J Biol Chem*, **281**, 33182-33191.

Farmery, M.R., Allen, S., Allen, A.J. and Bulleid, N.J. (2000) The role of ERp57 in disulfide bond formation during the assembly of major histocompatibility complex class I in a synchronized semipermeabilized cell translation system. *J Biol Chem*, **275**, 14933-14938.

Flood, F., Murphy, S., Cowburn, R.F., Lannfelt, L., Walker, B. and Johnston, J.A. (2005) Proteasome-mediated effects on amyloid precursor protein processing at the gamma-secretase site. *Biochem J*, **385**, 545-550.

Fraser, P.E., Levesque, G., Yu, G., Mills, L.R., Thirlwell, J., Frantseva, M., Gandy, S.E., Seeger, M., Carlen, P.L. and St George-Hyslop, P. (1998) Presenilin 1 is actively degraded by the 26S proteasome. *Neurobiol Aging*, **19**, S19-21.

Fukumori, A., Okochi, M., Tagami, S., Jiang, J., Itoh, N., Nakayama, T., Yanagida, K., Ishizuka-Katsura, Y., Morihara, T., Kamino, K., Tanaka, T., Kudo, T., Tanii, H., Ikuta, A., Haass, C. and Takeda, M. (2006) Presenilin-dependent gamma-secretase on plasma membrane and endosomes is functionally distinct. *Biochemistry*, **45**, 4907-4914.

Glenner, G.G., Wong, C.W., Quaranta, V. and Eanes, E.D. (1984) The amyloid deposits in Alzheimer's disease: their nature and pathogenesis. *Appl Pathol*, **2**, 357-369.

Glickman, M.H. and Ciechanover, A. (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev*, **82**, 373-428.

Goedert, M. and Spillantini, M.G. (2006) A century of Alzheimer's disease. *Science*, **314**, 777-781.

Gong, B., Cao, Z., Zheng, P., Vitolo, O.V., Liu, S., Staniszewski, A., Moolman, D., Zhang, H., Shelanski, M. and Arancio, O. (2006) Ubiquitin hydrolase Uch-L1 rescues beta-amyloid-induced decreases in synaptic function and contextual memory. *Cell*, **126**, 775-788.

Gotz, 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**, 1491-1495.

Grbovic, O.M., Mathews, P.M., Jiang, Y., Schmidt, S.D., Dinakar, R., Summers-Terio, N.B., Ceresa, B.P., Nixon, R.A. and Cataldo, A.M. (2003) Rab5-stimulated up-regulation of the endocytic pathway increases intracellular beta-cleaved amyloid precursor protein carboxyl-terminal fragment levels and Abeta production. *J Biol Chem*, **278**, 31261-31268.

Greenfield, J.P., Tsai, J., Gouras, G.K., Hai, B., Thinakaran, G., Checler, F., Sisodia, S.S., Greengard, P. and Xu, H. (1999) Endoplasmic reticulum and trans-Golgi network generate distinct populations of Alzheimer beta-amyloid peptides. *Proc Natl Acad Sci U S A*, **96**, 742-747.

Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y.C., Zaidi, M.S. and Wisniewski, H.M. (1986) Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *J Biol Chem*, **261**, 6084-6089.

Guo, Q., Fu, W., Sopher, B.L., Miller, M.W., Ware, C.B., Martin, G.M. and Mattson, M.P. (1999a) Increased vulnerability of hippocampal neurons to excitotoxic necrosis in presenilin-1 mutant knock-in mice. *Nat Med*, **5**, 101-106.

Guo, Q., Sebastian, L., Sopher, B.L., Miller, M.W., Ware, C.B., Martin, G.M. and Mattson, M.P. (1999b) Increased vulnerability of hippocampal neurons from presenilin-1 mutant knock-in mice to amyloid beta-peptide toxicity: central roles of superoxide production and caspase activation. *J Neurochem*, **72**, 1019-1029.

Guterman, A. and Glickman, M.H. (2004) Complementary roles for Rpn11 and Ubp6 in deubiquitination and proteolysis by the proteasome. *J Biol Chem*, **279**, 1729-1738.

Haass, C. and Selkoe, D.J. (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat Rev Mol Cell Biol*, **8**, 101-112.

Hansson, C.A., Frykman, S., Farmery, M.R., Tjernberg, L.O., Nilsberth, C., Pursglove, S.E., Ito, A., Winblad, B., Cowburn, R.F., Thyberg, J. and Ankarcrona, M. (2004) Nicastrin, presenilin, APH-1, and PEN-2 form active gamma-secretase complexes in mitochondria. *J Biol Chem*, **279**, 51654-51660.

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H. and Mizushima, N. (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature*, **441**, 885-889.

Hartmann, T., Bieger, S.C., Bruhl, B., Tienari, P.J., Ida, N., Allsop, D., Roberts, G.W., Masters, C.L., Dotti, C.G., Unsicker, K. and Beyreuther, K. (1997) Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides. *Nat Med*, **3**, 1016-1020.

He, G., Qing, H., Cai, F., Kwok, C., Xu, H., Yu, G., Bernstein, A. and Song, W. (2006) Ubiquitin-proteasome pathway mediates degradation of APH-1. *J Neurochem*, **99**, 1403-1412.

He, G., Qing, H., Tong, Y., Cai, F., Ishiura, S. and Song, W. (2007) Degradation of nicastrin involves both proteasome and lysosome. *J Neurochem*.

Holtzman, D.M. (2004) In vivo effects of ApoE and clusterin on amyloid-beta metabolism and neuropathology. *J Mol Neurosci*, **23**, 247-254.

Hoozemans, J.J., Veerhuis, R., Van Haastert, E.S., Rozemuller, J.M., Baas, F., Eikelenboom, P. and Scheper, W. (2005) The unfolded protein response is activated in Alzheimer's disease. *Acta Neuropathol (Berl)*, **110**, 165-172.

Hoshino, T., Nakaya, T., Araki, W., Suzuki, K., Suzuki, T. and Mizushima, T. (2007) Endoplasmic reticulum chaperones inhibit the production of amyloid-beta peptides. *Biochem J*, **402**, 581-589.

Hosoda, A., Kimata, Y., Tsuru, A. and Kohno, K. (2003) JPDI, a novel endoplasmic reticulum-resident protein containing both a BiP-interacting J-domain and thioredoxin-like motifs. *J Biol Chem*, **278**, 2669-2676.

Hsia, A.Y., Masliah, E., McConlogue, L., Yu, G.Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R.C., Nicoll, R.A. and Mucke, L. (1999) Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. *Proc Natl Acad Sci U S A*, **96**, 3228-3233.

Hsieh, H., Boehm, J., Sato, C., Iwatsubo, T., Tomita, T., Sisodia, S. and Malinow, R. (2006) AMPAR removal underlies Abeta-induced synaptic depression and dendritic spine loss. *Neuron*, **52**, 831-843.

Huse, J.T., Liu, K., Pijak, D.S., Carlin, D., Lee, V.M. and Doms, R.W. (2002) Beta-secretase processing in the trans-Golgi network preferentially generates truncated amyloid species that accumulate in Alzheimer's disease brain. *J Biol Chem*, **277**, 16278-16284.

Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., Petersen, R.C., Stevens, M., de Graaff, E., Wauters, E., van Baren, J., Hillebrand, M., Joosse, M., Kwon, J.M., Nowotny, P., Che, L.K., Norton, J., Morris, J.C., Reed, L.A., Trojanowski, J., Basun, H., Lannfelt, L., Neystat, M., Fahn, S., Dark, F., Tannenberg, T., Dodd, P.R., Hayward, N., Kwok, J.B., Schofield, P.R., Andreadis, A., Snowden, J., Craufurd, D., Neary, D., Owen, F., Oostra, B.A., Hardy, J., Goate, A., van Swieten, J., Mann, D., Lynch, T. and Heutink, P. (1998) Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature*, **393**, 702-705.

Iqbal, K., Alonso Adel, C., Chen, S., Chohan, M.O., El-Akkad, E., Gong, C.X., Khatoon, S., Li, B., Liu, F., Rahman, A., Tanimukai, H. and Grundke-Iqbal, I. (2005) Tau pathology in Alzheimer disease and other tauopathies. *Biochim Biophys Acta*, **1739**, 198-210.

Iwata, N., Tsubuki, S., Takaki, Y., Shirotani, K., Lu, B., Gerard, N.P., Gerard, C., Hama, E., Lee, H.J. and Saido, T.C. (2001) Metabolic regulation of brain Abeta by neprilysin. *Science*, **292**, 1550-1552.

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

Johnson, E.S., Ma, P.C., Ota, I.M. and Varshavsky, A. (1995) A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J Biol Chem*, **270**, 17442-17456.

Johnson, R.J., Xiao, G., Shanmugaratnam, J. and Fine, R.E. (2001) Calreticulin functions as a molecular chaperone for the beta-amyloid precursor protein. *Neurobiol Aging*, **22**, 387-395.

Jolly-Tornetta, C. and Wolf, B.A. (2000) Protein kinase C regulation of intracellular and cell surface amyloid precursor protein (APP) cleavage in CHO695 cells. *Biochemistry*, **39**, 15282-15290.

Kaether, C., Schmitt, S., Willem, M. and Haass, C. (2006) Amyloid precursor protein and Notch intracellular domains are generated after transport of their precursors to the cell surface. *Traffic*, **7**, 408-415.

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 beta-secretase and presenilin-1 requires APP. *Nature*, **414**, 643-648.

Kamenetz, F., Tomita, T., Hsieh, H., Seabrook, G., Borchelt, D., Iwatsubo, T., Sisodia, S. and Malinow, R. (2003) APP processing and synaptic function. *Neuron*, **37**, 925-937.

Karlstrom, H., Bergman, A., Lendahl, U., Naslund, J. and Lundkvist, J. (2002) A sensitive and quantitative assay for measuring cleavage of presenilin substrates. *J Biol Chem*, **277**, 6763-6766.

Katayama, T., Imaizumi, K., Manabe, T., Hitomi, J., Kudo, T. and Tohyama, M. (2004) Induction of neuronal death by ER stress in Alzheimer's disease. *J Chem Neuroanat*, **28**, 67-78.

Keller, J.N., Huang, F.F. and Markesbery, W.R. (2000) Decreased levels of proteasome activity and proteasome expression in aging spinal cord. *Neuroscience*, **98**, 149-156.

Kinoshita, A., Fukumoto, H., Shah, T., Whelan, C.M., Irizarry, M.C. and Hyman, B.T. (2003) Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. *J Cell Sci*, **116**, 3339-3346.

Koo, E.H. and Squazzo, S.L. (1994) Evidence that production and release of amyloid beta-protein involves the endocytic pathway. *J Biol Chem*, **269**, 17386-17389.

Kudo, T., Okumura, M., Imaizumi, K., Araki, W., Morihara, T., Tanimukai, H., Kamagata, E., Tabuchi, N., Kimura, R., Kanayama, D., Fukumori, A., Tagami, S., Okochi, M., Kubo, M., Tanii, H., Tohyama, M., Tabira, T. and Takeda, M. (2006) Altered localization of amyloid precursor protein under endoplasmic reticulum stress. *Biochem Biophys Res Commun*, **344**, 525-530.

Lazarov, O., Morfini, G.A., Lee, E.B., Farah, M.H., Szodorai, A., DeBoer, S.R., Koliatsos, V.E., Kins, S., Lee, V.M., Wong, P.C., Price, D.L., Brady, S.T. and Sisodia, S.S. (2005) Axonal transport, amyloid precursor protein, kinesin-1, and the processing apparatus: revisited. *J Neurosci*, **25**, 2386-2395.

Lee, M.C., Miller, E.A., Goldberg, J., Orci, L. and Schekman, R. (2004) Bi-directional protein transport between the ER and Golgi. *Annu Rev Cell Dev Biol*, **20**, 87-123.

Lee, M.S., Kwon, Y.T., Li, M., Peng, J., Friedlander, R.M. and Tsai, L.H. (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature*, **405**, 360-364.

Leem, J.Y., Saura, C.A., Pietrzik, C., Christianson, J., Wanamaker, C., King, L.T., Veselits, M.L., Tomita, T., Gasparini, L., Iwatsubo, T., Xu, H., Green, W.N., Koo, E.H. and Thinakaran, G. (2002) A role for presenilin 1 in regulating the

delivery of amyloid precursor protein to the cell surface. *Neurobiol Dis*, **11**, 64-82.
Lesne, S., Koh, M.T., Kotilinek, L., Kayed, R., Glabe, C.G., Yang, A., Gallagher, M. and Ashe, K.H. (2006) A specific amyloid-beta protein assembly in the brain impairs memory. *Nature*, **440**, 352-357.
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**, 1487-1491.
Li, J., Pauley, A.M., Myers, R.L., Shuang, R., Brashler, J.R., Yan, R., Buhl, A.E., Ruble, C. and Gurney, M.E. (2002) SEL-10 interacts with presenilin 1, facilitates its ubiquitination, and alters A-beta peptide production. *J Neurochem*, **82**, 1540-1548.
Lindholm, D., Wootz, H. and Korhonen, L. (2006) ER stress and neurodegenerative diseases. *Cell Death Differ*, **13**, 385-392.
Lindsten, K., de Vrij, F.M., Verhoef, L.G., Fischer, D.F., van Leeuwen, F.W., Hol, E.M., Masucci, M.G. and Dantuma, N.P. (2002) Mutant ubiquitin found in neurodegenerative disorders is a ubiquitin fusion degradation substrate that blocks proteasomal degradation. *J Cell Biol*, **157**, 417-427.
Ling, Y., Morgan, K. and Kalsheker, N. (2003) Amyloid precursor protein (APP) and the biology of proteolytic processing: relevance to Alzheimer's disease. *Int J Biochem Cell Biol*, **35**, 1505-1535.
Lippincott-Schwartz, J., Cole, N.B. and Donaldson, J.G. (1998) Building a secretory apparatus: role of ARF1/COPI in Golgi biogenesis and maintenance. *Histochem Cell Biol*, **109**, 449-462.
Lue, L.F., Kuo, Y.M., Roher, A.E., Brachova, L., Shen, Y., Sue, L., Beach, T., Kurth, J.H., Rydel, R.E. and Rogers, J. (1999) Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. *Am J Pathol*, **155**, 853-862.
Martoglio, B. and Dobberstein, B. (1998) Signal sequences: more than just greasy peptides. *Trends Cell Biol*, **8**, 410-415.
Mathews, P.M., Jiang, Y., Schmidt, S.D., Grbovic, O.M., Mercken, M. and Nixon, R.A. (2002) Calpain activity regulates the cell surface distribution of amyloid precursor protein. Inhibition of clapains enhances endosomal generation of beta-cleaved C-terminal APP fragments. *J Biol Chem*, **277**, 36415-36424.
McConlogue, L., Castellano, F., deWit, C., Schenk, D. and Maltese, W.A. (1996) Differential effects of a Rab6 mutant on secretory versus amyloidogenic processing of Alzheimer's beta-amyloid precursor protein. *J Biol Chem*, **271**, 1343-1348.
Menendez-Benito, V., Verhoef, L.G., Masucci, M.G. and Dantuma, N.P. (2005) Endoplasmic reticulum stress compromises the ubiquitin-proteasome system. *Hum Mol Genet*, **14**, 2787-2799.
Meusser, B., Hirsch, C., Jarosch, E. and Sommer, T. (2005) ERAD: the long road to destruction. *Nat Cell Biol*, **7**, 766-772.
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**, 345-347.
Naruse, S., Thinakaran, G., Luo, J.J., Kusiak, J.W., Tomita, T., Iwatsubo, T., Qian, X., Ginty, D.D., Price, D.L., Borchelt, D.R., Wong, P.C. and Sisodia, S.S.

(1998) Effects of PS1 deficiency on membrane protein trafficking in neurons. *Neuron*, **21**, 1213-1221.

Naslund, J., Haroutunian, V., Mohs, R., Davis, K.L., Davies, P., Greengard, P. and Buxbaum, J.D. (2000) Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. *Jama*, **283**, 1571-1577.

Ni, Y., Zhao, X., Bao, G., Zou, L., Teng, L., Wang, Z., Song, M., Xiong, J., Bai, Y. and Pei, G. (2006) Activation of beta2-adrenergic receptor stimulates gamma-secretase activity and accelerates amyloid plaque formation. *Nat Med*, **12**, 1390-1396.

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., Naslund, J. and Lannfelt, L. (2001) The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Abeta protofibril formation. *Nat Neurosci*, **4**, 887-893.

Nunan, J., Shearman, M.S., Checler, F., Cappai, R., Evin, G., Beyreuther, K., Masters, C.L. and Small, D.H. (2001) The C-terminal fragment of the Alzheimer's disease amyloid protein precursor is degraded by a proteasome-dependent mechanism distinct from gamma-secretase. *Eur J Biochem*, **268**, 5329-5336.

Nunan, J., Williamson, N.A., Hill, A.F., Sernee, M.F., Masters, C.L. and Small, D.H. (2003) Proteasome-mediated degradation of the C-terminus of the Alzheimer's disease beta-amyloid protein precursor: effect of C-terminal truncation on production of beta-amyloid protein. *J Neurosci Res*, **74**, 378-385.

Oddo, S., Caccamo, A., Kitazawa, M., Tseng, B.P. and LaFerla, F.M. (2003) Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiol Aging*, **24**, 1063-1070.

Paschen, W. and Frandsen, A. (2001) Endoplasmic reticulum dysfunction--a common denominator for cell injury in acute and degenerative diseases of the brain? *J Neurochem*, **79**, 719-725.

Paschen, W. and Mengesdorf, T. (2005) Endoplasmic reticulum stress response and neurodegeneration. *Cell Calcium*, **38**, 409-415.

Pasternak, S.H., Bagshaw, R.D., Guiral, M., Zhang, S., Ackerley, C.A., Pak, B.J., Callahan, J.W. and Mahuran, D.J. (2003) Presenilin-1, nicastrin, amyloid precursor protein, and gamma-secretase activity are co-localized in the lysosomal membrane. *J Biol Chem*, **278**, 26687-26694.

Patrick, G.N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P. and Tsai, L.H. (1999) Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature*, **402**, 615-622.

Paulsson, K. and Wang, P. (2003) Chaperones and folding of MHC class I molecules in the endoplasmic reticulum. *Biochim Biophys Acta*, **1641**, 1-12.

Paulsson, K.M. and Wang, P. (2004) Quality control of MHC class I maturation. *Faseb J*, **18**, 31-38.

Plutner, H., Davidson, H.W., Saraste, J. and Balch, W.E. (1992) Morphological analysis of protein transport from the ER to Golgi membranes in digitonin-permeabilized cells: role of the P58 containing compartment. *J Cell Biol*, **119**, 1097-1116.

Qing, H., Zhou, W., Christensen, M.A., Sun, X., Tong, Y. and Song, W. (2004) Degradation of BACE by the ubiquitin-proteasome pathway. *Faseb J*, **18**, 1571-1573.

Rao, R.V., Ellerby, H.M. and Bredesen, D.E. (2004) Coupling endoplasmic reticulum stress to the cell death program. *Cell Death Differ*, **11**, 372-380.

Rechards, M., Xia, W., Oorschot, V., van Dijk, S., Annaert, W., Selkoe, D.J. and Klumperman, J. (2006) Presenilin-1-mediated retention of APP derivatives in early biosynthetic compartments. *Traffic*, **7**, 354-364.
Rechards, M., Xia, W., Oorschot, V.M., Selkoe, D.J. and Klumperman, J. (2003) Presenilin-1 exists in both pre- and post-Golgi compartments and recycles via COPI-coated membranes. *Traffic*, **4**, 553-565.
Reinhard, C., Hebert, S.S. and De Strooper, B. (2005) The amyloid-beta precursor protein: integrating structure with biological function. *Embo J*, **24**, 3996-4006.
Reinstein, E. and Ciechanover, A. (2006) Narrative review: protein degradation and human diseases: the ubiquitin connection. *Ann Intern Med*, **145**, 676-684.
Roberson, E.D., Scearce-Levie, K., Palop, J.J., Yan, F., Cheng, I.H., Wu, T., Gerstein, H., Yu, G.Q. and Mucke, L. (2007) Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. *Science*, **316**, 750-754.
Rubinsztein, D.C. (2006) The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature*, **443**, 780-786.
Russo, C., Venezia, V., Repetto, E., Nizzari, M., Violani, E., Carlo, P. and Schettini, G. (2005) The amyloid precursor protein and its network of interacting proteins: physiological and pathological implications. *Brain Res Brain Res Rev*, **48**, 257-264.
Sadasivan, B., Lehner, P.J., Ortmann, B., Spies, T. and Cresswell, P. (1996) Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity*, **5**, 103-114.
Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multhaup, G., Condron, M.M., Teplow, D.B. and Haass, C. (2001) Presenilin-dependent gamma-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. *EMBO Rep*, **2**, 835-841.
Selivanova, A., Winblad, B., Farmery, M.R., Dantuma, N.P. and Ankarcrona, M. (2006) COPI-mediated retrograde transport is required for efficient gamma-secretase cleavage of the amyloid precursor protein. *Biochem Biophys Res Commun*, **350**, 220-226.
Selkoe, D.J. (2001) Clearing the brain's amyloid cobwebs. *Neuron*, **32**, 177-180.
Selkoe, D.J. (2002) Deciphering the genesis and fate of amyloid beta-protein yields novel therapies for Alzheimer disease. *J Clin Invest*, **110**, 1375-1381.
Sengupta, A., Kabat, J., Novak, M., Wu, Q., Grundke-Iqbal, I. and Iqbal, K. (1998) Phosphorylation of tau at both Thr 231 and Ser 262 is required for maximal inhibition of its binding to microtubules. *Arch Biochem Biophys*, **357**, 299-309.
Skovronsky, D.M., Doms, R.W. and Lee, V.M. (1998) Detection of a novel intraneuronal pool of insoluble amyloid beta protein that accumulates with time in culture. *J Cell Biol*, **141**, 1031-1039.
Skovronsky, D.M., Pijak, D.S., Doms, R.W. and Lee, V.M. (2000) A distinct ER/IC gamma-secretase competes with the proteasome for cleavage of APP. *Biochemistry*, **39**, 810-817.
Song, S., Kim, S.Y., Hong, Y.M., Jo, D.G., Lee, J.Y., Shim, S.M., Chung, C.W., Seo, S.J., Yoo, Y.J., Koh, J.Y., Lee, M.C., Yates, A.J., Ichijo, H. and Jung, Y.K. (2003) Essential role of E2-25K/Hip-2 in mediating amyloid-beta neurotoxicity. *Mol Cell*, **12**, 553-563.
Storrie, B., Pepperkok, R. and Nilsson, T. (2000) Breaking the COPI monopoly on Golgi recycling. *Trends Cell Biol*, **10**, 385-391.

Suh, Y.H. and Checler, F. (2002) Amyloid precursor protein, presenilins, and alpha-synuclein: molecular pathogenesis and pharmacological applications in Alzheimer's disease. *Pharmacol Rev*, **54**, 469-525.

Suzuki, N., Cheung, T.T., Cai, X.D., Odaka, A., Otvos, L., Jr., Eckman, C., Golde, T.E. and Younkin, S.G. (1994) An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. *Science*, **264**, 1336-1340.

Taguchi, J., Fujii, A., Fujino, Y., Tsujioka, Y., Takahashi, M., Tsuboi, Y., Wada, I. and Yamada, T. (2000) Different expression of calreticulin and immunoglobulin binding protein in Alzheimer's disease brain. *Acta Neuropathol (Berl)*, **100**, 153-160.

Ting, J.T., Kelley, B.G., Lambert, T.J., Cook, D.G. and Sullivan, J.M. (2007) Amyloid precursor protein overexpression depresses excitatory transmission through both presynaptic and postsynaptic mechanisms. *Proc Natl Acad Sci U S A*, **104**, 353-358.

Tsai, J., Grutzendler, J., Duff, K. and Gan, W.B. (2004) Fibrillar amyloid deposition leads to local synaptic abnormalities and breakage of neuronal branches. *Nat Neurosci*, **7**, 1181-1183.

Turner, P.R., O'Connor, K., Tate, W.P. and Abraham, W.C. (2003) Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. *Prog Neurobiol*, **70**, 1-32.

Turner, R.S. (2006) Alzheimer's disease. *Semin Neurol*, **26**, 499-506.

Uemura, K., Kitagawa, N., Kohno, R., Kuzuya, A., Kageyama, T., Chonabayashi, K., Shibasaki, H. and Shimohama, S. (2003) Presenilin 1 is involved in maturation and trafficking of N-cadherin to the plasma membrane. *J Neurosci Res*, **74**, 184-191.

Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J. and Selkoe, D.J. (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature*, **416**, 535-539.

van Anken, E. and Braakman, I. (2005) Versatility of the endoplasmic reticulum protein folding factory. *Crit Rev Biochem Mol Biol*, **40**, 191-228.

van Leeuwen, F.W., de Kleijn, D.P., van den Hurk, H.H., Neubauer, A., Sonnemans, M.A., Sluijs, J.A., Koycu, S., Ramdjielal, R.D., Salehi, A., Martens, G.J., Grosveld, F.G., Peter, J., Burbach, H. and Hol, E.M. (1998) Frameshift mutants of beta amyloid precursor protein and ubiquitin-B in Alzheimer's and Down patients. *Science*, **279**, 242-247.

Van Leeuwen, F.W., Hol, E.M., Hermanussen, R.W., Sonnemans, M.A., Moraal, E., Fischer, D.F., Evans, D.A., Chooi, K.F., Burbach, J.P. and Murphy, D. (2000) Molecular misreading in non-neuronal cells. *Faseb J*, **14**, 1595-1602.

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

Verhoef, L.G., Lindsten, K., Masucci, M.G. and Dantuma, N.P. (2002) Aggregate formation inhibits proteasomal degradation of polyglutamine proteins. *Hum Mol Genet*, **11**, 2689-2700.

Vetrivel, K.S. and Thinakaran, G. (2006) Amyloidogenic processing of beta-amyloid precursor protein in intracellular compartments. *Neurology*, **66**, S69-73.

Wild-Bode, C., Yamazaki, T., Capell, A., Leimer, U., Steiner, H., Ihara, Y. and Haass, C. (1997) Intracellular generation and accumulation of amyloid beta-peptide terminating at amino acid 42. *J Biol Chem*, **272**, 16085-16088.

Williams, D.R. (2006) Tauopathies: classification and clinical update on neurodegenerative diseases associated with microtubule-associated protein tau. *Intern Med J*, **36**, 652-660.

Wilson, C.A., Doms, R.W., Zheng, H. and Lee, V.M. (2002) Presenilins are not required for A beta 42 production in the early secretory pathway. *Nat Neurosci*, **5**, 849-855.

Wilson, C.M., Farmery, M.R. and Bulleid, N.J. (2000) Pivotal role of calnexin and mannose trimming in regulating the endoplasmic reticulum-associated degradation of major histocompatibility complex class I heavy chain. *J Biol Chem*, **275**, 21224-21232.

Wilson, R., Allen, A.J., Oliver, J., Brookman, J.L., High, S. and Bulleid, N.J. (1995) The translocation, folding, assembly and redox-dependent degradation of secretory and membrane proteins in semi-permeabilized mammalian cells. *Biochem J*, **307 (Pt 3)**, 679-687.

Wisniewski, T., Ghiso, J. and Frangione, B. (1991) Peptides homologous to the amyloid protein of Alzheimer's disease containing a glutamine for glutamic acid substitution have accelerated amyloid fibril formation. *Biochem Biophys Res Commun*, **179**, 1247-1254.

Yang, Y., Turner, R.S. and Gaut, J.R. (1998) The chaperone BiP/GRP78 binds to amyloid precursor protein and decreases Abeta40 and Abeta42 secretion. *J Biol Chem*, **273**, 25552-25555.

Zhang, K. and Kaufman, R.J. (2006) The unfolded protein response: a stress signaling pathway critical for health and disease. *Neurology*, **66**, S102-109.