Cell death and signal transduction pathways in Alzheimer's disease: The role of presenilin 1

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'There was a merchant in Baghdad who sent his servant to market to buy provisions and in a little while the servant came back, white and trembling, and said, Master, just now when I was in the market-place I was jostled by a woman in the crowd and when I turned I saw it was death that jostled me. She looked at me and made a threatening gesture; now, lend me your horse, and I will ride away from this city and avoid my fate. I will go to Samarra and there death will not find me. The merchant lent him his horse, and the servant mounted it, and he dug his spurs in its flanks and as fast as the horse could gallop he went. Then the merchant went down to the market place and he saw me standing in the crowd and he came to me and said, Why did you make a threatening gesture to my servant when you saw him this morning? That was not a threatening gesture, I said, it was only a start of surprise. I was astonished to see him in Baghdad, for I had an appointment with him tonight in Samarra.'

From 'To cut a long story short' - *'Death Speaks'* by Jeffrey Archer

CONTENTS

ABSTRACT

Mutated presenilins (PSs) may cause familial Alzheimer's disease (FAD) by altering neuronal signal transduction pathways, by increasing Aβ production or by triggering a number of pro-apoptotic mechanisms. The present thesis explores mechanisms by which PSs regulate signal transduction and cell death with relevance to AD.

Paper I explored the complex proteolytic processing of wild-type (WT) and FAD presenilin 1 (PS1) exon 9 deleted mutant (ΔE9 PS1) during apoptosis. PS1 was actively processed by both caspases and the proteasome during apoptosis in SH-SY5Y neuroblastoma cells non-transfected (NT) or transfected with WT PS1. Proteasome inhibitors blocked the degradation of full-length WT PS1 and caspase cleaved PS1 fragments. In contrast, the N-terminal and C-terminal fragments generated by PS1 endoproteolysis and the truncated full-length ΔE9 PS1 were not degraded by the proteasome during apoptosis.

Paper II aimed to investigate whether PS1 cleavage by caspases is an early or a late event in the activation of apoptotic cascades. Overexpression of ΔE9 PS1 sensitized SH-SY5Y neuroblastoma cells to death induced by calcium ionophore A23187. We found that PS1 alternative cleavage is an early apoptotic event in ΔE9 PS1 transfected cells, simultaneous with minimal caspase-3 activation and preceding cleavage of poly(ADP-ribose) polymerase (PARP) and gelsolin. PS1 ΔE9 perturbed Ca^{2+} homeostasis and buffering in SH-SY5Y cells, this being at least one of the mechanisms by which mutated PS1 sensitized cells to apoptosis. We concluded that alternative cleavage of PS1 is an early apoptotic event; therefore it may play a role for the regulation of the proteolytic cascades during apoptosis.

Papers III and IV aimed to characterize the role of PS1 in cholinergic muscarinic receptor signal transduction pathways. We studied the effect of three FAD PS1 mutants (ΔE9, M146V and L250S) and two dominant negative PS1 mutants (D257A and D385N) on basal and carbachol-stimulated phosphoinositide (PI) hydrolysis and intracellular calcium concentrations ($[Ca^{2+}]_i$) in SH-SY5Y neuroblastoma cells. We found a significant increase in basal PI hydrolysis in PS1 ΔE9 and PS1 M146V, but not in PS1 L250S, cells. All PS1 ΔE9, PS1 M146V and PS1 L250S cells showed a significant increase in carbachol-induced $[Ca^{2+}]_i$ as compared to non-transfected or wild-type PS1 transfected cells. The elevated carbachol-induced $[Ca^{2+}]_i$ signals were reversed by PLC inhibition and by ryanodine receptor blockade in all mutant PS1 cell lines. In PS1 M146V and PS1 L250S cells, pharmacological γ-secretase inhibition was also able to reverse the elevated carbachol-induced $[Ca^{2+}]_i$ signals. Cells expressing dominant negative PS1 had attenuated carbachol-stimulated PI hydrolysis and $[Ca^{2+}]_i$ responses. In NT or WT PS1 cells pharmacological inhibition of γ-secretase attenuated carbachol-stimulated PI hydrolysis and $[Ca^{2+}]_i$ responses to levels found in PS1 dominant negative cells. The findings of these reports suggest that PS1 can regulate PLC activity and that this function is γ-secretase activity dependent.

In conclusion, these studies provide evidence that PS1 regulates both cholinergic signal transduction pathways and apoptotic cell death and that PS1 mutations are responsible for both sensitizing neurons to death and altering neuronal signal transduction.

LIST OF ORIGINAL PUBLICATIONS

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

I. **B. O. Popescu, A. Cedazo-Minguez, L. M. Popescu, B. Winblad, M. Ankarcrona.**
The proteasome regulates presenilin 1 levels during apoptosis.
Manuscript.

II. **B. O. Popescu, A. Cedazo-Minguez, L. M. Popescu, B. Winblad, R. F. Cowburn, M. Ankarcrona.**
Caspase cleavage of exon 9 deleted presenilin-1 is an early event in apoptosis induced by calcium ionophore A23187 in SH-SY5Y neuroblastoma cells.
Journal of Neuroscience Research **66**:122-134 (2001).

III. **A. Cedazo-Minguez, B. O. Popescu, M. Ankarcrona, T. Nishimura, R. F. Cowburn.**
The presenilin 1 ΔE9 mutation gives enhanced basal phospholipase C activity and a resultant increase in intracellular calcium concentrations.
The Journal of Biological Chemistry **277**:36646-36655 (2002).

IV. **B. O. Popescu, A. Cedazo-Minguez, E. Benedikz, T. Nishimura, B. Winblad, M. Ankarcrona, R. F. Cowburn.**
γ-Secretase activity of presenilin 1 regulates acetylcholine muscarinic receptor mediated signal transduction.
In press in The Journal of Biological Chemistry, JBC online (18 nov2003).

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ABBREVIATIONS

Aβ	amyloid beta peptide
aCTF	alternative, caspase-generated, C-terminal fragment
Ach	acetylcholine
AD	Alzheimer's disease
ALG	apoptosis-linked gene
AM	acetoxymethyl
aNTF	alternative, caspase-generated, N-terminal fragment
APLP	amyloid precursor-like protein
ApoE	apolipoprotein E
APP	amyloid precursor protein
$[Ca^{2+}]_i$	intracellular calcium concentration
CCE	capacitative calcium entry
CSF	cerebrospinal fluid
CTF	C-terminal fragment
ΔE9	exon 9 deleted
DAG	diacylglycerol
DMEM	Dulbecco's modified essential media
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
FAD	familial Alzheimer's disease
GSK3	glycogen synthase kinase 3
HEK	human embryonic kidney
IP_3	inositol triphosphate
IP_3R	inositol triphosphate receptor
IPs	inositol phosphates
JNK	c-jun N-terminal kinase
kDa	kilodaltons
KHB	Krebs-Henseleit bicarbonate buffer
LDH	lactate dehydrogenase

MAPK	mitogen activated protein kinase
MEM	minimum essential media
mRNA	messenger ribonucleic acid
MTT	3-(4, 5-dimethyl-thiazol-3-yl)-2, 5-diphenyltetrazolium bromide
NGF	nerve growth factor
NO	nitric oxide
NFT	neurofibrillary tangles
NTF	N-terminal fragment
NT	non-transfected
PARP	poly(ADP-ribose)-polymerase
PBS	phosphate buffered saline
PHFs	paired helical filaments
PI	phosphoinositide
PIP_2	phosphatidylinositol 4,5-biphosphate
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PKC	protein kinase C
PLC	phospholipase C
PS1	presenilin 1
PS2	presenilin 2
PSs	presenilins
RIP	regulated intramembrane proteolysis
RyR	ryanodine receptors
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
SP	senile plaques
STS	staurosporine
TM	transmembrane
WT	wild-type

INTRODUCTION

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most frequent cause of age-related progressive dementia, which constantly leads to profound alteration of cognitive functions and premature death. A certain AD diagnosis has still to be confirmed post-mortem and relies on the presence of senile plaques (SP), neurofibrillary tangles (NFT) and widespread neuronal degeneration, the pathological hallmarks of the disease. A reduction in weight with 20 % or more is characteristic for AD brains and is due to atrophy, which is the macroscopic proof of cell loss and shrinkage. Both cell death and loss of synapses account for the clinical manifestations of the disease (Selkoe, 2002). Brain atrophy affects the temporal, parietal and frontal lobes, in this order. SP consist of extracellular core accumulations of beta amyloid peptide (Aβ), surrounded by morphologically altered nerve terminals, especially dendrites, with abnormal lysosomes and mitochondria. Aβ results from the β-amyloid precursor protein (APP), which is the substrate of a complex proteolytic processing by proteases, called secretases. NFTs are found within neurons and are formed by paired helical filaments (PHFs) of hyperphosphorylated microtubule-associated protein tau.

Based on genetic and epidemiological data, AD is classified as either sporadic or familial (FAD). Highly penetrant mutations in genes encoding presenilin 1 (PS1), presenilin 2 (PS2) or APP are associated with early onset FAD but account for only approximately 1% of all AD cases (St. George-Hyslop, 2000). In addition, carriers of apolipoprotein E (apoE) allele ε4 have a higher risk to develop AD and a lower age of onset of the disease, and brains of ε4 AD cases have more severe plaque and tangle pathology (Cedazo-Minguez and Cowburn, 2001). Other unidentified susceptibility genes have been suggested for AD, for instance localized to chromosomes 10 (Bertram et al., 2000) and 12 (Pericak-Vance et al., 1997) but they have not been cloned yet and their link to AD is not clearly established.

To date the cause and progression of AD are not fully understood. Two major pathogenic scenarios emerged in the last decade to explain the neuronal dysfunction and loss. These are the amyloid cascade hypothesis (Hardy and Higgins, 1992) and the cytoskeleton degeneration hypothesis (Braak and Braak, 1991) that will be detailed below.

AMYLOID PRECURSOR PROTEIN AND Aβ GENERATION

APP is an evolutionary conserved type I membrane protein with a wide tissue distribution, including neurons. APP has a short C-terminal domain located to the cytosol and a long N-terminal domain located to lumen or extracellular space (Figure 1).

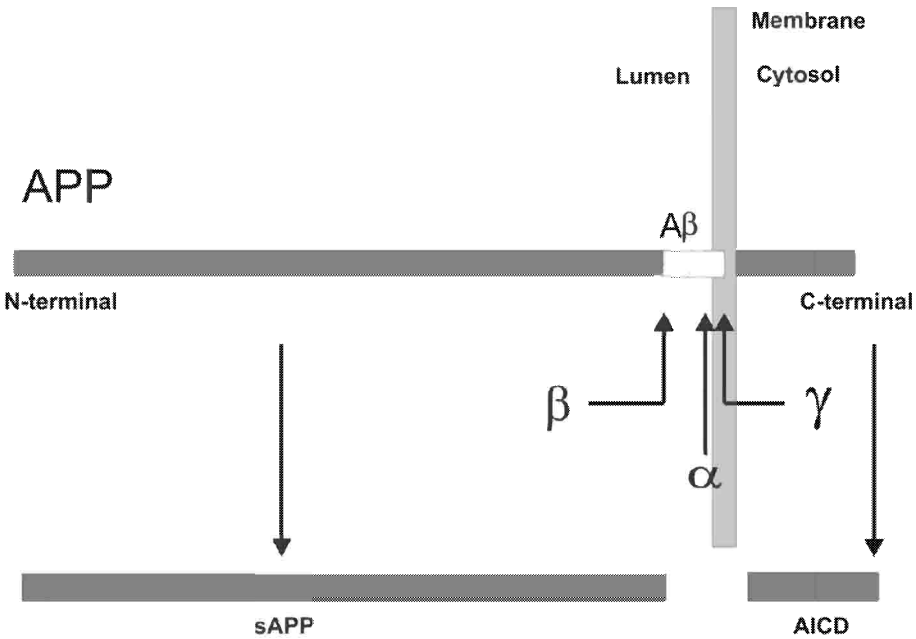

Figure 1. Proteolytic processing of the amyloid precursor protein (APP). APP is an integral type I protein with the N-terminal part laying in the lumen and the C-terminal part within the cytosol. In the non-amyloidogenic pathway, APP is cleaved by α-secretase, within the Aβ sequence. In contrast, in the amyloidogenic pathway APP cleavage by β- and γ-secretases generates Aβ. The cut of α- and β-secretases releases secreted APP (sAPP) and the γ-secretase cleavage gives rise to the APP intracellular domain (AICD), which translocates to the nucleus and regulates gene transcription.

Different APP mRNAs arise from the splicing of a gene located on chromosome 21 (APP-695, APP-751, APP-770). Other proteins with similar structure to APP exist and are called APLPs (Suh and Checler, 2002). APP physiological functions are not fully elucidated yet, but roles in axonal transport, synaptic plasticity, neuroprotection, cell growth and cell adhesion, were suggested (De Strooper and Annaert, 2000). However, mice homozygous for an APP null mutation have only discreet locomotor and behavioral deficits (Zheng et al., 1995), probably due to the redundancy of APLPs as far as double mutants homozygous for an APP deletion and an APLP2 deletion do not survive (Heber et al., 2000). APP normally undergoes proteolysis by α-secretase within the Aβ domain (Figure 1) to yield a secreted form (α-APPs), and further processing of the remaining CTF by γ-secretase results in a small peptide (p3) and an APP intracellular domain (AICD).

An alternative processing pathway involving β- and γ-secretases generate β-APPs, Aβ peptide and AICD (Figure 1). Two members of a disintegrin and metalloprotease family (ADAM), ADAM-10 and ADAM-17 (TACE) are candidates for α-secretases, a transmembrane aspartyl-protease, BACE (Asp-2) was identified as β-secretase and a high molecular weight multiprotein complex consisting of PSs, Nicastrin, Aph-1 and Pen-2 executes the γ-secretase activity (Suh and Checler, 2002, Edbauer et al., 2003). Presenilins are essential for the γ-secretase activity and production of Aβ. Cells from PS1/PS2 double-knockout mice do not produce any Aβ, by complete abolishment of the γ-secretase activity (Herreman et al., 2000, Zhang et al., 2000), which proves that presenilins are needed for APP processing and Aβ generation. However, another group reported that in mice fibroblasts deficient in both PS1 and PS2, Aβ production still takes place (Armogida et al., 2001). Mutations in genes encoding APP or PSs result in an enhanced production of longer, more fibrillogenic forms of Aβ (Suzuki et al., 1994, Scheuner et al., 1996, Citron et al., 1997).

Different functions have been attributed to each proteolytic product derived from APP. The soluble (secreted) forms of APP were proposed to be neuroprotective and to play a role in learning and memory (Turner et al., 2003). The small peptide p3 has no known function so far. The AICD activates gene expression by coupling with the nuclear adaptor protein Fe65 (Cao and Sudhoff, 2001) and this transcriptional activity is downregulated by activation of the NF-kappaB pathway (Zhao and Lee, 2003).

Some reports suggest a neurotoxic activity for C-terminal APP species (Suh and Checler, 2002). APP is also a substrate for caspase cleavage, and a resulting 31 amino acids CTF has been shown to be toxic (Lu et al., 2003). Finally, a large body of evidence supports that Aβ peptide is toxic. Aβ has been shown to be toxic to cells in culture both when applied extracellulary or after microinjection (Zhang et al., 2002). It seems that mainly the intracellular form and not the secreted one is responsible for the neurotoxicity (Echeverria and Cuello, 2002). Aβ promotes oxidative stress either directly or indirectly and oxidation of Aβ at methionine residue 35 can lead to further generation of reactive oxygen species (ROS) (Kanski et al., 2002). The oxidative effects of Aβ may also be mediated through interaction with redox-active metals since metal chelation treatment of Aβ significantly attenuates toxicity. It has also been argued that Aβ is an anti-oxidant and produced to protect cells from oxidative challenge (Rottkamp et al, 2002). Other mechanisms involved in Aβ neurotoxicity are alteration of Ca^{2+} homeostasis (Mattson et al., 1992, Vitek et al., 1994), enhancement of glutamate-mediated toxicity (Harkany et al., 2000), microglial activation with cytokine release (Hu et al., 1998, Lue et al., 2001), activation of mitogen-activated protein kinase (MAPK) with subsequent tau hyperphosphorylation (Rapoport and Ferreira, 2000). The amyloid cascade hypothesis is based on all these data and states that tau hyperphosphorylation is induced by altered APP processing and Aβ generation.

TAU PROTEIN AND NEUROFIBRILLARY TANGLES

Tau is a microtubule-associated phosphoprotein which is expressed in both central and peripheral nervous system, mainly in neurons. Tau binds to tubulin, promoting microtubules assembly and stability. The balance of tau phosphorylation / dephosphorylation modulates the stability of the cytoskeleton and the axonal morphology. In AD brains, tau becomes hyperphosphorylated mainly in its C-terminal region, which contains the microtubule-binding domain. The hyperphosphorylated tau forms paired helical filaments (PHFs) that in turn form the neurofibrillary tangles. More than 20 phosphorylation sites have been identified for tau (Hanger et al., 1998). Many different kinases have been

shown to phosphorylate tau, including cyclin dependent kinases 2 and 5, MAPK, Ca^{2+}-calmodulin-dependent protein kinase II, PKA, PKC, but the most important tau kinase in the brain seems to be glycogen synthase kinase-3 β (GSK-3β, Lovestone and Reynolds, 1997). Tau is dephosphorylated by both protein phosphatase 2A (PP2A) and 2B (PP2B), but PP2A is by far predominant (Billingsley and Kincaid, 1997). In AD, the balance between the activity of tau kinases and phosphatases is altered and the hyperphosphorylated tau leads to impaired axonal transport and finally to neurodegeneration. The neurofibrillary tangle pathology matches both the areas of neuronal loss and the degree of cognitive deterioration. However, evidence presented by a recent study has suggested that tangle formation could be secondary to altered APP processing (Oddo et al., 2003).

CELL DEATH IN ALZHEIMER'S DISEASE BRAIN

Neuronal death in specific brain regions is a common feature of neurodegenerative disorders. The cell death process probably begins several years before clinical signs such as cognitive impairment become apparent. As more and more neurons degenerate the patient decline progressively in cognition and dies within five to twenty years after the first diagnosis. The mechanisms of cell death in the AD brain are not fully elucidated however it is likely that several forms of cell death are involved. It seems that loss of synapses is the first morphological sign of neuronal injury in AD, preceding cell death. Recently, a triple-transgenic derived model of AD made possible the observation that synaptic loss appears earlier than plaques and tangles (Oddo et al., 2003). Cytosolic extracts from synaptosomes exposed to Aβ induced chromatin condensation and fragmentation of isolated nuclei showing that apoptotic signals can be generated locally in synapses (Mattson et al., 1998a, Mattson et al., 1998b). Neurons that lose synapses and therefore also contact and communication with other cells are still alive but do not function as before and do not survive in the long run, probably being secondary deprived of trophic factors. Such cells could however stay in the tissue as "ghost cells" before they are phagocytozed by astrocytes or microglia. One example of such "semi-dead cells" is

the so-called dark neurons. Morphological characteristics of dark neurons include: massive shrinkage; hyper-electron-dense karyoplasms, cytoplasm, dendrites; aggregation of nuclear chromatin and dilation of Golgi cisternae. Dark neurons either recover after an insult or they die through pathways distinct from apoptosis and necrosis (Csordas et al., 2003). However, some dark neurons were positive for apoptotic markers in a PS1 mutant transgenic mouse model (Tabira et al., 2002). In the same study an increase of TUNEL/Aβ42 positive neurons was detected in samples from AD brain (Tabira et al., 2002). There are also several other evidences for apoptosis in AD. Post-mortem analysis of AD brain showed TUNEL positive neurons and glia in hippocampus and cortex indicating DNA fragmentation, increased expression of Bcl-2 family members, as well as increased caspase activities and cleavage of caspase substrates (Table 1)

Table 1. Evidences for apoptosis in AD brain.

Apoptosis hallmark	References
DNA fragmentation	Su et al., 1994, Lassmann et al., 1995, Smale et al., 1995, Dragunow et al., 1995, Li et al., 1997, Lucassen et al., 1997, Sugaya et al., 1997
Increased expression of Bcl-2 family proteins	Su et al., 1997, Drache et al., 1997, MacGibbon et al., 1997, Kitamura et al., 1998, Giannakopoulos et al., 1999
Increased caspase activities	Yang et al., 1998, Uetsuki et al., 1999, LeBlanc et al., 1999, Chan et al., 1999, Stadelmann et al., 1999, Rohn et al., 2001, Pompl et al., 2003

Cells that are triggered to die by apoptosis (eg. have active caspase 8 and 9, which are initiator caspases), but fail to complete the process because executor caspases such as caspase-3 and -7 are not active, were described in AD brain (Raina et al., 2001). This phenomenon is called “abortosis” and is as an anti-apoptotic mechanism that might try to protect neurons from death. However, this process is probably finally over-ridden since many neurons still die in AD. There is also evidence for activation of cell cycle proteins in AD brain (Yang et al., 2003, Copani et al., 2003). This may be an attempt of the cells to try to survive bad conditions or toxic stimuli. However the neurons do not go through mitosis, instead they are stuck in a cycle they cannot complete and eventually die. Post

mitotic neurons do not normally divide, but it is possible that re-entry of the cell cycle is necessary for the completion of apoptosis. Normally proliferating cells are regularly checked throughout the cell cycle and taken aside to die by apoptosis when damaged. Maybe also post mitotic cells have to take this way to death.

A cell dying by apoptosis leaves no traces in the tissue since it is silently disassembled and phagocytosed. Therefore the main part of cells, which presumably have died by apoptosis during the course of AD, have already been cleared from the tissue at the time of autopsy. This is one of the difficulties with proving the impact of apoptotic cell death in AD. It has also been argued that the great difference in time spans between the disease process (approximately 20 years) and the apoptotic process (approximately 24 hours), rules out apoptosis as a mechanism for cell death in AD. However, if cell death is triggered at different times during the course of the disease it is very likely that cells die by apoptosis in AD.

It is also possible that neurons die by necrosis in AD if a violent insult overlaps on the chronic progressive course of the disease. Recent findings brought to attention a possible role of vascular injuries in AD progression (Jellinger and Attems, 2003). Glutamatergic neurotransmission, an important process in learning and memory, is severely disrupted in patients with AD. Glutamate can induce either necrosis or apoptosis depending on the concentration (Ankarcrona et al., 1995). In stroke models neurons in the focus of the ischemic area die by necrosis, while neurons in the penumbra zone die by apoptosis (Siesjö, 1992). Glutamate can also accumulate in the AD brain and the NMDA receptor antagonist memantine is now used to treat moderate to severe AD (Butterfield et al., 2003).

PRESENILINS (PSs)

PSs structure and expression

In 1995, genes encoding PS1 and PS2 were identified on chromosome 14 (Sherrington et al., 1995) and chromosome 1 (Levy-Lahad et al., 1995), respectively, and mutations of these genes were correlated with a large number of FAD cases. To date,

more than 100 pathological mutations of PS1 and only a few of PS2 were detected in FAD cases. All these are missense mutations that generate single amino acid substitutions in the protein primary structure, with the exception of PS1 exon 9 deletion splice mutation (Perez-Tur et al, 1995).

PS1 and PS2 are multipass membrane proteins sharing 60% amino-acid sequence homology and are predicted to contain 6 to 8 transmembrane (TM) domains (Doan et al., 1996). Between TM domains 6 and 7 they contain a large hydrophilic loop, on the cytosolic side of the membrane. The cytoplasmic loop includes the sites where different proteolytic cleavages of PSs occur (Figure 2). A physiological endoproteolytic cleavage by an elusive protease known as "presenilinase" occurs within the exon 9 encoded region of PSs, between residues Thr_{291} and Ala_{298} (Podlisny et al., 1997). An autoproteolysis hypothesis for PSs was proposed (Wolfe et al., 1999) but recent data make it less probable (Campbell et al., 2003). This cleavage results in a ~30 kDa N-terminal fragment and a ~20 kDa C-terminal fragment, which accumulate in cells with a 1:1 stoichiometry and represent the main PSs species in human tissues (Podlisny et al., 1997). The NTF and CTF form a functional heterodimer, which is a part of a high molecular weight complex consisting of several molecular partners (Nicastrin, Aph-1 and Pen-2) that executes the γ-secretase cleavage of APP described above (Edbauer et al., 2003). The levels of N-terminal and C-terminal PSs derivatives seem to be tightly regulated by limiting cellular factors (Thinakaran et al., 1996) and overexpression of PSs holoproteins does not increase correspondingly the levels of N- and C-terminal fragments.

PSs are highly conserved among species, from flies like *Drosophila melanogaster* (Hong and Koo, 1997, Boulianne et al., 1997) or worms like *Caenorhabditis elegans* (Levitan et al., 1996, Li and Greenwald, 1996) to mammals. There are no known proteins with a sequence similar to PSs in unicellular organisms.

PSs are ubiquitous proteins, being identified in most human organs, e.g. brain, lung, heart, liver and muscle (Okochi et al., 1998). Within the brain, PSs are present in variable amounts in all regions, mainly in the neocortex, hippocampal pyramidal neurons and magnocellular basal forebrain neurons (Lee et al., 1996, Lah et al., 1997), areas primarily affected by AD. PS1 and PS2 mRNAs were identified to a larger extent in

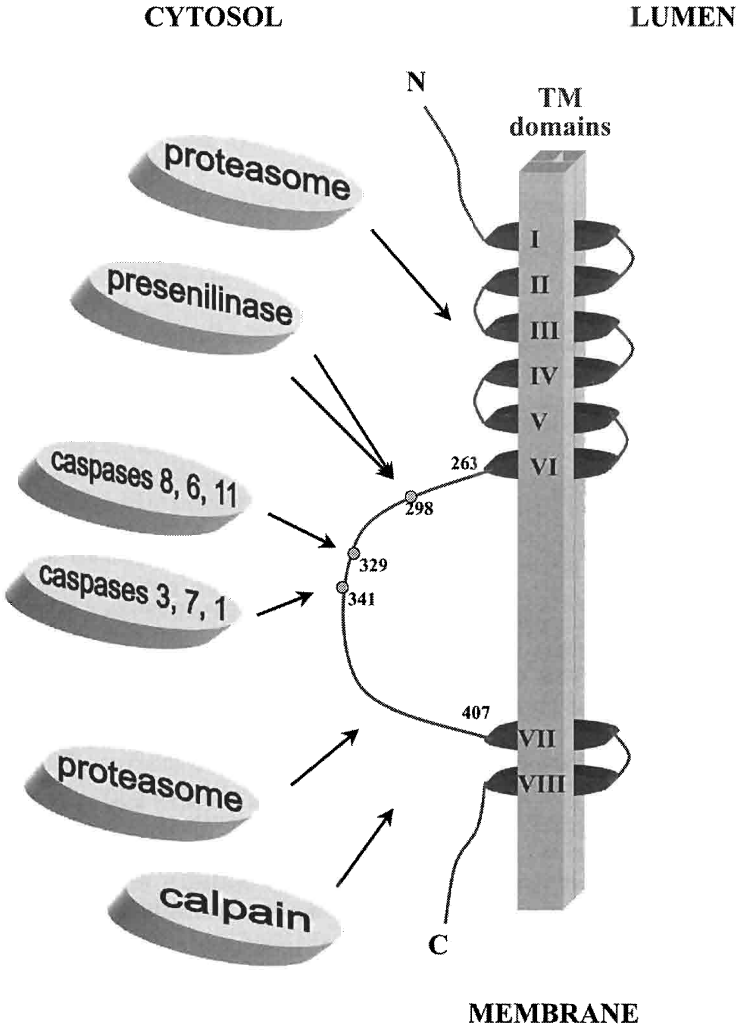

Figure 2. Topology and proteolytic processing of PS1. PS1 is an integral protein, which contains 6-8 transmembrane (TM) domains. A large hydrophilic loop (between amino acids 263-407) and the N-terminal and C-terminal regions of PS1 are located on the cytoplasmic side of the membrane. PS1 is physiologically processed by 'presenilinase' to N- and C-terminal fragments that are stable in heterodimeric complexes. Caspases cleave PS1 at two identified sites within the loop. N- and C-terminal fragments not included in heterodimers are degraded by the proteasome. Calpain, another protease activated during cell death, also cleaves the C-terminal fragment of PS1.

neurons but are also detectable in glial cells and no difference in distribution of the two related proteins was found (Lee et al., 1996).

Within the cells, PSs are mainly located to the endoplasmic reticulum (ER) and Golgi apparatus (Kovacs et al., 1996, Culvenor et al., 1997), but also to the nuclear envelope (Li et al., 1997), to the plasma membrane (Dewji and Singer, 1997) and to the mitochondrial inner membrane (Ankarcrona and Hultenby, 2002). PSs are not substrates for sulfation, acylation or glycosylation (Walter et al., 1996, De Strooper et al., 1997).

PSs physiological roles

Mice knocked-out for both PS1 and PS2 die before embryonic day 13.5 (Donoviel et al., 1999). PS1 can compensate for the loss of PS2 (PS1+/+, PS2-/- and PS1 +/-, PS2-/- embryos survive), while PS2 cannot fully compensate for the loss of PS1 (PS1-/-, PS2+/+ embryos die at birth; PS1-/-, PS2+/- embryos die during E 9.5 – E 13.5, Herreman et al., 1999). PS1 gene deletion is lethal and PS1-/- embryos show alterations in somatic development and massive neuronal loss (Shen et al., 1997). Not only wild type PS1 but also FAD mutant PS1 can rescue PS1 -/- embryos (Davis et al., 1998) indicating that FAD mutations do not affect the PS1 functions during embryogenesis. A wealth of data shows that the main function of PSs consists of enabling regulated intramembrane proteolysis (RIP) through γ-secretase activity. Presenilins are important during development since the cleavage of NOTCH and the subsequent generation of NICD, a C-terminal fragment of NOTCH involved in gene regulation, is dependent on functional presenilin (Struhl and Greenwald, 1999). Other functions were also suggested for PSs, like involvement in cell-to-cell adhesion (Georgakopoulos, 1999), facilitation of GSK-3β interaction with tau (Takashima et al., 1998) and of Erb-4 intranuclear signalling (Ni et al., 2001).

PSs and apoptosis regulation

Mutated PSs could cause FAD by triggering various pro-apoptotic mechanisms in agreement with the hypothesis that cell loss in AD is due to apoptosis. Many reports have

shown that different PSs FAD mutations sensitize cells to apoptosis triggered by several stimuli (Table 2). These data could explain why mutant PS FAD brains show extensive neurodegeneration at an early age.

Table 2. Presenilin mutations effects on apoptosis (adapted from Popescu and Ankarcrona, 2000)

	Mutation	Apoptosis inducer	Cells/animals	Reference
PS1	H115Y	Anti-Fas receptor antibody	Jurkat cells	Wolozin et al., 1998
	M146V	β-amyloid	PC12 cells	Guo et al., 1998b
	M146V	β-amyloid	PC6 cells	Guo et al., 1999a
	M146V	β-amyloid	Rat hippocampal neurons	Guo et al., 1999b
	M146V	Focal cerebral ischemia	PS1 mutant knock-in mice	Mattson et al., 2000
	M146V	Glucose deprivation	PS1 mutant knock-in mice	Xie et al., 2001
	M146V	Etoposide	PS1 mutant knock-in mice	Chan et al., 2002
	M146L	ER-Golgi toxins, staurosporine	Mouse cortical neurons	Terro et al., 2002
	A246E	None (spontaneous)	Rat hippocampal neurons	Wiehl et al., 1999
	A246E	None (spontaneous)	PC12 cells	Wiehl et al., 1999
	A246E	Staurosporine	H4 cells	Kovacs et al., 1999
	L250S	High glucose	SH-SY5Y cells	Tanii et al., 2000
	L286V	Trophic factor deprivation, β-amyloid	PC12 cells	Guo et al., 1997
	L286V	β-amyloid	PC12 cells	Guo et al., 1998a, Guo et al., 1998b
	L286V	3-nitropropionic acid malonate	PC12 cells	Keller et al., 1998
	L286V	Staurosporine	H4 cells	Kovacs et al., 1999
	L286V	β-amyloid	PC6 cells	Guo et al., 1999a
	L286V	etoposide	PC12 cells	Chan et al., 2002
	C410Y	None (spontaneous)	Rat hippocampal neurons	Weihl et al., 1999
	C410Y	None (spontaneous)	PC12 cells	Weihl et al., 1999
	Exon 9 deletion	Staurosporine	H4 cells	Kovacs et al., 1999
	Exon 9 deletion	High glucose	SH-SY5Y cells	Tanii et al., 2000
	Exon 9 deletion	Overexpression of PAG	HEK293 cells	Zhou et al., 2002
PS2	N114I	Trophic factor deprivation, β-amyloid	PC12 cells	Wolozin et al, 1996
	N114I	None (spontaneous)	HeLa cells	Janicki et al., 1997
	N114I	None (spontaneous)	HEK293 cells	da Costa et al., 2003

Various studies reported numerous proteins directly interacting with PSs (Van Gassen et al., 2000). Beside the interaction with the γ-secretase complex partners, which will be detailed later, PSs also bind to proteins involved in the mitochondrial and cytoplasmic phases of apoptosis.

PSs interact with Bcl-2 family proteins. The Bcl-2 protein and members of its family regulate apoptosis at the molecular level, by inhibiting or promoting adapters that activate the caspase proteolytic cascade (Adams and Cory, 1998). Alberici and colleagues reported in 1999 a direct interaction between Bcl-2 and PS1, using a yeast two-hybrid interaction system. Their hypothetical model proposes a macromolecular complex containing Bcl-2 and PS1, which disassembles in response to an apoptotic stimulus, suggesting a cross-talk between Bcl-2 and PS1 during apoptosis (Alberici et al., 1999). Bcl-2 is located to the outer membrane of mitochondria, to the cytosol and to the nuclear membrane, but a functional concept of homo- and hetero-dimerization between the Bcl-2 family members has been discussed with respect to the mitochondrial localization (Cory and Adams, 2002). Beside the location in ER and Golgi apparatus, PS1 seem also to be present in the mitochondrial inner membrane (Ankarcrona and Hultenby, 2002), which also supports the concept that Bcl-2 and PS1 could functionally interact during apoptosis. Moreover, another anti-apoptotic member of the Bcl-2 family, Bcl-X_L, has been found to interact with both PS1 and PS2 (Passer et al., 1999). In addition, a recently identified pro-apoptotic mitochondrial protein (PSAP) has been shown to interact with PS1 (Xu et al., 2002).

PSs interact with catenins. The catenins are a family of proteins characterized by repeats of an amino acid sequence motif related to a *Drosophila Armadillo* gene product. Within the cells, catenins function in at least two ways. First, they constitute between actin filaments and cadherins, connecting the cytoskeleton to intercellular adhesive junctions. Second, they are switch molecules in the Wingless/Wnt signaling pathway, which is involved in development of the nervous system and many other cell fate decisions (Dierick et al., 1999). Several reports showed that β-catenin interacts with PS1 (Zhou et al., 1997, Murayama et al., 1998) and the two proteins are found in high

molecular weight intracellular complexes. Levesque and co-workers identified the PS1 and PS2 sequences that interact with β-catenin as being located to the cytoplasmic hydrophilic loop (Levesque et al., 1999). It has been shown that not only β-catenin, but also other members of the catenin family, like p0071 (Stahl et al., 1999) and neural plakophilin-related armadillo protein (Levesque et al., 1999), interact with PSs. It has been reported that β-catenin is more stable in complexes with PS1, and PS1 mutations have a destabilizing effect, which potentiates apoptosis (Zhang et al., 1998). Furthermore, β-catenin levels are decreased in AD brains (Zhang et al., 1998). Interestingly, β-catenin is also a substrate for caspases, its cleavage concurring to cytoskeletal breakdown during apoptosis (Brancolini et al., 1997). β-catenin is also a substrate for GSK-3β phosphorylation, the main kinase involved in tau hyperphosphorylation (Aberle et al., 1997), which interacts with PS1 as well (Takashima et al., 1998).

Many studies have explored the mechanisms by which mutant PSs are able to sensitize cells to undergo apoptosis upon stressful conditions. These data are presented in Table 3.

Beside the deleterious effects of PSs mutations it is still under debate how the wild-type PSs participate in the regulation of apoptosis. Da Costa and colleagues have recently shown that the CTF of PS2 is pro-apoptotic and this function is not dependent on its NTF counterpart, being executed through activation of p53, down regulation of Bcl-2 and translocation of cytochrome c to the cytosol, with subsequent caspase activation (da Costa et al., 2003). This study is in agreement with previous ones, which have shown that PS2 is pro-apoptotic (Vito et al., 1996, Deng et al., 1996, Araki et al., 2000). Another mechanism proposed for the pro-apoptotic effect of PS2 was activation of p38-MAPK (Sun et al., 2001). The pro-apoptotic PS2 seems also to down-regulate PS1 during apoptosis, which was proposed to be anti-apoptotic (da Costa et al., 2002). Treatment of PC12 cells with nerve growth factor (NGF), which has a well-known anti-apoptotic effect in various models, increases the levels of PS1 NTF and CTF (Counts et al., 2001) and one study suggested that PS1 is anti-apoptotic by inhibiting the SAPK/JNK signaling pathway (Kim et al., 2001). However, previous studies have shown that the overexpression of wild-type PS1 leads to abnormal accumulation of full-length protein and to increased sensitivity of neurons or Jurkat cells to undergo apoptosis (Czech et al.,

Table 3. Cellular mechanisms triggered by mutated PSs in different apoptotic models (adapted from Popescu and Ankarcrona, 2000)

Mechanism	PS mutation	Apoptosis blocking agents	Reference
Ca^{2+} homeostasis alteration	PS1 M146V	Xestospongin, dantrolene	Mattson et al., 2000
	PS1 M146V	-	Chan et al., 2000
	PS1 M146V, L286V	Calbindin D28k	Guo et al., 1998b
	PS1 L286V	Nifedipine, dantrolene	Guo et al., 1996
	PS1 L286V	Bcl-2, cycloheximide	Guo et al., 1997
	PS1 L286V	Secreted β-APP	Guo et al., 1998a
	PS1 L286V	BAPTA-AM, xestospongin C	Chan et al., 2002
Oxyradicals overproduction	PS1 M146V, L286V	Mn superoxide dysmutase, uric acid	Guo et al., 1999a
	PS1 L286V	Propyl gallate, vitamine E	Guo et al., 1997
	PS1 L286V	17β-estradiol	Mattson et al., 1997
	PS1 L286V	Propyl gallate, glutathion	Keller et al., 1998
Activation of Jun kinase	PS1 H115Y	-	Wolozin et al., 1998
Caspase activation	PS1 M146V	Z-Val-Ala-Asp (OMe)-CH_2F	Guo et al., 1999b
	PS1 A246E, L286V, exon 9 deletion	Z-Val-Ala-Asp (OMe)-CH_2F	Kovacs et al., 1999
	PS1 L286V	Z-Val-Ala-Asp (OMe)-CH_2F	Keller et al., 1998
	PS1 L286V	Z-Val-Ala-Asp (OMe)-CH_2F	Chan et al., 2002
Calpain activation	PS1 L286V	Calpain inhibitors I and II	Chan et al., 2002
Mitochondrial permeability transition disruption	PS1 L286V	Cyclosporin A	Keller et al., 1998
Cytoskeleton breakdown (β-catenin destabilization)	PS1 I143T, M146V, H163R, C410Y	-	Zhang et al., 1998
Disruption of insulin-like growth factor-1 signaling	PS1 L250S, exon 9 deletion	Insulin-like growth factor-1	Tanii et al., 2000
Downregulation of Akt/PKB	PS1 A246E, C410Y	NGF, Akt	Weihl et al., 1999
Enhanced p53 induction	PS1 L286V	-	Chan et al., 2002
Alteration of chaperone protein expression	PS1 M146V	-	Maezawa et al., 2002
ER-Golgi stress	PS1 M146L	β-mercapto ethanol	Terro et al., 2002
Increased levels of pro-apoptotic transcription factors (Gadd 143)	PS1 M146V, L286V	-	Milhavet et al, 2002

1998, Wolozin et al., 1998). Interestingly, both PS1 and PS2 overexpression arrest cells in the G1 phase of the cell cycle, suggesting a possible mechanism of full-length PSs to sensitize cells to apoptosis (Janicki and Monteiro, 1999, Janicki et al., 2000). In addition, one recent study concluded that neither mutant nor wild-type PS2 enhance apoptosis triggered by staurosporine or H_2O_2 in different cellular systems (Gamliel et al., 2003) and another study has been similarly concluded for PS1 (Bursztajn et al., 1998). It has been also suggested that PS1 CTF is released into the extracellular compartment during apoptosis and could participate in intercellular apoptotic signaling (Benussi et al, 2001).

Both PS1 and PS2 are processed by caspases during apoptosis, two different caspase cleavage sites being identified for PS1 ($ENDD_{329}$ and $AQRD_{341}$) and one site for PS2 ($DSYD_{329}$). Caspases 8, 6 and 11 cleave PS1 after residues ENDD329 and caspases 3, 7 and 1 after AQRD341 (van de Craen et al., 1999). PS2 is cleaved at the mentioned site by caspases 8, 3, 1, 6 and 7 (van de Craen et al., 1999). In the same study it was shown that PS1 mutations do not alter the sensitivity of PS1 to caspase cleavages. PSs are also substrates for proteasomal degradation (Fraser et al., 1998, Steiner et al., 1998, Checler et al., 2000) and for calpain cleavage (Maruyama et al., 2000). The role of this complex proteolytic processing of PSs (Figure 2) is not fully understood yet and two scenarios are possible. The first asserts that PSs are only "innocent bystanders" in the way of activated proteolytic cascades during apoptosis. The second would imply that PSs alternative fragments play a role in signal transduction during apoptosis and data supporting this hypothesis have been generated. Both PS1 and PS2 caspase-cleaved, alternative CTFs (aCTFs) have been shown to be anti-apoptotic (Vezina et al., 1999, Vito et al., 1997). PS2 C-terminal fragment is phosphorylated in vivo at residues 327 and 330, in the immediate proximity of caspase cleavage site and this phosphorylation inhibits the caspase cleavage of PS2 (Walter et al, 1999). Phosphorylated PS2 was also shown to slow apoptosis in HeLa cells treated with staurosporine (Walter et al., 1999). Recently another report showed that PS1 has a phosphorylation site within the caspase-recognition motif, operated by PKC (Fluhrer et al., 2003). Phosphorylation of PS1 at residue 346 inhibits the caspase-cleavage of PS1 and reduces the progression of apoptosis (Fluhrer et al., 2003).

Ca^{2+} SIGNALING ALTERATIONS IN ALZHEIMER'S DISEASE

Calcium ion is broadly used as a signal transduction element by an enormous variety of cells, ranging from bacteria to neurons (Clapham, 1995). During the last decades it became a generally accepted fact that alteration of intracellular calcium concentration ($[Ca^{2+}]_i$) plays an important role in cell death and in AD. In neurons, the rise of $[Ca^{2+}]_i$ can trigger necrosis, apoptosis, or both (Ankarcrona et al., 1995), depending on the mitochondrial energy charge. There are multiple mechanisms mediated by Ca^{2+} in cell killing, e.g. mitochondrial dysfunction, activation of proteases, alteration of cytoskeletal network (Nicotera and Orrenius, 1998). Calcium signaling deficits in aging brain (Gibson and Peterson, 1987, Khachaturian, 1991, Verkhratsky and Toescu, 1998) and in AD brain (Tanzi, 1998, LaFerla, 2002) were reported by many groups and a theory stating that alterations of Ca^{2+} homeostasis could be a prime event in AD emerged a long time ago (Khachaturian 1989). This hypothesis is currently based on reports showing that Ca^{2+} dysregulation alters both Aβ formation (Mattson et al., 1992) and tau phosphorylation (Mattson et al., 1990). Increased $[Ca^{2+}]_i$ results in enhanced Aβ generation (Querfurth and Selkoe, 1994) whereas in turn cells treated with Aβ show increased $[Ca^{2+}]_i$ (Mattson et al., 1992). The mechanism by which Aβ increase $[Ca^{2+}]_i$ seems to be induction of calcium-permeable pores within the plasma membrane (Bhatia et al., 2000). In contrast, secreted APP has been shown to be neuroprotective (Goodman and Mattson, 1994, Guo et al., 1998a). However, recent data suggest that APP processing can also regulate calcium signaling through APP AICD-controlled gene transcription regulation (Leissring et al., 2002). Cells treated with ApoE have also been shown to react by increasing $[Ca^{2+}]_i$, and this increase is dependent on the ApoE isoform (ApoE4>ApoE3>ApoE2, Ohm et al., 2001) and on extracellular calcium concentration (Cedazo-Minguez et al., 2003). Activation of GSK-3β induced by both ApoE4 and Aβ (1-42) is also dependent partially on an enhancement of $[Ca^{2+}]_i$ (Cedazo-Minguez et al, 2003).

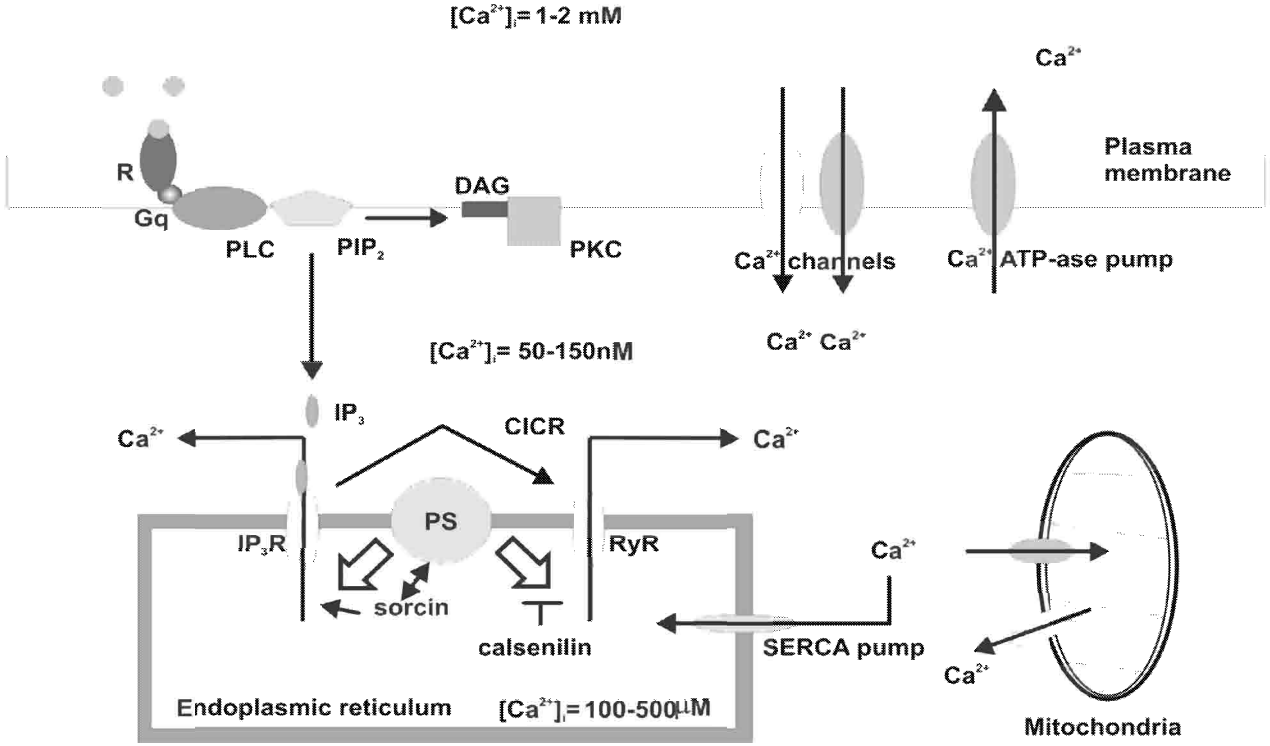

Figure 3. Ca^{2+} signaling pathways and regulation. Phospholipase C (PLC), which predominantly couples to muscarinic receptors (R) via G proteins, was shown to be essential for transduction of cholinergic signals in hippocampal and cortical neurons). Activated PLC hydrolyses plasma membrane phosphoinositides (PIP_2) to form the second messengers IP_3 and diacylglycerol (DAG), which release calcium from the endoplasmic reticulum (ER) and activate protein kinase C (PKC), respectively. PSs interfere with Ca^{2+} signaling and homeostasis by interacting with calsenilin, sorcin and ryanodine receptors (RyR). IP_3R = IP_3 receptors. CICR = Calcium-induced calcium release.

A large proportion of calcium mobilization and regulation in neurons can be attributable to cholinergic neurotransmission (Berridge, 1998). Phospholipase C (PLC), which predominantly couples to muscarinic receptors via G proteins, was shown to be essential for transduction of cholinergic signals in hippocampal and cortical neurons (Kim et al., 1997). Activated PLC hydrolyses plasma membrane phosphoinositides (PI) to form the second messengers IP_3 and diacylglycerol (DAG), which release calcium from the endoplasmic reticulum (ER) and activate protein kinase C (PKC), respectively (Rebecchi and Pentyala, 2000, Figure 3). Cholinergic signaling is impaired in AD at different levels (Auld et al., 2002) and muscarinic receptor stimulated PI hydrolysis is disturbed in vitro by both Aβ and apoE4 (Cedazo-Minguez and Cowburn, 2001), two crucial molecules involved in AD pathogenesis.

PSs and calcium signaling

In 1996, Guo and coworkers reported that the L286V PS1 mutation perturbs Ca^{2+} homeostasis in PC12 cells, suggesting that PS1 mutations result in a gain of altered function which causes dysregulation of Ca^{2+} signaling (Guo et al., 1996). These data were in agreement with older reports showing a significant enhancement of $[Ca^{2+}]_i$ in stimulated fibroblasts of AD patients as compared with fibroblasts from age-matched healthy subjects (Ito et al., 1994, Hirashima et al., 1996). During the last years, several other lines of evidence sustained the hypothesis that altered Ca^{2+} homeostasis could be one of the mechanisms by which PSs mutations sensitize neurons to cell death (Table 3). Recently, two Ca^{2+}-binding proteins, sorcin and calsenilin, have been shown to directly interact with PSs. Sorcin is a cytosolic protein identified in multidrug-resistant cells that share substantial homology with the light chain of calpain, a Ca^{2+} activated protease (Van der Blick et al., 1986). Sorcin is found in mammalian brain associated with ryanodine receptors (Pickel et al., 1997) and co-expressed with N-methyl-D-aspartate receptors (Gracy et al., 1999), both involved in Ca^{2+} signaling. PS2 interacts with sorcin (Pack-Chung et al., 2000) and this finding suggests a molecular connection between PS2 and

regulation of Ca^{2+} homeostasis. An interaction between PS2 and another calcium-binding protein, calmyrin, has been reported (Stabler et al., 1999). In addition, PS1 interacts with ryanodine receptors and mutant PS1 have been shown to increase the number of ryanodine receptors in transgenic mice brains (Chan et al., 2000). In order to search for new proteins that interact with PSs, Buxbaum and coworkers screened a human brain cDNA library and identified a new Ca^{2+}-binding protein, which they named calsenilin (Buxbaum et al., 1998). Calsenilin has been shown to bind to the C-terminal sequence of both PS1 and PS2. In the same study it was shown that when PS2 and calsenilin were co-expressed in neuroglioma cells, PS2 was processed to a C-terminal fragment of 20 kDa to an amount that paralleled the level of calsenilin expression, suggesting that calsenilin could influence PS2 processing. This C-terminal fragment of PS2 corresponded to the caspase-cleaved C-terminal fragment of PS2, also known as ALG-3, which had anti-apoptotic effect in one study (Vito et al., 1997). However, this data became controversial once other studies were recently reported, where the effect was opposite (da Costa et al., 2002, da Costa et al., 2003). Recently, another study demonstrated that calsenilin modulates Aβ production, in so far as calsenilin knock-out mice show reduced Aβ brain levels (Lilliehook et al., 2003).

PSs seem to be involved not only in alteration of $[Ca^{2+}]_i$ upon triggering of cell death, but also upon physiological stimulation. Serial reports by Leissring and co-workers have shown that mutant PS1 enhances calcium signaling mediated by caged IP_3 in Xenopus oocytes and that calsenilin reverses this enhancement (Leissring et al., 1999, Leissring et al., 2000a). By imaging calcium release with high-resolution line-scanning confocal microscopy they found that mutant PS1 induced an increase of ER calcium stores, without affecting the number or the sensitivity of IP_3 receptors (IP_3R) in the ER (Leissring et al., 2001). Moreover, besides being involved in regulating Ca^{2+} ER stores, PS1 is important for the capacitative calcium entry (CCE) and cells from mutant PS1 knockin mice show deficits in CCE (Leissring et al., 2000b). Finally, a recent study revealed that the phosphoinositide/calcium signaling pathway is dependent on the γ-secretase activity of PS1, since PS1 knockout cells or cells treated with γ-secretase inhibitors showed no calcium signals upon bradykinin stimulation (Leissring et al., 2002).

AIMS OF THE STUDY

Besides being essential for the γ-secretase cleavage of APP and other RIP substrates, PS1 has been shown to be involved in apoptosis and PS1 proteolytic products were suggested to regulate apoptosis. Also various studies suggested that PS1 could be important for signal transduction and regulation of Ca^{2+} homeostasis. The present project investigated the proteolytic processing of PS1 during apoptosis and the impact of PS1 on cholinergic muscarinic signal transduction, with emphasis on FAD mutations. The specific aims of each study were to:

- monitor the proteolytic processing of wild-type PS1 and a FAD-linked mutant PS1 during apoptosis in relation to cell viability and to identify the fate of PS1 resultant species

- investigate the time relationship between caspase cleavage of PS1 and caspase cleavages of other substrates and to describe mechanisms by which a PS1 FAD-linked mutation prone cells to apoptosis

- test the hypothesis that FAD-linked PS1 mutations influence cholinergic muscarinic receptor-stimulated signaling by comparing basal and carbachol stimulated phosphoinositide hydrolysis and $[Ca^{2+}]_i$ and to clarify the role of PS1 in this signal transduction pathway

MATERIALS AND METHODS

Cell culture, cell lines and transfections

Human SH-SY5Y neuroblastoma cells (papers I, II, III and IV) were purchased from the European collection of cell cultures, center for applied microbiology and research, Salisbury, Wiltshire, U.K.. Transfection of cDNA constructs for mutant PS1 into SH-SY5Y cells was performed using DOTAP Liposomal Transfection Reagent (Roche, IN, USA) in accordance to manufacturer's protocol. Stable transfectants were first selected in 500 μg/ml geneticin for several days and then the geneticin concentration was reduced to 200 μg/ml. Expression levels of the transfected PS1 were determined by Western blotting as previously described (Tanii et al., 2000). We also confirmed expression of mutant PS1 mRNA in SH-SY5Y cells by sequencing following reverse transcription. SH-SY5Y neuroblastoma cells transfected either with wild-type or the exon 9 deletion variant of PS1 were previously established in our lab (Tanii et al., 2000). SH-SY5Y cells were grown in DMEM supplemented with 10% (v/v) heat inactivated foetal calf serum, 1% (v/v) penicillin-streptomycin, 1% (v/v) L-glutamine, at 37°C in a humidified 5% CO_2 atmosphere. Transfected cells were additionally supplemented with 200 μg/ml geneticin. Cells were seeded at a density of 15-25 × 10^3 cells/cm^2.

Assessment of cell death and viability

MTT assay

In papers I and II cell viability was assessed by a modified version of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, that detects the cellular capacity to convert MTT tetrazolium salt to formazan (Mossman, 1983). Briefly, MTT powder was dissolved in MEM without phenol red at 0.3 mg/ml and added to the cells. After 1 hr at 37°C, the medium was removed and the formazan crystals were

dissolved in isopropanol. Aliquots were transferred to 96-well-plates and the optical density was read at 592 nm in a plate reader. Results were expressed as a percentage of viable cells compared with control (unexposed) cells.

Lactate dehydrogenase (LDH)-release quantification

In paper II, LDH-release was measured using the Cytotoxicity Detection Kit (Boehringer-Mannheim AB, Scandinavia), according to the manufacturer protocol. In brief, the assay evaluates the plasma membrane damage in a cell population exposed to a toxic treatment, by quantifying spectrophotometrically the LDH activity in the supernatant. Cytotoxicity (%) was calculated using the formula: sample absorbance (exposed cells) – low control absorbance (unexposed cells) / high control absorbance (lysed cells) – low control absorbance (unexposed cells) × 100.

ELISA detection of apoptotic mono- and oligonucleosomes

In paper II, mono- and oligonucleosomes resulting from apoptotic fragmentation of nuclei were detected with the Cell Death Detection ELISA PLUS Kit (Boehringer-Mannheim AB, Scandinavia). The test is based on a quantitative sandwich enzyme immunoassay and uses mouse monoclonal antibodies directed against DNA and histones. This makes possible the in vitro detection of characteristic apoptotic mono- and oligonucleosomes in the cell lysates, by a spectrophotometrical measurement at 405 nm.

Chromatin staining

In papers I and II, chromatin staining with propidium iodide was performed. Cells were cultured on glass coverslips, in 12-well-plates. After experiments, medium was removed and cells gently washed with 37°C PBS. Cells were subsequently fixed and stained with 80% methanol-20% propidium iodide (500 μg/ml in PBS) for 10 min in the dark. The coverslips were mounted on glass slides in glycerol:PBS (1:1) solution. In

paper II, cells that detached from the coverslips were collected by centrifugation and fixed and stained as above. After a second centrifugation cells were resuspended in glycerol:PBS (1:1) solution and mounted on glass slides with coverslips. Cells were visualized in a confocal scanning system using a 543 nm helium-neon laser. For cell counting, coverslips were examined under a fluorescence microscope (excitation 546 nm/ emission 617 nm). Normal and apoptotic cells (with bright, condensed and fragmented nuclei) were counted in at least five random fields per experiment and the results were expressed as percentage of apoptotic cells as compared to the total number of counted cells. At least a total of 350 cells was counted for each experiment.

Cell lysates, protein determination and immunoblot analyses

In papers I, II, II and IV, the Western blots were performed with a protocol modified after the generally described one (Laemmli, 1970). Specific details regarding for instance the antibodies and their dilutions or analyses of different cell populations are described in detail in each paper. In brief, SH-SY5Y neuroblastoma cells were harvested with a cell-scraper in the growth medium and collected by 3,000 rpm centrifugation for 3 min. After removal of the medium, cells were resuspended and washed with ice-cold Ca^{2+}- and Mg^{2+}-free PBS, and centrifuged again in the same conditions. Pellets were then resuspended in 100 µl of lysis buffer (20 mM Tris-Hcl, 137 mM NaCl, 2 mM EDTA, 2% NP-40, 2% Triton X-100 and 400 µg/ml protease inhibitors) and centrifuged at 11,000 rpm for 10 min at 4°C. Samples were stored at -20°C until use. Protein concentration was determined by a BSA protein assay kit (Pierce, Rockford, IL, USA) and equivalent amounts of protein/lane were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes at 100 mA current for 4 hr. Blocking was performed in TBS buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl) with 5% (w/v) dry milk powder. Membranes were incubated with the primary antibodies overnight. The immunoblots were subsequently washed 3 × 10 min in TBS containing 0.05-0.1% Tween-20 and incubated for 1 hr with HRP-linked secondary antibody. Bound antibody was detected by the ECL method. Immunoblots of some of the experiments were stripped as described by

the nitrocellulose membranes manufacturer (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and reblotted with different antibodies. In paper I, the equal protein loading was also checked by staining of the blots with Ponceau S solution. In papers II and III, to semi-quantify the proteins, the relative density of immunoreactive bands on Western blots was calculated from the optical density multiplied by the area of the selected band.

Immunocytochemistry

In paper III, immunocytochemistry was performed in order to study PS1 cellular localization. SH-SY5Y cells grown on coverslips were fixed at 4°C in 2% paraformaldehyde - 0.2% glutaraldehyde for 20 min and subsequently rinsed in PBS (3 × 10-min rinses). Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min, and then 10% goat serum was added for 30 min to block unspecific binding of antibodies. Cells were incubated overnight at 4 °C with primary antibodies diluted in 10% serum. Next day cells were rinsed in PBS (3 × 10-min rinses) before incubation with secondary antibodies conjugated with fluorescent dyes in PBS - 0.3% Triton X-100 for 1 h in the dark. Finally cells were rinsed in PBS (3 × 10-min rinses) and mounted in glycerol: PBS (1:1) on glass slides.

PI hydrolysis assay

In papers III and IV, PI hydrolysis was essentially measured as previously described (Fowler et al., 1987). One day prior to the experiment, SH-SY5Y cells were changed to serum-free media containing 5 μCi/ml *myo*-[2-^{3}H]inositol and incubated for 24 hr. Cells were harvested by scraping with a rubber policeman in PBS. Contents were centrifuged at 1,500 rpm for 15 min. Pellets were washed twice with 37 °C PBS and re-suspended in 37 °C Krebs-Henseleit bicarbonate buffer containing 10 mM LiCl (KHB/Li), gassed with 5% CO_2, 95% O_2 and centrifuged again (15,000 rpm, 15 min). Cell pellets were re-suspended in KHB/Li, regassed, and added to glass centrifuge tubes

containing KHB/Li buffer with or without 100 μM carbachol. The tubes were incubated at 37 °C under an atmosphere of 5% CO_2, 95% O_2 with gentle agitation for 25 min. Incubations were stopped by adding a chloroform:methanol mixture (1:2). Tubes were incubated on ice for 30 min and phases separated by adding chloroform and water followed by vortexing and centrifugation. The aqueous phase was removed and labeled inositol phosphates (IPs) separated from *myo*-[^{3}H]inositol by Dowex chromatography, as previously described (Berridge, 1982). The chloroform phase was removed, placed into scintillation vials, and allowed to evaporate before determination of 'lipid dpm' by scintillation spectroscopy. Results were expressed as dpm IPs/(dpm IPs + dpm lipid). This unit is independent of the number of cells aliquoted in each tube and upon the degree of labeling of inositol phospholipids (Fowler et al., 1987).

Intracellular calcium measurements

In papers II, III and IV apparent $[Ca^{2+}]_i$ was measured with the calcium sensitive dye Fluo-3 acetoxymethyl (AM) ester, using a protocol modified after the initial one (Grynkiewicz et al., 1985). Details of specific incubation times and experiments are given in each paper separately. Briefly, SH-SY5Y neuroblastoma cells were grown in 96-well-plates in the conditions described above. Growth medium was removed, cells washed with Ca^{2+}- and Mg^{2+}-free PBS and loading medium added. Loading medium was MEM without phenol red containing 5 μM Fluo-3 AM ester, 0.5% (V/V) Pluronic F-127 and 1 mM of the organic anion-transport inhibitor probenecid. Pluronic F-127 was used in order to partially permeabilize the cell membranes for Fluo-3 AM and probenecid to limit the leakage of the fluorescent indicator. The same concentration of probenecid was present in all incubation solutions during experiments. After incubation with the loading medium for 90 min in the dark cells were washed with Ca^{2+}- and Mg^{2+}-free PBS and then MEM without phenol red was added. Then cells were incubated for 120 min in the dark, to allow intracellular esterases to decompose the Fluo-3 AM ester. Subsequently, MEM was replaced with Ca^{2+}- and Mg^{2+}-free PBS and the fluorescent signal measured in different experimental paradigms in a multi-well fluorescent plate-reader, the excitation

and emission wavelengths being 485 and 530 nm, respectively. Apparent $[Ca^{2+}]_i$ corresponding to the fluorescent value F was calculated with the formula: $[Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F)$, where K_d for Fluo-3-Ca^{2+} was taken as 390 nM, as indicated by the manufacturer protocol (Molecular Probes, Europe BV, The Netherlands). F_{min} was determined by measuring the signal of the unloaded cells and F_{max} was determined by the addition to the cells of a 100 mM $CaCl_2$ buffer containing 1% (V/V) Triton X-100.

Statistical Analyses

Analyses of differences were performed in all papers by one-way analysis of variance (ANOVA) followed by Fisher's post-hoc test, using Stat View 4.12 statistical software. A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

THE EXPERIMENTAL MODEL

In all four studies we chose to use SH-SY5Y neuroblastoma cells because they are well characterized human, neural-type cells.

In paper I, we used SH-SY5Y neuroblastoma cells and induced apoptosis by staurosporine (STS) treatment because this experimental paradigm has been well characterized by previous studies (Boix et al., 1997, Lopez and Ferrer, 2000). Moreover, we found that the variability of cell death upon STS treatment assessed by MTT assay was low enough to enable us to search for differences in cell death between different cell lines and treatments. SH-SY5Y cell lines expressing comparable levels of wild-type (WT) or exon 9 deleted (ΔE9) PS1 were previously established in our lab (Tanii et al., 2000).

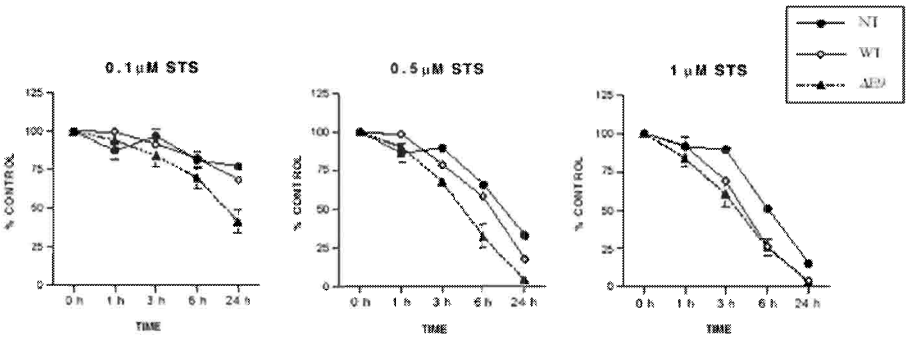

Figure 4. MTT assay of cell viability. SH-SY5Y cells non-transfected (NT) or transfected to express wild-type (WT) or exon 9 deleted (ΔE9) PS1 were exposed to 0.1, 0.5 or 1 μM staurosporine (STS) in growth medium up to the indicated time-points. Bars indicate standard error mean (SEM). Cell viability was compared and expressed as a percentage of cell viability in non-treated cells (exposed to vehicle, DMSO) within each cell line (control values, 100%, 0 h). Statistical analysis of the results was carried out using ANOVA followed by Fisher's posthoc test (N=8). A dose- and time-dependent loss of cell viability was seen for all cell lines. Treatment with 0.1 μM STS induced a similar decrease in cell viability in NT and WT PS1 cells, but a significantly higher decrease in ΔE9 PS1 cells at 24 h time-point ($p < 0.001$). Treatment with 0.5 μM STS induced a higher decrease in cell viability in both WT PS1 and DE9 PS1 cells at 24 h time-point as compared to NT cells ($p < 0.01$) and for this STS concentration a higher decrease in cell viability was seen in ΔE9 PS1 cells at 6 h and 24 h time-points as compared to both NT and WT PS1 cells ($p < 0.01$). Treatment with 1 μM STS induced a higher decrease in cell viability in both WT PS1 and ΔE9 PS1 cells as compared to NT cells at 3 h ($p < 0.01$), 6 h ($p < 0.01$) and 24 h ($p < 0.001$) and no significant differences were seen between WT PS1 and ΔE9 PS1 cells.

The presence of correct sequences of the constructs was confirmed by gene sequencing and the expression was checked by Western blotting with an N-terminal PS1 anti-serum directed against PS1 residues 2-12 (Mehta et al., 1998). We used the ΔE9 PS1 mutation not only because it is FAD-associated, but also because it does not undergo endoproteolysis, lacking the domain where the endoproteolytic cleavage occurs. Therefore it can serve as a control for WT PS1 in PS1 processing studies. As expected, in non-transfected cells we identified PS1 mainly as NTF (~30 kDa) and very low amounts of full length PS1 were detected (~50 kDa). Endoproteolysis is limited by unidentified cellular factors (Thinakaran et al., 1997) and therefore in cells overexpressing PS1, full-length PS1 is not entirely cleaved by proteolysis. As compared to WT PS1, ΔE9 PS1 accumulates even in higher amounts, due to lack of endoproteolysis. The presence of PS1 NTF in cells expressing ΔE9 PS1 is explained by endoproteolysis of endogenous PS1.

Further on, we confirmed that STS induced apoptotic cell death by MTT assay (Figure 4), chromatin staining with propidium iodide (Figure 5), caspase-3 activation and PARP cleavage.

In paper II, we used A23187 calcium ionophore to induce apoptosis because calcium dyshomeostasis is a well-established pathogenic event in AD (LaFerla, 2002) and A23187 has been previously used to induce apoptosis in neurons or neuroblastoma cells (Katayama et al., 1999). We exposed cells to different concentrations of A23187 for 30 min and then we replaced them in growth medium. As in paper I, we used NT cells and cells transfected to express either WT PS1 or ΔE9 PS1. Empty vector-transfected cells (VT) were also used to rule-out possible effects of the transfection method on susceptibility of cells to activate cell death.

In paper III, to test the hypothesis that FAD-linked PS1 mutations influence muscarinic receptor-stimulated signaling we used SH-SY5Y neuroblastoma cells stably transfected with either WT PS1 or ΔE9 PS1 to compare basal and muscarinic agonist carbachol-stimulated PI hydrolysis and $[Ca^{2+}]_i$. SH-SY5Y cells have been extensively used as a neuronal model to study PI hydrolysis and indirectly PLC activation (Fisher, 1995). We chose to study the ΔE9 PS1 mutation because in the previous study (paper II) we found that it gives an increased $[Ca^{2+}]_i$ when transfected into SH-SY5Y cells.

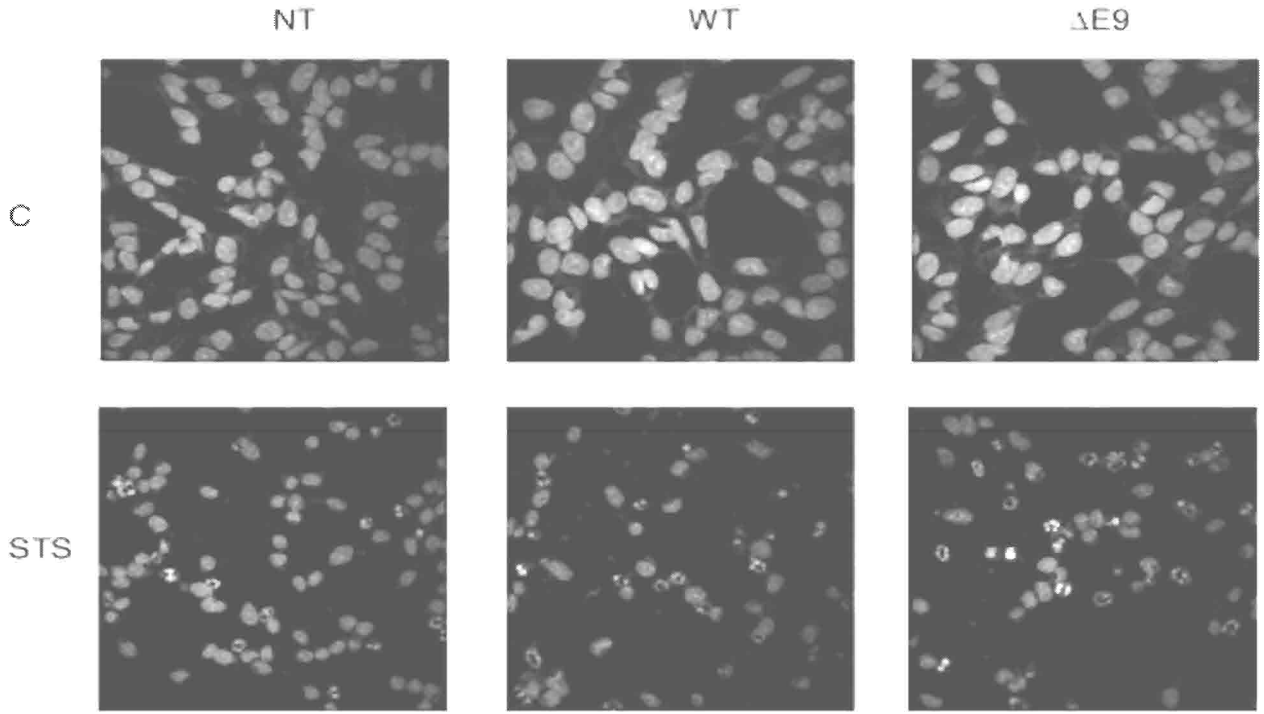

Figure 5. Fig.3. Confocal microscopy images of SH-SY5Y cells non-transfected (NT) or transfected to express wild-type (WT) or exon 9 deleted (DE9) PS1 stained with propidium iodide in control (C, cells exposed to vehicle, DMSO) conditions or after 6 h treatment with 1 μM staurosporine (STS). Typical nuclear morphological changes associated with apoptosis can be noticed in cells treated with STS for 6 h.

In paper IV, in order to investigate the pathways by which PS1 interferes with cholinergic signal transduction, we transfected SH-SY5Y neuroblastoma cells to express WT PS1, the FAD-causing M146V PS1 and L250S PS1 mutants or the dominant negative D257A PS1 and D385N PS1 mutants lacking γ-secretase activity (Kimberly et al., 2000). These cell lines were used to measure basal and carbachol-stimulated PI hydrolysis and $[Ca^{2+}]_i$ responses. We also used pharmacological inhibition of γ-secretase with the specific γ-secretase inhibitor DAPT (Dovey et al., 2001) and with pepstatin A, a general aspartyl protease inhibitor, which has been shown to be also a potent non-competitive γ-secretase inhibitor (Tian et al., 2002).

THE PROTEASOME AND CASPASES REGULATE PS1 LEVELS DURING APOPTOSIS (PAPERS I AND II)

PS1 has been shown to be substrate for a complex proteolytic processing. First, the full length protein has a very short half life, being rapidly cut down by an yet unknown protease to NTF and CTF, in a process called endoproteolysis (Podlisny et al., 1997). PS1 full-length protein which does not undergo endoproteolysis and NTF and CTF that are not incorporated in functional heterodimers are degraded by the ubiquitin-proteasome system, in a natural turnover (Checler et al., 2000). During apoptosis, PS1 is processed by caspases to aNTF and aCTF (Loetscher et al., 1997) and alternative PS1 fragments have been suggested to regulate apoptosis (Wolozin et al., 1998, Vezina et al., 1999, da Costa et al., 2003). Paper I characterized the proteolytic processing of PS1 during apoptosis induced by staurosporine in SH-SY5Y neuroblastoma cells.

Many studies showed that mutant PSs may cause FAD by different mechanisms, like production of a more toxic form of Aβ (Aβ 1-42, Borchelt et al., 1996), altered calcium homeostasis, accumulation of reactive oxygen species and mitochondrial dysfunction (Keller et al., 1998), downregulation of the unfolded protein response (Katayama et al., 1999) and enhanced caspase-3 activity (Kovacs et al., 1999). In order to understand whether caspase cleavage of PS1 plays a role in the apoptotic machinery, in paper II we wanted to classify the PS1 proteolysis as an early or late apoptotic event, as compared to morphologic and biochemical typical apoptotic changes.

ΔE9 PS1 sensitizes cells to death induced by Ca^{2+} overload and STS (papers I and II)

In paper II, we exposed the cells for 24 hr to three different concentrations of A23187 (1, 3 and 5 μM) in order to trace dose- and time-dependent cytotoxicity curves. MTT assay showed that cells overexpressing either WT PS1 or ΔE9 PS1 suffered a higher loss of cell viability as compared to NT and VT cells upon treatment with 3 or 5 μM A23187. Considering that MTT assay estimates cell viability only in cells that remain adherent over the time of the experiment, we further performed a quantification of LDH released in the medium by dead cells. Both 3 and 5 μM concentrations of A23187 caused a significantly higher LDH release in ΔE9 PS1 cells as compared to all control cell lines. The LDH release was partially prevented by the addition of the general caspase-inhibitor z-VAD-fmk proving that LDH release at least partially originates from cells undergoing apoptosis and then secondary necrosis in this cell culture paradigm. In order to confirm that ΔE9 PS1 FAD mutation sensitized cells to apoptosis and not to other forms of cell death, we also performed an ELISA assay that detects cytosolic mono- and oligonucleosomes characteristic for apoptotic nuclear fragmentation. Indeed, after 24 hr exposure to 3 μM A23187 ΔE9 PS1 cells showed significantly more apoptotic DNA fragmentation as compared to WT PS1 cells. These findings are in agreement with previous studies reporting that PS1 mutations sensitize cells to undergo apoptosis (e.g. Guo et al, 1996, Kovacs et al., 1999). With the MTT assay we did not detect any difference in cell viability between WT and mutated PS1 transfected cells. In contrast, LDH release and apoptotic DNA quantification revealed a significantly higher cell death in cells expressing mutant PS1. The difference in results could be explained by the fact that MTT assay estimates cell viability only for adherent cells and is not suitable to detect changes in non-adherent cells.

In paper I we also found that ΔE9 PS1 mutation sensitize cells to death induced by low concentrations of STS (0.1 and 0.5 μM) but not by high STS concentration (1 μM), as seen with the MTT assay (Figure 4).

PS1 processing during apoptosis (papers I and II)

In paper I, we checked the PS1 processing during STS-induced apoptosis in NT cells, two clones of WT PS1 cells (C 1-1 and C 1-14) and two clones of ΔE9 PS1 cells (C 1-2 and C 2-4), by a PS1 N-terminal antiserum. Six hr exposure to 1 μM STS did not affect the levels of NTF in any of the cell lines. Full-length PS1 was completely processed in both WT PS1 clones and only partially processed in ΔE9 PS1 clones upon STS treatment. An alternative ~ 36 kDa PS1 NTF generated by caspase cleavage (aNTF) was detected in ΔE9 PS1 clones, at higher levels in C 2-4 clone. In order to study processing of PS1 during apoptosis and its relation to cell viability and considering that PS1 processing was similar in different clones expressing the same PS1 variant, we used clones C 1-1 of WT PS1 and C 2-4 of ΔE9 PS1 for other experiments. This difference in full-length WT PS1 and ΔE9 PS1 processing during apoptosis was not specific only for the STS treatment, since a similar result was obtained in these cell lines after 96 hr of serum deprivation (data not shown) or in apoptosis induced by A23187 calcium ionophore (paper II).

To study the PS1 processing we also used a C-terminal PS1 antiserum, which identified only the ~20 kDa CTF derived from endoproteolysis in NT cells and both full length and CTF in WT PS1 and ΔE9 PS1 cells in control conditions. Figure 6 shows a time course experiment where PS1 processing was studied by both N-terminal and C-terminal antibodies. With the C-terminal PS1 antiserum we also showed a complete degradation of full-length WT PS1 during 6 hr of STS treatment. In WT PS1 cells, the levels of the PS1-CTF were progressively reduced during STS treatment giving rise instead to a ~14 kDa caspase-cleaved alternative CTF (aCTF) detectable at 3 and 6 hr. Small amounts of this fragment were also detected in NT cells after 6 hr STS treatment (Figure 6, the immunoblot of NT cells is overexposed as compared to immunoblots of WT PS1 and ΔE9 PS1 cells, which are of similar exposure). In ΔE9 PS1 cells truncated full-length PS1 was processed to give rise to aCTF that was detectable after 3 h STS treatment (Figure 6).

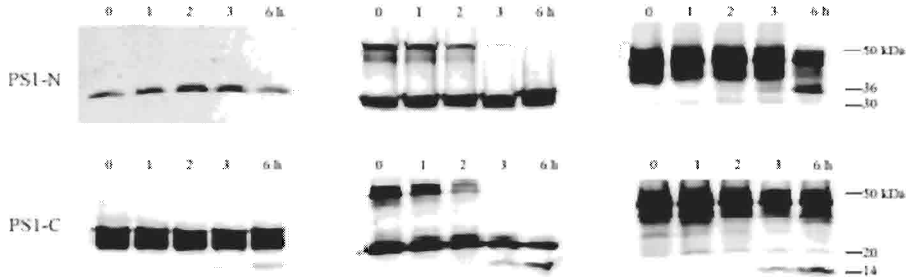

Figure 6. Time-course study of PS1 processing during apoptosis. SH-SY5Y cells non-transfected (NT) or transfected with either wild-type (WT) or exon 9 deleted (DE9) PS1 were exposed to vehicle (DMSO, control cells, 0 h) or to 1 μM staurosporine (STS) in growth medium up to the indicated time-points. PS1-N = PS1 N-terminal antibody immunoblot, PS1-C = PS1 C-terminal antibody immunoblot.

As expected, during STS-induced apoptosis a PS1 aCTF was generated in all cell lines as a result of caspase activation. Despite the very low amounts of full-length PS1 in NT cells, an aCTF was identified as well. This suggests that aCTF was generated from PS1 CTF in NT cells, a finding in agreement with previous reports (Grunberg et al., 1998). An aNTF was detected only in ΔE9 PS1 cells, since in this case the caspases cleave the truncated full-length protein. In the other cell lines the aCTFs are generated mainly from PS1 CTF and aNTF is produced in non-detectable amounts.

In paper II, in order to see how PS1 alternative cleavage is temporally related to caspase cleavages of other substrates and to morphological apoptotic changes, we performed a time-chase experiment after the exposure to the calcium ionophore (3μM). Low amounts of active caspase-3 were detectable on the immunoblots in ΔE9 PS1 cells immediately after the 30 min exposure to A23187 and at this time point the first PS1 aNTF of ~40 kDa has appeared on the PS1 immunoblots. In contrast, in WT PS1 cells the same A23187 concentration did not induce detectable caspase-3 activation or PS1 processing to alternative fragments, a result in agreement with higher apoptotic cell death seen in mutant PS1 bearing cells. PS1 alternative cleavage occurred in ΔE9 PS1 cells prior to cleavages of poly (ADP-ribose) polymerase (PARP) and gelsolin, other caspase substrates, which were evident at 1 hr after exposure to A23187. A second PS1 aNTF of

~36 kDa was identified at 1 hr after exposure to A23187 in ΔE9 PS1 cells. To confirm that all the proteolytic processing seen on the immunoblots was a result of caspase activation, we treated the cells with the general caspase inhibitor z-VAD-fmk, which was able to reverse these cleavages.

Considering that PS1 caspase cleavage occurred in ΔE9 PS1 cells before cleavage of other substrates and prior to any morphological changes, it is possible that alternative PS1 fragments play a role in regulating apoptosis signaling.

PS1 transfected cells undergoing apoptosis induced by calcium ionophore lose cell to cell adhesion (paper II)

In order to additionally check the cell death nature induced by A23187 in PS1 transfected cells we performed a chromatin staining with propidium iodide of adherent cells at different time points after exposure to calcium ionophore. Although MTT reduction showed an important reduction in cell viability and LDH release and apoptotic DNA quantification showed cell death, we found only a very low percentages of cells with nuclear apoptotic morphology. Phase-contrast microscopy showed instead that the cells were rounding up and detaching from the wells. Therefore, non-adherent cells were collected by centrifugation at 6 hr after exposure to A23187 and stained with propidium iodide (see Materials and methods). Almost all non-adherent cells had apoptotic morphology for both WT PS1 and ΔE9 PS1 cell lines. These results confirm that in apoptosis of cells with extracellular attachment there is a collapse of the cytoskeleton, which occurs before the nuclear chromatin condensation. Our data are in agreement with previous studies showing that alteration of cell-to-cell adhesion occurs during early phases of apoptosis (Ankarcrona et al., 1994, Brancolini et al., 1997, Kothakota et al., 1997, Mills et al., 1999).

Because we saw by chromatin staining that the non-adherent cells had apoptotic morphology we decided to study protein processing in this population with high enrichment in apoptotic cells. Therefore, non-adherent cells were collected by centrifugation at 6 hr after exposure to A23187 and immunoblots for PS1, caspase-3,

PARP and gelsolin performed for both adherent and non-adherent cell pools. Both non-adherent WT PS1 and ΔE9 PS1 cells showed activation of caspase-3, but the amount of caspase active fragment was three times higher in ΔE9 PS1 cells as compared to WT PS1 cells. In agreement with the results of paper I, the immunoblots with the PS1 N-terminal antiserum showed generation of the two predicted aNTFs in ΔE9 PS1 cells and a processing of the full length in WT PS1 cells, possibly by the proteasome, without generation of aNTFs. Also confirming the results of paper I, the endoproteolytically derived NTF levels were stable during apoptosis. The amounts of caspase-cleaved PARP fragment were also increased in ΔE9 PS1 cells as compared to WT PS1 cells. In contrast to the results pattern seen with PARP, gelsolin was cleaved to a similar extent in both non-adherent WT and ΔE9 PS1 cells, a result that further supports the finding that both cell lines show similar detachment. The similar cleavage of gelsolin in both cell lines could possibly be explained by the demonstration that not only caspase-3, but also calpain cleaves gelsolin, and the resulting fragments are apparently of similar sizes and caspase inhibition is able to block this cleavage (Wolf et al., 1999).

Proteolytic systems that process PS1 during apoptosis (paper I)

In order to search for proteases responsible for processing of PS1 during physiological turnover and during apoptosis, we used various protease inhibitors (caspase, calpain, proteasome, cysteine proteases and aspartic proteases inhibitors) and followed up the effects on accumulation of different PS1 species (paper I).

Under control conditions, none of the protease inhibitors changed the levels of PS1-NTF and PS1-CTF over 6 hr, showing a high stability of the fragments probably incorporated in functional PS1 heterodimers. Inhibition of the proteasome resulted in accumulation of full-length PS1 over 6 hr in WT PS1 and ΔE9 PS1 cells, in agreement with previous studies showing that non-processed full-length PS1 undergoes proteasomal degradation (Checler et al., 2000).

During apoptosis, inhibition of the proteasome by two different compounds during STS treatment resulted in accumulation of PS1 aCTF in all cell lines. These

inhibitors were also able to block the degradation of aNTF in ΔE9 PS1 cells. In addition, full-length PS1, which was degraded after STS treatment in WT PS1 cells, was partly restored in the presence of proteasome inhibitors. The caspase inhibitor Z-VAD-fmk was able to block the generation of PS1 aCTF in all cell lines, confirming that the ~14 kDa fragment was a result of caspase cleavage. In ΔE9 PS1 cells Z-VAD-fmk also completely reversed the generation of aNTFs from the truncated full-length PS1. Conversely, it had no effect on the processing of full-length PS1 in WT PS1 cells.

To rule out the possibility that processing of the full-length WT PS1 by the proteasome during apoptosis could have been consequent to a larger distribution of PS1 within the cells due to overexpression, we optimised the Western blotting to detect minimal levels of full-length PS1, which are normally found in non-transfected cells. Treatment of SH-SY5Y cells with 1μM STS for 6 hr induced a complete processing of full-length PS1, which was restored by addition of 10 μM of lactacystin to the STS treatment (data not shown).

The complete degradation of full-length WT PS1 cannot be explained by an inhibition of protein synthesis, as far as ΔE9 PS1 levels were not affected. Moreover, proteasome inhibition during STS treatment increased the full-length WT PS1 levels, showing that full-length PS1 is actively degraded by the proteasome during apoptosis. Interestingly, we found that ΔE9 PS1 was resistant to proteasome degradation during apoptosis as compared to WT PS1, being mostly processed by caspases. Two possibilities arise: either the proteasome is more active during apoptosis in WT cells or ΔE9 PS1 is a bad substrate for the proteasome. The latter assumption seems probable, as far as differences in ubiquitination of WT PS1 and ΔE9 PS1 may lead to differences in substrate accessibility. Out of 16 lysine residues present in PS1 amino acid sequence, 2 are located within the exon 9 corresponding domain, 2 are embedded within transmembrane domains, and 5 are in the close proximity of transmembrane domains. Therefore, the 2 lysine residues comprised by the exon 9 domain, which are located to the accessible PS1 loop, could be important for PS1 ubiquitination and hence responsible for differences in proteasome-mediated degradation between WT PS1 and ΔE9 PS1.

Interestingly, contrary to all other PS1 species, the levels of endoproteolytically derived PS1 NTF and CTF were not affected by proteasome inhibition in NT and WT

PS1 cells, showing a slow turnover and high stability during apoptosis of these fragments. The stability of the functional PS1 species (NTF and CTF PS1 heterodimer and ΔE9 PS1 variant) makes it possible that γ-secretase activity could take place during the cell death process, although no direct proof supports this hypothesis yet. As shown above, PS1 CTF is a substrate for caspase cleavage, and γ-secretase activity could be for caspases a target to hunt down, considering that secreted APP is neuroprotective (Turner et al., 2003) and gene regulation executed by APP AICD is not fully elucidated.

PS1 processing and cell viability (paper I)

In order to search for a correlation between the levels of different PS1 species and cell viability, in paper I we performed the MTT assay and the propidium iodide staining in the presence or absence of protease inhibitors which altered PS1 processing. The general caspase inhibition significantly improved cell viability estimated by MTT assay and significantly reduced the percentage of apoptotic cells estimated by chromatin condensation in all three cell lines. Calpain inhibition also significantly reduced the percentage of apoptotic cells in all cell lines. The proteasome inhibitors did not significantly affect cell viability estimated by MTT assay at in any of the cell lines. Based on previous reports (Vezina et al., 1999, da Costa et al., 2002) we expected that accumulation of PS1 aCTF would affect cell death. In our hands, proteasome inhibition was able to increase the levels of PS1 aCTF in apoptotic cells in all cell lines, but the accumulation of aCTF did not affect cell viability detected by MTT reduction. However, with the chromatin staining we found that proteasome inhibitors significantly increased the percentage of apoptotic cells in NT cells, but not in WT PS1 or ΔE9 PS1 cells treated with STS. Therefore it is possible that overexpression of full-length PS1 and/or accumulation of aCTF, which were common for both WT PS1 and ΔE9 PS1 cell lines, reversed the enhancement of STS-induced apoptosis by proteasome inhibitors seen in NT cells.

ΔE9 PS1 induces Ca^{2+} overload and dyshomeostasis (paper II)

Different reports have shown that PS1 FAD-linked mutations trigger alterations in Ca^{2+} homeostasis both during apoptosis (Guo et al., 1996, Guo et al., 1997) and signaling (Leissring et al., 1999, Leissring et al., 2000). Therefore we estimated $[Ca^{2+}]_i$ in NT, WT PS1 and ΔE9 PS1 cells upon basal conditions as well as during and following exposure to calcium ionophore. We did not see any significant differences in basal or ionophore-triggered $[Ca^{2+}]_i$ in WT PS1 cells as compared to NT cells. In contrast, ΔE9 PS1 cells exhibited significantly higher basal $[Ca^{2+}]_i$ as compared to both NT and WT PS1 cells. During the 30 min exposure to A23187, $[Ca^{2+}]_i$ showed a similar pattern but at different levels among the different cell lines. After a fast increase at 1 min of exposure, a decrease at 5 min followed, as a probable result of Ca^{2+} buffering by Ca^{2+} buffering proteins. Then up to 30 min exposure a continue increase in $[Ca^{2+}]_i$ followed in all cell lines, but at a significantly higher values in ΔE9 PS1 cells as compared to NT and WT PS1 cells. Moreover, even though a drop in $[Ca^{2+}]_i$ was present in all cell lines after stopping the exposure to ionophore, ΔE9 PS1 cells kept a higher $[Ca^{2+}]_i$ as compared to NT and WT PS1 cells. These results strongly suggest that ΔE9 PS1 alters Ca^{2+} homeostasis and buffering. Alteration of Ca^{2+} buffering by other PS1 FAD-linked mutations has been shown by a previous report, where the Ca^{2+}-binding protein calbindin was able to counteract the PS1 mutant effects (Guo et al., 1998). High $[Ca^{2+}]_i$ is important for execution of cell death by impairing the mitochondrial function, activation of proteases and cytoskeletal breakdown (Nicotera et al., 1990). We have shown in this study how in a PS1 FAD-linked mutant the dyshomeostasis of $[Ca^{2+}]_i$ correlated with caspase-3 higher activation and finally cell death, as compared to cells expressing WT PS1.

PS1 AND ACETYLCHOLINE MUSCARINIC RECEPTOR MEDIATED SIGNAL TRANSDUCTION (PAPERS III AND IV)

Beside sensitizing cells to apoptosis and altering processing of APP, PS1 mutations have been shown to alter calcium homeostasis. A few recent reports suggested

that PS1 could be involved in regulation of Ca^{2+} mobilization at the level of ER, being coupled with ryanodine receptors (RyR) and at the plasma membrane, altering CCE. Cholinergic signaling is one of the major sources of Ca^{2+} regulation and redistribution in neuronal cells (Berridge, 1998) and it is well known to be impaired in AD (Jope et al., 1997). Paper III aimed to clarify whether a FAD-linked PS1 mutant has impact on the signal transduction triggered by the cholinergic muscarinic receptors.

A recent study demonstrated that phosphoinositide/Ca^{2+} signaling pathway is dependent on the γ-secretase activity of PS1, since PS1 knockout cells or cells treated with γ-secretase inhibitors showed no Ca^{2+} signals upon bradykinin stimulation (Leissring et al., 2002). Paper IV tested the hypothesis that PS1-dependent γ-secretase activity regulates the muscarinic receptor mediated signal transduction.

Effects of FAD mutant PS1 on PI hydrolysis and $[Ca^{2+}]_i$ (papers III and IV)

Basal PI hydrolysis values were similar in NT, WT PS1 and L250S PS1 cells. In contrast, ΔE9 PS1 and M146V PS1 cells showed a significantly higher basal PI hydrolysis. As compared to NT and WT PS1 cells, carbachol-stimulated PI hydrolysis did not differ significantly in either M146V PS1 or L250S PS1 cells. In contrast, the carbachol-stimulated PI hydrolysis was higher in ΔE9 PS1 cells as compared to WT PS1 cells. All FAD mutant PS1 bearing cells (ΔE9 PS1, M146V PS1 and L250S PS1 cells) showed significantly higher basal $[Ca^{2+}]_i$ as compared to NT and WT PS1 cells. The apparent discrepancy between PI hydrolysis and $[Ca^{2+}]_i$ found in L250S PS1 cells can be due to different sensitivities of the two methods used. As compared to NT and WT PS1 cells, ΔE9 PS1, M146V PS1 and L250S PS1 cells showed a significantly higher carbachol-stimulated peak $[Ca^{2+}]_i$. Moreover, in these cell lines $[Ca^{2+}]_i$ showed a long tail-off effect and did not return to the baseline value for 10 min after carbachol was added.

In order to elucidate whether a high $[Ca^{2+}]_i$ is responsible for PLC high activity or increased PLC activity induces a high basal $[Ca^{2+}]_i$ in ΔE9 PS1 cells, in paper III we treated cells with neomycin, an inhibitor of PLC (Schacht, 1976) and then measured both

basal and carbachol-stimulated PI hydrolysis and $[Ca^{2+}]_i$. Pretreatment for 3 hr with 500 μM neomycin decreased the high basal and carbachol-stimulated PI hydrolysis seen in ΔE9 PS1 cells to the same level as for WT PS1 and NT cells. Pretreatment for 10 min with 100 and 500 μM neomycin before carbachol stimulation did not affect basal $[Ca^{2+}]_i$ in NT and WT PS1 cells, but significantly reduced the basal $[Ca^{2+}]_i$ in ΔE9 PS1 cells to levels seen in NT and WT PS1 cells. Neomycin also significantly reduced the carbachol elevation of $[Ca^{2+}]_i$ in all cell lines but did not reverse it in ΔE9 PS1 cells to the level seen in WT PS1 and NT cells and did not either abolish the long tail-off effect. These results suggested that the enhanced PI hydrolysis and $[Ca^{2+}]_i$ seen in ΔE9 PS1 cells are due to an initial increase in PLC activity and the rise in $[Ca^{2+}]_i$ is a secondary event. In order to further confirm that ΔE9 PS1 mutation increase $[Ca^{2+}]_i$ by primarily enhancing PI hydrolysis, we treated cells with a specific antagonist of IP_3R, xestospongin C (Gafni et al., 1997). Xestospongin C did not influence the basal $[Ca^{2+}]_i$ in WT PS1 and NT cells but reversed it in ΔE9 PS1 cells to levels seen in WT PS1 and NT cells. In ΔE9 PS1 cells xestospongin C also gave a dose-dependent reduction of carbachol-stimulated $[Ca^{2+}]_i$ to levels found in WT PS1 and NT cells and also abolished the long tail-off effect. Together these data are consistent with a mechanism whereby the ΔE9 PS1 mutation increases basal PLC activity to raise the level of IP_3 available to gate Ca^{2+} release via IP_3Rs.

In paper IV, neomycin did not significantly affect the basal apparent $[Ca^{2+}]_i$ in NT, WT PS1, M146V PS1 or L250S PS1 cell lines. Neomycin significantly reduced the peak $[Ca^{2+}]_i$ in NT, WT PS1, M146V PS1 and L250S PS1 cells in a dose-dependent fashion. Treatment with the higher neomycin concentration reduced the carbachol-induced peak $[Ca^{2+}]_i$ in M146V PS1 and L250S PS1 cells to the level seen in WT PS1 cells. Treatment with neomycin reduced but did not completely reverse the long tail-off effect in M146V PS1 or L250S PS1 cells.

Considering that different reports suggested that Ca^{2+} signal amplification induced by PS1 mutations is due to ryanodine receptors (Guo et al., 1996, Chan et al., 2000) we tested the effects of dantrolene, a specific RyR blocker (Zhao et al., 2001). In paper III, dantrolene used in concentrations of 100 nM, 1 μM and 10 μM did not induce any significant changes of basal $[Ca^{2+}]_i$ in WT PS1 and NT cells. In contrast, in ΔE9 PS1 cells, both 1 μM and 10 μM dantrolene significantly reduced basal $[Ca^{2+}]_i$ but to values

still significantly higher than that for either WT PS1 or NT cells. Dantrolene treatment of ΔE9 PS1 cells also abolished the long tail-off effect of carbachol stimulation, restoring a stimulation pattern seen in control cell lines. Ten μM dantrolene also significantly reduced the carbachol-induced peak of $[Ca^{2+}]_i$ in WT PS1 and NT cells, but not in ΔE9 PS1 cells.

The fact that the RyR antagonist dantrolene decreased basal $[Ca^{2+}]_i$ only in ΔE9 PS1 cells, but not down to a level similar to that found in WT PS1 and NT cells suggests that a component of the increased basal $[Ca^{2+}]_i$ in ΔE9 PS1 cells caused by enhanced PLC activity is mediated via RyRs. Dantrolene also abolished the long tail-off effect seen with carbachol stimulation in ΔE9 PS1 cells, which suggest that this alteration of the Ca^{2+} stimulation pattern is due to amplification through RyR, probably part of a calcium-induced calcium release mechanism.

In paper IV, dantrolene did not significantly change the basal apparent $[Ca^{2+}]_i$ values in NT, WT PS1, M146V PS1 or L250S PS1 cells. Treatment with 10 μM dantrolene reduced the carbachol-induced peak $[Ca^{2+}]_i$ in M146V PS1 cells and L250S PS1 to the level seen in WT PS1 cells. Ten μM dantrolene treatment also significantly inhibited the long tail-off effect seen in FAD mutant PS1 cells but a complete return to baseline $[Ca^{2+}]_i$ was seen only at 10 min after carbachol addition.

In agreement with previous reports (Leissring et al., 1999, Leissring et al., 2000a, Leissring et al., 2001, Guo et al., 1996) ΔE9 PS1, M146V PS1 and L250S PS1 cells showed significant increases in $[Ca^{2+}]_i$ upon carbachol stimulation as compared to WT PS1 and NT cells. It has been suggested that enhanced calcium signaling induced by PS1 mutants is due to elevated ER calcium content and can be reversed by dantrolene, a specific ryanodine receptor antagonist (Guo et al., 1996). We also found that dantrolene was able to reverse both the increased peak $[Ca^{2+}]_i$ values and the long tail-off effect seen in FAD PS1 mutant cells. It has been also shown that expression of the IP_3R is not changed by FAD mutant PS1 M146V (Leissring et al., 2001) but that the same PS1 mutant increases the expression of RyR (Chan et al., 2000). We were able to reverse the increase in $[Ca^{2+}]_i$ peak evoked by carbachol in our FAD PS1 mutant cells by the PLC inhibitor neomycin. However, neomycin was not able to fully reverse the long tail-off effect, which was sensitive to dantrolene. These data suggest that in FAD PS1 mutant

cells an amplification of calcium-induced calcium-release is mediated via ryanodine receptors.

In order to see whether altered calcium signaling in FAD mutant PS1 cells could be reversed by γ-secretase inhibition, we tested the effects of DAPT and pepstatin A on basal and carbachol-stimulated $[Ca^{2+}]_i$ in FAD mutant M146V PS1 and L250S PS1 cells (paper IV). DAPT did not affect the basal $[Ca^{2+}]_i$ values, but significantly reduced the peak $[Ca^{2+}]_i$ in both M146V PS1 and L250S PS1 cells. Pepstatin A showed similar effects to that of DAPT. The effects of both DAPT and pepstatin A on carbachol-stimulated $[Ca^{2+}]_i$ were similar to that seen with neomycin, including the incomplete reversal of the long tail-off effect. These data suggested that the enhancement of calcium signaling was dependent on γ-secretase activity.

PLC levels in NT, WT PS1 and ΔE9 PS1 cells (paper III)

Considering that an enhanced PI hydrolysis in ΔE9 PS1 cells could have been induced by a higher PLC level, we investigated the expression levels of two PLC isoforms, PLC-β1 and PLC-γ, in NT, WT PS1 and ΔE9 PS1 cells. We chose these two isoenzymes because they are expressed in the brain and in SH-SY5Y cells. The PLC-β1 N-terminal antibody recognized two immunoreactive bands, of ~150 kDa and of ~100 kDa. The 150 kDa band represents the full-length PLC-β1 and the 100 kDa represents the NTF of PLC-β1 after proteolytic cleavage by calpain (Park et al., 1993). The 150 kDa full-length PLC-β1 was decreased in ΔE9 PS1 cells as compared to both WT PS1 and NT cells. In contrast, both WT PS1 and ΔE9 PS1 cells showed a higher level of 100 kDa cleaved PLC-β1 as compared to NT cells. The PLC-γ antibody recognized in all cell lines only one immunoreactive band of ~148 kDa and we found no differences in PLC-γ expression between NT, WT PS1 and ΔE9 PS1 cells.

Both full-length and calpain-cleaved PLC-β1 have been shown to possess catalytic activity in purified enzyme preparations (Park et al., 1993) but it is currently unknown how much each species contributes to PI hydrolysis. The C-terminal region of PLC-β1 is responsible for the membrane association of the enzyme (Kim et al., 1996) and

the 150 kDa protein is present in the particulate fraction. In contrast, the 100 kDa cleaved PLC-β1 is mainly located to the cytosol and is more active but less stable than the full-length species (Park et al., 1993). Therefore, from our data it is tempting to speculate that the higher PLC activity seen in mutant PS1 cells could be a result of the higher proteolytic cleavage of PLC-β1.

Localization of PS 1 in NT, WT PS1 and ΔE9 PS1 cells (paper III)

For the hypothesis that PS1 could interact with PLC or PLC cleavage to be true it would be needed that PS1 is located to the plasma membrane. Even though PS1 is known to be principally located to ER and Golgi apparatus, recent reports have suggested that PS1 is also localized to the plasma membrane, where it has been shown to form complexes with components of the cadherin-catenin cell-to-cell adhesion system (Beher et al., 1999, Schwarzman et al., 1999, Georgakopoulos et al., 1999). We therefore investigated whether in the cells used in our study PS1 is located to the plasma membrane by double immunostaining with anti-N terminal PS1 and anti-cadherin antibodies and confocal microscopy. Indeed, we found that PS1 and N-cadherin colocalize at the level of the plasma membrane. We chose to examine immunoreactivity of N-cadherin, which is a member of the cell adhesion system, because is known to be concentrated at the plasma membrane at sites of cell-cell contact. From a direct immunoreactivity study for PLC-β1 would have been difficult to withdraw any conclusion, because both PS1 and PLC-β1 are widely distributed throughout the cell. Our findings are in agreement with previous reports, where cell surface PS1 has been suggested to regulate cell-cell adhesion and synaptic contacts (Georgakopoulos et al., 1999). Others have shown that PS1 can stabilize cadherin-catenin complexes to stimulate Ca^{2+}-dependent cell-to-cell adhesion (Baki et al., 2001). However, ΔE9 PS1 failed to stabilize cadherin-catenin complexes and stimulate cell-cell adhesion. Moreover, the same group has shown that the intracellular domain of E-cadherin is released to the cytosol after a PS1-dependent γ-secretase and regulates the disassembly of adherens junctions, the cleavage being induced by apoptosis or Ca^{2+} influx (Marambaud et al., 2002). Interestingly, the PLC-γ can be activated by integrins (Wrenn et al., 1996, Wossmeyer et al., 2002), another component of the cell-to-

cell adhesion complex, and integrin functions have been shown to be regulated by calpain (Leitinger et al., 2000, Sato and Kawashima, 2001).

Dominant negative PS1 and pharmacological inhibition of γ-secretase reduces PLC activity and Ca^{2+} signals (paper IV)

In this study we tested different clones of D257A PS1 and D385N PS1 cells. With the exception of clone D257A-2C, all other dominant negative PS1 clones showed significantly lower basal PI hydrolysis as compared to NT and WT PS1 cells. However, all D257A PS1 and D385N cell lines showed significantly lower carbachol-stimulated PI hydrolysis as compared to NT and WT PS1 cells. For further experiments, we selected PS1 D257A-2C and PS1 D385N-1B cells. These were the clones with the lowest level of PS1 expression among these cell lines. This was done in order to rule out potential artifacts due to ER stress that could in theory occur due to over expression on non-endoproteolysed protein.

In accordance to the basal PI hydrolysis data, D257A PS1 cells showed no difference in basal $[Ca^{2+}]_i$ as compared to NT and WT PS1 cells and D385N PS1 cells showed a significantly lower basal $[Ca^{2+}]_i$ as compared to NT and WT PS1 cells. In contrast to FAD mutant PS1 cells, both dominant negative PS1 cells showed very poor $[Ca^{2+}]_i$ increases upon carbachol stimulation, that were significantly lower as compared to NT and WT PS1 cells. Despite the poor responses seen in both D257A PS1 and D385N PS1 cells, the effects of carbachol on $[Ca^{2+}]_i$ increases were statistically significant as compared to basal values.

Considering that dominant negative PS1 reduced PLC signaling and calcium responses, we wanted to confirm that impairment of PLC activity was due to a lack of γ-secretase activity. For this, we pre-treated cells for 24 h with the specific γ-secretase inhibitor DAPT, and measured basal and carbachol-stimulated PI hydrolysis and $[Ca^{2+}]_i$ in NT, WT PS1, D257A PS1 and D385N PS1 cells.

Treatment with DAPT did not significantly affect basal PI hydrolysis in NT, D257A PS1 and D385N PS1 cells but significantly lowered basal PI hydrolysis in WT

PS1 cells. DAPT also significantly reduced carbachol-stimulated PI hydrolysis in both NT and WT PS1 cells but did not affect PI hydrolysis responses in D257A PS1 or D385N PS1 cells. Treatment with DAPT had no significant effect on basal $[Ca^{2+}]_i$ in NT, WT PS1, D257A PS1 or D385N PS1 cells). Corresponding to effects seen on PI hydrolysis, DAPT significantly reduced the carbachol-stimulated $[Ca^{2+}]_i$ peaks in NT and WT PS1 cells but had no effect in D257A PS1 or D385N PS1 cells. Pepstatin A gave a similar results profile to DAPT on basal and carbachol-stimulated $[Ca^{2+}]_i$ in all cell lines.

In conclusion, in this study we designed experiments to evaluate whether γ-secretase activity was important for regulating the signaling pathway either at the ER level or upstream at the level of PLC. All cell lines expressing dominant negative PS1 showed blunted carbachol-stimulated PI hydrolysis responses, suggesting that PLC activity is regulated by the γ-secretase activity of PS1. To confirm this hypothesis, we treated NT and WT PS1 cells with DAPT and pepstatin A and looked at effects of γ-secretase inhibition on PI hydrolysis and $[Ca^{2+}]_i$. Both inhibitors significantly reduced carbachol-stimulated PI hydrolysis responses in NT and WT PS1 cells. Corresponding to the PI hydrolysis data and in agreement with a previous report (Leissring et al., 2002), the calcium signals generated by carbachol in NT and WT PS1 cells treated with γ-secretase inhibitors and in dominant negative PS1 cells were significantly inhibited. Taken together these data strongly suggest that the γ-secretase activity of PS1 has a regulatory function on PLC activity.

CONCLUSIONS

Papers I and II

- PS1 is processed during apoptosis by multiple proteolytic systems, including the proteasome, caspases and calpains
- Caspase cleavage of PS1 is an early event in apoptosis, preceding cleavage of other caspase substrates and typical morphology of apoptosis
- Endoproteolytically-derived PS1 NTF and CTF are stable during apoptosis, probably being incorporated in a functional heterodimer
- During apoptosis, the proteasome process proteolytically full-length PS1 and the alternative PS1 fragments derived from caspase cleavage
- Truncated FAD-linked ΔE9 PS1 is resistant to proteasomal degradation during apoptosis as compared to WT PS1
- FAD-linked ΔE9 PS1 mutation sensitize neuronal type SH-SY5Y cells to cell death induced by the calcium ionophore A23187
- PS1 transfected cells undergoing apoptosis induced by calcium ionophore A23187 alter their cell to cell contacts and display typical protein caspase-cleavages
- One mechanism by which ΔE9 PS1 sensitize cells to apoptosis is alteration of Ca^{2+} homeostasis
- No direct relationship between PS1 processing and cell viability was found in the experimental paradigm employed by our studies

Papers III and IV

- FAD-linked ΔE9 PS1 and M146V PS1 mutations enhances basal PI hydrolysis and $[Ca^{2+}]_i$
- ΔE9, M146V and L250S FAD-linked PS1 mutants alter basal and carbachol-stimulated $[Ca^{2+}]_i$

- The Ca^{2+} homeostasis dysregulation induced by FAD-linked ΔE9 PS1, M146V PS1 and L250S PS1 is mediated by IP_3R and RyR amplification of Ca^{2+}
- ΔE9 PS1 mutation causes upstream changes in PI signaling which secondary lead to high $[Ca^{2+}]_i$
- PS1 is located not only to ER and Golgi apparatus, but also to the plasma membrane, where it could interfere with signal transduction pathways
- The Ca^{2+} homeostasis dysregulation induced by M146V and L250S PS1 mutants is reversed by pharmacological inhibition of γ-secretase
- Dominant negative PS1 and pharmacological inhibition of γ-secretase reduces PLC activity and Ca^{2+} signals, supporting the hypothesis that PS1 regulates PLC activity

FUTURE PERSPECTIVES

Last decades have seen an enormous progress in AD research, with genetic and cell biological studies offering new pathogenic hypotheses and new opportunities to design AD drugs. Identification of β- and –γ secretases with development of inhibitors of these proteases and initiation of Aβ vaccination are good examples. New derived transgenic animals seem to offer a better animal model for the disease that will probably be helpful to better understand the chronology of pathogenic events and to test drugs for AD. However a consensus regarding mechanisms of neurodegeneration in AD has not been reached yet.

A wealth of data shows that PSs are key molecules in AD pathogeny. First, mutations in PS1 or PS2 genes cause the majority of FAD cases. Second, PSs are part of the γ-secretase protease complex, which executes the final cut of APP in order to release Aβ peptide, and PSs seem to be essential for γ-secretase activity. Moreover, various reports suggest that PSs regulate apoptosis biochemistry.

In the first part of this thesis (papers I and II) there is data analyzing the complex proteolytic processing of PS1 during apoptosis in a time-relationship to other typical apoptotic biochemical and morphological events. PS1 is cleaved early during apoptosis making it possible that PS1 species regulate apoptosis cascades. PS1 is not cleaved only by caspases during apoptosis, being processed by the proteasome as well, with the exception of the very stable NTF and CTF, probably incorporated in functional high molecular weight complexes. In order to elucidate the exact role of different alternative PSs species in regulation of apoptosis, future studies are needed, employing models where this alternative PSs fragments are expressed separately.

Beside the role in cell death, which could correspond to the advanced stage of AD, PSs regulate signal transduction pathways, and alteration of cholinergic signal transduction occurs during the early stages of AD. In the second part of this thesis (papers III and IV) there is data showing how wild-type and mutant PS1 regulate cholinergic muscarinic signal transduction. Our data suggest that PS1 modulates phosphoinositide/Ca^{2+} signals, by regulating PLC activity. Moreover, mutant PS1 seems to lead to neuronal dysfunction and finally to neurodegeneration by generating a Ca^{2+}

overload upon cholinergic stimulation. However, the effects of γ-secretase inhibition on physiological muscarinic stimulation presented in paper IV raise questions about possible side effects of γ-inhibitors as therapeutic agents, which are added to a possible inhibition of Notch cleavage.

In conclusion, future research on PSs biological functions holds the key to establish whether they can be used as a therapeutic target in reducing γ-secretase activity and/or modulate neuronal apoptosis in AD.

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