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# A STUDY OF β-SECRETASE CLEAVED ALZHEIMER AMYLOID PRECURSOR PROTEIN

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Stockholm 2002

*To Gudrun and Gunnar Sennvik, with love*

# TABLE OF CONTENTS

# ABSTRACT

Alzheimer's disease (AD) is characterized by the degeneration and loss of neurons, intracellular neurofibrillary tangles and the accumulation of extracellular senile plaques consisting mainly of β-amyloid (Aβ). Aβ is generated from the amyloid precursor protein (APP) through sequential cleavage by proteases β- and γ-secretase. Alternatively, APP may be cleaved within the Aβ region by α-secretase, preventing intact Aβ formation. Both the α- and β-secretase cleavages result in the release of large soluble APP fragments called α- or β-sAPP, respectively. The work presented in this thesis describes the processing and secretion of differentially cleaved APP.

The purpose of the study were to investigate the β-secretase cleavage of APP. Paper I examined differentially cleaved APP as diagnostic markers for AD. It was concluded that soluble β-secretase cleaved APP (β-sAPP) levels in CSF do not change in AD, although soluble α-secretase cleaved APP (α-sAPP) and total sAPP decreases. Paper II and III provided insights into the mechanisms of the alternative APP cleavages during apoptosis in two different cell systems. In a primary rat cortical culture system, calcium homeostasis and caspase actions proved to be important effectors of the β-secretase cleavage. Expression of the Arctic APP mutation in human neuroblastoma cells increased the vulnerability to cell death and modified β-sAPP secretion, stressing the role of FAD mutations in apoptosis and APP processing. The localization and content of β-sAPP in brain was explored in paper IV. Altered β-sAPP staining patterns indicated abnormal processing and transport of APP in AD brain.

In summary, (i) β-sAPP in CSF, (ii) β-sAPP secretion from apoptotic neurons and (iii) β-sAPP in brain were analysed. The results indicated that altered processing and transport of APP takes place in AD and during apoptosis. Since APP processing is considered a key event in the pathological cascade leading to AD, the proteases that cleave APP and the regulation mechanisms of those proteases are prime therapeutic targets.

# LIST OF ORIGINAL PUBLICATIONS

I.     Sennvik K., Fastbom J., Blomberg M., Wahlund L-O., Winblad B., Benedikz E., *"Levels of α- and β-secretase cleaved amyloid precursor protein in the cerebrospinal fluid of Alzheimer's disease patients",* Neurosci Lett, 2000, 277:169-172.

II.    Sennvik K., Benedikz E., Fastbom J., Sundström E., Winblad B., Ankarcrona M., *"Calcium ionophore A23187 specifically decreases the secretion of β-secretase cleaved amyloid precursor protein during apoptosis in primary rat cortical cultures",* J Neurosci Res, 2001, 63(5):429-437.

III.   Sennvik K., Nilsberth C., Stenh C., Lannfelt L., Benedikz E., *"The Arctic Alzheimer mutation enhances sensitivity to toxic stress in human neuroblastoma cells".* Neurosci Lett, 2002. Accepted for publication.

IV.    Sennvik K., Bogdanovic N., Volkmann I., Fastbom J., Benedikz E., *"β-secretase cleaved amyloid precursor protein in Alzheimer brain: a morphologic study".* Manuscript.

Published papers were printed with permission from Elsevier Science (Neurosci Lett) and Wiley (J Neurosci Res).

# ABBREVIATIONS

| | |
|---|---|
| α-sAPP | soluble α-secretase cleaved amyloid precursor protein |
| β-sAPP | soluble β-secretase cleaved amyloid precursor protein |
| Aβ | β-amyloid |
| ACT | α1-antichymotrypsin |
| AD | Alzheimer's disease |
| APLP | amyloid precursor-like protein |
| apoE | apolipoprotein E |
| APP | amyloid precursor protein |
| APPArc | amyloid precursor protein with the Arctic mutation |
| APPDu | amyloid precursor protein with the Dutch mutation |
| APPFlem | amyloid precursor protein with the Flemish mutation |
| APPSwe | amyloid precursor protein with the Swedish mutation |
| APPwt | wild-type amyloid precursor protein |
| BACE1 | β-site amyloid precursor protein cleaving enzyme 1 |
| BACE2 | β-site amyloid precursor protein cleaving enzyme 2 |
| CSF | cerebrospinal fluid |
| ELISA | enzyme-linked immunosorbent assay |
| EOAD | early-onset Alzheimer's disease |
| ER | endoplasmic reticulum |
| FAD | familial Alzheimer's disease |
| HRP | horse-radish peroxidase |
| KPI | Kunitz protease inhibitor |
| MMSE | mini mental state examination |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| PBS | phosphate buffered saline |
| PI | propidium iodide |
| PKC | protein kinase C |
| PS1 | presenilin 1 |
| PS2 | presenilin 2 |
| sAPP | soluble amyloid precursor protein |
| TACE | tumor necrosis factor α-cleaving enzyme |
| TBS | tris buffered saline |
| TBS-T | tris buffered saline with Tween-20 |

# INTRODUCTION

## Alzheimer's disease

HISTORY

Alzheimer's disease is named after Dr Alois Alzheimer, who in 1907 published an account of rapidly deteriorating mental illness in a 51-year old woman [3]. After four and a half years, during which the patient suffered increasing memory impairment, disorientation, hallucinations and personality changes, she died in a completely demented state. At autopsy, her brain showed considerable global atrophy. Histological analysis with the Bielschowsky silver stain revealed dense bundles of fibrils within the neurons and numerous focal lesions in the cerebral cortex. The fibrils were later named 'neurofibrillary tangles' and the lesions 'senile plaques'. The combination of progressive presenile dementia with neurofibrillary tangles and senile plaques became known as Alzheimer's disease (AD).

EPIDEMIOLOGY

AD is the most common cause of dementia in the Western world, accounting for up to 50-60% of all late-onset dementia cases [66]. Advanced age and a family history of dementia increase the risk of developing AD as do vascular disease and head trauma [44]. Typically, clinical symptoms of AD begin with short-term memory impairment and loss of cognitive function [145]. In later stages, linguistic abilities and spatio-temporal orientation decline as well and later still, sensory and motor functions are lost. At the time of death, which usually occurs about 5-15 years after disease onset, the patient is bed-ridden and completely dependent on constant care and supervision [63].

CLINICAL DIAGNOSIS OF AD

A diagnosis of AD is currently based on a typical medical history in combination with the exclusion of other dementing illnesses. Vascular dementia, frontotemporal dementia, dementia with Lewy bodies and Parkinson's disease are among the more common illnesses that must be ruled out in order for the patient to be diagnosed with AD. One commonly used set of diagnostic criteria classifying the patient as having possible, probable or definitive AD has been described by the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [109]. While possible and probable AD may be diagnosed

according to NINCS-ADRDA by physical and mental examination alone, a definite diagnosis of AD also requires a histopathological confirmation. Other sets of criteria for clinically diagnosing AD are the International Classification of Disease, 10th revision (ICD-10) criteria [167] and the Diagnostic and Statistical Manual of Mental Disorders (DSM IV) definition of dementia [7]. For the neuropathological diagnosis of AD, the consortium to establish a registry of Alzheimer's disease (CERAD) criteria [112], Khachaturian's criteria [81], the Tierny criteria [159] and Braak staging [17] are employed. The Mini-Mental State Examination (MMSE) test is a widely used questionnaire designed to assess cognitive function [45].

PATHOLOGY

AD is neuropathologically characterised by the presence of degenerated and dead neurons, especially in the parietal and temporal lobes; extracellular senile plaques (Fig.1a) and intracellular neurofibrillary tangles (Fig.1b) in the brain. Additional features of AD are deficits in multiple neurotransmitter signalling systems and synaptic loss. The classical senile plaque in AD consists of a central core of radiating amyloid fibrils, surrounded by a rim of dystrophic neurites and activated microglia and astrocytes [103]. Amyloid is also deposited in cerebral blood vessels.

The term amyloid (=starchlike) refers to chemically heterogenous protein deposits which are (i) composed of straight unbranching protein fibrils of 6-10 nm diameter, (ii) present in an anti-parallel β-pleated sheet conformation and (iii) able to display green-red birefringence upon Congo Red staining [52]. Amyloid may be found in different tissues and is associated with several diseases [151].

The main component of the neurofibrillary tangles is hyper-phosphorylated tau protein, which assembles into paired helical filaments [85]. In the terminal stage of degeneration, when the neuron has dissipated, only the tangle remains. Both the amyloid load and the neurofibrillary tangles have been shown to correlate with the degree of dementia [17,30].
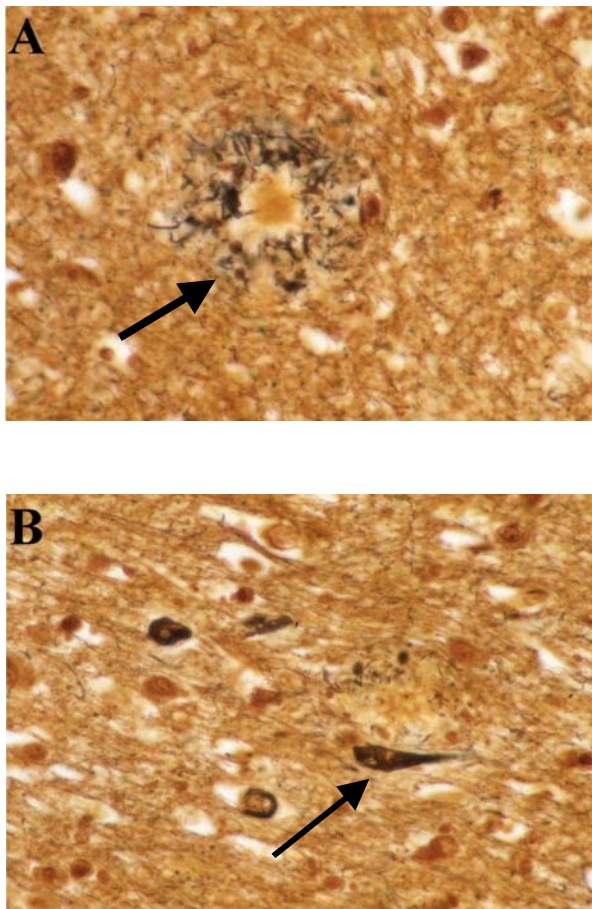
*Fig.1. a) senile plaque b) neurofibrillary tangle in AD brain (Palmgren silver stain).*

## Genetics

It was established as early as in the 1950's, through genealogical studies, that AD has a genetic component and that the hereditary form of AD generally has an earlier age of onset than sporadic AD [145]. The proportion of familial AD (FAD) has been suggested to constitute 25-50% of all AD cases. There may, however, be multiple genetic and environmental factors affecting age of onset and disease progression.

APP

Glenner and Wong reported the first partial amino acid sequence of β-amyloid (Aβ) purified from AD brains in 1984 [53]. Aβ was found to be a 39-43 residue cleavage product from a previously unknown transmembrane protein, the amyloid precursor protein (APP). In 1987, the first genetic defects leading to AD were identified in the amyloid precursor protein (APP) gene on chromosome 21q21 [149]. Certain mutations in the APP gene were then shown to co-segregate with early onset AD (EOAD) and to be inherited as an autosomal dominant trait [8,26,38,54,68,90,116,117]. Although the APP mutations are rare,

they are of great significance for understanding the molecular mechanisms of AD pathogenesis. The presence of Alzheimer-like plaques in Down's syndrome (DS) brains can most likely be explained by the additional copy of the APP gene in these individuals [123].

PRESENILIN 1 AND 2

In 1995, the highly homologous presenilin 1 and 2 (PS1 and PS2) genes were identified as FAD genes [96,129,142]. The PS1 gene was found to be located on chromosome 14q24.3 and the PS2 gene on chromosome 1q31-42. Over 100 FAD mutations have been reported in the PS1 gene and 6 FAD mutations in the PS2 gene. Taken together, the mutations in the PS genes account for over 50% of the familial EOAD cases [66].

The presenilins are conserved polytopic transmembrane proteins found mainly in intracellular membranous compartments [177]. Full-length PS1 and PS2 consist of 467 and 448 amino acids respectively. Most studies support a model with eight transmembrane domains and a large cytoplasmic loop between domain six and seven, although six to seven transmembrane topologies have also been suggested. During maturation, the presenilins are cleaved within the loop, resulting in the formation of a heterodimeric complex composed of the N-terminal and the C-terminal fragment. The presenilins are essential for the unusual intramembrane cleavage of several proteins, including APP [35], Notch [34] and ER stress sensor/resident transmembrane kinase Ire1p [122].

APOE

The apolipoprotein E (apoE) gene on chromosome 19q12-q13 was identified as a risk factor or susceptibility gene for AD in 1993 [153]. ApoE is a polymorphic protein playing a central role in the metabolism and transport of cholesterol and triglycerides. There are three major alleles of apoE, ε2, ε3 and ε4 which encode three different protein isoforms, E2, E3 and E4, respectively. Genetic analysis has shown the ε4 allele to increase the risk of late-onset AD in a dose-dependent manner, while the ε2 allele seems to have a protective effect [29,134]. The mechanism by which apoE contributes to AD pathogenesis is not known, but apoE has been shown to associate with Aβ in brain [118], and the three isoforms are believed to affect Aβ fibrillization and plaque formation differentially [181]. There are also variations in the ability of apoE isoforms to bind to tau [152] and in the manner in which they interact with the low-density lipoprotein [153].

TAU

Tau is a microtubule associated protein mainly produced by neurons [84]. Tau is present in both central and peripheral nervous systems. The function of tau is to promote microtubule assembly and stability [43]. The C-terminal region of tau contains either three or four tandem repeats, whose flanking regions are necessary for binding to microtubules [19,42,56]. In AD, the flanking regions are heavily phosphorylated by multiple kinases, weakening the binding [75]. Specific mutations in the tau gene have been shown to be causal for frontotemporal dementia [71] and Parkinsonism [148], but so far no tau mutations have been implicated in Alzheimer's disease pathogenesis.

OTHER GENETIC RISK FACTORS

A common polymorphism in α-1-antichymotrypsin (ACT) confers a significant risk for AD in individuals carrying the ApoEε4 allele [79,157]. ACT is a major component in amyloid plaques [1] and is believed to influence fibril formation [47]. Recently identified disease loci on chromosome 3 and 10 are also of interest, since they may be associated with Aβ degrading enzymes neprilysin and insulin degrading enzyme (reviewed in [138]). Although many other susceptibility genes have been reported, so far none of them seem to have the same magnitude of impact as the APP, PS and APOE genes.

## APP

APP is predicted to be a type I integral membrane glycoprotein, with a long luminal N-terminal segment, a single membrane spanning region and a short intracellular tail [80]. The Aβ region originates close to the membrane on the luminal side and ends 12-15 amino residues into the membrane. APP is an evolutionary conserved protein which is ubiquitously expressed in humans [55]. APP and the APP-like proteins (APLP:s) are highly homologous and are considered to belong to the same protein family, although the APLP:s lack the Aβ region [164]. Due to alternative splicing of three exons, APP exists as eight different isoforms, the most common being APP695, APP751 and APP770 [55].

In the extracellular part of APP, binding domains for copper, zinc, heparin, collagen and fibulin have been identified and the isoforms APP751 and APP770 also contain a Kunitz-type protease inhibitor (KPI) domain [83]. APP695, which lacks the KPI insert, is the splice product most common in neuronal cells [80]. Further heterogeneity is achieved as post-translational modifications are added: sulfation, phosphorylation and N- and O-

glycosylation (reviewed in [137]). Following trafficking to the cell surface, APP may be reinternalised and targeted to endosomes and then recycled to the cell surface or to lysosomal compartments. In polarised cells such as Madin-Darby canine kidney cells and hippocampal neurons, APP is transported to the basolateral compartment and to axons, respectively [141,150,158].

There is histochemical evidence for the presence of APP in senile plaques, cerebrovascular amyloid and granular amyloid deposits [155,156] in the brain, but there are also reports showing APP to be present only in a subset of senile plaques [74,131]. APP within the neuron has been detected in the cytoplasm [21,25], in the axon [141,144,150,158,172], in neurites and neuropil threads, but also in neuronal vesicles and on neuronal surfaces [25].

The normal function of APP and its homologues *in vivo* is still not fully understood, but it has been shown to be involved in cell adhesion, cell proliferation, neuroprotection and neurite outgrowth. Since APP structurally resembles a receptor and is targeted to the cell surface [80], it has been implicated in signal transduction, for example in the Reelin signalling pathway, which regulates neuronal migration and positioning [128]. In another study, APP was shown to function as a membrane receptor protein, linking kinesin-1 to vesicles moved by fast anterograde axonal transport [78].

The adapter protein Fe65 interacts with the YENPTY motif in the cytoplasmic domain of APP via a phosphotyrosine interaction domain [16] and initiates nuclear signalling [22,82]. Another effect of the APP-Fe65 interaction is to increase processing and cellular retention of APP and to regulate cell movement [132,133]. A few other APP binding proteins such as X11, with partner MINT-1, and mDAB compete for the same binding site and may regulate APP (reviewed in [33]). Additionally, activation of a $G_0$ trimeric complex binding to the cytoplasmic tail of APP, may induce cell death *in vitro* [121] .

 The A β fragment is generated beginning with an initial APP cleavage at amino acid residue 672 to define the N-terminus of Aβ (Fig.2) [140]. The cleavage is referred to as β-secretase cleavage and results in the secretion of the soluble β-secretase cleaved APP ectodomain (β-sAPP) from neurons and transfected cells. The remaining C-terminal fragment containing the transmembrane and cytoplasmic domains (C99) is further cleaved by γ-secretase, resulting in release of the 40 or 42 amino residue Aβ peptide [65,143].
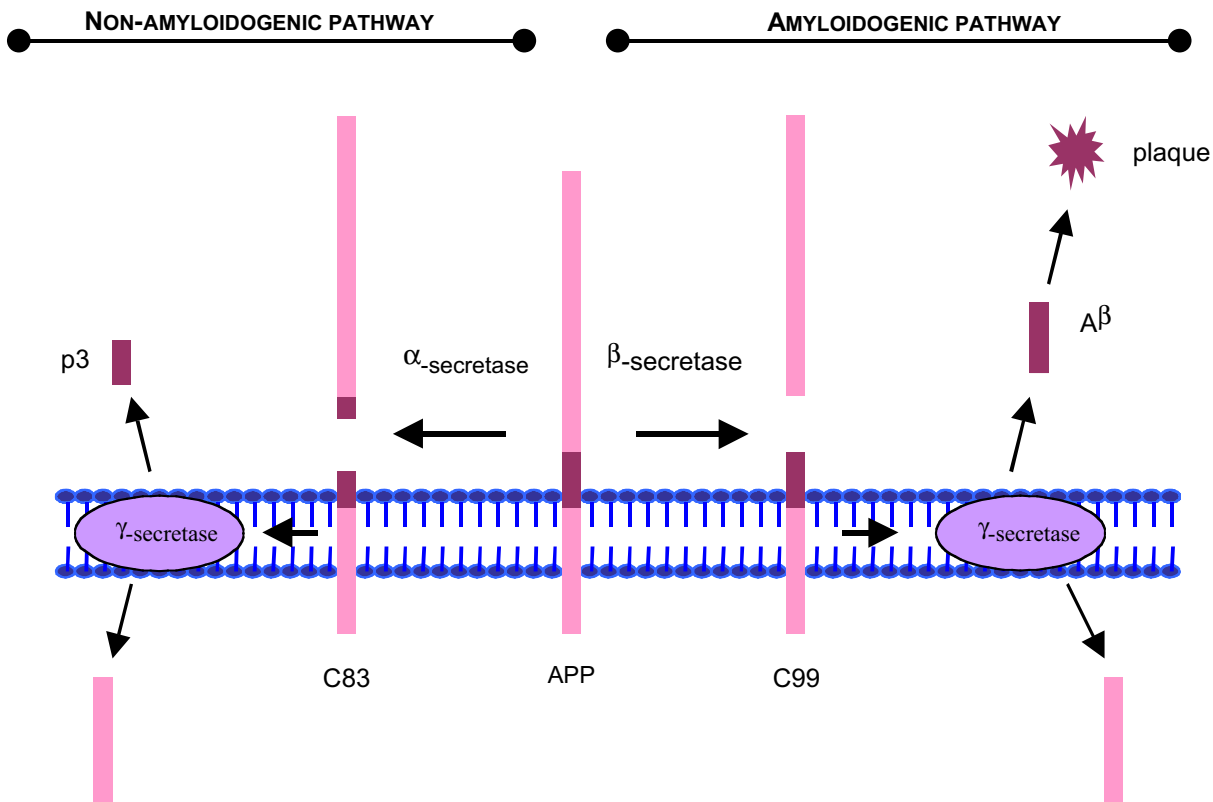
*Fig.2. The amyloidogenic and non-amyloidogenic processing of APP.*

Alternatively, APP may be cleaved between residue 16-17 of the Aβ domain [39]. The event is called α-secretase cleavage and precludes Aβ formation. The soluble α-secretase cleaved APP (α-sAPP) is secreted and the C-terminal fragment (C83) is processed by γ-secretase, resulting in a soluble 3-kDa peptide (p3).

FAD point mutations have been located at or in the vicinity of all three secretase cleavage sites (Fig.3) and are believed to interfere with normal endoproteolysis of APP (reviewed in [66]). The Swedish APP (APPSwe) KM670/671NL mutation is of particular interest as it introduces a double amino acid substitution near the β-secretase cleavage site [116]. The APPSwe mutation leads to over-production of Aβ by favouring β-secretase cleavage over α-secretase cleavage. APP mutations C-terminal to the Aβ domain tend to increase the longer 42-amino residue form of Aβ specifically, as do the PS mutations, whereas the APPSwe mutation increases all forms of Aβ.
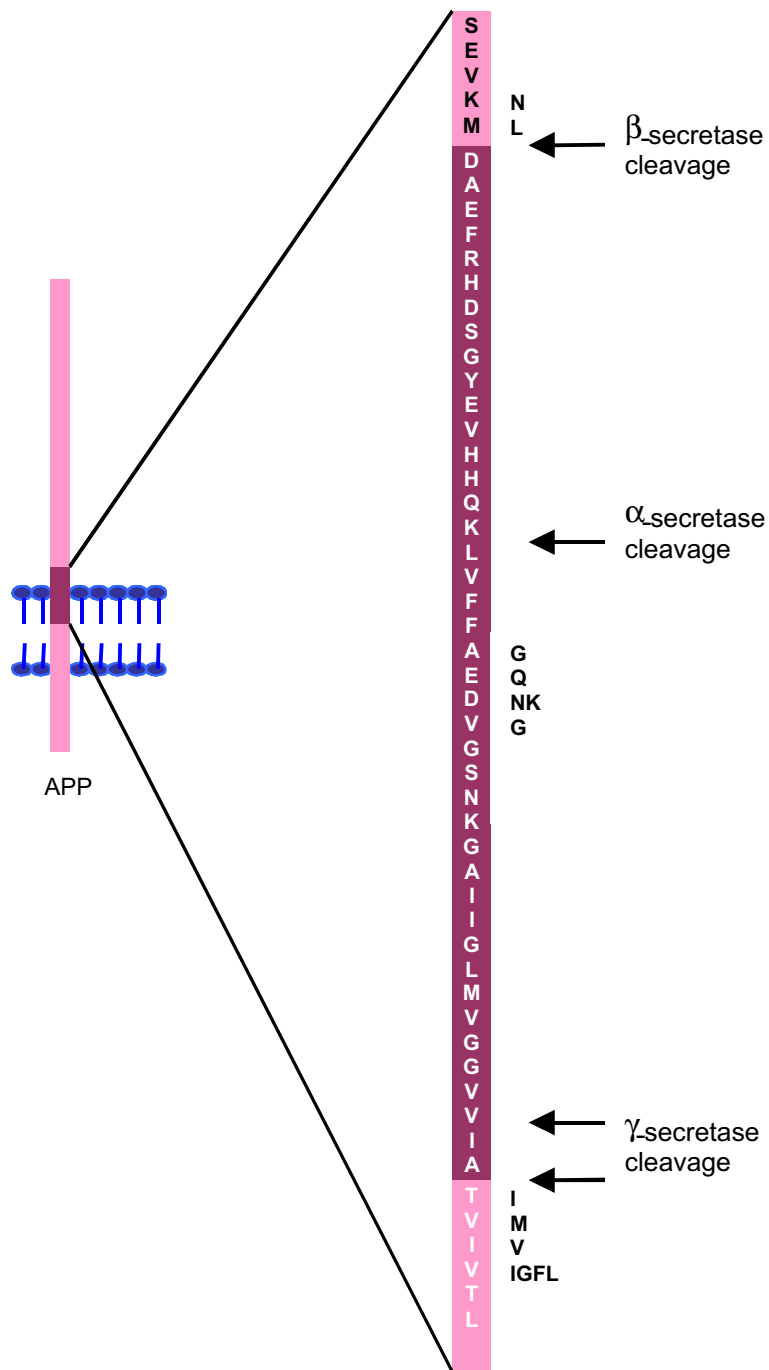
*Fig. 3. A part of APP containing the Aβ sequence. Pathogenic AD mutations to the right of the sequence.*

Most mutations within the Aβ sequence result in cerebral amyloid angiopathy and hemorrhagic stroke, while parenchymal plaques are not always noted [58,68,95,163]. The dissimilarities in clinical features may be due to differences in APP processing or Aβ fibrillisation kinetics. The Arctic (APParc) mutation, E693G, which lies within the Aβ sequence, is believed to cause early-onset AD in a Swedish family [120]. Subsequent fibrillisation studies suggested a molecular mechanism of disease where the amino acid

substitution in the Aβ peptide changes the aggregation kinetics by increasing formation and stability of protofibrils.

Although Aβ42 only accounts for about 10% of the total Aβ, it seems to be the more pathogenic of the Aβ species [176]. Having two additional hydrophobic residues, it is less soluble than Aβ40 and aggregates more easily [10,18]. Aβ42 is also the major constituent of senile plaques [72], while Aβ40 predominates in vascular amyloid [59,154]. There have been numerous reports on the possible neurotoxicity of Aβ, but although no clear consensus has been reached [168,174,175], it seems that multiple species of Aβ are neurotoxic [67,87]. Fibrillar Aβ has been shown to interact directly with a receptor for advanced glycation end products; an interaction which could mediate Aβ toxicity and lead to activation of microglia and production of reactive oxygen species [174]. Aβ has also been shown to directly disrupt membranes [110] or perturb membrane permeability through forming ionic pores [6,98].

## APP secretases

THE α-SECRETASES

Three related metalloproteases-disintegrins, ADAM9, ADAM10 and TACE/ADAM17 (tumor necrosis factor-α converting enzyme) have been shown to be involved in the α-secretase cleavage of APP [20,88]. ADAM10 and TACE are multidomain type I proteins with a propeptide domain and a zinc-dependent metalloprotease catalytic domain, a cysteine-rich sequence, a transmembrane domain and a cytoplasmic tail [15]. TACE contributes to the shedding of several transmembrane proteins other than APP, for example, the tumor necrosis factor after which it is named, the transforming growth factor α, L-selectin and Notch [20,40,125]. Rather than start a cascade reaction, TACE appears to cleave multiple substrates directly. These cleavage events may be induced by activation of the protein kinase C (PKC) signalling pathway.

Although various stimuli activating PKC have been shown to increase α-secretase cleavage at the expense of β-secretase cleavage [24,27], there also exists a basal α-secretase activity which cannot be regulated through PKC stimulation [146]. It is believed that TACE and ADAM10 are responsible for the PKC-regulated α-secretase activity in the trans-Golgi

network, while the unregulated α-secretase cleavage of APP is performed by another or several other proteases at the cell surface. Soluble APP species have been reported to have neuroprotective and neurotrophic properties [48,57,102,104].

THE β-SECRETASES

An aspartyl protease, β-site APP cleaving enzyme 1 (BACE1) or memapsin-2, was recently found to exhibit all known functional and characteristics of β-secretase [12,161]. BACE1 cleaves full-length APP at Asp1 of the Aβ sequence and, to a minor extent, at Glu11 [160]. Topologically, BACE1 is a 501 amino acid long membrane-bound type 1 protein with a lumenal active site. The N-terminal protease domain is followed by a connecting strand, a transmembrane region and a cytosolic domain [69]. An N-terminal signal sequence and a propeptide domain are removed post-translationally. Cleavage and maturation of BACE1 appears to be dependent on furin or a furin-like proprotein convertase [13].

BACE1 belongs to the pepsin protease family, but differs significantly from the other family members in the disulfide bridge structure and the arrangement of the substrate binding site [69]. A close homologue to BACE1, BACE2, has also been found, but the proteases differ in their tissue distribution [12] and specificity. BACE1 is abundantly expressed in brain and appears to represent the major β-secretase activity, while the highest expression levels of BACE2 are found in peripheral tissue. Although BACE2 is able to cleave APP at the β-secretase site, it cuts most efficiently at Phe19 and Phe 20 [173], functioning as an alternative α-secretase.

BACE1 is localised on chromosome 11q23.3. So far, no mutations in the BACE1 gene have been associated with AD. BACE2 maps to the obligate Down's syndrome region of chromosome 21, which suggests that the third copy of BACE2 could contribute to the high incidence of amyloid plaques in Down's syndrome patients.

THE γ-SECRETASE COMPLEX

PS1 and PS2 are required for γ-secretase cleavage of APP and Notch processing, either through performing the cleavage itself or through functional interactions with the protein that does perform it [97]. The two aspartate residues Asp257 or Asp385 within transmembrane regions of PS1 are essential for the γ-secretase cleavage [169]. The protein, nicastrin, which binds to the presenilins and modulates APP and Notch cleavage, is likely

another functional component of the γ-secretase complex [178]. It is unclear whether the same γ-secretase generates both Aβ40 and Aβ42 or if different proteases are reponsible for the two cleavages.

An axonal membrane compartment containing both APP, BACE1 and PS1 was recently identified, suggesting that amyloidogenic processing of APP can occur during axonal transport [77]. A study of polarised sorting of BACE1 in Madin-Darby kidney canine cells indicates that although some BACE1 is targeted apically, substantial amounts of BACE1 are also targeted basolaterally, away from the majority of the APP molecules [23]. Decreasing cholesterol concentrations inhibits β- and γ-secretase processing of APP [41], suggesting that lipid membrane microdomains are important determinants for correct exposure of APP to secretases.

## Apoptosis in Alzheimer's disease

Apoptosis is a tightly regulated physiological mechanism crucial to maintaining a balance between cell death and cell division, especially during development (reviewed in [11]). It is defined by a combination of morphological and biochemical criteria (reviewed in [105]). Typically, the cell body shrinks, the plasma membrane blebs and the nuclear DNA condenses and fragments, while organelle integrity is preserved until late stages of the process. The apoptotic cell is ultimately phagocytosed, ensuring that release of intracellular material and secondary inflammation will not occur in the surrounding tissue. In contrast, cells undergoing necrosis in response to acute insults swell and lyse. During apoptosis, a group of cysteine aspartate-specific proteases (caspases) is specifically activated [119]. Caspases cleave a multitude of substrates directly and also modulate other cellular systems involved in the apoptotic process.

Apoptosis is potentially important for the initiation and progression of neuronal loss in AD [31]. A number of apoptotic markers have been found in AD postmortem tissue [11]. Various pathogenic AD mutations in the APP and presenilin genes have also been shown to increase cellular sensitivity to toxic insults [37,62,170,171,180]. Further, increased Aβ levels may damage synapses, neurites and mitochondria, and subsequently cause caspase activation, setting off an apoptotic cascade reaction. Apoptotic mechanisms implicated in

AD include increased oxidative stress, perturbed calcium homeostasis, mitochondrial dysfunction and caspase activation [11,107,108].

Both APP and the presenilins have been reported to be subject to processing by caspases [166]. Non-secretase processing of APP occurs at two caspase-3 consensus sites in the extracellular domain (DNVD$_{197}$-S and DYAD$_{219}$ –G) and at one caspase-3, -6 and -8 consensus site in the cytoplasmic tail (VEVD$_{720}$-A) [51,93,124]. It has been proposed that caspase cleavage of APP at the VEVD$_{720}$-A site would give rise to increased Aβ levels with a corresponding decrease in α-APP. Furthermore, the APPSwe mutation at the β-secretase cleavage site improves a caspase recognition sequence (VKMD$_{653}$-A → VNLD$_{653}$-A) [51]. It is possible that caspase cleavage shifts APP processing toward the amyloidogenic pathway, although some recent evidence indicate that amyloidogenesis is independent of caspase cleavage [147].

## Biochemical markers and treatment for AD

There is presently no biochemical marker in clinical use to singly confirm a diagnosis of AD. Measurements of tau in CSF are used to complement neuroimaging and neuropsychological assessment. Elevated tau levels in CSF does however overlap with other neurodegenerative disorders and to a certain extent, also with cognitively healthy controls [50,73]. Of the various APP metabolites in CSF investigated for diagnostical purposes, Aβ42 in CSF has repeatedly been shown to be decreased in AD patients [49]. Decreased α-sAPP in CSF was found both for FAD patients carrying the Swedish mutation [89] and for sporadic AD patients [139].

Current pharmacological therapies against neurodegenerative disorders are largely based on replacement or potentiation of depressed neurotransmitter systems. Standard AD treatment enhances cholinergic neurotransmission through cholinesterase inhibitors, but although anticholinesterase treatment slows the clinical progression, it has little effect on improving memory and cognition [14,46,101]. Non-cognitive symptoms of AD, such as behavioural changes, psychotic symptoms, sleep disorders and motor dysfunction are treated separately with antidepressants, neuroleptics, sedatives, anticonvulsants and antiparkinsonism drugs [162].

Several emerging therapeutic approaches targets the production, clearance and aggregation of the Aβ peptide. The identification of the two crucial enzymes for Aβ generation provide therapeutic targets for their inhibitions. Aβ burden in APP transgenic mouse brain has been shown to be decreased by treatment with the metal chelator clioquinol [28] or an antibody against Aβ [9,36]. Immunisation with Aβ42 reduces Aβ levels, inhibits amyloid deposition and clear sexisting plaques in a mouse model of AD amyloidosis [136]. Nonsteroidal anti-inflammatory drugs [165] and cholesterol-lowering drugs [41] are able to reduce Aβ levels *in vitro* and in animal experiments. Clinical trials will tell whether any of these approaches can be successful in battling AD.

# AIMS OF THE STUDY

The work presented in this thesis describes the differential cleavage and secretion of sAPP. The specific aims were:

❊ To make a β-sAPP specific antibody

❊ To determine the presence of β-sAPP in cerebrospinal fluid

❊ To determine the levels of differentially cleaved sAPP in cerebrospinal fluid from sporadic AD patients and compare them to those of healthy controls

❊ To determine the effects of apoptosis on APP processing in primary rat cortical cell cultures

❊ To determine the effects of the APPArc mutation on APP processing in apoptotic human neuroblastoma cells.

❊ To determine the localisation and distribution of β-sAPP in AD brain and compare it to control brain.

# MATERIALS AND METHODS

The twenty-six subjects included in paper I were recruited through Huddinge University Hospital (see Table 1 for demographic data). All the AD patients (n = 13) in the study were sporadic, as AD patients with a family history of AD were excluded. The AD patients were diagnosed according to the NINCDS-ADRDA criteria. The healthy control subjects were not patients of the outpatient clinic at Huddinge Hospital, but were instead recruited from the Swedish Pensioner Society (n = 11) and among relatives and spouses of patients with dementia (n = 2). The degree of cognitive impairment for both AD patients and controls was assessed with MMSE. CSF was obtained by lumbar puncture in the forenoon with the patient sitting in an upright position. Of the CSF samples, 25 µl were taken, diluted 1:1 with Laemmli buffer and heated to 85°C for 5 min.

|  | *AD patients* | *Controls* |
|---|---|---|
| Number of subjects | 13 | 13 |
| Mean age | 64 ± 11 | 70 ± 8 |
| Gender | 7M / 6F | 5M / 8F |
| MMSE | 19 ± 6 | 29 ± 1 |

*Table 1. Clinical data for AD patients and controls in paper I.*

For paper IV, post-mortem brain tissue from sporadic AD patients and controls was obtained from available material in the Huddinge Brain Bank, Sweden. We selected nine individuals: three with neuropathological diagnoses of severe AD, three with mild AD and three controls. The mean age did not differ significantly between the groups. The AD patients fulfilled the clinical NINCDS criteria for probable and possible AD respectively, and the CERAD criteria for definitive and possible AD. The controls consisted of patients without a history of dementia or other neurological/psychiatric diseases.

| Subject status | Age | Gender |
| --- | --- | --- |
| Control | 64 | F |
| Control | 84 | F |
| Control | 86 | F |
| Mild AD | 85 | M |
| Mild AD | 85 | F |
| Mild AD | 85 | M |
| Severe AD | 65 | F |
| Severe AD | 90 | F |
| Severe AD | 83 | F |

*Table 2. Clinical data for patients and controls in paper IV.*

ANTIBODIES

The polyclonal rabbit antibody β939 was raised against the amino acid sequence immediately preceding the β-secretase cleavage site in APP, SEVKM. The antibody was first affinity purified against G-protein, then against the same peptide SEVKM and used at a concentration of 3 µg/ml. For paper I, the monoclonal antibody 22C11 (Boehringer) was used at a dilution of 1:1000 to detect total sAPP in CSF and the monoclonal antibody 6E10 (Senetec) was used at a concentration of 0.57 µg/ml to detect α-sAPP in CSF. The polyclonal rabbit antibody AP-180 was raised against amino acids 46-61 in APP, purified by the same methods as β939, and used at a concentration of 10 µg/ml to detect total sAPP in cell media (paper II and III). Secondary antibodies for Western blotting consisted of horse-radish peroxidase (HRP) conjugated anti-mouse antibody (Amersham) and anti-rabbit antibody (Sigma) diluted 1:5000 and 1:50,000 respectively. For Western blotting, all antibodies were diluted in 2.5% non-fat dry milk in Tris-buffered saline TBS (20 mM Tris, 0.5 M NaCl, pH 7.4). When used for immunohistochemical staining β939 antibody was diluted to 0.5 µg/ml in TBS, with a secondary biotinylated anti-rabbit antibody (DAKO) diluted to 5 µg/ml.

ANTIBODY CHARACTERISATION OF β939

A peptide ELISA was performed using the synthetic peptides CSEVKM and ISEVKMDAEFRH. The peptides were dissolved in sodium carbonate coating buffer pH 9.0 with concentrations ranging from 0 to 50 µg/ml. The ELISA plate was coated with 100

μl of peptide solution per well and incubated at 4°C overnight. The plate was then washed three times in TBS containing 0.05% Tween (TBS-T) and remaining binding sites were blocked with 5% milk in TBS for 30 min. The β939 antibody was added at a concentration of 10 μg/ml and the plate was incubated for 3h. After washing, HRP-conjugated anti-rabbit antibody was added for 1 h. The plate was washed again and 100 μl of *o*-phenylendiamine peroxide substrate per well was added. The reactions were stopped after 30 min, by addition of 100 μl of 2 M $H_2SO_4$ per well and the plate was analysed at $\lambda = 450$ nm in an ELISA spectrophotometer (Emax).

For immunoprecipitation of α-sAPP from CSF, 25 μl of CSF was diluted in 125 μl of TBS. The sample was pre-incubated with 25 μl of anti-mouse antibody coupled to agarose beads for 30 min, centrifuged and the supernatant collected. The 6E10 antibody (3.4 μg) was added incubated for 1 h on ice. Another 25 μl of anti-mouse antibody agarose was added and the sample was rotated at 4°C overnight. Following centrifugation, the supernatant was collected and mixed with an equal volume of 2 x Laemmli buffer. The precipitate was washed with TBS and twice with TBS-T, then dissolved in 60 μl of Laemmli buffer. Both the precipitate and the supernatant samples were heated to 85°C and analysed by Western blotting.

PREPARATION AND TREATMENT OF PRIMARY RAT CORTICAL CULTURES

Primary cortical cultures were prepared from the cortex of E15 foetal Sprague-Dawley rats according to [94]. Following dissection and dissociation by mechanical trituration, the cells were seeded on poly-L-lysine coated dishes, 12 well plates or glass coverslips at a density of 1,000,000 cells/60 mm dish, 400,000 cells/10mm well in the 12 well plates and 400,000 cells/10mm coverslip, respectively. The cells were kept in DMEM/HAM F12 (ratio 1:1) medium supplemented with 10% v/v FBS, 2 mM L-glutamine and 0.5% v/v penicillin-streptomycin for 5-6 days. Cells from each preparation were exposed to 0, 1, 2, or 4 μM of calcium ionophore A23187 for 30 min in Locke-5 buffer (2.3 mM $CaCl_2$, 5 mM KCl, 154 NaCl, 4 mM $NaHCO_3$, 5 mM HEPES, 5.0 mM glucose) pH 7.5, and then incubated in 2% FBS DMEM/F12 medium for 0, 3, 6, or 24 h; or else exposed to 0, 0.5, 1 or 2 μM colchicine in 2% FBS DMEM/F12 medium for 6, 24 or 48 h. For some experiments the general caspase inhibitor Z-VAD-FMK (25 μM) was added to the medium 30 min before exposure to either A23187 or colchicine and remained present during the entire incubation. Media and lysates from the cells were collected and the protein content assayed using

24

Pierce's bicinchoninic protein assay. Equal amounts of protein from the samples were diluted 1:1 with 2 x Laemmli sample buffer and heated to 85°C for 5 min.

TRANSFECTION AND TREATMENT OF HUMAN NEUROBLASTOMA CELLS

The Arctic APP mutation (APParc) was introduced into APP695 cDNA in the pcDNA3 vector using QuikChange Site-Directed Mutagenesis Kit, according to the manufacturer's instructions. SH-SY5Y cells were stably transfected with either APParc or wild-type APP (APPwt). Three cell lines of each were selected for the experiments and untransfected SH-SY5Y cells were used as baseline. APP expression was monitored by Western blotting throughout the course of the experiments. The SH-SY5Y cells were grown to 80-90% confluency on 100 mm plastic dishes, 12 well plates or 13mm glass coverslips in OptiMEM medium. The medium was supplemented with 5% v/v foetal bovine serum (FBS), 2 mM L-glutamine and, for transfected cells, 300 μM G418. The cells were exposed to either 0, 1, 2 or 5 μM of A23187 for 30 min in Locke-5 buffer, and then incubated in 2% FBS OptiMEM medium for 0, 3, 6 or 24 h; or to 0, 0.25, 0.5 or 1 μM colchicine in 2% FBS OptiMEM medium for 0.5, 3, 6 or 24 h; or to 0, 50, 100 or 200 μM $H_2O_2$ in 2% FBS OptiMEM for 0.5, 3, 6 or 24 h. Media and lysates from the cells were collected and the protein content assayed using Pierce's bicinchoninic protein assay. Equal amounts of protein from the samples were diluted 1:1 with 2 x Laemmli sample buffer and heated to 85°C for 5 min.

MTT ASSAY

Cell viability was measured by the colorimetric MTT tetrazolium salt assay [99,115]. A23187, hydrogen peroxide and colchicine do not reduce MTT directly. MTT tetrazolium salt was dissolved in serum-free culture medium to a final concentration of 0.3 mg/ml and added to the cells for 1 h at 37°C. The medium was then removed, isopropanol added and the absorbance detected at $\lambda$ = 592 nm in a spectrophotometer. The optical density values of the treated cells were normalized against the untreated controls.

ANNEXIN V AND PROPIDIUM IODIDE STAINING OF INTACT CELLS

Annexin V (green fluorescence) binds to phosphatidyl serine, which protrudes from the plasma membranes of apoptotic cells [135]. Propidium iodide (red fluorescence) is a cell impermeable chromatin dye, staining only necrotic cells, whose membrane integrity has been disrupted. With double staining, it is therefore possible to differentiate apoptotic and

necrotic cells. Cells grown on coverslips were washed three times with HEPES buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$), pH 7.4, stained with 50 µg/ml annexin V and 50 µg/ml PI in HEPES buffer for 10 min and washed three times again in HEPES buffer. The coverslips were mounted onto glass slides and examined in a Nikon fluorescence microscope and apoptotic and necrotic cells were scored.

PROPIDIUM IODIDE STAINING OF FIXED CELLS

Propidium iodide is also commonly used to visualise condensed DNA in fixed cells, as a way of quantifying apoptosis. Cells grown on coverslips were washed three times in phosphate buffered saline (PBS), pH 7.4, then stained and fixed with 2 µg/ml propidium iodide in methanol for 10 min and washed three times again in PBS. The coverslips were mounted onto glass slides and examined in a Nikon fluorescence microscope and apoptotic cells were scored.

WESTERN BLOTTING

The samples were separated on 8% sodium dodecyl sulphate polyacrylamide gels and the proteins transferred onto polyvinylidene difluoride membranes. Blotted membranes were blocked with a 5% w/w non-fat dry milk-TBS solution for 30 min, then stained overnight with the appropriate primary antibody. The membranes were washed 3 x 20 minutes in TBS-T, then incubated with HRP-coupled secondary antibody. After washing for 5 x 20 minutes in TBS-T, immunoreactivebands were visualised using an enhanced chemiluminiscence (ECL) detection system. The amount of APPs were quantitated from the films by densitometric evaluation, using ImageMaster1D (Pharmacia Biotech, Sweden), and expressed as OD units x $mm^2$.

BRAIN TISSUE PROCESSING  AND IMMUNOHISTOCHEMISTRY

Briefly,  4% formaldehyde-fixed tissue blocks were taken from frontal associative cortex (BA 9), medial temporal gyrus (BA 21) and primary occipital cortex (BA 17), embedded in paraffin and cut with a microtome to sections of 6 µm thickness and mounted onto coated slides (PlusGlas, SuperFrost). Following deparaffinization, masked epitopes were exposed by microwave treatment for 10 min in citrate buffer, pH 6.0. The sections were washed in TBS and incubated in DAKO protein block solution for 1 h, then incubated with the β939 antibody at a concentration of 0.5 µg/ml in TBS overnight. The sections were washed in TBS for 10 min and incubated in biotinylated anti-rabbit antibody at a concentration of 5

μg/ml in TBS for 30 min, washed in TBS for 10 min. Bound antibody was detected using the DAKO catalysed signal amplification system with diaminobenzidine as chromogen. Some of the sections were counterstained with either alkaline Congo Red or with hematoxyline. The Congo Red staining, which is used to detect the presence of amyloid, was visualised using a Nikon polarizing filter set and fluorescence microscopy. For control staining, the primary antibody was omitted.

STATISTICS

In paper I, each CSF sample was run in triplicate for each antibody, with one sample as internal control. The sAPP was quantitated from the films by densitometric evaluation. The median values were calculated and the group of values for the AD patients was compared to the group of values for the healthy controls, using the non-parametric Mann-Whitney test.

In paper II, the MTT assays were performed in triplicate for each sample. To evaluate apoptotic and/or necrotic cells, more than 400 cells from each sample were scored. On the Western blots, the samples were run in duplicates and normalized against the controls. All experiments were repeated on three different cell preparations, after which average values and standard error of the means were calculated. The MTT assay and annexin/PI staining data were analysed using a paired two-tailed t-test. For the Western blot data, group comparisons were made with the Kruskal-Wallis method. Subsequent post-hoc tests were performed using the Mann-Whitney test

In paper III, the MTT assays and scoring of apoptotic cells were performed as in paper II. The media samples were run in triplicate on Western blots. Treated samples were normalized against controls on the same blots. All data were presented as normalized average values $\pm$ SEM. Statistical analysis was carried out using the Kruskal-Wallis test, with the Mann-Whitney test as post-hoc test.

The statistical significance level was set at $p < 0.05$. All statistical analyses were conducted using StatView 4.51 (Abacus concepts, Berkeley, CA).

# RESULTS AND DISCUSSION

ANTIBODY CHARACTERISATION

In paper I, the rabbit polyclonal β939 antibody against β-sAPP was produced, purified and characterised. The β939 antibody's end-specificity was verified by ELISA, its non-cross-reactivity with α-APP with immunoprecipitation, its APP specificity by comparison with 22C11 and its overall specificity with recombinant proteins on Western blot, both alpha and beta-cleaved APP. The peptide ELISA showed that β939 bound to the sequence SEVKM only when the C-terminal methionine was free, confirming that only β-cleaved APP could be detected by this antibody (Fig 4a). Immunoprecipitation of sAPP from CSF using the α-sAPP specific antibody 6E10 resulted in a precipitate containing only α-sAPP and a supernatant depleted of α-sAPP, but containing β-sAPP. Both the precipitate and the supernatant were analyzed by Western blot. As expected, the 6E10 antibody stained only the precipitate (Fig 4b). The β939 antibody stained only the supernatant, proving the presence of β-sAPP in the CSF and the antibody's ability to detect it (Fig 4c). Since the β939 antibody did not stain the precipitate, the possibility of cross-reactions with α-sAPP could be ruled out.
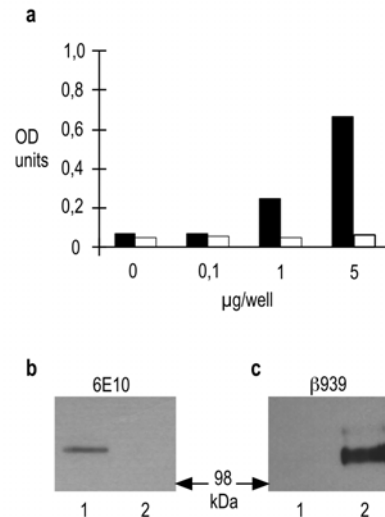


*Fig.4. Characterisation of the β-sAPP antibody β939. (a) Direct peptide ELISA with β939. Plates were coated with either the peptide CSEVKM ending at the β-secretase cleavage site (filled bars) or a peptide overlapping the cleavage site ISEVKMDAEFR (empty bars).(b,c) Immunoprecipitation of α-sAPP from CSF, using 6E10 antibody. The precipitate, lane 1, and supernatant, lane 2, were analysed by Western blot and stained with (b) 6E10 and (c) β939.*

Seeing that gray matter was extensively stained in paper IV, we performed control staining without primary antibody and without amplification system, respectively, and found that the staining, although intense, was specific.

For papers II and III, the AP-180 antibody was used to detect total sAPP in cell media. The rabbit polyclonal AP-180 antibody has been shown to stain APP on Western blots similarly to the commercial N-terminal APP-antibody 22C11 (data not shown).

sAPP IN CSF

The cerebrospinal fluid of thirteen patients with sporadic AD and thirteen healthy controls (Table 1), was analyzed on Western blot for comparisons of differential sAPP content in paper I. Using the β939 antibody, substantial amounts of β-sAPP were shown to be present in the CSF of AD patients and controls alike. Although comparison of the median values of β-sAPP yielded no statistically significant difference between AD patients and controls, the median values of the α-sAPP and total sAPP were found to be significantly lower for AD patients than for controls, $p = 0.017$ and $p = 0.0038$ respectively (Fig. 5). This resulted in a relative increase of β-sAPP in CSF from AD cases. Neither the MMSE scores or the age of the subjects correlated with any of the levels of the sAPPs.
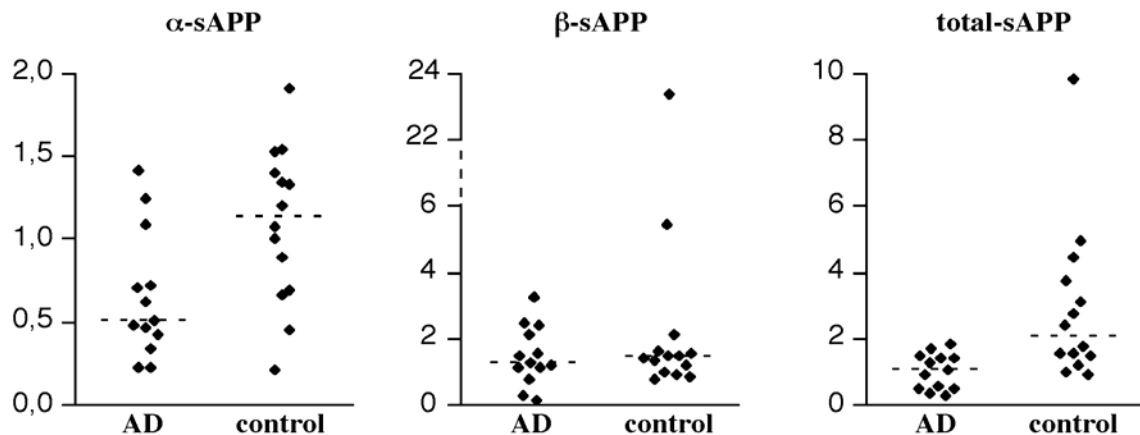


Fig.5. Median scores of α-sAPP, β-sAPP and total sAPP in CSF (OD units x mm$^2$) for AD cases and controls. Median value for each group is indicated by dotted line.

Decreased α-sAPP levels in CSF have previously been found in association with the APPSwe FAD mutation, reflecting increased Aβ production [89]. Our results indicate that the shift towards amyloidogenic pathways in FAD is a factor also in sporadic AD. Total sAPP, as well as different isoforms of APP, have been reported to be significantly lower in

the CSF of AD patients. This decrease has been attributed to increased proteolysis of APP, loss of functional neurons or dilution as the CSF volume increases during AD. However, unchanged or increased levels of total sAPP have also been reported. In one recent study, analysis of sAPP in CSF from rats showed decreases in α-sAPP and total sAPP in CSF with advancing age, although Aβ levels did not change [4]. The α-sAPP levels were found to have a positive correlation to spatial memory task performance. Decreased α-sAPP levels in carriers of the APPSwe FAD mutations also correlates with declining cognitive function [2]. We did not detect any correlation between sAPP levels and MMSE scores, possibly due to the limited number of subjects included in our study.

It is unclear whether the promoted neurodegeneration is due to a decreased neuroprotective effect of α-sAPP or a relative increase in β-sAPP, and subsequently Aβ. Another possible effector molecule is C99, the C-terminal fragment originating from the β-secretase cleavage. C99 has been implicated in cellular signalling and may be a mediator of apoptosis [127].

INDUCTION OF APOPTOSIS

In paper II and III, the β-sAPP secretion from apoptotic primary neurons or APParc/APPwt transfected SH-SY5Y cells was studied, using different apoptosis induction systems. The primary neurons were treated with either the $Ca^{2+}$ ionophore A23187 or colchicine. A23187 elevates intracellular $Ca^{2+}$ by allowing extracellular $Ca^{2+}$ to cross the plasma membrane and by causing release of $Ca^{2+}$ from intracellular stores. Destabilisation of $Ca^{2+}$ homeostasis is believed to be a major factor in neuronal apoptosis and degeneration [106]. Colchicine causes apoptosis by binding to and disrupting cellular microtubules. It has been shown that colchicine induces tau modifications and compromises cellular transport, particularly through the secretory pathway, which results in loss of delivery and secretion polarity [32]. The APP transfected SH-SY5Y cells were also treated with the oxidative stress inducer hydrogen peroxide, which has been demonstrated to trigger apoptosis and neurodegeneration.

The concentrations of A23187 and colchicine used in paper II and III was optimised for different purposes. In paper II, we chose the lowest concentration that reduced cell viability in primary cortical cultures by 20-40% over a time period of at least 6 h. In paper III, the objective was to find the concentration at which the cell viability of the APParc and APPwt

cells differed signfcantly. There are also inherent differences of the cells; the overexpression of APP in the neuroblastoma cells may affect their viability negatively, while the glial feeder layer of the primary cultures enhances survival. The concentration of Aβ in media from the primary cultures was below detection level.

PRIMARY RAT CORTICAL CULTURES: ASSESSMENT OF APOPTOSIS

Following treatment, the cell viability was assessed with the MTT assay. The MTT experiments showed that cell viability decreased significantly with increasing incubation times and concentrations of either A23187 or colchicine. The difference in temporal effects of A23187 and colchicine is based on the different mechanisms of the two agents. Cell death was induced by A23187 by a 30 min exposure. At the 24 h time point, the cell viability was approximately 50% and it is reasonable to conclude that early apoptotic changes had already exerted their influence on protein processing. Due to slow kinetics [5], cell viability was not impaired by colchicine at 6h, but at 24 h cell viability was significantly decreased. At the 48 h time point cell viability was decreased below 50%, implying cytoskeletal breakdown in a majority of the cells. Addition of the general caspase inhibitor Z-VAD-FMK, completely inhibited the decrease in viability for cells exposed to A23187 and partially so for cells exposed to colchicine after 24 h. Caspase inhibition did not have any effect on the viability of colchicine treated cells after 48 h.

To differentiate between apoptotic and necrotic cell death, the exposed cells were stained directly with annexin V and propidium iodide. The number of apoptotic cells was found to be significantly increased in the cell cultures exposed to 2 μM A23187 or 1 μM colchicine. The addition of  Z-VAD-FMK significantly reduced the number of apoptotic cells in the cell cultures exposed to A23187, but in those exposed to colchicine. After 48 h, the cells exposed to colchicine had also begun to die by secondary necrosis. At all earlier time points, the ratio of necrotic cells was less than 3%. This confirms that both A23187 and colchicine specifically induce apoptosis in primary rat cortical cultures, at concentrations of 2 μM and 1 μM, respectively, and that the appropriate time span for studying the cells lies within 6 and 24 hours.

The secretion of sAPP into the media was assayed by Western blotting. We found that cells exposed to 2 μM A23187 and incubated for 6 or 24 h (Fig 6a), secreted significantly less β-sAPP, $p = 0.0039$ and $p = 0.0065$, respectively. When a lower concentration of A23187, 1 μM, was used, the effect was less pronounced but still perceivable. The total sAPP levels were not affected by A23187 exposure (Fig 6b), indicating unimpeded protein transport. The addition of the caspase inhibitor Z-VAD-FMK inhibited the decrease in β-sAPP secretion, which shows that the levels of β-sAPP can be regulated by caspase actions.
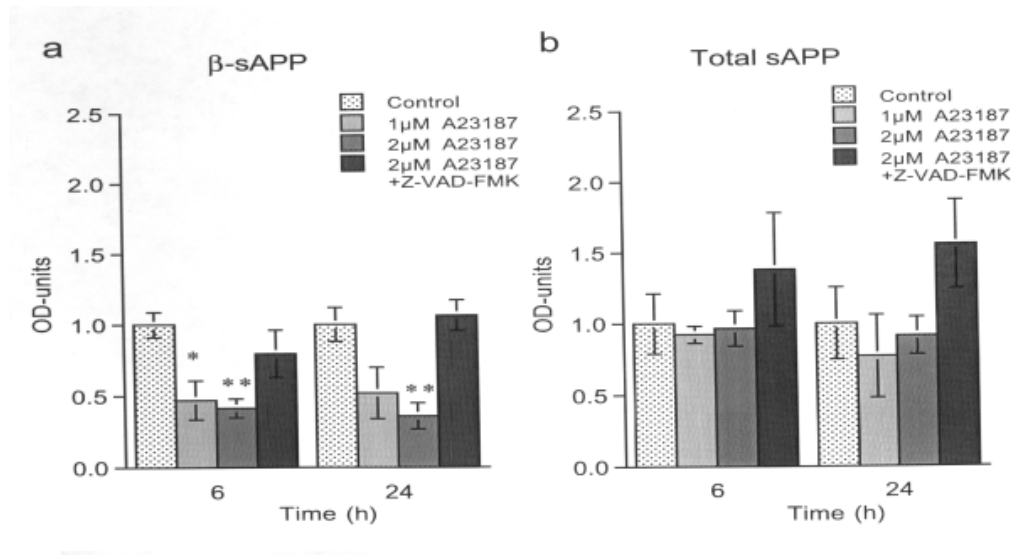


Fig.6. Levels of a) β-sAPP and b) total sAPP secreted from primary rat cortical cultures at 6 and 24 h after a 30 min exposure to 0, 1 or 2 μM A23187. * = p < 0.05 and ** = p < 0.01 in comparison to control levels.

The specific shift in APP processing after A23187 treatment levels indicates a decrease in the proteolytic generation of β-sAPP by BACE1, rather than a reduced availability of the precursor. Since BACE1 activity is regulated by intracellular calcium levels through furin cleavage of its propeptide in the ER [13], a reduced amount of BACE1 might account for the decrease in β-sAPP. However, the reduction in active BACE1 alone cannot account for the decrease in β-sAPP, at least not after an incubation time of only 6 h, as the half-life of BACE1 is approximately 16 h. Also, caspase inhibition restored both cell viability and the secreted β-sAPP to control levels. This indicates that the decrease in β-sAPP is connected to apoptotic events rather than to decreased amounts of BACE1, unless BACE1 itself is cleaved by caspases.

The decrease in secreted β-sAPP cells may seem counterintuitive, since increased Aβ formation has been linked to apoptosis as well as with AD pathogenesis [50, 91,179]. One possibility is that generation of the neuroprotective α-sAPP is upregulated in response to the perceived excitotoxic insult, at the expense of the β-secretase cleavage. Alternatively, α-sAPP generation may be stimulated by A23187, especially since there are multiple α-secretases, which can be selectively activated. A23187 have been shown to stimulate total sAPP secretion in several different cell types [60,76,100,113], but the evidence is conflicting, as it has also been shown to inhibit APP release [86,126]. Furthermore, there are a number of potential caspase cleavage sites located within the sequence of APP [51,93,124]. Caspase cleavage of APP may render it less susceptible to β-secretase cleavage, perhaps by altering APP trafficking or by inducing a shift in BACE1 cleavage from the β-secretase site to the other BACE1 cleavage site after residue 11 in APP.No significant differences in secreted β-sAPP (Fig 7a) or total sAPP (Fig 7b) were found between cells exposed to colchicine and controls at either time point or concentration, nor did caspase inhibition seem to affect the outcome. The large variations in sAPP between colchicine treated samples may be due to the breakdown in cellular transport system caused by the colchicine [92]. This indicates that in spite of the colchicine exposure, sAPP was delivered to the plasma membrane and secreted, possibly through the endosomal-lysosomal pathway or by diffuse transport throughout the cell.
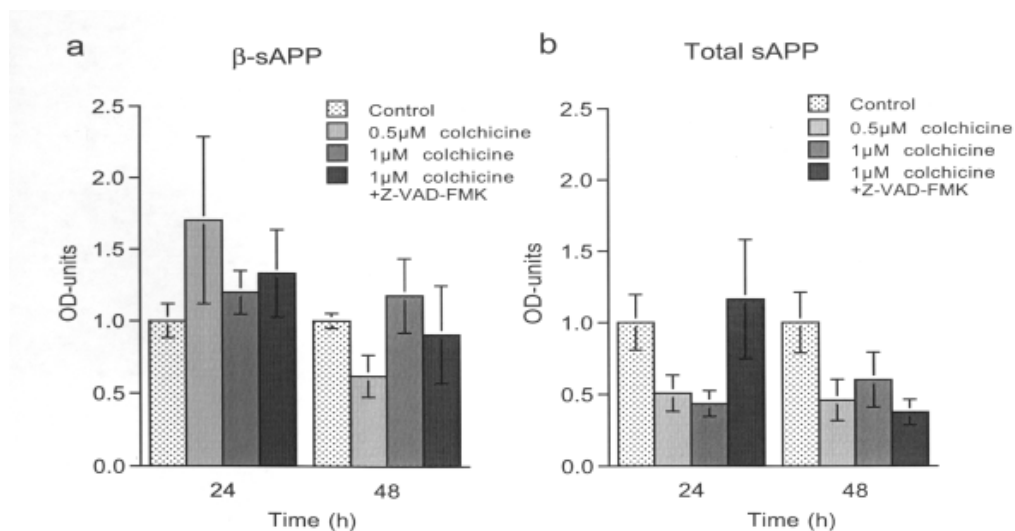


Fig.7. Levels of a) β-sAPP and b) total sAPP secreted from primary rat cortical cultures after exposure to 0, 0.5 and 1 µM colchicine for 24 and 48 h.

Exposure of human neurons to colchicine has been reported to decrease the secretion of both sAPP and Aβ but not to interfere with APP metabolism [92,114]. This would suggest that colchicine does not affect biosynthetic processing or enzymatic cleavage directly, only vesicular transport. In our study, the addition of caspase inhibitor did not affect the APP processing in colchicine exposed cells and only partially improved cell viability. The apoptosis induced by colchicine might be an event largely independent of caspase actions.

APPARC/APPWT TRANSFECTED SH-SY5Y CELLS: ASSESSMENT OF APOPTOSIS

The MTT assay was used to analyse the respective cell viability of APParc, APPwt and untransfected SH-SY5Y cells, following exposure to different concentrations of A23187, colchicine and $H_2O_2$. Time points were chosen for appropriate decrease in cell viability (data not shown). For each of the agents, a concentration at which APParc cells showed a statistically significant decrease in cell viability compared to APPwt transfected cells was found at 2 µM A23187, 0.5 µM colchicine and 100 µM $H_2O_2$. These concentrations were selected for further experiments. No significant differences in cell viability were found between untransfected and APPwt transfected cells.

Annexin V and PI staining in paper II was performed on living cells with intact membrane integrity. Due to the large number of cell lines in paper III, staining of living cells was not practically feasible. We opted for a fixation/staining method with a non-expensive dye, which would allow us to analyse large numbers of cells at a time. Using propidium iodide staining to detect chromatin condensation in apoptotic cells, we found that untreated APParc cells contained a significantly higher percentage of apoptotic cells than untreated APPwt cells. No statistically significant differences were found between control APPwt and untransfected cells. APParc cells exposed to A23187, colchicine or $H_2O_2$ contained a significantly larger percentage of apoptotic cells than corresponding APPwt transfected cells. No significant difference was found between APPwt transfected and untransfected cells, except for APPwt cells exposed to $H_2O_2$, which contained a larger percentage of apoptotic cells than untransfected cells. The statistical calculations of apoptotic cell percentages in treated cells were all carried out with appropriate corrections for baseline values.

Our results are concordant with a number of reports of enhanced sensitivity to apoptosis being linked to the APP Swedish double mutation (APPSwe), KM 670/671NL, which leads

to increased β-secretase cleavage [37], and to several APP717 and presenilin mutations, which affect the γ-secretase processing [62,170,171,180]. Basal enhanced sensitivity to apoptosis has been demonstrated for some of these mutations, but not all [62,180]. Since the APPSwe, the APP717 and the presenilin mutations all increase production and secretion of Aβ, the enhanced sensitivity to apoptosis in mutant cells could be due to increased Aβ exposure. The Arctic mutation, however, mainly serves to increase formation and stability of protofibrils and the enhanced sensitivity to toxic insults in APParc cells likely derives from Aβ protofibril accumulation.

APPARC/APPWT TRANSFECTED SH-SY5Y CELLS: α- AND β-sAPP SECRETION DURING APOPTOSIS

During apoptosis induced by any of the agents used in paper III, we found a significant decrease of β-sAPP secreted by APParc cells, but not in the APPwt cells (Fig. 8). The decrease was significant both in respect to the untreated APParc cells and to the APPwt cells. No significant decreases between APParc and APPwt cells were found for the α-sAPP, but the decrease for α-sAPP secretion by peroxide treated APPwt cells was significant compared to untreated APPwt cells.
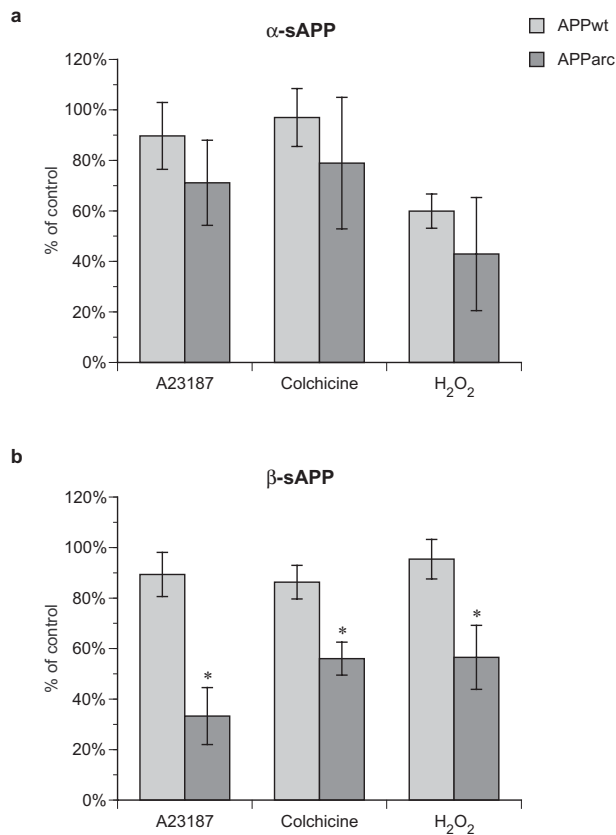
*Fig 8. Levels of a) α-sAPP and b) β-sAPP secreted from APPwt and APParc transfected SH-SY5Y cells exposed to 2 μM A23187, 0.5 μM colchicine or 100 μM $H_2O_2$. On the Western blots, the samples were run in triplicates and normalized against controls on the same blots. Data are presented as normalised average values ± SEM. \* = p < 0.05*

Again, the specific shift in APP processing after A23187 treatment indicates a decrease in the proteolytic generation of β-sAPP by BACE1. The decrease seems to be associated to the enhanced sensitivity to toxic stress in APParc cells. We hypothesise that the β-sAPP secretion decreases once an apoptotic threshold is reached. Vulnerable cells such as APParc cells and primary cortical cultures would reach their apoptotic threshold earlier than do APPwt and untransfected SH-SY5Y cells.

Apoptosis in APParc cells may render APP less susceptible to β-secretase cleavage, perhaps by altering trafficking or by inducing a shift in BACE1 cleavage from the β-secretase site to the other BACE1 cleavage site after residue 11 in APP. Alternatively, other species of N-terminally truncated Aβ peptides may be generated in the APParc cells during apoptosis, resulting in an apparent decrease in β-sAPP. Since the β939 antibody is specific for the β-secretase cleavage site, any shift in cleavage site preference would be interpreted as a decrease of β-sAPP in our detection system. Additional aspects of β-sAPP production or secretion may also be compromised during apoptosis.

DETECTION OF β-sAPP IMMUNOSTAINING IN BRAIN

In paper IV, the localisation and distribution of β-sAPP in brain was investigated. Both control and AD brains showed extensive granular β-sAPP immunostaining throughout the gray matter in frontal, temporal and occipital lobe (Fig 9a and 9b). The staining patterns were similar for all the investigated regions, although the staining intensity was lower in the occipital lobe. Granular white matter immunostaining was also found in all three regions (Fig 9a and 9b). The staining patterns of control and AD brain were similar, but the intensity differed, due to the additional pathological features in AD brain; the increase in astrocyte density and/or the larger amount of core and non-core neuritic plaques and amyloid-bearing vessels, all of which were found to be associated with β-sAPP. β-sAPP staining was also found surrounding cerebral blood vessels in gray and white matter in both AD and control brain.

β-sAPP IMMUNOSTAINING: GRAY MATTER

The main finding within gray matter was the immunostaining of astrocyte-like formations, characterized by distinctive ramification around a centrally placed cell soma. Counterstaining with hematoxyline confirmed the cell bodies to be of typical astrocyte origin (Fig 9c and 9d). While staining intensity varied among the astrocytes, it was most prominent in the subpial glial compartment of layer I and in the gray matter throughout layers II–IV. For the AD cases, the staining in the layers V-VI was notably more intense than in the controls, particularly on the border of gray and white matter (Fig 9a). The strong staining of the upper layers suggests β-sAPP involvement in neuronal plasticity. The high levels of β-sAPP in deep cortical layers correlates well with the reported expression pattern of BACE1 in the mouse brain [130]. The major source of the β-sAPP is likely neuronal, since white matter staining was much less extensive.

Both controls and AD cases showed strong and discrete staining patterns of β-sAPP in white matter, often with a single or an assembly of glial cells at the core (Fig 9e and 9f). White matter β-sAPP staining in AD brain was generally stronger than in controls, possibly due to influence of the vascular pathology and unspecific microgliosis in the white matter.

We found a granular linear staining pattern approximate to an axon-like distribution in both control and AD brain. Hematoxyline counterstaining revealed the linearly distributed granules to be intraaxonal, indicating presence and transport of β-sAPP in the axon. For these structures, staining was strongest in controls, as granules there were numerous and regularly distributed. In AD brains, the staining was often patchy and the distribution of granules was irregular (Fig 9e). Overall, axonal structures in AD brains contained a reduced amount of immunopositive granules. This reduction of β-sAPP may reflect disturbed axonal transport or processing of β-sAPP in the remaining axons. Loss of axonal network or neurodegenerative changes in the soma may have a further negative influence on axonal β-sAPP transport.

It has been reported that APP functions as a membrane receptor protein, linking kinesin-1 to vesicles moved by fast anterograde axonal transport [78]. An axonal membrane compartment containing both APP, β-secretase and presenilin-1 was recently identified, suggesting that amyloidogenic processing of APP can occur during axonal transport [77]. Overexpression of APPL causes axonal vesicle accumulation, which leads to neuronal apoptosis [61] and AD pathogenic mutations amplify the pro-apoptotic effect even more. Perineuronal staining could be seen in both controls and AD brain. In AD brain, there was also occasionally intraneuronal staining (Fig 9g).
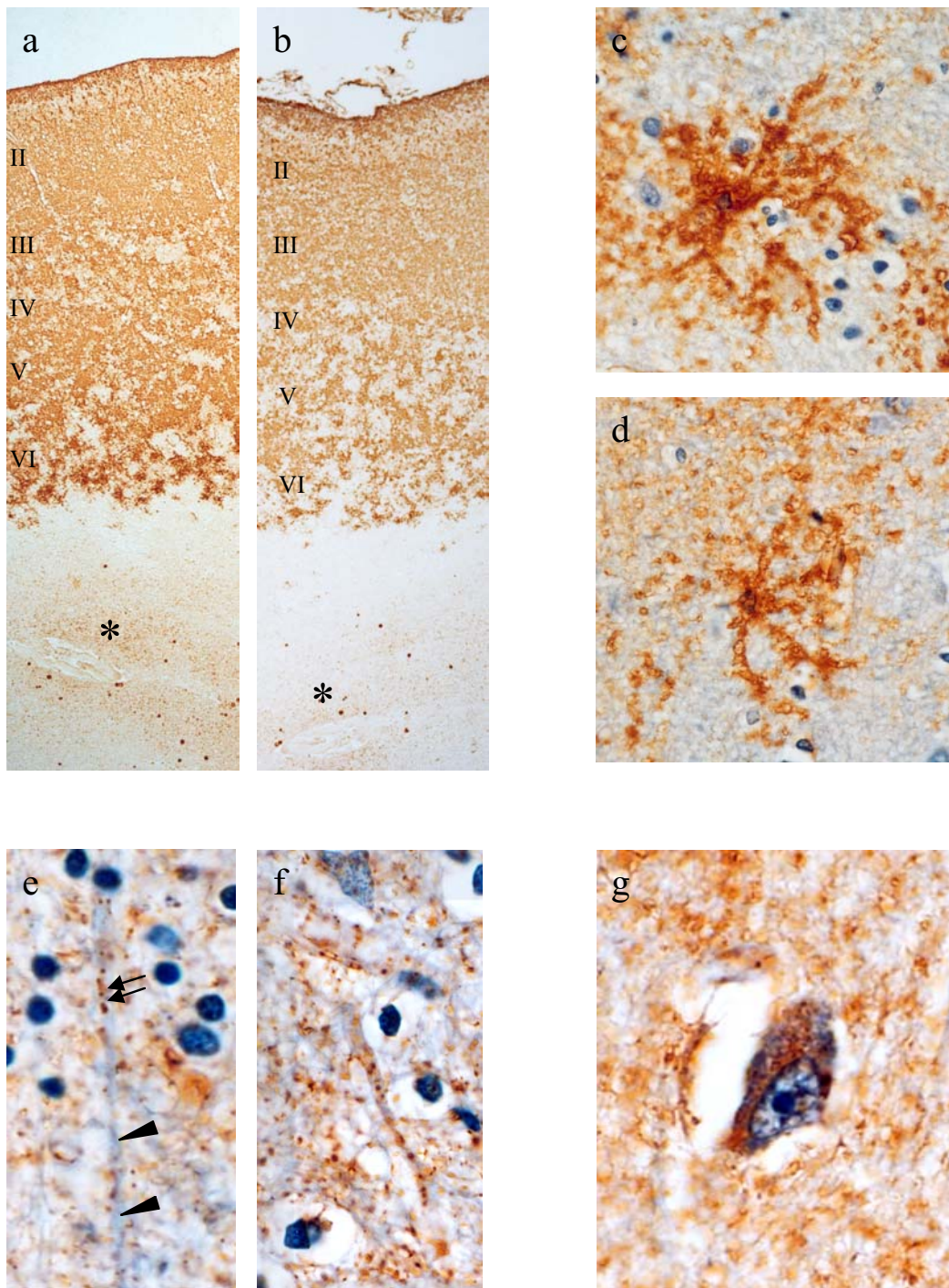
Fig.9 β-sAPP staining in a) AD and b) control brain. Gray matter stained strongly for β-sAPP. In AD brain most cortical layers were evenly stained. There was also characteristically more intensive staining at the border between gray and white matter in AD brain. In control brain, the most intense staining was found in superficial layers. Astrocyte-like cells stained strongly for β-sAPP in both c) AD and d) control brain. Also white matter stained strongly for β-sAPP in both e) AD and f) control brain. Granular staining in the axon-like structures indicates axonal transport of the β-sAPP. In AD brain, e) axon-like structures may be empty (arrowheads) or the intraaxonal immunostaining of β-sAPP (arrows) may be patchy and uneven (arrows). In controls, f) the white matter displays evenly distributed immunostaining. g) β-sAPP positive staining in one neuronal cell in the AD brain. Magnification: a) and b) were taken with a 4x objective, c)-g) with a 100x objective immersed in oil. c)-g) were also counterstained with hematoxyline. Roman numbers indicates cortical layers. * indicates perivascular space in white matter.

Pathological hallmarks of AD such as amyloid deposits and tangles could not be visualised and distinguished in brain based on β-sAPP immunostaining alone. When counterstaining with Congo Red, accentuated β-sAPP staining could be visualised around amyloid plaque cores in the outer plaque ring (Fig 10a) and paralleling Congo-positive fibrillary plaque formation in non-core plaques (Fig 10b). β-sAPP staining was also detected in the vicinity of Congo-positive subarachnoidal and penetrating blood vessels (Fig 10c) as well as extracellular tangle formation (Fig 10d). In none of these instances did the β-sAPP staining directly overlap with the Congo-positive structures.
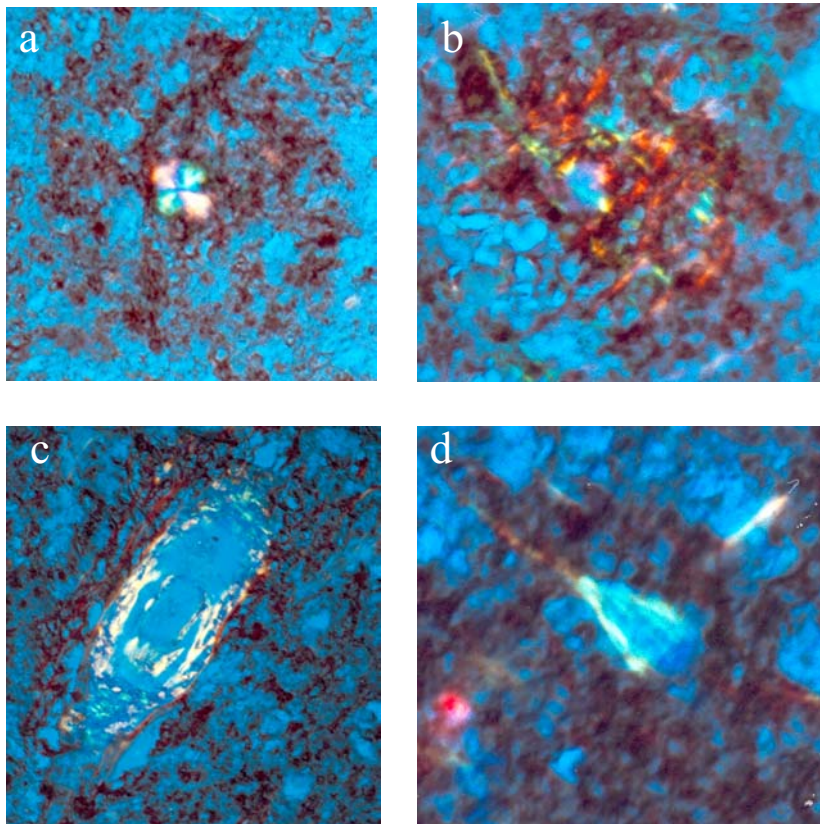


*Fig 10. β-sAPP staining around Congo-positive structures in AD frontal cortex. a) Characteristic senile plaque with congophilic core. β-sAPP immunostaining surrounds the core. b) β-APP immunostaining in the core-less, neuritic-like plaque. c) β-sAPP immunostaining around congophilic vessels. d) β-sAPP immunostaining around tangle-bearing, Congo-positive neuron. The β-sAPP staining does not overlap with the congophilic amyloid structure. All microphotographs were taken under polarizing and light microscopy. Magnification: 40x objective. Double staining was performed with alkaline Congo Red.*

# CONCLUSIONS AND FUTURE PERSPECTIVES

The main research tasks today are (i) to obtain reliable diagnostic markers for AD so that early intervention may be facilitated and (ii) to find therapeutic drug targets. Both of these tasks necessitate an increased understanding of AD pathogenesis and the molecular mechanisms by which AD initiates and progresses.

Though many questions remain to be answered, AD research has made significant advances in the last years. At the time of acceptance for paper I, none of the APP secretases had yet been identified. The identification and characterisation of the β-secretase BACE/memapsin/Asp2 by four independent research teams late in 1999 was a  major break-through. The exact composition of γ-secretase complex is still unknown, but one component after the other are slowly being identified. Several proteases with α-secretase activity have been investigated and characterised. Inhibitor design for both β- and γ-secretase is well underway.

Several recent reports indicate that aberrant APP transport and trafficking may contribute to the development of AD. Not only are different Aβ species generated in separate organelles, but the α- and β-secretase cleavage events also depend on differential sorting of APP, BACE1 and α-secretase [23,146]. Amyloidogenic processing of APP can take place in an axonal compartment [77] and impaired axonal transport of APP due to pathogenic AD mutations has been shown to cause apoptosis and neurodegeneration *in vitro* [61]. The relative contribution of apoptosis to the neuronal loss in AD is difficult to assess because of the chronic nature of the disease process. At any one time only a limited number of apoptotic neurons can be detected and dysfunction may be initiated prior to degeneration. It has been proposed that induction of some compensatory responses to apoptosis in AD brain is able to rescue the neurons from terminal apoptosis.

Paper I describes APP species secreted into cerebrospinal fluid (CSF). After raising and purifying a rabbit polyclonal antibody specific for soluble β-secretase cleaved APP (β-sAPP), we compared the levels of soluble α-secretase cleaved APP (α-sAPP), β-sAPP and total soluble APP (sAPP) in CSF from 13 sporadic AD patients and 13 healthy controls. The CSF samples contained substantial amounts of β-sAPP, but we found no statistically

significant difference in the β-sAPP levels between AD patients and controls. The levels of α-sAPP and total sAPP were found to be significantly decreased in AD, although there was considerable overlap between patients and controls. This was the first report of β-sAPP in CSF.

Paper II describes APP processing and secretion in primary rat cortical cultures, using either colchicine or the calcium ionophore A23187 to induce apoptosis. Exposure to A23187 significantly decreased the secretion of β-sAPP in a caspase dependent manner, although the secretion of total sAPP did not change and caspase inhibition restored cell viability to control levels. Exposure to colchicine did not change the amount of either secreted β-sAPP or total sAPP and caspase inhibition was only partially able to restore cell viability. It was concluded that calcium homeostasis is an important apoptotic effector specifically affecting the β-secretase cleavage of APP.

In paper III, the Arctic APP  E693G mutation was shown to decrease cell viability in human neuroblastoma cells. The cell viability was further compromised following exposure to calcium ionophore A23187, microtubule-binding colchicine or oxidative stress inducer hydrogen peroxide. During apoptosis, cells with the Arctic mutation decreased their secretion of β-sAPP. The enhanced sensitivity to toxic stress in cells carrying the Arctic mutation may contribute to the pathogenic pathway leading to AD.

In paper IV, content and localisation of β-sAPP was localised in brain tissue sections from the frontal, temporal and occipital lobe. Strong granular β-sAPP staining was found throughout the gray matter of all three areas, while white matter staining was considerably weaker. β-sAPP was found to be localized in astrocytes and in axons. In AD brain, β-sAPP was also found surrounding senile plaques and cerebral blood vessels. The overall β-sAPP immunostaining was stronger and more extensive in gray matter than in controls and axonal β-sAPP staining was patchy and unevenly distributed, indicating impaired axonal transport. The alterations in β-sAPP staining suggests abnormal processing and transport of APP in AD brain.

Paper II and III show decreases in β-sAPP in apoptotic cells treated with A23187, compared to non-treated cells, and in APParc transfected cells compared to APPwt. The

apparent shift in secretase activity, could be caused by a caspase cleavage precluding β-secretase action or by increasing preference for the second BACE1 cleavage or another alternative cleavage site. These events might then be coupled to impaired/altered transport of APP. However, in paper I, we presented evidence that the α-sAPP is the APP species mainly affected in CSF, and in paper IV, we found more intense staining for β-sAPP in AD brain, which suggests altered cellular transport rather than impaired BACE activity. Indeed, a paper in press [70] shows that the cellular location of BACE dramatically affects its cleavage site specificity.

In polarised cells, Aβ production has been reported to be regulated by differential targeting of BACE [23]. The main part of BACE along with a small amount of APP is sorted apically, while the main part of APP is sorted to the basolateral surface [64] where α- and β-secretase compete for substrate in [23]. Increasing the fraction of APP targeted to the apical surface could lead to more amyloidogenic processing.

The decreases in β-sAPP secretion during apoptosis may indeed be due to impaired cellular trafficking. Impaired axonal transport would be expected to have a negative effect on β-sAPP production. Unexpectedly, A23187, but not colchicine treatment of primary rat neurons resulted in decreased β-sAPP secretion. It is possible that the other effects of colchicine, ie, dispersion of the Golgi apparatus and the randomisation of transport, as evident by the disparate values for both sAPP and β-sAPP, preceded and drowned out a change in β-sAPP secretion. Since the neuroblastoma cells are not polarised, it is difficult to predict what the decreases in β-sAPP in APParc signify, except for a larger vulnerability within specific parts of the transport system. For the peroxide treated APParc cells, the decrease in α-sAPP secretion signals that the overall transport to the cell surface is impeded.

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The β-secretase cleavage of APP generates the N-terminal end of Aβ. It is considered the rate-limiting step of Aβ formation; increased β-secretase cleavage leads to increased levels of Aβ. The functional importance of the cleavage itself is evident; the functional importance of the resulting fragments β-sAPP and C99 is less clear. The N-terminal fragment β-sAPP has been shown to be neuroprotective, the neuroprotection conferred by the same amount of α-sAPP is over a hundred times more potent [48]. Assuming that α-

and β-secretase compete for the same pool of APP, even a minor shift in secretase activity could affect the neuron negatively. The C99 fragment may be further cleaved by γ-secretase or caspases, releasing a cytoplasmic domain which has been implicated in nuclear signalling [22] and neuronal apoptosis [111]. Aβ generation is almost certainly not the sole purpose and consequence of the β-secretase cleavage, but its other functions are yet to be clarified.

Further studies are required to map the mechanisms of APP processing and successive events leading to AD, and to critically examine the significance of these basic research findings as they are translated into therapeutic modalities in years to come.

# ACKNOWLEDGEMENTS

I would like to thank

* ❋ my supervisors Drs. Eirikur Benedikz and Johan Fastbom for scientific guidance and continuous support
* ❋ Dr. Maria Ankarcrona for multiple collaborations
* ❋ Prof. Bengt Winblad for introducing me to neuroscience and giving me the opportunity to work in his department
* ❋ all co-authors for their contributions to the work included in the thesis
* ❋ Drs. Richard Cowburn and Maria Ankarcrona for proof-reading the thesis
* ❋ past and present colleagues for maintaining a creative and friendly atmosphere at the department of NEUROTEC
* ❋ my family and friends for their loyalty and love

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