PROTEASES IN THE BRAIN

PROTEASES IN BIOLOGY AND DISEASE

Volume 1
PROTEASES IN TISSUE REMODELLING OF LUNG AND HEART
Edited by Uwe Lendeckel and Nigel M. Hooper

Volume 2
AMINOPEPTIDASES IN BIOLOGY AND DISEASE
Edited by Nigel M. Hooper and Uwe Lendeckel

Volume 3
PROTEASES IN THE BRAIN
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Preface

This, the third volume in the *Proteases in Biology and Disease* series, focuses on Proteases in the Brain. In all organs of the body proteases have critical roles to play both in normal development and functioning and in disease states. The brain is no exception to this, with proteases having emerging roles in synaptic plasticity, memory, neurodegenerative disorders such as Alzheimer's, Parkinson's and prion diseases, ischemia and traumatic brain injury, inflammatory and infectious diseases, and tumour progression. This volume brings together a wide range of topics under this central theme and highlights the large number of proteases involved in these normal and disease processes.

The first chapter by Hans-Gert Bernstein reviews the current knowledge about the impact of proteolytic processes in the development and progression of Alzheimer's disease and whether proteases may be promising new therapeutic targets for the treatment of this fatal disease. In Chapter 2, Stephen Crocker, Patrice Smith and David Park review the current hypotheses about how dopamine neurons of the substantia nigra pars compacta degenerate in Parkinson's disease, focusing attention on the recent evidence supporting a central role for calcium-dependent calpains in this process. The prominent role of the cysteine proteases of the calpain and caspase families in the pathogenesis of brain ischemia are discussed in Chapter 3 by Swapan Ray. A description of the use of calpain and caspase inhibitors for neuroprotection in brain ischemia is also presented. Multiple proteases, including the calpains, caspases, cathepsins, serine proteases and matrix metalloproteases have all been implicated in the mechanisms underlying traumatic brain injury. In Chapter 4, Susan Knoblach and Alan Faden review the involvement of these proteases in traumatic brain injury and the potential that inhibition of them may have in its treatment. The next chapter by Bernd Kieseier and Fabian Bernal describes the role of caspases,

calpains, cathepsins, matrix metalloproteases and ADAM proteases in the pathogenesis of inflammatory and infectious diseases of the central nervous system, with particular focus on multiple sclerosis and bacterial meningitis. In Chapter 6, Stefan Brocke and colleagues describe how dipeptidyl peptidase IV regulates T cell function in CNS inflammation. Proteases are known to be critically involved in a number of steps in tumour progression, such as tumour growth, invasion, migration and metastasis. In Chapter 7, Sajana Lakka and Jasti Rao discuss the role and regulation of proteases, including cathepsins, plasminogen and matrix metalloproteases in human glioma.

The role of proteases in the metabolism of the prion protein is discussed in Chapter 8 by Antonieta Valenzuela and colleagues, while in Chapter 9, Mathias Hallberg, Pierre Le Grevès and Fred Nyberg cover the role of proteases in the processing, conversion and inactivation of neuropeptides. In the next chapter, John Wright and Joseph Harding describe the role of matrix metalloproteases and their inhibitors (TIMPs) in the degradation and preservation, respectively, of the extracellular matrix during neuronal plasticity. They also discuss the potential roles of calpains, tissue plasminogen activator and matrix metalloproteases in memory consolidation. The theme of proteases in neuronal plasticity is continued by Nobuko Mataga and Takao Hensch in Chapter 11, where the roles for the serine proteases plasminogen activators in development and plasticity in the normal mammalian brain is reviewed, with emphasis on the experience-dependent plasticity in the visual cortex. In Chapter 12, Hiroshi Nakanishi discusses the regulation of proteases in the context of modulating synaptic activity. The role of proteinase-activated receptors in brain function is the focus of Chapter 13 by Barry Festoff, with particular emphasis on thrombin and the protease-activated receptors (PARs) in nervous system function and dysfunction. In the final chapter, Corey Ford and Gary Rosenberg discuss the role of matrix metalloproteases in the neuroinflammatory damage evident in multiple sclerosis and the potential therapeutic strategies being explored to control their activities.

We hope that, like the first two volumes in the *Proteases in Biology and Diseases* series, this third volume will prove to be a timely and useful source of information both for those well versed in the role of proteases in the brain and for those who are beginning to realize the important role of this family of enzymes in brain function and dysfunction. Finally, we would like to thank all the authors for their scholarly and timely contributions and apologise to them for editorial changes in the interests of consistency and clarity.

N.M. Hooper and U. Lendeckel
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Contents

Chapter 1

Proteases and Alzheimer's Disease: Present Knowledge and Emerging Concepts of Therapy

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1. INTRODUCTION

Nearly a century ago, Alois Alzheimer published a short note about the famous case of Auguste D., a 56 years old woman who had suffered from severe memory impairment and delusions (Alzheimer 1907). When morphologically analyzing her brain after death Alzheimer saw some of the characteristic structural alterations (compromised neurons, extracellular senile plaques, intraneuronal tangles), which now are commonly accepted as neuropathological hallmarks of the disease carrying his name. Alzheimer disease (AD) is currently the fourth leading cause of death and most common cause of dementia. Nosologically, AD is not a single disorder in spite of a common clinical phenotype. Etiologically, at least two different types exist. In a minority of 5% of the cases or even less; AD is due to mutations in certain genes, resulting in the permanent generation of Aβ fragments (see below). The majority of cases of AD are sporadic in origin, with old age as a main risk factor (Hoyer 2004). With age being an important risk factor for the development of AD, and with the population aging rapidly in developed countries, the number of persons suffering from the disease will dramatically grow during next decades. Suffering from AD clearly extends beyond the patient and immediate caregivers, challenging the society as a whole. Hence, many efforts have been directed towards revealing the cellular and molecular events behind the development of AD and to establish therapeutic strategies in order to cure the disease or, at least to decelerate its progression. Although the mechanisms leading to AD are

still far from being really understood, it became evident during the last years that proteases are prominently and in many ways involved in the pathobiology of the disease. The present article aims at reviewing current knowledge about the impact of proteolytic processes in the development and/or progression of AD and to ask, whether proteases may be part of an "avenue of hope" (Samuels and Grossman, 2003) taking us to promising new therapeutics of this treacherous, fatal disease.

2. PROTEASES AND NEUROPATHOLOGICAL HALLMARKS OF AD

AD is a progressive degenerative encephalopathy, which is clinically characterized by profound behavioural disorders, loss of memory and reasoning, and personality changes. Neuropathological hallmarks of AD are accelerated atrophy and loss of neurons from specific areas of the brain, reduction of synapses on surviving neurons, deposition of amyloid in neuritic plaques and within the walls of the cerebral microvasculature, and the increased appearance of neurofibrillary tangles (for overview, see Masliah and Terry, 1993; Selkoe, 1994; Bernstein *et al* 1996). Although some of these pathological changes may occasionally be observed in brains of aged patients without clinical signs of AD, there is no doubt that these hallmarks are highly indicative of AD, and that the degree of their expression correlates with the severity of the disease. A plethora of findings shows that protein-cleaving enzymes are active players in almost all of these processes, and one hardly can find a brain-associated protease which never has been suspected to be involved in AD. However, recent research has helped to draw a much clearer picture now, and I will try to relate specific proteases to specific AD-associated processes. Special emphasis will be given to the process of amyloidogenesis, which is regarded a core event in AD pathogenesis (Hardy and Selkoe, 2002).

2.1 Proteases and Amyloidogenesis in AD

A primary neuropathological hallmark of AD brains is the accumulation of β-amyloid peptide (Aβ) in amyloid (diffuse and later neuritic) plaques and, to a lesser extent, blood vessels. The neurotoxic Aβ peptides are derived by proteoloytic processing of the amyloid precursor protein (APP) by certain proteases called β- and γ- secretases, generating three forms of Aβ corresponding to Aβ (1-40), Aβ (1-42), and Aβ (1-43). A scheme showing APP cleavage by the secretases is shown in Fig. 1. The amyloid precursor protein (APP) gene is located on human chromosome 21 and

consists of 18 exons, which are alternatively spliced into several different transcripts (APP isoforms), named accordingly to their length in amino acids, APP_{695}, APP_{751}, and APP_{770} (Beyreuther and Masters, 1990). Two of them have an extra sequence homologous to the Kunitz-type trypsin inhibitor (Tanzi *et al* 1988, Selkoe, 1994). APPs are transmembrane type I proteins which are not only widely distributed within normal and AD brains, but also in non-neural tissues. Their physiological role in neural tissues is largely unknown but might be connected with nerve cell proliferation and survival as well as with synaptogenesis (for recent considerations, see Caille *et al* 2004).

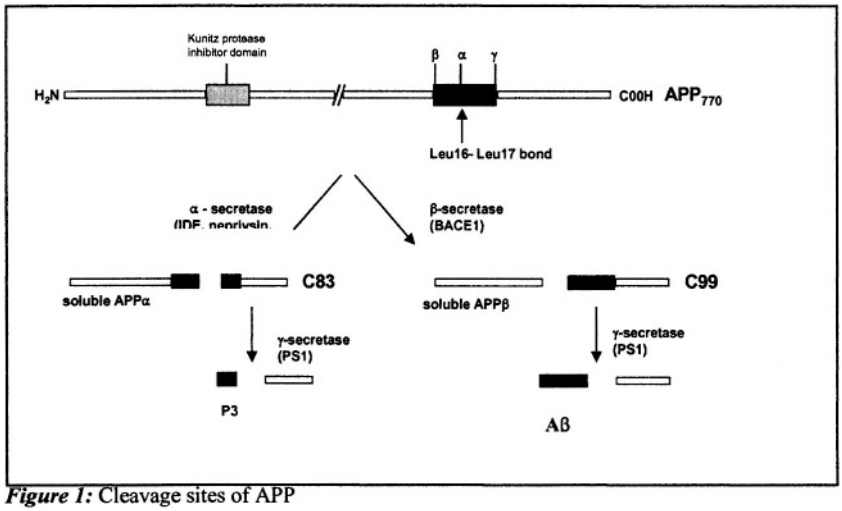

Figure 1: Cleavage sites of APP

2.1.1 Normal processing of APP by α-secretases: first cut is the deepest

In the normal brain, most APP is processed in a way that prevents the formation of potentially amyloidogenic fragments. This non-amyloidogenic secretory pathway is predominant over the amyloidogenic pathway and involves the obligate cleavage of APP by a so-called **α-secretase(s)** (Selkoe 1991; Asai *et al* 2003 and many others). By cleaving the APP molecule at the Leu16-Leu17 bond within the Aβ region of the APP molecule thereby producing a soluble α-APP fragment and an 83-residue COOH-terminal fragment, the formation of "dangerous" amyloidogenic fragments is precluded. Moreover, the large ectodomain released from the cell surface by the action of α-secretase has several neuroprotective properties (reviewed in Allinson *et al* 2003) Thus, the cut made by α-secretases determines whether or not amyloid formation may follow. During the past decade, several candidate

proteases have been identified, which are capable of acting as α-secretases. However, most data now favours one of the ADAMs (a Disintegrin and Metalloprotease) family of proteases, ADAM9 (EC 3.4.24.-), ADAM10 (Kuzbanian; EC 3.4.24.81) and ADAM17 (TNFα-converting enzyme, TACE; EC 3.4.24.86) as the physiological α-secretases.

ADAMs are membrane proteins containing a disintegrin and metalloprotease domain, which are widely distributed and play important roles in a plethora of cellular events (Wolfsberg *et al* 1995). At least 17 individual members of the ADAM family are expressed in the brain (Karkainen *et al* 2000). By the work of several groups ADAMs 9, 10, and 17 have been shown to be putative α-secretases (Koike *et al* 1999; Lammich *et al* 1999; Skovronsky *et al* 2000; Marcinkiewicz and Seidah, 2000; Lopez-Perez *et al* 2001; Hooper and Turner, 2002; Allinson *et al* 2003; Asai *et al* 2003). There is some recent evidence that ADAMs 9, 10, and 17 together might represent the "true" α-secretase (Asai *et al* 2003). Of these enzymes, ADAM 10 is currently the best studied one with regard to AD. ADAM 10 was found to be located in neurons and senile plaques of AD brains and brains of patients with Down syndrome (Fig. 2; Bernstein *et al* 2003). Together with ADAM 17, ADAM 10 is implicated in Notch signaling, which is disturbed in AD (reviewed in Hartmann *et al* 2001) and was shown to compete with β-secretase for cleavage of APP (Marcinkiewicz and Seidah, 2000). Alterations in ADAM 10 activity is a very early event in the progression of AD (Colciaghi *et al* 2004). Unfortunately, there are as yet no genetic studies linking mutations of ADAM enzymes with AD.

2.1.2 Processing of APP by β-secretase: opening the route to amyloid formation

Beta-secretase generates the NH_2-terminus of Aβ. By splitting APP it produces a soluble fragment of APP (β-APP_S) and a 99-residue COOH-terminal molecule (C99), which remains membrane-bound. Nowadays there is little doubt that the authentic β-secretase is a type 1 transmembrane protein belonging to the pepsin family, which is called **BACE-1** (for β-site APP-cleaving enzyme, EC 3.4.23.46) (Vassar 2001, 2004; Haass 2004; Hook and Reisine, 2003). It contains aspartyl protease activity. Its physiological function is yet poorly understood. There exists a close homologue to BACE-1, called **BACE-2,** which does not contribute to the amyloidogenic processing of APP (Haass 2004). Instead, BACE-2 seems to have α-secretase-like properties (Farzan *et al* 2000). Under normal conditions there is a competition between α-and β-secretases for the substrate APP (Asai *et al* 2003; Neve 2003). The gene coding for BACE-1 is located on chromosome 11, but no AD-causing mutation in this gene has been identified so far. However, polymorphisms in the BACE gene seem to

influence the risk for AD (Kirschling *et al* 2003). There is evidence that a high molecular complex variant of BACE has a higher β-secretase activity than the monomer (Marlow *et al* 2002).

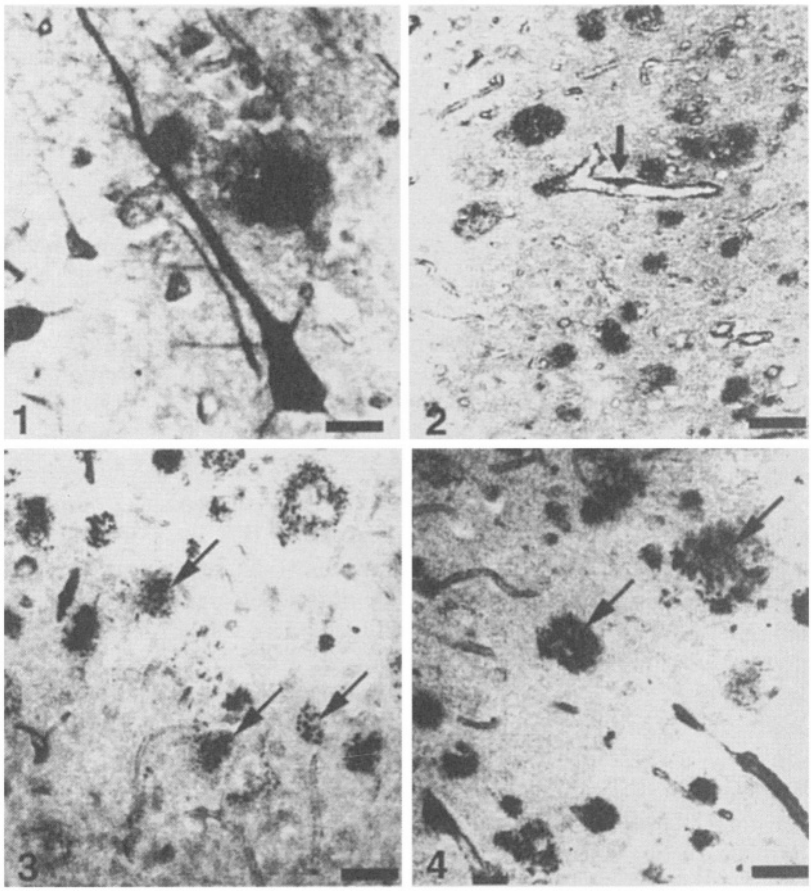

Figure 2: Immunohistochemical localization of proteases in AD brains. **1** and **2:** IDE-immunoreactivity in neurons and senile plaques in the neocortex of an AD patient (from Bernstein *et al* 1999 with kind permission of Elsevier). **3:** ADAM 10 immunoreactive material decorating multiple plaques in an AD brain. **4:** ADAM 10 immunoreactivity associated with plaques in an adult Downs patient (3 and 4 from Bernstein *et al* 2003 with kind permission of Kluwer Academic Publishers).

Interestingly, the BACE-2 homologue maps to chromosome 21, raising the possibility of an involvement of this protease in Down syndrome, but due to its minute brain concentrations and its α-secretase properties this possibility may be ruled out (Esler and Wolfe, 2001; Citron 2002). Beta-secretase mRNA is highly expressed in the brain as well as in other tissues. Intracellularly,

the enzyme is located in the Golgi apparatus, the endosomes, and at the cell membrane (for review see: Esler and Wolfe, 2001). BACE-1 appears to be co-localized with its cleavage product, β-APP_S and the putative α-secretase ADAM 10 in human cortical neurons (Marcinkiewicz and Seidah, 2000; Sennvik *et al* 2004). Strong support for the pivotal (and exclusive) role of BACE-1 as β-secretase comes from experiments with BACE-1-deficient mice: absolutely no Aβ is found in their brains (Cai *et al* 2001).

2.1.3 The γ-secretases: presenilins and beyond

Both the C83 (generated by α-secretase) and C99 (produced by β-secretase) APP fragments are substrates to γ-secretase, which performs a rather unusual proteolysis in the middle of the transmembrane domain to liberate the 4-kD Aβ from C99 and a 3-kD peptide (P3) from C83 (Esler and Wolfe, 2001; Haass 2004). Thus, only proteolysis of the C99 molecule yields amyloidogenic peptides. Hence, β-secretase initiated Aβ-generation is a prerequisite for "amyloidogenic" γ-secretase activity. A recently discovered ε-cleavage site within the APP intracellular domain does not influence the generation of Aβ by γ-secretase activity (Bergman *et al* 2003). The nature of the γ-secretase(s) has long been elusive. Now there is consensus that γ-secretase activity is largely due to the action of **presenilins** (EC 3.4.23.-) which are membrane-bound aspartyl proteases. There are two homologous presenilins, **PS1** and **PS2.** Clearly, PS1 and 2 have important biological functions in the brain other than to produce Aβ peptide (which was called a "rather unfortunate by-product" of PS action by Haass 2004). Among their natural substrates one can find cadherins, nectins and neuregulin receptors, erbBs 2 and 4 (reviewed in Bergman et *et al* 2003; Haass 2004).

Initially, presenilins were linked to familial (early-onset) AD (Sherrington *et al* 1995). Common to all known familial cases of AD are point mutations in the APP gene, which all seem to influence γ-secretase activity and bring about a shift in the ratio of γ-secretase generated Aβ fragments towards an increase of the highly amyloidogenic Aβ-42. This process is thought to significantly contribute to the aggressive progression of early-onset AD (Hardy and Selkoe, 2002; Haass 2004). Moreover, mutations in the presenilin molecule itself play a pivotal role in early-onset AD (Kim and Tanzi, 1997). Their role in sporadic AD is less clear. Good evidence for the dominant role of PS1 and PS2 in general amyloid formation comes from observations in PS1 knockout mice, which have drastically reduced Aβ generation (De Strooper *et al* 1998). Moreover, PS1/PS2 double knockout mice show no Aβ generation at all (Herreman *et al* 2000). It is now widely accepted that PS1 and PS2 alone do not explain γ-secretase activity. In search for additional players in the amyloid game new factors were identified: **nicastrin** (Yu *et al* 2000), **APH-1** (anterior pharynx-

defective phenotype) and **PEN-2** (PS-enhancer) (for recent considerations see De Strooper *et al* 2003). It is thought that these factors form a complex with the presenilins and are able to limit PS activity (Haass 2004).

2.1.4 Aβ degrading proteases

The following proteases have been shown, at least in vitro, to degrade the Aβ peptide:

- Insulin-degrading enzyme, IDE (insulinase, insulysin, insulin-glucagon protease; EC 3.4.24.11)
- Neprilysin (neutral endopeptidase, enkephalinase, acute lymphoblastic leukaemia antigen; EC 3.4.24.11)
- Plasmin(EC3.4.21.7)
- Thimet oligopeptidase (EC 3.4.24.15)
- Angiotensin-converting enzyme (ACE; EC 3.4.15.1)

Insulin-degrading enzyme (IDE) is an intracellular protein with important binding, regulatory and adaptive functions (Hamel *et al* 1998). Since IDE is capable of splitting insulin with high specificity the enzyme is regarded a reliable marker for insulin catabolism in various tissues including the brain (Dorn *et al* 1983, 1986; Reiser *et al* 1987). Besides insulin, IDE degrades other peptides, too (glucagon, β- and γ-endorphins and others, Safavi *et al* 1996). Work from several laboratories has demonstrated the Aβ degrading properties of the enzyme (Kurochkin and Goto 1994; Kurochkin 1998; McDermott and Gibson 1997; Qui *et al* 1998; Vekrellis *et al* 2000). Insulin-degrading enzyme has immunohistochemically been demonstrated to be present in normal and AD brains (Fig. 2). At the cellular level the enzyme was found in neurons and senile plaques (Bernstein *et al* 1999; Cook *et al* 2003). Although there are hints for an increased expression of IDE in neurons adjacent to senile plaques (Bernstein *et al* 1999), the overall Aβ-degrading capacity in AD brains is only about 50% of that of control brains (Perez *et al* 2000; Hoyer 2004). Moreover, mice lacking the IDE gene show a considerable accumulation of brain amyloid due to reduced degradation of Aβ (Craft and Watson, 2004), whereas transgenic animals overexpressing the enzyme have significantly reduced Aβ levels (Leissring *et al* 2004). On the other hand, rats carrying partial loss-of-function mutations of IDE show impaired degradation of Aβ (Farris *et al* 2004). Furthermore, IDE activity may depend on the intracellular metabolic state and the apolipoprotein-E genotype of the individual, which in itself may be a risk factor for AD (Cook *et al* 2003). In addition to that, genetic variants in the haplotype of IDE gene have been shown to be significantly associated with plasma levels of Aβ and risk for AD (Ertekin-Taner *et al* 2004). Interestingly enough, the main substrate of IDE, insulin, as well as brain

insulin receptors, have repeatedly been shown to be prominently involved in the pathophysiology of AD (Hoyer 1998, 2004; Frolich *et al* 1998; Craft and Watson, 2004 and many others). Hoyer (1998) was the first to come up with the idea that sporadic AD might be the brain type of non-insulin dependent diabetes mellitus.

And perhaps, brain insulin (either transported to brain tissue through the blood-brain barrier or locally synthesized) competes with Aβ for degradation by IDE. Consequently, permanent excess of one substrate, insulin (resulting from hyperinsulinaemia as observed in type II diabetes) would reduce degradation of the other, Aβ (Craft and Watson, 2004). Indeed, diabetes mellitus was found to double the risk for AD, with patients treated with insulin having the highest risk for dementia (Ott *et al* 1999).

Neprilysin is a zinc metalloendopeptidase which metabolizes substance P, somatostatin and other neuropeptides. Originally, the enzyme came into focus of AD research because of altered neuropeptide levels and degradation rates in AD brains (Weber *et al* 1992; Waters and David, 1995). Experimental work of Iwata and colleagues has clearly shown that neprilysin is an Aβ degrading enzyme, which significantly contributes to the metabolic regulation of brain Aβ (Iwata *et al* 2001; Carson and Turner, 2002). Neuropathologically, neprilysin is associated with neuritic plaques (Sato *et al* 1991). Moreover, there is evidence that formation of amyloid plaque is prevented by increased brain levels of neprilysin (Mohajeri *et al* 2002, 2004; Leissring *et al* 2004). A reduction of neprilysin levels has the opposite effect. Interestingly, region-specific reductions of enzyme levels seem to be part of the aging process of the brain – at least in the mouse (Iwata *et al* 2002). Finally, a recent genetic study showed that a newly discovered bi-allelic polymorphism of the neprilysin gene is associated with an increased risk for AD in an age-dependent manner (Clarimon *et al* 2003).

In some reports **plasmin**, **thimet oligopeptidase** and **ACE** have also been implicated in AD (for review see: Carson and Turner, 2002). Despite their property to cleave Aβ in vitro, comparatively little is known about the actual role of these enzymes in Aβ degradation in vivo. Here, further work is needed to better understand their impact.

Among the intrinsic factors modulating amyloidogenesis, the age-dependent activation of the endosomal-lysosomal system is the best studied one (Nixon 2001; Nakanishi 2003). During normal aging, and very prominently in AD brain, certain enzymes such as **calpains** (EC 3.4.22.-) and especially lysosomal cathepsins become dramatically upregulated in expression and activity. Years ago it has been shown that changes of the lysosomal cathepsins (cathepsin D and others) is an early and reliable cellular marker for AD (Bernstein *et al* 1989, 1990; Cataldo and Nixon, 1990). **Cathepsin D** (EC 3.4.23.5) has repeatedly been shown to contribute to the intracellular clearance of Aβ peptides and to be responsible for

degradation of Aβ which accumulates in neuronal lysosomes or is phagocytosed by microglia (for review see Nakanishi 2003). Thus, cathepsin D seems to play important roles as an enzyme that precludes amyloid formation by digestion of Aβ (Bendiske and Bahr, 2003). Curiously, cathepsin D possesses β-and γ-secretase activities as well, and its actual role in amyloid formation is far from being understood (for review see: Nixon 2002). However, a recent meta-analysis of the association of cathepsin D gene polymorphism with the risk of AD came to the conclusion that there is yet little support for the idea that cathepsin D is a major risk factor for the disease (Ntais *et al* 2004). It should be emphasized that the activation of the endosomal-lysosomal system does not only activate cathepsins, but may also lead to enhanced γ-secretase activity of the presenilin complex, as recent findings demonstrate the localization of nicastrin and PS at membranes of lysosomes (Pasternak *et al* 2004).

2.2 Proteases and Formation of Neurofibrillary Tangles in AD

Another core pathological feature of AD is the presence of intracellular neurofibrillary tangles (NFT), which are composed of microtubule-binding protein tau assembled into paired helical and straight filaments. It has been disputed for a long period of time, whether amyloid or tangle formation represents the primary pathological event in AD. Now there is some evidence that these pathological entities may be functionally linked (for review see Gamblin *et al* 2003). In AD brains, the tau present in NFTs is aberrantly (hyper)phosphorylated and often proteolytically truncated at the carboxy-terminus (Grundke-Iqbal *et al* 1986). Histological labeling of tau pathology has been introduced to trace and grade the progression of the disease (using the so-called Braak staging, Harding *et al* 2000). Truncation seems to be crucial for enhanced tau filament assembly in vitro and, possibly, under vivo conditions of AD (Abraha *et al* 2000). Recently it was found that **caspase-3** (EC 3.4.22.-; a member of the caspase family of cysteine proteases) is responsible for removal of amino acid residues from the C-terminus (Gamblin *et al* 2003). Interestingly, upregulation of caspase-3 and other members of the caspase family is observed in AD brains and plays a crucial role in Aβ-induced neuronal death (see below). Meanwhile, additional caspases (6, 7, 8 and 9) have been identified which also cleave tau in neurons undergoing Aβ-induced apoptosis (Gamblin *et al* 2003). The importance of this mechanism is underlined by the finding that tau-depleted neurons in knockout mice are resistant to Aβ-induced neurotoxicity (Rapoport *et al* 2002). Normally, tau protein is turned over by **calpains.** Hyperphosphorylated tau, however, is highly resistant against cleavage by calpain (Nixon 2002). The neuronal calpain system becomes activated early

in AD. Activated μ-calpain is detectable in neurites before any tau pathology is visible. At later stages of AD the enzyme is found in close association with tau-containing granules (the assumed precursor of neurofibrillary tangles in AD) as well as in neuropil threads with tangles (Grynspan *et al* 1997). Calpains are known to modulate phosphorylation and proteolysis of tau and might thus contribute to the formation of paired helical filaments in AD (Litersky and Johnson, 1995; Grynspan *et al* 1997). The described alterations in calpain activities might be the result of a profoundly disturbed neuronal calcium homeostasis, which is a key phenomenon of aging and AD (Braunewell *et al* 2001). Another typical manifestation of tauopathy-related events in AD is the accumulation of mutant ubiquitin as a morphological marker of proteasomal dysfunction (for review see Fischer *et al* 2003). However, the deposition of ubiquitin is not AD-specific. It can be also observed in other neurogenerative diseases (Fischer *et al* 2003). Interestingly, ubiquitin interacts with the Aβ degrading enzyme, IDE (Saric *et al* 2003), which might have consequences for the clearance of Aβ. In patients with sporadic AD or with Down syndrome dinucleotide deletions within the ubiquitin B gene can be found (Van Leeuwen *et al* 1998). The disturbed function of the **proteasome** leading to ubiquitin accumulation in AD may have fatal consequences for the neurons, since the proteasome is a major factor for protein degradation of the cell (Ding and Keller, 2003; Fischer *et al* 2003). It has been proposed that a chronic low-level inhibition of the proteasome complex is the functional basis for some of the observed neuropathological changes (Ding and Keller, 2003). Paired helical tau filament itself further contributes to proteasome inhibition in AD (Keck *et al* 2003). Finally, a certain contribution of cathepsins to tangle pathology cannot be ruled out, because cathepsins B and D are capable of splitting neurofilament proteins (reviewed in Bernstein *et al* 1996).

2.3 Proteases in Neuronal Loss and Synaptic Pathology of AD

Neuronal loss distinguishes AD from normal aging and correlates best with cognitive decline in AD individuals (LeBlanc *et al* 1999). The extent of nerve cell death in AD is high. So, in mild cases of AD, there is already a 50% loss of neurons in the entorhinal cortex, a region which is very early affected in the development of AD (Braak and Braak, 1996; Hyman and Gomez-Isla, 1996). The progressive nature of neuronal cell dysfunction and death in AD patients points to an apoptotic mechanism of neuronal cell death. Some researchers believe that the initiation of the apoptotic program in AD is prior to amyloid and tangle formation (Su *et al* 2002). Undoubtedly, both processes influence one another. Neuronal cell death leads to an increase of Aβ generation by activation of proteolytic enzymes.

Aβ in turn is neurotoxic and induces apoptosis (Dickson 2004). Thus, apoptosis-mediated increase in Aβ may be part of a cascade of events that further enhances neuronal cell death (for review see LeBlanc 1997). The main actors in apoptosis are the **caspases**. Caspases normally exist in the cytosolic fraction of the cell as inactive precursors that become activated by enzymatic cleavage during apoptosis. Several studies have shown that caspase-3 activation is both necessary and sufficient to trigger apoptosis (Mehmet 2000; Takuma *et al* 2004). Morphologically, elevated levels of activated caspase-3 immunoreactivity is found in neurons, astrocytes and blood vessels of AD patients (Su *et al* 2002). Moreover, these authors could show that the activation of apoptotic mechanisms takes place in selective compartments containing granules of granulovascuolar degeneration. These granules are rare in normal brain, but appear markedly increased in number in AD patients (Su *et al* 2002). They are suggested to arise from microautophagy and may be formed through lysosomal autophagy of intraneuronal substances (Okomato *et al* 1991). If this interpretation is correct, the caspase-mediated apoptotic pathway of neuronal (and glial?) (Takuma *et al* 2004) death would be linked to activation of the endosomal-lysosomal system (Nixon 2000; Nakanishi 2003), bringing the cathepsins into play again. Exciting new findings show that unsuccessful attempts to re-enter the cell cycle always precede nerve cell death in AD (Arendt 2003; Yang *et al* 2003; Reiser and Bernstein, 2004). A role of proteases in this process remains to be defined yet. Data from rat models of AD show that there is a calpain- and caspase-induced disruption of neurogenesis and perturbed neural progenitor cell homeostasis in these animals (Haughey *et al* 2002). Such pathomechanisms might play a role in humans, too. Another defining feature of AD is synaptic loss, whereby the hippocampus is the most affected brain region (for recent work see Honer 2003). As most other neuropathological hallmarks, synaptic injury is found very early in AD evolution. Loss of terminals is accompanied by the reduction of specific presynaptic proteins such as GAP-43, SNAP-25, synapsin (Honer 2003). Synaptic changes in AD are thought to mainly result from the neurotoxic attack of Aβ peptides (Lue *et al* 1996) or disturbed glutamate metabolism and/or transport (Masliah *et al* 1996; Lue *et al* 1999). There is evidence from aged mouse brain that neprilysin is presynaptically localized and protects the synapse from Aβ neurotoxicity by effectively clearing Aβ (Iwata *et al* 2004). It would be of interest to know, whether or not neprilysin is a component of all synapses. A similar synapse-specific role of the enzyme in normal and AD human brain has not been demonstrated yet. The immune processes which are implicated in destruction of cells and synapses in AD, will be regarded below.

3. PROTEASES AND INFLAMMATION IN AD

Neuroinflammation is a central feature in AD (McGeer and McGeer, 2001). The hypothesis that inflammatory processes might occur in AD brains emerged when activated microglia, expressing immunocompetent protein HLA-DR were detected in association with typical AD lesions (McGeer *et al* 1987). Although activation of microglia has long been considered as a secondary event following neuronal damage, there is increasing evidence for an independent role of microglia-related immune processes in AD (Nakanishi 2003). Various molecules acting as key mediators in peripheral immune reactions, are present at high concentrations in AD brains (Rogers *et al* 1988; McGeer and McGeer, 1995; Nakanishi 2003). The main result of neuroinflammation with regard to AD is a self attack by host defence mechanisms (autotoxicity), which finally leads to cell death. Proteases are believed to play significant roles in the inflammatory processes, since proteolytic mechanisms are involved in clearance of abnormal proteins, reformulation of extracellular matrix, facilitation of pathways for cell chemotaxis and other immune processes (for overview, see McGeer and McGeer, 2002). Amazingly little is known about the authentic proteases in neuroinflammation, however. Polymorphisms of two protease inhibitors, **α-2-macroglobulin** and **α-1-antichymotrypsin** seem to increase the risk for AD (Blacker *et al* 1998). Several observations suggest that activated microglia express and secrete lysosomal cysteine proteases, the **cathepsins S**, **L** and **B** (EC 3.4.22.27, EC 3.4.22.15 and EC 3.4.22.1; Banati *et al* 1993; Bernstein *et al* 1996; Petanceska *et al* 1996; Kingham and Pocock, 2001). Cathepsin S is cabable of degrading extracellular matrix proteins at neutral pH, which might play a certain role in neurodegeneration. Cathepsin B seems to be even more important in this context, because it has been identified to be the major causative factor of microglia-mediated neuronal apoptosis (Nakanishi 2003). The essential role of cathepsin B as a mediator of neuronal death induced by Aβ-activated microglial cells has recently been confirmed and extended by biochemical and functional genomics studies (Bohne 2004; Gan *et al* 2004). Nishioku and co-workers (2002) have demonstrated that that another cathepsin, the non-lysosomal aspartic protease **cathepsin E** (EC 3.4.23.37) is linked to the processing of exogenous antigens and MHC class molecules through the microglial endosomal-lysosomal system. This is of special interest, since cathepsin E immunoreactivity, which is fairly low in normal brain tissue (Nakanishi *et al* 1994), becomes sharply upregulated in AD brains (Bernstein and Wiederanders, 1994). In brains of AD patients this enzyme was seen in multiple microglial cells and "dying" neurons of Nuc. basalis of Meynert (Bernstein and Wiederanders, 1994). Additional support for a prominent impact of several cathepsins in microglia-mediated inflammatory processes

is lent by convincing findings in cathepsin B, L, and D single or double knockout mice (summarized in Nakanishi 2003). How far endogenous brain-associated inhibitors of cysteine proteases (cystatins A, B, and C) play a significant role in cathepsin-mediated inflammatory processes in AD remains to be established (Bernstein *et al* 1994; Nixon 2002; Nakanishi 2003). Thus, both lysosomal and non-lysosomal cathepsins appear to have central pathophysiological functions in neuroinflammation.

4. PROTEASE-BASED THERAPEUTIC CONCEPTS IN AD

Nobody will deny that the search for successful treatment strategies for AD is one of the most challenging tasks for contemporary medicine. Endeavours to cure AD certainly imply conceptual considerations, where proteases play an outstanding role. Unfortunately, most of the proteases which are implicated in AD pathobiology have also eminent functions in normal cell proteolysis. Hence, any effort to manipulate ("normalize") the expression and/or activity of these enzymes in AD will have considerable side effects. Thus, some of these manipulations might appear to be jumps out of the frying pan into the fire.

If assumed that AD represents the effects of a chronic imbalance between Aβ production and Aβ clearance, several therapeutic strategies may be proposed (Hardy and Selkoe 2002; Neve 2003; Ferrer *et al* 2004; Zlokovic 2004):

- Partial inhibition of either of the two Aβ-generating proteases, β-and γ-secretase(s)
- Partial enhancement of α-secretase(s) activity
- Prevention of the oligomerization of Aβ or enhancement of its clearance
- Depression of the Aβ-induced inflammatory response
- Anti-amyloid vaccination
- Modulation of the cholesterol homeostasis
- Chelation of Cu^{2+} and Zn^{2+} ions which contribute to Aβ deposition
- Prevention of synaptotoxic and neurodegenerative effects putatively triggered by Aβ accumulation
- Enhancement of clearance of amyloid through the blood-brain barrier

Of these approaches, the first two directly concern brain proteases. In the case of the β-secretase (BACE1) there is ongoing research for reliable small-molecule inhibitors that can fit into the large active site of this

aspartyl protease and still penetrate the blood-brain barrier (Citron 2002; Hardy and Selkoe, 2002; Vassar 2002, 2004). An important step towards reaching this goal was the development of the first BACE inhibitor which is based on the sequence around the β-secretase cleavage site of APP, where the Leu-Asp amide bond of EVNL/DAEF was replaced by a hydroxyethylene transition state analogue isostere (Roggo 2002). Another potent β-secretase inhibitor (hispidin) is a natural product. It was isolated from mycelial cultures of *Phellinus linteus.* Unfortunately, this inhibitor affects α-secretase activity as well (Park *et al* 2004). It has been suggested that an inhibitor against the BACE polymer would be even more effective in depressing intraneuronal β-secretase activity (Marlow *et al* 2002). In the case of γ-secretases, potent inhibitors already exist which work well in cellular model systems and mice. However, experience with AD patients is almost completely lacking. The first compounds applied in humans were described by Dovey *et al* (2000). Concerns with these inhibitors regard possible interference with the important Notch signaling system and other cell surface receptors (Selkoe 2001; Hardy and Selkoe 2002; Sisodia *et al* 2002; Lewis *et al* 2003). Inhibition of Notch processing seems to be responsible for some of the undesirable biological effects which occur after chronic treatment with γ-secretase inhibitors (for example, altered lymphopoiesis and intestinal cell differentiation, Wong *et al* 2004). Since the development of β-secretase inhibitors is not so easy, a promising way to reduce amyloid formation was the stimulation of α-secretases in order to facilitate the non-amyloidogenic APP pathway. Very recently a new signaling pathway was found in AD - sumoylation (Li *et al* 2003, Neve 2003). SUMOs (**s**mall **u**biquitin-like **mo**difiers) 1- and –2 are intraneuronal proteins which occur at higher concentrations in AD and Down brain. They are capable of mediating α-secretase, in preference to β-secretase-mediated cleavage of APP (Li *et al* 2003). ADAMs-related α-secretase activity may be upregulated by muscarinic agonists, steroid hormones, cholesterol-lowering drugs and certain metal ions (summarized by Hooper and Turner, 2002; Allinson *et al* 2003). Insulin-degrading enzyme's main substrate is insulin. Perhaps, attempts to metabolically lower "intracerebral hyperinsulinaemia" would help to increase the Aβ-degrading activity of IDE (for review see Hoyer 2004). Interestingly, light chains of certain anti-Aβ-antibodies were revealed that have Aβ-degrading activity. Application of these light chains might have an amyloid-clearing effect (Rangan *et al* 2003). Of note, partial blocking or activation of other putative APP-cleaving enzymes (cathepsins, calpain, caspases) might also have beneficial influence on the prevention of amyloid formation and, possibly, on other neuropathological signs of the disease as well. Cathepsins of the cysteine proteases type (i.e. B, H, L, and S) have endogenous inhibitors which are widely distributed in neural tissue, the cystatins. These cystatins become