Chapter 6

Amyloid β Degradation: A Challenging Task for Brain Peptidases

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Abstract: Amyloid β (Aβ) accumulates in the neuropil and within the walls of cerebral vessels in association with normal aging, dementia or stroke. Aβ is released from its precursor protein as soluble monomeric species yet, under pathological conditions, it self-aggregates to form soluble oligomers or insoluble fibrils that may be toxic to neurons and vascular cells. Aβ levels could be lowered by inhibiting its generation or by promoting its clearance by transport or degradation. Here we will summarize recent findings on brain proteases capable of degrading Aβ, with a special focus on those enzymes for which there is genetic, transgenic or biochemical evidence supporting a role in the proteolysis of Aβ *in vivo*.

Key words: Amyloid β, dementia, stroke, amyloidoses, Alzheimer's disease, brain proteases, insulin degrading enzyme.

1. INTRODUCTION

The progressive accumulation of amyloid β peptide (Aβ) in the form of soluble oligomers or insoluble fibrils is part of the normal process of brain aging (Glenner and Wong, 1984; Coria *et al.*, 1987; Wang *et al.*, 1999). Such accumulation reaches very high levels in the neuropil and cerebral microvessels in several neuropathological conditions characterized by dementia or stroke. These include Alzheimer's disease (AD), Down's syndrome beyond the age of 35 years, sporadic cerebral amyloid angiopathy (SCAA) and dementia pugilistica (reviewed in Morelli *et al.*, 2002). The

development of these sporadic disorders may be strongly influenced by genetic and environmental factors such as the apolipoprotein E (apoE) genotype (reviewed in Roses, 1997), chronic trauma or cholesterol metabolism (Hartman *et al.*, 2002; Hartmann, 2001; Pappolla *et al.*, 2003). In addition, the Aβ-associated disorders comprise rare genetic diseases known as familial early-onset AD, hereditary cerebral haemorrhage with amyloidosis (HCHWA) of Dutch and Italian types and dementia with amyloid angiopathy of Flemish, Arctic or Iowa origin. This group of autosomal dominant disorders is associated with mutations in the amyloid β-precursor protein (APP) and Presenilin (PS)1/2 genes (reviewed in Rocchi *et al.*, 2003; Miravalle *et al.*, 2000). From the study of APP and PS mutations using *in vitro* (synthetic peptides, cell cultures) and *in vivo* (transgenic mice) models, three basic mechanisms to understand Aβ accretion in the brain have emerged. The first mechanism is related to an increase in the rate of total Aβ production, as described with the Swedish double mutation (NL-APP) at the β-secretase site and with the Flemish mutation (A692G) (Citron *et al.*, 1992; De Jonghe *et al.*, 1998). While the former seems to accelerate the generation of the N-terminus of Aβ by β-secretase, the latter may impair the non-amyloidogenic α-secretase cleavage between positions 16-17 of Aβ. A second mechanism is the relative increase in the production of "long Aβ" species ending at Ala42 or Thr43 that are more hydrophobic and tend to aggregate more rapidly than "short Aβ" ending at Val40. This shift in the processing of Aβ C-terminus is related to a group of APP mutations close to the γ-secretase site and with most of the PS1/2 mutations studied so far (Suzuki *et al.*, 1994; Citron *et al.*, 1997). The third mechanism is associated with mutations that are clustered in the middle region of Aβ and result in the generation of Aβ variants with a high fibrillogenic potential under physiologic pH and ionic strength, such as the Dutch (E22Q), Arctic (E22G) and Iowa (D23N) species (reviewed in Miravalle *et al.*, 2000; Van Nostrand *et al.*, 2001). However, none of these mechanisms seem to participate in the accumulation of cerebral Aβ in the vast majority of cases, such as in sporadic AD, SCAA or normal aging, in which mutations are not in play. Recent studies in transgenic mice carrying human APP mutants suggest that the removal of Aβ from the brain is a very active process, raising the possibility that a defective clearance of Aβ may contribute to its accumulation under physiological and pathological situations (Dewachter *et al.*, 2000). After being released by secretases in the CNS, soluble Aβ can follow three possible main pathways (Figure 1): 1) it can be transported to the systemic circulation along perivascular drainage channels *via* the CSF or directly, across the blood brain barrier (Shibata *et al.*, 2000; Kuo *et al.*, 2001), 2) it can undergo extracellular or intracellular proteolytic degradation, and 3) it can self-assemble, aggregate and deposit in the form of brain

amyloid plaques or amyloid angiopathy. Since this third pathway is critically dependent on concentration, minor flaws in the two mechanisms of clearance (transport and proteolysis) may rapidly shift the equilibrium to Aβ oligomerization. In this Chapter we will briefly review the recent advances on the major proteases involved in Aβ clearance, their possible participation in disease and their potential significance from a therapeutic perspective in cerebral Aβ amyloidoses.

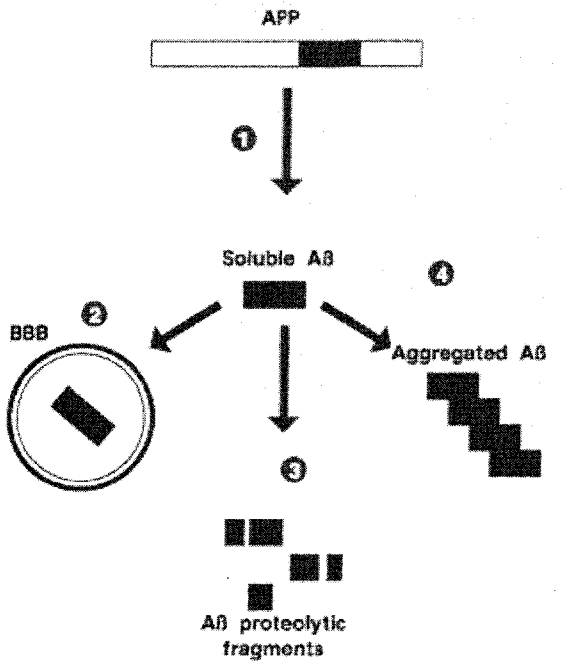

Figure 1. Schematic representation of Aβ pathways in the brain. 1) Aβ is released from APP by β and γ secretases; 2) soluble Aβ can be transported to the systemic circulation across the blood-brain-barrier (BBB) or along perivascular drainage pathways; 3) Aβ can be degraded by proteases in the intracellular or extracellular compartments. 4) Aβ that escapes from pathways 2 and 3 accumulates as aggregated species.

2. Aβ DEGRADATION PATHWAYS

The proteolytic degradation of soluble Aβ in the brain is believed to take place both in intracellular and extracellular compartments. Various mechanisms of Aβ internalization into neurons and glial cells have been described, including non-saturable uptake as well as receptor-mediated

endocytosis by the serpin-enzyme complex, the LDL-receptor related proteins (LRP-1 and LRP-2), scavenger receptors and the receptor for end glycation products (RAGE) (reviewed in Morelli *et al.*, 2002). The LRP pathway mediates the internalization of several ligands including apolipoprotein E, α-2 macroglobulin and APP, all related to the pathogenesis of AD, with LRP-1 being expressed mainly in neurons and LRP-2 in choroids plexus and ependymal cells. As a result of internalization, Aβ may be degraded by lysosomal proteases including cathepsins D, B and S, which have been shown to hydrolyze Aβ *in vitro* (Hamazaki, 1996; Liuzzo *et al.*, 1999; Gupta-Bansal *et al.*, 1995). In support of this mechanism, chloroquine treatment of cultured cells inhibited the degradation of internalized Aβ by the LRP receptor and co-infusion of synthetic Aβ with leupeptin in the rat brain resulted in the intra and extracellular accumulation of Aβ (Hammad *et al.*, 1997; Frautschy *et al.*, 1998). In AD brains, the high expression of lysosomal hydrolases in vulnerable neurons and extracellularly, in close association with Aβ deposits suggests a significant activation of the lysosomal system in the course of the disease (Cataldo and Nixon, 1990). Moreover, mice with an 80% reduction in LRP levels due to genetic disruption of its ligand RAP, when crossed with mice expressing mutant human APP show a significant increase in extracellular Aβ deposition, reinforcing the role of the LRP pathway in Aβ clearance (Van Uden *et al.*, 2002).

In addition, several lines of evidence support a role for the plasmin system in the degradation of Aβ. Plasminogen, tissue (tPA) and urokinase-like plasminogen activators (uPA) are synthesized by neurons, with high levels in the hippocampus (Chen and Strickland, 1997). Plasmin degrades Aβ *in vitro*, and a recent study in two models of APP transgenic mice showed that the plasmin system was compromised long before the onset of detectable amyloid deposition, further suggesting the importance of a degradative failure in this process (Melchor *et al.*, 2003). Moreover, an important reduction in the levels of plasmin and plasminogen found in the neocortex and hippocampus of AD patients raises the possibility that an initial up-regulation of the system may become exhausted in the course of the disease (Ledesma *et al.*, 2000). Other peptidases that may participate in the extracellular degradation of Aβ are matrix metalloproteinases (MMPs). MMP-9 has been detected in pyramidal neurons in human hippocampus, is found in neuritic plaques and seems to be overexpressed in AD brains as compared to controls (Backstrom *et al.*, 1996). MMP-9 can degrade Aβ *in vitro* and species ending at Gly_{37}, consistent with its activity, have been found in amyloid extracted from leptomeningeal and cortical vessels. Active forms of MMP-2 are capable of degrading Aβ1-40 and Aβ1-42 purified from vascular amyloid and treatment of primary hippocampal cultures with

synthetic Aβ increased the production of MMP-2, MMP-9 and MMP-3 (Deb *et al.*, 1996; 1999).

In addition to the proteolytic systems mentioned above, that may participate in the degradation of Aβ under pathological conditions, recent compelling evidence indicates that a group of zinc-metallopeptidases including neprilysin, endothelin-converting enzymes and insulin-degrading enzyme may regulate the steady state levels of Aβ in the mammalian brain (Table I).

Table I. Major Aβ-degrading proteases

Protease	Type	Cellular localization	Overexpression (transfected cells)	Knock out mice
Neprilysin	Neutral, Zn-metallo	Plasma membrane	↓ Aβ (EC,IC)	↑ Aβ* (gene-dose effect)
Endothelin - Converting Enzymes	Neutral / acidic Zn-metallo	Plasma membrane, Internal membranes	↓ Aβ (EC)	↑ Aβ* (gene-dose effect)
Insulin-Degrading Enzyme	Neutral, Zn-metallo	Cytosol, Membranes, Peroxisomes, Secreted	↓ Aβ (EC,IC)	↑ Aβ* (gene-dose effect)

EC, extracellular; IC, intracellular, * As determined in brain tissue homogenates

3. NEPRILYSIN

Neprilysin is also known as neutral endopeptidase (NEP), EC3.4.24.11, enkephalinase, CD10 and common acute lymphoblastic leukaemia antigen (CALLA). NEP is a widely expressed zinc metallopeptidase with a HEXXH active motif that is strongly inhibited by thiorphan and phosphoramidon, a property shared with other members of the M13 subfamily (reviewed in Turner and Tanzawa, 1997). NEP is a type II integral membrane protein with a large lumenal-extracellular domain that contains the active site (Oefner *et al.*, 2000). Such topology allows NEP to hydrolyse several peptides at the cell surface, including enkephalins, substance P, atrial

natriuretic peptide, somatostatin, endothelin and insulin B chain. Within the CNS, NEP predominates in synaptic terminals of the neuropil in which it may participate in terminating the action of neuropeptides (Schwartz *et al*, 1980; Turner *et al.*, 2001). Early reports on the possible relationship of NEP with Aβ metabolism included the staining of AD senile plaques with a monoclonal anti-NEP and the degradation of synthetic Aβ1-40 *in vitro* by NEP purified from rabbit kidney (Sato *et al.*, 1991; Howell *et al.*, 1995). More recently, it was shown that synthetic radiolabeled Aβ1-42 injected into the rat hippocampus was degraded by a thiorphan-sensitive NEP-like activity. Remarkably, the continuous infusion in these animals of thiorphan for several weeks resulted in the accumulation of endogenous Aβ42 in the form of Congo red-negative deposits that resembled diffuse plaques in the cortex and hippocampus (Iwata *et al.*, 2000). Mice with disruption of the NEP gene have a lower capability in degrading injected Aβ in a gene-dose dependent manner. Moreover, the accumulation of endogenous Aβ is highest in the hippocampus and lowest in the cerebellum, in parallel with the severity of pathology in AD and transgenic mice models of AD (Iwata *et al.*, 2001). In the human brain, NEP distribution shows an inverse correlation with the vulnerability to Aβ deposition, including a low expression in hippocampal neurons and smooth muscle cells in the microvasculature affected with amyloid angiopathy (Yasojima *et al.*, 2001; Carpentier *et al.*, 2002). The potential anti-amyloidogenic effects of NEP *in vivo* has been recently assessed by the injection of a lentiviral vector expressing this gene into the CNS of APP transgenic mice that develop amyloid deposition. A higher expression of NEP in the injected hemi-brain correlated with smaller plaques, a reduction of approximately 50% in Aβ burden and a mild improvement in MAP-2 dendritic staining that suggested less neuronal damage (Marr *et al.*, 2003).

4. ENDOTHELIN-CONVERTING ENZYMES

Endothelin-converting enzymes (ECEs) are type II integral membrane zinc metalloproteases belonging like NEP, to the M13 subfamily of mammalian neutral endopeptidases. One major physiologic role of ECEs is the cleavage of big endothelin to generate the potent vasoconstrictor peptides known as endothelins (Turner *et al.*, 1997). Several isoforms of ECEs have been identified, namely ECE-1, ECE-2, and ECE-3. Within each of the first two groups, several sub-isoforms derived from the splicing of single genes have also been determined (reviewed in D'Orleans-Juste *et al.*, 2003). ECE-1 isoforms are mainly located on the plasma membrane and widely expressed in endothelial cells and non-vascular cells, including neurons and astrocytes

in the CNS. ECE-2 isoforms, that predominate in intracellular membranes, also have a ubiquitous expression with high levels in the brain, (reviewed in Davenport *et al.*, 2000; Eckman *et al.*, 2001). Recently, Eckman *et al.* (2001) reported that treatment of cell lines that express ECE with phosphoramidon caused a 2-3-fold increase in extracellular Aβ steady-state levels and that overexpression of ECE-1 in CHO cells, which lack endogenous ECE activity, reduced dramatically extracellular Aβ concentration. Moreover, a recombinant soluble form of ECE was capable of cleaving synthetic Aβ1-40 at the amino- side of Leu_{17}, Val_{18} and Phe_{19}, consistent with the known preference of ECEs for hydrophobic residues at P1'. ECE-1 homozygous knock out mice have severe cardiac and cranial abnormalities that result in embryonic lethality, however, ECE-1 (+/-) and ECE-2 (-/-) animals are viable and healthy. The study of mice with these genotypes at 3-4 weeks of age showed a mild (20-30%) though significant increase in Aβ levels in a soluble fraction of brain homogenates. Interestingly, ECE-2 deficiency showed a gene-dose effect on Aβ accumulation (Eckman *et al.*, 2003). These results strongly support a key participation of ECEs in the physiological catabolism of Aβ in the mammalian brain.

5. INSULIN-DEGRADING ENZYME

Insulin-degrading enzyme (IDE) or Insulysin is a thiol zinc-metallopeptidase that belongs to the "inverzincin" family, name that describes an inverted active sequence HXXEH as compared to the typical zinc peptidases such as thermolysin, NEP or ECEs (Becker and Roth, 1992). IDE is highly conserved in evolution and has a ubiquitous expression including high levels in testis, ovary, kidney, liver, red cells, pancreas, muscle and brain (Kuo *et al.*, 1993). IDE has been implicated in broad physiological roles, such as cellular growth and differentiation, the modulation of proteasomal activity and steroid signaling. Although it is mainly found in the cystosol and peroxisomes, IDE has been also located in active form at the surface of many cell types, including neurons (reviewed in Duckworth *et al.*, 1998; Vekrellis *et al.*, 2000). Moreover, a secreted form of the protease has been found in the conditioned media of different cell lines and primary cultures (Safavi *et al.*, 1996; Gasparini *et al.*, 2001). The biochemistry of the association of IDE to membranes and the mechanism of its secretion remain to be studied since IDE seems not to have a typical signal peptide and no isoforms or post-translational modifications have been described so far.

5.1 IDE: an amyloid-degrading protease

IDE is known to degrade several peptides, many of which have a propensity to form amyloid fibrils *in vitro* and *in vivo* including insulin, glucagon, amylin, atrial natriuretic peptide, calcitonin and Aβ (reviewed in Duckworth *et al.*, 1998). Although IDE has no sequence specifity, it shows some preference for basic or hydrophobic residues on the carboxyl-side of the scissile bonds, suggesting a recognition of secondary or tertiary structure shared by amyloid-forming peptides. Aβ degradation by IDE was first shown with synthetic peptides and purified protease and subsequently demonstrated in a variety of cultured cells and brain homogenates (Vekrellis *et al.*, 2000; Kurochkin and Goto, 1994; Sudoh *et al.*, 2002; McDermott and Gibson, 1997; Perez *et al.*, 2000). With regard to the effect of Aβ oligomerization upon IDE activity and specificity, it has been proposed that IDE is capable of degrading monomeric Aβ more efficiently than oligomeric Aβ species, which are thought to be toxic to neurons or vascular cells (Walsh *et al.*, 2002). We have recently shown, in support of such notion, that a recombinant rat IDE degraded very efficiently Aβ monomers while SDS-resistant dimers were almost completely resistant to proteolysis (Figure 2).

IDE may recognize a motif on monomeric Aβ within positions 18-22, a hydrophobic stretch that is critical for amyloid formation (McDermott and Gibson, 1997; Soto *et al.*, 1995). It is possible that when these residues participate in peptide self-assembly, they are no longer accessible to IDE catalytic site. To confirm the effect of Aβ dimerization upon proteolysis, the study of peptide species isolated under native conditions is needed, yet, the possibility that Aβ dimers exist at physiological concentrations in the brain (Garzon-Rodriguez *et al.*, 1997) raises an important question about Aβ catabolism with potential therapeutic implications.

Recently, mice with targeted deletions of the IDE gene have been generated and characterized (Farris *et al.*, 2003; Miller *et al.*, 2003). Brain membranes and soluble fractions isolated from IDE (-/-) animals showed a significant decrease in synthetic Aβ degradation. Moreover, endogenous brain levels of Aβ were increased, with an inverse correlation with IDE activity (Miller *et al.*, 2003). Interestingly, the loss of activity of IDE in this animal model also resulted in the accumulation of an unphosphorylated form of the carboxyl-terminal domain of APP, hyperinsulinemia and glucose intolerance (Farris *et al.*, 2003).

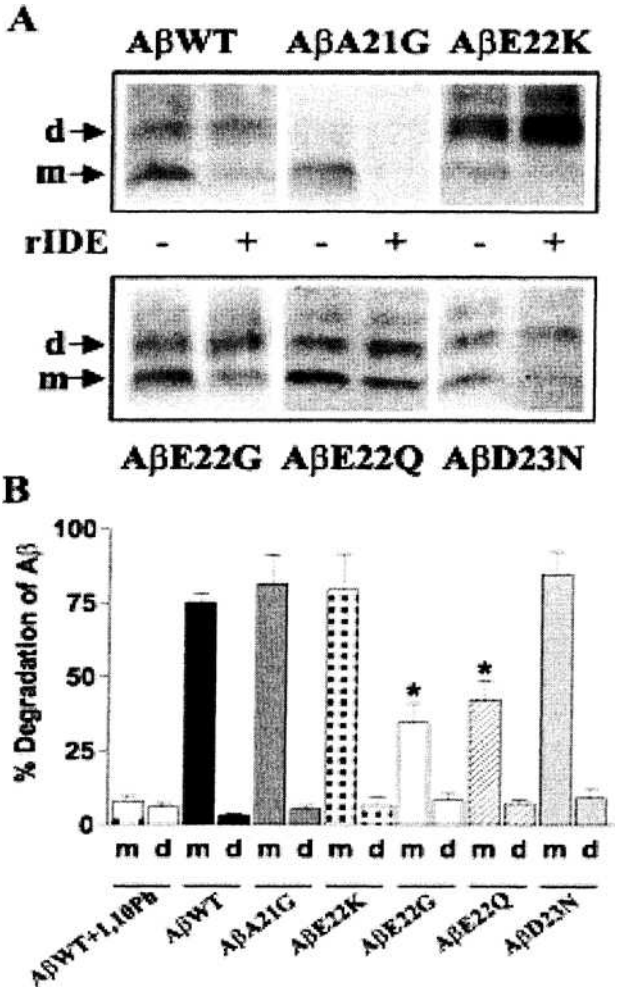

Figure 2. Degradation of Aβ variants by recombinant rat IDE. Panel A, western blot analysis with monoclonal 6E10 of degradation of AβWT and Aβ genetic variants by rIDE. m, Aβ monomers; d, Aβ dimers. Panel B, densitometric quantitation of the immunoreactivity of Aβ as in panel A. m, monomers; d, dimers. Bars represent the mean ± SE of three independent experiments. *, $p<0.01$ (Student's *t* test) as compared with AβWT. From Morelli *et al.*, 2003, by permission of the American Society for Biochemistry and Molecular Biology, Inc.

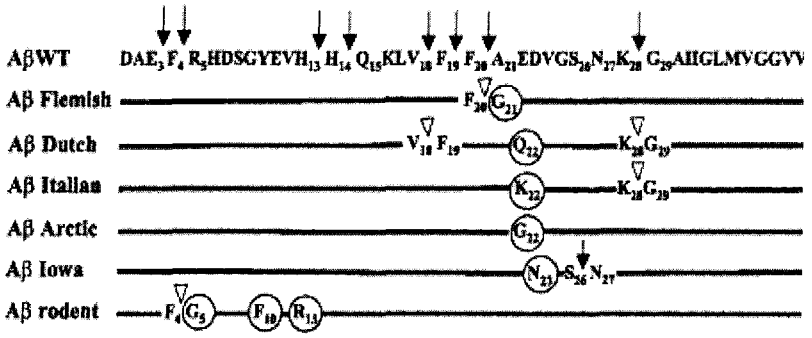

Figure 3. Schematic representation of cleavage sites within AβWT, Aβ rodent, and Aβ human genetic variants by rIDE. *Arrows* show the peptide bonds hydrolysed by rIDE. *Open arrowheads* (∇) indicate loss of cleavage under the experimental conditions tested. The *lines* represent identity of amino acid sequence and cleavage sites. Amino acid substitutions in each Aβ variant are shown in *circles*. From Morelli *et al.*, 2003, by permission of the American Society for Biochemistry and Molecular Biology, Inc.

5.2 IDE degrades Aβ genetic variants

A group of autosomal dominant mutations in the APP gene result in amino acid substitutions at positions 21, 22 or 23 of Aβ sequence. Although these Aβ variants present with a primarily vascular deposition, they translate into different clinical phenotypes. Aβ Arctic (E22G) and Aβ Iowa (D23N) are characterized by presenile dementia, Aβ Flemish (A21G) is associated with early onset dementia and cerebral hemorrhage, while Aβ Dutch (E22Q) and Aβ Italian (E22K) variants have a predominant vascular phenotype characterized by massive strokes (reviewed in Miravalle *et al.*, 2000). We have recently studied the degradation of Aβ WT and Aβ variants by recombinant IDE. Monomers of AβWT, AβA21G, AβE22K and AβD23N were readily degraded with an apparent similar efficiency. In contrast, AβE22Q and AβE22G monomers were substantially more resistant to proteolysis by rIDE (Figure 2). More studies are needed to clarify the mechanism of such resistance, including the influence of Aβ oligomerization and the kcat/Km for each Aβ species at low concentrations to minimize aggregation. Interestingly, amino acid substitutions did not affect substantially IDE specificity, with the exception of minor changes in the sites of cleavage in the Flemish and Iowa variants (Figure 3). These results support the concept that up-regulation of IDE activity may promote the removal of soluble Aβ in hereditary forms of Aβ diseases.

5.3 A pathogenic role of IDE in AD?

An early study by our group suggested that IDE activity was significantly reduced in a soluble fraction obtained from the temporal cortex of AD patients as compared to age-matched controls (Perez *et al.*, 2000). Subsequently, Cook *et al.* (2003) have shown that a decrease in IDE expression was more pronounced in the hippocampus in late-onset AD patients carrying the APOE ε4 allele. Genetic linkage studies indicate that a locus on chromosome 10q within 195 kilobases of the IDE gene is associated with late-onset Alzheimer's disease (LOAD) (Bertram *et al.*, 2000; Myers *et al.*, 2000; Ertekin-Taner *et al.*, 2000; Abraham *et al.*, 2001). So far, the analysis of all the coding exons, untranslated regions, and two intronic polymorphisms of the IDE gene has failed to reveal association with AD (Prince *et al.*, 2003; Boussaha *et al.*, 2002). However, a more recent study of five IDE polymorphisms stratified by APOE genotype raised the possibility that the lack of a minor IDE haplotype may be predictive of AD in APOE ε4-negative individuals (Edland *et al.*, 2003). Although these results need confirmation with larger numbers of AD patients and controls, they raise the interesting possibility that IDE expression levels are related to the APOE genotype. Moreover, together with biochemical and functional data, they suggest that IDE may participate in the pathogenesis of LOAD.

6. FUTURE PERSPECTIVES

The recent and significant advances on our knowledge about Aβ degradation raise the possibility that in the near future brain proteases may be manipulated to promote Aβ clearance. However, several aspects of Aβ metabolism remain to be clarified, both in normal conditions and disease. It is yet not clear which is the main compartment for Aβ degradation in the brain and whether the initial Aβ oligomerization takes place intra or extracellularly. The apparent redundancy in Aβ proteolysis suggests that the process is ubiquitous, with Aβ degradation taking place, at a given time, in different compartments and by different peptidases. Yet, protease efficiency may be strongly dependent upon Aβ oligomerization. This process is influenced, in turn, by the primary structure of the peptide, postranslational modifications, or interaction of Aβ with other proteins. Moreover, several aspects related to protease manipulation need to be carefully addressed in animal models. The list includes the impact on basic aspects of brain physiology such as insulin signaling, neuropeptide transmission, blood flow, clot formation or extracellular matrix integrity. As an example, fibrinolytic therapy of myocardial infarction with tPA has been associated with cerebral

hemorrhage in patients with SCAA (Wijdicks and Jack, 1993), thus, the pharmacological activation of plasmin may be particularly problematic in this group of patients. Several proteolytic systems seem to be chronically stimulated in AD brains and therefore, increasing their expression or activity may have little impact on Aβ metabolism. On the other hand, the promotion of the activity of proteases that are thought to be decreased in AD such as NEP or IDE may have profound effects on the steady-state levels of soluble Aβ. With the transgenic animal models currently available, the proteases that degrade Aβ can be up-modulated and the consequences upon Aβ levels and its purported toxicity assessed *in vivo* (Marr *et al.*, 2003). These experiments may be decisive to clarify the potential of treatments based on Aβ degradation for the management of AD and related disorders.

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