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Key Enzymes and Proteins in Amyloid-Beta Production and Clearance

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1. Introduction

Last century, in 1907, Alois Alzheimer first described the disease that now bears his name, relating a 54-year-old female case with presenile dementia. Alzheimer's disease (AD), an irreversible progressive neurodegenerative disorder, is actually the most common form of dementia. AD affects more than 24 million people all over the world, and is predicted to double every 20 years, becoming one of the medical burdens of our days. More than 90% of AD cases are sporadic late-onset, and have a complex idiopathic aetiology. The small number of early onset AD cases, is related to hereditary monogenic defects, and has provided important clues for understanding the AD pathology (Bertram, et al. 2007). Although the available drugs are able to delay the symptoms and progression of the disease and to positively influence the quality of life of the patients, at present there is still no cure for AD.

AD is characterized clinically by progressive decline in cognitive function and neuropatologically by the presence of neuropil threads and neuron loss, in addition to the molecular hallmarks of neurofibrillar tangles and neuritic (or senile) plaques in the brain. Neuritic plaques are extracellular amyloid deposits found abundantly in the hippocampus, in the neocortex and in the amygdala of AD brains. These pathological brain changes may occur 20 to 30 years prior to the onset of the clinical symptoms and the symptomatic phase of AD can last from 5 to 12 years (DeKosky and Marek 2003). The extracellular neuritic plaque deposits of amyloid were first investigated by (Glenner and Wong 1984), when they purified microvascular amyloid deposits from AD brains, and provided a partial sequence of a 4kDa subunit protein, that they named amyloid-beta (Aβ) peptide. Around the same time, the hyperphosphorylated tau (p-tau), a microtubule assembly protein, was identified as the main constituent of the neurofibrillar tangles (NFTs) that accumulate inside many neurons in AD brains (Grundke-Iqbal, et al. 1986). Amyloid deposition and neurofibrillar tangles, occur with some frequency in brains of young adults with Down's syndrome (Schochet, et al. 1973). The discovery that amyloid deposits in the brain from Down syndrome were composed of Aβ peptide (Glenner and Wong 1984), as well as the cloning of the beta amyloid precursor protein (APP), with its localization to the chromosome 21

(Korenberg, et al. 1989), led the scientists to search AD-causing mutations in the APP gene. Since that, several mutations associated with familial early onset forms of AD have been described, either in the APP gene (Kowalska 2003) or in preselin 1 (PS1) or preselin 2 (PS2) genes (Bertram et al. 2007). Either PS1 or PS2 can be the catalytic subunit of γ-secretase, which is the final endoprotease in the pathways that generate the Aβ peptide (see section 2). All these findings led to the amyloid cascade hypothesis, articulated by John Hardy and others (Hardy and Higgins 1992), in which the accumulation of Aβ peptide, generated from the proteolytic cleavage of APP in the brain, could trigger a complex downstream cascade that results in the symptoms of AD. This hypothesis states that gradual accumulation and aggregation of the hydrophobic Aβ peptide initiates a cascade that leads to synaptic alterations, astrocytic and microglial activation, the modification of the soluble tau protein into insoluble paired helical filaments, and progressive neuronal loss associated with multiple neurotransmitter deficiencies and cognitive failure (Hardy and Selkoe 2002). The cascade hypothesis suggests that stopping or slowing formation of the Aβ plaques would delay the onset of the disease symptoms. Aβ is found in the extracellular fluids of the brain, including cerebrospinal fluid (CSF), and in the interstitial fluid surrounding neurons and glial cells in brain lobes (Seubert, et al. 1992; Vigo-Pelfrey, et al. 1993). Over the last years, several key proteins have been described as being implicated in Aβ production and clearance, but further elucidation of the mechanisms involved in the process will be important for identifying new potential therapies to reduce Aβ accumulation and combat AD. This book chapter reviews the production of Aβ from APP and the proteins involved in its degradation and clearance .

2. Generation of amyloid beta peptides

The β amyloid precursor protein, APP, takes a central position in AD pathogenesis, as it is processed by the sequential action of β- and γ-secretase, generating the Aβ peptide, which is deposited as amyloid plaques in brains of AD individuals. APP is an integral membrane protein, with a large N-terminal extracellular domain and a short C-terminal cytoplasmatic domain, which is expressed ubiquitously in neuronal and non-neuronal cells.
The human APP gene is located on chromosome 21 (Korenberg et al. 1989) and alternative splicing results in protein isoforms of various lengths: two isoforms predominant in non-neuronal tissues (751- and 770-), and the 695-amino acid form, that is the predominant isoform in neurons (Kang and Muller-Hill 1990). APP belongs to a protein family that includes APP-like protein 1 (APLP1) and 2 (APLP2) (Eggert, et al. 2004), a group of type-I transmembrane proteins that are processed in the same fashion. APP is hydrolyzed into different fragments (Figure 1) during its intracellular trafficking, and these metabolites mediate various functions (Haass 2004; Haass and Selkoe 1993). APP is first cleaved by either α- or β-secretase at the α- or β-sites, respectively, which lie in the extracellular domain of the APP. These proteases compete for APP, originating: soluble APPα (sAPPα, for α-secretase) or soluble APPβ (sAPPβ, for β-secretase), which are released to the extracellular space, and a membrane anchored C-terminal end (C83 for α-secretase or C99 for β-secretase). Subsequently, in the lipid bilayer, γ-secretase acts in the C-terminal end, C83 or C99. The γ-cleavage of C83 generates the APP intracellular domain (AICD), with 6kDa, and the N-terminal peptide with 3kDa (p3) into the extracellular space. γ-cleavage of C99, in a specific sequence (Aβ domain) generates Aβ peptide and the AICD. This pathway of APP processing by β-secretase followed by γ-secretase leading to Aβ peptide is called the

amyloidogenic pathway. Aberrant and/or cumulative Aβ production, have been postulated to be the main etiological basis of AD. The alternative pathway of APP processing by α-secretase followed by γ-secretase, in which no Aβ is formed, is termed nonamyloidogenic pathway.
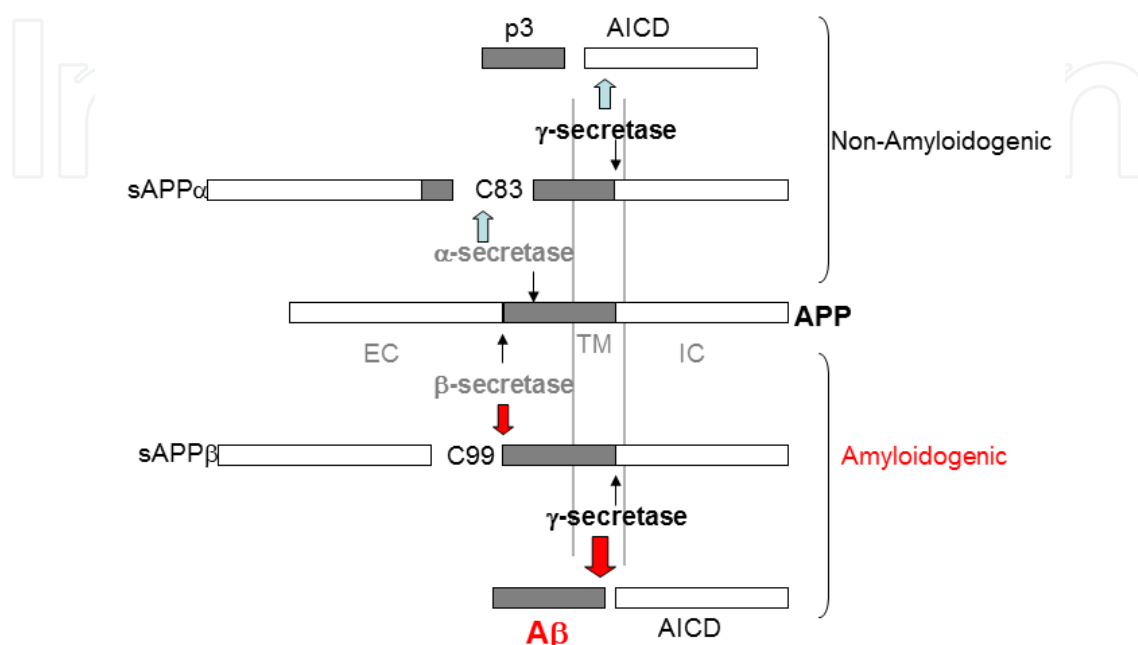

Fig. 1. Schematic diagram of APP processing (not drawn in scale). TM-transmembrane;EC-extracelular; IC- intracellular

Aβ is a ~4kDa peptide with 38 to 43 amino acids, depending on the site of γ-secretase cleavage. Aβ peptide is normally produced by cells and under physiological conditions there are two major species: Aβ40 and Aβ42. The major species produced is Aβ40 and corresponds to 90% of the total Aβ peptide. The minor species produced, Aβ42, is more prone to aggregation due to two additional hydrophobic amino acids, and therefore it is the predominant species accumulated in AD brain plaques.

A number of proteins influence the subcellular trafficking itinerary of APP and β-secretase between the cell surface, endosomes and the Trans-Golgi-Network (TGN). APP is synthesized in the endoplasmic reticulum (ER) and is transported through the Golgi apparatus to the TGN, where the highest concentration is found in steady state neurons (Greenfield, et al. 1999). From TGN, the APP can be transported, in TGN-derived vesicles, to cell surface where it is either cleaved by α-secretase to produce sAPPα, or internalized via the endosomal-lysosomal pathway (Caporaso, et al. 1994). The Aβ peptide is generated either in the ER and TGN (Greenfield et al. 1999), as in the endosomal/lysosomal system (Haass and Selkoe 1993). Available evidence suggests that co-residence of APP and β-secretase in the endosomal compartments promotes amyloidogenesis. Retrograde transport of APP, out of the endosome to the TGN, reduces Aβ production, while APP routed to, and kept at the cell surface, enhances its non-amyloidogenic processing (Tang 2009).

Very little is known about the physiological function of APP and its proteolytic products. APP knockout mice (KO) are viable and fertile, showing a slight abnormal phenotype

(Dawson, et al. 1999). APLP1 and APLP2 KO mice are also viable and fertile, but APP/APLP2 and APLP1/APLP2 double null mice and APP/APLP1/APLP2 triple null mice show early postnatal lethality (Heber, et al. 2000; von Koch, et al. 1997). APP/APLP1 double null mice are viable, suggesting redundant functions of amyloid precursor protein family members (Heber et al. 2000). Putative suggested roles for APP include trafficking, neurotrophic signalling, control of cell adhesion, neuritic outgrowth and synaptogenesis, apoptosis and transcription regulation (Zheng and Koo 2006). As APP is proteolysed in the cell, the net effect of full-length APP on cellular activity may be a combination of the function of its proteolytic products, depending on the proportion levels of each of them.

2.1 Alpha-secretase

APP is cleaved by α-secretase in the center of Aβ domain, precluding Aβ peptide generation, and a soluble domain of APP is released: sAPPα. Three related proteases, all from the ADAM family, had been suggested to exert the α-secretase activity: ADAM-9, ADAM-10 and ADAM-17 (Asai, et al. 2003). Like full length APP, members of the ADAM family (a desintegrin and metalloprotease family) are type-I transmembrane proteins, possessing both potential adhesion and protease domains. Several studies suggested α-secretase activity for ADAM-9. However, as RNAi of ADAM-9 has no effect in sAPPα generation (Kuhn, et al. 2010), ADAM-9 seems to be involved only in regulated α-cleavage and not in constitutive α-secretase activity. Different studies strongly suggest that ADAM-10 is the main constitutive α-secretase that is active at the cell surface (Lammich, et al. 1999) (Jorissen, et al. 2010), but there may exist some functional redundancy in α-cleavage by the ADAM protease family. ADAM-17 is an 824 amino acid polypeptide containing a secretory signal sequence, a desintegrin domain and a metalloprotease domain, that also seems to be involved in regulated α-secretase activity (Merlos-Suarez, et al. 2001).

sAPPα has important roles in neuronal plasticity/survival, stem cell proliferation in CNS, and is able to rescue the abnormalities of APP deficient mice, indicating that most of APP´s physiological function is mediated by sAPPα (Zhang, et al. 2011).

2.2 Beta-secretases: BACEs

β-secretase cleavage is the first critical step in the APP amyloidogenic pathway, and increased β-secretase activity levels have been correlated with brain Aβ deposition in late onset AD patients (Li, et al. 2004). Aβ peptide is generated from APP by a sequential two-step proteolytic process involving β- and γ-secretases (Haass 2004), being BACE 1 (β-site APP cleaving enzyme 1) the major β-secretase in the cell (Vassar, et al. 1999). BACE1 is a member of the pepsin family of aspartyl proteases, and its activity on APP generates the membrane bound C-terminal fragment (CTFβ or C99). BACE1 requires acidic environment for optimal activity and cleaves APP at the known β-site locations, Asp1 and Glu11. Overexpression of BACE1 induces cleavage of APP at β-sites and is mainly found in the early Golgi, late Golgi/early endosomes and endosomes with acidic environment. BACE1 is also found at the cell surface (Huse, et al. 2002; Vassar et al. 1999).

BACE2 is an additional β-secretase, mapped in 21q22.3 region (Solans, et al. 2000), that also cleaves β-secretase substrates. However, BACE2 expression in neurons is lower than BACE1 (Bennett, et al. 2000). BACE1 null mice died in the first weeks and those that survived, were smaller, presented hyperactive behaviour, were affected by hypomyelination of peripheral nerves and had altered neurological behaviours such as elevated pain sensitivity

(Dominguez, et al. 2005). BACE2 KO mice are healthy overall, while a deficiency of both BACE1 and BACE2 enhanced the BACE1 KO lethality phenotype, suggesting functional redundancy (Dominguez et al. 2005).

Cathepsin B has been proposed as another β-secretase. Although its inhibition has been found to reduce Aβ production, its physiological activity is not well established (Hook, et al. 2009).

2.3 The gamma secretase complex

γ-secretase is a protein complex, of high molecular weight, responsible for the membrane cleavage of the APP C-terminal remnants after cleavage by either α- or β-secretase (C83 and C99, respectively). The cleavage of C83 and C99 by γ-secretase generates p3 and the Aβ peptide, respectively. In addition to C83 and C99 peptides, several non-APP substrates are cleaved by γ-secretase and all of them are type-I transmembrane proteins that require ectodomain shedding as a prerequisite to γ-secretase cleavage (Haapasalo and Kovacs 2011). γ-secretase complex comprises four core components which include: presenilin (PS1 or PS2), nicastrin, anterior pharynx-defective-1 (APH-1), and presenilin enhancer-2 (PEN2) (Kimberly and Wolfe 2003; Takasugi, et al. 2003). All of these four core components of γ-secretase are necessary for the enzymatic activity of the complex. PS1 and PS2 are two presenilin homologs, and several mutations in the corresponding genes have been described as the major cause of familial AD cases (Bertram et al. 2007). Different kinds of experimental evidences suggest that presenilins are transmembrane proteins with crucial catalytic roles in γ-secretase activity (Zhang et al. 2011). Nicastrin, other component of γ-secretase, is a type-I transmembrane glycoprotein considered the scaffolding protein of the complex (Vassar and Citron 2000). Finally, the other two components of γ-secretase are: APH-1, that interacts with nicastrin to form a stable intermediate in the early assembly stages of the complex and PEN-2, that regulates PS endoproteolysis (Vassar and Citron 2000). Besides the four components of γ-secretase complex, some factors, playing a modulatory role, have been described. Example of that is CD147 that when down regulated increases Aβ production and TMP21/p23 that regulates γ-cleavage (Zhang et al. 2011).

Experimental evidences support the idea that γ-secretase resides in ER, Golgi-TGN, endosomes and intermediate compartments, most of which (except TGN) do not correspond to the main localizations of APP (Cupers, et al. 2001).

3. Enzymes and peptides involved in amyloid-beta degradation and clearence

The clearance of Aβ in the human central nervous system (CNS), is roughly 8% of total production per hour. It is controlled by Aβ degradation in the brain and by its efflux from the CNS to the peripheral circulation through the blood-brain barrier or the blood-cerebrospinal fluid barrier (Zlokovic 2004). The high clearance of Aβ is in part due to the presence of cryptidases in several cellular compartments and reduction of Aβ degradation by these enzymes may be implicated in the progression of AD cases. Aβ accumulation, and the concomitant formation of amyloid plaques observed in AD brains occurs in the extracellular space. It is also in this compartment, that Aβ can be degraded by cell surface and/or secreted cryptidases, such as insulin degrading enzyme and neprylisin. The subcellular distribution of Aβ degrading cryptidases is a strong indicator that Aβ degradation can be controlled at multiple subcellular compartments, such as the mitochondria, ER, Golgi, endosomes and lisossomes (Malito, et al. 2008).

In the brain, Aβ metabolism is mainly regulated by the activity of neprilysin (NEP) and insulin-degrading enzyme (IDE), but presequence peptidase, endothelin converting enzyme (ECE), angiotensin-converting enzyme (ACE), the uPA/tPA- plasmin system and matrix metalloproteinases are also involved in the process (Table 1). In addition, some proteins as transthyretin (TTR), gelsolin, alpha2-macroglobulin and apolipoprotein E do also play an important role in Aβ clearance and degradation. Some of them do actually have a direct function in Aβ catabolism such as TTR, plasmin, and gelsolin, which have the capacity to cleave the peptide, others because their interaction with Aβ enable its degradation or prevent its neurotoxicity as it is the case of alpha-2-macroglobulin and apolipoprotein E (Table 2).

Enzymes	Function	Brain distribution	References
IDE	Hydrolises peptide bonds of Aβ40 and Aβ42	Neurons, microglia, endothelial cells, choroid plexus epithelial cells	(Bora et al. 2010; Bora and Prabhakar 2010; Malito et al. 2008)
NEP	Hydrolises peptide bonds of Aβ42	Neurons, microglia, endothelial cells, choroid plexus epithelial cells	(Malito et al. 2008; Meilandt et al. 2009)
PreP	Hydrolises peptide bonds of Aβ40 and Aβ42 in mitochondria		(Falkevall et al. 2006)
ECE	Hydrolises peptide bonds of Aβ40 and Aβ42	Neurons, astrocytes, endothelial cells	(Miners et al. 2008b)
ACE	Converts Aβ42 to Aβ40 Cleaves Aβ40	Neurons, endothelial cells, choroid plexus epithelial cells	(Miners et al. 2008a; Zou et al. 2007)
Plasmin	Degrades aggregated and non-aggregated Aβ40 and Aβ42 Inhibits Aβ fibrillogenesis	Neurons, microglia	(Tucker et al. 2002; Tucker et al. 2000b)
MMP-2	Hydrolises peptide bonds of Aβ40 and Aβ42 Beta-secretase activity	Microglia, astrocytes, Schwann cells	(Miners et al. 2008a)
MMP-9	Hydrolises peptide bonds of Aβ40 and Aβ42 and aggregated Aβ fibrils	Neurons, endothelial cells	(Miners et al. 2008a; Yan et al. 2006)

Table 1. Functions and brain distribution of key enzymes in Aβ clearance.

Key players	Function	Brain distribution	References
TTR	Hydrolises peptide bonds of Aβ40 and Aβ42, and aggregated Aβ oligomers and fibrils	choroid plexus epithelial cells meninges	(Costa et al. 2008; Schwarzman et al. 1994)
Gelsolin	Inhibits fibrillization of Aβ and defibrillates preformed fibrils	Oligodendrocytes, microglia, choroid plexus epithelial cells	(Chauhan et al. 2008; Ray et al. 2000)
Alpha-2 macroglobulin	Aβ carrier, binds Aβ protecting it from proteolysis, reduces Aβ aggregation and fibril formation	astroglia	(Du et al. 1998; Du et al. 1997)
Apo E	Binds Aβ enhancing the proteolytic activity of NEP, and IDE	Astrocytes, neurons, microglia	(Jiang et al. 2008)
MT-2	Diminishes Aβ binding to TTR Prevents copper mediated aggregation of Aβ	Cortical neurons	(Chung et al. 2010; Martinho et al. 2010)
MT-3	Increases Aβ binding to TTR Inhibits formation of Aβ aggregates	Cortical neurons	(Irie and Keung, 2001, 2003; Martinho et al. 2010)

Table 2. Functions and brain distribution of key proteins in Aβ clearance.

3.1 IDE – Insulin degrading enzyme

Insulin degrading enzyme (IDE) is a ~ 110 kDa zinc-containing metalloendopeptidase that degrades monomeric forms of Aβ peptides and insulin, which has also high nanomolar affinity for other substrates with different sequences and structures. The common features of IDE substrates are that they are all amyloidogenic in nature. So, besides Aβ and insulin, IDE also cleaves, insulin-like growth factor 2, atrial natriuretic peptide, bradykinin, endorphin, and glucagon. However, *in vivo* relevance of this degrading activity has only been demonstrated for Aβ and insulin (Malito et al. 2008). Interestingly, patients with type 2 diabetes are under an increased risk of AD. In addition, Aβ is a direct competitive inhibitor of insulin binding and action, and this is a likely justification for the increased levels of Aβ observed in insulin resistant AD patients (Xie, et al. 2002). This dual effect of IDE stresses the importance of the development of inhibitors and activators of its activity for the treatment of diabetes and AD, respectively.

The first evidences of the capacity of IDE to degrade Aβ were reported by Kurochkin and Goto (1994) who identified a protein of 110,000 Da present in cytosol fractions from rat brain and liver that cross-linked to ^{125}I-labeled synthetic Aβ. A few years later IDE was actually identified as the main soluble Aβ degrading enzyme in human brain and in neuronal cell cultures, where its action takes places in the extracellular milieu (McDermott and Gibson 1997).   Besides its ability to degrade Aβ, IDE activity is also associated with oligomerization of synthetic Aβ at physiological levels in the conditioned media of cultured cells (Qiu, et al. 1998). In addition, evidence that membrane-associated and secreted IDE isoforms carry out the degradation and clearance of Aβ secreted by neurons and microglia was provided a couple of years later (Bertram, et al. 2000).

IDE is composed of four homologous domains that share 15–24% sequence similarity. These domains form two functional N- and C-terminal domains that are joined by an extended 28 amino acid residue loop, creating a large catalytic chamber which can accommodate substrates of the order of 6 kDa. Substrate binding is assisted by the C-terminal domain and, its hydrolysis occurs at the N-terminal domain (Li, et al. 2006). The active site of IDE encompasses the His-Glu-aa-aa-His sequence and requires zinc. IDE enzyme hydrolyses several peptide bonds of both Aβ40 and Aβ42, but is particularly efficient at hydrolysing the Lys28-Gly29 peptide bond followed by the Phe19-Phe20 and His14-Gln15 bonds of these substrates (Bora, et al. 2010; Bora and Prabhakar 2010).

IDE is expressed by cortical and subcortical neurons, and has been detected in the cytoplasm of the three major components of the vascular wall: endothelial cells, pericytes and smooth muscle cells (Dorfman, et al. 2010; Gao, et al. 2004). IDE is also expressed towards the apical surface of the choroid plexus tissue where its inhibition leads to disrupted metabolism of Aβ and its concurrent accumulation at the blood-CSF barrier (Behl, et al. 2009). Besides the cytoplasm, IDE is found in endosomes, on the cell surface, and in the extracellular milieu. The type of cell in the nervous system expressing IDE establishes whether it is secreted or associated with the cell surface. Primary mouse microglia and the BV-2 cell line are found to secrete IDE, but in primary hippocampal neurons and differentiated PC12 cells only membrane associated IDE has been found. IDE is also present in mitochondria, and in the dendrites of neurons (Malito et al. 2008).

Evidence that IDE activity in AD brains is reduced compared to age-matched controls has been giving support to the hypothesis that reduced IDE activity may contribute to Aβ accumulation in the brain (Perez, et al. 2000). For example, membrane-bound IDE protein concentrations and activity decrease during the conversion from mild cognitive impairment (MCI) to mild-severe AD in the hippocampus, which correlates negatively with brain Aβ42 content in MCI and in AD brain (Zhao, et al. 2007). Still, other studies indicate that IDE may be less important in the process of Aβ clearance (Wang, et al. 2010). There is also evidence that in transgenic mice brain, Aβ plaques induce cortical mRNA and protein levels of IDE in parallel with increased Aβ40 and Aβ42 production, suggesting a positive feedback regulatory mechanism of Aβ regulation (Vepsalainen, et al. 2008).

3.2 NEP – Neprilysin

Neprilysin (NEP) is a 90–110 kDa plasma membrane glycoprotein of the neutral zinc metalloendopeptidase family. It consists of a short N-terminal cytoplasmic tail, a single transmembrane helix and a large C-terminal extracellular ectodomain. The ectodomain of neprilysin is largely made of α-helices with six disulfide bridges and at least three N-

glycosylation sites. The ectodomain encompasses the catalytic site, with the conserved HExxH motif necessary for zinc coordination and a proteolytic chamber (Malito et al. 2008). NEP is widely expressed in several tissues such as the brush-border of intestinal and kidney epithelial cells, neutrophils, thymocytes, lung, prostate, testes, and brain. In the brain, it is expressed on the plasma membranes of neurons, pre- and post-synaptically, and is most abundant in the nigrostriatal pathway, and in brain areas vulnerable to amyloid plaque deposition, such as the hippocampus (Wang, et al. 2006). It is also expressed in the tunica media and endothelium of cortical and leptomeningeal blood vessels where it participates in the regulation of the vascular tone, and in pyramidal neurons. NEP is also involved in the regulation of neuropeptide signalling (Dorfman et al. 2010; Miners, et al. 2008b).

NEP has been implicated in the degradation of Aβ. It degrades Aβ42 *in vivo,* and when NEP is inhibited, Aβ degradation in rat hippocampus is blocked with a concomitant increase of Aβ42 plaques in this brain region, and in the cortical region outside the hippocampus. In addition, the capacity to degrade Aβ42 of exogenous origin is severely compromised in NEP knockout homozygote mice in which the levels of Aβ40 and Aβ42 remain very high. These studies are corroborated by correlative studies in patients with sporadic AD and healthy age matched controls. Overexpression of NEP in brains of human amyloid precursor protein (hAPP) transgenic mice decreases overall Aβ levels and amyloid plaque burdens by 50% and effectively prevented early Aβ deposition in the neocortex and hippocampus. However, it did not reduce levels of Aβ oligomers or improved deficits in spatial learning and memory. The differential effect of NEP on plaques and oligomers suggests that NEP-dependent degradation of Aβ affects plaques more than oligomers and that these structures may form through distinct assembly mechanisms (Meilandt, et al. 2009). Moreover, the expression of NEP on the surface of leukocytes, trough lentivirus transplantation of bone marrow cells, reduced soluble brain Aβ levels by ~30% and lowered the accumulation of Aβ peptides by 50–60% when transplantation was performed at both young and early adult age. This peripheral NEP expression reduced amyloid dependent performance deficits of these animals in the Morris Water Maze (Guan, et al. 2009). *Ex-vivo* gene delivery of a soluble form of NEP, via fibroblasts, into transgenic APP mice also demonstrated increased clearance of plaques. Interestingly, mRNA and protein levels of NEP can be induced by intracranial injections of Aβ42, which also reduced the accumulation of amyloid plaques (Malito et al. 2008). Microglia from old PS1-APP mice, but not from younger mice, have a twofold to fivefold decrease in expression of NEP, compared with their littermate controls (Hickman, et al. 2008). Despite all the evidence sustaining the proteolytic action of NEP on Aβ, the molecular basis of the interaction between NEP and Aβ remains largely unexplained because the volume of the proteolytic chamber of NEP is about half the size of Aβ (Malito et al. 2008).

3.3 Presequence peptidase

Presequense peptidase (PreP) is a 110 kDa metalloprotease, ubiquitously expressed, but with higher abundance in heart and skeletal muscle. It is responsible for degradation of targeting peptides, which have been cleaved off inside the mitochondrial matrix after protein import, but it also cleaves other unstructured peptides up to 65 amino acids (Stahl, et al. 2002). More recently, PreP was shown to completely degrade Aβ40 and Aβ42, which are present in mitochondria. More relevance is added to this action of PreP as it has been shown that hPreP is actually the only protease responsible for degradation of Aβ in the

mitochondria (Falkevall, et al. 2006) and Aβ-induced mitochondrial toxicity has been associated with AD (Tillement, et al. 2011).

Human PreP consists of 4 domains, creating two halves connected by a hinge region. The two halves can create a large catalytic chamber of 10 000 Å where Aβ fits. The inverted zinc-binding motif is located in the N-terminal region, but also includes residues located in the C-terminal half, about 800 amino acids distant from the zinc-binding motif (Johnson, et al. 2006). Unlike IDE, PreP cannot degrade insulin, making PreP a better therapeutic agent candidate than IDE as its use would preclude deleterious side effects associated with the degradation of insulin. Another interesting feature of PreP is that its proteolytic activity against Aβ is abolished under oxidizing conditions, probably due to the formation of a disulphide bridge between Cys90 and Cys527 that inhibits the substrate from entering the catalytic chamber. These findings indicate a possible inhibition of hPreP under elevated ROS production in mitochondria implicated in AD, and might therefore be relevant in this disease (Alikhani, et al. 2009).

3.4 ECE – Endothelin converting enzyme

Endothelin-converting enzymes 1 and 2 (ECE-1 and ECE-2) were originally implicated in the processing of pro-hormone forms of endothelin. They are type II integral membrane zinc metalloendopeptidases that are primarily localized to the endothelium throughout the human vasculature. They share common catalytic substrates and are responsible for cleaving big endothelins to produce potent vasoconstrictor endothelins (Miners, et al. 2008a; Miners et al. 2008b).

ECE sequences and domain organization are similar to NEP's and are also capable of degrading Aβ *in vitro* and *in vivo* (Eckman, et al. 2001). Homozygous knockouts for ECE-1 are lethal and heterozygous animals show an increased amount of Aβ40 and Aβ42 in the brain. Homozygous ECE-2 knockout mice show a gene dose dependent increase of both forms of Aβ in the brain as well (Eckman, et al. 2006; Eckman, et al. 2003).

ECE-1 is present in neurons, specifically in pyramidal neurons of the hippocampus and layer V of the neocortex, and to a less extent in astrocytes (Sluck, et al. 1999). In mice, ECE-2 is largely confined to the brain; in the rat brain, ECE-1 occurs in the cerebrovascular endothelium, whereas ECE-2 is predominantly neuronal, with special incidence on hippocampal pyramidal neurons (Miners et al. 2008a; Miners et al. 2008b).

There is evidence of significant decrease in ECE-2 gene expression in AD patients (Weeraratna, et al. 2007). The contribution of ECE-1 to the accumulation of Aβ or reduction in local microvascular blood flow in AD seems to be detrimental, with abnormal production of ET-1 being more likely to reflect Aβ -mediated upregulation of ECE-2 (Palmer, et al. 2010).

3.5 ACE – Angiotensin-converting enzyme

Angiotensin-converting enzyme (ACE) is a membrane-bound zinc metalloprotease expressed in blood vessels throughout the body. ACE is extremely important for the regulation of fluid homeostasis and blood pressure. It converts angiotensin I to the potent vasoconstrictor angiotensin II. Within the brain, ACE has been detected in cortical pyramidal neurons and in the cerebral vasculature. Its highest levels occur in circumventricular organs, such as the subfornical organ, area postrema, and the median eminence but was detected in other areas such as the caudate nucleus, putamen, substantia

nigra pars reticularis, nucleus of the solitary tract, dorsal motor nucleus, median preoptic nucleus, and choroid plexus in rat, human, rabbit, sheep and monkey (Miners et al. 2008a).

ACE has two homologous domains, each having a functional active site. The N-domain of ACE is responsible for converting Aβ42 to Aβ40, whereas the angiotensin-converting activity is found predominantly in the C-domain of ACE. N-linked glycosylation is essential for both Aβ42 to Aβ40 conversion and angiotensin-converting activities and protects ACE from proteolysis (Zou, et al. 2009).

The first correlations between ACE and AD were found in 1985 by Zubenko and colleagues (Zubenko, et al. 1985) who showed that the CSF of patients with moderate degrees of senile dementia of the AD type exhibited about half the ACE activity of age and sex-matched control individuals, raising the possibility that ACE activity in the CSF could be an index of AD. In a posterior study, however, no differences in ACE activity in the CSF were found between AD patients and age matched controls raising some controversy on this issue (Konings, et al. 1993), but other studies reported reduced activity of ACE in the parietal cortex of AD brains (Ichai, et al. 1994).

ACE was found to significantly inhibit Aβ aggregation in 2001 (Hu, et al. 2001). The inhibition of aggregation was specifically blocked by preincubation of ACE with an ACE inhibitor, lisinopril. ACE degraded Aβ by cleaving Aβ40 at the site Asp(7)-Ser(8). Compared with Aβ40, aggregation and cytotoxic effects of the degradation products Aβ-(1-7) and Aβ-(8-40) peptides were reduced or virtually absent. These findings led to the hypothesis that previous associations of ACE genotype with AD susceptibility (Farrer, et al. 2000; Hu, et al. 1999; Isbir, et al. 2001; Yang, et al. 2000) could rely on its capacity to degrade Aβ and prevent the accumulation of amyloid plaques *in vivo*. Besides Aβ40 degradation, cellular expression of ACE also promotes degradation of naturally secreted Aβ42, and leads to the clearance of both species (Hemming and Selkoe 2005). In addition, ACE also converts Aβ42 to Aβ40 which is less neurotoxic (Zou, et al. 2007).

Pharmacological inhibition of ACE with a widely prescribed drug, captopril, promotes the accumulation of cell-derived Aβ in the media of APP expressing cells and questions if prescribed ACE inhibitors against hypertension could elevate cerebral Aβ levels in humans (Hemming and Selkoe 2005). To address this question the ACE inhibitor captopril was administered to two lines of APP transgenic mice presenting low or high levels of Aβ with associated plaque deposition. In both models, captopril did not affect cerebral Aβ levels nor plaque deposition or peripheral Aβ levels (Hemming, et al. 2007).

Epidemiological data obtained from a male population, however, suggests that angiotensin receptor blockers are more efficient in reducing the incidence and progression of AD and dementia compared with ACE inhibitors or other cardiovascular drugs (Li, et al. 2010). However, there are strong indications that ACE inhibitors may reduce the risk for and progression of dementias (Shah, et al. 2009). Still, these effects remain rather controversial, and prescription of ACE inhibitors against hypertension should be regarded with caution (Hajjar, et al. 2008; Kehoe and Wilcock 2007; Shah et al. 2009). Alternatively, ACE inhibitors could be designed to specifically target the angiotensin-converting C-domain, without inhibiting the Aβ42 to Aβ40-converting activity of ACE or increasing neurotoxic Aβ42 (Zou et al. 2009).

3.6 uPA/tPA-plasmin system

Plasmin is a serine protease generated from the proteolytic cleavage of inactive plasminogen, by tissue-type (tPA) or by urokinase-type plasminogen activator (uPA). In the

brain, plasminogen is synthesized in neurons, whereas uPA and tPA expression can be induced in neurons as well as in microglia. This system is involved in many normal neural functions, such as neuronal plasticity, learning, and memory (Wang et al. 2006; Zhao and Pei 2008).

uPA and tPA are induced by Aβ treatment in primary rat embryonic cortical cultures *in vitro* as well as in a murine model of Aβ accumulation *in vivo* (Tucker, et al. 2000b). In addition, it plasmin degrades both non-aggregated and aggregated Aβ40 (Tucker, et al. 2000a), and also degrades Aβ42, preventing Aβ42 aggregation into beta-pleated sheet structures (Exley and Korchazhkina 2001). Plasmin also inhibits Aβ toxicity, reduces Aβ deposition *in vitro*, and inhibits Aβ fibrillogenesis (Tucker, et al. 2002). The anti-amyloidogenic effect of plasmin is further enhanced by its capacity to increase the processing of human APP, and efficiently degrading secreted amyloidogenic and non-amyloidogenic APP fragments. Consistent with these observations, brains from AD patients contain reduced levels of plasmin (Ledesma, et al. 2000). Moreover, Aβ injected into the hippocampus of mice lacking either tPA or plasminogen persists causing activation of microglial cells and neuronal damage. Conversely, Aβ injected into wild-type mice is rapidly cleared and does not cause neuronal degeneration (Melchor, et al. 2003). Assembly of Aβ42, on the other hand, seems to promote the up-regulation of the tPA/plasminogen proteolytic system, which can modulate the deposition of amyloid plaques *in vivo*, in a negative feedback mechanism (Lee, et al. 2007).

3.7 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are zinc- and calcium dependent endopeptidases, produced by neurons and glial cells. Metalloproteinase 2 (MMP-2) can be detected in the walls of some blood vessels and scattered white matter glia. Metalloproteinase 3 (MMP-3) is present in and around some neurons and within occasional amyloid plaques, and metalloproteinase 9 (MMP-9) is present in many neurons.

MMP-2 and MMP-9 all have Aβ-degrading activity *in vitro*, although the Aβ degrading activity of these MMPs has not received so much attention *in vivo*. However MMP-2 and MMP-9 activity are elevated in homogenates of hippocampal tissue from AD brains, and several cell types (glial, neuronal and vascular) up-regulate endogenous MMP-2 -3 and -9 expression in response to Aβ stimulation (Deb and Gottschall 1996; Miners et al. 2008b; Gottschall 1996).

MMP-2 also known as gelatinase A/type IV collagenase/matrix hydrolyses Aβ40 and Aβ42 peptides at Lys 16-Leu 17, at Leu 34-Met 35, and Met 35-Val 36 peptide bonds (Roher, et al. 1994). Besides its Aβ degrading activity, MMP-2 has also beta-secretase activity (LePage, et al. 1995). In AD brains, MMP-2 is present in the reactive microglia located in the center of senile plaques, and in Schwann cells (Yamada, et al. 1995). Aβ stimulates the expression and activation of MMP-2 to a large extent due to the increased expression of membrane type-1 (MT1)-MMP expression, the primary MMP-2 activator (Jung, et al. 2003).

There seems to be a complex regulation of MMP2 expression by oligomeric Aβ in astrocytes. Oligomeric Aβ directly down-regulates MMP2 expression and activation in astrocytes. However, it also induces the production of pro-inflammatory cytokines which stimulate production of MMP2 (Li, et al. 2011).

Increased MMP-9 immunolabelling has been detected in neurons of AD patients, as well as in neurofibrillary tangles, plaques and blood vessel walls, but not in granular neurons or glial cells. MMP-9 is also detected in the vicinity of extracellular amyloid plaques (Backstrom, et al. 1996). MMP-9, unlike ECE-1, NEP and IDE, is capable of cleaving aggregated Aβ fibrils (Miners et al. 2008b). It cleaves Aβ at several sites, predominantly at Leu34-Met35 within the membrane-spanning domain (Backstrom et al. 1996; Yan, et al. 2006). The presence of the apoE4 significantly dampens Aβ-induced MMP-9 in primary cultures of rat astrocytes. This effect may affect Aβ clearance and promote Aβ deposition in AD brains (Guo, et al. 2006). There is also evidence that reduction of Aβ levels through the action of MMP-9 may also result from the direct processing of cell surface APP with an alpha-secretase like activity, substantially reducing the levels of secreted Aβ peptide (Talamagas, et al. 2007).

Aged APP/presenilin 1 mouse astrocytes surrounding amyloid plaques, showed enhanced expression of MMP-2 and MMP-9. Astrocyte-conditioned medium obtained from these animals degraded Aβ, producing several small fragments. In the brains of MMP-2 and MMP-9 KO mice, significant increases in the Aβ levels were found in comparison to wild-type controls. This study reinforces previous *in vitro* evidences that MMP-2 and -9 may contribute to extracellular brain Aβ clearance through the degradation of Aβ (Yin, et al. 2006).

Several MMPs polymorphisms have been examined in relation to the risk of developing dementia with controversial results (Baig, et al. 2008; Helbecque, et al. 2007; Reitz, et al. 2008; Reitz, et al. 2010). MMPs may also be considered potential plasmatic and cerebrospinal fluid markers of AD as the levels of MMP-3 in the plasma and CSF of AD patients was found higher than in controls, whereas MMP-2 was significantly decreased in CSF but unchanged in plasma (Horstmann, et al. 2010).

3.8 Transthyretin

Transthyretin (TTR) is a 55 kDa homotetrameric protein secreted mainly by the liver and choroid plexus into the plasma and CSF, respectively (Soprano, et al. 1985). The name "transthyretin" discloses its dual physiological role as a carrier for thyroid hormones (Woeber and Ingbar 1968) and retinol, the latter through the binding to retinol-binding protein (Goodman 1985). TTR plasma concentration is age dependent and in healthy newborns it is about half of that in adults (Stabilini, et al. 1968; Vahlquist, et al. 1975), varying from 20 to 40 mg/ dL. In spite of the low TTR levels in CSF (~2 mg/ dL), the choroid plexus is presented as the major site of TTR expression, expressed as a ratio of tissue/mass, corresponding to a 30-fold higher than that found in plasma and represents 20% of the total CSF proteins (Weisner and Kauerz 1983).

Over a hundred TTR mutations have been associated with Familial Amyloid Polyneuropathy (FAP), some of which are very common in Portuguese patients, such as the valine at position 30 substituted by a methionine (TTR V30M) (Saraiva, et al. 1984), others provide a very aggressive phenotype, such as the TTR L55P (Jacobson, et al. 1992). Curiously, TTR T119M was described as an example of a non-aggressive mutation that inclusively has a protective role against the disease (Almeida, et al. 2000).

The first report that associates TTR to Aβ and AD as a protective molecule is from Schwarzman et al. who describes the capacity of normal CSF to inhibit amyloid formation and concluded that TTR was the major Aβ binding protein in the CSF, that could also

decrease the aggregation state of the peptide and its toxicity (Schwarzman, et al. 1994). Prior to this finding, TTR was found associated to senile plaques, NFTs and microangiopathic lesions (Shirahama, et al. 1982). The sequestration hypothesis was raised suggesting that normally produced Aβ is sequestered by certain extracellular proteins, thereby preventing amyloid formation and Aβ cytotoxicity; when sequestration fails amyloid formation occurs (Schwarzman and Goldgaber 1996). The observation that TTR is reduced in the CSF of AD patients further supported the idea of a TTR protective role in this pathology (Serot, et al. 1997). Authors also observed a decrease in TTR levels with age which could be related to the epithelial atrophy in the choroid plexus. Along time several reports described TTR decrease in CSF of AD patients (Davidsson, et al. 2002; Serot et al. 1997; Gloeckner, et al. 2008; Hansson, et al. 2004) although it remains unclear whether this reduction is restricted to AD, or on the contrary is common to other neurodegenerative disorders (Chiang, et al. 2009). It is also uncertain if the TTR decrease in the CSF happens early in disease development (or even before symptoms appears) or if it is a latter event, and thus studies involving patients with mild cognitive impairment (MCI) and early-staged probable AD patients are needed.

Other studies using a transgenic model of *Caenorhabditis elegans* expressing Aβ42 supported a TTR role in AD as administration of TTR rescued the neurodegeneration observed in this model (Link 1995). Mammalian models used to recapitulate AD features were never completely successful as AD transgenic mice did not show NFTs and demonstrate little or no neuronal cell loss (Holcomb, et al. 1998; Hsiao, et al. 1996; Irizarry, et al. 1997a; Irizarry, et al. 1997b; Stein and Johnson 2002). However, in some of the models, animals showed increased TTR expression in the hippocampus (Stein and Johnson 2002); TTR was then described to be a survival gene (Stein and Johnson 2002) and although this work is controversial because TTR expression is thought to be confined to the choroid plexus and meninges (in the case of the brain), authors further showed that when a chronic infusion of an antibody against TTR was applied into the hippocampus of mice expressing human APP, an increase of Aβ, tau phosphorylation, neuronal loss and apoptosis were observed (Stein, et al. 2004). Underlying these observations is, according to authors, sAPPα that leads to increased expression of protective genes, such as TTR, to confer neuroprotection (Stein et al. 2004). Other studies, using transgenic APP mice hemizigous for endogenous TTR showed accelerated Aβ deposition (Choi, et al. 2007), while double transgenic mice for APP and TTR presented lower deposition (Buxbaum, et al. 2008). However, in other models, TTR was described to have the opposite effect and was associated with increased vascular Aβ deposition (Wati, et al. 2009).

Regarding the nature of TTR/Aβ interaction, different researchers confirmed TTR binding to Aβ (Carro, et al. 2002; Liu and Murphy 2006; Costa, et al. 2008) not only to the monomer but also to Aβ oligomers and fibrils, raising the hypothesis that TTR may be involved in the formation of senile plaques (Costa et al. 2008); TTR was also able to inhibit and to disrupt Aβ fibrils. However, which TTR conformation binds Aβ peptide is still controversial. Du Murphy et al. showed that TTR tetramers interact preferably with Aβ aggregates rather than Aβ monomers enhancing Aβ aggregation, whereas TTR monomers arrest Aβ aggregate growth (Du and Murphy 2010). On the other hand, studies by Costa et al. (Costa et al. 2008) showed that soluble Aβ binds to different TTR variants correlating negatively with the amyloidogenic potential of the TTR mutant. Thus, TTR119M presented the highest affinity to Aβ, contrarily to what was observed by Du and Murphy (Du and Murphy 2010). Other

studies also indicated a different relation between TTR variant/amyloidogenic potential and binding to the peptide, with amyloidogenic mutants binding less to Aβ peptide and inhibiting less its aggregation (Schwarzman, et al. 2004). In this work it was also shown that TTRs were functional tetramers. Hence, other studies are necessary to completely understand the nature of the TTR/Aβ interaction, concerning both Aβ and TTR species involved. Structural analysis, obtained from computer-assisted modeling (Schwarzman et al. 2004) predicted the existence of an Aβ binding domain on the surface of each TTR monomer; residues 30-60, especially the 38-42 region of TTR seemed to be the key structure of the Aβ binding domain (Schwarzman and Goldgaber 1996; Schwarzman, et al. 2005). Du and Murphy, identified the A strand, in the inner β-sheet of TTR, as well as the EF helix, as regions of TTR that are involved with Aβ association (Du and Murphy 2010).

The discussion on the TTR interaction with Aβ and consequent inhibition of aggregation and toxicity reduction raised the hypothesis that mutations in the TTR gene or conformational changes in the protein induced by aging, could affect the sequestration properties. A study was conducted with the aim of identifying mutations in the TTR gene in the AD population but no correlation was found (Palha, et al. 1996). Finally, and concerning the mechanism underlying TTR protective role in AD, Costa and colleagues found that TTR is able to proteolytically process Aβ peptide (Costa et al. 2008). Regarding TTR ability to degrade Aβ, several cleavage sites were identified and the newly generated Aβ peptides shown to have decreased amyloidogenic potential, when compared to the full length counterpart (Costa et al. 2008); TTR is also able to degrade aggregated forms of the peptide and inhibition of the TTR activity resulted in increased Aβ fibril formation (Costa et al. 2008).

3.9 Gelsolin

Gelsolin can be found both as an intrinsic cytoplasmic protein and as a secreted protein in plasma and CSF (Kwiatkowski, et al. 1985). Intracellular gelsolin regulates actin polymerization by binding to actin, and it also caps and breaks the actin filaments (Janmey, et al. 1985; Matsumoto, et al. 2003). Secreted (plasma/CSF) and intracellular gelsolin originate from the alternative splicing of a single gene, but their disulphide structure is different with recognised functional implications. All of the five cysteine (Cys) residues present in human cytoplasmic gelsolin are free thiols, whereas in plasma/CSF gelsolin, three Cys residues are free thiols and the other two Cys residues are disulfide-linked (Wen, et al. 1996). The five free thiol groups are likely to confer anti-oxidant properties to the molecule. Secreted gelsolin has an extension of 25 amino acids at its N-terminal, which is absent in the cytoplasmic form (Chauhan, et al. 2008).

Gelsolin found in the CSF may originate from oligodendrocytes or microglia (Chauhan et al. 2008). It was also suggested that choroid plexus may be responsible for the presence of gelsolin in CSF (Matsumoto et al. 2003), where it may have also an important function counteracting the neurotoxicity of Aβ (Antequera, et al. 2009; Vargas, et al. 2010).

Plasma and cytosolic gelsolin both bind Aβ and the Aβ -gelsolin complex exists in the plasma and in the cytosol (Chauhan, et al. 1999; Ji, et al. 2008). Both inhibit the fibrillation of Aβ, and defibrillate preformed fibrils (Ray, et al. 2000). In addition it has been demonstrated that when administered to transgenic mouse models of AD reduces the Aβ40/Aβ42 and the amyloid load (Hirko, et al. 2007; Matsuoka, et al. 2003). Therefore, gelsolin may also be looked as a potential therapeutic agent against AD.

3.10 Alpha2-macroglobulin

Alpha2-macroglobulin (a2M) is a 720 kDa soluble glycoprotein composed of four identical 180 kDa subunits, each encoded by a single-copy gene on human chromosome 12. Each subunit contains at least five binding sites: the bait region, the internal thiol ester, the receptor binding site, the Aβ binding site, and the zinc binding site. The bait region, the internal thiol ester and the receptor binding site are crucial for the activation and internalization of a2M. The bait region binds any known protease. The four bait regions in the tetramer are in close contact and get cleaved by the bound proteases, triggering activation of a2M. This conformational change results in the exposure of the four thiol esters, and of the four receptor binding sites, to the extracellular environment. The receptor binding site of each monomer, a 27 residue consensus sequence located at their C-terminal tail, will then bind to the receptor and mediate the internalization of the complex. The Aβ binding site is located between the bait and the receptor binding regions of each monomer (Borth 1992; Du, et al. 1997; Hughes, et al. 1998; Kovacs 2000).

Binding to Aβ42 occurs with high affinity (Kd= 3.8×10^{-10} M) and protects the peptide from proteolysis by exogenous trypsin, suggesting that a2M may function as a carrier protein for Aβ and may serve to regulate clearance of Aβ from such tissues as the brain (Du et al. 1997; Hughes et al. 1998). a2M co-incubated with Aβ significantly reduces aggregation and fibril formation *in vitro*, and cultured fetal rat cortical neurons are less vulnerable to the toxic actions of Aβ following pretreatment with a2M, being likely that a2M has the capacity to keep Aβ in a soluble state, preventing fibril formation and associated neurotoxicity (Du, et al. 1998). a2M also inhibits both Aβ fibril formation and Aβ -induced cytotoxicity in PC12 cells (Monji, et al. 2000). The inhibition of the formation of amyloid fibrils are probably due to the interaction of a2M with prefibrillar species to maintain the solubility of Aβ (Yerbury, et al. 2009), and therefore their aggregation state in the extracellular milieu. In addition, Qiu et al described a serine protease that binds to a2M to form a stable high molecular weight complex capable of efficiently cleaving Aβ (Qiu, et al. 1996). This serine protease-a2M complex was capable of proteolytically digesting both Aβ40 and Aβ42, resulting in disruption of the central region of the peptide (residues 10–35), which is believed to mediate the conformational change that underlies Aβ self-aggregation (Qiu et al. 1996).

a2M is also a physiological ligand for the low-density lipoprotein receptor-related protein (LRP) abundantly expressed in the CNS. The a2M/Aβ complexes can be degraded by glioblastoma cells and fibroblasts via LRP, but the degradation of free Aβ must be mediated via an LRP-independent pathway. These results suggest that LRP can function as a clearance receptor for Aβ via a2M (Narita, et al. 1997). The effect of self-aggregation and LRP-1 ligands on the elimination of human Aβ 40 from the rat brain across the blood-brain barrier has been investigated recently. In the first instance it was demonstrated that the elimination rate of [125]I hAβ40 dimer was 92.7% decreased compared to the [125]I hAβ40 monomer. When pre-incubated with LRP-1 ligands, such as activated a2M, apolipoprotein E2 (apoE2), apoE3, apoE4, and lactoferrin, the elimination of [125]I hAβ40) was reduced. There seems that dimerization and LRP-1-ligand complex formation prevents the elimination of Aβ40 from the brain across the blood-brain barrier (Ito, et al. 2007).

There is also an over-representation of a common a2M polymorphism, Val1000 (GTC)/Ile1000 (ATC) in AD patients, which correlates with an increase in Aβ burden (Kovacs 2000; Liao, et al. 1998), further sustaining the importance of a2M in Aβ clearance.

3.11 Apolipoprotein E

From all the proteins and peptides associated with AD and amyloid beta metabolism, apolipoprotein E (ApoE) is probably the most thoroughly studied, and several recent reviews give excellent and comprehensive overviews about its structure and function (Zhong and Weisgraber 2009a, b), and the pathways in which it is involved in AD (Kim, et al. 2009), either Aβ dependent or Aβ independent (Huang 2010; Mahley, et al. 2006). Therefore, this section will just give a brief overview about ApoE and will focus only on its association with AD via Aβ dependent pathways.

ApoE is a 34,2 kDa glycoprotein containing 299 amino acids. The protein contains two structural domains that are responsible for different functions of apoE. The amino-terminal domain (residues 1-191) contains the lipoprotein receptor binding region (residues 136-150), and the carboxyl-terminal domain (residues 216-299) contains the major lipoprotein (lipid) binding domain.

There are three common isoforms of ApoE, ApoE2, ApoE3 and ApoE4, which are encoded by three alleles (e2, e3 and e4, respectively) of a single gene. Sequence differences among the ApoE isoforms reside only on amino acids 122 or 158 which may be cysteine or arginine, but are sufficient to determine significant functional consequences (Huang 2010; Mahley et al. 2006).

ApoE4 is the major known genetic risk factor for AD and as much as 65–80% of all AD patients are ApoE4 carriers. This allele is over-represented in late-onset familial AD in several populations and in late-onset sporadic AD (Farrer, et al. 1997). In addition, as the number of Apo E alleles increases, the risk of onset of AD also increases while the age of onset decreases; as the number of ApoE4 alleles increases from 0 to 2, the risk of developing late-onset AD increases from 20% to 90%, and the mean age of onset decreases from 84 to 68 years (Corder, et al. 1993; Frangione, et al. 1996). The N and C terminal domains of ApoE interact in ApoE4 (called domain interaction), which is mediated by the formation of a salt bridge between Arg-61 in the N- terminus and Glu-255 in the C- terminus of ApoE4. This interaction might be the molecular basis for the detrimental effects of ApoE4 in AD pathogenesis (Huang 2010).

ApoE is expressed in several organs but is mainly expressed in the liver, followed by the brain. Non-neuronal cells, mainly astrocytes and to some extent microglia, are the major cell types that express apoE in the brain (Boyles, et al. 1985; Pitas, et al. 1987). Neurons also produce ApoE under certain conditions, particularly in response to brain injury (Boschert, et al. 1999) .

ApoE (E2 and E3) are important for the distribution of lipids among cells throughout the body and within the CNS, where the principal apolipoproteins are, and where they transport lipids and cholesterol for cell repair and neurite outgrowth. These properties are not shared by Apo E4 which seems to counteract the features of Apo E2 and ApoE3 with detrimental effects (Mahley et al. 2006).

ApoE is also essential for astrocytes to bind, internalize and degrade Aβ deposits (Koistinaho, et al. 2004). ApoE binds Aβ with high affinity. It was demonstrated that lipid-free ApoE4 and ApoE3 form stable complexes with Aβ, with ApoE4 being more effective in the formation of the complex, but inducing a pathological β-sheet conformational change in Aβ ((Wisniewski and Frangione 1996). This interaction requires the N-terminal and the C-terminal domains, and there are strong indications that the interaction is affected by the lipid content (Weisgraber and Mahley 1996).

ApoE, but not ApoE4 plays a role in facilitating the proteolytic clearance of soluble Aβ from the brain. The endolytic degradation of Aβ peptides within microglia by NEP and related enzymes is dramatically enhanced by ApoE. Similarly, Aβ degradation extracellularly by IDE is facilitated by ApoE. The capacity of ApoE to promote Aβ degradation is dependent upon the ApoE isoform and its lipidation status. The enhanced expression of lipidated ApoE, through the activation of liver X receptors, stimulates Aβ degradation (Jiang, et al. 2008).

In contrast, Apo E4 inhibits Aβ clearance and stimulates Aβ deposition (Huang, et al. 2004). It also enhances Aβ production and potentiates Aβ induced chromosomal leakage and apoptosis (Mahley and Huang 2006). In addition, ApoE4 enhances Aβ42 oligomer induced toxicity (Manelli, et al. 2007). Taken together these observations sustain the effects of ApoE4 in the enhancement of the overall Aβ burden via interaction with the peptide.

3.12 Metallothioneins 2 and 3

Metallothioneins (MTs) were discovered as cadmium binding proteins in equine kidney cortex. MTs is a generic name for a superfamily of low molecular weight cysteine- and metal-rich proteins with high affinity for divalent metals, such as zinc, cadmium and copper with four major isoforms, MT-1 to MT-4, identified in humans. They occur in all living organisms from the simplest forms of life, such as prokaryotes to the most complex, such as higher plants and vertebrate animals. It is clear that MTs are multipurpose proteins with unquestionable metal binding and anti-oxidant properties. In addition, there is increasing evidence that MT-1 and MT-2 (MT-1/2), and MT-3 display such diverse physiological actions as inhibition of pro-apoptotic mechanisms, enhancement of cell survival, tissue regeneration, and have anti-inflammatory properties. Concurrent with this wide array of functions, MT-1/2 have been implicated in neuroprotection and neurodegeneration, and particularly in AD (Hidalgo, et al. 2009; Penkowa, et al. 2006).

It is interesting that a considerable body of work has related an increase of MT-1/2 brain levels with aging and AD. In AD patients elevated levels of cytokines and IL-1 may induce MT-1/2 production in astrocytes. Studies in animal models of AD, showed that the MT-1/2 levels were higher, when compared to WT mice, while MT-3 levels were unaltered or reduced, suggesting that these proteins may have a relevant role in providing long term protection against inflammation (Hidalgo et al. 2009). This up-regulation of MT-1/2 in animal models of AD may have detrimental consequences in Aβ clearance as MT-2 diminishes the binding of TTR to Aβ (Martinho, et al. 2010). Considering TTR an Aβ scavenger as explained above, a less efficient removal of Aβ would be expected when MT-2 levels are increased, and this appears to be the case in AD. Furthermore, inhibition of homeodomain interacting protein kinase 2 activity in AD patients, might be involved in p53 misfolding, most likely through MT-2A upregulation (Lanni, et al. 2010). In addition, it was also shown that MT-2A may also prevent copper-mediated Aβ aggregation and neurotoxicity, by a mechanism which primarily involves a specific metal exchange interaction between Zn7.MT-2A and Cu(2).Aβ, and subsequent inhibition of H_2O_2 generation (Chung, et al. 2010). Furthermore, a metal swap between Zn7.MT-3 and soluble and aggregated Aβ1–40–Cu(II) abolishes ROS production and related cellular toxicity (Meloni, et al. 2008).

These studies relating metal exchange and Aβ aggregation, conducted to a recent interest in the use of metal-chelation drugs as a potential therapy for AD. For example, the

administration of the copper and zinc chelating drug, clioquinol, prevents plaque formation in transgenic AD mice (Hegde, et al. 2009).

Since the discovery of MT-3 as a growth inhibitory factor, with reduced expression levels in AD brains compared to age-matched controls, several studies provide evidence that MT-3 is related to the aetiology of AD. Some studies indicate that MT-3 may potentially promote the clearance of Aβ plaques, while others show an opposite trend or even no differences (Howells, et al. 2010). MT-3 alone antagonizes the toxic effect of Aβ because it inhibits the formation of SDS-resistant Aβ aggregates, thereby protecting cortical neurons from its toxic effects. Both the full-length and the N-terminal domain of MT-3 promote neuron survival at low concentrations but inhibited it at high concentrations. These observations suggest that the anti- Aβ activity of MT-3 is different from its neuronal growth inhibitory activity. Other possible mechanisms underlying the protection of MT-3 from Aβ toxicity may be related to its interaction with TTR by improving its Aβ degrading capacity (Martinho et al. 2010).

4. Conclusion

The extracellular neuritic plaque deposits of amyloid found in AD brains contain Aβ, and according to the amyloid cascade hypothesis, the accumulation of Aβ peptide, can trigger gradual synaptic alterations, astrocytic and microglial activation, and modification of the soluble tau protein into insoluble paired helical filaments, with progressive neuronal loss and cognitive failure (Hardy and Selkoe 2002). The cascade hypothesis suggests that stopping or slowing formation of the Aβ plaques would delay the onset of the disease symptoms. Therefore, it is crucial to thoroughly elucidate the regulation of Aβ production and clearance to design new and effective therapies against AD.

APP, takes a central position in AD pathogenesis, as Aβ arises from its proteolytic cleavage through the sequential action of β- and γ-secretase. APP is first cleaved by either α- or β-secretase at the α- or β-sites, respectively, which lie in the extracellular domain of APP. These proteases originate: soluble APPα (sAPPα, for α-secretase) or soluble APPβ (sAPPβ, for β-secretase), which are released to the extracellular space, and a membrane anchored C-terminal end (C83 for α-secretase or C99 for β-secretase). γ-cleavage of C99, generates the Aβ peptide. This pathway is known as the amyloidogenic pathway. Misregulation of all the intervenients in this process may lead to Aβ accumulation. If APP cleavage by β-secretase or γ-cleavage of C99 is more effective than α-secretase, then the all process shifts into the accumulation of Aβ. Also if APP synthesis is enhanced, as it is the case of several mutated forms of APP associated with early onset AD, the production of Aβ is also increased with similar results (Ertekin-Taner 2007). So, alterations in APP synthesis and processing by both secretases, or downstream in the γ-secretase complex action, may account for more severe forms of disease. Another good example of that are the mutations associated with presenilins 1 and 2 , which are part of the γ-secretase complex, known to be responsible for early onset inherited forms of AD (Ertekin-Taner 2007).

Once Aβ has been produced, other important players come into action (Figure 2). These are all the enzymes and proteins that, either by directly cleaving Aβ into less harmful peptides or making them more prone to proteolysis or less susceptible to aggregation through protein-protein interactions, will reduce the Aβ load in vulnerable areas of the brain.

Reduced degradation of Aβ by proteases is generally accepted to enhance plaque pathology in AD brains, and may depend on several factors such as decreased mRNA expression or decreased protein levels or activity, either in the brain cortex, hippocampus or in brain

microvessels; and post-translational modifications, such as oxidation and deposition of the enzymes in the diseased brain with consequent loss of its native structure and functionality (Dorfman et al, 2010.).

IDE and NEP are well accepted as Aβ degrading enzymes, but although few studies compare the relative contribution of each of these enzymes to the overall Aβ load, NEP seems to be the major protease involved in Aβ degradation. In transgenic mice models of familial AD, over-expression of IDE or NEP prevents amyloid plaque pathology and consequent early death (Leissring, et al. 2003) indicating that degradation of Aβ by these metalloproteinases may be of high therapeutic interest for AD patients. A recent study carried out in AD patients showed that, NEP mRNA, protein levels, and activity are decreased compared to normal controls without any cognitive impairment. In these patients IDE activity was unchanged, though mRNA levels increased. In the same study ECE-1 expression or activity in AD brains was not different from age-matched controls. Correlation analyses suggested that NEP expression was correlated with Aβ accumulation and clinical diagnosis, being lower in AD than in controls, whereas no correlations of IDE and ECE-1 with Aβ levels or clinical diagnosis has been found. These findings provide additional support for NEP as the major protease involved in Aβ degradation (Wang et al. 2010).

Age is the major risk factor for AD. Aβ -synthesizing enzyme activities increase with age, coinciding with declining soluble Aβ and increasing insoluble Aβ (Miners, et al, 2008b.). In addition, there is an overall ageing-related down regulation of Aβ degrading proteases which is particularly relevant for NEP but not for IDE in transgenic Tg2576 mice brains (Dorfman et al., 2010). Nevertheless further studies assessing the relative contribution of each of these enzymes , IDE, NEP, Prep, ECE, ACE and plasmin are necessary to understand why the system redundancy is not always effective, and which of these enzymes may be more adequate as candidate therapeutic targets.

Regarding proteins with the capacity to degrade Aβ or of interfering in the process, TTR is probably the most promising, with potential as a biomarker of the disease and even as a putative therapeutic agent. The literature shows that TTR plays an important role in the modulation of Aβ aggregation and toxicity. The use of TTR as a sera biomarker for diagnosis purposes in AD patients should also be explored, with obvious advantages, for both patients and researchers, and there are already indications that sera TTR is decreased in AD patients when compared to age-matched controls (Han et al., 2011). Moreover, it is important to investigate the factors that affect TTR/A-Beta binding and/or TTR alterations that lead to its decreased in AD, such as protein oxidation (Biroccio et al., 2006). Further studies are necessary to unravel the mechanism underlying TTR protective role in AD, to establish if TTR decline is a cause or consequence of disease, and the cellular pathways involved.

Other proteins as gelsolin, a2M and Apo E counteract Aβ deposition through interactions with the peptide, but apparently none of them seem to have the capacity to cleave the peptide. In general these proteins bind Aβ and the effects of these interactions translate into preventing Aβ fibrillation, and eventually on disaggregation of pre-existent fibrils (gelsolin and a2M); binding of enzymes conferring them the capacity to degrade Aβ (a2M) or may enhance the capacity of Aβ proteases, as NEP and IDE to cleave Aβ (ApoE). MT ½ and MT3 bind TTR affecting its capacity to cleave Aβ and prevent metal associated Aβ aggregation or inhibit their formation.

The scientific achievements acquired over the past decade on all the pathways and key players involved in the amyloid cascade will hopefully contribute to the development of more adequate therapies against AD in a closer future.
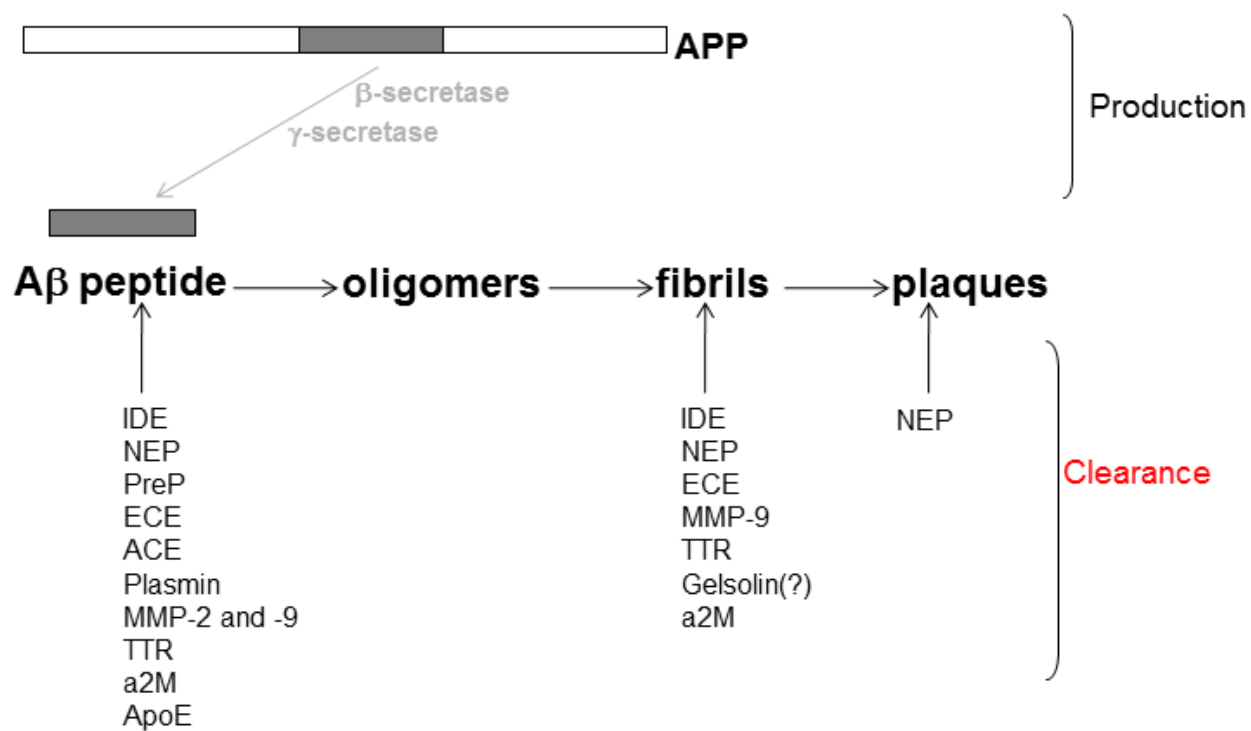

Fig. 2. Schematic diagram depicting APP processing: key proteins in amyloid-beta production and clearance (not drawn in scale). IDE- Insulin degrading enzyme; NEP- Neprilysin; PreP- Presequence peptidase; ECE –Endothelin-converting enzyme; ACE- Angiotensin-converting enzyme; MMP- Matrix metalloproteinase; TTR-Transthyretin; a2M- Alpha2-macroglobulin; ApoE- apolipoprotein E.

5. Acknowledgments

6. References

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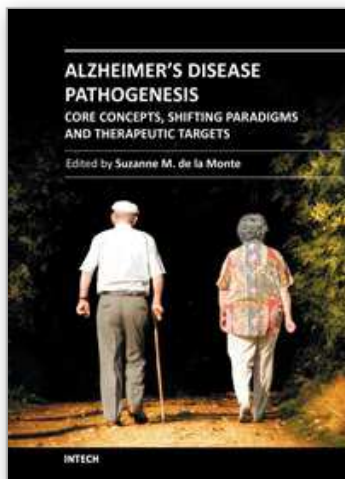
Alzheimer's Disease Pathogenesis-Core Concepts, Shifting Paradigms and Therapeutic Targets

Edited by Dr. Suzanne De La Monte

Alzheimer's Disease Pathogenesis: Core Concepts, Shifting Paradigms, and Therapeutic Targets, delivers the concepts embodied within its title. This exciting book presents the full array of theories about the causes of Alzheimer's, including fresh concepts that have gained ground among both professionals and the lay public. Acknowledged experts provide highly informative yet critical reviews of the factors that most likely contribute to Alzheimer's, including genetics, metabolic deficiencies, oxidative stress, and possibly environmental exposures. Evidence that Alzheimer's resembles a brain form of diabetes is discussed from different perspectives, ranging from disease mechanisms to therapeutics. This book is further energized by discussions of how neurotransmitter deficits, neuro-inflammation, and oxidative stress impair neuronal plasticity and contribute to Alzheimer's neurodegeneration. The diversity of topics presented in just the right depth will interest clinicians and researchers alike. This book inspires confidence that effective treatments could be developed based upon the expanding list of potential therapeutic targets.

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