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REVIEW

β-Amyloid catabolism: roles for neprilysin (NEP) and other metallopeptidases?

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Abstract

The steady-state level of amyloid β-peptide (Aβ) represents a balance between its biosynthesis from the amyloid precursor protein (APP) through the action of the β- and γ-secretases and its catabolism by a variety of proteolytic enzymes. Recent attention has focused on members of the neprilysin (NEP) family of zinc metalloproteinases in amyloid metabolism. NEP itself degrades both $A\beta_{1-40}$ and $A\beta_{1-42}$ *in vitro* and *in vivo*, and this metabolism is prevented by NEP inhibitors. Other NEP family members, for example endothelin-converting enzyme, may contribute to amyloid catabolism and may also play a role in neuroprotection. Another metalloproteinase, insulysin (insulin-degrading enzyme) has also been advocated as an amyloid-degrading enzyme and may contribute more generally to metabolism of amyloid-forming peptides. Other candidate enzymes proposed include angiotensin-converting enzyme, some matrix metalloproteinases, plasmin and, indirectly, thimet oligopeptidase (endopeptidase-24.15). This review critically evaluates the evidence relating to proteinases implicated in amyloid catabolism. Therapeutic strategies aimed at promoting Aβ degradation may provide a novel approach to the therapy of Alzheimer's disease.

Keywords: amyloid, endopeptidase, insulysin, neprilysin, neurodegeneration, neuroprotection.

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Alzheimer's disease (AD) is characterized by two types of proteinaceous deposits in the brain: extracellular plaques and intracellular neurofibrillary tangles. The main constituent of the plaques is the hydrophobic amyloid β-peptide (Aβ) (Glenner and Wong 1984), which is a 4-kDa peptide derived by proteolytic cleavage of the amyloid precursor protein (APP) (Fig. 1). Much attention has focused on the enzymes involved in the amyloidogenic (β- and γ-secretases) and non-amyloidogenic (α-secretase) pathways. Although β-secretase has been unequivocally identified as a novel, membrane-bound aspartyl proteinase (BACE or Asp-2) (Hussain *et al*. 1999; Sinha *et al*. 1999; Vassar *et al*. 1999; Yan *et al*. 1999; Howlett *et al*. 2000), the precise identities of α- and γ-secretase remain equivocal. α-Secretase is most likely to be a member of the ADAMs (a disintegrin and metalloprotease) family and ADAMs 9, 10 and 17 have all been implicated in this proteolytic event (Hooper and Turner 2002). The presenilins are clearly involved in the γ-secretase cleavage process but the jury is still out on whether these transmembrane proteins are themselves the catalytic agents or whether they act upstream of γ-secretase itself (Armogida *et al*. 2001; Checler 2001; Small 2001; Wolfe 2001). Nevertheless, both β- and γ-secretases represent viable therapeutic targets for the treatment and/or prevention of AD. As the steady state levels of all peptides *in vivo* are a direct consequence of the balance between their anabolism and catabolism, peptide accumulation can arise not only from increased production but also from decreased breakdown. Studies of Aβ in AD have primarily focused on formation of the neurotoxic peptide and on its polymerization into fibrils. The degradation of Aβ peptide has been regarded, at best, as a minor and irrelevant pathway. However, more recently a number of candidate Aβ peptide-degrading enzymes have emerged. Enhancement of a key enzyme in Aβ catabolism

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Abbreviations used: Aβ, amyloid β-peptide; ACE, angiotensin-converting enzyme; AD, Alzheimer's disease; ADAMs, a disintegrin and metalloprotease; CHO, Chinese hamster ovary; DINE, damage-induced neuronal endopeptidase; ECE, endothelin-converting enzyme; ECEL1, endothelin-converting enzyme-like-1; IDE, insulin-degrading enzyme (insulysin); MMP, matrix metalloproteinase; NEP, neprilysin (neutral endopeptidase-24.11); NL, neprilysin-like.

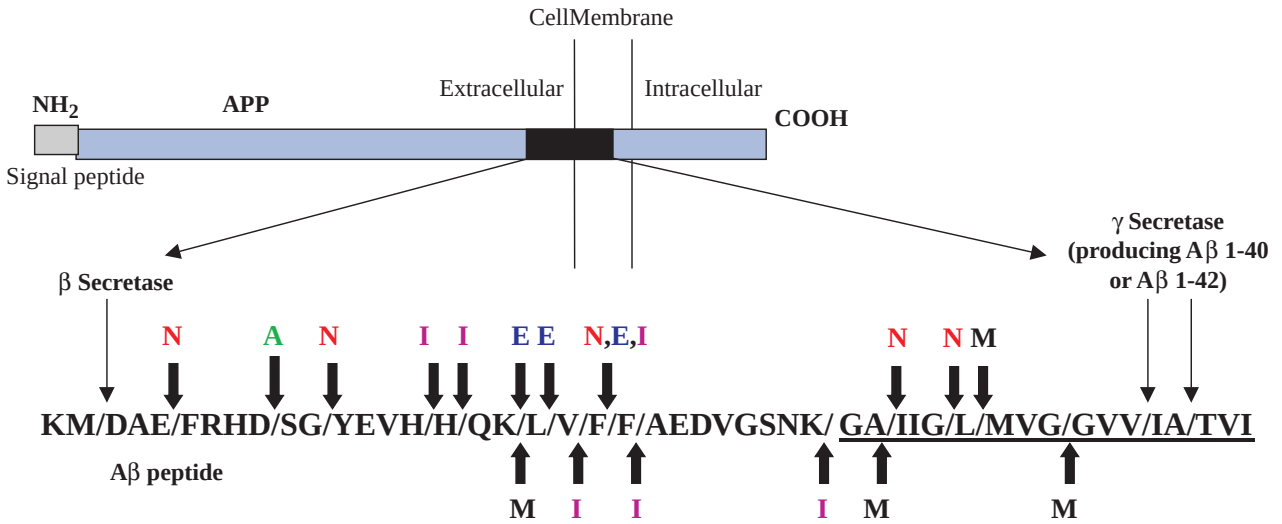

Fig. 1 The Aβ peptide is located within the transmembrane and extracellular domains of APP (shown as a black box). Release of Aβ occurs when β-secretase, a membrane-bound aspartyl protease, cleaves APP at the N-terminal end of Aβ. This results in the release of a soluble fragment known as sAPPβ and a membrane-associated C-terminal fragment, C99. γ-Secretase then cleaves C99 at the C-terminus of Aβ, within the transmembrane domain of APP, to release Aβ. Arrows shown above the Aβ peptide sequence and the symbol / between residues show the major cleavage sites determined for the following metallopeptidases: A, angiotensin-converting enzyme; N, NEP (Howell *et al.* 1995); E, ECE-1 (Eckman et al. 2001); M, MMP-9 (Backstrom *et al.* 1996) and I, IDE (Mukherjee et al. 2000). The Aβ peptide sequence is represented by the amino acid single letter code. The underlined region represents the portion of the peptide originally contained within the transmembrane domain.

could clearly provide an alternative therapeutic target to slow AD progression. This review evaluates the evidence relating to a number of Aβ-degrading enzymes, in particular the neprilysin (NEP) family of zinc metallopeptidases. The involvement of this family in neuronal protection is also highlighted. Other enzymes implicated in Aβ catabolism include insulin-degrading enzyme (IDE), angiotensin-converting enzyme (ACE) and thimet oligopeptidase, all of which are also zinc peptidases, as well as the serine proteinase, plasmin. A number of criteria must clearly be established for any candidate enzyme, of which the ability to degrade Aβ peptide *in vitro* is clearly a necessary but minimal requirement. The peptidase must also be active at the appropriate location and with the correct topology so as to be accessible to the substrate. Finally, inhibition of the enzyme (or its knock-out) should lead to accumulation of the peptide *in vivo*.

The NEP family in the nervous system

NEP, a 90–110-kDa plasma membrane glycoprotein, is the prototype and best-characterized member of the M13 zinc metallopeptidase family (reviewed in Turner *et al.* 2001). Seven members have been identified to date in humans, each of which is composed of a short N-terminal cytoplasmic region, a membrane-spanning section and a large C-terminal extracellular, catalytic domain, which contains the typical HExxH zinc-binding motif. Physiological substrates have been identified for only a few family members. NEP is identical with the neutrophil, cluster-differentiation antigen CD10, and is also known as the common acute lymphoblastic leukaemia antigen (CALLA) (LeTarte *et al.* 1988). It exists as an ectoenzyme preferentially hydrolysing extracellular oligopeptides (< 5 kDa) on the amino side of hydrophobic residues, which makes it suitable to play a role in the degradation of the small, hydrophobic 40–42 amino acid Aβ peptide. NEP is typically inhibited by the compounds phosphoramidon and thiorphan at nanomolar concentrations. The recent structural solution at 2.1 Å of the extracellular domain of human NEP complexed with phosphoramidon has led to a greater understanding of substrate specificity and the catalytic mechanism for this enzyme family (Oefner *et al.* 2000) (Fig. 2). In particular, it reveals a restricted active site cleft preventing access of large peptides and proteins, explaining its oligopeptidase character. NEP is primarily expressed in kidney where it comprises 4% of brush border membrane protein and functions to inactivate atrial natriuretic peptide. However, it occurs at much lower levels in many other tissues, including brain, where it is located on neuronal membranes, both pre- and postsynaptically (Barnes *et al.* 1992). It is most abundant in a nigrostriatal pathway, but is also found in some areas of amyloid plaque deposition, such as hippocampus (Barnes *et al.* 1995). NEP is pivotal in the hydrolysis of neuropeptides at the synapse, including substance P and the enkephalins (Turner and Tanzawa 1997; Turner *et al.* 2001).

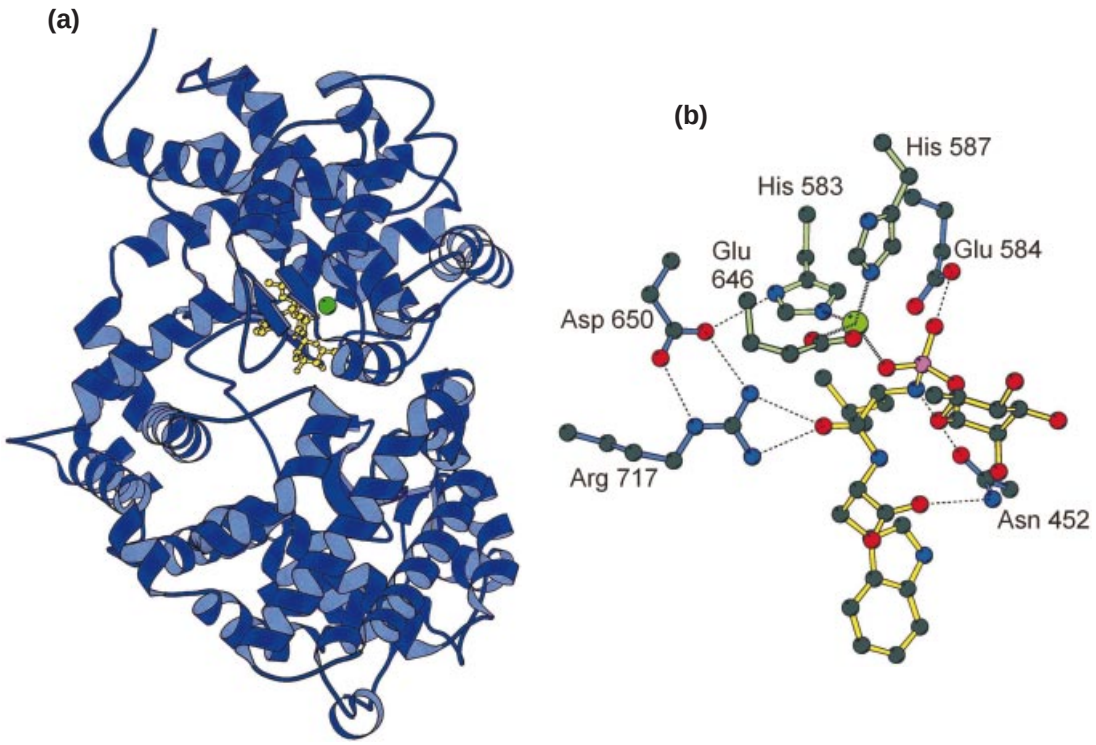

Fig. 2 Crystal structure of neprilysin complexed with phosphoramidon (Oefner *et al.* 2000: PDB code 1DMT). (a) Ribbon diagram of the overall fold of neprilysin (residues 52–749). The location of the active site is indicated by the ball-and-stick representation of the bound phosphoramidon (yellow) and by the zinc ion (green sphere). (b) The active site region of neprilysin. The zinc ion is shown as a green sphere and its three protein ligands are also in green. Other protein side chains with roles in substrate binding or catalysis are shown in blue. The bound phosphoramidon is shown in yellow. Metal co-ordination and hydrogen bonds are indicated by dashed lines.

Searching for the proteolytic activity that processed the inactive big endothelin-1 into the potent vasoconstrictor, endothelin-1 (ET-1), led to the discovery and subsequent cloning of endothelin-converting enzyme (ECE-1) (Takahashi *et al*. 1993; Shimada *et al*. 1994), which shares many similarities with NEP. ECE-1 appears to have a more restricted substrate specificity than NEP, although it can hydrolyse bradykinin, substance P and neurotensin (Hoang and Turner 1997; Johnson *et al*. 1999). A key distinction between NEP and ECE-1 is in inhibitor sensitivity, with ECE-1 showing a much reduced sensitivity to phosphoramidon and being virtually insensitive to thiorphan. Four distinct human isoforms of ECE-1 have been isolated, designated 1a, 1b, 1c and 1d. The four isoforms differ only in part of their cytoplasmic N-terminal regions and are derived from a single gene through the use of alternative promoters (Valdenaire *et al*. 1999). Immunofluorescence microscopy analysis has shown distinct subcellular localizations for the ECE-1 isoforms. ECE-1a, -1c and -1d can be predominantly localized to the cell surface, whereas ECE-1b is exclusively intracellular and shows significant colocalization with marker proteins for the trans-golgi network. The four isoforms identified to date in man are all conserved in rat. This conservation suggests that the multiplicity may be of physiological importance (Valdenaire *et al*. 1999). A second ECE-like gene (ECE-2) has been cloned (Emoto and Yanagisawa 1995) and differs from ECE-1 by being optimally active at acidic pH, but its functional importance remains to be elucidated. ECE-2 is most abundant in the nervous system. Other mammalian NEP homologues expressed in brain include: (i) the phosphoramidon- and thiorphan-sensitive 'soluble secreted endopeptidase' (SEP) (Ikeda *et al*. 1999) or 'neprilysin-like 1' (NL1) (Ghaddar *et al*. 2000) from mouse (of which the human and rat orthologues have been termed MMEL2 and NEP II, respectively) (Tanja *et al*. 2000; Bonvouloir *et al*. 2001), and (ii) the human orphan peptidase 'ECE-like 1' (ECEL1) (previously known as X-converting enzyme; XCE), critical for the neural control of respiration (Schweizer *et al*. 1999; Valdenaire *et al*. 2000) and separately identified in rat as 'damage-induced neuronal endopeptidase' (DINE) (Kiryu-Seo *et al*. 2000). The recently discovered NEP homologues are all enzymes in search of a function.

Neuroprotective role of the NEP family

The expression of a number of NEP family members is modified following nerve injury, suggesting a possible

protective role for the enzymes. For example, Wallerian degeneration leads to remarkable changes in the distribution of membrane peptidases, with NEP expression particularly reflecting the changed morphology of Schwann cells (Kenny and Bourne 1991). NEP histochemical staining in motor neurones can be seen to decrease in response to transection of the hypoglossal nerve, the reduced levels lasting for up to five weeks (Back and Gorenstein 1994). In contrast, after hypoglossal nerve injury, expression of mRNAs for ECE-1 and ECE-2 is enhanced in injured motor neurones and astrocytes, respectively (Nakagomi *et al*. 2000). This increase reaches a maximum at 5 days post-transection (the time point at which significant activation of astrocytes is observed morphologically) and remains elevated above normal levels for a period of five weeks. Endothelins might therefore play a role in communication between injured neurones and astrocytes in their response to nerve injury. ECE-1 is also involved in ventilatory responses to hypoxia at birth (Renolleau *et al*. 2001) and contributes to neuronal protection following hypoxia by affecting the surrounding cerebral blood flow (Park and Thornhill 2001). In turn, reactive oxygen species, especially superoxide, regulate ECE activity (López-Ongil *et al*. 2000). Neuronal expression levels of rat DINE were also seen to increase dramatically following various types of nerve injury, which included nerve transection and ischaemia (Kiryu-Seo *et al*. 2000). DINE expression also correlated with enhanced expression and activity of antioxidant enzymes, thereby diminishing the impact of oxidative stress induced by nerve injury (Kiryu-Seo *et al*. 2000). This cohort of studies implicating NEP-like enzymes in neuroprotective events has coincided with recent studies discussed below suggesting that these enzymes can attenuate the neurotoxic effects of β-amyloid by metabolizing the peptide. Whether these effects are inter-related in any way remains to be evaluated, as do the signalling pathways that may be involved.

Amyloid catabolism

The groundbreaking studies showing that immunization of rodents with β-amyloid could result in plaque disposal (Schenk *et al*. 1999) have focused attention on the removal of deposited amyloid as a viable therapeutic strategy in AD. Hence, interest has recently refocused on proteases that may contribute to this process. However, much earlier and neglected studies had pioneered this concept, particularly in relation to NEP. Philippe Crine and colleagues (Howell *et al*. 1995) recognized that NEP was an attractive candidate peptidase in the degradation of Aβ peptide for several reasons. These included the observed NEP immunoreactivity in amyloid plaques (Sato *et al*. 1991) (although this could well be a non-specific effect and is common to many proteins), the extracellular location of the catalytic site and the broad substrate specificity of the enzyme, with a particular preference for hydrophobic residues. There are 13 potential cleavage sites for NEP in the Aβ peptide, of which purified NEP was shown to be able to cleave five (Fig. 1) in a phosphoramidon-sensitive manner (Howell *et al*. 1995) and with a K_m of 2.8 μM for $A\beta_{1-42}$ (Takaki *et al*. 2000). This relatively high affinity may well reflect the fact that Aβ contains a tachykinin-like sequence (Yankner *et al*. 1990; Howell *et al*. 1995) and tachykinins are among the best known substrates for NEP *in vitro* (Matsas *et al*. 1984). Studies *in vivo* have complemented the *in vitro* studies on Aβ degradation. Catabolism of radiolabelled $A\beta_{1-42}$ injected into the rat hippocampus could be attributed predominantly to the action of a phosphoramidon-sensitive enzyme (Iwata *et al*. 2000). Other phosphoramidon-sensitive NEP family members could conceivably contribute to Aβ metabolism. Indeed, Eckman *et al*. (2001) have recently shown that ECE-1 could also hydrolyse both $A\beta_{1-40}$ and $A\beta_{1-42}$, although at distinct sites from NEP-susceptible bonds (Howell *et al*. 1995) (Fig. 1). A number of cell lines, including a neuroblastoma line (SHSY-5Y), and a neuroglioma (H4) have been shown to secrete increased levels of Aβ when treated with phosphoramidon, but not thiorphan, which is consistent with an ECE contribution (Fuller *et al*. 1995; Eckman *et al*. 2001). Furthermore, overexpression of ECE-1 in Chinese hamster ovary (CHO) cells almost eliminates extracellular Aβ levels, and this process is also inhibited by phosphoramidon treatment (Eckman *et al*. 2001). However, caution is needed in extrapolating from experiments in transformed cell lines and non-neural cells. Nevertheless, in support of a potential role of ECE-1 in AD, decreased endothelin levels have been reported in the cerebrospinal fluid of AD patients (Yoshizawa *et al*. 1992). Additionally sib-pair analyses of genetic factors contributing to late onset AD have not excluded the region on chromosome 1 where the ECE-1 gene is located (Kehoe *et al*. 1999). ECE-2 is also expressed in the CNS and therefore could play a role in the regulation of Aβ peptide levels in the brain.

Shirotani *et al*. (2001) have compared the potential for some other thiorphan- and phosphoramidon-sensitive NEP family members to degrade Aβ, and showed that NEP was by far the most efficient *in vitro* of those tested. A number of studies therefore focus on NEP itself as the most relevant of the NEP family in amyloid catabolism. Perhaps most convincingly, in NEP deficient mice, endogenous $A\beta_{1-40}$ and $A\beta_{1-42}$ were increased to levels comparable with the effects on $A\beta_{1-42}$ levels resulting from presenilin mutations (Iwata *et al*. 2001). Substantial reductions were also seen in the degradation of exogenous $A\beta_{1-42}$ in the NEP knock-out mice, although metabolism was not completely eliminated suggesting that other enzymes can participate, particularly in the absence of NEP. Chronic administration of thiorphan to rats has been shown to cause an accumulation of Aβ in hippocampal regions (Iwata *et al*. 2000), implicating the involvement of NEP rather than ECE in this process and

supporting NEP immunodepletion experiments that arrived at a similar conclusion (Takaki *et al*. 2000). Finally, reduced NEP levels have been reported in high plaque areas of Alzheimer brain (Yasojima *et al*. 2001).

Other candidates as amyloid-degrading enzymes

Despite this wealth of data, both *in vitro* and *in vivo*, suggesting that NEP and its relatives are major contributors to Aβ turnover, particularly of the (1–42) peptide, there is also evidence favouring other pathways, particularly involving IDE, now named insulysin (Roth 1998). However, whether IDE does play a specific role in insulin metabolism *in vivo* is still debatable. Coincidentally, NEP was also originally detected as the major insulin B-chain degrading enzyme of kidney. IDE is a neutral thiol-dependent, zinc metalloprotease expressed in all mammalian tissues, which was originally shown to hydrolyse Aβ by Kurochkin and Goto (1994). The sites of hydrolysis of Aβ by NEP, ECE, IDE and other peptidases are compared in Fig. 1. Unlike the ectoenzyme topology of NEP, IDE is principally a cytosolic protein, although it has been detected within peroxisomes, where it may contribute to the degradation of oxidized proteins (Morita *et al*. 2000). There is also some evidence for a secreted form of IDE, a surprising observation given that it contains no signal sequence for secretion. IDE differs from the NEP family in that it contains an inverted zinc consensus sequence (HxxEH), a feature found in a number of other prokaryotic and eukaryotic zinc peptidases (Roth 1998). IDE has an unusual substrate specificity that appears to be directed towards hydrolysis on the amino side of hydrophobic and basic residues (Song *et al*. 2001), although it has been proposed that substrate recognition is, at least in part, dependent upon adoption of a β-sheet conformation upon substrate binding (Kurochkin 1998, 2001). This hypothesis is based on the ability of IDE to degrade a number of amyloid-forming peptides, including insulin itself, atrial natriuretic peptide, amylin and Aβ. Thus, IDE may protect against the toxic effects of amyloids by functioning as an amyloid-scavenging enzyme. More recent studies have developed further the concept of IDE as an Aβ-degrading enzyme. The screening of a microglial cell line (BV-2) for proteases capable of degrading secreted Aβ led to the identification of IDE as a major candidate (Qiu *et al*. 1998). Immunodepletion of conditioned medium with an IDE antibody completely removed its Aβ-degrading activity. The Aβ-degrading activity of a secreted component of IDE has also been demonstrated in PC12 cells and in primary cortical neurones (Vekrellis *et al*. 2000). Unlike the microglial cells, IDE remained membrane-attached in differentiated PC12 cells, as demonstrated by cell-surface biotinylation (Vekrellis *et al*. 2000). The mechanism by which the IDE is released from cells remains unexplained, but might arise from the occurrence of distinct isoforms of the enzyme with different targetting sequences. Over-expression of presenilin-1 in CHO cells has been shown to stimulate the degradation of $A\beta_{1-42}$ by IDE, apparently by stabilizing the enzyme against proteolytic inactivation (Pérez *et al*. 2001). The anatomical distribution of IDE has been compared in normal and AD human brains. Paradoxically, immunostaining for IDE in AD brains appeared stronger than in control brains, with the staining labelling not only neurones but also senile plaques (Bernstein *et al*. 1999). However, in a separate study (Pérez *et al*. 2000), cytosolic IDE activity from AD brain fractions was significantly lower (50% reduction) than for controls, although the number of samples examined was very limited. Genetic evidence for the linkage of AD in some late-onset families with chromosome 10q, close to the IDE gene, may also be indicative of a protective role for the enzyme (Bertram *et al*. 2000).

Another thiol-dependent, zinc peptidase implicated in Aβ catabolism is the predominantly cytosolic thimet oligopeptidase (EC 3.4.24.15; E-24.15), previously erroneously identified as a β-secretase (Papastoitsis *et al*. 1994). However, conditioned medium from E-24.15 antisense-transfected SKNMC neuroblastoma cells exhibited significantly higher levels of Aβ than in controls, indicating the involvement of E-24.15 in Aβ catabolism as opposed to anabolism, at least in these cells. Pre-treatment of the conditioned medium with serine-protease inhibitors reduced Aβ catabolism and hence Yamin *et al*. (1999) hypothesized that E-24.15 played an indirect role by activating an Aβ-degrading serine proteinase. The matrix metalloproteinases MMP-2 and MMP-9 had earlier been implicated in amyloid catabolism (Roher *et al*. 1994; Backstrom *et al*. 1996) and, most recently, ACE is proposed as a candidate. Hu *et al*. (2001) have claimed that ACE can inhibit Aβ aggregation, and that this effect is mediated by hydrolysis of the (1–40) peptide at the Asp7–Ser8 bond (Fig. 1), a surprising observation given the known specificity of ACE (Turner and Hooper 2002). Further work is needed to substantiate these claims, for example in ACE-deficient animals, especially since Iwata *et al*. (2000, 2001) observed no effects of ACE inhibitors on Aβ metabolism *in vivo*. Previous genetic linkage studies have associated an ACE polymorphism with longevity (Schächter *et al*. 1994), although this has been disputed more recently (Blanché *et al*. 2001).

Finally, the serine peptidase plasmin has been shown *in vitro* to cleave both $A\beta_{1-40}$ (Van Nostrand and Porter 1999) and $A\beta_{1-42}$ preventing the aggregation of $A\beta_{42}$ (Exley and Korchazhkina 2001). In cultured hippocampal neurones, plasmin is associated with lipid rafts in neuronal membranes where it can process APP preferentially at the nonamyloidogenic, α-secretase site; whereas plasmin in the medium degraded APP fragments, including Aβ (Ledesma *et al*. 2000). Aβ aggregates, in turn, are a potent stimulator of the plasmin system substituting for fibrin aggregates in the activation process (Van Nostrand and Porter 1999; Tucker

et al. 2000). Brain tissue from Alzheimer's disease patients is reported to have reduced levels of plasmin (Ledesma *et al*. 2000). Thus, the plasmin system may be another regulator of Aβ levels, especially in pathological conditions.

Concluding remarks

How can all these disparate observations be explained? It is likely that amyloid catabolism is a continuous event, occurring in both intracellular and extracellular compartments, and no single enzyme is likely to be uniquely responsible at all locations. The rates of amyloid catabolism are likely to differ in different brain regions and be dependent upon the complement of peptidases located therein and the oligomeric state of the amyloid substrate. The *in vivo* data in rodents, including the use of $NEP^{-/-}$ mice (Iwata *et al*. 2001), are compelling but some caution is required in their interpretation. For example, the experiments of Iwata *et al*. (2000) were principally focused on Aβ metabolism in the hippocampus, a known location for NEP (Barnes *et al*. 1995). However, little NEP is present in cortical areas where Aβ deposition also occurs. Here, IDE or other peptidases may be the principal contributors to metabolism. The development of more selective inhibitors for the various enzymes described here would aid elucidation of the relative importance of distinct catabolic pathways. Whatever the normal mechanism for removal of Aβ peptides, it is clear that in AD the enzymic mechanisms are overwhelmed. Whether up-regulation of one or more of the described metallopeptidases can help to redress the balance remains to be evaluated. In the case of NEP, its down-regulation has been described in a number of distinct cancers (Papandreou *et al*. 1998) and in neuroblastoma cell lines (Medeiros *et al*. 1991). In contrast, NEP is up-regulated in gliomas and glioma cell lines (Carrel *et al*. 1982; Medeiros *et al*. 1991) and elucidation of the underlying regulatory mechanisms may be valuable in understanding how to manipulate levels of the enzyme *in vivo*.

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Note in press

Insulysin has also recently been shown to degrade the intracellular domain of APP (AICD) efficiently and may therefore regulate the biological activity of this fragment of the APP molecule (Edbauer *et al*. 2002).

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