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REVIEW ARTICLE

Aβ-Degrading Enzymes: Potential for Treatment of Alzheimer Disease

James Scott Miners, PhD, Neil Barua, BMedSci, BMBS, MRCS, Patrick Gavin Kehoe, PhD, Steven Gill, MBBS, FRCS, and Seth Love, PhD, FRCP, FRCPath

Abstract

There is increasing evidence that deficient clearance of β-amyloid (Aβ) contributes to its accumulation in late-onset Alzheimer disease (AD). Several Aβ-degrading enzymes, including neprilysin (NEP), insulin-degrading enzyme, and endothelin-converting enzyme reduce Aβ levels and protect against cognitive impairment in mouse models of AD. The activity of several Aβ-degrading enzymes rises with age and increases still further in AD, perhaps as a physiological response to minimize the buildup of Aβ. The age- and disease-related changes in expression of more recently recognized Aβ-degrading enzymes (e.g. NEP-2 and cathepsin B) remain to be investigated, and there is strong evidence that reduced NEP activity contributes to the development of cerebral amyloid angiopathy. Regardless of the role of Aβ-degrading enzymes in the development of AD, experimental data indicate that increasing the activity of these enzymes (NEP in particular) has therapeutic potential in AD, although targeting their delivery to the brain remains a major challenge. The most promising current approaches include the peripheral administration of agents that enhance the activity of Aβ-degrading enzymes and the direct intracerebral delivery of NEP by convection-enhanced delivery. In the longer term, genetic approaches to increasing the intracerebral expression of NEP or other Aβ-degrading enzymes may offer advantages.

Key Words: Aβ-degrading enzymes, Alzheimer disease treatment, Cathepsin B, Convection-enhanced delivery, Mouse models, Neprilysin.

INTRODUCTION

The accumulation of the β-amyloid (Aβ) peptide in the brain is thought to be central to the pathogenesis of Alzheimer disease (AD), at least in its early stages. Rare familial autosomal dominant forms of AD, which account for fewer than 5% of all AD cases, occur as a result of gene mutations in the amyloid precursor protein (APP) and presenilin genes (*PSEN1, PSEN2*) that increase $A\beta_{1–42}$ or the ratio of $A\beta_{1–42}$ and $A\beta_{1–40}$ (1–4). In all forms of AD, the accumulation of Aβ must reflect an imbalance between its production and clearance, but for most of all AD cases (~95%–99%), which are of late-onset and sporadic in nature, the cause of that imbalance is unclear. In healthy individuals, the production and turnover of Aβ are rapid (estimated at ~7.6% and ~8.3%, respectively, of the total volume of Aβ per hour [5]), suggesting that small changes in Aβ production or clearance can cause abnormal accumulation of Aβ. To date, there is little evidence to suggest that an increase in the overall production of Aβ is responsible for the development of sporadic AD in most cases (6). Recent research suggests strongly that clearance rather than production of Aβ is impaired in late-onset sporadic AD (7).

Clearance of Aβ from the brain is mediated by multiple diverse processes. These include drainage along perivascular basement membranes, possibly to cervical lymph nodes and into the cerebrospinal fluid (CSF) (8–10); transport across vessel walls into the circulation, mediated by low-density lipoprotein receptor-related protein 1 (11) or the P-glycoprotein (PgP/MDR1/ABCB1) efflux pump (12–14); the sequestration of Aβ by soluble low-density lipoprotein receptor-related protein 1 receptor in the circulation to promote the efflux of soluble Aβ out of the CNS (15); microglial phagocytosis (16); and enzyme-mediated degradation of Aβ (17–19) (Fig. 1).

Enzyme-mediated degradation of Aβ has received a great deal of attention during the past decade. Many enzymes are capable of cleaving full-length Aβ in vitro, producing fragments that are generally less neurotoxic and more easily cleared. The biologic relevance of several but not all of these enzymes to Aβ clearance has been established in vivo in mouse models of AD. As described below, overexpression of genes encoding the relevant enzymes in mice transgenic for mutant forms of human APP (hAPP) that cause familial AD was shown to reduce Aβ accumulation and, in many cases, to ameliorate cognitive and motor deficits. Interventions that can selectively increase Aβ-degrading protease activity constitute a potential strategy for treatment of AD. However, these interventions may have unwanted adverse events, as discussed below.

Recent studies on postmortem human brain tissue revealed elevation of the activities of several candidate Aβ-degrading enzymes in both late-onset AD and Down syndrome (25, 26). The activities of these enzymes tended to increase with disease progression, as indicated by Braak tangle stage. Aβ-degrading enzyme activities were also reported to increase in hAPP mice at about the time of Aβ deposition (27–29). Lastly, in vitro exposure to Aβ upregulated several of these enzymes in human neuronal, glial, and vascular cell lines (29–36). Together, these data led to the hypothesis that Aβ-degrading enzymes protect

From the Dementia Research Group (JSM, PGK, SL); and Department of Neurosurgery (NB, SG), Institute of Clinical Neurosciences, Clinical Science at North Bristol, University of Bristol, Frenchay Hospital, Bristol, UK.
Send correspondence and reprint requests to: James Scott Miners, PhD, Dementia Research Group, Clinical Science at North Bristol, University of Bristol, Frenchay Hospital, Bristol BS16 1LE, UK; E-mail: scott.miners@bristol.ac.uk
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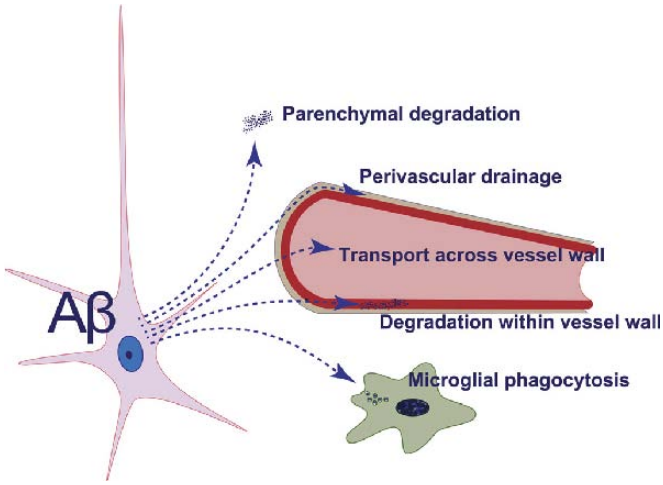

FIGURE 1. β-Amyloid (Aβ) clearance from the brain. The clearance of neuronally produced Aβ from the brain involves several processes. The best characterized of these are illustrated in this diagram: enzymatic degradation within the brain parenchyma (17–19); perivascular drainage of Aβ along basement membranes to cervical lymph nodes and into the cerebrospinal fluid (9, 10, 20); transport across vessel walls into the circulation, mediated by binding of Aβ to lipoprotein receptor–related protein 1 (11, 21, 22) or P-glycoprotein (PgP/MDR1/ABCB1) efflux pump (12–14); enzymatic degradation at the vessel wall (23); and microglial phagocytosis (16, 24).

against the development of AD through upregulation in response to Aβ.

This review focuses on lessons learned from in vitro and animal models about the contribution of enzymatic degradation to the clearance of Aβ and discusses the practical implementation and potential pitfalls of Aβ degradation therapies in AD.

CLEAVAGE OF Aβ IN VITRO

Amyloidogenic processing of APP through sequential cleavage by β- and γ-secretases (37–39) releases Aβ from APP. The predominant species of Aβ thereby produced are $Aβ_{1–40}$ and, in lesser amounts, $Aβ_{1–42}$. Cleavage of APP by α-secretase within the Aβ segment (nonamyloidogenic processing) prevents the formation of Aβ (40–42). When present in excess, extracellular $Aβ_{1–42}$, which is more prone to aggregate than $Aβ_{1–40}$ (43), tends to precipitate within the brain parenchyma, forming plaques, whereas $Aβ_{1–40}$ is more likely to reach the cerebral blood vessels and to accumulate within the vascular and perivascular extracellular matrix, leading to cerebral amyloid angiopathy (CAA), present in more than 90% of patients with AD (44).

A large number of enzymes have now been identified, which cleave either at a single site or at multiple sites within Aβ (Fig. 2). Enzymes that have Aβ-degrading activity in vitro include members of the zinc metalloendopeptidase family, which are constitutively expressed, and include neprilysin (NEP), NEP-2, endothelin-converting enzymes (ECE-1 and ECE-2), angiotensin-converting enzyme (ACE), and the closely related thiol-dependent metalloendopeptidase insulin-degrading enzyme (IDE). A number of serine proteases, released as inactive zymogens, also have Aβ-degrading activity on activation, that is, metalloendopeptidase matrix proteins MMP-2 and MMP-9, as well as plasmin, myelin basic protein, and acyl peptide hydrolase (APEH), so too do some cysteine proteases, including cathepsin B, D, and S (Table 1) (19, 74, 127–129). These enzymes all cleave peptide bonds; some also have carboxypeptidase activity and sequentially cleave single amino acids from the carboxyl terminus. The nature of Aβ cleavage, involving many enzymes acting at multiple cleavage sites, some shared, results in a multitude of different peptide fragments, some of which have yet to be characterized for their pathogenicity, tendency to aggregate, and ease of clearance.

Aβ exists in a dynamic equilibrium of soluble monomeric, oligomeric, protofibrillar, and fibrillar states (130). Evidence from in vivo and in vitro studies suggests that soluble oligomeric forms of Aβ are the major toxic species in AD (130–133), although in postmortem brains, there is considerable overlap in the level of soluble oligomeric Aβ in the cerebral cortex between AD patients and controls (134–136). The cellular location and relative ability of many Aβ-degrading enzymes to cleave monomeric, oligomeric, and fibrillar Aβ have been reasonably well characterized (Table 1). These data suggest that enzymes acting at specific locations within the cell are preferentially active against specific assemblies of Aβ. For example, intracellular enzymes such as IDE (112, 137), which is predominantly cytosolic, and the lysosomal-endosomal proteases cathepsin B (CTSB) (138), ECE-1, and ECE-2 (92, 96) preferentially degrade soluble monomeric or oligomeric Aβ (138) that is internalized within neurons and microglia. Plasma membrane–bound enzymes and their membrane-cleaved soluble forms such as NEP, ACE, and APEH act mainly on soluble and oligomeric Aβ, probably released at the synapse, whereas serine proteases that can degrade fibrillar Aβ, such as plasmin, MMP-2, and MMP-9, are secreted by activated astrocytes, microglia, and neurons and likely participate in the digestion of amyloid within plaques. Understanding how each protease functions within the CNS and how its function is affected in AD are key to delineating opportunities for therapeutic enhancement of enzyme-mediated degradation in this disease.

All known Aβ-degrading enzymes are capable of cleaving monomeric Aβ, but many have restricted ability to cleave oligomeric and fibrillar Aβ. Modeling the 3-dimensional structure of full-length $Aβ_{1–42}$ has given insights into the proteolytic degradation of Aβ (73) (Fig. 2B). On fibrillization of Aβ, amino acid residues 18 to 42 form a β-strand–turn-β-strand motif containing 2 intermolecular parallel in-register β sheets. Most cleavage sites that are accessible when Aβ is in an unstructured aqueous soluble monomeric state likely become inaccessible to proteolytic cleavage on oligomerization and fibrillization. Crouch et al (61) suggested that the accumulation of fibrillar Aβ may result from this decrease in susceptibility to proteolytic cleavage.

In general, enzyme-mediated degradation of Aβ diminishes its neurotoxicity. The products of Aβ proteolytic cleavage by ACE (cleavage between Asp^7-Ser^8) are less neurotoxic, less likely to aggregate, and are more easily cleared than full-length Aβ (56). Insulin-degrading enzyme–mediated cleavage of $Aβ_{1–40}$ and $Aβ_{1–42}$ eliminates the toxic effects of these proteins on rat cortical cells (52) and CTSB cleavage of $Aβ_{1–42}$

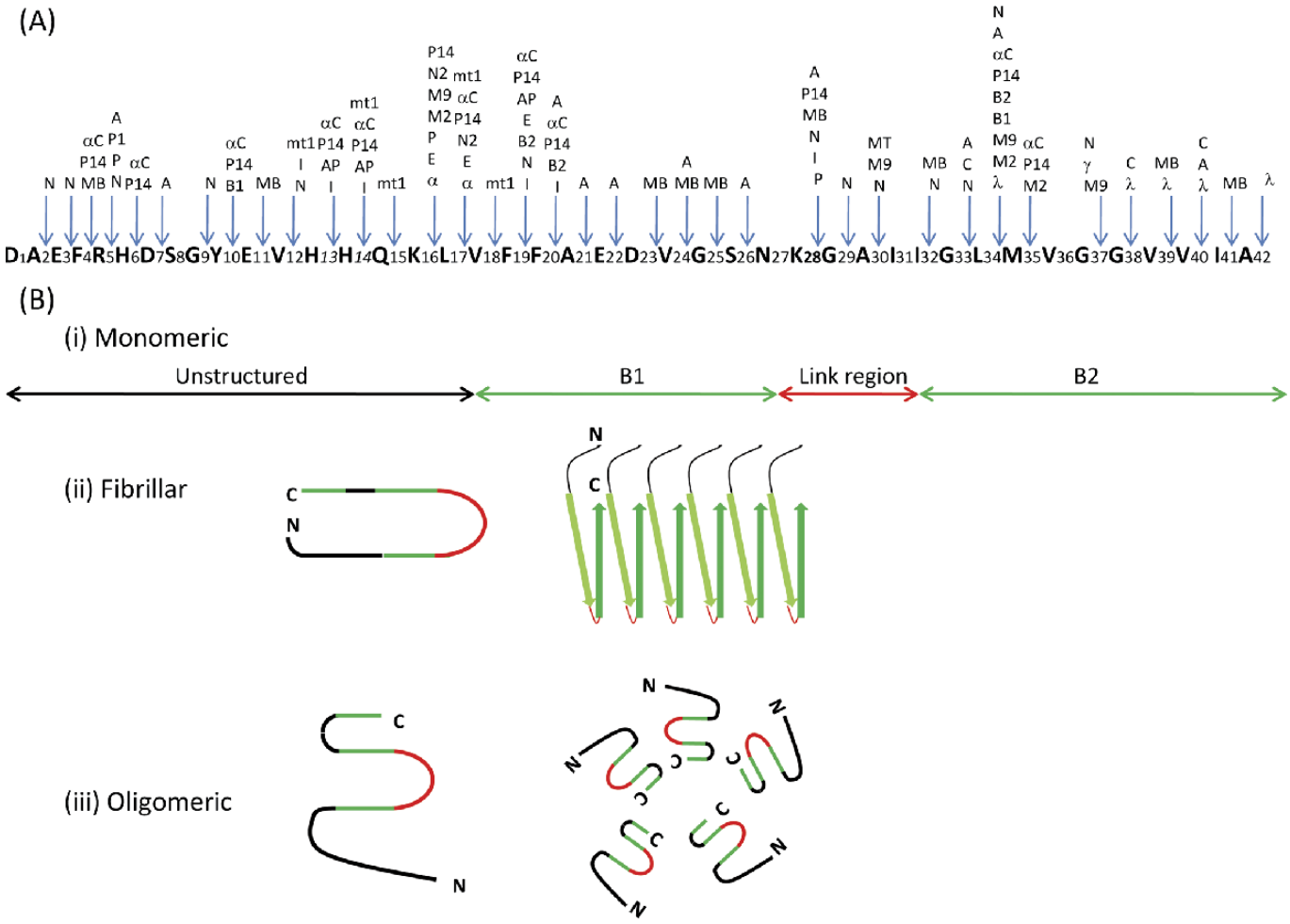

FIGURE 2. Sequence and structure of β-amyloid (Aβ) and known cleavage sites. **(A)** The primary sequence of Aβ and the putative Aβ-degrading enzymes and their cleavage sites. N, neprilysin (45–49); E, endothelin-converting enzyme (49–51); I, insulin-degrading enzyme (47, 48, 52–55); A, angiotensin-converting enzyme (56–60); M2, matrix metalloproteinase 2 (61–63); M9, matrix metalloproteinase 9 (64, 65); P, plasmin (28, 45, 51, 66, 67); C, CTSB (66); Mt1, membrane-type metalloproteinase 1 (68); MB, myelin basic protein (69); AP, acyl peptide hydrolase (70, 71); N2, neprilysin 2 (72); P14, protease XIV (73); αC, α-chymotrypsin (73). These data are summarized from Carson and Turner (74), Wang et al (75), and Eckman and Eckman (76). **(B)** Schematic representation of molecular models of the monomeric units (i), fibrillary (ii), and oligomeric (pentameric) $A\beta_{42}$ (reprinted with permission from Macmillan Publishers Ltd: *Nature Structural & Molecular Biology* [77], copyright 2010). All known Aβ-degrading enzymes are capable of cleaving monomeric Aβ. In fibrillar Aβ, amino acids (aa) 1 to 17 (black line) are unstructured; aa 18 to 42 form a β-turn-β fold containing hydrophobic regions B1 (aa 17–27) and B2 (aa 32–42) (green) that are inaccessible to enzyme-mediated cleavage. The Lys 28 loop region (red) between the 2 β-sheets is an accessible cleavage site and is a key determinant of the cytotoxicity of degradation products (73). (iii) In pentameric Aβ, the link region is probably inaccessible to enzyme-mediated cleavage.

yields C-terminally truncated peptides ($A\beta_{1–40}$, $A\beta_{1–38}$, and $A\beta_{1–33}$), which all show less toxicity and less fibrillogenic capacity than full-length $A\beta_{1–42}$ (32). However, a recent study revealed that the morphology of Aβ degradation products influences their neurotoxicity and that a linking region between the 2 β-pleated sheets is a key morphologic determinant (73). Degradation products resulting from proteolytic cleavage at Lys 28 by protease XIV were more neurotoxic than products of cleavage by α-chymotrypsin, which did not cleave at Lys 28. The cleavage products were also morphologically distinct, protease XIV cleavage products resembling oligomers (morphologically distinct from Aβ fibrils) (139), and those from α-chymotrypsin degradation having fibrillar characteristics. Aβ peptide fragments $A\beta_{25–35}$ (140) and $A\beta_{22–35}$ (141) also have similar in vitro neurotoxicity and aggregation profiles to their full-length Aβ counterparts (142) and $A\beta_{17–20}$ and $A\beta_{30–35}$ peptide fragments, which themselves do not self-aggregate spontaneously promote the aggregation and neurotoxicity of full-length $A\beta_{1–40}$ (143). The direct toxicity of not only $A\beta_{1–40}$ and $A\beta_{1–42}$ but also their degradation products, therefore, likely depends on how they combine to form assemblies in vivo because conversion from oligomers to protofibrils to fibrils is associated with a reduction in directly mediated damage to synapses and neurons (77). The general assumption that cleavage of Aβ is beneficial is probably overly simplistic inasmuch as the neurotoxic profiles and aggregating potential

of most of the common degradation products have not been fully explored.

CLEAVAGE OF Aβ IN VIVO

Neprilysin, the prototypic Aβ-degrading metalloendopeptidase, has been shown to be the most efficient of a range of thiorphan- and phosphoramidon-sensitive endopeptidases in degrading Aβ in vitro (144). Neprilysin has also been extensively studied in vivo (17, 19). Neprilysin was initially identified as a regulator of Aβ level when infusion of thiorphan, a NEP inhibitor, into rat striatum elevated the level of exogenously administered radiolabeled Aβ (45). Genetic ablation of the NEP gene (*NEP*) in hAPP mice confirmed the importance of NEP in preventing Aβ accumulation; endogenous Aβ increased, and clearance of exogenously administered Aβ was impaired, although not completely inhibited (46).

Mice with inactivation of *NEP* (46), *NEP-2* (145), *ECE-1*, *ECE-2* (92), or *IDE* genes (114, 146) all showed a moderate (1.5- to 2-fold) increase in endogenous Aβ, thereby confirming that these enzymes contribute to the regulation of Aβ level in vivo. However, these effects were much more modest than those observed after infusion of the metalloendopeptidase inhibitors, thiorphan and phosphoramidon, which produced a ~30- to 50-fold increase in Aβ (45, 147), suggesting the existence of unidentified NEP-like endopeptidases (145). Genetic knockout of plasmin, urokinase plasminogen activator, or tissue plasminogen activator, other candidate proteases that cleave Aβ in vitro, did not result in alterations in endogenous Aβ (148), nor did the absence of *ACE* alter steady-state levels of Aβ (149, 150). The possible roles of more recently identified Aβ-degrading proteases, including myelin basic protein (69), APEH (70), MT1-MMP (68), and CTSB (32), in regulating Aβ in vivo remain unknown.

Inactivation of *NEP* in hAPP mice not only increased Aβ, including oligomeric Aβ (82), causing plaque-like deposits in the brain, but also impaired synaptic plasticity, caused behavioral and cognitive abnormalities (82, 151) and CAA (152). Moreover, the severity of CAA was greater in $NEP^{-/-}$ than $NEP^{+/-}$ mice (152). Infusion of the NEP inhibitor thiorphan in rats was also associated with impairment of cognitive performance (153, 154).

Overexpression of human NEP (~8-fold increase) in hAPP (Swe/Ind) transgenic mice markedly reduced cerebral Aβ, largely preventing plaque formation, and significantly improved life expectancy (47, 155). Overexpression of human NEP (~30-fold increase in NEP activity) in hAPP (Swe/Ind) transgenic mice also substantially (~70%) reduced both triton-soluble and SDS-soluble $Aβ_{1–40}$ and $Aβ_{1–42}$ and improved performance in the Morris water maze (155). However, not all groups have demonstrated benefit. Meilandt et al (156) did not show improvement in spatial learning and memory in NEP/hAPP double transgenic mice (~11-fold increase in NEP) despite a 50% reduction in soluble Aβ and prevention in plaque formation; the authors suggested that the lack of improvement reflected an inability of NEP to cleave trimers and Aβ*56. Other authors have reported that NEP is able to cleave dimers, trimers, tetramers, and other oligomeric species of Aβ (82, 83).

Several other experimental strategies have provided indirect or direct evidence of the pathologic and clinical benefit of increasing intracerebral NEP in mouse models of AD. The reduction in Aβ and improvements in behavior and memory noted in hAPP mice treated with somatostatin (SST) (157) or environmental enrichment (158) correlated closely with upregulation of NEP. Unilateral injection of viral vectors encoding human NEP into the hippocampus of hAPP mice reduced Aβ level and plaque pathology (159, 160). Long-term gene transfer of human NEP in hAPP mice (producing a moderate [~3-fold] but sustained increase in NEP) reduced intracellular and extracellular Aβ and improved behavior and memory (161, 162). These mice also had evidence of reduced oxidative stress and inflammation (162) and less synaptic and dendritic damage (161).

Aβ-DEGRADING ENZYMES IN HEALTH AND DISEASE

When the role of Aβ-degrading proteases in preventing Aβ accumulation was first established in vivo, it was hypothesized that an age-associated decline in Aβ-degrading protease activity would contribute to Aβ accumulation, particularly in patients with late-onset AD, in whom the decline might be steeper. Earlier studies supported such a relationship and revealed reduced NEP and IDE mRNA and protein immunolabeling in AD compared with control brains (80, 163–165). Furthermore, the reduction in both NEP and IDE in AD was more pronounced in brain tissue from *APOE* ε4-positive patients (80, 106, 166, 167), whereas Hellstrom-Lindahl et al (168) reported decreased NEP protein (measured by immunoblot analysis) in AD and with age.

However, in several studies, the correlation between the immunohistochemical assessment of NEP (usually involving limited numbers of subjects) and its measurement in brain tissue homogenates (usually by densitometry of Western blots) was poor (80, 164, 168). Furthermore, studies on AD were based on the examination of relatively small series of brains with severe, late-stage disease, and most did not address the question of whether any observed reduction was simply secondary to late-stage neuronal damage and loss rather than a primary mediator of the pathology. Another consideration is the validity of mRNA and protein concentrations as surrogate markers of enzyme activity. Many Aβ-degrading enzymes are highly glycosylated and contain sites of potential posttranslational modification that may influence enzyme activity and become modified during disease (Table 2). The measurement of enzyme activity in biological tissues poses several challenges not least because some Aβ enzymes have both membrane-bound and soluble isoforms, but nonetheless provides the most relevant measurement of Aβ-degrading enzyme capacity with respect to their contribution to Aβ homeostasis. We have optimized several immunocapture-based fluorogenic assays of Aβ-degrading enzyme activity for use on postmortem brain tissue and CSF (169, 170). To avoid the problems of overlapping substrate specificity, overlapping pH optima, and nonspecific inhibition, we introduced an immunocapture step to isolate the enzyme of interest (e.g. NEP or IDE) from the biological samples. We showed that the assays have high sensitivity and greatly improved specificity to allow high-throughput analysis in multiwell plates.

TABLE 1. Distribution, Subcellular Location, Physiological Function and Form of β-Amyloid (Monomeric, Oligomeric and Fibrillar) Preferentially Cleaved By Candidate Degrading Enzymes

Family	Member (Aliases)	Subcellular Location	Expression Within the Human Brain	Aβ Preference	Physiological Function and Common Substrates* in Addition to Aβ Cleavage
M13 Zinc-binding membrane metalloendopeptidases:	NEPRILYSIN (NEP, CD10, CALLA, EC.3.4.24.15)	• Plasma membrane	• Neuronal (78, 79) • Vessel (vascular smooth muscle cells) (80, 81)	• Soluble Ab (73) • Oligomeric Ab (82, 83)	• Neuropeptide signaling (enkephalins, endorphins, substance P, neuropeptide Y, somatostatin, neurotensin) • Vascular tone (bradykinin, angiotensin I, endothelin 1) (reviewed by Skidgel et al [84])
(Type II integral membrane bound glycoproteins)	NEPRILYSIN-2 (NEP-2, SEP, MMEL1/2)	• Plasma membrane (85) • Endoplasmic reticulum (85) • Secreted (85, 87)	• Neuronal (Miners, unpublished observations)	• Unspecified	• Similar substrate specificity to neprilysin (86); however, NEP-2 has a more restricted substrate specificity (substance P and angiotensin-I) (85)
	Endothelin-converting enzyme 1 (ECE-1, EC.3.4.24.71)	• Plasma membrane (ECE-1 a, c, d [88]) • Trans-Golgi and acidic intracellular compartments (ECE-1 b) (92, 93)	• Vascular endothelial (89, 90) • Limited neuronal (94)	• Soluble monomeric (51)	• Involved in maintenance of vascular tone (Big endothelin, endothelin 1, angiotensin 1, bradykinin [91]) • Neuropeptide signaling (substance P, neurotensin) • Cleaves oxidized insulin β chain (49, 95)
	Endothelin-converting enzyme 2 (ECE-2, EC.3.4.24.71)	• Intracellular acidic compartment (96) • Plasma membrane (98)	• Pyramidal neurons (31) • Astrocytes/microglia in AD (31)	• Soluble monomeric (51)	• Similar substrate specificity to ECE-1 (Big endothelin 1, neurotensin, bradykinin, angiotensin I, substance P (reviewed by Mzhavia et al [97])
Membrane-bound zinc metalloproteinase	Angiotensin-converting enzyme (ACE) (dipeptidyl carboxypeptidase, EC.3.4.15.1)	• Plasma membrane-bound glycoprotein • Perinuclear region	• Pyramidal neurons (99, 100)	• Soluble Aβ (56, 57, 101)	• Vascular tone (angiotensin I, bradykinin) • Neuropeptide signaling (enkephalin, substance P, neurotensin, enkephalin, corticotrophin) (reviewed by Skidgel and Erdos [102])
Thiol-metalloendopeptidase:	Insulin-degrading enzyme (Insulysin; IDE, EC.3.4.24.56)	• Cytosolic (103–105) • Exosome (111) • Peroxisome (108, 115) • Plasma membrane (55, 118) • Rough endoplasmic reticulum (109, 118) • Secreted (54)	• Neuronal (106, 107) • Astrocyte and microglia (54) • Vessel (endothelial) (116, 117)	• Soluble monomeric only • No activity towards oligomeric (73, 112, 113) • No activity toward fibrillar Aβ	• Insulin metabolism (insulin [108, 109], amylin [104], insulin-like growth factors I and II [110], and glucagon) • Atrial natriuretic factor, β-endorphin, TGFa, and APP (114)
Matrix metalloproteinases	Matrix metalloproteinase-9 (MMP9, gelatinase-B, EC.3.4.24.35)	• Secreted • Plasma membrane (64)	• Neuronal (65) • Astrocyte and microglia (119)	• Soluble Aβ (65) • Aβ fibrils (64)	• Turnover of extracellular matrix proteins (type IV collagen, gelatin, aggrecan, α-crystallin β chain, a-synuclein) • Vascular tone (Big endothelin 1; substance P)
	Matrix metalloproteinase-2 (MMP-2, gelatinase-A, EC.3.4.24.24)	• Secreted	• Blood vessels and glia (120)	• Soluble Aβ (62) • Oligomeric Aβ (119) • Partial digestion of fibrillar Aβ (63, 121)	• Turnover of extracellular matrix proteins (actin, aggrecan, collagen-α_1 chain etc) • Vascular tone (Big endothelin 1)
	Type-1 transmembrane MMP (MT1-MMP, EC.3.4.24.80)	• Plasma membrane	• Neuronal • Astrocyte • Microglia • Vessel	• Soluble • Fibrillar Aβ (68)	• Turnover of extracellular matrix proteins (aggrecan, fibrinogen, myelin basic protein, etc) • Activation of MMP-2 • Autocatalysis (Mt1-MMP) • APOE

Serine proteases	Myelin basic protein (MBP, EC.2.1.1.126)	• Plasma membrane (122)	• Oligodendrocyte (white matter)	• Soluble • Fibrillar Ab (69)	• Autocatalysis (123)
	Plasmin (tissue plasminogen activator for tPA; urokinase-type plasminogen activator for uPA, EC.3.4.21.7)	• Secreted • Cytoplasm (124)	• Neuronal (124)	• Aβ monomers and fibers (28) • Oligomers (47, 113)	• Degrades plasma proteins (fibrin, plasminogen, and other matrix proteins)
	Acyl peptide hydrolase (APEH, EC.3.4.19.1)	• Intracellular and secreted (71)	• Unknown distribution	• Monomeric and oligomeric Aβ (preferentially degrades dimeric and trimeric assemblies) (70)	• Unknown substrates
Cysteine proteases	Cathepsin B (CTSB, EC.3.4.22.1)	• Lysosomal • Secreted (125)	• Neuronal • Microglia	• Soluble monomeric and nonfibrillar (32) • Fibrillar Aβ (32)	• Lysosomal cysteine protease

The physiological function and substrate specificity (if known) of enzymes most likely to be affected in Alzheimer disease are indicated, but the ranges of functions and substrates of these enzymes are wide, and the information in this table is not comprehensive. For more information, the reader is referred to the MEROPS database (http://merops.sanger.ac.uk/) (126).
NS, not specified.

We used these immunocapture-based assays to examine a large number of brains from normal controls across a wide age spectrum (16–95 y) and from AD patients with intermediate as well as late-stage disease and found that NEP and IDE activities rose with age in the normal brain (171) but were higher still in AD (26), rising progressively with increasing AD severity, according to Braak tangle stage. The activity of ACE was similarly increased in AD (33, 99, 100). Interestingly, NEP and IDE levels decreased with age specifically within the AD cohort (26). The levels and activities of NEP, ACE, and IDE were unaffected by postmortem delay (26, 33).

The levels and activities of NEP and ACE were also elevated in Down syndrome (which is associated with the development of AD-type neuropathologic changes in midlife) and correlated positively with the level of insoluble Aβ (25). Disease progression was also associated with increased NEP level (172) and a trend toward increasing ACE level in human CSF (33). The activity of IDE was elevated in AD, although the protein level did not differ significantly between AD and controls (26). Together, these findings argue strongly against a deficit in the activity of any of these Aβ-degrading enzymes in AD.

The levels of several other candidate Aβ-degrading proteases, namely, ECE-2 (31), MMP-2 and MMP-9 (64, 173), CTSB (32, 174, 175), and APEH (70), were reported to be elevated in AD; however, in other studies, the concentrations and activities of MMP-2, MMP-3, MMP-9, and plasmin were not increased (120, 124).

Increases in Aβ-degrading protease activity in AD are, therefore, more likely to be a protective response to the accumulation of Aβ (18). This hypothesis is supported by a large number of reports of induction of proteases, including NEP (176), ECE (30), ACE (33), ECE-2 (31), IDE (29), MMP-2, -3, and -9 (34–36), and CTSB (32), after exposure to Aβ, particularly fibrillar forms. Further support comes from the rise in IDE level (29), tissue plasminogen activator and urokinase plasminogen activator activity (28), and MMP-2 and -9 activity (27) in aged hAPP mice and the dose-dependent induction of neuronal NEP in hAPP mice given intracerebral injections of synthetic fibrillar Aβ (177, 178). Vepsalainen et al (179) reported that the earliest disparity in NEP and IDE levels between hAPP and wild-type control mice coincided with the onset of $A\beta_{1–40}$ and $A\beta_{1–42}$ deposition. Interestingly, Leal et al (29) found that the increases in IDE protein level and activity were not mirrored by the mRNA level, which remained unchanged, suggesting that the increases were mediated by posttranslational modification.

NEP AND CEREBRAL AMYLOID ANGIOPATHY

The most common form of CAA is caused by the accumulation of Aβ in the walls of leptomeningeal and cerebrocortical blood vessels, particularly arterioles (180, 181). The Aβ accumulates initially in the basement membranes but eventually causes the death of cerebrovascular smooth muscle cells (CVSMCs), replacement of the tunica media, and, particularly in larger leptomeningeal vessels, patchy replacement of the adventitia (8). The prevalence and severity of sporadic CAA increases with age and in AD (44, 182, 183). The Aβ

TABLE 2. Predicted Posttranslation Modification Sites Within β-Amyloid-Degrading Enzymes

	Potential Phosphorylation*	Potential *N*-Linked *N*-Glycosylation+ Sites†	Potential *O*-Linked *O*-Glycosylation Sites (S or T)‡	Potential Sumoylation Sites (Contain ΨKXE/D Sequence)§
Neprilysin (NEP)	15 S 12 T 12 Y	5 predicted N sites (145N +, 311N +, 325 N ++, 335N + 628N+)	None	3 high-probability sumoylation sites (K708, K289, K3)
Angiotensin-converting enzyme	17 S 11T 10 Y	4 predicted N residues (31N ++, 96N +, 260N ++, 369N ++)	8 predicted T residues (T38, T41, T46, T47, T51, T52, T53, T57)	2 high-probability sumoylation sites (K125, K338)
Insulin-degrading enzyme	18 S T 8 Y 15	2 predicted N (732 N+++, 994 N+)	None	4 high-probability sumoylation sites (K562, K523, K999, K488)

Full-length NEP (accession number NP009220.2), angiotensin-converting enzyme (accession number AAH36375.1), and insulin-degrading enzyme (accession number AAH96336).

*NetPhos 2.0 prediction of phosphorylation sites (http://www.cbs.dtu.dk/services/NetPhos/).

†NETNGly 1.0 prediction of *N*-glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/).

‡NETOGlc 3.1 prediction of *O*-glycosylation sites (http://www.cbs.dtu.dk/services/NetOGlyc/).

§SUMOplot prediction (http://www.abgent.com/doc/sumoplot) software was used to assess the likelihood of posttranslational modification: +, low probability; ++, medium probability; +++, high probability.

K, lysine; N, asparagines; S, serine; T, threonine; Y, tyrosine.

that accumulates in CAA is thought to be predominantly, if not exclusively, of neuronal origin (184–186). The isoform that is most abundant in vessel walls in CAA is $A\beta_{1-40}$ (187), but it is unclear whether this is also the isoform of Aβ that is most toxic to CVSMCs.

Cerebrovascular accumulation of Aβ may be caused by increased production of this peptide in familial forms of AD and CAA. However, in most cases, decreased removal, particularly as a result of the effects of age on vessel walls, is likely to be the major cause of CAA. Arteriosclerosis and age-related changes to the composition of the arterial basement membranes may impede the perivascular drainage of Aβ, as does preexisting CAA (188, 189). Yet another consideration is the effect of age on the degradation of Aβ within the vessel wall itself. Neprilysin is expressed by CVSMCs within larger arterioles in the cerebral cortex and leptomeninges (80, 81), and ECE-1 (89) and IDE (116, 117) are expressed by endothelial cells. Neprilysin immunolabeling of CVSMCs is reduced in AD, particularly in patients who have moderate to severe CAA (80).

We recently showed that NEP activity is reduced in CAA, in both the leptomeninges (within which the only source of NEP is the CVSMCs in meningeal blood vessels) and vessel-enriched preparations of cerebral cortex (23). The observed reduction was still present after adjustment for the smooth muscle actin content of the samples, that is, it was not simply a consequence of death of CVSMCs, although loss of CVSMCs, whether from arteriosclerosis or as a consequence of CAA, would be expected to exacerbate any preexisting deficiency in vessel-associated NEP. Neprilysin immunolabeling and enzyme activity in CVSMCs varied according to *APOE* genotype, being significantly higher in the absence of the *APOE* ε4 allele (23, 80). Indeed, in our recent study, the relationship between *APOE* genotype and vessel-associated NEP activity mirrored that between *APOE* genotype and risk of AD; after adjustment for smooth muscle actin, NEP activity was highest in blood vessels from patients with a *APOE* ε2/ε3 genotype and decreased stepwise through the *APOE* ε3/ε3, ε3/ε4, and ε4/ε4 genotype groups.

Cerebrovascular smooth muscle cells are sensitive to the cytotoxic effects of Aβ, particularly $A\beta_{1-42}$ (190, 191). The activity of NEP seems to be important in preventing Aβ-mediated death of CVSMCs. We demonstrated that the death of CVSMCs on exposure to $A\beta_{1-42}$ was increased by siRNA-mediated knockdown or thiorphan-mediated inhibition of NEP activity. Conversely, death of CVSMCs was reduced (i.e. the cells were protected) when NEP activity was increased by either the addition of SST or the transfection with NEP complementary DNA. Our findings provide strong evidence that NEP protects CVSMCs from Aβ toxicity and protects cerebral blood vessels from the development and complications of CAA. A reduction in NEP activity within the cerebral vasculature, either with age or as a consequence of *APOE* genotype, may predispose to the development of CAA.

THE ROLE OF MICROGLIA IN THE REMOVAL OF Aβ

The accumulation of Aβ in sporadic AD is likely to be multifactorial in etiology, reflecting the impairment of several processes, the contributions of each of which may vary between individuals. One means of Aβ clearance that has not received much attention is the uptake and removal of Aβ by microglia, the intrinsic phagocytic cells of the CNS. Microglia are activated by fibrillar Aβ (fAβ) in vitro and are found in large numbers near Aβ plaques (190, 191). Activated microglia express receptors that promote the uptake and clearance of fAβ (e.g. CD36 and the receptor for advanced glycation end products [192]) and may restrict plaque formation by phagocytosis of Aβ (193). Levels of these receptors on microglia are significantly reduced in aged mice (194), suggesting that the ability of microglia to clear fAβ may decline with age.

Microglial uptake of Aβ, initiated by binding of anti-Aβ antibody to Fc receptors (195, 196) or by Fc-independent mechanisms (197, 198), is thought to be a major route of plaque removal after Aβ immunization in mouse models of AD (196, 199) and in humans (200). However, activated microglia produce a range of proinflammatory cytokines and neurotoxic factors that can themselves cause neuronal damage and death (201–204). Recent studies indicate that environmental stimuli largely determine whether microglia adopt a phagocytic ''protective'' response or a damaging ''neurotoxic'' response (205, 206) and that a proinflammatory response is associated with reduced microglial phagocytosis (207).

Modulation of the microglial response by either suppressing the proinflammatory response or boosting the anti-inflammatory response has potential therapeutic potential. Administration of rosiglitazone (a peroxisome proliferator-activated receptor agonist) facilitates Aβ removal in hAPP mice by switching on the phagocytic phenotype of microglia, a process that is mediated by the suppression of proinflammatory mediators (208). Heneka et al (209) reported that the addition of noradrenaline to cultures of microglia exposed to fibrillar $A\beta_{1-42}$ suppressed cytokine and chemokine production and promoted cell migration and phagocytosis of Aβ. In contrast, ablation of the noradrenergic locus coeruleus in hAPP mice, a procedure that suppresses neuroinflammation, reduced microglial migration and phagocytosis of Aβ. In vitro, the anti-inflammatory cytokines transforming growth factor β (TGF-β) and interleukin (IL)-4 increased microglial clearance of Aβ, including oligomeric Aβ (210, 211), and overexpression of TGF-β in hAPP mice resulted in a 70% reduction in parenchymal Aβ load (210).

On receptor-mediated phagocytosis, fAβ is transported within the endosomal-lysomal pathway (212–215). Because fAβ has shown considerable resistance to proteolytic cleavage, the extent to which internalized fAβ is efficiently degraded remains unclear (216, 217). Degradation of internalized Aβ in primary mouse microglia was mediated by acidification of lysosomes (to pH ~5–6) after activation by the proinflammatory mediators, macrophage-stimulating colony factor and IL-6 (218). This suggests that Aβ-degrading proteases such as cathepsins B and D (219, 220) and ECE-2 (98), which work optimally within a mildly acidic pH of 5.5 rather than neutral endopeptidases (NEP, IDE, ECE-1) may be more important in microglial clearance of Aβ. Cathepsins S, D, and B were reported to participate in the intracellular degradation of Aβ peptides in human and rat brains (32, 221, 222).

In contrast, uptake and clearance of soluble Aβ is rapid and involves macropinocytosis and rapid delivery to lysosomes (223). Soluble Aβ is sensitive to many proteases including NEP and IDE, although whether NEP and IDE are involved in the intracellular degradation of soluble Aβ has yet to be established (192).

Microglia probably also participate in the extracellular degradation of Aβ. Microglia express IDE, which gets incorporated within exosomes, and these are then secreted (111). Tamboli et al (224) reported that statins promote microglial removal of Aβ by inducing the release of exosomal IDE. Norepinephrine (noradrenaline), which suppresses neuroinflammation (see previous paragraphs), was also found to promote uptake of Aβ by induction of mouse Aβ formyl peptide receptor but also to clear Aβ through production of IDE (225). Both NEP and IDE expression were increased in rat microglia by treatment with IL-4 (211).

THERAPEUTIC POTENTIAL OF Aβ-DEGRADING ENZYMES IN AD

Although it remains unproven that a decline in Aβ-degrading enzyme activity contributes to the accumulation of Aβ in AD (25, 26, 171), it is clear that overexpression of Aβ-degrading enzymes, neprilysin in particular, can reduce amyloid deposition and ameliorate cognitive decline in AD animal models (155, 161, 162). There are several ways in which these findings could be translated into therapeutic strategies that might be suitable for use in humans. These include the administration of agents that upregulate Aβ-degrading enzyme activity, various forms of gene therapy that increase the expression of Aβ-degrading enzymes in the periphery or within the brain, direct delivery of the enzymes into the brain, and, lastly, approaches based on the delivery of stem cells.

Administration of Agents That Increase NEP Expression and Activity

The peripheral administration of agents that enhance the activity of Aβ-degrading enzymes has the appeal of ease of administration. A number of agents that increase the expression of NEP in vitro have been identified, but there is a paucity of evidence of their ability to do so in vivo. The neuropeptide SST upregulates NEP activity in vitro (157) through activation of the SST receptors, SSTR2 and SSTR4, suggesting that Aβ levels might be controlled by SST receptor agonists (226). Reduction in SST (227) and SST receptor levels (228) may contribute to Aβ pathology in AD (229). Infusion of Aβ resulted in impaired SST signaling (230) and reduction in NEP (231). These abnormalities were prevented by administration of minocycline (231) and insulin-like growth factor 1 (232), suggesting that enhancement of SST signaling might be used to increase NEP activity.

Several agents are known to increase transcription of *NEP*. The APP intracellular domain binds to the *NEP* promoter and upregulates NEP transcription in human neuroblastoma cell lines (NB7 and SH-SY-5Y) and is associated with enhanced Aβ degradation (233, 234). The tyrosine kinase inhibitor, imatinib mesilate (Gleevec), increases APP intracellular domain and NEP and lowers cellular Aβ (235). Transcriptional regulation of NEP is regulated by histone acetylation; histone deacetylase inhibitors valproate and trichostatin A raised NEP enzyme activity in SH-SY-5Y cells, which normally express NEP only at low levels (234). Several findings highlight the potential therapeutic use of valproate in AD (236): not only does it upregulate NEP, it also upregulates plasmin (237), and its safety and efficacy have been extensively documented in the treatment of epilepsy and psychosis–related behavioral problems (238).

Postmenopausal estrogen deficiency is thought to be a risk factor for AD (239), and estrogen use in postmenopausal women was found to delay the onset and reduced the risk of

AD (240). Estrogen was reported to promote Aβ degradation of SH-SY-5Y human neuroblastoma cells by increasing NEP expression (241). Reduced Aβ levels associated with a 2.9-fold increase in NEP gene transcription were observed in SK-N-SH cells transfected with hAPP (Swe) after incubation by gensenoside Rg3, a major component of ginseng (242). Other substances reported to increase NEP activity include green tea extracts (243) and red wine (244).

Gene Therapy

Genetic approaches to reducing Aβ accumulation in hAPP mice have mostly used viral vector–mediated transfection to increase the expression of NEP or other Aβ-degrading enzymes either peripherally or within the brain. The potential of gene therapy for treatment of AD was the subject of an extensive recent review by Nilsson et al (245).

Adenoassociated virus-mediated transfection of mouse NEP into the hind limb of 3×Tg-AD mice (harboring PS1M146V, hAPP(Swe), and tauP301L transgenes) produced a 60% reduction in soluble Aβ and 50% reduction in plaque deposits within the brain at 6 months (246). Peripherally expressed NEP did not affect levels of physiological substrates of NEP within the CNS, including substance P, Leu-Enk, and bradykinin. Lebson et al (247) used peripheral bone marrow cells (CD11b+ monocytes) to deliver NEP in the brain. CD11b+ monocytes transfected with human soluble NEP tracked toward compact Aβ plaques within the brain and completely arrested plaque development in hAPP transgenic mice. The shorter-than-expected half-life of these cells in the circulation (~90 minutes) and within the tissue (~3 days) may limit their practical use for long-term chronic conditions such as AD. Intraperitoneal injection of a lentivirus vector-expressing NEP fused with the ApoB transport domain (allowing active transport across the blood-brain barrier) was associated with lower Aβ levels and a reversal of memory deficits in mThy1-hAPP751 mice (248).

Unilateral injection of lentivirus or adenovirus encoding human *NEP* into the hippocampus of hAPP mice reduced Aβ load and significantly improved performance in memory tasks (159, 160). However, this approach is associated with systemic and local immune responses that can neutralize the therapeutic effects (249). Overexpression of NEP by neurons in a *Drosophila* model of AD decreased $A\beta_{1-42}$ accumulation and neuronal loss but also led to an age-dependent axonal degeneration and shortened the lifespan of the flies (250), probably caused by the effects on physiological neuropeptide substrates of NEP, including enkephalin, substance P, glucagon-like peptide 1, and galanin (246, 251, 252).

Long-term gene transfer of human NEP in hAPP mice, producing a moderate (~3-fold) but sustained increase in NEP, reduced intracellular and extracellular Aβ and improved behavior and memory (161, 162). The mice also had evidence of reduced oxidative stress and inflammation (162) and less synaptic and dendritic damage (161). Hippocampal transplantation of fibroblasts transfected with complementary DNA encoding soluble NEP reduced plaque burden in hAPP mice (253). So too did transplantation of NEP-expressing leukocytes into 3×Tg AD mice, which had lower levels of soluble (30%) and plaque-associated Aβ (50%–60%) and improved cognitive performance (254). This therapeutic strategy was applied to AD in a phase 1 clinical trial of ex vivo NGF gene therapy, in which autologous fibroblasts genetically modified to express NGF were neurosurgically implanted into the basal forebrains of 8 patients (255).

Convection-Enhanced Delivery of NEP as a Potential Treatment for AD

Although viral vector-mediated NEP gene delivery has proven effective in transgenic mouse models, there are significant barriers to the translation of this approach to the treatment of patients (256–258). Many of the limitations of viral vectors might be overcome by delivery of NEP directly to the brain. This approach also has advantages in patient safety, in that the delivery of NEP could rapidly and reliably be adjusted or discontinued in the event of adverse effects.

Convection-enhanced delivery (CED) is a relatively novel neurosurgical method of direct drug delivery to the brain through ultrafine microcatheters. Historically, intracerebral drug injection has relied on diffusion of a therapeutic agent along a concentration gradient resulting in limited distribution and is associated with tissue damage and reflux of infusate along the outer surface of the needle or catheter. Convection-enhanced delivery relies on a pressure gradient established at the tip of a very fine infusion catheter resulting in bulk flow of the infused fluid through the extracellular and perivascular spaces (259).

Convection-enhanced delivery has been used in clinical trials for a number of neurodegenerative and neuro-oncological diseases such as CED of glial cell–derived neurotrophic factor for Parkinson disease (260–265). It was also used for enzyme replacement in preclinical models of Gaucher disease in which preservation of enzyme function after CED was later demonstrated (266, 267).

The challenges of applying CED to CNS disorders in which the pathology is predominantly focal, such as brain tumors or Parkinson disease, differ from those presented by AD, in which the pathology is more widespread. Early in vivo studies of CED attributed the distribution of macromolecules to interstitial flow and neuronal transport (267–269). However, as research progressed to include the delivery of liposomes and viral vectors, it became clear that agents delivered by CED tended often to accumulate in close association with blood vessels and perivascular spaces (270, 271). Convection-enhanced delivery of Aβ-degrading proteases may therefore be particularly effective for the clearance of Aβ in perivascular drainage pathways and treatment of CAA (9, 10, 272–274).

Stem Cell Therapy

Stem cells are a potential vehicle for delivery of Aβ-proteases into the CNS. One in vitro study reported significant upregulation of NEP (692 ± 226-fold increase of mRNA levels) after neuroectodermal conversion of adult mesenchymal stem cells (275). However, clinical translation of stem cell therapies faces many obstacles, including those of cell delivery in adequate numbers, prediction and control of cell

migration, and, in the case of nonautologous transplants, a lifelong requirement for immunosuppression (276, 277).

CONCLUSIONS

Deficient clearance of Aβ from the brain probably contributes to late-onset AD. The routes of Aβ clearance are diverse and numerous and include its enzyme-mediated cleavage, resulting in fragments that are generally less toxic, less likely to aggregate, and more easily cleared. It remains unclear whether deficiencies in these enzymes contribute to Aβ accumulation in AD. In cell culture systems, most Aβ-degrading enzymes are upregulated by fibrillar Aβ, and their expression tends to increase in transgenic mouse models of AD once Aβ plaques start to form. The activity of several Aβ-degrading enzymes also rises with age in the human brain and in AD and correlates with the level of fibrillar Aβ. Together, these findings suggest that upregulation of Aβ-degrading enzymes is a physiological response to Aβ, perhaps serving to minimize further buildup of this peptide.

In animal models of AD, by increasing the activity of some of the Aβ-degrading enzymes, NEP in particular, Aβ accumulation can be reduced or prevented and cognitive performance improved. The early initiation of therapy is likely to be critical. Strategies directed at the removal of Aβ in advanced disease, particularly Aβ immunotherapy in human clinical trials, have resulted in the clearance of Aβ plaques without improvement in cognitive performance (278), suggesting that downstream pathogenic processes eventually become independent of the continued accumulation of Aβ (136). Of the various potential approaches to translating these findings into a treatment of AD in man, we suggest that the most promising, at least in the short to medium term, are the peripheral administration of drugs that increase NEP activity and the direct CED of NEP into the brain.

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