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SYMPOSIUM: Clearance of Aβ from the Brain in Alzheimer's Disease

Aβ-Degrading Enzymes in Alzheimer's Disease

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Abstract

In Alzheimer's disease (AD) Aβ accumulates because of imbalance between the production of Aβ and its removal from the brain. There is increasing evidence that in most sporadic forms of AD, the accumulation of Aβ is partly, if not in some cases solely, because of defects in its removal—mediated through a combination of diffusion along perivascular extracellular matrix, transport across vessel walls into the blood stream and enzymatic degradation. Multiple enzymes within the central nervous system (CNS) are capable of degrading Aβ. Most are produced by neurons or glia, but some are expressed in the cerebral vasculature, where reduced Aβ-degrading activity may contribute to the development of cerebral amyloid angiopathy (CAA). Neprilysin and insulin-degrading enzyme (IDE), which have been most extensively studied, are expressed both neuronally and within the vasculature. The levels of both of these enzymes are reduced in AD although the correlation with enzyme activity is still not entirely clear. Other enzymes shown capable of degrading Aβ *in vitro* or in animal studies include plasmin; endothelin-converting enzymes ECE-1 and -2; matrix metalloproteinases MMP-2, -3 and -9; and angiotensin-converting enzyme (ACE). The levels of plasmin and plasminogen activators (uPA and tPA) and ECE-2 are reported to be reduced in AD. Reductions in neprilysin, IDE and plasmin in AD have been associated with possession of *APOE* ε4. We found no change in the level or activity of MMP-2, -3 or -9 in AD. The level and activity of ACE are increased, the level being directly related to Aβ plaque load. Up-regulation of some Aβ-degrading enzymes may initially compensate for declining activity of others, but as age, genetic factors and diseases such as hypertension and diabetes diminish the effectiveness of other Aβ-clearance pathways, reductions in the activity of particular Aβ-degrading enzymes may become critical, leading to the development of AD and CAA.

INTRODUCTION

Aβ accumulation is thought to be central to the pathogenesis of Alzheimer's disease (AD) (62); evidence includes the finding that familial autosomal dominant forms of AD all result from gene mutations that increase either both $A\beta_{1–40}$ and $A\beta_{1–42}$, or the ratio of $A\beta_{1–42}$: $A\beta_{1–40}$. The mechanism of Aβ accumulation in cases of late-onset sporadic AD (LOAD) is less clear. Although some groups have found increased levels or activity of β-secretase (the rate-limiting enzyme in Aβ synthesis) in LOAD, other evidence suggests that this is secondary to Aβ accumulation rather than a primary abnormality. The level of Aβ within the brain depends not only on the rate of Aβ production, but also on the rate of its removal through various clearance pathways and by enzyme-mediated degradation. Several candidate Aβ peptidases are expressed both neuronally and within the cerebral vasculature. This review will focus on studies on human post-mortem brain tissue of the potential relevance of some of these peptidases to the pathogenesis of AD.

Aβ ACCUMULATION IN AD

Aβ production results from amyloidogenic processing of amyloid precursor protein (APP): the sequential cleavage of APP by β- and γ-secretases (51, 95, 131). The predominant species of Aβ thereby produced are $A\beta_{1–40}$ and, in lesser amounts, $A\beta_{1–42}$. Cleavage by α-secretase of APP within the Aβ segment (non-amyloidogenic processing) prevents the formation of Aβ (24, 79, 84). When present in excess, extracellular $A\beta_{1–42}$, which is more prone to aggregate than $A\beta_{1–40}$ (71), tends to precipitate within the brain parenchyma, forming plaques, whereas $A\beta_{1–40}$ 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 over 90% of patients with AD.

Excessive accumulation of Aβ in early-onset familial AD (EOAD) and hereditary CAA, results from increased amyloidogenic processing of APP, often associated with an increased ratio of $A\beta_{1–42}$: $A\beta_{1–40}$ (115, 128). This occurs as a result of

autosomal dominant mutations in the genes encoding APP (*APP*), presenilin-1 (*PSEN-1*) or presenilin-2 (*PSEN-2*) (141), or from increased production of APP, in trisomy 21 (16, 40) or duplication of the APP locus on chromosome 21 (25, 121). APP gene mutations that cause a decrease in the $A\beta_{1–42}$: $A\beta_{1–40}$ ratio (ie, a relative excess of $A\beta_{1–40}$) usually cause familial CAA(68).

These data constitute strong evidence that Aβ accumulation is central to the pathogenesis of not only AD but also CAA. To date, there is however, little evidence to suggest that an increase in neuronal synthesis of Aβ, or an increase in the overall level of Aβ production via amyloidogenic processing, is responsible for the development of the majority of LOAD and sporadic CAA cases. Several groups have found that the level of β-secretase-1 (BACE-1) activity, the rate-limiting enzyme in Aβ synthesis, is increased in AD cases compared with controls (reviewed in Stockley and O'Neill (138). Tyler *et al* (148) observed increased BACE-1 activity, and a concurrent decrease in α-secretase activity in tissue homogenates from the temporal cortex in AD. Increased BACE-1 activity in AD was found to result from a rise in the maximum rate of enzyme activity, mediated by a post-translational mechanism, rather than a change in BACE-1 protein levels, which were reduced in AD, especially in cases with advanced disease (139). A study of two strains of mouse transgenic for mutant human APP (one causing early and one late accumulation of Aβ) revealed an increase in the amount of BACE-1 only after the commencement of Aβ plaque formation (168). Elevated BACE-1 levels appeared to be restricted to the perimeter pf plaques, and occurred without any change in BACE-1 mRNA level. These findings argue against a primary role for BACE-1 in the formation of plaques but suggest that BACE-1 activation, via a positive feedback loop initiated by parenchymal accumulation of Aβ, could exacerbate Aβ production in AD.

Evidence obtained over the past decade points to deficiencies in Aβ clearance or enzyme-mediated Aβ degradation as factors potentially responsible for Aβ accumulation, particularly in LOAD and CAA. Intact, soluble Aβ is probably cleared from the brain by several routes: low-density lipoprotein receptor-related protein-1 (LRP-1)-mediated transport across vessel walls into the circulation (134), transport of Aβ across the blood–brain barrier, from the abluminal to the luminal side via the P-glycoprotein (PgP/MDR1/ABCB1) efflux pump (81, 83, 154), and drainage along perivascular basement membranes, possibly into the cerebrospinal fluid (CSF) (114, 158, 159). Aβ can potentially gain access to the CNS from the blood, through binding to receptor for advanced glycation end products (RAGE) in endothelial cells (38, 92). Zlokovic *et al* recently showed that Aβ within the blood stream binds to soluble LRP-1; this acts as a peripheral sink, sequestering the circulating Aβ and promoting the passage of soluble Aβ out of CNS (123). The above studies are discussed in detail in accompanying papers in this symposium.

While some soluble Aβ is therefore cleared from the brain still intact, Aβ also undergoes enzymatic degradation (43, 156). Some of the candidate Aβ peptidases can cleave only soluble monomeric Aβ. Others are able to degrade Aβ oligomers and even fibrillar aggregates, that is, forms of Aβ that cannot directly be cleared into the blood or CSF.

CANDIDATE Aβ PEPTIDASES IN THE CNS

Multiple enzymes have been identified that can cleave at either a single or multiple sites within Aβ. The cleavage products are less likely to aggregate and less neurotoxic than Aβ itself. The contributions of these enzymes to the normal homeostasis of Aβ by neurons *in vitro* and within the CNS of experimental animals have been the subject of a large number of studies. Although some of this information is touched upon below, the emphasis in the present paper is on findings that have been validated by post-mortem examination of human brain tissue.

Neprilysin

Neprilysin (NEP, also known as neutral endopeptidase-24.11, EC.3.4.24.11, enkephalinase, neutrophil cluster-differentiation antigen 10 or common acute lymphoblastic leukemia antigen) is a 90–110 kDa plasma membrane glycoprotein of the neutral zinc metalloendopeptidase family (145–147). Within the brain, NEP is expressed at pre- and post-synaptic membranes and is involved in the regulation of neuropeptide signaling (13, 57, 135). It is also expressed in the tunica media and endothelium of cortical and leptomeningeal blood vessels (29, 98), where it is involved in the regulation of vascular tone (31, 118).

The first evidence of deficiencies in NEP in AD came from two studies showing that NEP mRNA and protein levels were significantly lower in regions of brain with high plaque burdens, and mRNA levels were lower in hippocampus from AD than control brains despite preservation of MAP-2 (neuronal) mRNA (4, 165). Subsequent studies yielded inconsistent findings: non-significant reduction in NEP mRNA in the frontal cortex and hippocampus in AD (29), significant reduction in NEP mRNA in the frontal cortex in AD (122), and no difference in NEP mRNA levels between cases and controls (65).

Antibodies to NEP label neurons within the cerebral cortex (4), particularly pyramidal neurons (29, 98). Carpentier *et al* (29) reported diminished immunolabeling of hippocampal pyramidal neurons in AD. We found significantly reduced NEP labeling in frontal and temporal neurons in AD and a non-significant reduction in the amount of NEP in homogenates of frontal cortex (98).

Carpentier *et al* (29) also noted an inverse relationship between the amounts of Aβ and NEP in the cerebral vasculature. We confirmed this and showed that vessel-associated NEP is significantly reduced in AD, the reduction being inversely related to CAA severity. In cases of AD with severe CAA, NEP levels were equally reduced in the Aβ-laden and Aβ-free vessels (98). The findings indicated that the reduction in cerebrovascular NEP in AD is not simply due to replacement of tunica media by Aβ. Carpentier *et al* (29) also observed reduced NEP in vessels in which the tunica media had not been destroyed, pointing to global reduction in vessel-associated NEP as a potential cause of CAA.

Further support for a role for NEP in normally preventing CAA comes from the finding that NEP cannot cleave the abnormal forms of Aβ that result from Dutch, Flemish, Italian or Arctic mutations in APP and which are all associated with severe CAA (142). It is also of note that NEP cleaves monomeric $A\beta_{1–40}$ more efficiently than it does $A\beta_{1–42}$ (73). A deficiency in NEP would therefore be

expected to decrease the ratio of $A\beta_{1–42}$: $A\beta_{1–40}$, favoring vascular deposition of Aβ.

We found a significant association between NEP labeling and *APOE* genotype, the only well-established genetic risk factor for both AD and CAA (30, 33, 53, 129, 140). Possession of *APOE* ε4 was associated with reduced immunolabeling of neurons and vessels for NEP (98). Colinearity of ε4 with the presence of moderate to severe CAA precluded assessment of the independence of this association from NEP levels. However, logistic regression analysis showed low NEP labeling to be a significant independent predictor of moderate to severe CAA. The precise relationship between NEP immunolabeling and enzyme activity, particularly in the vascular tunica media, remains to be established, although the likelihood is that these are directly related.

Correlation between immunolabeling studies and measurements of NEP protein levels in human brain tissue homogenates (usually by densitometry of Western blots) has generally been poor. In most studies NEP levels have not differed significantly between AD cases and controls (65, 98, 122). Wang *et al* (155) found significantly reduced NEP levels in the mid-frontal cortex in AD (but not frontotemporal degeneration, suggesting that the reduction in NEP in AD was not simply secondary to neuronal loss). NEP levels correlated directly with brain weight and synaptic protein levels and inversely with plaque counts, formic acid-extractable $A\beta_{1–40}$ and $A\beta_{1–42}$, neurofibrillary tangle counts and phospho-tau levels. Hellstrom-Lindahl *et al* (65) also reported a significant inverse relationship between NEP levels and insoluble $A\beta_{1–40}$ and $A\beta_{1–42}$ in the temporal and frontal cortex, in both control and AD brains.

The levels of NEP in post-mortem brains were shown to decline with age (65). It may be of relevance that the levels of somatostatin, which up-regulates NEP activity, also fall with age and in AD (124). Somatostatin levels in the frontal cortex were significantly lower in AD cases positive for *APOE* ε4 (61). An *APOE*-dependent genetic association between AD and three single nucleotide polymorphism (SNPs) within the somatostatin gene (3q27.3) was reported in a Finnish population (151). The biological link between somatostatin, NEP and *APOE* ε4 merits further exploration.

NEP enzyme activity in CSF is significantly reduced in AD and patients with mild cognitive impairment (MCI) [a prodromal form of cognitive decline that has a high later conversion rate to AD (153)] compared with controls (94). However, when the AD patients were subdivided according to severity of disease, the most severely affected patients showed a significant increase in NEP activity, with levels similar to those in the controls. It is not clear whether NEP activity in the CSF parallels that in brain tissue but these data suggest that (i) CSF measurement of NEP activity has potential as a biomarker of early AD, and (ii) there may be late-stage up-regulation of NEP activity (eg, as a response to elevated Aβ). We (98) and others (155) used fluorogenic peptide substrates to measure NEP activity in brain homogenates and found this to be significantly reduced in AD. However, when applied to brain homogenates, the specificity of this method for NEP is questionable (100). We have recently developed immunocapture-based fluorogenic peptide cleavage assays (100) that allow the measurement of NEP and IDE enzyme activity levels in brain homogenates with much greater specificity (Figure 1).

Some genetic studies have shown strong associations between polymorphisms in the gene-encoding NEP (*MME*) and AD (64, 125, 133) whereas others have found no associations (111, 137).

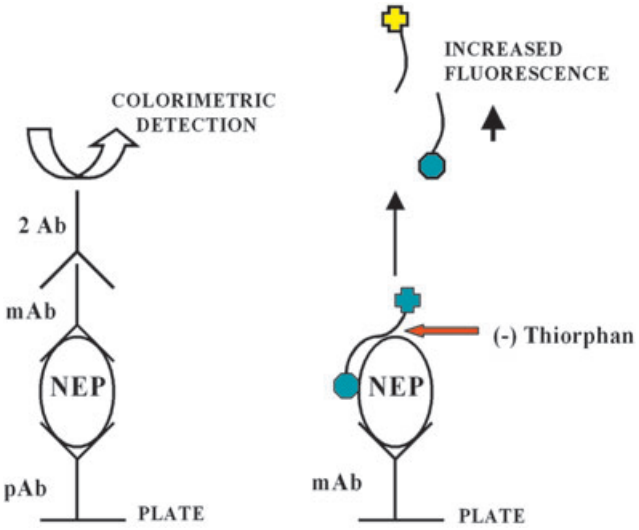

Figure 1. Schematic representation of immunocapture-based fluorometric enzyme activity assay for the measurement of neprilysin (NEP) and insulin-degrading (IDE) enzyme activity within brain tissue homogenates. The initial immunocapture phase (similar to a standard indirect sandwich ELISA method), prior to the addition of the fluorogenic substrate, allows the specific measurement of NEP enzyme activity in biological tissues separate from other closely related enzymes. Specificity is demonstrated by almost complete inhibition of fluorogenic peptide substrate cleavage in the presence of thiorphan (200 nM), a NEP-specific inhibitor. The assay combines high sensitivity and specificity and can be adapted to a 96-well plate format to permit high-throughput analysis. ELISA = Enzyme-linked immunosorbent assay.

Yamada *et al* (163) reported that a GT repeat polymorphism in the enhancer/promoter region of NEP was associated with CAA severity.

Insulin-degrading enzyme

Insulin-degrading enzyme (IDE) (insulysin, insulinase, EC3.4.24.56) is a zinc metalloendopeptidase that is highly expressed in the liver, testis, muscle and brain (82). It is a single polypeptide with a molecular weight of 110 kDa and is encoded by a gene (*IDE*) on chromosome 10q23–q25 (2). IDE is predominantly cytosolic (3, 17, 41), with smaller amounts in peroxisomes (8, 103), rough endoplasmic reticulum and plasma membranes (60, 132). A secreted form of IDE is present within extracellular compartments such as CSF (117). IDE degrades a wide range of substrates that share a common amyloidogenic secondary protein structure and include insulin (42, 60, 78), amylin (17), insulin-like growth factors I and II (101) and Aβ (96, 113, 117).

IDE is predominantly expressed neuronally within the brain (18, 32) and has been demonstrated in Aβ plaques and endothelial cells (102). In AD, immunolabeling suggested that neuronal and plaque-associated IDE was increased in AD (18) but quantitative *in situ* hybridization demonstrated reduced neuronal IDE mRNA in the dentate granule cells, hilus and CA2-3 fields of the hippocampus, and Western blot analysis of hippocampal homogenates showed decreased IDE protein levels. The reduction of IDE mRNA and

protein in AD was *APOE* ε4 dependent (32). Our immnuolabeling studies have shown that neuronal IDE expression within the pyramidal cells of the hippocampus is highest in CA3/4 neurons and lowest in the CA1 subfield and is significantly reduced in AD cases compared with controls (Figure 2). In our cohort IDE immunolabeling levels varied with APOE genotype but not significantly, and were lowest in patients with advanced Braak tangle stages and high temporal Aβ plaque load.

Reduced levels of cystolic IDE in AD were reported by Perez *et al* (113). More recently, Zhao *et al* (169) demonstrated that hippocampal IDE protein and activity were reduced in MCI and further reduced in AD; in contrast to Perez *et al* (113), the reductions were within the membrane fraction and not the cytosol. The reduction in membrane-bound IDE was region-specific (not seen in the occipital cortex) and correlated inversely with $A\beta_{1–42}$ load. Region-specific alterations were also reported by Caccamo *et al* (26): reduced levels in IDE in the hippocampus and cerebral cortex but increased levels in the cerebellum.

IDE has also been detected immunohistochemically in pericytes, endothelial and cerebrovascular smooth muscle cells (59, 102). IDE was present within occasional vessels in AD but not controls (18). Measurement of IDE levels in vessel-enriched preparations of brain tissue showed cases with severe CAA to have elevated IDE levels but reduced IDE activity (102), raising the possibility that inactivation or inhibition of IDE activity may contribute to the development of CAA.

There is evidence of genetic linkage of AD to chromosome 10 in the region to which *IDE* is mapped (19, 104). Several studies observed an association between *IDE* haplotypes (20, 21, 50, 56, 116), and SNPs within the *IDE* gene, with AD (152). Other studies (1, 23, 126) have not found significant associations. Genetic variation in close proximity to the *IDE* gene was found to be associated with clinical disease severity, plaque and neurofibrillary tangle density (116) and plasma Aβ42 levels in AD patients, (50) providing further support for a genetic association with AD. Naturally occurring splice variants of IDE associated with AD were reported to have reduced catalytic activity (54, 55).

Endothelin-converting enzymes

Endothelin-converting enzymes ECE-1 and ECE-2 (EC 3.4.24.71) are type II integral membrane zinc metalloendopeptidases (144) that are primarily localized to the endothelium throughout the human vasculature (36). They share common catalytic substrates and are responsible for cleaving big endothelins to produce potent vasoconstrictor endothelins (48, 162). In man, four isoforms of ECE-1 are encoded by a single gene on chromosome 1 (1p36). These differ in their subcellular location: ECE-1a, 1c and 1d are located predominantly in the plasma membrane (130, 150); ECE-1b is predominantly intracellular. ECE-2 is present intracellularly and has an acidic pH optimum in contrast to the neutral pH optimum of ECE-1 (48).

Immunolabeling of rat (14) and bovine brain (48) has revealed ECE-1 in neurons, specifically within pyramidal neurons of the hippocampus and layer V of the neocortex (136). A few astrocytes were also labeled (136). In mice, ECE-2 labeling is largely confined to the brain (164); in the rat brain, ECE-1 and ECE-2 mRNA was localized to neurons (105).

Limited information is available on the expression of ECE-1 and -2 in human brain. ECE-1 immunoreactivity was seen within neurons and their processes in the cerebral cortex (37). We found the strongest labeling for ECE-1 to be within the cerebrovascular endothelium, whereas ECE-2 was predominantly neuronal, with strong labeling of hippocampal pyramidal neurons (Figure 3).

Although several experimental studies have highlighted a role for ECE-1 and ECE-2 in degradation of Aβ (44–46) there are few data from studies on human tissue. A recent microarray study, looking at changes in gene expression in the inferior parietal lobe in LOAD, revealed a highly significant decrease in ECE-2 gene expression, confirmed by RT-PCR (157). Immunolabeling showed loss of ECE-2 from neurons; similar loss was not seen in Parkinson's disease dementia, hippocampal sclerosis or dementia lacking distinctive histological features. Yoshizawa *et al* (167) found ET-1 levels in human CSF to be lower in AD patients than controls; it remains to be established whether or not the lower ET-1 levels are caused by reduced ECE activity.

A SNP in ECE-1 (chromosome 1p36) was associated with increased hippocampal neuronal ECE-1 expression and protection from AD in a large case–control study (58). No studies have assessed genetic associations of ECE-2 with AD.

Angiotensin-converting enzyme

Angiotensin-converting enzyme (ACE; peptidyl-dipeptidase A; EC 3.4.1.5.1) is a membrane-bound zinc metalloprotease that is widely expressed in the vasculature throughout the body. ACE is encoded by a gene (*ACE*) on chromosome 17q23.3 and has an important role in the regulation of fluid homeostasis and blood pressure (49, 119). The two major physiological substrates for ACE are angiotensin I (which is converted by ACE to the vasoconstrictor angiotensin II) and bradykinin. Within the human brain, ACE has been detected predominantly in pyramidal neurons in the cortex (layer V) and within the cerebral vasculature (99, 127).

Human genetic studies have provided strong evidence of an association between ACE and AD. Several studies, including a large meta-analysis, revealed that possession of an insertion (I) polymorphism within intron 16 of *ACE* is strongly associated with AD risk whereas the deletion (D) variant is protective (47, 75–77, 90, 107). In a study concerned with the genetic basis of hypertension, serum ACE protein levels were found to be lower in people with the I/I (higher AD risk) genotype (120). These findings have been replicated in human post-mortem CSF samples from our own cohort; ACE protein levels were significantly decreased in CSF from AD cases compared with controls and were significantly lower in CSF from individuals with the I/I genotype (Figure 4). *In vitro* studies have shown that ACE can cleave $A\beta_{1–40}$ and $A\beta_{1–42}$ (66, 69, 110) and converts $A\beta_{1–42}$ to $A\beta_{1–40}$ (170). However, Lendon *et al* (91) found no relationship between *ACE* genotype and either Aβ load or CAA severity. Other studies have not shown *ACE* genotype to be associated with vascular dementia or vascular pathology (80, 90).

The conclusions from animal studies of the effects of ACE on Aβ homeostasis are not entirely clear. Most have found inactivation of the mouse *ACE* gene or inhibition of ACE in mice not to affect Aβ levels (46, 67) but Zou *et al* (170) reported that administration of the ACE inhibitor captopril to mice transgenic for the Swedish APP double mutation increased $A\beta_{1–42}$ deposition.

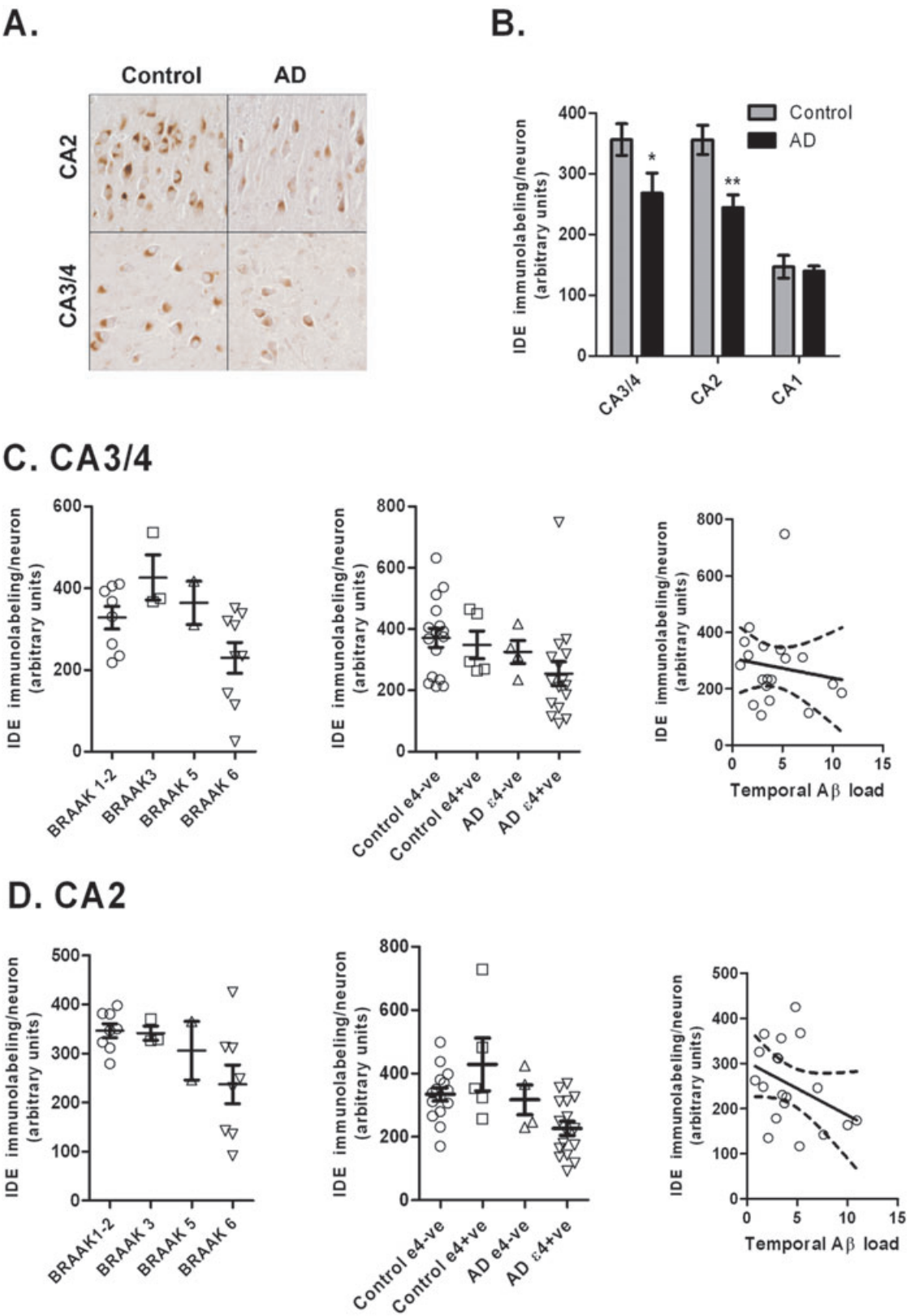

Figure 2. (**A**) Insulin-degrading enzyme (IDE) immunolabeling of neurons within the CA3-4 and CA2 regions of the hippocampus in control cases and Alzheimer's disease (AD). (**B**) IDE immunolabeling of neurons is significantly reduced in AD cases (n = 20) compared with age-matched controls (n = 22) (CA3-4, $P < 0.05$ and CA2, $P < 0.01$). Reductions in IDE neuronal labeling in (**C**) CA3-4 and (**D**) CA2 hippocampal regions are inversely related to Braak tangle stage, *APOE*ε4 genotype, and Aβ plaque load in the temporal lobe. The error bars indicate standard error of mean.

In post-mortem brain tissue, ACE protein levels and activity are raised in AD. Savaskan *et al* (127) described increased immunolabeling of ACE in neurons and blood vessels in the parietal cortex in AD. ACE levels were also raised in hippocampal homogenates (12) and ACE activity increased in the caudate nucleus and cerebral cortex in AD (6). We also found ACE immunolabeling and activity to be elevated in the frontal cortex in AD (99); the activity correlated directly with parenchymal Aβ plaque load, raising the possibility that intracerebral ACE levels are up-regulated as a response to Aβ accumulation within the brain.

In our series, ACE immunolabeling in frontal and temporal cortex was predominantly perivascular and was significantly increased in moderate to severe CAA. Perivascular ACE co-localized with the extracellular matrix marker proteins fibronectin and decorin. Angiotensin II (ANG II), the primary cleavage product of ACE, is expressed alongside ACE in the cerebral vasculature (127). The significance of perivascular ACE in CAA is unclear but it may be relevant that we have found strong labeling of blood vessels for ANG II receptor in some patients with CAA (Figure 5). ANG II stimulates the production of ECM, fibronectin in particular (34, 112), and also induces expression of transforming growth factor β (TGF-β) and its receptors (28, 88). TGF-β stimulates the synthesis of ECM and is associated with pathological accumulation of ECM in various inflammatory and fibrotic diseases (22, 106) and is elevated in AD (160). Overexpression of TGF-β by astrocytes or neurons in transgenic mice resulted in deposition of ECM and accumulation of Aβ perivascularly (149, 161).

ACE enzyme activity was increased in the CSF in a series of patients with either MCI or AD compared with controls. ACE activity levels were significantly higher in MCI cases than in AD (63). It remains unclear, however, whether enzyme activity

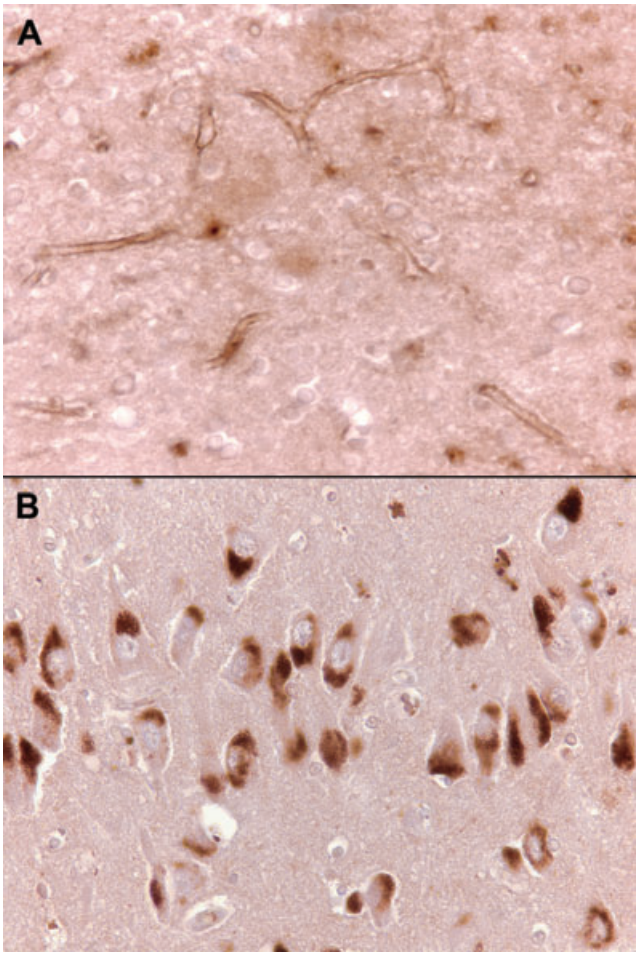

Figure 3. *Endothelin-converting enzyme (ECE-1) in sections of temporal lobe.* **A.** Immunolabeling ECE-1 is largely confined to the endothelium. **B.** Neurons in the CA2 subfield of the hippocampus are strongly immunopositive for ECE-2.

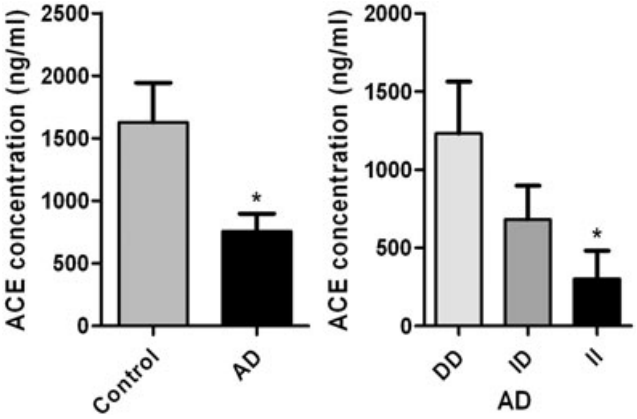

Figure 4. Angiotensin-converting enzyme (ACE) protein levels in human post-mortem cerebrospinal fluid (CSF) samples. The bars in the left chart show the mean values and standard error of the mean in Alzheimer's disease (AD) and control samples. In the right chart the levels are subdivided according to ACE (I/D) genotype. ACE levels (measured by sandwich ELISA) were significantly reduced in AD cases (n = 122) compared with controls (n = 20) ($P < 0.05$). ACE CSF protein levels were significantly lower in AD cases of I/I genotype (n = 29) (ie, the genotype associated with increased risk of AD) than in D/D (protective genotype) (n = 44) ($P < 0.05$) and lower, but not significantly, in I/D (n = 34) cases. The number of control CSF samples (I/I = 2, I/D = 8, D/D = 7) in this small series was too small for meaningful assessment of the influence of I/D genotype. ACE CSF levels did not vary according to age or gender but showed a weak positive association with post-mortem delay (which was adjusted for in the statistical analysis). ELISA = Enzyme-linked immunosorbent assay. * $P < 0.05$.

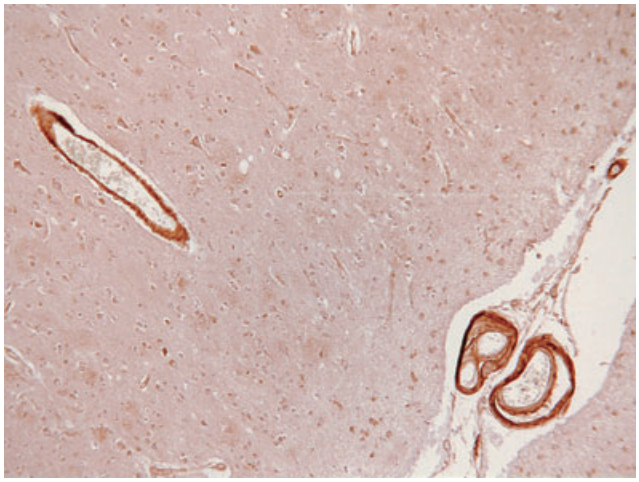

Figure 5. Strong labeling of angiotensin II receptor in arteriolar walls in an Alzheimer's disease (AD) patient with cerebral amyloid angiopathy (CAA).

parallels protein levels; another study found that ACE protein levels in CSF did not vary between AD patients and controls (109).

Plasmin

The serine protease, plasmin (EC 3.4.21.7), which is generated from inactive plasminogen, cleaves Aβ at multiple sites (143) and prevents the aggregation of $A\beta_{1-42}$ into β-pleated sheets (52). Generation of plasmin from plasminogen results from proteolytic cleavage by either tissue-type (tPA) or urokinase-type plasminogen activator (uPA). *In vivo* data from two separate human APP transgenic mouse models revealed that reduced activity of the tPA system may account for enhanced Aβ levels. In one APP(SI) transgenic mouse model, a decrease in tPA activity (with a corresponding increase in the level of tPA inhibitor) correlated with Aβ accumulation in 14-month-old mice (27). Elevated Aβ in another human APP transgenic mouse strain also correlated with increased levels of tPA inhibitor (97). In contrast, in 22-month-old transgenic mice, the levels of tPA and uPA activity were increased. Incubation of fibrillar (not soluble) Aβ with primary cortical neuronal cultures caused an increase in tPA and uPA mRNA levels (143). The plasmin system may participate in a feedback mechanism in which fibrillary Aβ induces tPA and uPA synthesis and formation of plasmin which in turn degrades the Aβ.

In human post-mortem tissue, plasmin levels were significantly reduced in the hippocampus and frontal cortex in AD (86, 87). The reduction was confined to *APOE* ε4-positive cases. The finding that several Aβ-degrading enzymes (NEP, IDE and plasmin) show a similar relationship between *APOE* genotype and reduced levels or activity suggests a common pathway through which *APOE* mediates at least some of its influence on the risk of developing AD and CAA through effects on Aβ degradation.

The *PLAU* gene which encodes uPA which has been mapped to 10q22 and which lies within the reported region of linkage to AD has not been found to be associated with AD(141).

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are zinc- and calcium-dependent endopeptidases, several of which are produced by neurons and glial cells. MMP-2 (EC3.4.24.24), -3 (EC 3.4.24B6) and -9 (EC 3.4.24.35) all have Aβ-degrading activity *in vitro* (10). Although the Aβ-degrading activity of these MMPs has not yet been explored *in vivo*, MMP-9, unlike ECE-1, NEP and IDE, is capable of cleaving aggregated Aβ-fibrils (25). Asahina *et al* (7) reported increased MMP-9 immunolabeling of neurons in AD, as well as labeling of neurofibrillary tangles, plaques and vessel walls. Backstrom *et al* (9) found MMP-2 and -9 activity to be elevated in homogenates of hippocampal tissue from AD brains. Several cell types (glial, neuronal and vascular) up-regulate endogenous MMP-2 -3 and -9 expression in response to Aβ stimulation (39, 72, 89).

We looked at MMP-2, -3 and -9 levels and activity in the frontal cortex in AD (11). MMP-2 was detected immunohistochemically in the walls of some blood vessels and scattered white matter glia, MMP-3 in and around some neurons and within occasional plaques, and MMP-9 in many neurons. In our series, the level and activity of these three MMPs did not differ significantly between AD and control brains and were not related to Aβ plaque load. Analysis of polymorphisms in the *MMP-3* (-1171 5A/6A) and *MMP-9* (C-1562T) genes indicated that the -1171 6A *MMP-3* allele (with reduced promoter activity) was associated with AD; the *MMP-9* polymorphism was not.

UP-REGULATION OF Aβ PEPTIDASES WITH AGE AND IN RESPONSE TO Aβ

IDE levels (85), tPA and uPA activity (143) and MMP-2 and -9 activity (166) were all shown to be increased in aged mice that were transgenic for mutant human APP. Neprilysin levels were also increased, although not significantly, in aged Tg2567 mice (5). Increases in the levels of Aβ peptidases in the transgenic mice seem to parallel increases in Aβ load–somewhat surprisingly given the reduction in levels of these enzymes in AD. An observation in several studies on aged human APP transgenic mice (5, 85, 166) and post-mortem tissue (29) is the presence of NEP, IDE or MMP-2- or -9-positive astrocytes in close proximity to Aβ plaques, suggesting up-regulation by Aβ of these enzymes in astrocytes and, possibly, a role for astrocytes in the removal of Aβ, as suggested by Nicoll and Weller (108).

There is *in vitro* evidence that incubation of neuronal, microglial and vascular smooth muscle cells with fibrillar (not soluble) Aβ or fragments of APP can cause significant elevation in the levels of MMP-2, -3 and -9 (39, 72, 89), IDE (85) and plasminogen activators (143). These studies, together with others noted earlier, suggest that Aβ-degrading enzymes are part of a normal regulatory process that act at several stages after Aβ synthesis, up to and including plaque formation and CAA, to limit Aβ accumulation within brain parenchyma and blood vessels (Figure 6). Reduction in the level or activity of an Aβ peptidase may be offset, or at least partially compensated for, by consequent up-regulation of other Aβ-degrading enzymes. However, as age, environmental factors, genetic factors, diseases such as hypertension and diabetes impact on the effectiveness of other Aβ-clearance pathways, impairments in

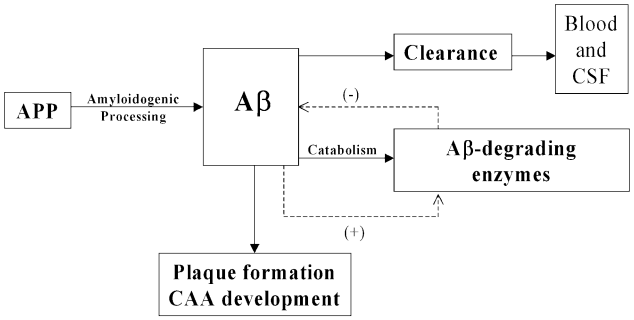

Figure 6. Steady-state Aβ levels are maintained by a balance between amyloidogenic processing of amyloid precursor protein (APP) and the removal of soluble Aβ by clearance pathways and enzyme-mediated degradation. Reduced activity of Aβ-degrading enzymes favors Aβ accumulation. This probably initiates compensatory upregulation (+) of Aβ-degrading enzymes, increased degradation of Aβ and reduction (–) in its accumulation. However, reduction in the level or activity of Aβ-degrading enzymes as a result of age, genetic and environmental factors, and a decline in the efficiency of other Aβ clearance pathways, causes excessive accumulation of Aβ, plaque formation and deposition of Aβ within vessel walls.

the activity of particular Aβ-degrading enzymes may become critical (Figure 6).

THERAPEUTIC CONSIDERATIONS

Virus-mediated delivery of human NEP, in mouse models of AD, has demonstrated the therapeutic potential of exogenous Aβ peptidases for reduction of Aβ levels (67, 70, 93). Most Aβ-degrading enzymes have multiple physiological roles. NEP and ACE, for example, are involved peripherally in the regulation of blood pressure and vascular tone (118). Drugs that have been developed for the treatment of hypertension and which inhibit NEP, ACE and ECE-1 (15, 35) have the potential to act adversely on the development of AD and this will require careful assessment in animal studies and, if given to patients, close clinical monitoring and post-mortem evaluation. Current evidence suggests, however, that agents routinely used in the treatment of hypertension, which have the ability to impact on Aβ levels if they penetrate the blood–brain barrier, offer some protection against cognitive decline in MCI and AD [reviewed in Kehoe and Wilcock (74)].

CONCLUSIONS

Aβ-degrading enzymes are involved in the physiological regulation of Aβ levels in the brain. Altered levels or activity of several of these enzymes have been demonstrated in both AD and CAA and may be influenced by *APOE* genotype. The multiplicity of enzymes capable of degrading Aβ within the brain and the availability of other pathways of Aβ removal probably limit the immediate impact of deficiency of any particular Aβ-degrading enzyme. However, as other pathways of Aβ removal decline in efficiency with age or disease, a reduced capacity for enzymatic degradation of Aβ may become critical, resulting in its accumulation within the brain parenchyma and blood vessels and the development of AD and CAA.

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