Aβ-degrading enzymes: modulators of Alzheimer’s disease pathogenesis and targets for therapeutic intervention

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Abstract
The accumulation of Aβ (amyloid β-protein) peptides in the brain is a pathological hallmark of all forms of AD (Alzheimer’s disease) and reducing Aβ levels can prevent or reverse cognitive deficits in mouse models of the disease. Aβ is produced continuously and its concentration is determined in part by the activities of several degradative enzymes, including NEP (neprilysin), IDE (insulin-degrading enzyme), ECE-1 (endothelin-converting enzyme 1) and ECE-2, and probably plasmin. Decreased activity of any of these enzymes due to genetic mutation, or age- or disease-related alterations in gene expression or proteolytic activity, may increase the risk for AD. Conversely, increased expression of these enzymes may confer a protective effect. Increasing Aβ degradation through gene therapy, transcriptional activation or even pharmacological activation of the Aβ-degrading enzymes represents a novel therapeutic strategy for the treatment of AD that is currently being evaluated in cell-culture and animal models. In this paper, we will review the roles of NEP, IDE, ECE and plasmin in determining endogenous Aβ concentration, highlighting recent results concerning the regulation of these enzymes and their potential as therapeutic targets.

Introduction
AD (Alzheimer’s disease) is the most prevalent progressive neurological disease in the elderly, affecting millions of individuals throughout the world. Aβ (amyloid β-protein) abnormally deposits in the brain in all forms of AD, but in normal brain this accumulation is prevented at least in part by the rapid proteolytic degradation of the peptide. With only a fraction of AD cases currently attributable to known risk factors other than age, it is quite possible that distinct subgroups of AD patients exist for whom decreased Aβ clearance is the precipitating factor for the disease. Recently, considerable data has emerged indicating that NEP (neprilysin), IDE (insulin-degrading enzyme), ECE-1 (endothelin-converting enzyme 1) and ECE-2, and plasmin each play a role in regulating the accumulation of Aβ in the brain (Figure 1).

NEP is a key player in Aβ catabolism
In a seminal study, Iwata et al. [1] investigated the catabolism of Aβ in rat brain by injecting radiolabelled Aβ42 into the hippocampal region of the brain in the presence of various protease inhibitors. The authors observed that degradation of the radiolabelled Aβ42 was inhibited by the metalloprotease inhibitor phosphoramidon and the more selective NEP inhibitor thiorphan, but not by inhibitors of other classes of proteases. Infusion of thiorphan directly into the hippocampus of rats for 30 days resulted in the local deposition of Aβ42, demonstrating the dramatic pathological effects of inhibiting Aβ catabolism in vivo. In a subsequent report, Iwata et al. [2] conducted similar studies using NEP-knockout mice and showed that degradation of exogenously administered Aβ42 was decreased in the NEP-deficient mice compared with wild-type controls. Additionally, the endogenous levels of Aβ40 and Aβ42 were significantly elevated in the brains of NEP-knockout mice, confirming that NEP is a physiologically relevant Aβ-degrading enzyme [2].

NEP activity is regulated by factors that influence AD
Several recent reports demonstrate that NEP expression and activity are complexly regulated by many factors that have ties with AD, including aging, oestrogen, and exercise and environmental enrichment. Aging is the most significant risk factor for the development of AD, and it has been hypothesized that the heightened risk of AD with increasing age might in many cases be due to down-regulation of NEP and other Aβ-degrading enzymes. In support of this hypothesis Fukami et al. [3] reported significant age-related reductions in NEP expression in amyloid-vulnerable regions of transgenic mouse brain. Similar results were obtained by Caccamo et al. [4] who showed that the levels of both NEP and IDE decrease with age in the murine hippocampus but not in cerebellum, which is resistant to amyloid deposition. An intriguing report from Saito et al. [9] indicates that NEP activity is regulated by...
somatostatin, a neuropeptide known to be significantly reduced with aging and in the brains of AD patients [5–8]. The regulatory effect of somatostatin was discovered in a cell-based screen for activators of NEP activity. In these studies, somatostatin treatment produced a dose-dependent increase in NEP activity that was accompanied by a significant decrease in the concentration of Aβ42 in the conditioned medium [9]. To determine whether somatostatin regulates NEP activity in vivo, the investigators next examined the brains of somatostatin precursor-knockout mice and found that NEP activity was decreased, and Aβ42 concentration was concomitantly elevated, in hippocampus but not in cerebellum. These results suggest a potential mechanism for the observed region-specific decrease in NEP activity with aging [10].

Oestrogen is another factor associated with AD that has recently been shown to regulate NEP activity in the brain. Epidemiological studies indicate that oestrogen replacement therapy may prevent or delay the onset of AD in post-menopausal women [11,12]. In an animal model expressing endogenous levels of APP (amyloid precursor protein), oestrogen depletion caused by ovariectomy resulted in increased levels of Aβ accumulation in the brain that were significantly reduced by supplementation with oestriadiol-17β [13]. Huang et al. [14] analysed the brains of ovariectomized rats and found that NEP activity was significantly decreased in the hippocampus, cerebellum, and caudate of rats deprived of oestrogen. The observed decrease in NEP activity was completely prevented by oestrogen replacement. These results implicate decreased Aβ degradation by NEP as a potential mechanism for the elevated Aβ levels observed in ovariectomized animals, and suggest that oestrogen may contribute to the maintenance of normal NEP levels in pre-menopausal women.

Epidemiological studies show a reduced risk for dementia, including AD, in individuals who exercise, socialize, and participate in leisure activities later in life, promoting a ‘use it or lose it’ hypothesis [15]. Recent studies have sought to investigate this hypothesis in mouse models of amyloid deposition by providing the mice with exercise and environmental enrichment in large cages with running wheels and toys. A recent study by Lazarov et al. [16] showed dramatic reductions in amyloid deposition in transgenic mice that were regularly removed from their standard cages and placed in large enrichment cages. Interestingly, among many changes detected, NEP activity was found to be significantly elevated in the brains of the enriched mice compared with standard housing controls. Furthermore, NEP activity was inversely correlated with amyloid burden in the enriched mice. These results support the hypothesis that the significant reduction in amyloid deposition in mice exposed to an enriched environment was due, at least in part, to the up-regulation of NEP activity.

**Figure 1** | Known cleavage sites for each of the Aβ-degrading enzymes [30,38,47–49]

![Diagram showing cleavage sites for Aβ-degrading enzymes](image)

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**IDE regulates the levels of Aβ in the brain**

IDE (insulysin) is another protease that plays an important role in regulating Aβ levels in the brain. In similar studies, Farris et al. [17] and Miller et al. [18] reported that the endogenous levels of both Aβ40 and Aβ42 were significantly elevated in the brains of IDE-knockout mice. Defective Aβ degeneration was clearly demonstrated in brain membrane and soluble fractions, as well as in primary cultured neurons derived from the IDE-deficient mice [17]. In a subsequent report, Farris et al. [19] characterized Aβ degradation in the GK rat, an inbred model of Type II (non-insulin-dependent) diabetes mellitus that bears two missense mutations in the IDE gene that result in decreased proteolytic activity. In primary cultured GK fibroblasts and neurons, and in soluble and membrane fractions of brain, degradation of both Aβ and insulin was found to be significantly impaired. As a result, Aβ accumulation was increased in the conditioned medium of primary neuronal cells derived from GK rats compared with controls. However, steady-state levels of Aβ in the intact brain were not elevated, suggesting that other mechanisms of Aβ clearance could compensate for decreased IDE function in this model at least at the ages tested. In the human brain, where the accumulation of Aβ may occur slowly over years or even decades before the development of AD symptoms, partial loss-of-function of IDE or any other Aβ-degrading enzyme may affect long-term Aβ accumulation in ways that may not be detected during the relatively short lifespan of the rodent [19].

**The increased risk for AD associated with diabetes and hyperinsulinaemia may be mediated in part by IDE**

Both the IDE-knockout mouse and the GK rat model show that impaired IDE function can be associated with both diabetic phenotype and reduced Aβ clearance. There is a growing body of evidence that diabetes, and hyperinsulinaemia in particular, are risk factors for developing AD, and the IDE region of chromosome 10 has been genetically linked to both late-onset AD [20] and Type II (non-insulin-dependent) diabetes mellitus [21]. In APP transgenic mice, diet-induced insulin resistance results in increased amyloid deposition associated with decreased IDE activity and increased Aβ production [22].

The effect of IDE variants on AD pathogenesis may depend on the APOE (apolipoprotein E) status of the individual. Hyperinsulinaemia was shown to be associated with
AD in APOE-ε4 negative but not APOE-ε4 positive individuals [23]. In light of this report, Edland et al. [24] re-examined several IDE polymorphisms that had previously been reported to lack any association with late-onset AD. When the data were stratified by APOE genotype, significant associations were found for three IDE variants in APOE-ε4 negative subjects with late-onset AD, but not in APOE-ε4 positive subjects. Further studies are needed to confirm these results in a larger population, and to determine whether these variants affect IDE activity levels.

The APOE-ε4 allele is a highly significant risk factor for AD [25]. While IDE variants were not found to be associated with AD in carriers of this allele, a recent study indicates that IDE expression is altered in APOE-ε4 positive AD brains. Cook et al. [26] reported a significant reduction in hippocampal IDE levels in APOE-ε4 positive AD patients compared with APOE-ε4 negative AD patients or APOE-ε4 positive controls, suggesting that the effect of APOE on AD risk may be mediated in part by IDE function.

**The plasmin system degrades Aβ and can be induced by Aβ aggregates**

In fibrinolysis, fibrin aggregates induce the synthesis of tPA (tissue-type plasminogen activator) and uPA (urokinase-type plasminogen activator). tPA is further activated by binding to fibrin, while uPA is constitutively active. Both tPA and uPA cleave plasminogen to generate the active serine protease plasmin, which, in turn, degrades the fibrin aggregates that elicit the proteolytic cascade [27]. In recent years, studies by several groups have shown that Aβ can similarly activate and be degraded by the plasmin system. Aggregated Aβ binds to and stimulates tPA [28,29], and induces expression of tPA and uPA mRNA both in vitro and in APP transgenic mice [30].

Aβ injected into the hippocampus of plasmin-deficient mice is removed more slowly than in wild-type mice, providing additional evidence for the role of this enzyme in Aβ clearance [31]. However, unlike mice deficient in NEP, IDE, ECE-1 and ECE-2, mice deficient in plasmin (plasminogen-knockout mice) do not have elevated levels of endogenous Aβ [32]. This result suggests that plasmin does not contribute to the regulation of steady-state Aβ levels under non-pathogenic conditions in the mouse brain. Given the evidence that the plasminogen system can be activated by Aβ, and that plasmin can cleave Aβ, it is likely that plasmin plays a more important role in Aβ clearance after aggregation is initiated. Further support for a role for plasmin in AD comes from genetic linkage studies. The uPA gene (PLAU) maps to a familial AD locus on chromosome 10 [20,33,34] and single nucleotide polymorphisms are associated with plasma Aβ42 concentration and late-onset AD [35]. As in plasminogen-knockout mice, the levels of endogenous Aβ were found to be unchanged in the brains of uPA-knockout mice. However, as predicted by the association of PLAU variants with plasma Aβ42 in late-onset AD families, plasma Aβ levels were in fact significantly elevated in these mice [35]. Collectively, these results provide strong evidence for a role of the plasmin system in Aβ catabolism.

**Aβ degradation by the ECEs**

ECE-1 and ECE-2 are homologous enzymes belonging to the M13 family of zinc metalloendoproteinases that also includes NEP [36]. The ECEs are named for their ability to hydrolyse a family of biologically inactive intermediates, big endotoxins (big ETs), exclusively at a Trp21–Val/Ile22 bond to form the potent vasoconstrictors endothelins. In addition to the specific cleavage of big ETs, ECE has been reported to hydrolyse several biologically active peptides in vitro, including bradykinin, neurotensin, substance P, and oxidized insulin B chain by cleaving on the amino side of hydrophobic residues [37]. The ability of ECE to degrade Aβ was discovered as a result of our observation that the metalloprotease inhibitor phosphoramidon caused a rapid increase in the accumulation of Aβ by cell lines that expressed ECE, but not by those known to be devoid of ECE expression [38]. When ECE-1 was overexpressed in the cells that lacked endogenous expression, the accumulation of Aβ in the conditioned medium was reduced by approx. 90%. Subsequent in vitro experiments with recombinant soluble ECE-1 demonstrated that the enzyme cleaves Aβ at multiple sites with a favourable kinetic profile.

**ECE-1 and ECE-2 contribute to the regulation of steady-state Aβ levels in the brain**

Both ECE-1 and ECE-2 are expressed in the brain in regions that are relevant to AD pathology [39,40]. To determine the physiological relevance of ECE-mediated Aβ cleavage, the level of endogenous Aβ was examined in the brains of ECE-1 and ECE-2-knockout mice. Because mice completely deficient in ECE-1 die in utero or shortly after birth, Aβ levels were analysed in ECE-1 (+/−) mice, which are healthy and have approximately a 27% reduction in ECE-1 activity. This modest reduction in activity resulted in a significant increase in the levels of both Aβ40 and Aβ42 in the brain, indicating that Aβ is a physiologically relevant substrate of ECE-1 [41]. Aβ is also a substrate for ECE-2 in the brain, as evidenced by increased Aβ40 and Aβ42 levels in the brains of ECE-2 (−/−) mice, which are viable and healthy. The concentration of Aβ in the brains of ECE-2 heterozygous mice was intermediate between that observed in the wild-type controls and the complete nulls, suggesting a gene–dosage effect.

**An ECE-1 promoter polymorphism that increases expression is associated with reduced risk for AD**

The role of ECE-1 in AD pathogenesis was recently explored in a case-control study of late-onset AD. Four isoforms of human ECE-1 differing only in the cytoplasmic tail are produced by a single gene through the use of alternative promoters [42]. The four isoforms cleave big ETs with equal efficiency but differ primarily in their subcellular localization and tissue distribution. Funalot et al. [39] screened the ECE1
gene for common polymorphisms and identified a single nucleotide polymorphism in the promoter region controlling transcription of ECE-1b and ECE-1d. The C/A substitution, which creates an E2F transcription factor-binding site, resulted in a significant increase in transcription in promoter-reporter gene assays. Additionally, ECE-1 mRNA expression was 2-fold higher in human prefrontal cortex from carriers of the A allele compared with non-carriers. While ECE-1 protein and activity levels were not determined in these samples, the increase in mRNA levels suggests that this may be a functional polymorphism. To determine whether this polymorphism might protect against AD, the authors performed a genetic association study in a large group of late-onset AD cases versus age- and sex-matched control subjects. The frequency of AA homozygotes was significantly reduced in the AD group compared with controls, indicating that increased expression of ECE-1 may be protective against AD.

Transgenic overexpression of NEP or IDE reduces amyloid deposition in mice

The up-regulation of any enzyme capable of degrading Aβ may be an effective strategy for the prevention or treatment of AD. Because each of the Aβ-degrading enzymes performs additional physiological functions, the side effects of up-regulation will need to be carefully evaluated. To determine whether chronic overexpression of NEP or IDE could be tolerated, Leissring et al. [43] generated transgenic mice expressing either of these enzymes under control of the developmentally delayed, neuron specific CaM kinase II (Ca2+/calmodulin-dependent protein kinase II) promoter. Both the NEP and IDE overexpressing mouse lines were healthy and had reduced cerebral Aβ levels compared with non-transgenic mice. To investigate whether chronic overexpression of these enzymes would alter the course of amyloid deposition, the NEP and IDE overexpressing lines were then crossed with APPswe/ind transgenic mice that develop amyloid plaques in an age-dependent manner. At 14 months of age, amyloid plaque burden was greatly reduced in the brains of mice expressing 2-fold levels of IDE, and essentially absent from the brains of mice expressing 8-fold levels of NEP. Remarkably, the increased expression of either IDE or NEP also significantly reduced the premature lethality phenotype of the APP transgenic mice.

Viral transduction of Aβ-degrading enzymes reduces Aβ accumulation in the brain

Gene therapy may be a means of increasing the expression of Aβ-degrading proteases in the brain, and viral vectors are an efficient way to transduce cells in the central nervous system. Two recent papers provide proof of concept for the notion that viral gene transfer of Aβ-degrading enzymes may be an effective therapeutic strategy for AD. In the first report, Marr et al. [44] injected a lentiviral vector expressing human NEP unilaterally into the hippocampus of APP transgenic mice. One month later, amyloid burden was found to be significantly reduced on the injected hemisphere and existing plaques were significantly smaller than those found on the contralateral side. There was no effect of injecting an inactive NEP construct, indicating that the reduced amyloid deposition was due to increased NEP activity. In a subsequent report, the effect of NEP gene transfer via AAV (adeno-associated virus) vectors was examined in APP transgenic mice. Iwata et al. [45] showed that NEP gene transfer could eliminate the increased concentration of Aβ in the hippocampus of NEP-knockout mice, reduce Aβ concentration in young APP transgenic mice, and also reduce amyloid deposition in aged transgenic mice. In contrast with the study of lentiviral gene transfer, in which NEP immunoreactivity was detected mainly in the cell bodies of infected neurons, AAV-mediated gene transfer in this study resulted in the localization of NEP at presynaptic sites and, interestingly, increased NEP activity on the contralateral side. The small increase in activity on the contralateral hippocampus, presumably a result of axonal transport, was sufficient to significantly reduce Aβ levels. These results show that even modest up-regulation of the activity of Aβ-degrading enzymes may be sufficient to reduce Aβ accumulation in the brain.

IDE is an allosteric enzyme with the potential for pharmacological up-regulation

Gene therapy is not the only means of increasing the activity of Aβ-degrading enzymes. The discovery that IDE is an allosteric enzyme led to the finding that small peptide substrates can augment the activity of the protease towards Aβ but not insulin [46]. Song et al. [46] demonstrated that IDE exists as a mixture of dimers and tetramers, with the dimer being the more active form. Through a series of experiments with mixed dimers consisting of one wild-type subunit and one mutant subunit, the investigators showed that substrate binding to one subunit is likely to cause a conformational change that activates the adjacent subunit. Consistent with this hypothesis, the small peptide substrate dynorphin B-9 increased the rate of Aβ hydrolysis 2.5-fold. Interestingly, insulin cleavage was actually inhibited by the peptide. This result may be explained by the fact that insulin, itself a dimer of A and B chains, can occupy both subunits of an IDE dimer with high affinity. Overall, the results of these studies suggest a new therapeutic strategy to develop small-molecule peptide analogues as activators of IDE activity towards Aβ [46]. Future studies are warranted to determine whether the other Aβ-degrading enzymes can be similarly activated.

Summary

There is now compelling evidence that the activities of multiple proteases help to regulate the accumulation of Aβ in the brain. Each of the Aβ-degrading enzymes is complexly regulated, indicating that multiple factors can influence the levels of these enzymes in the brain. While decreased
Aβ-degrading activity appears to occur with aging and other significant risk factors for AD. Increased Aβ-degrading activity has been found to be associated with conditions that are protective against AD. Recent in vitro and in vivo studies demonstrate that the up-regulation of Aβ-degrading enzymes can significantly reduce the accumulation of the peptide, providing proof of concept for this therapeutic strategy. However, further studies are required to determine the most effective strategy for up-regulation and also to determine the physiological consequences of increased protease activity. Animal studies so far indicate that increased expression of NEP or IDE is tolerated in the brain, suggesting that this may be an effective treatment for AD alone or in combination with other AD therapies such as immunotherapy and secretase inhibitors.

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References

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