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Enhanced Proteolysis of β-Amyloid in APP Transgenic Mice Prevents Plaque Formation, Secondary Pathology, and Premature Death

Report

Malcolm A. Leissring,[1] Wesley Farris,[1] Alice Y. Chang,[1] Dominic M. Walsh,[1] Xining Wu,[1] Xiaoyan Sun,[1] Matthew P. Frosch,[2] and Dennis J. Selkoe[1,*]

[1]Center for Neurologic Diseases
Department of Neurology
Brigham and Women's Hospital
Harvard Medical School
[2]Center for Aging, Genetics, and Neurodegeneration
Massachusetts General Hospital and
Department of Neurology
Harvard Medical School
Boston, Massachusetts 02115

Summary

Converging evidence suggests that the accumulation of cerebral amyloid β-protein (Aβ) in Alzheimer's disease (AD) reflects an imbalance between the production and degradation of this self-aggregating peptide. Upregulation of proteases that degrade Aβ thus represents a novel therapeutic approach to lowering steady-state Aβ levels, but the consequences of sustained upregulation in vivo have not been studied. Here we show that transgenic overexpression of insulin-degrading enzyme (IDE) or neprilysin (NEP) in neurons significantly reduces brain Aβ levels, retards or completely prevents amyloid plaque formation and its associated cytopathology, and rescues the premature lethality present in amyloid precursor protein (APP) transgenic mice. Our findings demonstrate that chronic upregulation of Aβ-degrading proteases represents an efficacious therapeutic approach to combating Alzheimer-type pathology in vivo.

Introduction

Alzheimer's disease (AD) is the most prevalent example of a class of human neurodegenerative disorders that involves progressive accumulation of misfolded, β sheet-rich proteins (Caughey and Lansbury, 2003). Genetic mutations that increase the production or stability of these proteins result in accelerated and severe forms of the disorders, signifying that accumulation of the proteins in the nervous system can actually cause the respective clinical syndromes. In the case of AD, the mechanisms of Aβ production, including the effects of causative mutations in the APP and presenilin genes, have received great attention, whereas far less is known about how Aβ is normally degraded and cleared (Selkoe, 2001). Among the proteases that have been found to be capable of degrading Aβ in vitro, several (IDE, NEP, and endothelin-converting enzyme-1 and -2) have been confirmed via genetic deletion in mice to contribute to the regulation of endogenous brain Aβ levels in vivo (Eckman et al., 2003; Farris et al., 2003; Iwata et al., 2001; Miller et al., 2003). Whether genetic defects in one or another of these proteases can actually initiate AD is unknown, although several laboratories have recently reported evidence of genetic linkage and/or allelic association of the IDE gene to conventional (late-onset) AD in various populations (Ait-Ghezala et al., 2002; Bertram et al., 2000; Edland et al., 2003; Prince et al., 2003).

Whether or not Aβ-degrading proteases are ultimately implicated in causing AD, chronic upregulation or disinhibition of one of these proteases, using various strategies, including small molecule activators, represents an attractive but unexplored therapeutic approach. Compounds that inhibit the β- or γ-secretases that generate Aβ have been identified and are being prepared for clinical trials, and an immunotherapeutic approach using Aβ vaccination has undergone initial evaluation in humans (for review, see Selkoe and Schenk, 2003). As both of these strategies have significant theoretical and practical risks, the alternative strategy of enhancing Aβ degradation ought to undergo rigorous preclinical evaluation to determine whether it is a safe and efficacious approach for lowering Aβ in man. To this end, we have examined the consequences in mice of enhancing the activities of either IDE or NEP, the two Aβ-degrading proteases that have received the most experimental confirmation. Our results show that chronically upregulating the activity of either protease in neurons results in striking decreases in brain Aβ levels, plaque formation and its associated cytopathology, and premature death in APP transgenic mice, in the absence of detectable adverse effects.

Results

We generated transgenic mice overexpressing human IDE or NEP using a promoter with developmentally delayed, neuron-specific expression (CaM kinase II; Mayford et al., 1996; Figure 1A). From among numerous potential founder animals, we obtained two stable mouse lines, one that expressed ~2-fold IDE (2xIDE) and another that expressed ~8-fold NEP (8xNEP), relative to nontransgenic littermate controls, as determined by quantitative Western blotting and protease activity assays (see below). The higher-fold increase in protease expression attained in the 8xNEP transgenic line is likely due to lower basal (endogenous) levels of brain NEP than IDE and also to more efficient translation of the NEP transgene, because the human NEP cDNA contains a better Kozak consensus sequence for translation initiation than does the IDE cDNA (Kozak, 1986). Consistent with the latter explanation, the 2xIDE transgenic line was found to express ~4 times more transgene RNA than the 8xNEP transgenic mice, as judged by real-time RT-PCR using primers common to the two transgenes (data not shown).

To examine the consequences for Aβ accumulation and amyloid plaque formation, the 2xIDE and 8xNEP transgenic mice were each crossed to transgenic mice

*Correspondence:dselkoe@rics.bwh.harvard.edu

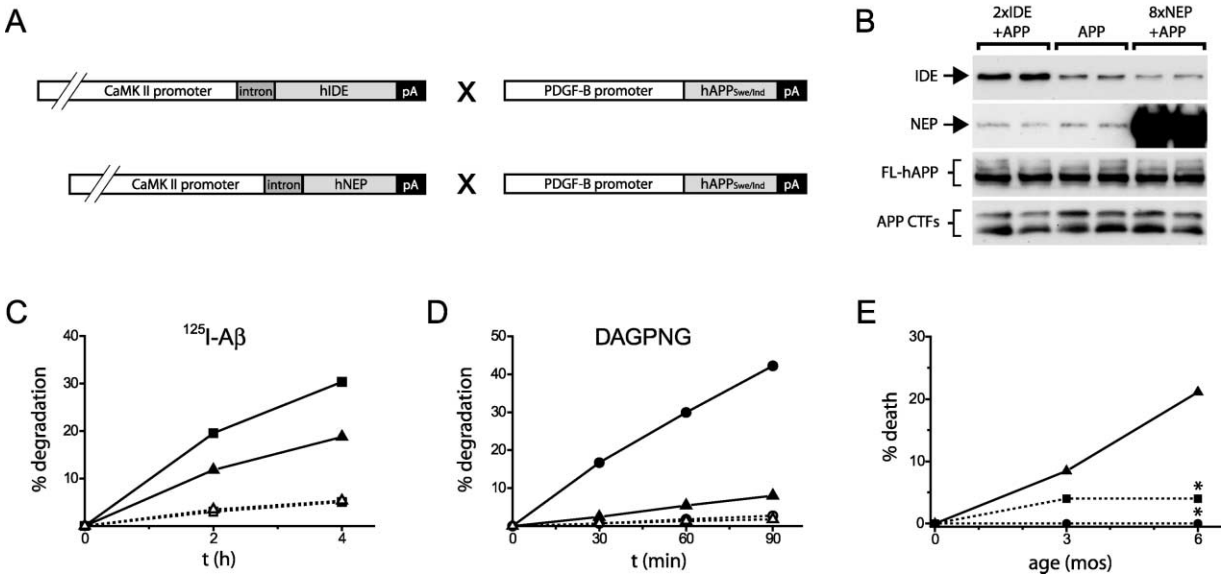

Figure 1. Generation and Characterization of IDE and NEP Transgenic Mice

(A) Construction of the IDE and NEP transgenes and overview of experimental design. Littermates expressing only the mutant APP transgene were used as controls.

(B) Western blot analysis of IDE, NEP, full-length APP, and APP carboxy-terminal fragments in the 2xIDE+APP and 8xNEP+APP transgenic mice and littermate APP transgenic controls.

(C) IDE activity in 2xIDE+APP transgenic mice (■) versus controls (▲) as determined by ^{125}I-Aβ degradation (Farris et al., 2003). Open symbols show degradation in the presence of 25 μM insulin, a strong competitive inhibitor of IDE.

(D) NEP activity in 8xNEP+APP transgenic mice (●) versus controls (▲), as determined by DAGPNG degradation. Open symbols show degradation in the presence of 20 nM thiorphan, a strong NEP inhibitor.

(E) Survival of transgenic mice. The premature death present in APP transgenic mice (▲) is significantly reduced in 2xIDE+APP (■) and 8xNEP+APP (●) transgenic mice at 6 months of age (*$p = 0.0345$ and 0.0348 by Fisher exact test for 2xIDE+APP and 8xNEP+APP transgenic mice versus APP transgenic mice, respectively; $n = 16–71$ mice per condition). All mice are littermates derived from the same crosses.

overexpressing high levels of human APP harboring both the KM670/671NL ("Swedish") and the V717F ("Indiana") AD-causing mutations (Mucke et al., 2000; Figure 1A). The degree of overexpression of each protease in the crossed mice was determined by quantitative Western analysis on amino acid-analyzed brain membrane fractions (Figure 1B). Interestingly, a small but statistically significant decrease in endogenous IDE levels was observed in the 8xNEP+APP transgenic mice (normalized densitometry values of 0.70 ± 0.15 in the 8xNEP+APP transgenic mice versus 1.00 ± 0.04 in APP transgenic controls; $p < 0.05$ by two-tailed Student's t test), and a similar, albeit nonsignificant, decrease in NEP levels was present in some IDE transgenic mice (Figure 1B). These results raise the possibility of cross-regulation between the two proteases, perhaps mediated by a common substrate. Importantly, the levels of full-length APP and APP carboxy-terminal fragments (C83 and C99) were unchanged in the 2xIDE+APP and 8xNEP+APP transgenic mice, excluding a detectable effect of these proteases on the α-, β-, and γ-secretase processing steps (Figure 1B).

Using a well-characterized iodinated Aβ degradation assay, we confirmed that the 2xIDE+APP mice had an ~100% increase in insulin-inhibitable Aβ-degrading capacity in isolated, washed brain membranes compared to their APP transgenic littermates (Figure 1C). 8xNEP+APP mice also exhibited a large increase in thiorphan-inhibitable Aβ degradation using this assay (data not shown), although accurate quantification was confounded by the fact that the vast majority of Aβ-degrading activity in these brain membrane samples is attributable to IDE. We therefore used the more selective and well-characterized NEP substrate DAGPNG (Florentin et al., 1984) and determined that NEP activity was increased ~700% in the 8xNEP+APP mice relative to controls (Figure 1D). These functional assays thus closely reflect the relative increases in the respective proteins we observed by Western blotting.

$APP_{Swe/Ind}$ single transgenic mice raised in our colony suffer an abnormally high rate of premature death (≤6 months) that cosegregates with the APP transgene. Such premature lethality is not unique to $APP_{Swe/Ind}$ mice, having been observed in other APP transgenic mouse models (e.g., Hsiao et al., 1995; D. Games and D. Schenk, personal communication). Remarkably, the incidence of premature death at 6 months of age was significantly attenuated in the 2xIDE+APP and the 8xNEP+APP transgenic mice relative to their APP transgenic littermate controls (Figure 1E). The fact that this effect was observed following upregulation of two different Aβ-degrading proteases that otherwise are not believed to have substrates in common strongly suggests that progressive Aβ build-up is responsible for the enhanced mortality observed in our APP transgenic mice. Moreover, the fact that premature death can occur as early as 3 months of age (Figure 1E), well prior to plaque deposition (see also Hsiao et al., 1995), implies that the responsible Aβ species are soluble.

To determine the effects of protease overexpression

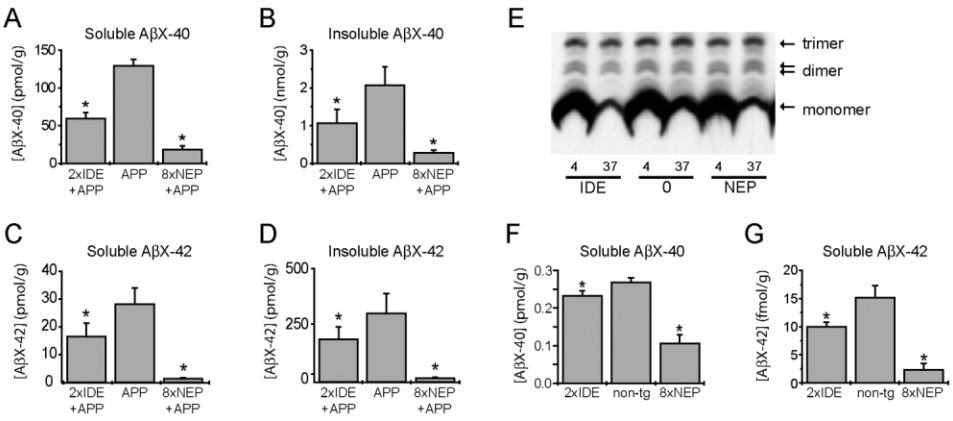

Figure 2. Decreased Aβ Levels in the 2xIDE+APP and 8xNEP+APP Transgenic Mice versus APP Transgenic Controls

(A–D) Cerebral concentrations of soluble (A and C) and insoluble (B and D) AβX-40 (A and B) and AβX-42 (C and D). Data are mean ± SEMs from age-matched 6- to 10-month-old mice (*$p < 0.05$; $n = 8$ mice per condition).

(E) Degradation of naturally secreted Aβ species by IDE and NEP. Note that both proteases preferentially degrade monomeric Aβ.

(F and G) Levels of soluble endogenous AβX-40 (F) and AβX-42 (G) are decreased in 2- to 3-month-old 2xIDE and 8xNEP single transgenic mice relative to nontransgenic controls (*$p < 0.05$ by one-tailed Student's t test; $n = 6$ 2xIDE and nontransgenic mice; $n = 3$ 8xNEP mice).

on Aβ accumulation, we quantified cerebral Aβ levels in 6- to 10-month-old mice using a sensitive and specific ELISA (Johnson-Wood et al., 1997). Soluble and insoluble AβX-40 and AβX-42 brain levels were significantly decreased by ~50% and ~90% in the brains of the 2xIDE+APP and 8xNEP+APP crossed transgenic mice, respectively, relative to APP transgenic controls (Figures 2A–2D). Consistent with data from gene-targeted animals (Farris et al., 2003; Iwata et al., 2001; Miller et al., 2003), the finding that soluble Aβ levels are reduced in the crossed mice, particularly the X-40 forms that are less prone to aggregation, suggests that IDE and NEP are acting at the level of soluble, monomeric Aβ species, prior to overt deposition. To examine this issue more thoroughly, we added recombinant IDE or NEP to conditioned medium containing both monomeric as well as oligomeric Aβ species naturally secreted from cells overexpressing APP (Walsh et al., 2002a); consistent with previous reports (Figure 6 of Qiu et al., 1997; Figure 3e of Walsh et al., 2002a), IDE degraded Aβ monomers but not oligomers, and NEP showed a similar preference (Figure 2E). To assess the effects of IDE and NEP overexpression on monomeric Aβ in vivo, in the absence of plaque deposition, we quantified endogenous murine Aβ levels in 2- to 3-month-old 2xIDE and 8xNEP single transgenic mice (i.e., not crossed to APP transgenics). The two transgenic lines each showed significant reductions in steady-state levels of soluble AβX-40 and AβX-42 that correlated with the magnitude of Aβ burden in APP transgenic crosses (Figures 2F and 2G), providing strong evidence that the proximal effect of these proteases is to reduce steady-state levels of monomeric Aβ.

Whereas NEP is well known to be a membrane-anchored, cell-surface protease (Saito et al., 2003; Turner, 1998), the subcellular localization of IDE has remained controversial despite reports documenting the presence of a portion of IDE molecules on the surface of neurons and other cell types (Seta and Roth, 1997; Vekrellis et al., 2000). Our finding that overexpression of IDE reduces both soluble and insoluble Aβ (Figures 2A–2D) shows definitively that IDE is localized to cellular compartments relevant to Aβ degradation in vivo. Given that only ~1%–2% of endogenous IDE protein is associated with membranes and therefore physically capable of interacting with Aβ (Duckworth et al., 1998), it is remarkable that a mere doubling of this subpopulation leads to a >50% reduction in brain Aβ burden in this particular AD mouse model, which harbors an ~10,000-fold increase in Aβ over endogenous mouse brain levels (Mucke et al., 2000). These results indicate that relatively small changes in the activity of IDE and other Aβ-degrading proteases can dramatically impact steady-state cerebral Aβ levels, suggesting that modest increases in proteolytic clearance of Aβ in humans might be sufficient to effect significant changes in the overall economy of brain Aβ.

In agreement with the ELISA data, Aβ plaque burden was decreased ~50% in the 2xIDE+APP double transgenic mice (Figures 3A and 3D) relative to age-matched APP transgenic littermate controls (Figures 3B and 3E). Strikingly, Aβ plaques were essentially completely absent in 8xNEP+APP double transgenic mice up to 14 months of age (Figures 3C and 3F), with only the smallest hint of diffuse Aβ immunoreactivity evident in a subset of animals (see Figure 3C, inset). The decrease in extracellular Aβ deposition was accompanied by corresponding reductions in multiple markers of secondary pathology, including astrocytosis (as marked by a GFAP antibody; Figures 4A–4C), dystrophic neurites (as marked by the 8E5 APP antibody that detects altered periplaque neurites; Figures 4D–4I), and microgliosis (as marked by a CD-45 antibody; Figures 4J–4L).

It is of special interest that chronic neuronal overexpression of IDE or NEP beginning postnatally did not itself have obvious deleterious consequences. Mice expressing only the 2xIDE or 8xNEP transgenes were born in the expected Mendelian ratios, were healthy and fertile, and showed no overt phenotypic abnormalities. Careful neuropathological analysis revealed no detectable differences between the brains of the protease

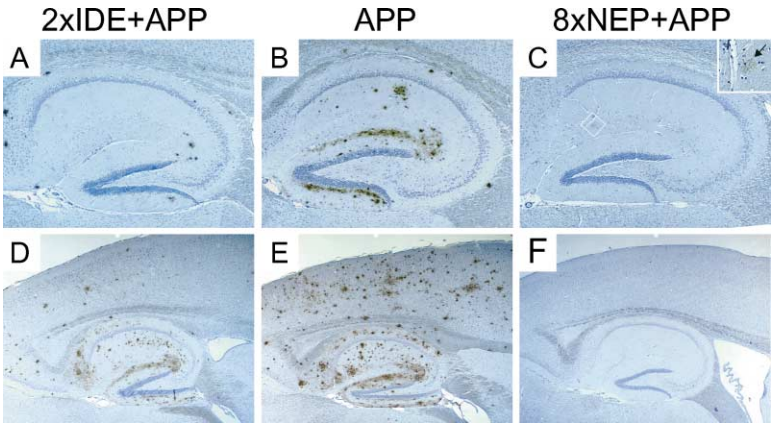

Figure 3. Decreased Aβ Plaque Burden in 2xIDE+APP and 8xNEP+APP Transgenic Mice versus Controls

(A–F) Aβ immunoreactivity in the brains of 8-month-old (A–C) and 14-month-old (D–F) transgenic mice. Plaque burden is greatly decreased in 2xIDE+APP transgenic mice (A and D) relative to APP transgenic littermate controls (B and E) and is essentially absent in 8xNEP+APP transgenic mice (C and F). The inset in (C) shows the very light Aβ immunoreactivity detected in a subset of 8xNEP+APP mice. Shown are brain sections representing the median degree of pathology at each time point as judged by two independent observers from among 8 to 12 mice per condition.

transgenic mice and their nontransgenic littermates (data not shown).

Discussion

Work from several laboratories, including ours, has increasingly implicated Aβ oligomers rather than monomers or mature amyloid fibrils as the species principally responsible for cell toxicity and neuronal dysfunction in various experimental models of AD and in the human disease itself (Gong et al., 2003; Hsia et al., 1999; Mucke et al., 2000; Selkoe, 2002; Walsh et al., 2002a, 2002b). It appears that relatively stable oligomeric intermediates in Aβ fibrillogenesis are initially formed intracellularly and are released in part into the extracellular space, where further aggregation into fibrous polymers may occur (Walsh et al., 2000, 2002a). The fact that IDE and NEP preferentially degrade monomeric species and not oligomeric or higher-order Aβ assemblies (Qiu et al., 1997; Saito et al., 2003; Turner, 1998; Walsh et al., 2002a; Figure 2E) has raised questions about the potential therapeutic utility of Aβ-degrading enzymes. Indeed, lentiviral overexpression of NEP in the brains of adult APP transgenic mice was recently found to only partially lower Aβ deposition (Marr et al., 2003), leaving open the possibility that existing aggregates and/or a subset of newly generated Aβ molecules might exist that are impervious to degradation by Aβ-degrading proteases (Walsh et al., 2000, 2002a). However, our finding that transgenic overexpression of NEP essentially completely prevented Aβ deposition and downstream pathology in an APP transgenic mouse with particularly high levels of AβX-42 (Mucke et al., 2000) is consistent with the concept that the pathway leading from Aβ production to neuronal dysfunction necessarily includes a protease-sensitive monomeric phase and suggests that

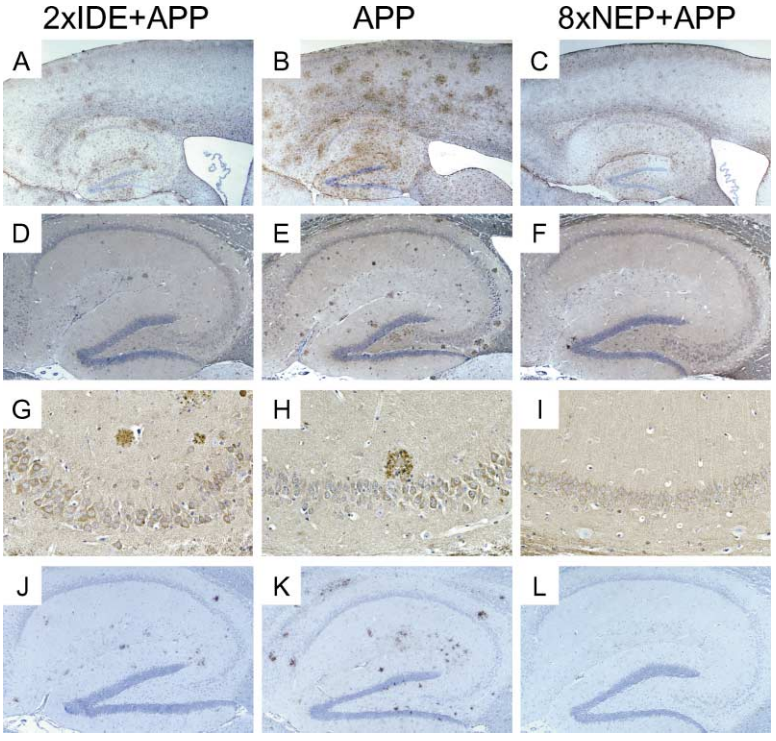

Figure 4. Aβ-Mediated Secondary Pathology

(A–C) Astrocytosis is greatly reduced in the cortex and hippocampus of 14-month-old 2xIDE+APP (A) and 8xNEP+APP (C) transgenic mice, relative to age-matched APP littermate controls (B).

(D–I) Reduction of neuritic dystrophy in the hippocampus of 2xIDE+APP (D and G) and 8xNEP+APP transgenic mice (F and I) versus controls (E and H). Dystrophic neurites were detected with 8E5, an antibody raised against the APP ectodomain, and visualized at 2.5× (D–F) and 20× (G–I) magnification.

(J–L) Reduced microgliosis in the hippocampus of 2xIDE+APP (J) and 8xNEP+APP (L) transgenic mice relative to APP transgenic controls (K). Shown are serial sections from the same 14-month-old mice selected for Figures 3D–3F.

therapies selectively targeting monomeric Aβ species ought to be sufficient to prevent the development of AD-type pathology in vivo. However, because IDE and NEP each have a number of substrates, the present study cannot exclude the possibility that increased proteolysis of substrates other than Aβ could somehow influence Aβ deposition and/or its downstream consequences.

What about mature amyloid deposits that already exist at the time of initiation of Aβ protease activation? While upregulation of IDE or NEP would not remove Aβ aggregates directly, the sharp decrease in Aβ monomer levels would be expected to lead to gradual disaggregation of polymers into monomers under an equilibrium reaction and the subsequent clearing of the liberated monomers by the protease. It is also plausible that other endogenous clearance mechanisms might be sufficient to clear preformed aggregates once monomer levels fall and the burden of ongoing Aβ deposition decreases. In support of the latter possibility, lesions of the perforant pathway that prevent APP delivery to and Aβ deposition in the hippocampus lead to a striking removal of preexisting Aβ deposits (Lazarov et al., 2002).

It is important to emphasize that our study provides no information about the relative roles of NEP and IDE in the normal regulation of cerebral Aβ levels nor does it speak to their relative efficacies as therapeutics. This is because the stable mouse lines we happened to generate had very different degrees of elevation of the respective proteases. Indeed, it is a principal message of our work that it may make little difference which Aβ-degrading protease is chosen as a therapeutic target, since it is already known that several are capable of degrading Aβ in vivo (Eckman et al., 2003; Farris et al., 2003; Iwata et al., 2001; Miller et al., 2003). Therefore, one could choose to pursue this strategy by focusing on that Aβ-degrading protease the upregulation of which is associated with the least adverse effects.

From a therapeutic perspective, upregulation of Aβ degradation appears to compare favorably to clearance-based therapeutics in mice based on immunization against Aβ. While Aβ-directed immunotherapies have proven effective in removing brain Aβ in mouse models (Bard et al., 2000; DeMattos et al., 2001; Hock et al., 2002; Schenk et al., 1999; Weiner et al., 2000), and perhaps even in blunting cognitive decline in some AD patients (Hock et al., 2003), this approach has also triggered deleterious immune-mediated reactions in a subset of patients (Nicoll et al., 2003). Based on the present findings, we propose that pharmacological upregulation of a single Aβ-degrading protease (e.g., either IDE or NEP) might achieve the same therapeutic benefit, while avoiding potentially adverse immune responses. Furthermore, such an approach would have the special advantage of acting *catalytically* to remove Aβ, rather than stoichiometrically, as antibodies do. The finding that chronic upregulation of these proteases was not accompanied by detectable adverse effects in mice up to ~14 months of age suggests that additional preclinical studies of the safety and efficacy of a proteolytic approach are worthy of pursuit. We are currently screening compound libraries for small molecules that could activate the IDE-mediated degradation of Aβ.

Experimental Procedures

Animals

Full-length cDNAs encoding unaltered human IDE or NEP (transcript variant 1) were cloned into a vector containing the 5′ regulatory region of the CaMKII gene, an upstream artificial intron, and a downstream SV40 polyadenylation signal (gift of J. Shen). The transgenic constructs were microinjected into the male pronucleus of C57Bl/6 single-cell embryos, which were implanted into pseudopregnant Swiss-Webster foster dames. Founder mice were identified from tail DNA and further characterized by Southern blotting to confirm that each transgenic was integrated at a single locus in each line, as described previously (LaFerla et al., 2000). Routine genotyping was performed by PCR using either IDE-Seq0 (5′-TTGATACCATTG GCCAGCTCTAG-3′) or NEP-Seq0 (5′-GTCGAGTCCATCGCTGT-3′) together with the common primer CaMKII Fwd: (5′-GGGATCCACT AGTTCTAGAGCG-3′). $APP_{Swe/Ind}$ transgenic mice (Mucke et al., 2000; J20 line; gift of Lennart Mucke, Gladstone Institute, UCSF) were maintained on a hybrid background (C57Bl/6 × DBA2) and genotyped by PCR, as described previously (Games et al., 1995; Mucke et al., 2000). All mice in the study were F1 progeny derived from a single cross between the J20 line and NEP or IDE transgenic mice.

For biochemical analysis, mice were anesthetized with Avertin, and their brains were removed, divided sagittally, and snap frozen. For immunohistochemical analysis, one brain hemisphere was post-fixed in 10% phosphate-buffered formalin for 2 hr, washed in phosphate-buffered saline, pH 7.4 (PBS), dehydrated, and embedded in paraffin, as described (Weiner et al., 2000). These studies were approved by the Harvard Medical School Standing Committee on Animals.

ELISA of Cerebral Aβ

To extract soluble cerebral Aβ, frozen cerebrums were homogenized in 5 volumes (w:v) 50 mM Tris-HCl, pH 7.4, and centrifuged at 100,000 × *g* for 1 hr, and the resulting supernate was retained as the soluble fraction. To extract Tris-insoluble Aβ, the resulting pellet was dissolved in 5 volumes (v:v) of 6 M guanidine hydrochloride, incubated at room temperature for 30 min, and spun at 100,000 × *g* for 1 hr. ELISAs were performed as described (Johnson-Wood et al., 1997; Weiner et al., 2000). We used antibodies that recognize species referred to as X-40 and X-42, which are heterogeneous Aβ peptides that begin anywhere in the amino-terminal region of Aβ and end at either residue 40 or 42.

Protease Activity Assays

Brain homogenates were prepared as described above, in the absence of protease inhibitors. After centrifugation at 100,000 × *g* for 1 hr, the membrane pellet was washed in PBS and recentrifuged two times. For some preparations, nonintegral membrane proteins were removed by washing in 100 mM Na_2CO_3 (pH 11.3), as described (Farris et al., 2003). Absolute protein concentrations were determined by amino acid analysis, and ^{125}I-Aβ degradation assays were performed as described (Leissring et al., 2003). NEP activity was also quantified using the NEP-specific fluorogenic peptide substrate, *N*-dansyl-D-Ala-Gly-p-(nitro)-Phe-Gly (DAGPNG; Sigma; Florentin et al., 1984). Briefly, 50 μg brain membrane protein was incubated with 50 μM DAGPNG and 20 μM elanapril (Sigma) dissolved in 0.5 ml Tris-HCl, pH 7.4, with or without 20 nM thiorphan. At each time point, 100 μl aliquots were diluted in 0.4 ml Tris-HCl, pH 7.4, and immediately boiled to terminate proteolytic activity. Following centrifugation at 3000 × *g* for 5 min, the supernatant was removed, and fluorescence (340 nm excitation, 520 nm emission) was read on a Victor2 multilabel plate reader (PerkinElmer). All activity assays were determined at 37°C.

Immunohistochemistry

Nine-micrometer brain sections were deparaffinized through a graded alcohol series and washed in PBS. Endogenous peroxidase activity was quenched by pretreatment with a 3% hydrogen peroxide/methanol solution for 25 min. Sections were blocked in 10% normal goat serum for 30 min, washed, and incubated with primary antibodies in 3%BSA/PBS overnight at 4°C in a humid chamber. After washing in PBS, the sections were incubated with the appro-

priate biotinylated secondary antibody for 30 min. The tissues were washed once again and developed using the avidin/biotin/HRP/DAB colorimetric method (ABC Elite Kit; Vector Labs; Burlingame, CA). Sections were counterstained with Harris hematoxylin, dehydrated, and affixed with coverslips. All brain sections chosen for staining were on a similar sagittal plane and contained approximately the same area of hippocampus. Serial sections from each animal were stained with each of the following antibodies: our pAb DW14 (1:2500) for human Aβ detection (Walsh et al., 2002a); mAb GFAP (1:500; Sigma, St. Louis, MO) for detection of reactive astrocytes; mAb 8E5 (1:10,000; gift from Elan Pharmaceuticals) reactive for full-length human APP, for detection of dystrophic neurites; and mAb anti-CD45 (1:5000; Serotec Ltd, Oxford, UK), for detection of microglia. The degree of amyloid pathology in the transgenic mice was assessed semiquantitively by two independent observers in a blinded fashion, using two sagittal brain sections per mouse (~1 mm apart) stained in parallel for Aβ in a total of 8 to 12 mice per transgenic line.

Western Blotting

Western blots were performed as described (Farris et al., 2003), using amino acid-analyzed brain membrane protein from the same animals used for the immunohistochemistry, Aβ ELISA, and protease activity assays. Antibodies used for Western blots included IDE-1 (Vekrellis et al., 2000), 8E5, and C8 (reactive with the carboxy terminus of APP), and the following anti-NEP antibodies: 56C6, CD10(C-17), CD10(N-14), CD10(H-321) (Santa Cruz Biotechnology, Inc.), and NEP11-A (Alpha Diagnostic Intl.). Densitometric analyses were performed using the AlphaEase software package according to the manufacturer's recommendations (Alpha Innotech Corp.). Immunoprecipitation of Aβ and incubations with recombinant proteases were performed as described (Walsh et al., 2002a). Recombinant NEP was the generous gift of Guy Boileau and Philippe Crine. Recombinant IDE was generated as described (Leissring et al., 2003).

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References

Ait-Ghezala, G., Abdullah, L., Crescentini, R., Crawford, F., Town, T., Singh, S., Richards, D., Duara, R., and Mullan, M. (2002). Confirmation of association between D10S583 and Alzheimer's disease in a case–control sample. Neurosci. Lett. *325*, 87–90.

Bard, F., Cannon, C., Barbour, R., Burke, R.L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., et al. (2000). Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat. Med. *6*, 916–919.

Bertram, L., Blacker, D., Mullin, K., Keeney, D., Jones, J., Basu, S., Yhu, S., McInnis, M.G., Go, R.C., Vekrellis, K., et al. (2000). Evidence for genetic linkage of Alzheimer's disease to chromosome 10q. Science *290*, 2302–2303.

Caughey, B., and Lansbury, P.T., Jr. (2003). Protofibrils, pores, fibrils, and neurodegeneration: Separating the responsible protein aggregates from the innocent bystanders. Annu. Rev. Neurosci. *9*, 9.

DeMattos, R.B., Bales, K.R., Cummins, D.J., Dodart, J.C., Paul, S.M., and Holtzman, D.M. (2001). Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. Proc. Natl. Acad. Sci. USA *98*, 8850–8855.

Duckworth, W.C., Bennett, R.G., and Hamel, F.G. (1998). Insulin degradation: progress and potential. Endocr. Rev. *19*, 608–624.

Eckman, E.A., Watson, M., Marlow, L., Sambamurti, K., and Eckman, C.B. (2003). Alzheimer's disease beta-amyloid peptide is increased in mice deficient in endothelin-converting enzyme. J. Biol. Chem. *278*, 2081–2084.

Edland, S.D., Wavrant-De Vriese, F., Compton, D., Smith, G.E., Ivnik, R., Boeve, B.F., Tangalos, E.G., and Petersen, R.C. (2003). Insulin degrading enzyme (IDE) genetic variants and risk of Alzheimer's disease: evidence of effect modification by apolipoprotein E (APOE). Neurosci. Lett. *345*, 21–24.

Farris, W., Mansourian, S., Chang, Y., Lindsley, L., Eckman, E.A., Frosch, M.P., Eckman, C.B., Tanzi, R.E., Selkoe, D.J., and Guenette, S. (2003). Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. Proc. Natl. Acad. Sci. USA *100*, 4162–4167.

Florentin, D., Sassi, A., and Roques, B.P. (1984). A highly sensitive fluorometric assay for "enkephalinase," a neutral metalloendopeptidase that releases tyrosine-glycine-glycine from enkephalins. Anal. Biochem. *141*, 62–69.

Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., Gillespie, F., et al. (1995). Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. Nature *373*, 523–527.

Gong, Y., Chang, L., Viola, K.L., Lacor, P.N., Lambert, M.P., Finch, C.E., Krafft, G.A., and Klein, W.L. (2003). Alzheimer's disease-affected brain: Presence of oligomeric A{beta} ligands (ADDLs) suggests a molecular basis for reversible memory loss. Proc. Natl. Acad. Sci. USA *100*, 10417–10422.

Hock, C., Konietzko, U., Papassotiropoulos, A., Wollmer, A., Streffer, J., von Rotz, R.C., Davey, G., Moritz, E., and Nitsch, R.M. (2002). Generation of antibodies specific for beta-amyloid by vaccination of patients with Alzheimer disease. Nat. Med. *8*, 1270–1275.

Hock, C., Konietzko, U., Streffer, J.R., Tracy, J., Signorell, A., Muller-Tillmanns, B., Lemke, U., Henke, K., Moritz, E., Garcia, E., et al. (2003). Antibodies against beta-amyloid slow cognitive decline in Alzheimer's disease. Neuron *38*, 547–554.

Hsia, A.Y., Masliah, E., McConlogue, L., Yu, G.Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R.C., Nicoll, R.A., and Mucke, L. (1999). Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. Proc. Natl. Acad. Sci. USA *96*, 3228–3233.

Hsiao, K.K., Borchelt, D.R., Olson, K., Johannsdottir, R., Kitt, C., Yunis, W., Xu, S., Eckman, C., Younkin, S., Price, D., et al. (1995). Age-related CNS disorder and early death in transgenic FVB/N mice overexpressing Alzheimer amyloid precursor proteins. Neuron *15*, 1203–1218.

Iwata, N., Tsubuki, S., Takaki, Y., Shirotani, K., Lu, B., Gerard, N.P., Gerard, C., Hama, E., Lee, H.J., and Saido, T.C. (2001). Metabolic regulation of brain Abeta by neprilysin. Science *292*, 1550–1552.

Johnson-Wood, K., Lee, M., Motter, R., Hu, K., Gordon, G., Barbour, R., Khan, K., Gordon, M., Tan, H., Games, D., et al. (1997). Amyloid precursor protein processing and A beta42 deposition in a transgenic mouse model of Alzheimer disease. Proc. Natl. Acad. Sci. USA *94*, 1550–1555.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell *44*, 283–292.

LaFerla, F.M., Sugarman, M.C., Lane, T.E., and Leissring, M.A. (2000). Regional hypomyelination and dysplasia in transgenic mice with astrocyte-directed expression of interferon-gamma. J. Mol. Neurosci. *15*, 45–59.

Lazarov, O., Lee, M., Peterson, D.A., and Sisodia, S.S. (2002). Evidence that synaptically released beta-amyloid accumulates as extracellular deposits in the hippocampus of transgenic mice. J. Neurosci. *22*, 9785–9793.

Leissring, M.A., Lu, A., Condron, M.M., Teplow, D.B., Stein, R.L.,

Farris, W., and Selkoe, D.J. (2003). Kinetics of amyloid beta-protein degradation determined by novel fluorescence- and fluorescence polarization-based assays. J. Biol. Chem. *278*, 37314–37320.

Marr, R.A., Rockenstein, E., Mukherjee, A., Kindy, M.S., Hersh, L.B., Gage, F.H., Verma, I.M., and Masliah, E. (2003). Neprilysin gene transfer reduces human amyloid pathology in transgenic mice. J. Neurosci. *23*, 1992–1996.

Mayford, M., Bach, M.E., Huang, Y.Y., Wang, L., Hawkins, R.D., and Kandel, E.R. (1996). Control of memory formation through regulated expression of a CaMKII transgene. Science *274*, 1678–1683.

Miller, B.C., Eckman, E.A., Sambamurti, K., Dobbs, N., Chow, K.M., Eckman, C.B., Hersh, L.B., and Thiele, D.L. (2003). Amyloid-beta peptide levels in brain are inversely correlated with insulysin activity levels in vivo. Proc. Natl. Acad. Sci. USA *100*, 6221–6226.

Mucke, L., Masliah, E., Yu, G.Q., Mallory, M., Rockenstein, E.M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K., and McConlogue, L. (2000). High-level neuronal expression of abeta 1–42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. J. Neurosci. *20*, 4050–4058.

Nicoll, J.A., Wilkinson, D., Holmes, C., Steart, P., Markham, H., and Weller, R.O. (2003). Neuropathology of human Alzheimer disease after immunization with amyloid-beta peptide: a case report. Nat. Med. *9*, 448–452.

Prince, J.A., Feuk, L., Gu, H.F., Johansson, B., Gatz, M., Blennow, K., and Brookes, A.J. (2003). Genetic variation in a haplotype block spanning IDE influences Alzheimer disease. Hum. Mutat. *22*, 363–371.

Qiu, W.Q., Ye, Z., Kholodenko, D., Seubert, P., and Selkoe, D.J. (1997). Degradation of amyloid beta-protein by a metalloprotease secreted by microglia and other neural and non-neural cells. J. Biol. Chem. *272*, 6641–6646.

Saito, T., Takaki, Y., Iwata, N., Trojanowski, J., and Saido, T.C. (2003). Alzheimer's disease, neuropeptides, neuropeptidase, and amyloid-beta peptide metabolism. Sci. Aging Knowledge Environ. *2003*, PE1.

Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., et al. (1999). Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature *400*, 173–177.

Selkoe, D.J. (2001). Clearing the brain's amyloid cobwebs. Neuron *32*, 177–180.

Selkoe, D.J. (2002). Alzheimer's disease is a synaptic failure. Science *298*, 789–791.

Selkoe, D.J., and Schenk, D. (2003). ALZHEIMER'S DISEASE: Molecular understanding predicts amyloid-based therapeutics. Annu. Rev. Pharmacol. Toxicol. *43*, 545–584.

Seta, K.A., and Roth, R.A. (1997). Overexpression of insulin degrading enzyme: cellular localization and effects on insulin signaling. Biochem. Biophys. Res. Commun. *231*, 167–171.

Turner, A.J. (1998). Neprilysin. In Handbook of Proeolytic Enzymes, A.J. Barrett, N.D. Rawlings, and J.F. Woessner, eds. (San Diego: Academic Press).

Vekrellis, K., Ye, Z., Qiu, W.Q., Walsh, D., Hartley, D., Chesneau, V., Rosner, M.R., and Selkoe, D.J. (2000). Neurons regulate extracellular levels of amyloid beta-protein via proteolysis by insulin-degrading enzyme. J. Neurosci. *20*, 1657–1665.

Walsh, D.M., Tseng, B.P., Rydel, R.E., Podlisny, M.B., and Selkoe, D.J. (2000). The oligomerization of amyloid beta-protein begins intracellularly in cells derived from human brain. Biochemistry *39*, 10831–10839.

Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J., and Selkoe, D.J. (2002a). Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature *416*, 535–539.

Walsh, D.M., Klyubin, I., Fadeeva, J.V., Rowan, M.J., and Selkoe, D.J. (2002b). Amyloid-beta oligomers: their production, toxicity and therapeutic inhibition. Biochem. Soc. Trans. *30*, 552–557.

Weiner, H.L., Lemere, C.A., Maron, R., Spooner, E.T., Grenfell, T.J., Mori, C., Issazadeh, S., Hancock, W.W., and Selkoe, D.J. (2000). Nasal administration of amyloid-beta peptide decreases cerebral amyloid burden in a mouse model of Alzheimer's disease. Ann. Neurol. *48*, 567–579.