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Biochemical Identification of the Neutral Endopeptidase Family Member Responsible for the Catabolism of Amyloid β Peptide in the Brain[1]

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Amyloid β peptide (Aβ) is a physiological peptide that is constantly catabolized in the brain. We previously demonstrated that an endopeptidase sensitive to phosphoramidon and thiorphan conducts the initial rate-limiting proteolysis of Aβ *in vivo*, but the exact molecular identity of the peptidase(s) has remained unknown because of the molecular redundancy of such activity. We analyzed the brain-derived enzyme by means of immuno-depletion and gene disruption, and demonstrate here that neprilysin accounts for the majority of the Aβ-degrading activity. Furthermore, kinetic analysis, giving a K_m value of 2.8 ± 0.76 μM, indicated that $Aβ_{1-42}$ is a relevant substrate for neprilysin.

Key words: Alzheimer's disease, amyloid β peptide, catabolism, neprilysin, neutral endopeptidase.

The accumulation of amyloid β peptide (Aβ) in the brain triggers the pathological cascade leading to the development of Alzheimer's disease (AD) (*1*). Aβ is a physiological peptide (*2, 3*), the steady-state level of which is determined by the balance between anabolism and catabolism. Therefore, a reduction in the catabolism would elevate the Aβ level in the brain and thus could cause deposition. However, the mechanism of Aβ catabolism has been less well understood than that of anabolism, although a number of peptidases have been judged to be potential Aβ-degrading enzymes using either a test tube or tissue culture paradigm (*4–10*). The problem is that almost any peptidase would be capable of proteolyzing Aβ to some extent if the reaction is carried out under optimal conditions. To be a physiologically relevant Aβ degrader, a peptidase needs to be active in an appropriate location with the correct topology so as to be accessible to the substrate *in vivo*.

In a search for the physiologically relevant Aβ-degrading system, we established a novel *in vivo* experimental paradigm, in which an internally $^3H/^{14}C$-radiolabeled $Aβ_{1-42}$ peptide was injected into the rat hippocampus and its metabolic fate was analyzed by tracing the labels, and discovered that a neutral endopeptidase sensitive to phosphoramidon and thiorphan plays a major rate-limiting role. We also demonstrated that inhibition of the peptidase leads to the accumulation of Aβ in the brain. However, the molecular identity of the Aβ-degrading endopeptidase remains unclear because, as is always the case with almost any peptidase activity, there is some molecular redundancy of such activity. A group of neutral endopeptidases, belonging to the M13 family represented by neprilysin, are type II membrane-associated metalloendopeptidases with the active site facing the extracytoplasmic side (*11*). They include endothelin-converting enzymes (ECEs), a phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PEX), X-converting enzyme (XCE), neprilysin-like peptidases, and possibly other unidentified peptidase(s) (*12–17*).

The aim of the present study is to determine the exact molecular identity of the Aβ-degrading endopeptidase present in the brain. We first examined the presence of phosphoramidon- and thiorphan-sensitive endopeptidase activity in soluble and insoluble fractions of rat brain. Because we could only detect such activity in the insoluble fraction (data not shown), the majority of the Aβ-degrading peptidase relevant to the *in vivo* paradigm (*18–20*) seems to be associated with the membranes. When the membrane fraction solubilized with Triton X-100 was subjected to anion-exchange chromatography, the inhibitor-sensitive Aβ-degrading activity was eluted at similar elution volumes to the inhibitor-sensitive Z-Ala-Ala-Leu-pNA–degrading neprilysin activity and anti-neprilysin antibody immunoreactivity (Fig. 1). Although the data indicate that these three activities are closely associated with one another, it is also possible some other peptidase activity present in these

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Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β peptide; NEP, neprilysin, neutral endopeptidase 24.11; PEX, phosphate-regulating gene with homologies to endopeptidases on the X chromosome; XCE, X-converting enzyme; Z-Ala-Ala-Leu-pNA, carbobenzoxy-L-alanyl-L-alanyl-L-leucine-*p*-nitroanilide; MES, 2-(*N*-morpholino)ethanesulfonic acid; IP, immunoprecipitation.

HPLC fractions may have conducted the degradation of Aβ, because NEP-like proteases such as soluble secreted endopeptidase (SEP) (*16*) and neprilysin II (*17*) have isoelectirc points similar to that of neprilysin.

To define the identity of the peptidase, we performed immuno-depletion experiments with a specific antibody raised against the N-terminal sequence of neprilysin. We also used an anti-PEX antibody as a control. Because the N-terminal sequences are not homologous among the M13 family members, these antibodies do not cross-react with peptidases other than the target peptidase. Figure 2A shows that treatment of the enzyme sample with the anti–NEP-N antibody resulted in almost full removal of the Z-Ala-Ala-Leu-pNA–degrading activity, whereas the anti-PEX antibody did not have any effect. In accordance with this, the anti-neprilysin monoclonal antibody-reactive protein was also pulled down specifically by the anti–NEP-N antibody (Fig. 2B). We used these antibody-treated samples to examine the Aβ-degrading activity. As shown in Fig. 2C, the enzyme sample alone conducted thiorphan-sensitive limited proteolysis of the radio-labeled $A\beta_{1-42}$. This activity was almost fully abolished when the sample was immuno-depleted using the anti–NEP-N antibody. These results indicate that the majority of the Aβ-degrading activity can be accounted for by neprilysin among all the M13 family members.

In order to further confirm the above assumption, we analyzed the endopeptidase activity in neprilysin-deficient mice. Figure 3A shows the ion-exchange HPLC profiles of the detergent-extracted membrane fractions from wild-type and neprilysin-deficient mice. The data indicate that thiorphan-sensitive Z-Ala-Ala-Leu-pNA–degrading activity was almost completely abolished in the neprilysin-deficient mice. The data again indicate that neprilysin accounts for the majority of the neutral endopeptidase activity in the brain. Accordingly, the enzyme fraction from the neprilysin-deficient mice nearly completely failed to proteolyze the radio-labeled $A\beta_{1-42}$, whereas the sample from the wild-type mice was able to proteolyze it in a manner similar to the rat sample (Fig. 3B). The results also indicate the presence of minor non-neprilysin peptidase activity that may contribute to Aβ degradation since a small fraction of Aβ was still degraded by the samples from neprilysin-deficient mice. These observations rule out the involvement of non-neprilysin endopeptidase activity, *i.e.* a major peptidase, in Aβ degradation and indeed establish that neprilysin is the responsible peptidase.

We also performed kinetic analysis to determine whether or not $A\beta_{1-42}$ is a relevant substrate for neprilysin. As shown in Fig. 4, we examined proteolysis of the radio-labeled $A\beta_{1-42}$ at varying concentrations and time points. The K_m and V_{max} values were determined to be 2.8 ±0.76 μM and 7.6 ±0.26 nM min^{-1}, respectively. The K_m value is comparable to those for the known neprilysin substrates such as substance P and atrial natriuretic factor and even smaller than that for enkephalin (20 μM) (*21*), suggesting that Aβ is likely to be a physiological substrate. Besides, the fact that the majority of Aβ is associated with insoluble fractions in the brain (*18*) indicates that membrane-associated peptidase with an extracytoplasmic active site, *i.e.* neprilysin, is in an appropriate location. Furthermore, independent reports describing the association of Aβ (*22*) and

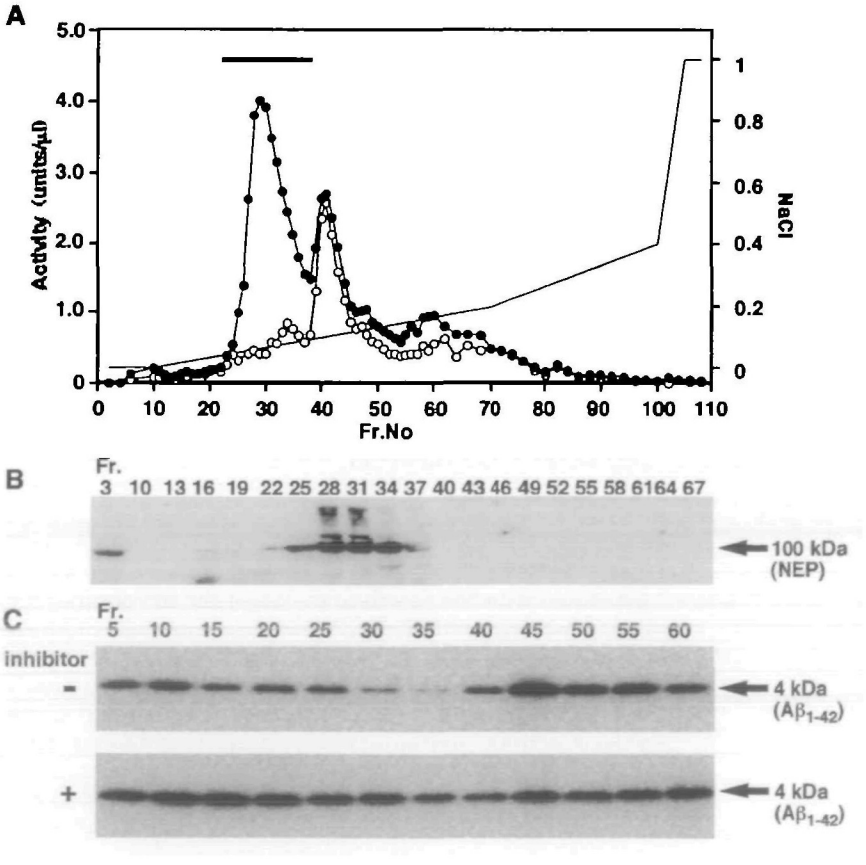

Fig. 1. Anion-exchange chromatography of membrane proteins from rat brain. A: DEAE-Toyopearl 650S chromatography. Membrane proteins were eluted with a linear gradient of 0–0.2 M NaCl (shown by solid lines). The hydrolytic activity of Z-Ala-Ala-Leu-pNA with (open circles) or without (closed circles) phosphoramidon is shown in units per μl. The fractions indicated by the bar were pooled and used as the enzyme sample for the following experiments. B: Western blot analysis of the DEAE fractions with the anti-neprilysin monoclonal antibody, 56C6. C: Aβ degradation assay. Synthetic $A\beta_{1-42}$ was incubated with the DEAE fractions in the presence or absence of thiorphan as indicated and then subjected to Western blot analysis with the anti-Aβ antibody, 4G8.

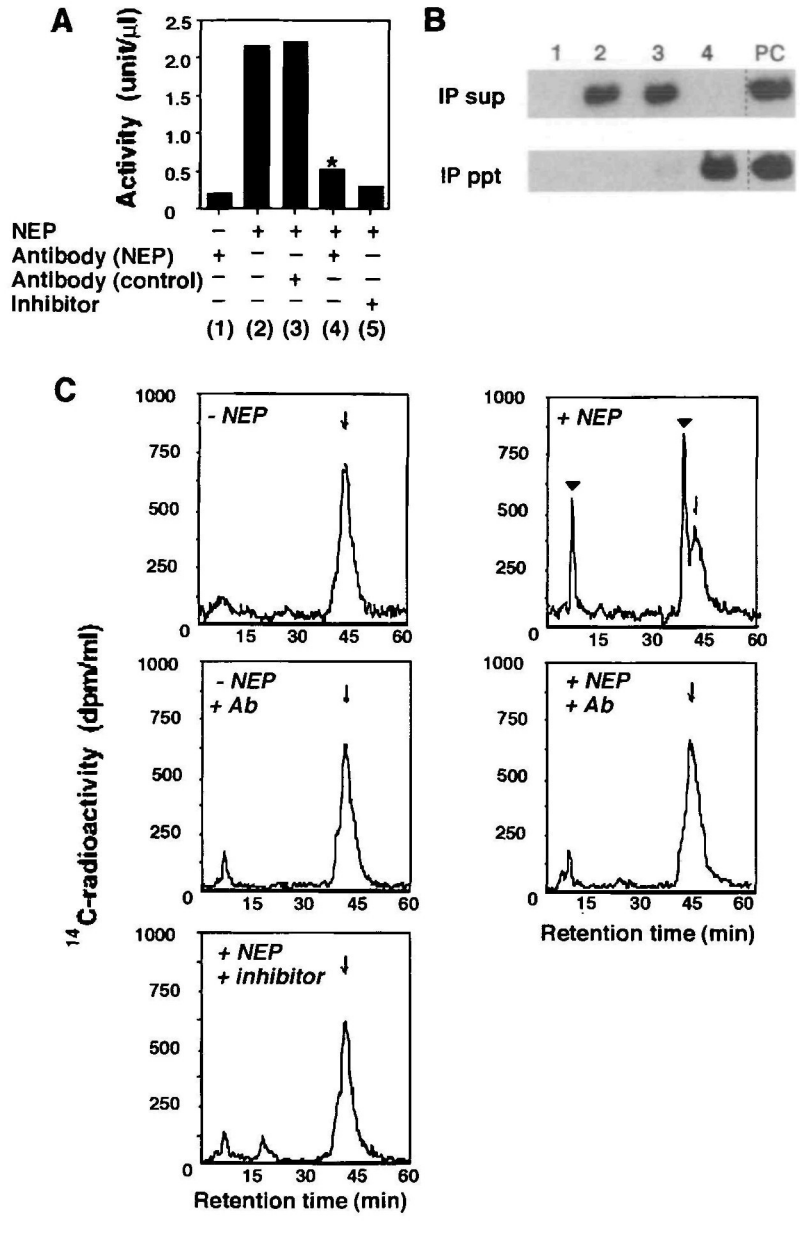

Fig. 2. Immuno-depletion of neprilysin. The enzyme sample was immuno-depleted using anti-NEP-N and control antibodies. A: Z-Ala-Ala-Leu-pNA–hydrolyzing activity. The activities of samples treated as indicated are shown in units per μl. B: Western blot analysis with the anti-neprilysin monoclonal antibody, 56C5. The supernatants and precipitates after immunoprecipitation (IP sup and IP ppt, respectively) were analyzed. Lanes 1–4 correspond to lanes 1–4 in panel A. PC: positive control (neprilysin sample). C: Aβ degradation activity. Enzyme samples treated as indicated were incubated with the $^{3}H/^{14}C$-radiolabeled Aβ$_{1-42}$ for 8 h and then subjected to radio-HPLC analysis. The arrow and arrowheads indicate intact Aβ$_{1-42}$ and proteolytic products, respectively. The elution profiles in the ^{14}C- and ^{3}H-modes were essentially identical.

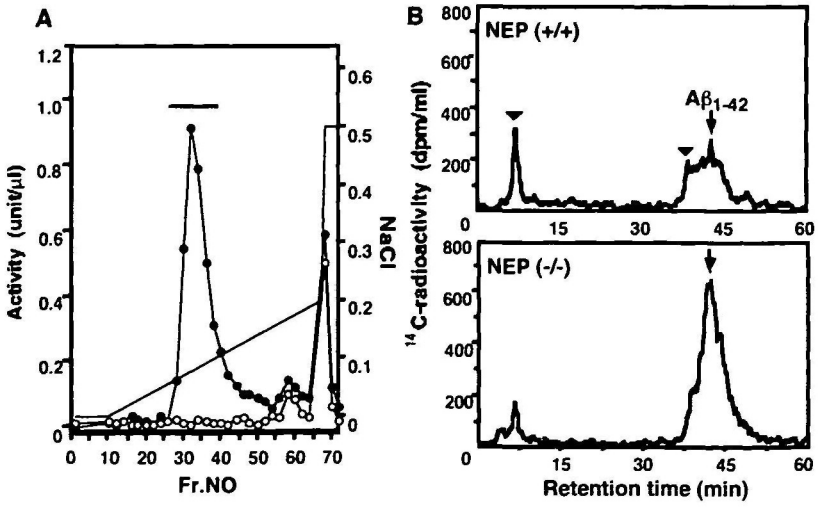

Fig. 3. Analysis of neprilysin-deficient mouse brain. A: MonoQ HR 5/5 column chromatography. Membrane proteins from the brains of NEP-deficient mice or control wild-type mice were separated by ion-exchange chromatography. The Z-Ala-Ala-Leu-pNA–hydrolyzing activities of neprilysin-deficient (−/−) and wild-type (+/+) mice are shown by open circles and closed circles, respectively. B: Aβ degradation activity. Equal amounts of the pooled fractions, as indicated by the bar in panel A, were incubated with $^{3}H/^{14}C$-radiolabeled Aβ$_{1-42}$ for 4 h. See the legend to Fig. 2.

A

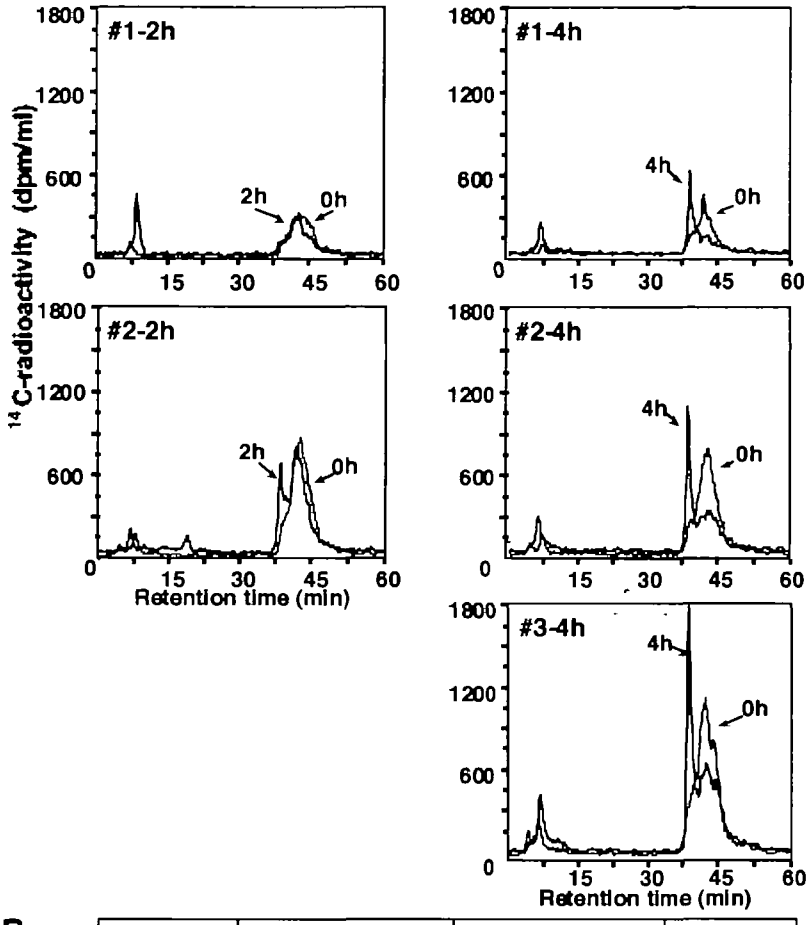

Fig. 4. Kinetic analysis of $A\beta_{1-42}$ proteolysis by neprilysin. A: Aβ degradation with various substrate concentrations and time points. 0.17 μg (#1), 0.33 μg (#2), or 0.5 μg (#3) of $^{3}H/^{14}C$-$A\beta_{1-42}$ was incubated with 74 units of NEP fraction for 2 or 4 h, as indicated. The HPLC profiles before and after digestion are shown as dotted and solid lines, respectively. B: Parameters used for kinetic analysis. See "MATERIALS AND METHODS."

B

	#1		#2		#3
S_0 (μM)	0.67		1.35		2.02
time (h)	2	4	2	4	4
S_0-S_t (%)	9.63	27.5	11.9	33.3	31.5
S_0/S_t	1.28	2.08	1.25	2.02	1.73

neprilysin (*23*) with the detergent-insoluble glycolipid-enriched domain implied that Aβ and neprilysin may even be colocalized in a specific cellular compartment in a concentrated manner. This assumption is also supported by the presence of neprilysin in neuronal rather than glial cells (*24, 25*).

Because the molecular identity of the Aβ-degrading endopeptidase candidate has been determined, it is now of particular interest to determine whether or not reduction of neprilysin that could be caused by aging would cause Aβ accumulation and AD development in the human brain. In enzymatic studies, it will be necessary to measure the phosphoramidon- or thiorphan-sensitive endopeptidase activity associated with membranes in order to define the neprilysin activity. Therefore, such studies measuring the bulk peptidase activity of brain homogenates containing the cytoplasmic fraction without the use of a detergent (*26*) are likely to fail to detect specific activity by picking up too broad a spectrum of various enzyme activities.

It may also be relevant to measure the mRNA level *versus* the Aβ load in aged human brains. Several studies have indicated that there are at least three different mRNA transcripts of the neprilysin gene (*27, 28*), among which type 1 seems to be the major form in the brain (*28*), suggesting that the gene expression is regulated in a region-specific manner. Furthermore, as the neprilysin genome region covering exon 1 seems to regulate neuron-specific gene expression, possible polymorphisms therein are interesting targets for the search for genetic risk factor(s). It is therefore possible that aging may cause down-regulation of neprilysin activity specifically in the brain through transcriptional regulation.

Our studies also suggest that we may be able to reduce the Aβ level in the brain by up-regulating neprilysin activity. The most straightforward approach would be to directly introduce neprilysin into the brain by, for instance, gene

therapy. An attractive feature of neprilysin is its non-destructive nature, because neprilysin is only capable of proteolyzing peptides smaller than 4–5 kDa (*11*). Another advantage is that NEP is capable of degrading even an unphysiologically large amount of Aβ, as demonstrated by the *in vivo* injection experiments (*18*). More detailed analysis of the neprilysin action *in vivo* will be necessary to confirm such possibilities.

MATERIALS AND METHODS

Materials—Pepstatin A, leupeptin, phosphoramidon and Z-Ala-Ala-Leu-pNA were purchased from the Peptide Institute (Osaka), thiorphan from Sigma (St. Louis, MO, USA), protease inhibitor cocktail "Complete, EDTA-free" from Roche Diagnostics (Mannheim, Germany), human amyloid β-peptide (1-42) from Bachem (Bubendolf, Switzerland), 4G8 monoclonal antibody specific for $A\beta_{17-24}$ from Senetek (Maryland Heights, Missouri), and 56C6 monoclonal antibody against NEP from Novacastra (Tyne, UK). All other chemicals were of the highest grade available. $^3H/^{14}C$-labeled $A\beta_{1-42}$ was synthesized as described previously (*18*). NEP-N polyclonal antibody was generated against a synthetic peptide, MGRSESQMDITDINAPKPKKKQC, and then affinity purified (*29*). PEX polyclonal antibody was also produced using synthetic peptide, CPANSTMNRGADSCRLW. NEP-deficient mice were as described previously (*30*).

Isolation of NEP from Rodent Brain—C57BL/6 male mice or Sprague-Dawley male rats aged 8–9 weeks were perfused intracardially with cold PBS prior to dissection. Each brain, without the cerebellum or brain stem, was homogenized in 9 volumes of ice-cold Tris buffer (10 mM Tris, pH 8, containing 0.25 M sucrose, 1× complete EDTA-free and 10 μM leupeptin) with a Teflon-glass homogenizer. The homogenate was centrifuged at 8,000 ×*g* for 15 min and the supernatant was recentrifuged at 40,000 ×*g* for 30 min. The resulting pellets were solubilized in the Tris buffer containing 1% Triton X-100 (v/v) for 1 h at 4°C. The solubilized membranes were centrifuged at 100,000 ×*g* for 1 h. The supernatant was subjected to anion-exchange chromatography, for which the column was equilibrated with 10 mM Tris (pH 8) containing 0.1% Triton X-100 and 10 μM leupeptin. Elution was conducted with a linear gradient of 0–0.2 M NaCl. Eight-millilter DEAE-Toyopearl 650S (Tosoh, Tokyo) and 1-ml MonoQ HR 5/5 (Amersham Pharmacia Biotech, Buckinghamshire, UK) columns were used for the rat and mouse brain homogenates, respectively. The endopeptidase activity was assayed as follows (*31*, *32*). Each sample was incubated with 50 μM Z-Ala-Ala-Leu-pNA at 37°C for 1 h in 50 mM MES (pH 6.5) in the presence or absence of 1 mM thiorphan or phosphoramidon, in a total volume of 100 μl. Similar results were obtained when the reaction was performed at pH 7.5. Ten microliters of a phosphoramidon (0.1 mM) and leucine aminopeptidase (0.1 mg/ml) (L-5006; SIGMA, St. Louis, MO) mixture was then added, and the reaction mixture were further incubated at 37°C for 20 min. After centrifugation, the absorbance of the liberated pNA was measured at 405 nm. One unit of the enzyme activity was defined as the amount that liberated 1 pmol of pNA per min.

Analysis of Aβ Degradation—To assay Aβ proteolysis, 5 μl of a HPLC sample prepared as described above was incubated with cold $A\beta_{1-42}$ (50 ng) at 37°C for 16 h in 10 μl of 50 mM MES (pH 6.5) containing Complete EDTA-free at the concentration suggested by the manufacturer, 1 mM leupeptin and 100 μM pepstatin, with or without 10 μM thiorphan. Similar results were obtained when the reaction was performed at pH 7.5. The reaction was terminated by the addition of 10 μl of solubilizing buffer [0.125 M Tris (pH 6.8), 14% glycerol, 4% SDS, 60% dimethyl sulfoxide, 0.01% bromophenol blue] followed by boiling at 99°C for 5 min. Eight-microliter aliquots were subjected to Western blot analysis with 4G8 antibodies. To analyze Aβ degradation in a more quantitative manner, 5 μl of a sample was incubated with 34 pmol of $^3H/^{14}C$-labeled $A\beta_{1-42}$ in a total volume of 50 μl of 50 mM HEPES (pH 7.2) buffer containing 1× complete EDTA-free, 1 mM leupeptin, and 100 μM pepstatin. The reaction was stopped by the addition of 250 μl of 0.05% triethylamine (pH 10.2) containing 10 mM betaine and 0.1 mM EDTA, followed by boiling for 5 min. The products were analyzed by radio-HPLC as previously described (*18*).

Immunoprecipitation of NEP—A HPLC sample with endopeptidase activity hydrolyzing 120 pmol of Z-Ala-Ala-Leu-pNA per min (120 units) was incubated with 0 or 5 μg of polyclonal antibodies (NEP-N or control antibodies) overnight at 4°C in 50 μl of 10 mM Tris (pH7.4) buffer containing 0.15 M NaCl and 0.1% Triton X-100. Protein A Sepharose 4FF (Amersham Pharmacia Biotech, Buckinghamshire, UK) pre-washed in the same buffer was added, followed by gentle agitation at 4°C for 1 h. After removing the gel by centrifugation, 10 μl of the supernatant was subjected to the Aβ degradation assay using 0.17 μg $^3H/^{14}C$-labeled $A\beta_{1-42}$ as described above. The gel pellets were washed in 10 mM Tris (pH7.4) buffer containing 0.15 M NaCl five times and the used for Western blot analysis.

Kinetic Analysis—A NEP fraction with endopeptidase activity of 74 units was incubated with various amounts (0.17–0.5 μg) of $A\beta_{1-42}$ at 37°C for the indicated times (t). The relative peak areas, determined on radio-HPLC analysis, were used to determine the initial Aβ concentration (S_0) and Aβ concentration at time t (S_t). K_m and V_{max} were determined based on the following first degree equation (*33*):

$$2.303/t \log S_0/S_t = V_{max}/K_m - (S_0 - S_t)/t K_m$$

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