Effects of Neprilysin Chimeric Proteins Targeted to Subcellular Compartments on Amyloid β Peptide Clearance in Primary Neurons*

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Neprilysin (NEP) is a rate-limiting amyloid β peptide (Aβ)-degrading enzyme in the brain. We demonstrated previously that overexpression of neprilysin in primary cortical neurons remarkably decreased not only extracellular but also intracellular Aβ levels. To investigate the subcellular compartments where neprilysin degrades Aβ most efficiently, we expressed neprilysin chimeric proteins containing various subcellular compartment-targeting domains in neurons. Sec12-NEP, β-galactoside α2,6-sialyltransferase-NEP, transferrin receptor-NEP, and growth-associated protein 43-NEP were successfully sorted to the endoplasmic reticulum, trans-Golgi network, early/recycling endosomes, and lipid rafts, respectively. We found that intracellularly, wild-type neprilysin and all the chimeras showed equivalent Aβ40-degrading activities. Aβ40 was more effectively cleared than Aβ42, and this tendency was greater for intracellular Aβ for extracellular Aβ. Wild-type and trans-Golgi network-targeted ST-NEP cleared more intracellular Aβ42 than the other chimeras. Wild-type neprilysin cleared extracellular Aβ more effectively than any of the chimeras, among which endoplasmic reticulum-targeted Sec12-NEP was the least effective. These observations indicate that different intracellular compartments may be involved in the metabolism of distinct pools of Aβ (Aβ40 and Aβ42) to be retained or recycled intracellularly and to be secreted extracellularly, and that the endogenous targeting signal in wild-type neprilysin is well optimized for the overall neuronal clearance of Aβ.

Accumulation of amyloid β peptide (Aβ)1 in the brain is a triggering event leading to the pathological cascade of Alzheimer’s disease (AD) (1). Aβ is a physiological peptide, the steady-state level of which is strictly determined through a balance between the anabolic and catabolic activities. Changes in the metabolic balance are closely associated with Aβ accumulation, because an increase in Aβ anabolic activity causes Aβ deposition in almost all cases of familial AD (2, 3). In sporadic AD brains, where elevation of anabolism seems to be rare, a reduction in catabolic activity involving Aβ-degrading enzyme(s) has been a candidate cause that may account for Aβ accumulation. Recently, several proteases, such as neprilysin (4), insulin-degrading enzyme (5, 6), and endothelin-converting enzymes 1 and 2 (7), have been identified as Aβ-degrading enzymes in the brain by reverse genetic studies. Deficiencies of these proteases were associated with an elevation of brain Aβ levels in each case, demonstrating that a reduction in the catabolic activity will also contribute to AD development by promoting Aβ accumulation. Although these peptidases are likely to contribute to overall Aβ clearance in the brain by complementing each other in a subcellular, cell type- and/or brain region-specific manner, neprilysin seems to play the primary role among all the Aβ-degrading enzymes thus far examined (8, 9). It is also important that the in vivo action of neprilysin in the brain is quite selective for Aβ; neprilysin deficiency does not elevate the levels of such “neprilysin substrate” neuropeptides as enkephalin (10), cholecystokinin, neuropeptide Y, and substance P,2 indicating the presence of redundant buffering mechanisms to metabolize these peptides. Moreover, brain neprilysin expression was selectively reduced not only in aged rodents (11) but also in the early stages of sporadic AD cases (12, 13), suggesting that up-regulation of neprilysin in the brain may prevent AD development by increasing Aβ clearance.

Neprilysin is a type II membrane protein with a catalytic site on the extracytoplasmic side and emerges on the cell surface. Aβ is generated in several organelles, including endoplasmic reticulum (ER), trans-Golgi network (TGN), and endosome (14, 15). Recent evidence indicates that lipid rafts, in which neprilysin is detectable (16, 17), could be involved in Aβ generation (18, 19). We showed that not only extracellular but also cell-associated Aβ levels were reduced by overexpression of neprilysin in primary cortical neurons using Sindbis viral vector (20), suggesting that neprilysin may degrade Aβ through a secretory pathway and possibly on the cell surface.

In this study, we constructed several neprilysin chimeric proteins for targeting to particular organelles, such as ER, TGN, and early/recycling endosomes, and lipid raft fractions, and we investigated the sites of neuronal subcellular compartments that might be closely associated with Aβ metabolism. For this purpose, we used well characterized organelle-targeting domains derived from type II transmembrane proteins or a transmembrane-associated protein, which show the same ori-

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1 The abbreviations used are: Aβ, amyloid β peptide; AD, Alzheimer’s disease; ER, endoplasmic reticulum; TGN, trans-Golgi network; ST, β-galactoside α2,6-sialyltransferase; TIR, transferrin receptor; GAP43, growth-associated protein 43; SFV, Semliki Forest virus; BiP, immunoglobulin heavy chain-binding protein; MAP-2, microtubule-associated protein-2; NEP, neprilysin; ELISA, enzyme-linked immunosorbent assay; APP, amyloid precursor protein; MES, 2-(N-morpholino)ethanesulfonic acid.

2 N. Iwata and T. C. Saido, unpublished data.
entation of peptide sequence on the membrane as neprilysin. We replaced the transmembrane domain of neprilysin with that of Sec12p, which is essential for retention in ER (21). For TGN and endosome targeting, we fused the extracellular domain of neprilysin to the intracellular and transmembrane regions derived from β-galactoside α₂,6-sialyltransferase (ST) or transferrin receptor (TFR), which contains the TGN (22) or early/recycling endosome retention motif, respectively. Finally, the N-terminal domain of growth-associated protein 43 (GAP43) (24) was fused to the N terminus of neprilysin for delivery to the lipid raft fraction. In addition to these neprilysin chimeric proteins, we expressed wild-type and inactive mutant neprilysin as positive and negative controls, respectively, in primary cortical neurons by use of the Semliki Forest virus (SFV) gene expression system, and we examined their effects on Aβ clearance.

EXPERIMENTAL PROCEDURES

Antibodies—Mouse monoclonal (56C6) and rabbit polyclonal (H-321) antibodies to the extracellular domain of human neprilysin were purchased from Novocastra Laboratories (Newcastle, UK) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), respectively. Anti-immunoglobulin heavy chain-binding protein (BiP) and anti-adaptin γ mouse monoclonal antibodies were from BD Transduction Laboratories (Lexington, KY); anti-syntaxin 13 mouse monoclonal antibody was from StressGen Biotechnologies Corp. (British Columbia, Canada), and fluorochrome-conjugated secondary antibodies were from Molecular Probes (Eugene, OR). Rabbit anti-microtubule-associated protein-2 (MAP-2) antisera was described previously (25).

Construction of Recombinant SFV Vectors and Infection—Human wild-type and inactive mutant neprilysin cDNAs in pBluescript II KS (+) were prepared as described previously (20, 26, 27). The cDNAs encoding neprilysin chimeric proteins for targeting of neprilysin enzyme activity to a particular organelle or lipid raft fraction, Sec12-neprilysin (NEP) to ER, ST-neprilysin to TGN, TFR-neprilysin to early/recycling endosomes, and GAP43-NEP to lipid raft fraction were constructed as follows. Neprilysin cytoplasmic domain fragment encoding amino acid residues 1–27 (NEP/CD1–27) was amplified by PCR using two oligonucleotide primers (5′-GGAGAATTCGGATGCAAGAAGAGAAGAAAGAGTCAAGAAA-GT-3′ and 5′-GGGGCCGGCAATGAGGTCTACTGTTT-3′), and then digested with HindIII and SmaI. Finally, three DNA fragments, NEP/CD1–27, ST/neprilysin (ST-NEP) encoding amino acid residues 5–750 (5′-GGAGGGAATTCTACCCCTTTACAATAGC-3′), and 5′-GGCCCGGGATTTGCAAGTCATCAGA-TCCAGTGCATTCATAGTAATCTCT-3′ and 5′-TACAGTGCATTTGCTTTTCATATT-TAGCTAGTCCATAT-3′ and 5′-ATAGGAGAAGCTTGAATTAATGAA-GAAGCCCAAATAGGAAGGTTGTAAGAAGG-3′). Neprilysin extracellular domain encoding amino acid residues 5–750 (5′-CCGGGTTCTACCCCTTTACAATAGC-3′) was fused to the N terminus of neprilysin by ligation of a PCR product using two oligonucleotide primers (5′-GGCCCCGGTATTGGAAGAAGAAGAAGAAAGAGTCAAGAAA-GT-3′ and 5′-TACAGTGCATTTGCTTTTCATATT-TAGCTAGTCCATAT-3′) and an XbaI-digested fragment of neprilysin cDNA in pBluescript II, and then digested with HindIII and SmaI. Finally, three DNA fragments, NEP/CD1–27, SEc1750, were ligated, resulting in Sec12-NEP (Fig. 1) in primary neurons were analyzed by Western blotting at 16 h after the transfection with pSFV-Helper1 RNA into baby hamster kidney cells using the Gene Pulser electroporation system (Bio-Rad) according to the manufacturer's instructions. The conditioned medium containing the recombinant viral vectors was collected at 24 h after transfection and stored at −80 °C until use. Primary cortical neurons were prepared from embryonic C57BL/6JScic mice as described previously (20). Infection was carried out by adding the viral vector solution to culture medium of cortical neurons (3–4 × 10⁶ cells), which had been cultured for 7–9 days. At 16 h after the infection, the conditioned medium and the cell lysate were subjected to assay. When neurons were moved from in vivo conditions to in vitro conditions, the expression level of neprilysin declined for unknown reasons.

Western Blot Analysis and Assay for Neutral Endopeptidase Activity—Infected neurons were extensively washed with PBS, collected, and lysed with 1% Triton X-100 lysis buffer. Aliquots of the cell lysates were subjected to Western blot analysis using 56C6 and to assay for neutral endopeptidase activity using benzoylargininbenzamid-Leu-Arg-p-nitrtolel (Peptide Institute, Osaka, Japan) as a substrate, as described previously (26, 28). Neprilysin-dependent neutral endopeptidase activity was evaluated from the decrease in the rate of digestion caused by 10 μM thiorphan, a specific inhibitor of neprilysin. Protein concentrations were determined using a BCA protein assay kit (Fierce). For Western blot-based quantitative analysis, the intensities of immunoreactive bands were measured with a densitometer, LAS-3000 and Science Lab 2001 Imaging Software for a Fujifilm Image-slides were infected with recombinant viral vectors and processed for double immunofluorescence staining after the cells had been treated with PBS containing 0.2% Triton X-100 as described previously (20), using the following sets of primary antibodies and appropriate fluorochrome-conjugated secondary antibodies as follows: 56C6 anti-NEP MAP-2 antibody and Alexa 568-conjugated goat anti-mouse IgG/Alexa 488-conjugated goat anti-rabbit IgG, anti-BiP antibody/H-321, anti-adaptin γ antibody/H-321 or anti-syntaxin 13 antibody/H-321, and Alexa 488-conjugated goat anti-mouse IgG/Alexa 568-conjugated goat anti-rabbit IgG. The immunofluorescence signals were observed under an IX70 inverted microscope in a confocal laser scanning system (Olympus, Tokyo, Japan). The digital images were captured with Fluoview, version 3.2 software (Olympus Optical), using a ×100 objective. Eight-bit gray scale images were saved as digitized tagged image format files to retain maximum resolution. Colocalized areas of immunoreactive neprilysin and each marker protein in neurons were quantitated using image analysis software, MetaMorph, version 6.1 (Nippon Roper, Chiba, Japan). Each set of experiments was repeated at least twice times.

Lipid Raft Fractionation—Virus-infected neurons (1 × 10⁶ cells) were extensively washed with PBS, collected, and homogenized in MES-buffered saline (MBS: 25 mM MES, pH 6.5, 0.15 M NaCl) containing 1% Triton X-100, proteinase inhibitors mixture (Complete™ Mini EDTA-free, Roche Diagnostics), and 0.7 μM/ml peptatin A (Pepstatin Insect) through a 22-gauge needle (20 passages) on ice, followed by incubation at 4 °C for 30 min. After centrifugation at 2,000 × g for 10 min, 2 ml of the supernatant was mixed with an equivalent volume of 80% sucrose solution, and overlaid sequentially with 4 ml of 35% sucrose and 4 ml of 80% sucrose. The gradient was centrifuged at 39,000 rpm for 2 h with an SW41 rotor, and then 12 fractions (1 ml each) were collected from top to bottom. The pellet (fraction 13) was resuspended in 1 ml of MBS by sonication. Aliquots of all fractions were subjected to Western blot analysis to evaluate targeting of GAP43-NEP chimeric protein to the lipid raft fraction.

Expression of Aβ—Extracellular Aβ in conditioned medium and intracellular Aβ extracted with 6 M guanidine HCI from cultured neurons were measured by sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (20, 29, 30), using BNT77/B2A27 and BNT77/BC05 monoclonal antibodies for Aβ40 and Aβ42, respectively.

RESULTS

Expression of Neprilysin Chimeric Proteins in Primary Cortical Neurons—Expression levels and relative molecular masses of wild-type, inactive mutant neprilysin, and the neprilysin chimeric proteins (Fig. 1) in primary neurons were analyzed by Western blotting at 16 h after the transfection with SFV vector (Fig. 2, A and B). Anti-neprilysin antibody 56C6 used here recognizes the extracellular domain of neprilysin, which is shared by all the constructs. Sec12-NEP showed a
molecular mass of $\sim 100$ kDa, which is similar to that of the wild-type and mutant neprilysin. The molecular masses of ST-NEP, TF-NEP, and GAP43-NEP were slightly altered from that of the wild type in accordance with the changes in the amino acid residues (see Fig. 1). The mutant, ST-NEP, TF-NEP, and GAP43-NEP chimeric proteins were expressed at similar levels to the wild-type, but the expression level of Sec12-NEP was 60% lower than those of the others (Fig. 2B).

We next determined the efficiency of each viral vector to primary neurons using double immunofluorescence staining for neprilysin and a neuronal marker, MAP-2. The efficiencies, defined as the ratio of neprilysin-positive neurons to MAP-2-positive neurons, were 67.8 ± 12.3% for the wild-type, 69.1 ± 11.9% for the mutant, 74.6 ± 14.4% for Sec12-NEP, 54.8 ± 5.4% for ST-NEP, 64.7 ± 19.5% for TF-NEP, and 62.0 ± 13.2% for GAP43-NEP infectants, indicating that all viral vectors had similar titers. Therefore, the reduced expression of Sec12-NEP was not caused by inefficient infection. We speculate that it probably was a consequence of rapid turnover of the expressed protein retained in ER rather than a reduction in transcription or translation, because Sec12-NEP and wild-type neprilysin share identical N-terminal amino acid sequences (see “Discussion” for further speculation).

Levels of thiorphan-sensitive endoproteolytic activity to hydrolyze benzoylloxycarbonyl-alanyl-alanyl-leucine-$p$-nitroanilide were essentially consistent with the protein expression levels of the wild-type or neprilysin chimeric proteins except for Sec12-NEP (Fig. 2C). ST-NEP, TF-NEP, and GAP43-NEP infectants showed peptidase activities comparable with that of the wild-type infectants. The mutant showed no enzymatic activity, as reported previously (20, 31). The activity in Sec12-NEP infectants was detectable and statistically significant ($p < 0.05$ as compared with the inactive mutant) but much lower than the others. However, this relatively low activity still seems to be sufficient to clear intracellular Aβ (see below).

Localization of Neprilysin Chimeras in Neurons—We performed double immunofluorescence microscopy using organellar markers to confirm sorting of neprilysin chimeric proteins to an appropriate organelle (Fig. 3A). The wild-type neprilysin showed a partial colocalization with each organellar marker protein (Fig. 3A, panels 1, 3, and 5), indicating that wild-type neprilysin is not exclusively transported to or retained in a particular compartment. Expression of our targeting constructs successfully sorted Sec12-NEP, ST-NEP, and TF-NEP to the expected organelle in neurons as follows. Sec12-NEP was extensively distributed in cell somas, showing localization similar to that of ER marker BiP (Fig. 3A, panel 2). ST-NEP was detected overlapping with a TGN marker adaptin γ appearing as speckles near the nucleus (Fig. 3A, panel 4). TR-NEP was detected as vesicular structures throughout the cytoplasm, colocalizing with an early/recycling endosomes marker syntaxin 13 (Fig. 3A, panel 6). The distributions of Sec12-NEP, ST-NEP, and TF-NEP in the target organelles were increased 2.1-, 3.1-, and 2.1-fold, compared with those of wild-type neprilysin, respectively ($p < 0.05$). We next examined targeting of GAP43-NEP to the lipid raft fraction by using sucrose gradient fractionation after 1% Triton X-100 extraction at 4 °C (Fig. 3B). Flotillin-1, a marker protein of the lipid raft fraction, was detected as Triton X-100-insoluble proteins in fractions 4 and 5 (data not shown). GAP43-NEP as well as wild-type neprilysin were detected in the lipid raft fraction (Fig. 3B). The amount of GAP43-NEP in the raft fraction was 1.3-fold higher, compared
with the case of wild-type neprilysin (p < 0.05). We did not employ the raft-targeting strategy described by Cailler et al. (32), in which a raft-targeting strategy was added at the C terminus of neprilysin, because such a modification may alter the conformation of the protein and thus may also modify the enzymatic properties of the peptidase.

We also investigated whether the expressed proteins appeared on the cell surface or not by performing immunofluorescence in the absence of Triton X-100. Wild-type neprilysin, ST-NEP, TIR-NEP, and GAP43-NEP, but not Sec12-NEP, were observed on the cell surface (data not shown).

**Effect of Targeting of Neprilysin to Particular Intracellular Compartments on Intracellular and Extracellular Aβ Levels**—We measured the amounts of Aβ extracted with guanidine hydrochloride from the primary neurons expressing wild-type neprilysin, inactive mutant neprilysin, and neprilysin chimeric proteins (Fig. 4) in order to assess how much of the endogenously generated intracellular Aβ was degraded. Both Aβ40 and Aβ42 were significantly decreased by the overexpression of wild-type neprilysin but not by that of inactive mutant neprilysin, as reported previously (20). All the chimeric proteins degraded Aβ40 to an extent indistinguishable from that of wild-type neprilysin. Aβ42 was much less degraded than Aβ40 in all cases (0–10% versus 50–60%). Our speculation on the reason for the preferential clearance of intracellular Aβ40 as compared with Aβ42 is that Aβ40 may be retained intracellularly for a longer time than Aβ42, for instance through a recycling mechanism. This assumption is supported by the observation that ER-targeted neprilysin degrades Aβ40 as sufficiently as wild type and other chimeras (see “Discussion” for further speculation). Most interesting, only wild-type neprilysin and ST-NEP degraded intracellular Aβ42 to a significant degree. This is presumably because Aβ42 is not generated in or recycled back to the ER (see “Discussion”) and because endosomes are somewhat too acidic for neprilysin, the optimum pH for which is neutral, to more efficiently degrade Aβ than in TGN.

We next examined the levels of Aβ40 and Aβ42 in the conditioned media of the infected cells (Fig. 5). Overexpression of the wild-type neprilysin, ST-NEP, TIR-NEP, and GAP43-NEP significantly decreased the amounts of extracellular Aβ40 and Aβ42 in a comparable manner (p < 0.01). ST-NEP and TIR-NEP differed from the wild-type neprilysin by clearing Aβ40 in a slightly but significantly less efficient manner (p < 0.01). Again, Aβ42 was cleared to a lesser extent than Aβ40 (−60 versus 80%), but the difference was smaller than in the case of intracellular Aβ clearance. In contrast, Sec12-NEP, targeted to ER, exhibited much weaker activity to reduce the extracellular Aβ (~20%).
The in vivo interesting, infusion of a cell-impermeable neprilysin inhibitor, that the substrate present in a larger amount has a greater the A

FIG. 5. Effects of neprilysin targeting to particular organelles or lipid raft fractions on the extracellular Aβ40 and Aβ42 levels. The Aβ40 and Aβ42 levels in the conditioned medium of infected neurons were measured by sandwich ELISA. The solid line shows the Aβ levels of cells expressing inactive mutant neprilysin. Each column with an error bar represents the mean ± S.D. of at least four independent experiments. *, p < 0.01, significantly different from wild-type neprilysin; †, p < 0.001, significantly different from inactive mutant neprilysin by the Mann-Whitney U test.

DISCUSSION

Despite a large number of cell biological studies of Aβ metabolism and transport, it still remains unclear exactly where in neurons Aβ is generated from amyloid precursor protein (APP). One of the aims of the present study was to obtain a clue to this question by targeting an Aβ-degrading enzyme to particular cellular components.

The major technical limitation of our experimental paradigm employed here is that, although we can control the efficiency of infection by altering the amount of the viral vectors, the amount of protein expressed per cell cannot be controlled. This limitation makes it somewhat difficult to interpret the results in a quantitative manner. This assumption probably applies to the overexpression paradigm in most cell biological studies of APP and Aβ metabolism thus far performed. However, because the Aβ clearance in each case, intracellular or extracellular, remained unsaturated (Figs. 4 and 5), the following qualitative considerations should at least be valid.

We would like to comment on the difference in the ratio of Aβ40 and Aβ42 levels between the in vitro and in vivo situations. The ratio is ~7:1 in vitro and 3:1 in vivo (27). This difference is presumably due to the difference in the volume of the extracellular space and in the complexity of axo-dendritic structure and connections. Therefore, a simple enzymatic explanation for the observation indicating that Aβ40 was more efficiently degraded than Aβ42 in the present in vitro experimental paradigm (Figs. 4 and 5) is that the affinity of neprilysin for Aβ42 is indistinguishable from that for Aβ40 (26), and that the substrate present in a larger amount has a greater chance of encountering the enzyme. In contrast, Aβ40 and Aβ42 are degraded to an essentially identical extent in the brain in vivo as indicated by reverse genetic evidence (4). Most interesting, infusion of a cell-impermeable neprilysin inhibitor, thiorphan, into the brain elevated Aβ42 but not Aβ40 in the insoluble fraction (33). These previous observations, together with the finding that the relative efficiency of Aβ42 degradation versus Aβ40 degradation is greater extracellularly than intracellularly, imply that Aβ40 and Aβ42 are somewhat differentially metabolized in spatial terms. Degradation of Aβ42 seems to be more closely associated with the outside surface of the cell than that of Aβ40. The “outside surface” probably corresponds to the presynaptic membrane in vivo (27, 34).

Among the four chimeric proteins examined in the present study, only Sec12-NEP, targeted to ER, was significantly less abundantly detected than wild-type neprilysin in terms of both protein quantity and enzymatic activity. As discussed under “Results,” this is likely to have been caused by metabolic instability of this chimera, which may be accounted for by premature glycosylation, because Sec12-NEP, but not the others, was sensitive to endoglycosidase H treatment (data not shown). Nevertheless, expression of Sec12-NEP reduced intracellular Aβ40 in a manner comparable with that of wild-type neprilysin and the other chimeras. This indicates that the relatively small activity of neprilysin expressed in ER was sufficient to degrade intracellular Aβ40, because wild-type neprilysin and all the other chimeras reside at least transiently in the ER after synthesis. Considering the previous reports describing Aβ generation in ER, TGN, and endosome (14, 15, 35), our observation implies that a certain pool of intracellular Aβ40 is retained in or recycled back to the ER in cultured neurons, as it is unlikely that the ER is a major site of Aβ generation from APP (36). If aging causes such a pool of intracellular Aβ to remain abnormally unmetabolized, pathological intraneuronal Aβ accumulation (37–39) may arise, possibly leading to ER stresses. Part of this ER-resident Aβ may be transported to the cytoplasm and undergo degradation catalyzed by proteosome or insulin-degrading enzyme (40). Of particular interest is the fact that intracellular Aβ42 was cleared significantly only by wild-type neprilysin and TGN-targeted ST-NEP (Fig. 4). This observation is consistent with our speculation that metabolism of Aβ42 takes place somewhat later than that of Aβ40 in the intracellular trafficking processes.

In clear contrast to intracellular Aβ, extracellular Aβ was poorly degraded by Sec12-NEP, which did not appear on the cell surface. Because the wild-type neprilysin and all the other chimeras were detected on the outside of neurons, the results indicate that the majority of extracellular Aβ degradation takes place on the cell surface in the present experimental paradigm. It was rather unexpected that GAP43-NEP, targeted to lipid rafts, failed to degrade Aβ more efficiently than wild-type neprilysin, because a number of studies has indicated that Aβ generation takes place in this microdomain (18, 19). The results can be accounted for by assuming that the extracellular Aβ was proteolysed by neprilysin after being secreted into the conditioned medium in the present in vitro paradigm. We would like to draw attention to the fact that although the ratio of soluble Aβ to insoluble Aβ is ~400:1 in vitro, the ratio in vivo is 3:1 (27). Therefore, there still remains a possibility that targeting neprilysin to lipid rafts may improve the efficiency of Aβ degradation under in vivo conditions, in which both neprilysin and Aβ are axonally transported to presynapses (27, 34, 41–44), and Aβ is likely to associate with such raft components as ganglioside and cholesterol (45, 46) and such raft-interacting proteins as apolipoproteins J and E (47–50). One way to further examine this possibility would be to utilize the in vivo gene transfer approach (27).

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