Neurobiology of Disease

Presynaptic Localization of Neprilysin Contributes to Efficient Clearance of Amyloid-β Peptide in Mouse Brain

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A local increase in amyloid-β peptide (Aβ) is closely associated with synaptic dysfunction in the brain in Alzheimer's disease. Here, we report on the catabolic mechanism of Aβ at the presynaptic sites. Neprilysin, an Aβ-degrading enzyme, expressed by recombinant adeno-associated viral vector-mediated gene transfer, was axonally transported to presynaptic sites through afferent projections of neuronal circuits. This gene transfer abolished the increase in Aβ levels in the hippocampal formations of neprilysin-deficient mice and also reduced the increase in young mutant amyloid precursor protein transgenic mice. In the latter case, Aβ levels in the hippocampal formation contralateral to the vector-injected side were also significantly reduced as a result of transport of neprilysin from the ipsilateral side, and in both sides soluble Aβ was degraded more efficiently than insoluble Aβ. Furthermore, amyloid deposition in aged mutant amyloid precursor protein transgenic mice was remarkably decelerated. Thus, presynaptic neprilysin has been demonstrated to degrade Aβ efficiently and to retard development of amyloid pathology.

Key words: **Alzheimer; axonal transport; axoplasmic transport; gene; hippocampus; presynaptic; projection; endopeptidase; amyloid-β peptide; gene transfer; adeno-associated viral vector; degradation; perforant path**

Introduction

Alzheimer's disease (AD) is characterized by extracellular deposition of amyloid-β peptide (Aβ), intracellular neurofibrillary tangles, and synaptic and neuronal loss (Hardy and Selkoe, 2002). Aβ deposition is a triggering event that causes a long-term pathological cascade of AD and is closely associated with the metabolic balance between Aβ anabolic and catabolic activities (Hardy and Selkoe, 2002; Saido, 2003; Saido and Nakahara, 2003). As almost all familial AD mutations cause an increase in the anabolism of a particular form of Aβ, $Aβ_{1-42}$, leading to Aβ deposition and accelerating AD pathology, a chronic reduction in the catabolic activity would also promote Aβ deposition (Hardy and Selkoe, 2002; Saido, 2003; Saido and Nakahara, 2003). Neprilysin (EC 3.4.24.11), previously called neutral endopeptidase-24.11, enkephalinase, or NEP, is a rate-limiting peptidase participating in Aβ catabolism, as proven by *in vivo* experiments tracing the catabolism of radiolabeled Aβ in brain and by reverse genetics studies (Iwata et al., 2000, 2001). Neprilysin-specific inhibitors and neprilysin gene disruption in mice abolished this function. Neprilysin gene disruption caused a gene dosage-dependent elevation of the endogenous Aβ levels in the mouse brain, suggesting that even a subtle reduction of neprilysin activity will contribute to AD development by promoting Aβ deposition (Iwata et al., 2001). This conclusion was reinforced by the observation that expression levels of neprilysin were particularly low in regions vulnerable to senile plaque development, such as hippocampus and midtemporal gyrus (Yasojima et al., 2001a,b). The neprilysin levels in these regions were selectively reduced by ~50% in sporadic AD patients, compared with age-matched controls. In addition, in normal aging of laboratory mice, neprilysin levels were selectively decreased at the outer molecular layer of the dentate gyrus and the stratum lucidum, the terminal zones of the lateral perforant path (Iwata et al., 2002), which are highly vulnerable regions to AD pathology (Gomez-Isla et al., 1996). Thus, age-related decline of neprilysin activity in these specific regions is likely to promote the pathogenesis of sporadic AD.

Much attention has been directed to a possible role of Aβ in synaptic dysfunction observed in brains of AD patients and several lines of mutant amyloid precursor protein (APP) transgenic mice (Hartley et al., 1999; Hsia et al., 1999; Lue et al., 1999; Mucke et al., 2000; Buttini et al., 2002; Walsh et al., 2002; Wang et al., 2002). An aberrant increase in Aβ at presynaptic sites has been suggested to give rise to synaptic dysfunction (Terry et al., 1991; Sze et al., 1997; Selkoe, 2002). Aβ generated from axonally transported APP is released from presynaptic sites and contributes to

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extracellular amyloid deposition (Buxbaum et al., 1998; Lazarov et al., 2002; Sheng et al., 2002). However, much less is known about the catabolic mechanism of Aβ at the presynaptic sites. Neprilysin was mainly detected in presynapses and on or around axons in the hippocampal formation by double immunostaining with presynaptic and axonal marker proteins (Fukami et al., 2002) and by subcellular fractionation and electron microscopic immunocytochemistry (Barnes et al., 1988, 1992), leading us to the notion that neprilysin regulates Aβ concentration around presynaptic sites and that a reduction of neprilysin activity may elicit local elevation of Aβ concentration in the extracellular space close to synapses, possibly affecting the local pathology during the course of AD development.

In the present study, we attempted to express neprilysin in the hippocampal formation of mice *in vivo* by means of viral vector-mediated gene transfer to confirm the pathological significance of presynaptic neprilysin. For this purpose, we selected a recombinant adeno-associated virus (rAAV) vector, because it offers the advantages of the ability to infect nondividing cells, affording a non-pathogenic, long-term transgene expression without a substantial inflammatory response (Okada et al., 2002). Because inflammatory responses diversely affect clearance of the amyloid burden (Weninger and Yankner, 2001), it is important to avoid inducing such a response to viral infection to elucidate properly the effect of neprilysin gene transfer. In addition, we used expression of an inactivated form of neprilysin as a negative control. Recently, Marr et al. (2003) reported successful reduction of Aβ by neprilysin gene transfer with a lentiviral vector to the brain of mutant APP transgenic mice, in which immunoreactive neprilysin was detected in cell bodies in most cases. However, the significance of Aβ degradation by neprilysin at the presynaptic sites remains unclear. Here, we show that neprilysin expressed in the neurons was axonally transported to presynaptic sites and presynaptic neprilysin efficiently degraded Aβ, eventually decelerating Aβ deposition.

Materials and Methods

Recombinant AAV vector production. The AAV vector plasmid contains human neprilysin cDNA or its mutant (inactivated form) at the C terminus of the human cytomegalovirus immediate-early promoter and the human growth hormone first intron at the N terminus of the simian virus 40 polyadenylation signal sequence between the inverted terminal repeats of the AAV serotype 5 genome (Chiorini et al., 1999). rAAV5 vectors were produced with AAV vector plasmid and AAV helper plasmids encoding rep and cap sequences from AAV serotype 5 and pHelper plasmid from the AAV Helper-Free System (Stratagene, La Jolla, CA). In brief, subconfluent human embryonic kidney 293 cells were transiently transfected by the calcium phosphate method. Seventy-two hours after transfection, the cells were collected and subjected to three cycles of freeze–thaw lysis. rAAV vectors were purified on two sequential continuous cesium chloride density gradients, and the final particle titer was estimated by quantitative DNA dot-blot hybridization. Before administration, rAAV vectors were diluted in HEPES-buffered saline to 0.1–2.0×10^{10} genome copies/0.6 μl.

Animals and surgical procedure. All animal experiments were performed in compliance with the institutional guidelines. Wild-type, neprilysin-deficient (Lu et al., 1995), young and aged Tg2576 (Hsiao et al., 1996) mice in littermates were evenly assigned to rAAV-NEP vector-injected group and three negative control groups that were injected with rAAV-NEPinactive vector, rAAV-*LacZ* vector, and the vehicle, respectively. A 26 S gauge needle equipped with a 1.0 μl motorized Hamilton syringe was inserted into the dentate gyrus (stereotaxic coordinates: anteroposterior, 2.4 mm; mediolateral, 2.0 mm; dorsoventral, 2.1 mm), the frontal isocortex (−2.3, 2.0, 1.5 mm), or the lateral entorhinal cortex (4.1, 4.5, 2.3 mm) of anesthetized mice. Two minutes after the insertion, 0.6 μl of a virus solution or vehicle was injected at a constant flow rate of 0.06 μl/min, and the needle was left in this configuration for an additional 2 min, to prevent reflux of the injected material along the injection track, before being slowly retracted.

Immunohistochemistry. The mouse brain was fixed by a transcardial perfusion with phosphate-buffered 4% paraformaldehyde and then embedded in paraffin. Four-micrometer-thick sections were mounted onto aminopropyltriethoxysilane-coated glass slides. Immunostaining for neprilysin was performed using the high-temperature antigen unmasking technique and the fluorescence-indirect tyramide signal amplification (TSA) method and anti-neprilysin monoclonal antibody (56C6; 1:200 dilution, Novocastra, Newcastle, UK) according to our previous report (Fukami et al., 2002). The immunostaining for β-galactosidase was performed using anti-β-galactosidase antibody (AB1211; 1:1500 dilution; Chemicon, Temecula, CA). Sections were observed under an Axiophot 2 fluorescence microscope (Zeiss, Göttingen, Germany), and the digital images were captured with a 2.5× objective and a CCD camera (ORCA-ERII; Hamamatsu Photonics KK, Shizuoka, Japan) using image analysis software, MetaMorph, version 6.1 (Universal Imaging Corporation, Downington, PA).

Multiple immunofluorescence staining of neprilysin and marker proteins, SV2A (119002; 1:500 dilution; Synaptic Systems, Göttingen, Germany), tau (Tau1; 1:200 dilution; Chemicon), MAP2 (M121; 1:10 dilution; Leinco Technologies, Manchester, UK), or GFAP (L1812; prediluted; Dako, Carpinteria, CA) was performed as described previously (Fukami et al., 2002). When we used anti-GAP43 (AB5220; 1:2000 dilution; Chemicon), anti-SNAP-25 (SC7538; 1:25 dilution; Santa Cruz, CA), anti-GluR1 (AB1504; 1:400 dilution; Chemicon), or anti-Aβ antibody, which recognizes the C-terminal of Aβ42 (C42) (Saido et al., 1995), we used a fluorescence-direct TSA method combined with horseradish peroxidase-labeled dextran polymer-conjugated goat anti-rabbit IgG (Envision plus; Dako) (Fukami et al., 2002). The sections labeled with immunofluorescence were observed under an IX70 inverted microscope incorporating a confocal laser scanning system FV300 with argon, helium–neon (G) and helium–neon (R) lasers (Olympus Optical Co., Tokyo, Japan). FITC/Alexa 488, Texas Red, and Alexa 633 were excited with 488, 543, and 633 nm laser beams and observed through 510–530, 560–600, and 660 nm bandpass emission filters, respectively. All of the single images were acquired by a triple scan with Fluoview, version 4.2 software (Olympus Optical) using a 100× oil immersion objective, then merged and saved as digitized tagged-image format files to retain maximum resolution.

Quantitative assessment of Aβ deposition. Three months after unilateral injection of rAAV-NEPinactive or rAAV-NEP (1.3×10^{10} genome copies) into the hippocampal formation of 18-month-old Tg2576 mice, the brain sections were immunostained using anti-Aβ antibody C42 and visualized by the avidin–biotin–peroxidase complex procedure (Vectastain ABC kits; Vector Laboratories, Burlingame, CA) using 3,3-diaminobenzidine as a chromogen. Sections were observed with a light microscope, Provis AX80 (Olympus Optical), using a 4× objective and a 1.5× digital zoom, and digital images were captured with a DP70 digital microscope camera (Olympus Optical). Densities of immunoreactive Aβ deposits in various areas of the hippocampal formation and cerebral cortex were measured using MetaMorph by an investigator "blinded" to sample identity, after the raw images had been inverted using image-editing software (Adobe Photoshop 7.0; Adobe Systems, Inc., Mountain View, CA). To reduce the variance among tissue sections, we used the average of data from three sections per mouse as an individual value.

Assay of neprilysin-dependent neutral endopeptidase activity. Triton X-100-solubilized membrane fractions from hippocampal formations and cerebral cortices of intact, buffer-, or rAAV-vector-injected mice were prepared to assay neutral endopeptidase activity as described previously (Iwata et al., 2002). The neprilysin-dependent neutral endopeptidase activity was fluorometrically assayed using 0.1 mM succinyl-Ala-Ala-Phe-MCA (Bachem, Bubendorf, Switzerland) as a substrate and determined from the fluorescence intensity (excitation, 390 nm; emission, 460 nm), based on 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 (Pierce, Rockford, IL).

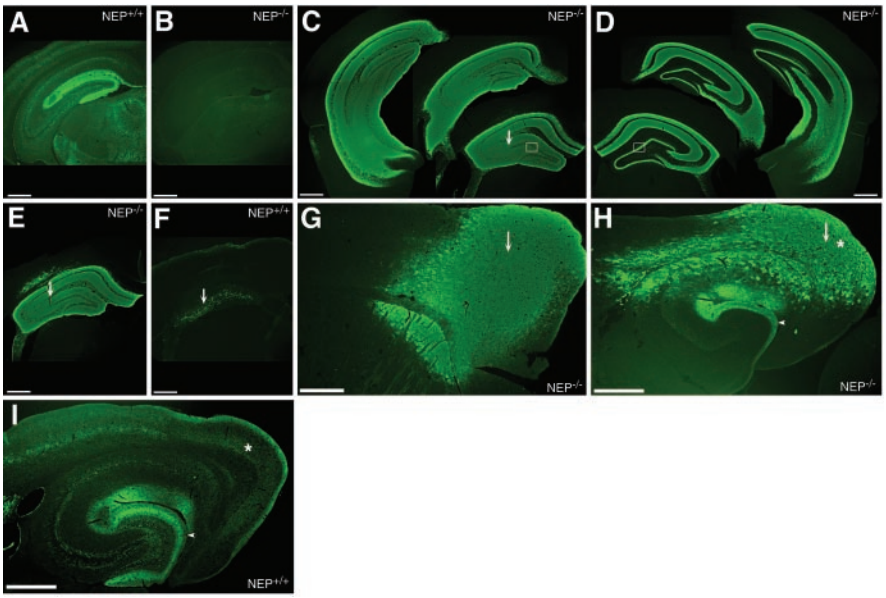

Figure 1. Expression profiles of neprilysin in brain after the vector injection. Immunohistochemical staining for neprilysin (*A–E,G–I*) or β-galactosidase (*F*) was performed 4 weeks after the injection of rAAV vectors (1.3×10^{10} genome copies). rAAV-NEP (*C*) or rAAV-NEPinactive (*E*) was unilaterally injected into the dentate gyrus of *neprilysin* $^{-/-}$ mouse. *C*, *E*, and *F* show the injection (ipsilateral) sides; *D* is the contralateral side to *C*. Three immunostained sections from anterior to posterior are shown in *C* and *D*. *F* shows the expression pattern of β-galactosidase in the dentate gyrus of wild-type mouse injected with rAAV-*LacZ*. *A*, *I*, and *B* show typical immunostaining patterns for *neprilysin* $^{+/+}$ and *neprilysin* $^{-/-}$ mouse brains, respectively. rAAV-NEP was injected into the frontal isocortex (*G*) and lateral entorhinal cortex (*H*) of *neprilysin* $^{-/-}$ mice. Arrows, arrowheads, and asterisks indicate the injection sites, the outer molecular layer of the dentate gyrus, and the lateral entorhinal cortex, respectively. Boxed areas were analyzed for subcellular localization of neprilysin as shown in Figure 2. Scale bars, 500 μm.

Aβ quantitation. The hippocampal formations and cerebral cortices were homogenized in 50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl and the protease inhibitor cocktail (TBS) with a Teflon-glass homogenizer and centrifuged at 200,000 × *g* for 20 min at 4°C. The supernatant was defined as the soluble (TBS-extractable) fraction, and guanidine–HCl was added to give 0.5 M (final concentration) before application to ELISA. The pellet was solubilized by sonication in 6 M guanidine–HCl buffer containing the protease inhibitor cocktail. The solubilized pellet was centrifuged at 200,000 × *g* for 20 min at 4°C, after which the supernatant was diluted 12 times to reduce the concentration of guanidine–HCl and used as the insoluble fraction. The amounts of $A\beta_{X\text{-}40}$ and $A\beta_{X\text{-}42}$ in each fraction were determined by sandwich ELISA using monoclonal antibodies BNT77/BA27 and BNT77/BC05, respectively.

Statistical analysis. All data were expressed as means ± SEM. For comparisons of the means between two groups, statistical analysis was performed by applying Student's *t* test after confirming equality between the variances of the groups. If the variances were unequal, Mann–Whitney *U* tests were performed (StatView 5.0 software; SAS, Cary, NC). Comparisons of the means among more than three groups were done by a one-way ANOVA followed by Dunnett's multiple-range test (StatView 5.0 software). *P* values of <0.05 were considered to be significant.

Results

Expression profiles of neprilysin in the brain after rAAV-mediated gene transfer

To evaluate the expression pattern of neprilysin, we injected an rAAV vector into the brain of neprilysin-deficient mouse and examined the expression profile by means of specific immunochemical staining for neprilysin. This staining gave specific signals of endogenous neprilysin in wild-type mice, but not in neprilysin-deficient mice (Fig. 1*A*,*B*). Expression of neprilysin after a single injection of rAAV-NEP vector into the unilateral dentate gyrus was spread over the bilateral hippocampal formations in the neprilysin-null background (Fig. 1*C*,*D*). The localization of neprilysin in the contralateral side of the hippocampal formation was similar to the pattern of afferent projection from the ipsilateral neurons, that is, the commissural, associational, and associational/commissural projections (Amaral and Witter, 1995). Intrahippocampally, intense signals of immunoreactive neprilysin were detected at the stratum lucidum (the projection site from the granular cells of the dentate gyrus) of the ipsilateral side 1 week after rAAV-NEP vector injection (data not shown), although they could not be clearly seen at 4 weeks after injection because of the more intense signal over the entire hippocampal formation (Fig. 1*C*). The inactivated form of neprilysin, one of the negative control proteins, was expressed to a similar extent and with a similar spatial pattern to that of wild-type neprilysin after rAAV vector-mediated gene-transfer (Fig. 1*E*). The expression of β-galactosidase, another negative control protein, was almost wholly restricted to the injection site, and its subcellular localization was limited to the neuronal somas (Fig. 1*F*), in contrast to neprilysin. The apparent difference of gene expression level of β-galactosidase can be simply explained in terms of the detection sensitivity of immunostaining, but the difference of distribution appears to be attributable to the nature of the expressed protein.

Expression of neprilysin was also detected after injections of rAAV-NEP vector into isocortex (Fig. 1*G*) and allocortex (Fig. 1*H*) regions, such as the entorhinal cortex. In particular, localization of neprilysin after the injection of rAAV-NEP vector into the lateral entorhinal cortex was observed in the outer molecular layer of the dentate gyrus, which is consistent with the projection of the perforant path. This distribution pattern of neprilysin was observed in intact wild-type mice (Fig. 1*I*). Taken together with the findings in the case of hippocampal rAAV-NEP injections, this result indicates that neprilysin expressed after the injection of rAAV-NEP vectors can be transported through appropriate projection pathways, as would be required for a widespread presynaptic distribution of exogenous neprilysin.

Next, we examined the subcellular localization of neprilysin in the contralateral hippocampal formation by confocal double immunostaining for neprilysin and several marker proteins, after the injection of the rAAV-NEP vector into the hippocampal formation of neprilysin-deficient mice. Neprilysin was well colocalized with presynaptic markers, SV2A (Fig. 2*A*), GAP43 (Fig. 2*B*), and SNAP-25 (data not shown), and also with an axonal marker tau (Fig. 2*C*), but not with a somatodendritic marker MAP2, a postsynaptic marker GluR1, or an astrocytic marker GFAP (Fig. 2*D–F*), in agreement with the colocalization patterns of neprilysin observed in the brain of intact wild-type mice (Fukami et al., 2002). Similarly, neprilysin observed at the ipsilateral hippocampal formation was present at the presynaptic terminals in various intrahippocampal circuits, but not in somatodendritic areas or astrocytes (data not shown). These results demonstrate that rAAV-NEP-derived neprilysin is axonally transported to the pre-

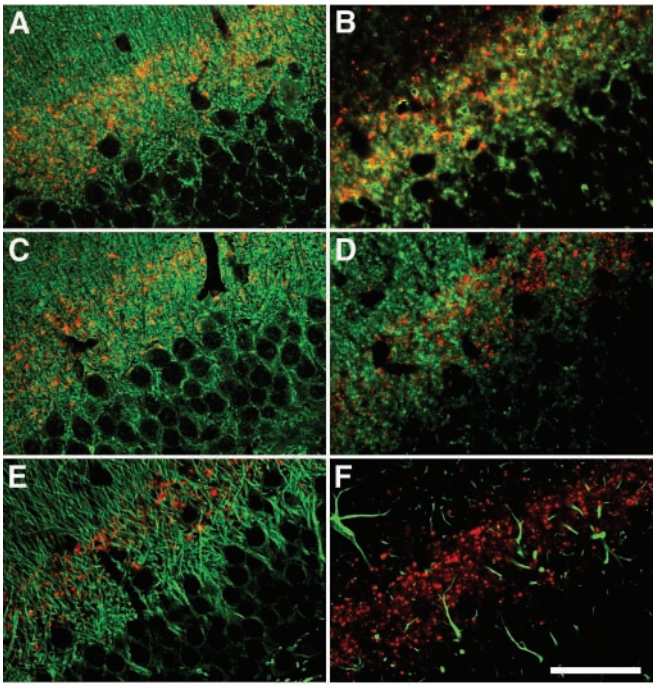

Figure 2. Subcellular localization of neprilysin expressed in the hippocampal formation after gene transfer. The subcellular localization of neprilysin expressed in the dentate gyrus of the contralateral hippocampal formation of *neprilysin* $^{-/-}$ mice by rAAV-mediated gene transfer was analyzed by double immunofluorescence staining for neprilysin (red) and several marker proteins (green). The merged images are shown here. *A*, SV2; *B*, GAP-43; *C*, tau; *D*, GluR1; *E*, MAP2; *F*, GFAP. Scale bar, 20 μm.

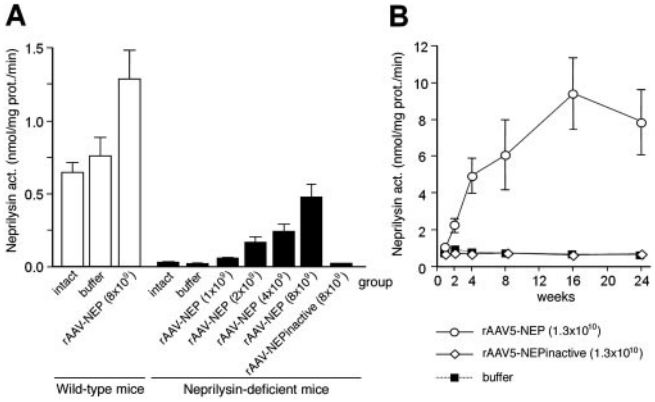

Figure 3. Functional expression of neprilysin after gene transfer. *A,* Levels of neprilysin-dependent endopeptidase activities in ipsilateral hippocampal formations of intact, buffer-injected, rAAV-NEP-injected, or rAAV-NEPinactive-injected *neprilysin* $^{+/+}$ and *neprilysin* $^{-/-}$ mice. Each column with a bar represents the mean ± SE of three mice. *B,* Duration of neprilysin gene expression. The endopeptidase activities in ipsilateral hippocampal formations were measured 1, 2, 4, 8, 16, and 24 weeks after unilateral injection of rAAV-NEP, rAAV-NEPinactive, or buffer into the hippocampal formation of *neprilysin* $^{+/+}$ mice. Each column with a bar represents the mean ± SE of three mice.

synaptic terminals that are supposed to be primary sites of Aβ catabolism, as expected from the fact that neprilysin is a type II membrane-associated protein.

Functional expression of neprilysin

We next investigated the levels of neprilysin gene expression by the measurement of neprilysin activity. One week after the single injection of rAAV-NEP vector into the hippocampal formation of neprilysin-deficient mice, the neprilysin activity was increased in a vector-dose-dependent manner, while the activity in the buffer- or rAAV-NEPinactive-injected group remained at the level observed in intact neprilysin-deficient mice (Fig. 3*A*). The injection of rAAV-NEP vector into the wild-type mice increased neprilysin activity over that in the intact or buffer-injected group to a degree which was similar to that in neprilysin-deficient mice injected with the equivalent amount of rAAV-NEP vector (Fig. 3*A*). In a time course experiment, the expression of neprilysin (based on activity measurement) was increased by a maximum of tenfold at 16 weeks after the infection, compared with the rAAV-NEPinactive-injected group, and continued to be increased up to 24 weeks (Fig. 3*B*).

Rescue of neprilysin-deficient mice from Aβ accumulation

We investigated changes in Aβ levels in both soluble (TBS-extractable) and insoluble (GuHCl-extractable) fractions extracted from hippocampal formations 4 weeks after bilateral injection of either rAAV vectors or buffer into the hippocampal formations of 10-week-old neprilysin-deficient mice (Fig. 4*A*). The Aβ levels in both fractions of intact neprilysin-deficient mice were increased up to approximately twofold, compared with those of wild-type mice, as reported previously (Iwata et al., 2001). After the injection of buffer, rAAV-*LacZ* vector, or rAAV-NEPinactive vector into neprilysin-deficient mice, the Aβ levels in each fraction remained unchanged, but the injection of rAAV-NEP vector significantly decreased Aβ to the wild-type levels, thus almost completely abolishing the increase of Aβ levels induced by neprilysin deficiency. In the experiments using wild-type mice, the neprilysin gene transfer further decreased the $A\beta_{x\text{-}40}$ levels in both fractions (Fig. 4*B*). Although we examined $A\beta_{x\text{-}42}$ levels, most of the values were below the reliable determination range of the standard curve in ELISA (data not shown).

Efficient degradation of Aβ by presynaptic neprilysin

In the Aβ measurement shown in Figure 4, we analyzed the hippocampal formations of both sides after bilateral injections of rAAV5 vectors or buffer, because the use of unilateral injection did not provide reliable data. However, this did not allow us to clarify the effect of exogenous neprilysin that was axonally transported to the presynaptic terminals. Therefore, we investigated mutant APP transgenic (Tg2576) (Hsiao et al., 1996) mice after the unilateral injection of rAAV5-NEP vector to demonstrate the significance of axonal transport and presynaptic localization of neprilysin for efficient Aβ degradation. Eight weeks after a unilateral injection of rAAV-NEP vector, the Aβ levels in the ipsilateral hippocampal formation of Tg2576 mice were significantly reduced, particularly in the soluble fraction (50 and 40% reduction for $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$, respectively) (Fig. 5*A*), compared with those after injection of rAAV-NEPinactive. In the contralateral hippocampal formation (Fig. 5*B*), Aβ levels in the soluble fraction were reduced to a comparable level to the ipsilateral side (33 and 28% reduction for $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$, respectively), although the increase in neprilysin activity in the rAAV-NEP-injected group was 2.6-fold and much lower than the increase in the ipsilateral side, which amounted to 20-fold relative to the negative controls (Fig. 5*C*). This result suggests that a small increase in neprilysin activity, as observed at the contralateral side, may be enough for substantial removal of soluble Aβ. The Aβ levels and neprilysin activities of the rAAV-NEPinactive group remained unchanged between the ipsilateral and the contralateral sides (Fig. 5*A,B*). In addition, we observed no marked change in either Aβ levels or neprilysin activity in the cerebral cortices of both sides, to which no exogenous neprilysin or its mutant was found

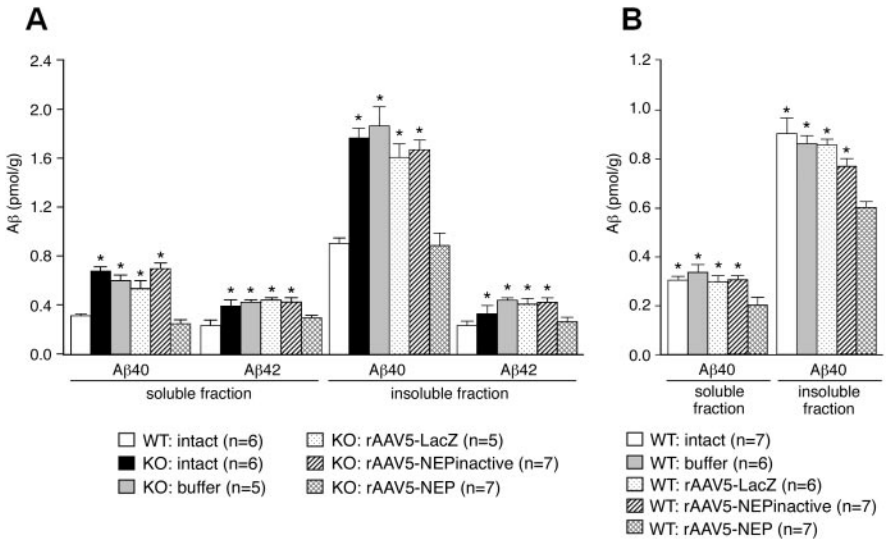

Figure 4. Reduction in intrahippocampal Aβ levels in neprilysin-deficient and wild-type mice after gene transfer. *A*, Aβ levels in both soluble and insoluble fractions of bilateral hippocampal formations were determined by sandwich ELISA 4 weeks after bilateral injection of rAAV-*LacZ* (1.3×10^{10} genome copies), rAAV-NEPinactive (1.3×10^{10}), rAAV-NEP (1.3×10^{10}), or buffer into the hippocampal formations of 10-week-old *neprilysin* $^{-/-}$ mice. Each column with a bar represents the mean ± SE of 5–7 mice. *B*, $A\beta_{x\text{-}40}$ levels were determined 4 weeks after bilateral injection of rAAV vectors or buffer into the hippocampal formations of 10-week-old *neprilysin* $^{+/+}$ mice under the same conditions as for *A*. The amount of amyloid precursor protein in all groups remained unchanged as analyzed by Western blotting (data not shown). Each column with a bar represents the mean ± SE of 6 or 7 mice. *$p < 0.05$, significantly different from rAAV-NEP-injected group.

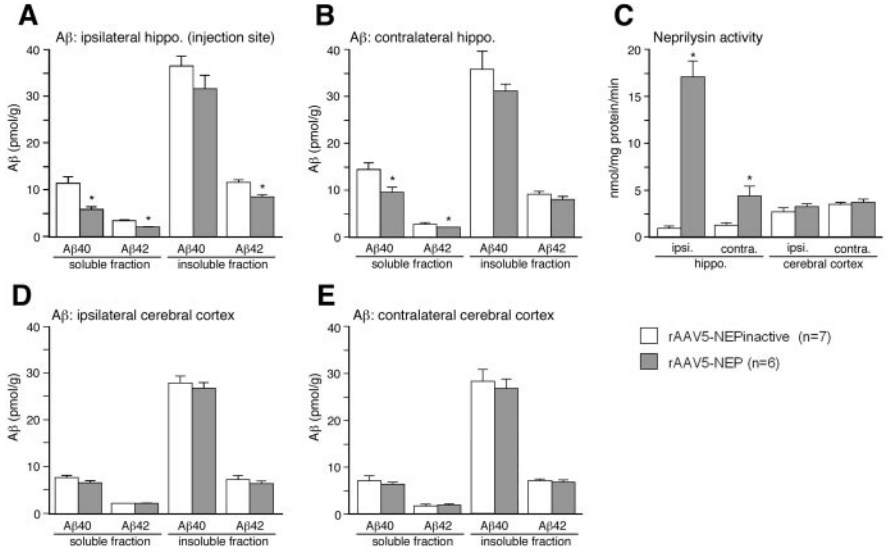

Figure 5. Changes in Aβ levels or endopeptidase activity in young Tg2576 mice after neprilysin gene transfer. Aβ levels in both soluble and insoluble fractions (*A, B, D, E*) and endopeptidase activities (*C*) of the ipsilateral (*A*) and contralateral (*B*) hippocampal formations, and the ipsilateral (*D*) and contralateral and (*E*) cerebral cortices were determined 8 weeks after unilateral injection of rAAV-NEPinactive (open column) and rAAV-NEP (shadowed column) (1.3×10^{10} genome copies) into the hippocampal formation of 10-week-old Tg2576 mice, respectively. Each column with a bar represents the mean ± SE of 6 or 7 mice. *$p < 0.05$, significantly different from rAAV-NEPinactive-injected group.

to be transported (Fig. 5*D*,*E*). These findings indicate that neprilysin degrades soluble forms of Aβ with high efficiency.

Deceleration of Aβ deposition in aged mutant APP transgenic mouse brain

We examined whether neprilysin gene transfer decelerated amyloid deposition in aged Tg2576 mouse brain. Brain sections were immunostained with anti-Aβ antibody 12 weeks after unilateral viral injection into the hippocampal formation of 18-month-old Tg2576 mice. The Aβ deposition in the ipsilateral hippocampal formation, especially the stratum radiata, was remarkably decreased by neprilysin expressed after injection of rAAV-NEP, compared with that after injection of rAAV-NEPinactive (Fig. 6*A*,*B*). Quantitative assessment revealed that Aβ deposits in the entire hippocampal formation and the stratum radiata of the ipsilateral side after injection of rAAV-NEP were reduced by 25 and 49%, respectively, compared with those after rAAV-NEPinactive (Fig. 6*C*). Aβ loads in the cerebral cortices were similar in the rAAV-NEP and rAAV-NEPinactive-injected groups (Fig. 6*C*).

We further immunostained brain sections from Tg2576 mice that had received neprilysin gene transfer to analyze the spatial association of neprilysin with Aβ plaques (Fig. 7*A–E*). Robust synaptic components displaying immunoreactivity with anti-SNAP-25 antibody were detected around amyloid plaques, and neprilysin-positive immunoreactivity corresponded well with the SNAP-25-positive components. Neprilysin and SNAP-25 immunoreactivity frequently overlapped with Aβ immunoreactivity inside Aβ plaques, as shown by triple labeling. Such colocalization was seen in many amyloid plaques. Thus, immunoreactive neprilysin was presynaptic or derived from presynaptic components. On the other hand, we detected GFAP–positive astrocytes surrounding Aβ plaques, but they did not colocalize with neprilysin or GFAP (data not shown).

Discussion

Neprilysin is supposed to be involved in the catabolism of various neuropeptides, such as enkephalin, substance P, somatostatin, and kinins (Turner, 1998), and is currently regarded as a major Aβ-degrading enzyme (Iwata et al., 2000, 2001; Takaki et al., 2000; Shirotani et al., 2001; Carson and Turner, 2002; Saido, 2003; Saido and Nakahara, 2003; Saito et al., 2003). In the present study, we expressed neprilysin in the hippocampal formation of mice using rAAV-based vectors and successfully reduced Aβ levels in that region; neprilysin gene transfer abolished the increase in Aβ levels in neprilysin-deficient mice, reduced Aβ in young mutant APP transgenic mice and was also effective in the deceleration of Aβ deposition in the brain of aged mutant APP transgenic mice. Recently, in addition to neprilysin, several peptidases, such as endothelin-converting enzymes (ECEs) 1, 2, and insulin-degrading enzyme (IDE), have been identified as Aβ-degrading enzymes by reverse genetics studies (Eckman et al., 2003; Farris et al., 2003; Miller et al., 2003). Although these peptidases are likely to contribute to overall Aβ clearance in brain by complementing each other in a subcellular, cell type-

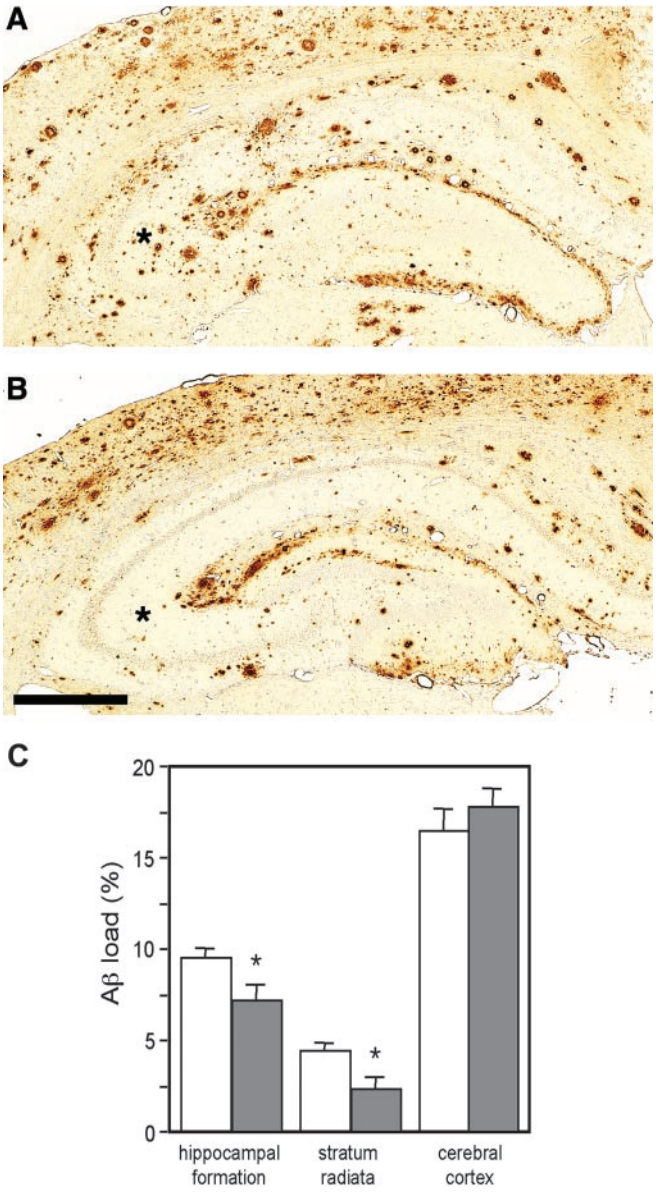

Figure 6. Deceleration of Aβ deposition in aged Tg2576 brains by neprilysin gene transfer. Twelve weeks after unilateral injection of rAAV-NEPinactive (*A*) or rAAV-NEP (*B*) (1.3×10^{10} genome copies) into the hippocampal formation of 18-month-old Tg2576 mice, brain sections were immunostained with anti-Aβ antibody, which recognized the C-terminal of Aβ42. Asterisks, stratum radiata. Scale bar, 500 μm. *C*, Aβ deposits in various areas of sections from the rAAV-NEPinactive-injected (shadowed column, $n = 7$) or rAAV-NEP-injected (open column, $n = 5$) brains were quantitated as described in Materials and Methods. Each column with a bar represents the mean ± SE. *$p < 0.05$, significantly different from rAAV-NEPinactive-injected group.

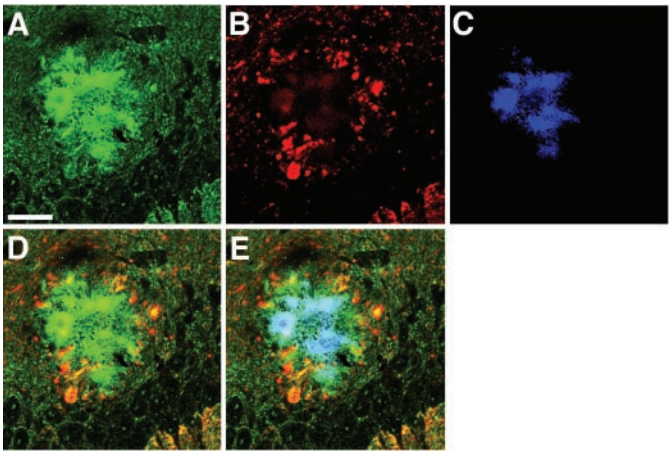

Figure 7. Detection of immunoreactive neprilysin from neuritic components surrounding amyloid plaques. Sections from rAAV-NEPinactive-injected brains of Tg2576 mice were immunolabeled with anti-SNAP-25 (*A*; green), anti-neprilysin (*B*; red), and anti-Aβ antibodies (*C*; blue). *D*, Merged images of sections labeled with anti-SNAP-25 and anti-neprilysin antibodies. *E*, Merged images of triply labeled section. Experimental conditions are the same as in the case of Figure 6. Scale bar, 20 μm.

and/or brain region-specific manner, the brain Aβ-elevating effect of neprilysin deficiency is greater than that of a deficiency of any other known Aβ-degrading enzyme (Iwata et al., 2001; Eckman et al., 2003; Farris et al., 2003; Miller et al., 2003; Saido and Nakahara, 2003; Saito et al., 2003). Neprilysin, a membrane-bound zinc metallopeptidase with its active site at the lumen side (Turner, 1998; Carson and Turner, 2002; Saido and Nakahara, 2003), is implicated in degradation of both intracellular and extracellular (membrane-associated) Aβ (Hama et al., 2001). The neprilysin-dependent degradation of exogenously administered synthetic $A\beta_{1-42}$ peptide *in vivo* (Iwata et al., 2000) demonstrates that neprilysin plays a larger role in the degradation of Aβ after secretion. In addition, because neprilysin was observed at the presynapses and on/around axons by double immunostaining with presynaptic markers (Fukami et al., 2002) or by subcellular fractionation and electron microscopic immunocytochemistry in brain (Barnes et al., 1988, 1992), it may play a key role in Aβ catabolism at and around neuronal synapses. The present report provides direct evidence for this.

In an earlier study of neprilysin gene transfer using a lentiviral vector, neprilysin expressed in the infected neurons was detected in cell bodies in most cases, as shown by immunostaining of the hippocampal formation (Marr et al., 2003). This is quite different from the results obtained with our vector; we found that that neprilysin was localized at the presynaptic sites, but not in the cell bodies. Therefore, Marr et al. (2003) probably observed mainly the intracellular degradation process of Aβ by neprilysin. Application of the rAAV vector-mediated gene transfer system, however, enabled us to evaluate catabolism of synaptic cleft-associated, presynaptic membrane-associated, and intravesicular Aβ by neprilysin around synaptic sites. Furthermore, in contrast to the study using the lentiviral vector, our gene transfer system produced remarkable effects on Aβ degradation not only on the injection side, but also in the contralateral hippocampus, suggesting that the rAAV-NEP vector offers substantial advantages for extensive reduction of Aβ levels. It is noteworthy that the relatively small increase of neprilysin activity in the contralateral hippocampal formation was sufficient to yield a significant reduction of Aβ. Our approach seems to enable widespread and efficient promotion of Aβ degradation, because exogenous neprilysin was axonally transported from the ipsilateral to the contralateral hippocampal formation and also to the intrahippocampal formation via appropriate neuronal circuits. Such axonal transport also occurs to the lateral entorhinal cortex-outer molecular layer of the dentate gyrus projection via the perforant path. Analysis of the hippocampal formation contralateral to the injection side in mutant APP transgenic mice in our work supported a possible role of neprilysin in Aβ degradation at the presynaptic sites, whereas Marr et al. (2003) examined the effects of neprilysin gene transfer on amyloid deposition in the ipsilateral hippocampal formation relative to the contralateral side, based on Aβ immunostaining. APP also undergoes axonal transport to

presynaptic terminals (Buxbaum et al., 1998), during which Aβ is generated through sequential cleavage by β- and γ-secretases (Hardy and Selkoe, 2002). Aβ released from presynaptic sites contributes to extracellular amyloid deposits, as demonstrated by *in vivo* experiments (Lazarov et al., 2002; Sheng et al., 2002). This process provides a rationale for the effective Aβ reduction by axonally transported rAAV-derived neprilysin. Although earlier studies found that dystrophic neurites in senile plaques were immunostained positively for neprilysin in AD brains and in brain of another strain of mutant APP-transgenic mouse (Akiyama et al., 2001; Fukami et al., 2002), neprilysin gene transfer more clearly visualized dystrophic neurites and amyloid plaques containing immunoreactive neprilysin. Thus, the sites for release and degradation of Aβ seem to be closely related to each other, and synaptic localization of neprilysin would be of particular importance for a connection with AD pathology.

We found that neprilysin gene transfer reduced both soluble and insoluble Aβ to similar extents in wild-type and neprilysin-deficient mouse hippocampal formations, whereas it reduced soluble Aβ more effectively than insoluble Aβ in the case of either the ipsilateral or the contralateral side of mutant APP transgenic mice. These results indicate that neprilysin preferentially attacks soluble Aβ, when Aβ is present at higher levels. Recent evidence suggests that soluble forms of Aβ are highly neurotoxic (Kuo et al., 1996; Lambert et al., 1998; Walsh et al., 1999; Hartley et al., 1999; Walsh et al., 2002), and may be more directly responsible for the pathological changes of AD, such as synaptic dysfunction and subsequent neuronal death (Kuo et al., 1996). The soluble forms of Aβ are composed of monomeric, oligomeric, and protofibrillar forms. Because neprilysin only degrades peptides smaller than 5 kDa, based on the size of its catalytic cavity (~20 Å) (Turner, 1998; Oefner et al., 2000), it is believed to degrade monomeric Aβ. However, there is a dynamic equilibrium between monomeric and oligomeric Aβ and further between oligomeric and protofibrillar Aβ (Walsh et al., 1999), and the deceleration of Aβ deposition by neprilysin gene transfer in aged mutant APP transgenic mice suggests that extensive removal of monomeric Aβ may reduce the levels of all of the soluble forms, and even insoluble forms, depending on the duration of the infection.

Synaptic dysfunction is indeed a typical and early function-related event in the AD cascade (Terry et al., 1991; Sze et al., 1997; Selkoe, 2002; Hardy and Selkoe, 2002), and is associated with a locally enhanced concentration of Aβ at the presynaptic terminals and inside synaptic vesicles in the AD brain. An increase in soluble Aβ levels rather than Aβ plaque load appears to cause such synaptotoxicity. Levels of soluble Aβ rather than insoluble Aβ are well correlated with synaptic changes and neuronal loss in AD brains (Lue et al., 1999; McLean et al., 1999). In several lines of mutant APP transgenic mice, structural changes in presynaptic terminals or neuronal loss are correlated with an increase in Aβ concentration in the brain, but not with plaque load (Hsia et al., 1999; Mucke et al., 2000; Buttini et al., 2002). Recent studies have indicated that particular forms of soluble Aβ, such as oligomeric or protofibrillar forms, cause dysfunction or modification of synaptic transmission and/or long-term potentiation *in vivo* (Walsh et al., 2002; Wang et al., 2002). The presence of neprilysin at the presynaptic sites would contribute to removal of a toxic form of Aβ through degrading monomeric Aβ, like clearance of neurotransmitters, in the extracellular space close to synapses, thereby eventually decelerating AD pathology.

The present data also suggest the presence of Aβ, either in particular compartments or of specific forms, which neprilysin cannot attack. In fact, the degree of Aβ removal in wild-type mice by neprilysin gene transfer was relatively low compared with that in neprilysin-deficient mice, and the degree of soluble Aβ removal by neprilysin gene transfer was similar in the ipsilateral and contralateral hippocampal formations of young APP transgenic mice, although the increases in neprilysin activity differed by [~10-fold. Because ECEs are supposed to degrade predominantly intracellular Aβ, which is present on the lumen side of a secreting vesicle, based on the acidic pH optima (Carson and Turner, 2002; Eckman et al., 2003; Saido and Nakahara, 2003), ECEs, as well as IDE, may be involved in degradation of Aβ in particular compartments, in which neprilysin does not play a major role.

In conclusion, we have demonstrated that presynaptic neprilysin efficiently and extensively degrades Aβ and decelerates Aβ deposition *in vivo*. These results indicate that upregulation of neprilysin activity at sites where a reduction in the neprilysin level may be caused by aging, might protect the synaptic function of AD-vulnerable neuronal circuits from Aβ pathology via a reduction of Aβ levels. We suggest that upregulation of neprilysin activity is a promising strategy for therapy and prevention of AD.

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