

Peripherally Expressed Neprilysin Reduces Brain Amyloid Burden: A Novel Approach for Treating Alzheimer's Disease

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A number of therapeutic strategies for treating Alzheimer's disease have focused on reducing amyloid burden in the brain. Among these approaches, the expression of amyloid β peptide ($A\beta$)-degrading enzymes in the brain has been shown to be effective but to date not practical for treating patients. We report here a novel strategy for lowering amyloid burden in the brain by peripherally expressing the $A\beta$ -degrading enzyme neprilysin on leukocytes in the 3xTg-AD mouse model of Alzheimer's disease. Through transplantation of lentivirus-transduced bone marrow cells, the $A\beta$ -degrading protease neprilysin was expressed on the surface of leukocytes. This peripheral neprilysin reduced soluble brain $A\beta$ peptide levels by ~30% and lowered the accumulation of amyloid β peptides by 50–60% when transplantation was performed at both young and early adult age. In addition, peripheral neprilysin expression reduced amyloid-dependent performance deficits as measured by the Morris water maze. Unlike other methods designed to lower $A\beta$ levels in blood, which cause a net increase in peptide, neprilysin expression results in the catabolism of $A\beta$ to small, innocuous peptide fragments. These findings demonstrate that peripherally expressed neprilysin, and likely other $A\beta$ -degrading enzymes, has the potential to be utilized as a therapeutic approach to prevent and treat Alzheimer's disease and suggest that this approach should be explored further. © 2008 Wiley-Liss, Inc.

Key words: gene therapy; peptidases; amyloid clearance

Alzheimer's disease (AD) is the most common cause of dementia among the elderly. The accumulation of amyloid β peptides ($A\beta$) and their subsequent aggregation is a fundamental event in AD (Selkoe, 1994). Current therapeutic approaches for treating AD have

included strategies aimed at preventing the formation and/or accumulation of $A\beta$ and reducing its cytotoxic and proinflammatory actions. Recent interest has emerged in utilizing $A\beta$ clearance as a target for treating AD. Although there has been considerable effort in this area, a successful approach has yet to emerge. Immunizing with synthetic $A\beta$ (Schenk et al., 1999; Janus et al., 2000; Morgan et al., 2000; Weiner et al., 2000; Das et al., 2001) or passive administration of anti- $A\beta$ antibodies (Bard et al., 2000; DeMattos et al., 2001, 2002) reduced behavioral impairment and plaque formation in mouse models of AD. These results led to a clinical trial that initially appeared to produce beneficial results (Hock et al., 2003). However, a phase II clinical trial was aborted after several patients developed encephalitis (Greenberg et al., 2003).

A finding that emerged from immunological studies is that there is an efflux of $A\beta$ from the brain into

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plasma (DeMattos et al., 2001, 2002), which could be trapped by anti-A β antibodies producing a peripheral “sink effect.” Similarly, adding A β -binding compounds to the plasma of PS/APP transgenic mice lowered brain A β levels by 50% or more (Matsuoka et al., 2003). Additionally, peripheral administration of a soluble form of the receptor for advanced glycation end products (sRAGE), which bind A β , led to a lowering of brain A β (Deane et al., 2003). However, a problem associated with immunotherapy and A β -binding compounds is the inability to clear the A β directly. In addition, passive immunization increases the incidence of microhemorrhage (Pfeifer et al., 2002; Wilcock et al., 2004; Racke et al., 2005) associated with increased vascular amyloid and cerebral amyloid angiopathy (Wilcock et al., 2004).

We have explored altering brain-periphery dynamics by peripheral expression of the peptidase neprilysin (NEP) as a way to lower plasma A β levels and as a consequence reduce brain A β . NEP is zinc metallopeptidase localized to the plasma membrane, placing it in an ideal location to hydrolyze extracellular A β . NEP has been shown to play a major role in the clearance of A β in the brain (for review see Hersh and Rodgers, 2008). Over-expressing NEP in the brain of hAPP transgenic mice caused a significant reduction of preformed amyloid deposits (Marr et al., 2003; Hemming et al., 2007). To test the effect of peripheral expression, NEP and an inactive NEP mutant (NEPx) were expressed on hematopoietic cells, and their effect on brain A β burden was determined in the 3xTg-AD (Oddo et al., 2003a,b) mouse model of AD.

The results of this study show that expression of NEP in peripheral blood reduces brain amyloid burden. This finding demonstrates for the first time the potential for using peripherally expressed NEP and other A β -degrading enzymes in the prevention and treatment of AD.

MATERIALS AND METHODS

Animals

All experimental procedures and protocols involving animals were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and performed in accordance with University guidelines. Bone marrow donor mice (C57Bl6/CD45.1) were from Jackson Laboratory (Bar Harbor, ME). The 3xTg-AD mice (129/C57Bl6/CD45.2) contain, in addition to a PS1M146V knock-in gene, a human APP695 gene carrying the Swedish double mutation and a human four-repeat tau harboring the P30L mutation, both under the control of the Thy1.2 gene cassette. This transgenic AD mouse model develops both plaque and tangle pathology in AD-relevant brain regions (Oddo et al., 2003a,b).

Primary Antibodies

PE-conjugated anti-mouse CD45.1 mAb (clone A20) was from BD PharMingen (San Jose, CA). Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human NEP mAb (clone CLB-CALLA/1,4F9) was from Research Diagnostics

Inc. (Flanders, NJ), and mouse anti-human NEP mAb (clone SS2/36) was from Dako (Carpinteria, CA). Mouse anti-human A β mAb (clone 6E10, 4G8) was from Signet Laboratories (Dedham, MA); goat anti-human APP (R8666) was kindly provided by Dr. Maria Kounnas of Torrey Pines Pharmaceutical Inc. (La Jolla, CA). Mouse anti-human A β mAb Ab9 (McGowan et al., 2005), and anti-human A β 42 M26-2.1.3 (Murphy et al., 2007) have been described elsewhere.

Production of Lentiviral Vectors

Lentivirus constructs were prepared as previously described (Marr et al., 2003), with virus produced in HEK293FT cells (Invitrogen, Carlsbad, CA) using a four-plasmid transfection system (Dull et al., 1998; Miyoshi et al., 1998). Lentivirus vectors expressing the human NEP gene and an inactive E585V point mutant of human NEP (NEPx) were under control of the cytomegalovirus (CMV) promoter. The NEP E585V mutant exhibits less than 0.25% of the activity of the wild-type enzyme (Devault et al., 1988). A GFP-NEP fusion protein was generated by insertion of a wild-type hNEP cDNA into the pEGFP-C3 plasmid (Clontech, Mountain View, CA). The cDNA was then cut with AgeI and ApaI and subcloned into the lentiviral expression vector pCSC-SP-PW for lentivirus production.

Transduction of Bone Marrow Cells and Bone Marrow Transplantation

Bone marrow was harvested from 8-week-old C57Bl6/CD45.1 mice by flushing femurs and tibiae. Bone marrow cells were prestimulated overnight with Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) containing 10 ng/ml of mIL3, mIL6, and hFlt3 ligand and 50 ng/ml hSCF (R&D, Minneapolis, MN) and then transduced with lentiviral vectors twice at 24-hr intervals at a multiplicity of infection of 100. On day 3, cells were washed twice with PBS, and transduction efficiency was measured by FACS analysis. Cells (10^6 cells/mouse) were injected into the tail vein of recipient 3xTg-AD mice that had been sublethally irradiated (6 Gy) to allow engraftment of the transplanted hematopoietic stem cells.

Leukocyte Separation

Leukocytes were isolated by the dextran sedimentation method (Bretz and Baggiolini, 1974; Klempner and Gallin, 1978). Briefly, equal volumes of PBS, 2% dextran 200, and blood were mixed and then incubated at 37°C without shaking for 20 min. The upper leukocyte-containing layer was collected, and contaminating erythrocytes were lysed with 155 mM NH₄Cl, 0.13 mM EDTA, and 12 mM NaHCO₃.

Leukocyte Counts

Total leukocyte counts were determined using the Heska CBC-Diff Veterinary Hematology System (Heska Corporation, Loveland, CO). Cellular differentials were determined from blood smear slides stained with Wright stain (LeukoStat; Fisher Scientific, Pittsburg, PA) and viewed under

the microscope (Doeing et al., 2003). Differentials were based on a count of 100 viewed cells.

Fluorescence-Activated Cell Sorting

Blood was collected via orbital sinus venipuncture, and erythrocytes and leukocytes were separated by dextran sedimentation as described above. After washing three times with FACS buffer (0.5% FBS in DPBS), 1 μ l of either erythrocytes or leukocytes from 100 μ l of blood were stained with fluorescein-conjugated anti-NEP or phycoerythrin (PE)-conjugated anti-CD45.1. For dual-color FACS analysis of GFP-hNEP, cells were stained with mouse anti-hNEP and then PE-conjugated anti-mouse IgG. The GFP signal was directly visualized. Analysis was performed on a FACSCalibur flow cytometer in CellQuest software.

In Vitro A β Degradation Assays

Leukocytes were separated from mice 2 months after transplantation of bone marrow cells and washed three times with PBS. Cells (10^7) were incubated with DMEM containing A β 1–40 (2 ng/ml; Biosource International, Camarillo, CA) at 37°C for 4 hr. The medium was gently centrifuged to avoid cell contamination and then assayed for A β 1–40 by sandwich ELISA. The rate of degradation was calculated based on the decrease of the A β 1–40 concentration in the media.

Brain A β Peptide Extraction and Quantification

Four or six months after transplantation, mice were killed by CO₂ narcosis and their brains removed and placed on ice. Brains were divided by midsagittal dissection, and one hemibrain was used for biochemical analysis. Briefly, each hemibrain was homogenized (150 mg wet wt/ml) in Radioimmunoprecipitation Assay Buffer (RIPA; 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 \times protein inhibitor cocktail, pH 8.0). The supernatant was collected after centrifugation at 20,000g for 30 min, representing the detergent-soluble fraction. The pellet was then sonicated with 70% formic acid (FA) and centrifuged at 100,000g for 1 hr. The aqueous layer was collected as the detergent-insoluble fraction. Total A β in each extract was determined by sandwich ELISA as described previously (McGowan et al., 2005). Ab9 (anti-A β 1–16) was used as the capture antibody and 4G8 (anti-A β 17–24) as the detection antibody for total A β analysis.

Immunohistochemistry

Cryostat coronal sections (16 μ m) from frozen hemibrains were fixed in 3% paraformaldehyde in PBS. After blocking with PBS containing 0.1% Triton X-100, 0.1% BSA, and 2% normal horse serum for 1 hr at room temperature, sections were incubated overnight at 4°C with antibody M26 for A β or R8666 for APP immunohistochemistry. Sections were washed three times in PBS, incubated in secondary antibody for 1 hr at room temperature, and developed with horse anti-mouse conjugated to peroxidase (1:2,500; Vector Laboratories, Burlingame, CA) and diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA) as substrate for visualization of A β . Adjacent sections were stained with 1% thioflavin-S for

10 min to visualize fibrillar A β . A β immunoreactivities were analyzed with Image-Pro Plus 6.2 software (Media Cybernetics, Silver Spring, MD), and relative A β immunoreactivities were calculated based on those of control mice receiving non-transduced bone marrow cells. Six slices from each mouse brain were analyzed with a 160- μ m interval.

Brain NEP Extraction and Activity Assay

NEP was extracted and activity measured from frozen brain samples as described elsewhere (Huang et al., 2004). Briefly, brain tissue was homogenized in 10 volumes of ice cold 10 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose and a protease inhibitor cocktail (complete, EDTA-free; Roche Diagnostics, Indianapolis, IN) using a Teflon-glass homogenizer. The homogenate was centrifuged first at 1,000g for 20 min and then at 100,000g for 1 hr. The pellet containing NEP was resuspended in Tris-HCl buffer with 0.1% Triton X-100. NEP activity was determined with glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide (Sigma, St. Louis, MO) as substrate (Huang et al., 2004). Reaction mixtures (200 μ l) contained 100 μ M substrate, 25–50 μ g brain membrane fraction, 1 μ g recombinant puromycin-sensitive aminopeptidase (Thompson et al., 2003), and 20 mM MES buffer, pH 6.5. Reactions were initiated by the addition of the membrane fraction and monitored at 37°C at an excitation wavelength of 340 nm and an emission wavelength of 425 nm. Phosphoramidon (50 μ M) and thiorphan (10 μ M), two NEP inhibitors, reduced the observed activity by more than 90%. The specific activity of NEP is expressed as nmol product formed/min/mg protein and represents an average of at least two separate measurements performed in duplicate. Protein concentration was measured with the BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

Behavioral Testing

3xTg-AD mice were behaviorally assessed using an adaptation of the Morris water maze (MWM), with acquisition training beginning 4 months posttransplantation, when the mice were 12 months old (Verbois et al., 2003; Davis et al., 2008). Animals were trained with a submerged platform for 4 days, and each day of testing consisted of four 60-sec trials with a 5-min intertrial interval (ITI). On the fifth day, the platform was removed from the pool, and a 30-sec probe trial was performed. A video camera (Panasonic CCTV camera MV1410) was used to record swimming during training and probe tests. Training data were analyzed by a two-way repeated-measures ANOVA (treatment \times day), and probe data were analyzed by one-way ANOVA, followed by Tukey's multiple-comparisons test.

Blood Pressure Measurements

Systolic blood pressure was measured on conscious, restrained mice at the same time of day, 5 days per week, on a 37°C heated stage using the tail cuff method (Cassis et al., 2005). Mice were subjected to 10 preliminary and 10 recorded measurements. A minimum of five measurements on each mouse was used.

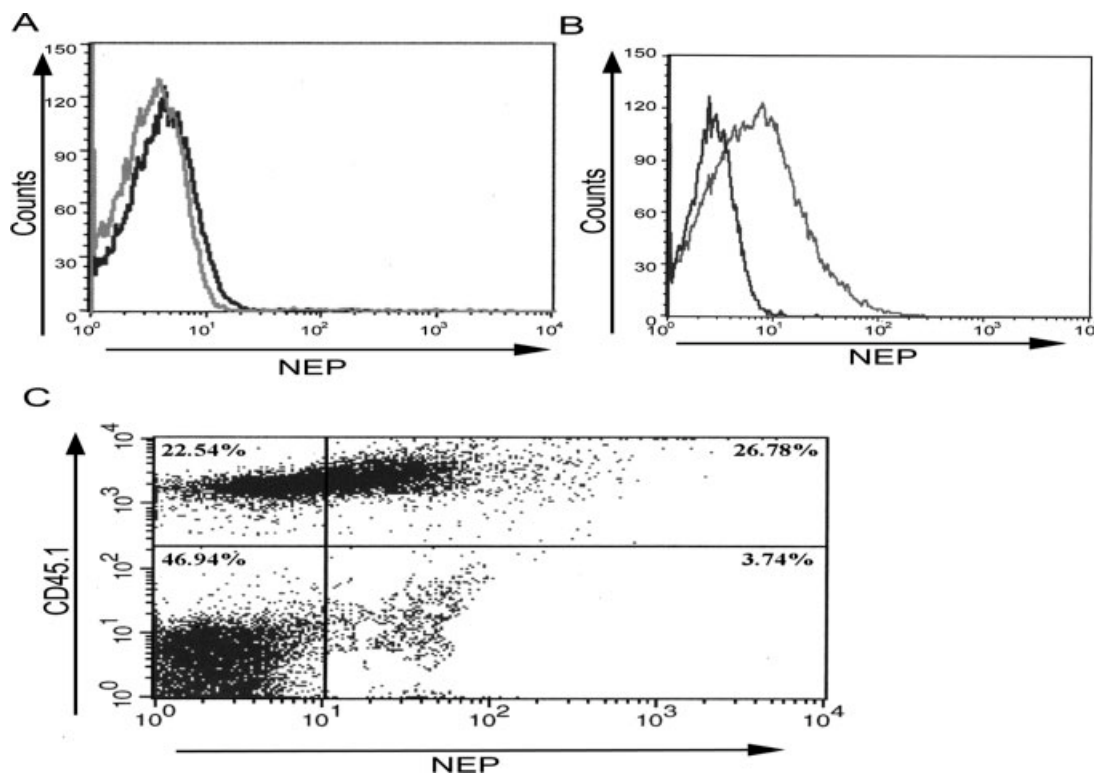


Fig. 1. Bone marrow transduction and transplantation results in peripheral NEP expression on leukocytes, but not on erythrocytes. Bone marrow cells harvested from CD45.1⁺ donor mice were transduced with lentivirus expressing NEP and transplanted into CD45.1⁻ recipient mice. Peripheral blood samples were obtained from recipient mice 4 weeks after transplantation and tested for cell surface NEP expression by FACS analysis using anti-NEP antisera. **A:** FACS analysis of NEP expression on erythrocytes using fluorescein-conjugated anti-NEP. No NEP-positive cells were observed. The black line represents control mice; the gray line represents mice that received NEP lentivirus-transduced bone marrow cells. **B:** FACS analysis of NEP expression on leukocytes using fluorescein-conjugated anti-NEP shows 25–40% NEP-positive cells. The lines are as described

for **A**. **C:** Dual-color FACS analysis of CD45.1 and NEP expression on leukocytes after bone marrow transplantation. Cells were screened for CD45.1 antigen or NEP as described in Materials and Methods. Cells in the upper two quadrants represent cells expressing CD45.1 (~50%), whereas cells in the right two quadrants represent cells expressing NEP. Those cells in the upper right quadrant (~27%) express both CD45.1 and NEP. Thus, ~50% of the recipient mouse's leukocytes express the CD45.1 antigen, and, of these CD45.1⁺ cells, ~50% express NEP. In repeated experiments, we found that 30–60% of the recipient mice's leukocytes express the CD45.1 antigen and that 50–80% of the CD45.1⁺ transplanted cells expressed NEP. The data shown are representative of one mouse from 18 mice involved in three independent experiments.

Statistical Analysis

Data are expressed as the mean \pm SEM and compared using a one-way or two-way ANOVA, followed by Tukey's multiple-comparisons test. Values are considered to be statistically significant at $P < 0.05$.

RESULTS

Bone Marrow Transduction and Transplantation Yields Peripheral NEP Expression

In vivo gene transfer of NEP was accomplished through lentiviral transduction of primary bone marrow cells derived from donor mice (C57Bl6/CD45.1). The efficiency of lentiviral transduction was 50–80% as determined by FACS analysis 24 hr after cell transduction and prior to transplantation. Transduced bone marrow cells (10⁶ cells/mouse) were transplanted into sublethally irra-

diated 2-month-old 3xTg-AD mice (129/C57Bl6/CD45.2). Peripheral blood samples were obtained 4 weeks after transplantation and tested for cell surface NEP expression. Although FACS analysis showed that erythrocytes did not express NEP (Fig. 1A), the leukocytes were found to contain 30–40% NEP-positive cells (Fig. 1B). This result was confirmed by CD45.1 and NEP dual FACS analysis, which showed that ~50% of the recipient mouse's leukocytes express the CD45.1 antigen. Among these CD45.1⁺ cells, 50–80% express NEP (Fig. 1C).

Leukocyte NEP expression gradually declined from 33.1% NEP-positive leukocytes at 1 month after transplantation to ~3% at 4 months after transplantation and remained at this reduced level throughout the experiment. The total amount of NEP present on leukocytes at the time of sacrifice (6 months posttransplantation) corresponded to ~1.6 ng NEP protein. The observed

decline in NEP expression could be due to the induction of antibody against NEP, although we failed to detect such antibodies by ELISA, or might have resulted

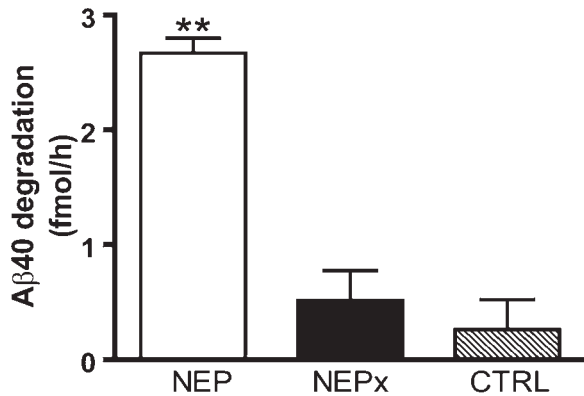


Fig. 2. NEP expressed on leukocytes is enzymatically active and degrades Aβ. Leukocytes were separated from mice 2 months after transplantation with control bone marrow cells, NEP lentivirus-transduced bone marrow cells, or inactive NEPx lentivirus-transduced bone marrow cells; $n = 5$ for each group. Aβ1–40 (2 ng/ml) was then incubated with 10^7 leukocytes from each group at 37°C, and the Aβ remaining was measured by Aβ ELISA. Rates were calculated as the difference between the starting amount of Aβ and the amount of Aβ remaining after 4 hr. Error bars represent SEM (** $P < 0.01$ relative to NEPx and the control).

from a decrease in cytomegalovirus (CMV) immediate early promoter activity (Scharfmann et al., 1991; Kay et al., 1992; Ramezani et al., 2000). However, as noted below, the expression level of NEP achieved was sufficient to lower brain amyloid burden.

To determine further that the peripherally expressed NEP is functional, leukocytes were isolated from mice after transplantation and incubated with Aβ1–40. Approximately 10% of the leukocytes expressed NEP or an inactive form of NEP designated NEPx, but only those leukocytes expressing active NEP were able to cleave Aβ1–40 (Fig. 2).

Leukocyte NEP Prevents Aβ Accumulation in Brain and Ameliorates the Performance Deficits in 3xTg-AD Mice

We first tested whether peripheral NEP could prevent Aβ accumulation in the brains of young 3xTg-AD mice. Bone marrow transplantation was performed at 2 months of age with eight NEP-expressing mice and eight control NEPx-expressing mice. An additional eight control mice received nontransduced bone marrow cells. Six months posttransplantation, the 3xTg-AD mice were sacrificed and their brains removed for immunohistochemical analysis and Aβ quantification by ELISA. Immunohistochemistry using an Aβ42-specific antibody, M26 (Fig. 3A), revealed few Aβ deposits in the

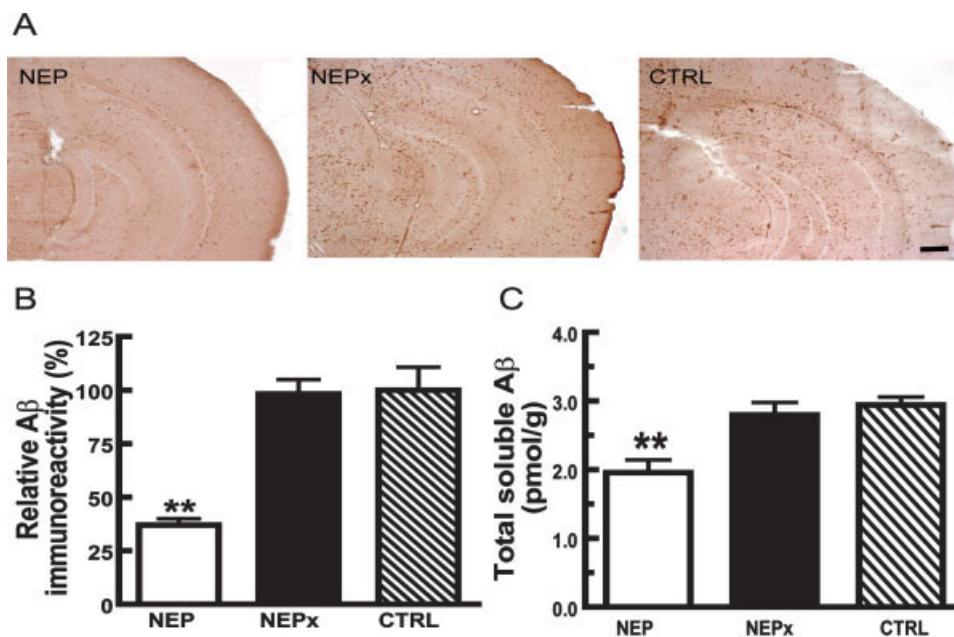


Fig. 3. NEP bone marrow transplantation in young 3xTg-AD mice prevents Aβ accumulation in brain. Bone marrow transplantation was conducted on 2-month-old 3xTg-AD mice, and Aβ levels were determined 6 months posttransplantation, when the mice were 8 months old. **A:** Aβ immunoreactivity in brain sections of mice expressing active NEP compared with mice expressing inactive NEPx and control 3xTg-AD mice. **B:** Quantitative analysis of Aβ immunoreactivity from six slices of each mouse brain reveals an ~60% reduction of Aβ

deposits in the cortex and hippocampus of NEP-expressing mice compared with NEPx-expressing mice. **C:** Detergent-soluble Aβ levels are decreased in the brain of mice expressing active NEP compared with the levels in mice expressing inactive NEPx and control untreated 3xTg-AD mice. Error bars represent SEM ($n = 8$; ** $P < 0.01$ relative to NEPx or the control). Scale bar = 500 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

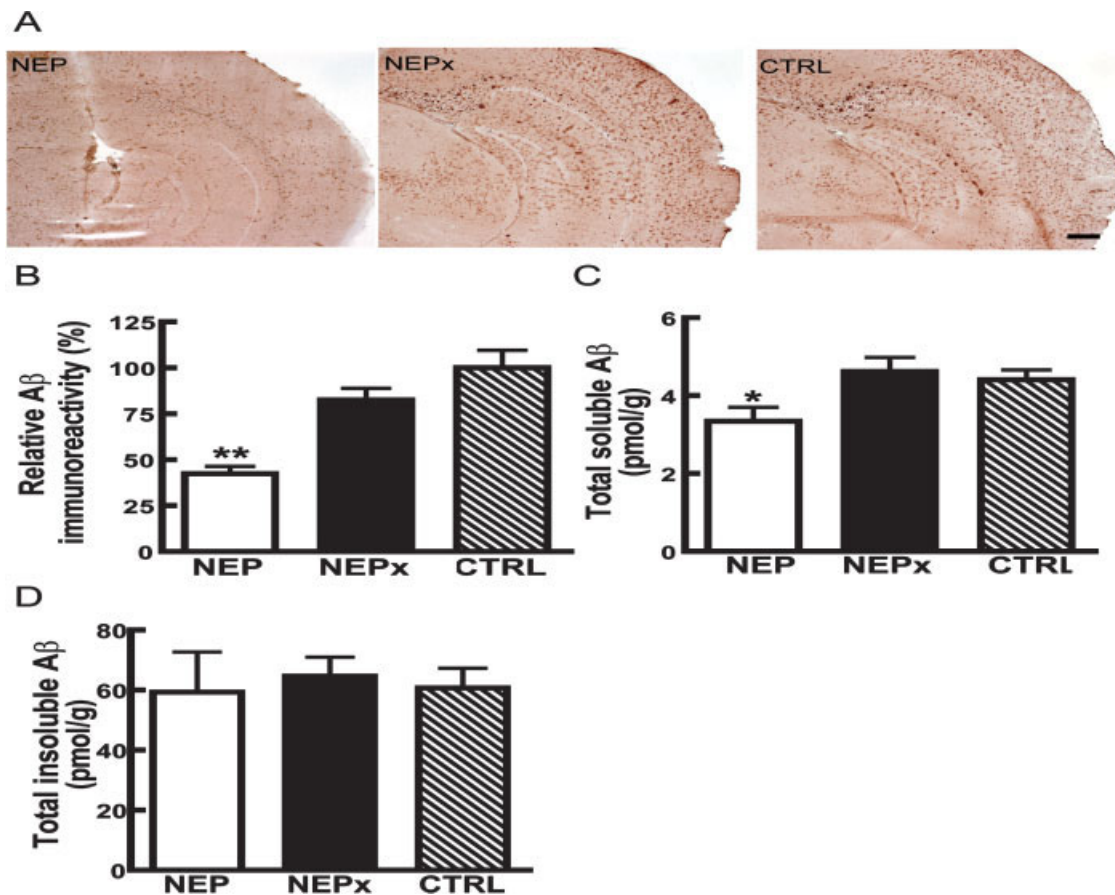


Fig. 4. NEP bone marrow transplantation prevents A β accumulation in the brain of adult 3xTg-AD mice. Bone marrow transplantation was conducted on 8-month-old 3xTg-AD mice, and A β levels were determined 4 months posttransplantation, when the mice were 12 months old. **A:** A β immunoreactivity in the brain of mice expressing active NEP compared with mice expressing inactive NEPx and control mice. **B:** Quantitative analysis of A β immunoreactivity from each mouse brain (six slices per mouse) reveals an ~50% reduction of A β deposited in the cortex and hippocampus of NEP-expressing mice compared with NEPx-expressing mice. **C:** De-

tergent-soluble A β levels are decreased in the brain of mice expressing active NEP compared with the levels in mice expressing inactive NEPx and control untreated 3xTg-AD mice. **D:** Detergent-insoluble A β levels are unaffected in the brain of mice expressing active NEP compared with the levels in mice expressing inactive NEPx and control untreated 3xTg-AD mice. Error bars represent SEM ($n = 10$ for NEP and NEPx expressing mice, 12 for control mice; * $P < 0.05$, ** $P < 0.01$ relative to NEPx or the control). Scale bar = 500 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

hippocampal region of the NEP-expressing mice, although there were considerably more A β deposits in the hippocampus of the inactive NEPx-expressing or control mice. Staining with an antibody specific for the C-terminus of hAPP, antibody R8666, showed no difference in hAPP levels between active NEP-expressing mice and inactive NEPx-expressing mice (data not shown). These results show that the reduction in brain A β burden produced by peripheral NEP is not due to changes in hAPP expression.

Quantitative analysis of A β immunoreactivity was used to determine the A β deposit load (percentage of area covered by A β). In the cerebral cortex and hippocampus A β deposit load was reduced ~60% by NEP (Fig. 3B). There was no significant thioflavin S-positive plaque formation in any group, consistent with the

report that in these mice thioflavin S-positive plaques are not significant at this age (Oddo et al., 2006).

As shown in Figure 3C, those mice peripherally expressing active NEP exhibited a significant reduction in total detergent-soluble A β (RIPA buffer-extractable A β) relative to mice expressing inactive NEPx ($P < 0.01$) or control mice ($P < 0.01$; NEP = 1.96 ± 0.18 pmol soluble A β /g, NEPx = 2.80 ± 0.18 pmol soluble A β /g, and control = 2.94 ± 0.12 pmol soluble A β /g brain). Detergent-insoluble A β was not detectable at this age in any group.

Based on the results obtained with expressing peripheral NEP in young 3xTg-AD mice, we studied a second group of mice in which we conducted bone marrow transplantation on 8-month-old mice and sacrificed these mice 4 months later at 12 months of age ($n = 10$ for NEP- and NEPx-expressing mice, $n = 12$

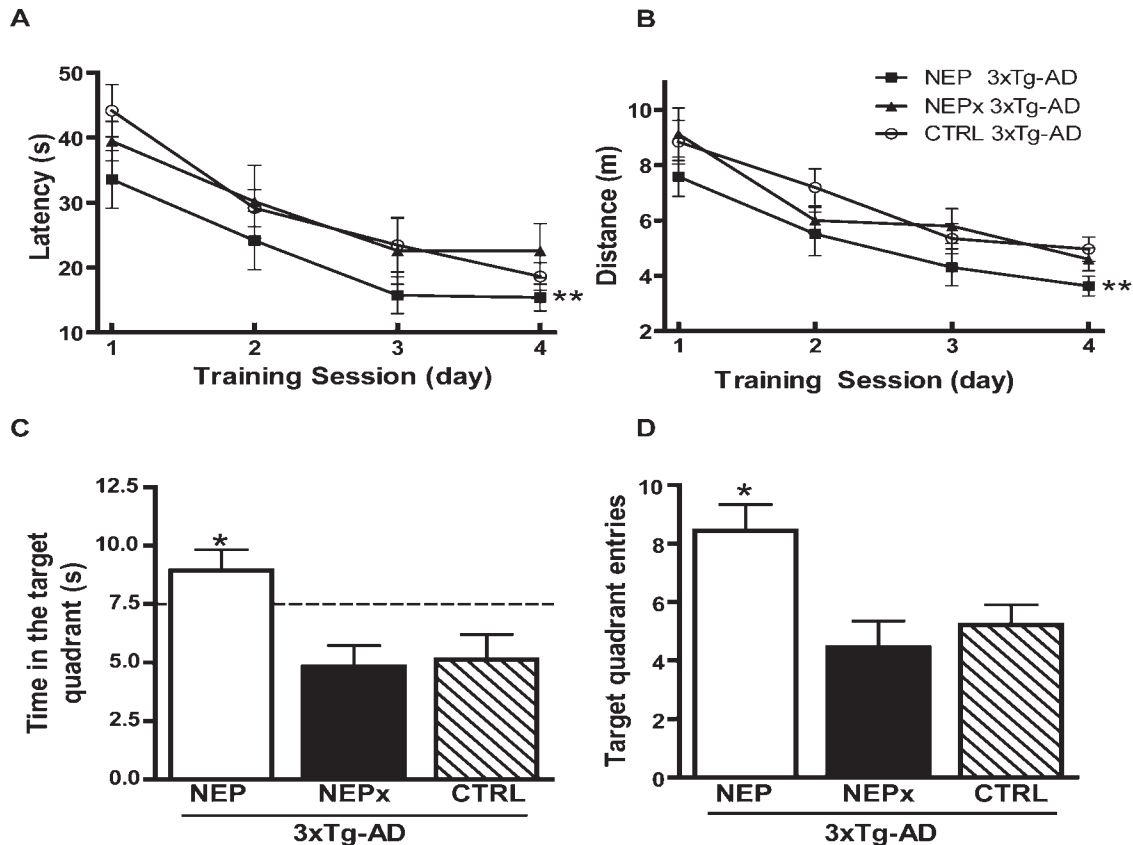


Fig. 5. NEP bone marrow transplantation in young adult 3xTg-AD mice ameliorates performance deficits in 3xTg-AD mice. **A,B:** Spatial learning phase of the Morris water maze assay that includes 4 days of training with the submerged platform. 3xTg-AD mice expressing active NEP showed significantly decreased latency (A) as well as distance traveled (B) to the platform compared with 3xTg-AD mice expressing inactive NEPx or control untreated 3xTg-AD mice. **C,D:** Memory portion of the Morris water maze assay with the platform removed to evaluate the time that animals spent in the

target quadrant (C) and entries into the target quadrant (D) where the platform was located. 3xTg-AD mice expressing active NEP showed significantly increased time spent in the target quadrant (C) and the number of target quadrant entries (D) compared with 3xTg-AD mice expressing inactive NEPx or untreated 3xTg-AD control mice. Horizontal line at 7.5 sec represents random for a 30-sec probe test. Error bars represent SEM ($n = 10$ for NEP- and NEPx-expressing mice and non-Tg mice, $n = 12$ for control mice; * $P < 0.05$, ** $P < 0.01$ relative to NEPx or the control).

for control mice). Immunohistochemical analysis again showed a decrease in brain A β staining in the mice peripherally expressing active NEP compared with mice peripherally expressing inactive NEPx or control mice (Fig. 4A). Quantification revealed that peripheral expression of NEP produced a ~50% reduction in A β immunoreactivity (Fig. 4B) and caused a significant reduction in soluble brain A β (NEP = 3.34 ± 0.36 pmol soluble A β /g, NEPx = 4.62 ± 0.36 pmol soluble A β /g, and control = 4.41 ± 0.25 pmol soluble A β /g brain; Fig. 4C). In contrast, we did not observe a significant change in the insoluble A β levels among these groups (NEP = 59.3 ± 13.4 pmol insoluble A β /g, NEPx = 64.6 ± 6.36 pmol insoluble A β /g, and control = 60.7 ± 6.56 pmol insoluble A β /g brain; Fig. 4D).

These mice were also tested in the Morris water maze before sacrifice to examine their spatial learning and memory and to determine whether the decrease of brain A β produced by peripheral NEP resulted in ameli-

oration of the performance deficits seen in 3xTg-AD mice (Fig. 5). The expression of NEP in the periphery significantly decreased the latency to enter the target quadrant (Fig. 5A) as well as the distance mice traveled to reach the platform (Fig. 5B). These data suggest that peripheral NEP expression improved spatial learning, whereas mice expressing NEPx showed no improvement compared with untreated 3xTg-AD mice. Similar effects were observed on the last day of the test, when the platform was removed from the water maze (the probe trial), as assessed by the measurement of the time animals spent in the target quadrant (Fig. 5C) and the number of entries into the target quadrant (Fig. 5D). The NEP-expressing mice also showed a longer distance swum in the target quadrant and more platform crossing times compared with inactive NEPx-expressing and untreated 3xTg-AD mice. All of these data indicate that peripheral NEP, by preventing A β accumulation in brain, ameliorates performance deficits in 3xTg-AD mice.

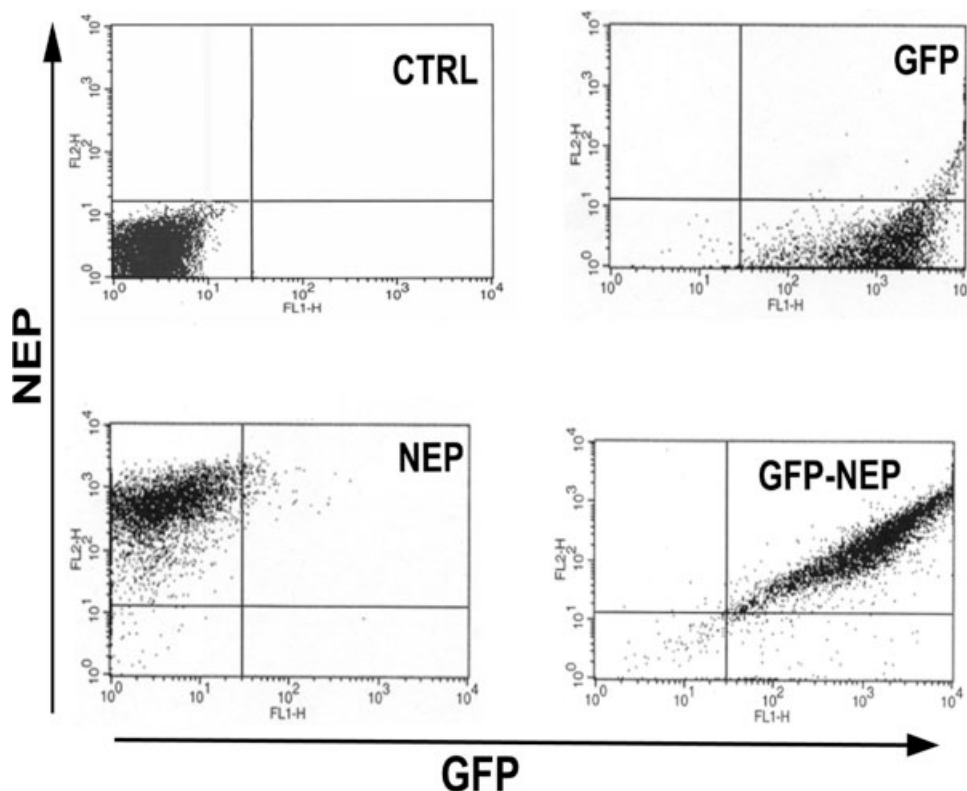


Fig. 6. Addition of a GFP tag to the N-terminus of hNEP does not change the cell membrane location of hNEP. HEK293T cells were transduced with lentivirus carrying NEP, GFP, or GFP-NEP and subjected to FACS analysis. The NEP signal was visualized with PE-conjugated antibody, and the GFP signal was directly visualized. Cells in the lower left quadrant represent cells negative for NEP and GFP; cells in the upper left quadrant are NEP-positive cells identified with

anti-NEP antibody. Cells in the lower right quadrant are directly visualized GFP-positive cells; cells in the upper right quadrant express both NEP and GFP as shown by antibody staining for NEP and direct visualization for GFP. Note that the intensity of GFP signal is well correlated with the intensity of NEP signal and that virtually all cells show both NEP and GFP signals. The data shown are representative of three independent experiments.

The Therapeutic Effect of NEP Bone Marrow Transplantation Is Not Due to the Integration of Bone Marrow-Derived Cells Into the Brain

It has been reported that bone marrow-derived cells can be detected in the brains of mice as early as 3 days after transplantation (Eglitis and Mezey, 1997; Brazelton et al., 2000; Mezey et al., 2000; Simard et al., 2006), although the actual contribution of these cells to total cell number is low. The same phenomenon was also observed in humans transplanted with bone marrow cells (Mezey et al., 2003; Crain et al., 2005). In addition, blood-derived microglia can be attracted to amyloid deposits and can eliminate amyloid deposits by a cell-specific phagocytic mechanism (Quaschnig, 2005).

To evaluate whether significant numbers of bone marrow-derived NEP-expressing cells integrated into brain, we generated a GFP-NEP fusion protein. When 293T cells were transfected with this GFP-NEP, cell surface NEP was detected by FACS analysis (Fig. 6), showing that the GFP tag did not change the cell surface location of NEP. The GFP signal correlated well with

the NEP signal in dual-color FACS analysis, so detecting GFP expression is indicative of NEP expression. In addition, the GFP-NEP fusion protein exhibited the same enzymatic activity as native NEP. When 3xTg-AD mice were transplanted with bone marrow cells transduced by lentivirus carrying the GFP-NEP fusion protein, no GFP signal was detected at 1 month after transplantation in the brain either by directly observing GFP fluorescence or by immunohistochemistry using anti-GFP antibody. On the other hand, ~30% of the leukocytes from these mice expressed cell surface GFP-NEP.

More importantly, when we compared the NEP activity in the brains of active NEP-expressing mice with the activity in inactive NEP \times -expressing mice or with control mice using a highly sensitive fluorogenic assay, no difference was detected (Fig. 7). There was also no difference in brain NEP levels as determined by Western blot analysis (data not shown). In contrast, these mice showed significant leukocyte expression of NEP. These data show that, under our experimental conditions, any NEP-expressing cells that might have migrated

to the brain did not significantly increase brain NEP activity and thus would not have had a significant impact on A β degradation.

NEP Expression Has No Effect on Leukocyte Counts and Differentials

To determine whether NEP expression on leukocytes had an effect on total leukocyte count or their differentials, leukocytes were separated from mice 2 months posttransplantation with control bone marrow cells or NEP or NEPx lentivirus transduced bone marrow cells, and total leukocytes and differential counts were performed. We found no difference between control mice and mice expressing NEP or NEPx. The values obtained were $2.38 \pm 0.32 \times 10^6$ leukocytes/ml with 62.2% lymphocytes and 37.4% neutrophils for control mice, $2.26 \pm$

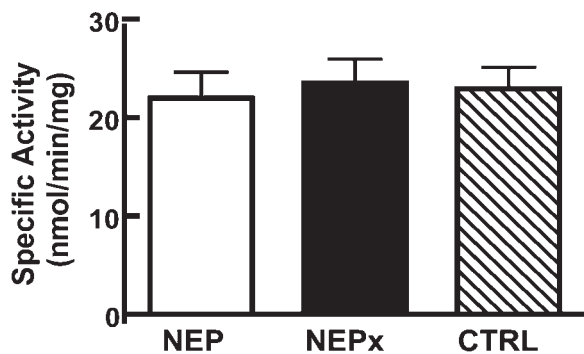


Fig. 7. NEP activity in mouse brain is unaffected after bone marrow transplantation. Extracts were prepared from mouse brain following transplantation with NEP or NEPx lentivirus-transduced bone marrow cells or untransduced control bone marrow cells. NEP activity was determined as described in Materials and Methods. No statistically significant differences are observed in the specific activities of NEP from brains of mice expressing active NEP or inactive NEPx and control 3xTg-AD mice. Error bars represent SEM ($n = 5$).

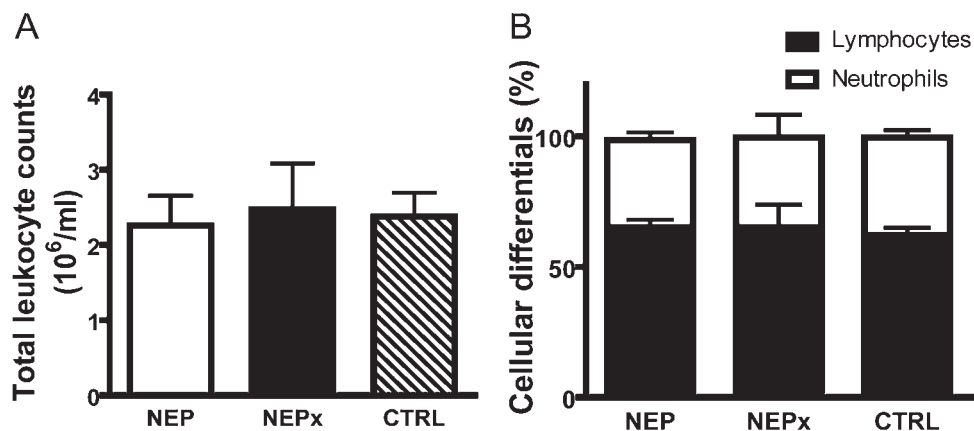


Fig. 8. NEP expression on leukocytes does not affect total and differential leukocyte counts. Blood samples were collected 2 months after transplantation of NEP or NEPx lentivirus-transduced bone marrow cells or untransduced control bone marrow cells and leukocyte counts (A) and their differential (B) determined as described in Materials and Methods. No differences were observed among these three groups. Error bars represent SEM ($n = 5$).

0.40×10^6 leukocytes/ml with 65.2% lymphocytes and 33.4% neutrophils for NEP-expressing mice, and $2.48 \pm 0.61 \times 10^6$ leukocytes/ml with 65.2% lymphocytes and 34.4% neutrophils for NEPx-expressing mice (Fig. 8).

NEP Bone Marrow Transplantation Has No Effect on Systolic Blood Pressure

NEP is involved in the degradation of several vasoactive peptides, including the natriuretic peptides, bradykinin, atrial natriuretic peptide, and angiotensin. Combined NEP and angiotensin-converting enzyme inhibitors, the so-called vasopeptidase inhibitors, are capable of producing vasodilatation by inhibiting the production of angiotensin II and the degradation of natriuretic peptides and bradykinin. This would result in a decrease in peripheral vascular resistance and an increase in blood pressure (Shibata et al., 2000; Weber, 2001). NEP degradation of these peptides occurs primarily in the liver, not in plasma. However, to determine whether the expression of NEP on leukocytes might affect peptide levels, we conducted a functional assay in which we directly determined whether the NEP in peripheral blood affected blood pressure. We measured systolic blood pressure (SBP) of mice receiving control bone marrow cells or bone marrow cells expressing NEP or NEPx 1 month after transplantation and again 6 months after transplantation. As shown in Figure 9, the systolic blood pressure of mice expressing NEP or NEPx is the same or slightly lower than control mice, and all are within the normal range. If NEP had significantly lowered vasoactive peptide levels by hydrolysis, blood pressure would have risen. Thus leukocyte NEP did not significantly affect natriuretic peptide levels. It is further noted that, during the course of these studies and at autopsy, we did not observe any general physical or behavioral abnormalities in mice expressing peripheral NEP or inactive NEPx.

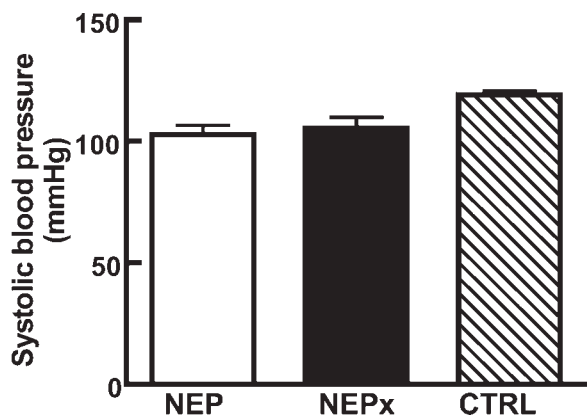


Fig. 9. NEP bone marrow transplantation does not affect systolic blood pressure in 3xTg-AD mice. Systolic blood pressure in 3xTg-AD mice was determined as described in Materials and Methods 1 month following transplantation with NEP or NEPx lentivirus-transduced bone marrow cells or untransduced control bone marrow cells. As shown, peripheral NEP expression does not alter systolic blood pressure. Data are the mean \pm SEM of weekly measurements ($n = 8$).

DISCUSSION

In this study, we have transplanted lentivirus-transduced bone marrow cells as a means to obtain peripheral expression of NEP. The results reported here show that peripherally expressed neprilysin is able to degrade A β and prevent brain A β accumulation in the 3xTg-AD mouse model of Alzheimer's disease. In addition brain A β levels were reduced by \sim 30%, and brain A β deposition decreased by \sim 50–60%.

It has been proposed that A β is transported from the brain to the blood via the LRP-1 receptor and is modulated by the LRP-1 ligands ApoE and α 2-macroglobulin (Shibata et al., 2000). A β can reenter the brain from plasma via the RAGE receptor (Deane et al., 2003). Thus the observed reduction in brain A β accumulation by peripheral NEP is likely a consequence of NEP-dependent hydrolysis of A β in blood preventing A β reentry into the brain and producing a one-way pathway for A β to exit the brain.

We did not detect any adverse effects of expressing peripheral NEP on leukocytes, nor did we observe an effect on blood pressure, showing a lack of effect on natriuretic peptide levels. This likely reflects the fact that liver NEP is primarily responsible for vasoactive peptide degradation and that A β is actively transported into and out of the brain, whereas other bioactive peptides are not actively transported. It is also worth noting that we found that overexpression of NEP in brain does not affect the levels of brain-derived neurotrophic factor, neurotrophin, substance P, or Met-enkephalin (J.B. Rose, E. Rockenstein, A. Adame, M. Mante, L. Crews, L.B. Hersh, F.H. Gage, B. Spencer, R.A. Marr, and E. Masliah, manuscript submitted).

Because the number of leukocytes expressing NEP declined over a 6-month period, the observed decrease

in brain A β accumulation might have resulted from an initial large drop in brain A β produced during the first 1–3 months, when NEP levels were their highest, followed by a rise in A β to the observed levels over the 3-month period when NEP levels declined. Alternatively, the lower sustained levels of leukocyte NEP might have been sufficient to decrease brain A β accumulation.

The exclusion of central nervous system (CNS) effects leads to the conclusion that increasing the peripheral activity of NEP represents a new strategy to treat Alzheimer's disease. An alteration of peripheral/brain A β dynamics represents a therapeutic target of Alzheimer's disease, as suggested by other studies (DeMattos et al., 2001, 2002; Matsuoka et al., 2003). Compared with passive immunization or A β -binding molecules, the strategy of increasing peripheral NEP activity as a treatment for AD has several advantages. First, this strategy is not based on immunomodulation, so it is unlikely to induce an adverse immune response as might occur with immunotherapy. Second, unlike sequestering compounds, NEP degrades A β in situ into innocuous peptide fragments. Thus, instead of increasing the plasma A β concentration, as other sequestering compounds do, NEP directly decreases A β levels in blood. Third, NEP can degrade plasma A β and promote transport of brain-derived A β into blood, thereby reducing A β levels and amyloid deposits in the brain. Similarly, NEP can degrade plasma A β and therefore reduce the transport of peripheral-derived A β back into the brain.

Here we show that peripheral expression of NEP in 3xTg-AD mice can prevent A β accumulation in the brain of both young and adult 3xTg-AD mice. These results establish the principal that peripheral expression of NEP and other A β -degrading peptidases can be developed as an alternative strategy for treating AD. To this end, we have recently shown that erythrocytes have the potential to carry peripheral NEP and other A β -degrading peptidases (Liu et al., 2007). In addition to AD, there are significant implications of this study for using peripheral expression of NEP for treating vascular amyloidosis and other amyloidosis.

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