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Peripherally expressed neprilysin reduces brain amyloid burden: A novel approach for treating Alzheimer's disease

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Abstract

A number of therapeutic strategies for treating Alzheimer's disease have focused on reducing amyloid burden in the brain. Amongst 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 3 $\times$ Tg-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 amyloid β 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 amyloid β peptide levels in blood, which cause a net increase in peptide, neprilysin expression results in the catabolism of the amyloid β peptide to small innocuous peptide fragments. These findings demonstrate that peripherally expressed neprilysin, and likely other amyloid β peptide degrading enzymes, has the potential for being utilized as a therapeutic approach to prevent and treat Alzheimer's disease and suggest this approach should be further explored.

Keywords

Gene therapy; peptidases; amyloid clearance

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Introduction

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 $A\beta$ (Das et al., 2001; Janus et al., 2000; Morgan et al., 2000; Schenk et al., 1999; Weiner et al., 2000) or passive administration with anti- $A\beta$ antibodies (Bard et al., 2000; DeMattos et al., 2001; DeMattos et al., 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 plasma (DeMattos et al., 2001; DeMattos et al., 2002), which could be trapped by anti- $A\beta$ antibodies producing a peripheral "sink effect". Similarly, adding $A\beta$ -binding compounds to the plasma of PS/APP transgenic mice lowered brain $A\beta$ 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 binds $A\beta$, led to a lowering of brain $A\beta$ (Deane et al., 2003). However, a problem associated with immunotherapy and $A\beta$ -binding compounds is the inability to directly clear the $A\beta$. In addition passive immunization increases the incidence of microhemorrhage (Pfeifer et al., 2002; Racke et al., 2005; Wilcock et al., 2004) 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 $A\beta$ levels and as a consequence reduce brain $A\beta$. NEP is zinc metallopeptidase localized to the plasma membrane placing it in an ideal location to hydrolyze extracellular $A\beta$. NEP has been shown to play a major role in the clearance of $A\beta$ in the brain (reviewed in Hersh and Rodgers, 2008). Overexpressing 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 $A\beta$ burden determined in the 3 $\times$ Tg-AD (Oddo et al., 2003a, 2003b) 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 $A\beta$ -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 3 $\times$ Tg-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 P301L 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, 2003b).

Primary antibodies

PE-conjugated anti-mouse CD45.1 mAb (clone A20) was from BD PharMingen (San Jose, CA). 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, while 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.

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, Mountainview, CA). The cDNA was then cut with *Age*I and *Apa*I 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% FCS containing 10 ng/mL of mIL3, mIL6, hFlt3 ligand and 50 ng/mL hSCF (R&D, Minneapolis, MN) and then transduced with lentiviral vectors 2 times at 24-h intervals at a multiplicity of infection of 100. On day 3, cells were washed twice with PBS and transduction efficiency measured by FACS analysis. Cells (10^6 cells/mouse) were injected into the tail vein of recipient 3 $\times$ Tg-AD mice that had been sublethally irradiated (6 Gy) to allow engraftment of the transplanted hematopoietic stem cells.

Leukocytes 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 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 (FACS)

Blood was collected via orbital sinus venipuncture, and erythrocytes and leukocytes separated by dextran sedimentation as described above. After washing 3 times with FACS buffer (0.5% FBS in DPBS), either 1 μ l of 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 using 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 medium containing A β 1–40 (2 ng/mL; Biosource International, Camarillo, CA) at 37°C for 4 hrs. 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 sacrificed by CO₂ narcosis and their brains removed and placed on ice. Brains were divided by midsagittal dissection, and 1 hemibrain used for biochemical analysis. Briefly, each hemibrain was homogenized (150 mg wet wt /mL) in Radio-Immunoprecipitation Assay Buffer (RIPA, 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1x Protein Inhibitor Cocktail, pH = 8.0). The supernatant was collected after centrifugation at 20,000 $\times$ g for 30 min, representing the detergent soluble fraction. The pellet was then sonicated with 70% formic acid (FA) and centrifuged at 100,000 $\times$ g for 1h. The aqueous layer was collected as the detergent insoluble fraction. Total A β in each extract were 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 h 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 h at room temperature, and developed with horse anti-mouse conjugated to peroxidase (1:2500; Vector Laboratories, Burlingame, CA) and DAB (Vector Laboratories, Burlingame, CA) as substrate for visualization of A β . Adjacent sections were stained with 1% thioflavin-S for 10 minutes to visualize fibrillar A β . A β immunoreactivities were analyzed with Image-Pro Plus 6.2 software (Media Cybernetics Inc., 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 (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 1,000 $\times$ g for 20 min and then at 100,000 $\times$ g for 1 h. 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 of brain membrane fraction, 1 μ g of 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) or thiorphan (10 μ M), two NEP inhibitors, reduced the observed activity by more than 90%. The specific activity of NEP is expressed as nmols 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

3×Tg-AD mice were behaviorally assessed using an adaptation of the Morris water maze (MWM) with acquisition training beginning 4 months post-transplantation when the mice were 12-months old (Davis et al., 2008, Verbois et al., 2003). Animals were trained in a submerged platform for 4 days and each day of testing consisted of four 60-s trials with a 5 min inter-trial interval (ITI). On the fifth day, the platform was removed from the pool and a 30-s 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 using a two-way repeated measures analysis of variance (ANOVA) (Treatment $\times$ Day), while probe data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test.

Blood pressure measurements

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

Statistical analysis

Data are expressed as the mean $\pm$ SEM and compared using a one-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. Values are considered to be statistically significant with a $p < 0.05$.

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 h after cell transduction and prior to transplantation. Transduced bone marrow cells (10^6 cells/mouse) were transplanted into sub-lethally irradiated 2-month-old 3×Tg-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. Of 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 (six months post transplantation) corresponded to ~1.6 ng of 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 may have resulted from a decrease in cytomegalovirus (CMV) immediate early promoter activity (Kay et al., 1992; Ramezani et al., 2000; Scharfmann et al., 1991). However, as noted below the expression level of NEP achieved was sufficient to lower brain amyloid burden.

To further determine that the peripherally expressed NEP is functional, leukocytes were isolated from mice after transplantation and incubated with A β 1–40. 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 A β 1–40 (Fig. 2).

Leukocyte NEP prevents A β accumulation in brain and ameliorates the performance deficits in 3 $\times$ Tg-AD mice

We first tested whether peripheral NEP could prevent A β accumulation in the brains of young 3 $\times$ Tg-AD mice. Bone marrow transplantation was performed at 2 months of age with eight NEP expressing mice and eight control inactive NEP (NEPx) expressing mice. An additional eight control mice received non-transduced bone marrow cells. Six months post-transplantation the 3 $\times$ Tg-AD mice were sacrificed and their brains removed for immunohistochemical analysis and A β quantification by ELISA. Immunohistochemistry using an A β 42 specific antibody M26 (Fig. 3A) revealed few A β deposits in the hippocampal region of the NEP-expressing mice, while there were considerably more A β deposits in the hippocampus of the inactive NEPx-expressing or control mice. Staining 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 (percent 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 Fig. 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 3 $\times$ Tg-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 four months later at 12 months of age ($n = 10$ for NEP and NEPx expressing mice, $n = 12$ for control mice). Immunohistochemical analysis again showed a decrease in brain A β staining in the mice peripherally expressing active NEP compared to mice peripherally expressing inactive NEPx or control mice, Fig. 4A. Quantification revealed that peripheral expression of NEP produced a ~50% reduction in A β (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 determine if the decrease of brain A β produced by peripheral NEP resulted in amelioration of the performance deficits seen in 3 $\times$ Tg-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 NEP dependent improved spatial learning, while mice expressing NEPx showed no improvement compared to untreated 3 $\times$ Tg-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 swam in the target quadrant and more platform crossing times compared with inactive NEPx-expressing and untreated 3 $\times$ Tg-AD mice. All of these data indicate that peripheral NEP, by preventing A β accumulation in brain, ameliorates performance deficits in 3 $\times$ Tg-AD mice.

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 (Brazelton et al., 2000; Eglitis and Mezey, 1997; 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 (Crain et al., 2005; Mezey et al., 2003). 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 3 $\times$ Tg-AD mice were transplanted with bone marrow cells transduced by lentivirus carrying the GFP-NEP fusion protein, no GFP signal was detected at one 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 to the activity in inactive NEPx-expressing mice, or to 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 made 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 post transplantation 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 $2.38 \pm$

0.32×10^6 leukocytes/mL with 62.2% lymphocytes and 37.4% neutrophils for control mice; $2.26 \pm 0.40 \times 10^6$ 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 (Weber, 2001, Shibata et al., 2000). NEP degradation of these peptides occurs primarily in the liver, not in plasma. However, to determine if 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 one month after transplantation and again six months after transplantation. As shown in Fig. 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.

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 3 $\times$ Tg-AD mouse model of Alzheimer's disease. In addition brain A β levels were reduced by ~30% and brain A β deposition decreased by 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 alpha2-macroglobulin (Shibata et al., 2000). A β can re-enter 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 re-entry 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 reflecting 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, while other bioactive peptides are not actively transported. It is also worth noting that we found that over expression of NEP in brain does not affect the levels of brain derived neurotrophic factor, neurotrophin, substance P, or Met-enkephalin (JB Rose, E Rockenstein, A Adame, M Mante, L Crews, L B Hersh, FH Gage, B Spencer, RA Marr, and E Masliah – manuscript submitted).

Since the number of leukocytes expressing NEP declined over a six month period the observed decrease in brain A β accumulation may have resulted from an initial large drop in brain A β produced during the first 1–3 months when NEP levels were their highest, and

followed by a rise in A β to the observed levels over the three month period when NEP levels declined. Alternatively, the lower sustained levels of leukocyte NEP may 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; DeMattos et al., 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. Firstly, this strategy is not based on immunomodulation, so it is unlikely to induce an adverse immune response as may occur with immunotherapy. Secondly, unlike sequestering compounds, NEP degrades A β *in situ* into innocuous peptide fragments. Thus, instead of increasing the plasma A β concentration, as do other sequestering compounds, NEP directly decreases A β levels in blood. Thirdly, 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 3 $\times$ Tg-AD mice can prevent A β accumulation in the brain of both young and adult 3 $\times$ Tg-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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Abbreviations

Aβ	amyloid β peptides
AD	Alzheimer's disease
FA	formic acid
FACS	fluorescence activated cell sorting
NEP	neprilysin
nonTg	non-transgenic
RIPA	Radio-Immunoprecipitation Assay Buffer

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Figure 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 are observed.

The black line represents control mice while the grey 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 in 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 methods. Cells in the upper two quadrants represent cells expressing CD45.1 (~50%), while 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 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.

Figure 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. Aβ 1–40 (2 ng/mL) was then incubated with 10^7 leukocytes from each group at 37°C, and the Aβ remaining measured by Aβ ELISA. Rates were calculated as the difference between the starting amount of Aβ and the amount of Aβ remaining after one hour.

Error bars represent SEM (** $p < 0.01$ relative to NEPx and the control).

Figure 3. NEP bone marrow transplantation in young 3 $\times$ Tg-AD mice prevents A β accumulation in brain

Bone marrow transplantation was conducted on 2-month old 3 $\times$ Tg-AD mice and A β levels were determined 6 months post-transplantation when the mice were 8 months old.

(A). A β immunoreactivity in brain sections of mice expressing active NEP compared with mice expressing inactive NEPx and control untreated 3 $\times$ Tg-AD mice. Scale bar, 500 μ m.

(B). Quantitative analysis of A β immunoreactivity from 6 slices of each mouse brain reveals a ~60% reduction of A β deposits in the cortex and hippocampus of NEP expressing mice compared with NEPx expressing mice.

(C). Detergent 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 3 $\times$ Tg-AD mice.

Error bars represent SEM (n = 8) (** p < 0.01 relative to NEPx or the control).

Figure 4. NEP bone marrow transplantation prevents A β accumulation in the brain of adult 3 $\times$ Tg-AD mice

Bone marrow transplantation was conducted on 8-month old 3 $\times$ Tg-AD mice and A β levels were determined 4 months post-transplantation 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. Scale bar, 500 μ m.

(B). Quantitative analysis of A β immunoreactivity from each mouse brain (6 slices per mouse) reveals a ~50% reduction of A β deposited in the cortex and hippocampus of NEP expressing mice compared with NEPx expressing mice.

(C). Detergent 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 3 $\times$ Tg-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 3 $\times$ Tg-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).

Figure 5. NEP bone marrow transplantation in young adult 3xTg-AD mice ameliorates performance deficits in 3xTg-AD mice (A and 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 (B) traveled 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 of animals spent in the target quadrant (C) and entries into target quadrant (D) where the platform was located. 3xTg-AD mice expressing active NEP showed significantly increased time mice spent in 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 seconds represents random for a 30 second probe test.

Error bars represent SEM (n = 10 for NEP and NEPx expressing mice and nonTg mice, 12 for control mice) (** p < 0.01 relative to NEPx or the control).

Figure 6. The 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 while 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 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.

Figure 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 3×Tg-AD mice. Error bars represent SEM (n = 5).

Figure 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 ($n = 5$).

Figure 9. NEP bone marrow transplantation does not affect systolic blood pressure in 3×Tg-AD mice

Systolic blood pressure in 3×Tg-AD mice was determined as described in Materials and methods one 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 ($n = 8$).