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**Brain Neprilysin Activity and Susceptibility to
Transgene-Induced Alzheimer Amyloidosis**

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ABSTRACT

Neprilysin (NEP) is a zinc metalloproteinase that degrades enkephalins, endothelins, and the Alzheimer's disease amyloid β ($A\beta$) peptides. NEP-deficient mice possess increased levels of brain $A\beta^{1-40}$ and $A\beta^{1-42}$. The objective of this study was to determine whether tissue NEP specific activity differs according to age and/or across mouse strains, especially those strains predisposed toward formation of $A\beta$ -amyloid plaques following overexpression of the human Alzheimer amyloid precursor protein (APP). The C57Bl/6J mouse strain appears to be relatively susceptible to cerebral amyloidosis, whereas the Swiss Webster (SW) strain appears more resistant. We investigated whether NEP specific activity in brain and kidney homogenates from SW and C57 mice of 6, 40, and 80 weeks old varied according to mouse strain, age, and gender. Among the variables tested, NEP specific activity varied most dramatically across mouse strain, with the kidney and brain of SW mice displaying the highest activities. Aging was associated with a reduction in brain NEP specific activity in both strains. Gender-specific differences were identified in kidney but not in brain. We conclude that aging- and strain-dependent differences in NEP specific activity may play a role in the differential susceptibility of some mouse strains for developing cerebral amyloidosis following human APP overexpression.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by the accumulation of senile plaques and neurofibrillary tangles in the brain [1]. The senile plaques are composed of amyloid- β peptides ($A\beta$; $A\beta^{1-40}$ and $A\beta^{1-42}$) that are generated by the sequential enzymatic cleavage of the amyloid precursor protein by β - and γ -secretases [for review, see 9]. $A\beta$ deposition is associated with dysfunction and/or death of neurons in the human central nervous system, leading to deterioration of memory and higher cognitive function [29]. Research toward developing treatments for AD are focused on preventing the formation of the $A\beta$ via development of β - and γ -secretase inhibitors or strategies for directly antagonizing $A\beta$ accumulation, using either "plaque-buster" drugs or immunotherapy to aid in clearing the brain of plaques [reviewed in 9]. Another potential therapeutic target focuses on activation of specific proteases that metabolize $A\beta$ peptides, thereby clearing them from the brain. Candidate proteases included insulin degrading enzyme (IDE) [30, 33] and neprilysin (NEP) [17].

Neprilysin (NEP) is a 97 kDa type II transmembrane zinc-metalloproteinase that is also known as neutral endopeptidase, enkephalinase, EC 3.4.24.11, CD10, and common acute lymphoblastic leukemia antigen [6,16]. NEP is expressed in the mammalian CNS and peripheral tissues, including kidney, lung, and reproductive organs [6 15, 16]. Structurally, NEP is a type II transmembrane protein, displaying a long extracellular domain that contains the catalytic site, an intramembranous domain, and a short N-terminal intracellular domain. By virtue of this structure, NEP acts as an ectoenzyme,

catalyzing cleavage in the extracellular space [25]. Recently, NEP has drawn much attention because of its ability to degrade A β 40 and A β 42 *in vitro* and *in vivo* [13, 17, 27, 28, 32], cleaving the A β ¹⁻⁴² peptide between the Glu³-Phe⁴, Gly⁹-Trp¹⁰, Phe¹⁹-Phe²⁰, Ala³⁰-Ile³¹, Gly³³-Leu³⁴ [13] or Gly³⁷-Gly³⁸ [17] peptide bonds. Injection of NEP inhibitors into the mouse brain results in an accumulation of A β peptides [17, 25], and transgene-induced overexpression of NEP decreases A β levels and plaque load [24]. Additionally, NEP knockout mice show increased levels of both A β 40 and A β 42 peptides [26].

In the current study, we sought to test further the hypothesis that alterations in endogenous NEP specific activity might be modulated by age, gender [3, 18] and/or strain, and that modulation of NEP specific activity as a function of these variables might contribute to differential brain A β deposition following overexpression of human APP in transgenic mice.

MATERIALS AND METHODS

Reagents

Phosphoramidon, enalapril and the fluorogenic substrate N-dansyl-D-Ala-Gly-p-nitro-Phe-Gly were purchased from Sigma (St. Louis, MO, USA)[6,7,12]. Recombinant human neprilysin (rhNEP) [14] was purchased from R&D (Minneapolis, MN, USA). Phosphoramidon and enalapril were dissolved in water; the substrate peptide was dissolved in methanol and then brought to 2 mM with Tris (50 mM, pH 7.4).

Preparation of Kidney and Brain Homogenates

Mice derived from the Swiss Webster (SW; 6 wk old mice from Charles River; 40 wk old mice from Taconic) and C57Bl/6J strains (6 and 40 wk old mice from Jackson Labs) were sacrificed by cervical dislocation. SW and C57 mice of 52 wks of age were purchased from Taconic and Jackson Labs, respectively, and aged in our facility. All mice were maintained with free access to food and water, but without environmental enrichment [21]. Upon sacrifice, kidneys and brain were immediately removed and placed into 1 ml of ice-cold 50 mM Tris-HCl, pH 7.4. The tissue was homogenized by 30 strokes in a dounce homogenizer and sonicated (BranSonic sonifier) on ice for 15 sec (2x). The homogenates were transferred to a sterile microcentrifuge tube for centrifugation at 10,000 x g at 4° C for 15 min to remove cell debris and nuclei. These samples were transferred to another sterile microcentrifuge tube, and the centrifugation was repeated. Final protein concentrations were determined using the BioRad[®] protein assay kit (Hercules, CA, USA), utilizing bovine serum albumin as the standard. The brain and kidney homogenates were stored at -80° until use.

NEP activity

Mouse tissue homogenates of brain or kidney and a standard curve of rhNEP (5 ng/ml – 5 µg/ml) were pre-incubated with enalapril, an angiotensin-converting enzyme (ACE) inhibitor, for 30 minutes at 37°C to prevent fluorogenic substrate (N-dansyl-D-Ala-Gly-p-nitro-Phe-Gly) cleavage by ACE [20], in the presence or absence of phosphoramidon, a specific NEP inhibitor. Following this pre-incubation, the fluorogenic substrate was added, and samples were incubated for an additional 1-2 hours at 37°C. The final concentrations were 12.5 µM for enalapril, 12.5 µM for phosphoramidon, and 500 µM for the substrate, in a reaction volume of 100 µl. At each indicated time point, fluorescence absorbance was recorded using a Perkin-Elmer Victor³ Reader (Forster City, CA, USA) with an emission wavelength of 535 nm and an excitation wavelength of 355 nm. Arbitrary fluorescence units for each sample were compared with a standard curve prepared using rhNEP in order to determine the NEP specific activity per mg protein.

Statistical Analyses

Analysis of variance (2-way ANOVA) was used to compare NEP specific activity in SW mice across ages and genders for both brain and kidney samples, and across age and strain (SW and C57B/6J). For all analyses, the confidence limit was set at $p < 0.05$. When a significant difference was determined by the 2-way ANOVA, an analysis of variance (1-way ANOVA) or Student's t-test (confidence limits of $p < 0.05$) was also performed. All statistical analyses were performed using Prism[®] 3.0 software. The data are presented as means \pm SEM.

RESULTS

rh Neprilysin Standardization

The activity of recombinant human neprilysin (rhNEP) was determined at 4 concentrations (0.005, 0.05, 0.5 and 5 $\mu\text{g/ml}$; $n=3$) following incubation for 1, 2 and 4 hours in the absence or presence of phosphoramidon, a specific NEP inhibitor. At the lowest concentration, 0.005 $\mu\text{g/ml}$ (5 ng/ml), NEP activity was undetectable. As the concentration of rhNEP was increased, the amount of NEP activity increased in a concentration-dependent manner, as indicated by an increase in fluorometric emission. Further, NEP activity displayed a time dependency at the 0.5 $\mu\text{g}/\mu\text{l}$ concentration. Phosphoramidon blocked the activity of rhNEP at all concentrations and times of incubation (data not shown).

Neprilysin activity in kidney and brain as a function of age, gender and strain

a) Kidney

NEP activity was assayed in the kidney of young (6 wk), old (40 wk) and aged (80 wk) male and female SW mice (Fig 1A). Kidney homogenate from female SW mice displayed a neprilysin specific activity (sp act) equivalent to 6.35 ± 0.29 ng rhNEP/mg of kidney protein. In male SW mice, the neprilysin activity was equivalent to 2.51 ± 0.21 ng rhNEP/mg kidney protein. A two-way ANOVA demonstrated that kidney neprilysin

specific activity varied significantly according to gender ($p < 0.0001$; Fig 1A) but was not apparently altered with aging from 6 to 80 wk of age ($p = 0.85$).

We also sought to determine whether kidney NEP specific activity varied according to background mouse strain. NEP specific activity was assayed in kidney homogenates of male mice from the two strains (SW and C57Bl/6J, $n = 5$) at two ages (6 and 40 weeks). Kidney NEP specific activity was significantly different between these mouse strains (ANOVA two-way, $p < 0.05$). SW displayed higher specific activity (2.68 ± 0.23 ng rhNEP/mg kidney protein) compared to C57Bl/6J (2.05 ± 0.13 ng/mg kidney protein) (ANOVA one-way, $p < 0.05$; C57Bl/6J vs SW). Neither strain showed an effect of aging on kidney NEP specific activity (Fig 1B).

b) Brain

In contrast to kidney, brain NEP specific activity in SW mice did not vary according to gender but rather varied in both genders according to age (ANOVA two-way; age, $p = 0.0001$; gender, $p = 0.53$) (Fig 2A). NEP specific activity at 80 weeks was significantly (~75%) reduced in both males and females relative to 6 weeks (one-way ANOVA, $p < 0.0002$). NEP specific activity in brain was also significantly different as a function of background mouse strain (ANOVA two-way, $p < 0.001$) (Fig 2B). In the male mouse brain, NEP specific activity was significantly lower in the C57Bl/6J strain when compared to activity in the SW mouse brain at the ages of both 6 and 40 weeks. By the age of 80 weeks, however, both strains displayed a significant decrease in NEP specific

activity, such that activities between the two strains was no longer significantly different. A two-way ANOVA indicated that both age ($p < 0.0001$) and strain ($p < 0.01$) affected NEP specific activity. Furthermore, a one-way ANOVA analysis within strains showed that at the age of 80 weeks, NEP specific activity was significantly different compared to the activity at ages of 6 or 40 weeks (SW $p < 0.01$; C57 $p < 0.01$ and $p < 0.05$. Fig. 2B).

DISCUSSION

In humans, the propensity toward cerebral amyloidosis varies according to gender, genetic background, organ, and aging. In the current study, we sought to test in mice the hypothesis that endogenous alterations in tissue NEP specific activity might be modulated by aging, gender or strain, and that modulation of NEP specific activity as a function of these variables might contribute to differential brain A β deposition following overexpression of human APP in transgenic mice. As reviewed below, it is worth noting that data already exist that implicate the mouse background strain and aging in modulating the relative propensity toward A β accumulation.

Role of genetic background in specifying the propensity toward cerebral A β amyloidosis

Unsurprisingly, the various A β plaque-forming mice reported in the literature have been created in a variety of genetic background strains. Of these, the only line containing the Swiss-Webster strain background is the PDAPP mouse, which is SW X C57Bl/6J X DBA and expresses APP at approximately 10-fold relative to endogenous levels [19]. Despite such exceptional levels of APP holoprotein expression, A β deposition occurs in only 50% of the animals despite identical transgene dose. PDAPP mice that fail to develop A β plaques are designated “low pathology” mice [7], a heritable trait that might plausibly be attributable to the SW and/or DBA components of the background strain. Our lab’s experience is consistent with this formulation. We created a line of APP^{SWE} SW mice that demonstrated a 5-fold overexpression of the APP transgene relative to endogenous APP levels. This level of APP fold-increase is clearly associated with plaque accumulation in

C57Bl/6J mice in other labs [2], yet our APP^{SWE} SW mice demonstrated no plaque accumulation, even at 24 months of age, and even after back-crossing with C57Bl/6J for one or two generations (Gandy and Ehrlich, unpublished data).

In recent work, Lamb and colleagues have proposed that the propensity toward A β deposition is at least partially controlled by genetic background [23]. In their studies, the Lamb group showed that YAC-APP transgenic mice on a congenic C57Bl/6J background showed A β plaque deposition as early as age 13 months, while YAC-APP transgenic mice on the DBA/2J background failed to produce plaques despite an identical level of overexpressed mutant APP [23].

Role of gender in specifying the propensity toward cerebral A β amyloidosis

Gender-related differences in plaque deposition in transgenic mice are also well recognized [e.g., 22]. Female APP^{SWE} transgenic mice generated in a mixed strain background (C57Bl/6 X SJL) demonstrate greater amyloid deposition than do their male littermates, and this becomes more apparent with age [4, 22, 34]. In the current study, gender differences were examined only in the SW strain, and we observed that female kidney NEP specific activity was significantly higher than corresponding male kidney NEP specific activity; however, NEP specific activity was similar in males and females in the brains of these same animals. Follow-up studies of the effect of estrogen and testosterone on NEP specific activity are underway since other studies along this line suggest that hormones are important regulators of NEP activity [14].

Role of aging in specifying the propensity toward cerebral A β amyloidosis

In SW mice, and to a lesser extent in C57Bl/6J mice, we found that brain NEP specific activity is dramatically diminished in the aged brain, implying that this is not a strain-specific occurrence. These results agree with other studies that reported an aging-dependent diminution in brain NEP activity [3, 18]. In C57Bl/6 male mice, NEP specific activity and protein levels are reported to decrease in the hippocampus by 20% over the lifespan from 10 to 132 weeks, while a much smaller decrease can be demonstrated in the neocortex [18]. In a mixed C57Bl/6/129 background, neprilysin protein levels are reported to decrease by greater than 50% in the hippocampus between 2 and 18 (78 weeks) months [3]. Of note, however, the decrease in C57Bl/6J was far greater than that shown in the study by Iwata *et al.* [18], implying that there may be differences between sub-strains of C57Bl, and/or that the differences in the total brain may be greater than in the hippocampus alone. The latter formulation appears unlikely, however, based on the correlation between our results and those from LaFerla and colleagues [3].

Cerebral A β clearance and the role of neprilysin

The literature supports the hypothesis that there might plausibly be direct links between levels of NEP activity and that of A β pathology. For example, injection of a lentivirus expressing the NEP gene decreased AD pathology in plaque-forming PDAPP and TASD41 transgenic mice [25, 26]. The inhibitors of NEP, thiorphan and

phosphoramidon, were shown to block the proteolyzing activity of brain derived NEP on $^3\text{H}/^{14}\text{C}$ $\text{A}\beta^{1-42}$ peptide [12]. When transgenic mice overexpressing NEP are crossed with plaque forming mice, brain $\text{A}\beta$ levels and plaque deposition both decrease [24].

Despite recent advances in defining the role of NEP in cerebral $\text{A}\beta$ clearance, the molecular and cellular mechanisms underlying clearance of brain $\text{A}\beta$ are incompletely understood. Both $\text{A}\beta^{1-40}$ and $\text{A}\beta^{1-42}$ are cleared rapidly from the blood and localized to the liver, spleen, kidney, urine, and stomach [11] with the liver accounting for 60% of $\text{A}\beta$ concentration [12]. Therefore, reductions in NEP specific activity in the periphery may also play a role in the accumulation of soluble $\text{A}\beta$ in AD brains by shifting the concentration gradient away from the circulation and toward brain absorption and subsequent plaque deposition. The “peripheral sink” phenomenon associated with $\text{A}\beta$ immunotherapy [7] dovetails well with such a concept, and the apparent success of that strategy in mouse models supports the notion that concomitant augmentation of both peripheral and central $\text{A}\beta$ clearance may be an attractive approach to treatment or prevention of cerebral amyloidosis in AD.

Conclusions

In summary, we have investigated the role of background mouse strain, gender, and aging on the specific activity of NEP in two tissues. Among the variables tested, NEP specific activity varied most dramatically across mouse strain, with the highest activities being observed in the kidney and brain of SW mice. Aging was associated with a reduction in

brain NEP specific activity in both strains. We conclude that aging- and strain-dependent differences in NEP specific activity may explain, at least in part, the roles that aging and background mouse strain play in differential specification of the susceptibility toward the development of cerebral A β amyloidosis.

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FIGURE LEGENDS

Fig 1.: Kidney NEP as a function of gender, aging, and mouse strain.

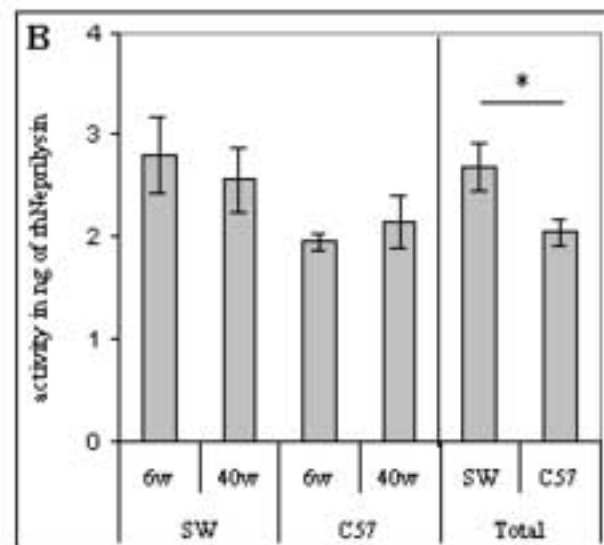
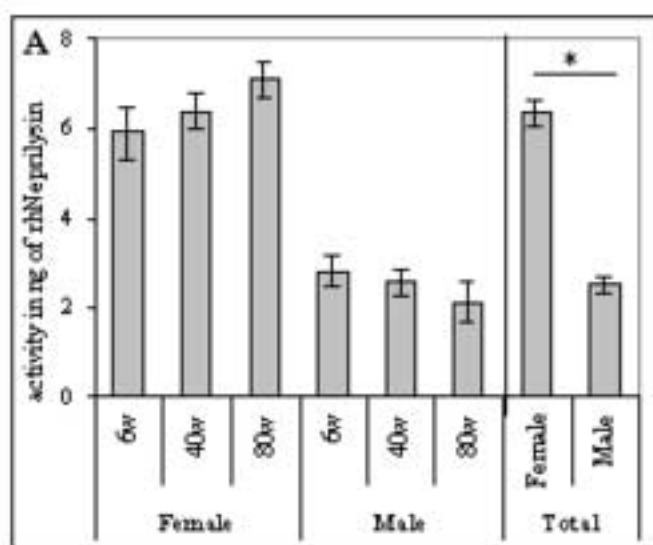
A) Neprilysin specific activity is higher in female than in male, but independent from age (ANOVA two-way, $p=0.85$ for age, $p<0.0001$ for gender). Samples evaluated for gender only (Student's t-test * $p<0.0001$). The data are expressed as mean \pm SEM ng/mg kidney protein. B) Neprilysin activity in males of 2 different strains: Swiss Webster and C57/6J (ANOVA two-way, $p=0.39$ for age, $p<0.05$ for strain). Samples from A evaluated for strain only (Student's t-test * $p<0.05$). The rhNEP specific activity is expressed as mean \pm SEM ng/mg kidney protein.

Fig 2.: Brain NEP in SW mice as a function of gender, aging and mouse strain.

A) Neprilysin activity does not differ in Swiss Webster female or male but decreases with age (ANOVA two-way, $p<0.0001$ for age, $p=0.53$ for gender). Samples evaluated for age only (ANOVA One-way * $p<0.01$, ** $p<0.001$). The rhNEP specific activity is expressed as mean \pm SEM pg/mg brain protein. B) Neprilysin activity in males of 2 different strains: Swiss Webster and C57/6J (ANOVA two-way, $p<0.0001$ for age, $p<0.01$ for strain). Samples evaluated for one strain only (ANOVA One-way: $p<0.001$ for SW, $p<0.01$ for C57. Bonferroni analysis: * $p<0.01$, ** $p<0.05$). The rhNEP specific activity is expressed as mean \pm SEM pg/mg brain protein.

Figure

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