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Oxidized neprilysin in aging and Alzheimer's disease brains

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Abstract

Deposition of amyloid in the brain is important in the pathogenesis of Alzheimer's disease (AD), but it remains to be determined if deposition is due to increased production or decreased clearance of fibrillogenic forms of β-amyloid (Aβ). Except for rare genetic forms of AD, there is little evidence for increased production of Aβ, but decreases in enzymes involved in the clearance of Aβ are increasingly being investigated. Neprilysin (NEP) is a major enzyme for degradation of Aβ and changes in amount or activity of NEP may play a role in Aβ deposition in AD. Since oxidative damage to proteins, including formation of adducts such as 4-hydroxynonenal (HNE), has been reported in AD, it was of interest to determine if NEP might be susceptible to oxidative modification. To address this question, monoclonal antibody immunoprecipitates of NEP were probed with polyclonal antibodies to NEP and HNE. The results showed decreased NEP in AD compared to normal controls. NEP in both AD and controls had HNE-modification and the ratio of oxidized to total NEP was greater in AD than in controls. These findings suggest that decreased NEP may contribute to Aβ deposition in AD and that age-related oxidative damage to NEP may play a role in age-related cerebral amyloidosis that is exacerbated in AD.

Keywords: Neprilysin; CD10; Common acute lymphoblastic leukemia antigen; 4-Hydroxynonenal; Oxidization; Alzheimer's disease; Amyloid; Aging

The amyloid cascade hypothesis of Alzheimer's disease (AD) posits that generation and deposition of β-amyloid (Aβ) leads to neuronal and synaptic degeneration and loss. It remains to be determined if abnormal Aβ accumulation in AD is due to increased Aβ production, decreased Aβ degradation or both. Although the mechanisms involved in Aβ production have been extensively studied, there is little evidence to suggest that increased generation of Aβ is important in sporadic AD. Accordingly, the role of Aβ degradation is increasingly being addressed in AD and several enzymes have been described with varying abilities to degrade Aβ [1–3]. Among them, neprilysin (NEP), which has been considered the most important enzyme for degradation of fibrillar Aβ, has been the most extensively studied [4–7].

NEP [also known as neutrophil cluster-differentiation antigen 10 (CD10) or common acute lymphoblastic leukemia antigen (CALLA)] is a 90- to 110-kDa plasma membrane glycoprotein that is composed of a short N-terminal cytoplasmic region, a membrane-spanning section, and a large C-terminal extracellular, catalytic domain, which contains a HExxH zinc-binding motif [8]. It exists as an ectoenzyme preferentially hydrolyzing extracellular oligopeptides (<5 kDa) on the amino side of hydrophobic residues, which makes it a suitable candidate for degradation of the small, hydrophobic 40–42 Aβ peptide. In the brain, it is expressed on neuronal membranes, both pre- and post-synaptically [9] and is most abundant in the nigrostriatal pathway, as well as in brain areas vulnerable to amyloid plaque deposition, such as the hippocampus [10].

If NEP plays a role in the deposition of Aβ in AD, decreased NEP activity could be due to either decreased expression of the enzyme or decreased enzyme activity, or both. Previous studies have indicated that NEP mRNA and protein are significantly decreased in AD compared to age-matched normal brains [11,12]. An age-related decline in NEP has also been reported in mouse hippocampus [13]. Recent data from our laboratory also

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confirm decreases in NEP in AD, but not in pathological aging, which is a term we use for cases with high amyloid loads, but minimal or no neuritic pathology [14]. This raises the possibility that NEP may be modified and functionally down-regulated in AD, but not in aging.

Abnormal post-translation protein modification is increasingly recognized as a common age-related phenomenon that may produce dysfunctional structural proteins and enzymes. Because a variety of proteins, including some enzymes, can be substrates of oxidative damage in AD brains [15], it was of interest to determine if NEP might be susceptible to oxidative modification. Several studies have shown that 4-hydroxynonenal (HNE), a by-product of lipid peroxidation that can form covalent adducts with proteins through histidine and lysine residues, is increased in AD and may contribute to cytopathological effects observed as a consequence of oxidative stress [16]. It was thus of interest to determine if NEP might be a substrate for HNE-modification. The present study shows for the first time that NEP in both controls and AD human brains can be modified by HNE and suggests that oxidative damage to NEP may be a factor in the accumulation of Aβ in AD.

Materials and methods

Antibodies and reagents. Monoclonal anti-human NEP antibody was purchased from DAKO (Carpinteria, CA) and polyclonal anti-NEP antibody was purchased from Chemicon International (Temecula, CA). Polyclonal anti-HNE antibody was purchased from A.D. Scientific (San Diego, CA). Anti-NEP monoclonal antibody 56C6 was from Novocastra Lab. (Newcastle, UK). HRP-conjugated secondary antibodies against mouse or rabbit immunoglobulins were the products of Southern Biotechnology Associates (Birmingham, AL). Protein G-conjugated agarose was purchased from Pierce (Rockford, IL). ECL system was purchased from Amersham Biosciences (Piscataway, NJ). Protease inhibitor cocktail and all other chemical reagents were purchased from Sigma (St. Louis, MO) unless specified.

Human brain cases. Frozen postmortem human brains were from the Mayo Clinic Jacksonville brain bank. A total of 5 cases of AD, 6 cases of Lewy body disease, and 15 age-matched normal controls (84 ± 4.5, 81 ± 9.9, and 87 ± 11 years, respectively) were used in the study. They were matched for postmortem interval (13 ± 9.9, 6.6 ± 2.4, and 9 ± 7.2 h, respectively). Partially thawed gray matter samples from mid-frontal gyrus and visual cortex (area 17) in the occipital lobe were carefully dissected from white matter and meninges, then immediately frozen on dry ice, and stored at −80 °C until use.

Immunoprecipitation of NEP. To determine if HNE-modified NEP is present in human brain, NEP was immunoprecipitated from gray matter of the mid-frontal gyrus with monoclonal anti-human CD10 antibody and immunoblotted with polyclonal antibodies to NEP and to HNE. Briefly, frontal gray matter from frozen postmortem AD and normal brains was homogenized on ice in TBS containing complete protease inhibitor cocktail at a concentration of 100 mg/ml (w/v) as described previously [17]. Brain homogenates containing an equivalent of 1 mg of gray matter were extracted with 200 μl of immunoprecipitation buffer (20 mM sodium phosphate, pH 7.5, 500 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, and 0.02% sodium azide) with a protease inhibitor cocktail at room temperature for 10 min, followed by centrifugation at 1000*g* at 4 °C for 10 min to remove any possible insoluble elements in the samples. Then 5 μg of anti-human CD10 monoclonal antibody was added to the supernatant of each sample. Following further incubation at 4 °C overnight with gentle end-to-end mixing, 50 μl protein G-conjugated agarose was added to each sample and incubated at room temperature for 2 h with constant end-to-end mixing. After washing with immunoprecipitation buffer six times, immobilized protein G-bound complex was boiled in 50 μl SDS–PAGE sample buffer for Western-blot analysis.

Western-blots of NEP, HNE, and protein quantification. Ten microliters from each sample of immunoprecipitation with anti-CD10 monoclonal antibody was resolved on 12% SDS–PAGE gels. After transfer to nitrocellulose membranes, specific protein bands were detected with anti-HNE (1:1000) and anti-NEP antibody (1:1000) with a standardized Western-blot protocol [17]. For negative controls, primary antibody was replaced with normal rabbit serum diluted in TBST (1:1000) followed by the same steps as in anti-NEP and anti-HNE blotting. Signal was detected with ECL system. The density of the specific 97-kDa band from each sample was measured with ImageJ [18] software and expressed as arbitrary optic density units. Data were analyzed by using Sigma Stat for Microsoft Windows, version 2.03 (SPSS Science, Chicago, IL), and significance levels were set as $p < 0.05$.

To eliminate the possibility of cross-reaction of anti-HNE antibody to NEP, human neprilysin expressed in 293 human embryonic cell line (NEP/293) was probed with anti-HNE antibody [6]. The same cell line transfected with empty vector was used as a negative control. Monoclonal anti-NEP antibody 56C6 was used for a positive control in the Western-blot.

The relative levels of NEP and HNE–NEP from occipital gray matter from the same panel of cases were measured directly from brain homogenates by Western-blot as described above. Briefly, brain homogenates dissolved in SDS–PAGE sample buffer at the concentration of 10 mg wet brain/ml were boiled for 5 min followed by centrifugation at 100,000*g* at 22 °C for 30 min to remove DNA and other possible insoluble elements. Samples containing 200 μg original wet brain from each case were resolved in two 12% SDS–PAGE gels in duplicate. After electrotransfer to nitrocellulose membranes under identical conditions, one set of samples was used for the Western-blotting for NEP and the duplicate set of samples was used for Western-blotting for HNE–NEP. The density of a specific 97-kDa band from each blot was measured as described above.

NEP and HNE–NEP were also measured by direct Western-blot from frontal cortex of 6 Lewy body disease and 10 normal cases for an additional comparison to evaluate if increased HNE–NEP in AD is disease-specific.

Results and discussion

In initial experiments, NEP was immunoprecipitated from a normal and an AD brain with mouse monoclonal

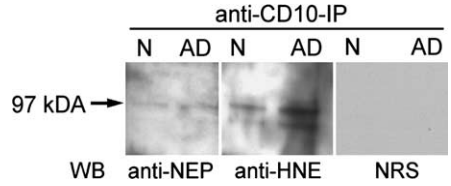

Fig. 1. Immunoprecipitation of NEP with anti-CD10 monoclonal antibody followed by immunoblotting with polyclonal antibodies to HNE and NEP. In negative control, primary antibody was replaced with 1:1000 diluted normal rabbit serum (NRS). N, normal brain; AD, Alzheimer's disease; MB, Western-blot; MW, molecular weight; and kDa, kilodalton.

anti-human CD10 (NEP) antibody followed by Western-blotting with rabbit polyclonal anti-NEP and anti-HNE antibodies. Both anti-HNE and anti-NEP antibodies detected a band at 97-kDa from the anti-CD10 immunoprecipitates (Fig. 1) consistent with the presence of HNE–NEP conjugates. To exclude the possible cross-reaction of anti-HNE antibody with NEP, human NEP expressed in embryonic 293 cell lines was probed with the anti-HNE antibody. Although both monoclonal anti-NEP antibody 56C6 and polyclonal anti-NEP antibody detected the same 97-kDa NEP band, the anti-HNE antibody failed to detect the same band (Fig. 2). Short of mass-spectroscopic analysis of the eluted protein, we believe that the evidence strongly favors the conclusion that the 97-kDa band detected in anti-NEP immunoprecipitates by anti-HNE antibody represents HNE-modified NEP. The HNE adduct would not be predicted to cause a significant shift in estimated molecular weight in the gel system used in this analysis. Although repeated efforts were made, the anti-HNE antibody could not be used for immunoprecipitation to confirm the results with cross-immunoprecipitation.

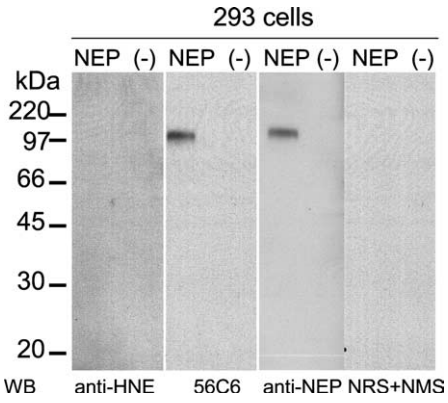

Fig. 2. Anti-HNE antibody does not cross-react with recombinant human NEP. Homogenates of human embryonic kidney 293 cells transfected with full-length human recombinant NEP DNA (lane NEP) were blotted with anti-HNE antibody, monoclonal anti-NEP antibody 56C6, and anti-NEP polyclonal antibody. The results show that both monoclonal and polyclonal anti-NEP antibodies detected the 97-kDa NEP band, but the anti-HNE antibody did not, consistent with the conclusions that anti-HNE antibody does not cross-react with NEP. The same amount of protein from 293 cells transfected with a mock vector was loaded as a negative control. Normal rabbit serum (NRS) and normal mouse serum (NMS) were used as negative control for secondary antibody. These results show no cross-reaction of anti-rabbit and anti-mouse IgG secondary antibodies.

To confirm these observations in a larger set of cases, HNE–NEP levels were measured in a series of 5 AD and 5 normal control brains that were matched for post-mortem delay. The same procedure, namely anti-CD10 immunoprecipitation followed by anti-HNE and anti-NEP immunoblotting, was performed (Fig. 3A). All measurements were performed under identical experimental conditions. The average NEP level in control brains was almost three times that in AD (16.6 ± 4.1 vs 4.9 ± 1.0, $p < 0.001$) (Fig. 3B). Both AD and controls had evidence of HNE-modified NEP, and given the significantly greater amount of NEP in controls, it was not surprising that HNE–NEP levels were also higher in controls (AD, 5.6 ± 2.4 arbitrary units; controls, 9.6 ± 2.6 arbitrary units; $p < 0.05$, Fig. 3C). With the

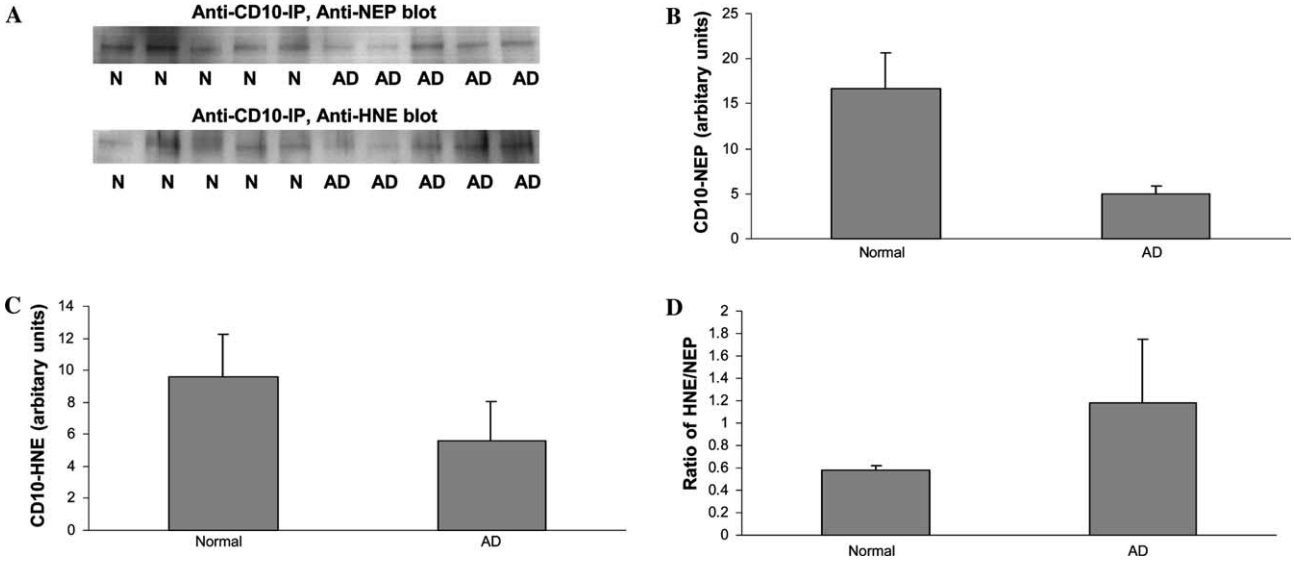

Fig. 3. Immunoprecipitation (IP) with anti-CD10 monoclonal antibody from frontal gray matter of normal and AD brains. IP proteins were resolved on 12% SDS–PAGE gels and immunoblotted with polyclonal antibodies to NEP (A, upper panel) and HNE (A, lower panel). The density of the 97-kDa band from NEP and HNE blot was measured with ImageJ software and expressed in arbitrary optical density units. The average levels of NEP (B) and HNE (C) are significantly greater in normals compared with AD (NEP $p < 0.001$ and HNE $p < 0.05$). In contrast, the ratio of HNE/NEP (D) is significantly greater in AD than normals ($p < 0.05$).

hypothesis that oxidized proteins would be increased in AD relative to controls and given the decreases in NEP protein in AD compared to controls, it was of interest to determine the proportion of HNE-modified NEP to total NEP. Consistent with the idea that oxidative damage is increased in AD, we found that the proportion of the NEP–HNE to total NEP was twice as much in AD as in controls (AD, 1.2 ± 0.6; controls, $0.6 \pm 0.0\%$, $p = 0.047$; Fig. 3D). These results suggest that not only is the NEP level decreased in AD, but also that oxidative damage to NEP is increased, which may further compromise its enzymatic activity.

To explore the relationship of HNE–NEP to AD pathology, both NEP and HNE–NEP were measured in the visual cortex, where there is much less AD type pathology than in mid-frontal cortex of the same cases. While the average level of NEP in normal brains was about 25% higher than in AD brains, the difference was not statistically significant. The average HNE–NEP in AD was only about 10% higher than in normals, which was also not statistically significant (Fig. 4A). The ratio of HNE–NEP/NEP in AD was more than twice that of the normals (0.26 vs 0.11), but not statistically significant (Fig. 4B). The results are consistent with the idea that HNE-modified NEP is increased in brain regions with greater AD type pathology. Since there is less difference in the amount of AD type pathology in visual cortex between AD and normals, the differences for NEP and HNE–NEP were also not significantly different. From these results it is not possible to determine if the decrease in NEP in areas with AD type pathology is specific or merely due to neuronal and synaptic loss that is greater in these areas, but in other experiments we have shown that decreases in NEP are not found in non-AD degenerative disorders with comparable degrees of neuronal and synaptic loss (unpublished results). These results imply that the decreases in NEP and the relative increases in HNE-modified NEP are indeed specific.

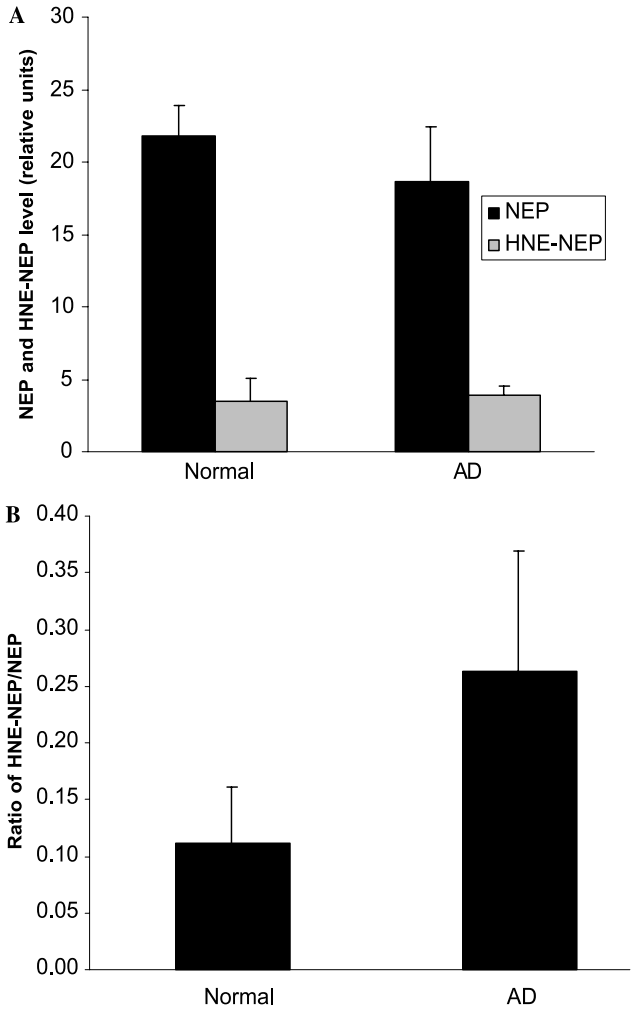

Fig. 4. NEP and NHE-NEP levels in the visual cortex of the occipital lobe of AD and normals. (A) NEP and HNE–NEP levels were not statistically different between normals and AD. (B) The ratio of HNE–NEP/NEP in the occipital lobe in AD is not significantly different from normals.

In order to further explore the specificity of these observations to AD type pathology, NEP and HNE–NEP were measured in frontal cortex from 6 LBD and 10 normal brains (Fig. 5). Although NEP was decreased in LBD when compared to normals (LBD vs normal: 28.0 vs 40.1; $p = 0.03$; Fig. 5A), the level of HNE–NEP (normal vs LBD: 15.3 vs 14.4; $p > 0.05$; Fig. 5A) and the ratio of HNE–NEP/NEP (normal vs LBD: 0.41 vs 0.54; $p > 0.05$; Fig. 5B) were similar in LBD and normals. The LBD cases used in this study had minimal AD type pathology, which confirms the specificity of the results to AD. The results also suggest that oxidative damage to NEP may both be a consequence of AD type pathology and also contribute to increased Aβ deposition in AD, possibly due to oxidative damage to a key Aβ degrading enzyme.

Surprisingly, HNE-modified NEP was detected not only in AD, but also in elderly control brains. These results suggest that oxidization of NEP may be an age-related process that is exaggerated in AD. The age range in both the AD and control groups used in this study was too narrow to detect an age-related effect in the present study. Clearly, analysis of additional cases of young controls with a range of ages is needed to determine the relationship of HNE-modification of NEP to aging.

The present findings are significant in light of experimental evidence that inhibition of NEP leads to biochemical and pathological deposition Aβ42 in animal models [4]. Moreover, augmenting NEP levels (e.g., lentiviral vector gene transfer) in amyloid-depositing transgenic mice expressing mutant human amyloid precursor protein (hAPP) decreases Aβ deposition and may even remove existing plaques [7]. Given that both mRNA and protein of NEP are reduced in brain regions vulnerable to AD pathology in animal models [13] and in AD [11,12], the present results support the notion that

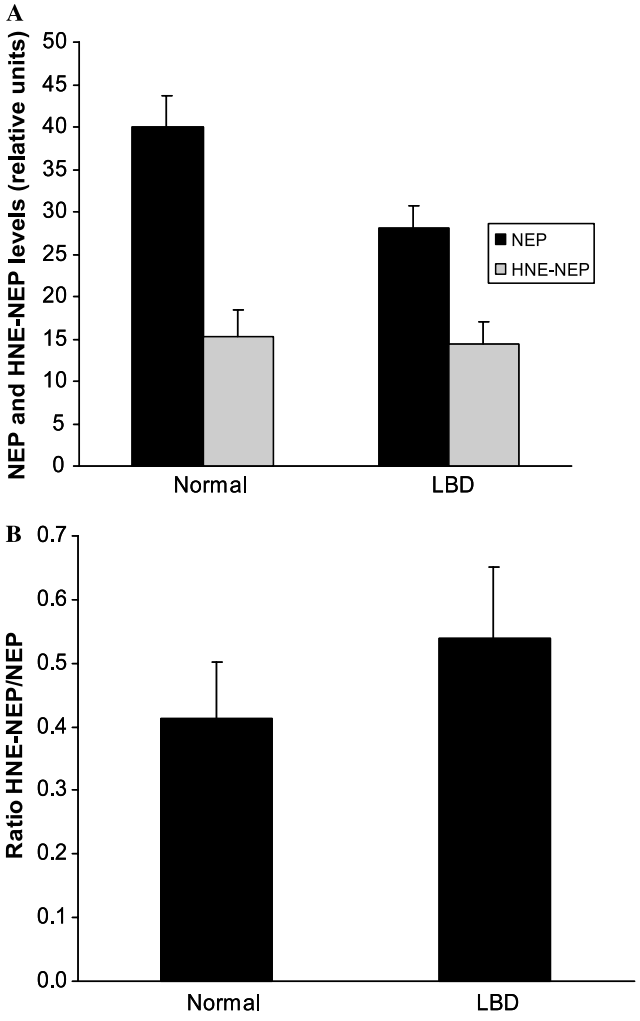

Fig. 5. NEP and HNE–NEP levels in frontal cortex of LBD and normals. (A) NEP levels in LBD are about 42% lower than in normals ($p = 0.03$), but HNE–NEP levels are similar in LBD and normals. (B) The ratio of HNE–NEP/NEP in LBD is not significantly different from normals.

decreases in Aβ clearance, in part mediated by decreases in NEP, play a role in pathogenesis of amyloid deposition in AD.

The present study suggests a possible mechanism for age-related decreases in NEP activity, although the functional consequence of HNE-modification of NEP remains to be determined. Interestingly, previous studies by Sayre and colleagues [19] have shown that HNE-modified proteins are increased not only in neurofibrillary tangle-bearing neurons, but also in apparently "normal" pyramidal neurons in AD. Taken together with the present findings, it is reasonable to speculate that age-related oxidative stress, which may be exaggerated in AD, may lead to dysfunction of key structural and enzymatic proteins, including NEP. The logical inference from the present observations is that reduction of oxidative stress, through anti-oxidants, for example, may decrease oxidative damage to NEP (and other proteins) and promote Aβ clearance, which may slow down degenerative processes related to Aβ deposition, as well as oxidative stress.

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