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Anti-amyloid activity of neprilysin in plaque-bearing mouse models of Alzheimer's disease

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Abstract Abnormally high concentrations of β-amyloid peptide (Aβ) and amyloid plaque formation in Alzheimer's disease (AD) may be caused either by increased generation or by decreased degradation of Aβ. Therefore, activation of mechanisms that lower brain Aβ levels is considered valuable for AD therapy. Neuronal upregulation of neprilysin (NEP) in young transgenic mice expressing the AD-causing amyloid precursor protein mutations (SwAPP) led to reduction of brain Aβ levels and delayed Aβ plaque deposition. In contrast, a comparable increase of brain NEP levels in aged SwAPP mice with pre-existing plaque pathology did not result in a significant reduction of plaque pathology. Therefore, we suggest that the potential of NEP for AD therapy is age-dependent and most effective early in the course of AD pathophysiology.

Key words: Transgenic mouse; Alzheimer's disease; β-Amyloid peptide; Protein stability; Therapy; Neprilysin

1. Introduction

Accumulation of β-amyloid peptide (Aβ) in the brain is considered the first pathological event leading to Alzheimer's disease (AD) [1]. High levels of fibrillary β-amyloid are deposited in diseased brain, which is associated with loss of synapses, impairment of neuronal functions and loss of neurons [2–5]. An imbalance between Aβ production and clearance may trigger AD [1]. Therefore, reduction of Aβ production or its accelerated clearance from the brain have been proposed as therapeutic targets for anti-Aβ drug development [2,6–8]. The enzyme neprilysin (NEP) has convincingly been shown to degrade Aβ in vivo [2,9–12]. NEP expression is down-regulated in wild-type and transgenic mice overexpressing the AD-causing Swedish mutations of human amyloid precursor protein (APP) in neurons (SwAPP mice) as a result of aging [13,14], as well as in affected areas of AD brains [15]. Aged SwAPP mice exhibit numerous compacted amyloid plaques consisting mainly of transgenically derived human Aβ [16]. We recently showed that injections of aggregated $Aβ_{42}$ into brains of young pre-symptomatic SwAPP mice led to an up-regulation of endogenous NEP expression that resulted in a reduction of Aβ levels and a delay of amyloid plaque deposition in these brains [12]. Therefore, to evaluate the effects of aging on NEP-related Aβ clearance in vivo, we performed similar injections in old SwAPP mice with profound amyloid plaque pathology to study whether a similar mechanism will be triggered in aged mice as was shown in the young SwAPP mice. We report here that, as in young mice, $Aβ_{42}$ aggregates caused sustained increases of NEP levels in brains of old SwAPP mice. These increases, however, were not associated with significant reduction of brain concentrations of Aβ, or with removal of pre-existing brain amyloid plaques in vivo.

2. Materials and methods

2.1. Animals and tissue preparation

Synthetic $Aβ_{1–42}$ ($Aβ_{42}$) and $Aβ_{42–1}$ (AβR) were both purchased from Bachem and reconstituted in phosphate-buffered saline (PBS, pH 7.4) by shaking at 1000 rpm for 48 h at 37°C. At the age of 1 year, SwAPP mice [12] received a unilateral injection of 1 μl 350 μM stock concentration fibrillar $Aβ_{42}$ ($n = 5$, Aβ high dose group) or 35 μM stock concentration ($n = 4$, Aβ low dose group) as described [12]. Control groups consisted of SwAPP littermates which received injections of 1 μl 350 μM stock concentration AβR ($n = 6$, AβR high dose group) or did not receive any injection ($n = 4$, un-injected). Twenty weeks after the injections mice were deeply anesthetized, blood was collected prior to perfusion and serum was prepared. Mice were then perfused transcardially with ice-cold PBS. The frontal parts of the brain containing interaural regions 6–3 were divided into the sagittal halves and were frozen immediately. These brain parts lay outside the injected brain area. The contralateral tissue to the injection site was homogenized for Western blotting and enzyme-linked immunosorbent assay (ELISA). The rest of the brains were fixed for 48 h in 4% paraformaldehyde at 4°C.

2.2. Histology

For immunohistochemistry, tissue was washed several times in PBS and embedded in paraffin. Five μm thick frontal adjacent sections were probed with antibodies exclusively recognizing $Aβ_{42}$ (Signet), $Aβ_{40}$ (Sigma) or both (4G8, Serotec). Sections were also stained simultaneously with 4G8 combined with antibodies against ionized calcium binding adapter molecule 1 (Iba-1) as a microglial marker [17,18] or glial fibrillary acidic protein (GFAP) as an astrocytic marker (Advanced Immunochemicals). The numbers of amyloid plaques stained with 4G8 were determined in parietal cortex and hippocampi of all mice on frontal sections 250 μm apart, beginning with a random section in interaural region 3, and the average number of plaques per section for each mouse was calculated as described [12].

2.3. Biochemical analysis

For combined Western blotting and Aβ ELISA, frontal brain tissue from the sagittal half of the brains contralateral to the injection sites,

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Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; NEP, neprilysin; Iba-1, ionized calcium binding adapter molecule 1; GFAP, glial fibrillary acidic protein

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containing interaural regions 6–3, was homogenized in a 20-fold wet weight amount of lysis buffer containing 100 mM Tris, 150 mM NaCl, 1% Triton X-100 and 1×complete proteinase inhibitor cocktail (Roche) pH 7.8. NEP Western blotting was performed as published [12]. After stripping [12], the blots were probed for β-actin (Abcam) as loading controls and for normalization of densitometric readings of NEP bands. Optical densities of immunoreactive protein bands were corrected by background subtraction and quantified using the NIH Image software.

For quantification of Aβ levels by ELISA, brain homogenates were centrifuged at 100 000×*g* for 45 min at 4°C. Supernatant fluids were removed and used for determination of soluble Aβ and the resulting pellets were dissolved in 70% formic acid for quantitation of aggregated Aβ. Consequently, the amounts of total Aβ and $Aβ_{42}$ were measured in both preparations by ELISA as described [12,19]. Furthermore, the $Aβ_{42}$ levels were determined in blood sera collected before sacrificing the mice [19].

In addition, the anti-Aβ antibody titers were determined in sera collected before sacrificing the mice and compared to the corresponding levels of SwAPP mice at 1 year of age, as described [12]. Moreover, the NEP enzymatic activity was measured in the brain homogenates according to published methods [12,20].

2.4. Statistical analysis

Data were collected by investigators blinded to the treatment of the mice. Comparison of the groups was done by Kruskal–Wallis test and the pairwise comparisons of the significance were done post-hoc with non-parametric Mann–Whitney *U*-test. In all graphs means ± S.E.M. are given.

3. Results

3.1. Dose-dependent $Aβ_{42}$-induced brain concentrations of NEP

To determine whether aging affects the regulation of the endogenous brain NEP levels in vivo, as demonstrated in the young SwAPP mice [12], aggregated Aβ or AβR was injected into the brains of SwAPP mice. We also tested whether the elevation of NEP levels in the brain depended on the dose of injected fibrillar Aβ by injecting either a high dose that induced the observed effect in the young SwAPP mice [12] or a 10-fold smaller dose in the same volume. Twenty weeks after the injections, control littermates, i.e. un-injected or SwAPP mice injected with high dose of AβR peptide, exhibited similar amounts of NEP in brain homogenates. A trend towards higher NEP levels was observed in brains of mice injected with low dose Aβ. In contrast, all SwAPP mice injected with high dose Aβ showed a marked elevation of NEP protein levels in the brain (Fig. 1A). β-Actin Western blotting was performed as loading control and for normalization of the NEP protein amounts. Quantitatively, when normalized to corresponding β-actin levels, NEP protein levels in brains of the un-injected group were similar to the AβR or the low dose Aβ injected group, whereas NEP levels were on average 11-fold higher in mice injected with high dose Aβ (Fig. 1A,B). NEP enzymatic activities in brains of mice injected with high dose Aβ were significantly higher when compared to other groups (Fig. 1C). The elevation of NEP enzymatic activity in brains of mice injected with low dose Aβ did not reach statistical significance. Moreover, no changes of NEP levels between control injected SwAPP mice and their un-injected SwAPP or non-transgenic littermates were observed (unpublished observations). These data show that NEP protein levels and enzymatic activity were increased following the Aβ injections and that this increase depended on the dose of injected Aβ. The NEP enzyme inhibitor thiorphan blocked these increase to background levels (Fig. 1C), confirming the specificity of the enzymatic assay.

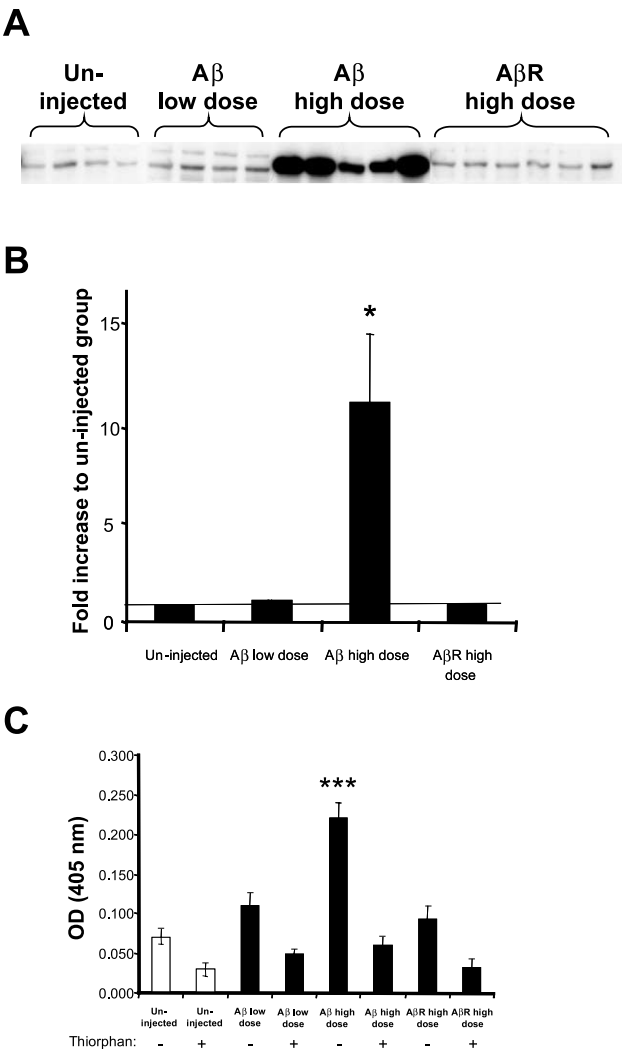

Fig. 1. A: Western blot analysis of all mice involved in this study. Twenty weeks after the injections, NEP protein levels were elevated in brains of all mice injected with high dose $Aβ_{42}$ (Aβ high dose, $n=5$), whereas in comparison to un-injected littermates ($n=4$), NEP levels were not changed in mice injected with low dose $Aβ_{42}$ (Aβ low dose, $n=4$) or with reversed $Aβ_{42}$ at the same high concentration (AβR high dose, $n=6$). B: Semiquantitative densitometric quantification of NEP immunoblots (as presented in A), normalized against the corresponding β-actin bands. The corresponding value of the un-injected group was set as 100%. Despite a large variability in individual mice, brain NEP protein levels were significantly higher after Aβ injections ($P \leq 0.05$) when compared with the un-injected transgenic littermates. Changes in brain NEP levels of animals in other groups did not reach statistical significance. C: When compared to un-injected littermates, only mice that received injections of high dose Aβ exhibited significant elevation of NEP peptidase activity in brain protein extracts ($P=0.001$), whereas no significant changes were observed in other groups. NEP enzymatic activities were blocked to background level by thiorphan.

3.2. Resistance of aggregated Aβ to NEP-dependent degradation

To determine whether the high brain NEP levels were associated with reduced concentrations of transgenic Aβ, we measured the concentrations of total Aβ and $Aβ_{42}$ in brain tissue by ELISA systems that specifically recognized intact human Aβ [12,19]. Surprisingly, neither the levels of detergent-soluble (Fig. 2A,B) nor the amounts of aggregated (i.e. formic acid-

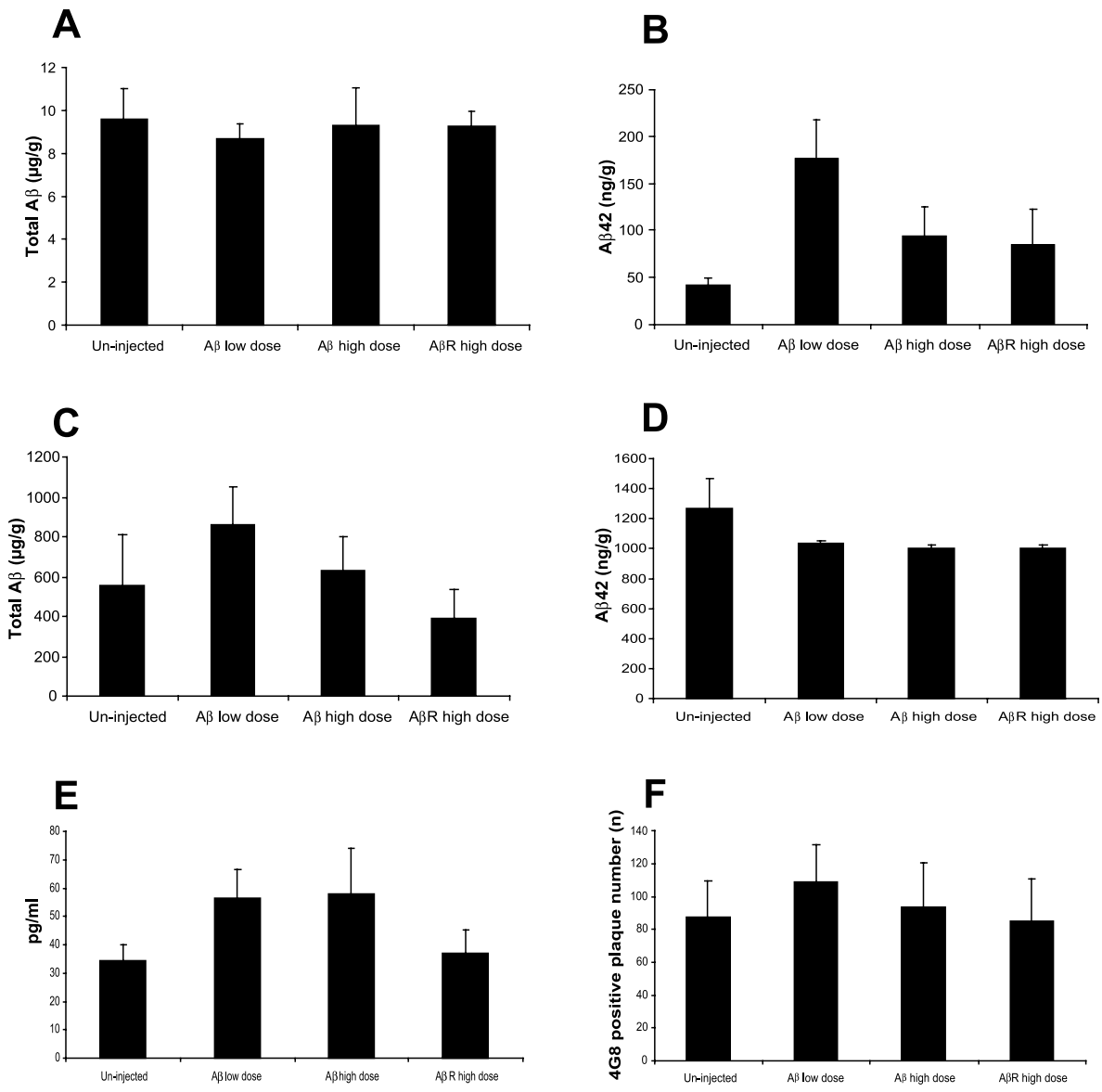

Fig. 2. Amyloid plaque pathology was not changed by $A\beta_{42}$ injections. Brain levels of detergent-soluble total Aβ (A) and $A\beta_{42}$ (B) were measured in brain homogenates of frontal brain tissue. Additionally, levels of formic acid-extractable total Aβ (C) and $A\beta_{42}$ (D) were also quantified in the same brain preparations. When compared to the un-injected group, no consistent, significant change of total Aβ or the more amyloidogenic $A\beta_{42}$ could be found in treated groups. E: $A\beta_{42}$ levels in sera of all mice were similar and did not differ due to our treatment. F: The elevation of NEP was insufficient to alter the average number of 4G8-positive plaques in cortical and hippocampal brain areas of the treated mice. G–L: All mice exhibited numerous amyloid plaques at the end of the experiment that were immuno-labeled with the 4G8 antibody as well as antibodies that specifically recognize either $A\beta_{40}$ or $A\beta_{42}$. Scale bar: 250 μm.

soluble) (Fig. 2C,D) total brain Aβ (Fig. 2A,C) and $A\beta_{42}$ (Fig. 2B,D) were significantly altered in SwAPP mice injected with high dose Aβ when compared to the other groups. In addition, the levels of serum $A\beta_{42}$ were similar for all groups (Fig. 2E).

We then tested whether the higher brain NEP levels were associated with reduced amyloid plaque pathology. In agreement with results obtained for the brain Aβ levels, no significant differences in numbers of 4G8-immunoreactive plaques could be found in any group (Fig. 2F). SwAPP mice (19/19) tested at the end of the experiment exhibited varying numbers of 4G8-positive amyloid plaques. We observed mice with high and low amyloid plaque load in all groups. Finally, to answer the question whether the treatment specifically affected one Aβ species, we stained the sections with antibodies recognizing $A\beta_{40}$ or $A\beta_{42}$ only. Again, plaque staining with these antibodies did not seem to change with our treatment (Fig. 2G–L) even though Aβ-treated mice showed a less intense staining of the plaques.

An antibody-mediated immune response against Aβ after peripheral immunization led to removal of amyloid plaques of AD mouse models [21] and AD patients [22]. Therefore, we examined whether endogenous antibodies were generated in treated mice. Very low amounts of anti-Aβ antibodies were found in sera of all mice which did not change in any treated group. In addition, no mouse-specific anti-Aβ antibodies were found in any brain by immunochemical methods. These data are in line with published data that no anti-Aβ antibody was produced by our treatment [12].

Activation of glial cells is often found as an accompanying

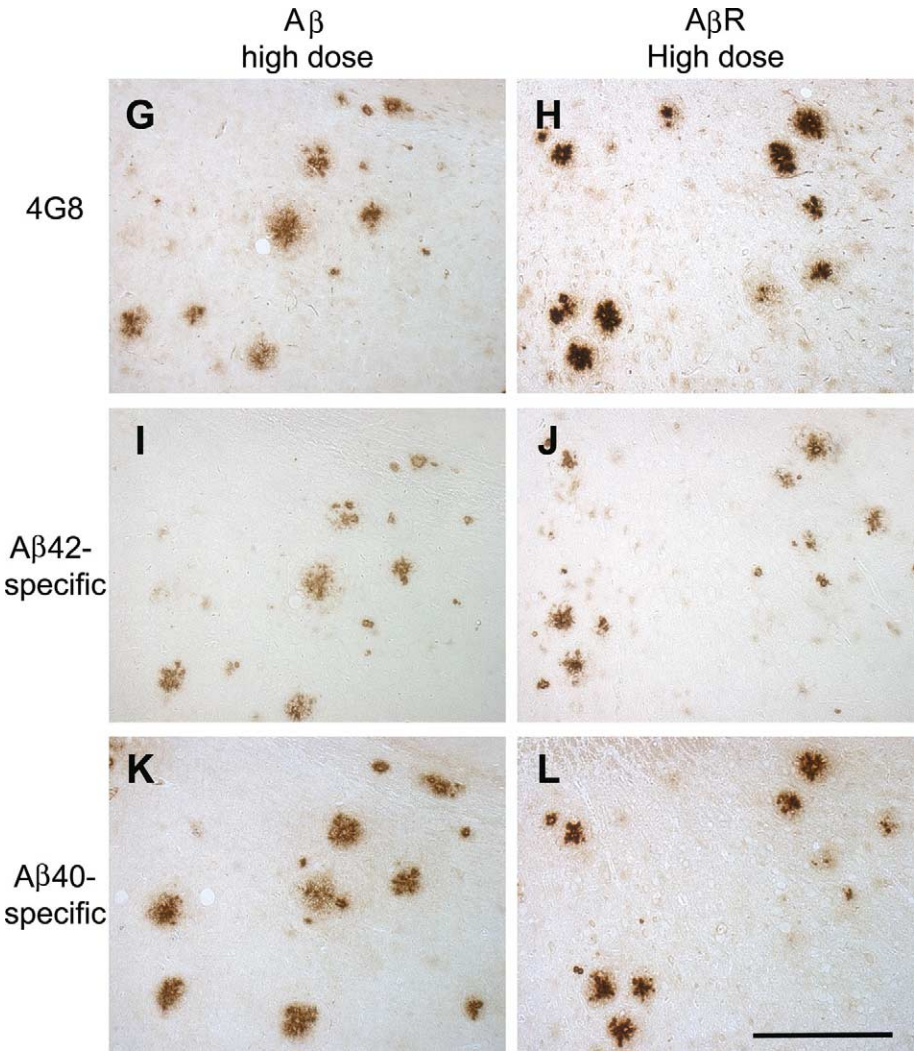

Fig. 2 (*Continued*).

event of the brain amyloidosis in SwAPP mice and in AD patients. Moreover, microglia and astrocytes are implicated in the removal of Aβ from brains of AD mouse models [23,24] and in human patients under therapy conditions [22]. Therefore, we analyzed the presence of activated glial cells in response to our treatments by immunohistochemistry. The degree of astrogliosis and microglial staining depended on the plaque pathology in individual mice but did not correlate with the treatment (Fig. 3).

4. Discussion

The aim of this study was to determine whether high NEP protein levels can reduce the pre-existing plaque pathology in brains of aged SwAPP brains. In a previous study, we showed that injections of synthetic fibrillar $A\beta_{42}$ into young SwAPP mouse brains, at an age before the onset of amyloid plaque deposition, resulted in neuronal up-regulation of NEP, cleavage and removal of Aβ and consequently led to a delay of amyloid plaque deposition [12]. The results of the present study show that similar treatment of aged SwAPP mice, already containing numerous amyloid plaques, also caused a sustained increase of NEP. Unlike the young mice, however, this treatment could not effectively reduce brain Aβ levels or remove the existing amyloid plaques from brains of aged mice.

The failure to remove Aβ from the brain is crucial for the development of AD [5,25]. Therefore, defining the temporal window for removal of Aβ from the brain is of prime importance for designing any intervention to ameliorate AD pathology. NEP levels are reduced in affected AD brains and in brains of aged mice [15,25,26]. Moreover, the heptapeptide spinorphin, the endogenous NEP inhibitor in human brain [2,27], as well as the endogenous mouse NEP inhibitor sialorphin [28] affect amyloid metabolism during aging [2,27,28]. It is therefore conceivable that the modulation of biological NEP activity by amyloidogenic factors with increasing age may be responsible for the observed differences in NEP biological activity in the young vs. aged mice in vivo.

Mechanistically, stereotaxic deposition of highly insoluble Aβ into brains stimulated a transcriptional activation of NEP gene expression [12]. This finding is further strengthened by results of the current study: the elevation of brain NEP level was highly specific to Aβ-injected brains. Only mice that were injected with high dose, but not with low dose Aβ or AβR in high concentrations, exhibited an elevated NEP protein and

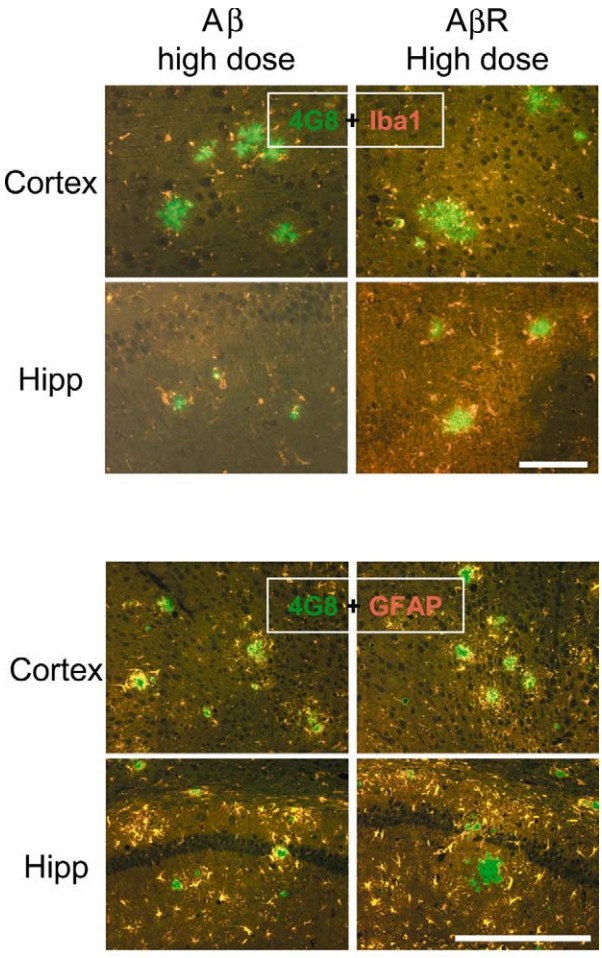

Fig. 3. In all groups reactive astrocytes and microglia were distributed in the brain and were mostly found colocalized with the amyloid plaques. Representative double immunohistochemical staining of β-amyloid plaques (4G8, green) in combination with a microglia cell marker (Iba-1, red) or with an astrocytic marker (GFAP, red) are shown. Scale bar: 200 μm.

enzymatic activity. These results emphasize that the observed NEP elevation is Aβ-specific and dose-dependent.

Unlike the young SwAPP mice that received the same treatment [12], and despite high NEP levels, no significant alteration of AD pathology was found in aged mice already exhibiting amyloid plaques at the beginning of the experiment. We therefore conclude that elevated brain NEP levels could not reduce the aggregated brain Aβ as efficiently as the reduction of the detergent-soluble Aβ pool observed in young SwAPP mice. This finding can be explained by the possibility that an increase in NEP did not suffice to digest plaque-associated fibrillar Aβ, because in the aged mice the pre-existing compacted plaques may be far less accessible to the NEP enzyme than soluble Aβ of young SwAPP mice with no fibrillar Aβ pathology at the time of injection [12,29]. Another explanation for the differences of the effects of high cerebral levels of NEP on young vs. aged SwAPP mice may include an age-dependent modulation of the activities of endogenous inhibitors such as sialorphin. The first hypothesis is supported by the observation that 20 weeks after injection, the injected fibrillar Aβ was detectable in brains of young SwAPP mice which showed an inhibition of Aβ plaque pathology [12], indicating that the fibrillar Aβ was resistant to cleavage by up-regulated NEP. Taken together, these data suggest that high levels of murine NEP were not able to degrade aggregated Aβ efficiently to affect the overt amyloid pathology of the aged SwAPP mice and are in agreement with studies demonstrating that NEP degrades monomeric or low oligomeric forms of Aβ [2,10,20].

Our data appear in contrast to a study by Marr and colleagues demonstrating that the injection of a lentivirus expressing human NEP resulted in a reduction of amyloid plaque load in a transgenic AD mouse model [11]. Whereas in their study the efficacy of virally expressed human NEP was tested, our study aimed at the long-term therapeutic efficacy of high NEP levels. There are two other substantial differences between our study and the above study. First, in our study the murine endogenous NEP was up-regulated, whereas in that study the human NEP was expressed. Secondly, we found the NEP expression to be neuronal, whereas in their study neurons and astrocytes must be assumed to be transduced by lentiviral application because NEP expression was driven by the CMV promoter. Therefore, the reasons for the above-mentioned discrepancy may be found in the experimental set-up. It is possible that mechanisms targeted by viral injections, i.e. massive ectopic transgenic expression of human NEP by neurons and astrocytes, may be conceptually different from the targeted long-term presence of elevated levels of murine NEP in neurons. It also must be stressed that human and mouse NEP proteins are not identical. Blast analysis shows that these two enzymes exhibit differences in many amino acids, including some in close vicinity to the active center of the enzyme [30]. Moreover, human Aβ differs from murine Aβ in three amino acids, one of which is a cleavage site for human NEP (Phe10→Tyr). Because human Aβ is expressed in the mice utilized in both studies, the different enzymatic activity and substrate specificity of human vs. murine NEP could cause the apparent observed discrepancy.

Further research is necessary to characterize the mechanisms of NEP function in brain amyloid formation and the role of physiological regulators, including endogenous NEP inhibitors, related enzymes and the above-mentioned substrate specificity. In particular, non-invasive strategies of increasing NEP levels, or activating its functions, may prove helpful to treat subjects at risk of AD.

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