Aβ42-induced Increase in Neprilysin Is Associated with Prevention of Amyloid Plaque Formation in Vivo*

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Brain β-amyloid plaques are principal targets for the development of treatments designed to slow the progression of Alzheimer’s disease. Intracranial injections of synthetic β-amyloid peptide (Aβ42) in transgenic mice expressing the Alzheimer’s disease-causing Swedish APP double mutations increased neuronal levels of neprilysin, a metalloendopeptidase that degrades Aβ in vivo, on mRNA and protein level. This increase was associated with significant reductions in brain levels of Aβ and with almost complete prevention of amyloid plaque formation throughout the brain. In addition, astrogliosis normally associated with amyloidosis was significantly reduced. Our results suggest that up-regulation of neprilysin in brain may represent an opportunity to reduce or prevent amyloid plaque formation in vivo.

Alzheimer’s disease (AD) is the most common cause of dementia in the elderly. AD is characterized immunohistologically by the deposition of extracellular fibrillary β-amyloid in brain, which is believed to be associated with impairment of neuronal functions and loss of neurons (1–3). β-Amyloid deposits are composed of several species of β-amyloid peptides (Aβ) including Aβ42 that is deposited early and progressively in amyloid plaques. Genetic evidence suggests that increased amounts of Aβ42 are produced in many, if not all, genetic conditions that cause familial AD (4–7), suggesting that amyloid formation may be caused either by increased generation of Aβ42, decreased degradation, or both (8). Therefore, reducing Aβ production in brain or the activation of mechanisms that accelerate its clearance from brain has become major targets for the development of drugs designed to reduce amyloid plaque formation and ameliorate AD pathology (1, 6, 9). Experimental models that express the disease-causing Swedish double mutation of APP (SwAPP) under the control of the hamster PrP promoter as well as into non-transgenic littermates (18, 19). At this age, our SwAPP mice had not developed amyloid plaques (18, 19). We report here that Aβ42 aggregates caused sustained increases in brain levels of NEP and that these increases were associated with dramatically reduced brain concentrations of Aβ as well as with prevention of brain amyloid plaque formation and reduced astrogliosis.

**EXPERIMENTAL PROCEDURES**

**Animals**—Transgenic mice expressing the AD-causing Swedish double mutant of the human APP (SwAPP) were generated and bred as described previously (18, 19). All mice used in this study were the progeny of a single SwAPP male cross-bred to non-transgenic (non-tg) littermates. The colony was housed under a light cycle of 12 h with dry food and water ad libitum. The presence of the transgene was determined by PCR on genomic DNA isolated from tail biopsies using specific primers 5’-GTG GAT AAC CCC TCC CCC AGC CTA GAC CA-3’ and 5’-CTG ACC ACT CGA CCA GGT TCT GGT T-3’ to the transgene (18). Fibrillar aggregated synthetic Aβ42 was prepared by resuspending lyophilized Aβ42 (Bachem) in phosphate-buffered saline (PBS), pH 7.4, by shaking for 48 h at 37 °C. At 11 weeks of age, SwAPP (n = 5) and non-tg littermates (n = 4) were anesthetized, 1 μl of fibrillar Aβ42 (350 ng each) was stereotaxically injected unilaterally into the parietal cortex −1.7-mm anterior, 1-mm lateral, and 1-mm ventral to the bregma above the hippocampus within 1 min (20). After the injection, the needle was left in place for one more minute and then was slowly withdrawn over a period of 1 min. Control groups consisted of SwAPP mice and non-tg mice that received PBS injections (n = 4 each). In a second set of experiments, we used C57BL/6 mice purchased from RCC Ltd. (Biotechnology & Animal Breeding (Fullingsdorf, Switzerland) at the age of 6 weeks and housed for 5 weeks in our animal facility. At 11 weeks of age, these mice received bilateral injections of Aβ42, reversed Aβ42-peptide (Aβ42-R, Bachem, aggregated as described for Aβ42), bovine serum albumin (BSA, 1.61 mg/ml), or PBS as described above and were sacrificed 1, 3, 12, and 20 weeks post-injection for combined analysis of mRNA and protein levels (n = 4 for each group and time).

**Tissue Preparation**—For combined Aβ-ELISA and immunohistochemistry, SwAPP and non-tg littermates injected with Aβ42 aggregates or PBS were deeply anesthetized 20 weeks following the injections by intraperitoneal injection of a mixture containing 60 μg/g

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‡The abbreviations used are: AD, Alzheimer’s Disease; Aβ, β-amyloid peptide; Aβ42, reversed Aβ42-peptide; IDE, insulin-degrading enzyme; ACE, angiotensin-converting enzyme; NEP, neprilysin; SWAPP, Swedish double mutation of APP; non-tg, non-transgenic; PBS, phosphate-buffered saline; GFAP, glial fibrillary acidic protein; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; pNA, p-nitroanilide.

3.4.24.15) (12), and neprilysin (NEP) also known as neutral endopeptidase (EC 3.4.24.11) (1). Among these enzymes, only NEP was shown to degrade Aβ42 in vivo. NEP is a 94-kDa type 2 membrane-bound Zn-metallopeptidase implicated in inactivation of several biologically active peptides including enkephalins, tachykinins, bradykinin, endothelins, and atrial natriuretic peptide (13–16). NEP is present in peptidergic neurons in the central nervous system (14, 15), and its expression in brain is regulated in a cell-specific manner. Although type 2 NEP transcripts are absent from the central nervous system, types 1 and 3 transcripts are localized in neurons and in oligodendrocytes of the corpus callosum, respectively (16, 17).

To characterize mechanisms involved in Aβ clearance in vivo, we injected aggregated Aβ42 into brains of 11-week-old transgenic mice that expressed the disease-causing Swedish double mutation of APP (SwAPP) under the control of the hamster PrP promoter as well as into non-transgenic littermates (18, 19). At this age, our SwAPP mice had not developed amyloid plaques (18, 19). We report here that Aβ42 aggregates caused sustained increases in brain levels of NEP and that these increases were associated with dramatically reduced brain concentrations of Aβ as well as with prevention of brain amyloid plaque formation and reduced astrogliosis.

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Ketaminol (Veternia, Zurich, Switzerland) and 20 μg/g Rompun (Provet, Lyssach, Switzerland) body weight and were perfused transcardially using ice-cold PBS, pH 7.4, followed by 4% paraformaldehyde and post-fixed in the same solution overnight. Tissue was washed several times in PBS, and the caudal parts of the brains reaching from interaural 0 to 1 excluding the cerebellum (20) were dissected and prepared for Aβ-ELISA. The remaining parts of the brain containing both hippocampi were embedded in paraffin and processed for IHC.

For combined Western blotting and reverse transcription-PCR, mice were killed and the frontal parts of the brain containing interaural region 6–4 were processed immediately for RNA isolation. The rest of the brains excluding the cerebellum were homogenized in lysis buffer containing 250 mM sucrose, 10 mM Tris, 1% Triton X-100, 1% SDS, and 5-μm thick paraffin sections were probed with antibodies against Aβ (4G8 and GE10, Serotec; 6H1 and 9G10, Evotec), NEP, IDE, angiotensin-converting enzyme (ACE), 24.15 protein, and GAPF according to providers’ protocols and counterstained with Nuclear Fast Red (Sigma).

For Western blotting as described previously (1). Total RNA was isolated by thepeqGOLD RNApure™ kit (Axon Laboratories) according to the manufacturer’s protocol. NEP mRNA levels were determined by quantitative real time reverse transcription-PCR performed on total RNA in the LightCycler (Roche Diagnostics) using the NEP-specific primers 5'-TAA GCC GCA GTC TCA GCC GAA ACT ACA A-3' and 5'-GAC TAC AGG GCC TCC ACT CAT TAT CCA CTC A-3' and CYBRGreen (Roche Molecular Biochemicals) in the reaction mixture. As a standard for the quantification of β-actin was amplified in same samples using 5'-TGG AAC GTG GAA GGT GAC A-3' and 5'-GGC AAG GGA CTT CCT GTA A-3' primers. The levels of NEP mRNA were normalized to the corresponding amount of β-actin mRNA in each mouse. The average was calculated for each group and compared with levels of uninjected mice for each time point.

**Western Blot Analysis**—Protein concentration of brain extracts were determined with the Bradford protein assay (Bio-Rad), and equal amounts of protein (50 μg) were loaded to each lane of a Novex 10–20% Tricine/SDS-PAGE (Invitrogen). After the electrophoresis, proteins were blotted on nitrocellulose membranes (Amersham Biosciences). Membranes were probed for NEP using the monoclonal antibody 56C6 at a dilution of 1:75. Blots were stripped in a buffer containing 2% SDS, 62.5 mM Tris, pH 6.8, and 100 mM β-mercaptoethanol at 50 °C for 30 min and probed for β-actin (1:5000) and glyceraldehyde-3-phosphate dehydrogenase (1:1000) as loading controls. Similar blots were probed with IDE-1 (1:500), ACE (1:200), and the polyclonal antibody to 24.15 protein (1:600). The antibodies to NEP, ACE, GFAP, β-actin, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Novacast, QBioscience, ImmunoGenex, Abcam, and Biodesign, respectively. The polyclonal antibodies to IDE (IDE-1) was a gift from Drs. Vekrellis and Selkoe (11), and the antibodies to 24.15 protein were gifts from Dr. C. Abraham (12).

Aβ-ELISA—For quantitation of Aβ content in mouse brain, protein extracts of brain tissue reaching from the stereotaxic coordinates interaural 0 to interaural 1 were prepared. This area lies outside of the site of the brain that was injected with Aβ42. Tissue samples were homogenized in a 25-fold wet weight amount of 70% formic acid, homogenates were centrifuged at 200,000 × g for 1 h at 4 °C, and supernatant fluids were neutralized by adding the 20-fold volume of 1 M Tris base. To measure the total content of brain Aβ, microtiter plates (Maxi Sorb, Nunc, Belgium) were coated with 150 μl of monoclonal mouse antibody 22C4 directed against the C terminus of Aβ40 and Aβ42 (amino acids 30–42) at a concentration of 20 μg/ml PBS. Plates were blocked with 1% BSA, 1% gelatin in 100 mM Tris, 5 mM EDTA, and 0.1% Tween 20, pH 7.6, for 4 h at 37 °C and washed three times with PBS containing 0.02% Tween 20. 150-μl diluted samples or standards (Aβ40, Bachem) were incubated overnight, washed, and detected with biotinylated monoclonal mouse antibody 6H11 directed against amino acids 1–17 of human Aβ (Evotec), incubated at a concentration of 1 μg/ml in blocking buffer for 4 h at 37 °C, and visualized with a peroxidase reaction using 3,3′,5,5′-tetrathymethylenbenzidine as substrate and detected at 450 nm. The linear range (r² = 0.99) of the used ELISA system was between 2.5 and 100 ng of Aβ/ml. Serial dilutions of samples were used for measurements in the linear range. In parallel, the concentrations of Aβ42 in each sample were determined using a commercially available kit (Innogenetics) according to provider’s instructions.

**Titer Assays**—Blood was taken from the tail veins at the times of injections and before sacrificing the mice. Anti-Aβ antibody titers were measured by incubating of serial dilutions of blood sera on Aβ40-coated microplates that were blocked with 100 mM Tris-HCl, pH 7.6, 5% milk powder, and 0.1% Tween-20 and washed three times with PBS containing 0.02% Tween 20. Serum samples were diluted in blocking buffer and incubated overnight at 4°C while shaking. Serial dilutions of monoclonal anti-Aβ antibody 6E10 were used as positive control. Plates were washed four times and incubated with goat anti-mouse biotinylated IgG (heavy and light) antibody (Vector Laboratories) in PBS with 1% BSA for 2 h at 37 °C. Plates were then washed four times and incubated with peroxidase-conjugated streptavidin (Jackson Laboratories) for 30 min at room temperature. After five washes, plates were incubated with 3,3′,5,5′-tetrathymethylenbenzidine for 5 min at room temperature, and the reactions were stopped with 1 M sulfuric acid. Optical densities were read at 450 nm in a microplate reader (Victor2 Multilabel, EG&G Wallac, Turku, Finland).

**NEP Peptidase Activity Assay**—Brain protein extracts were dialyzed against 4000 volumes of PBS for 24 h at 4 °C by using Slide-A-Lyzer dialysis cassettes (Pierce) with a 10-kDa cut off. Three independent samples of 10 μg of protein from each brain were incubated for 1 h at 37 °C with 50 μM Z-Ala-Ala-Leu-p-nitroanilide (ZAA-pNA, Bachem) in 50 mM HEPES buffer, pH 7.2. Thereafter, 0.4 milliunits of leucine aminopeptidase (Sigma) were added to the reaction mixtures, incubated for additional 20 min at 37 °C (21), and optical densities were measured at 405 nm. To inhibit NEP and related enzyme activities, 10 μM thiorphan (Sigma) was added for 5 min at room temperature before the addition of ZAA-pNA (21).

**Statistical Analysis**—Data were collected by investigators blinded to the genotype and treatment of the mice and were analyzed by non-parametric Mann-Whitney U tests.
**RESULTS**

**Aβ_{42} Increased Brain Concentrations of NEP**—To determine whether Aβ_{42} is involved in the regulation of NEP levels in vivo, we injected 1 μl of PBS or a suspension of aggregated synthetic Aβ_{42} into the brains of SwAPP mice and non-tg littermates and analyzed NEP by immunohistochemistry. Strongly stained NEP-immunopositive neurons were present at 20 weeks following one single injection of Aβ_{42} (Fig. 1). In these mice, NEP immunoreactivity was high in cellular compartments of many pyramidal cells of the cerebral cortex in all cortical layers (Fig. 1, h, d, i, and k) and in the cell bodies of pyramidal hippocampal neurons (Fig. 1, f, h, j, and l). In contrast, neuronal NEP staining was weak and diffused in cerebral cortex (Fig. 1, a and c) and hippocampus (Fig. 1, e and g) of non-tg littermates (Fig. 1, a and e) and SwAPP mice (Fig. 1, c and g) injected with PBS. Together these data show that Aβ_{42} injections increased neuronal levels of NEP protein in most brain regions. For detailed analysis of tissue levels of NEP, we injected wild type mice bilaterally with Aβ_{42} and analyzed them by Western blotting at 1, 3, 12, and 20 weeks following the injections. Control mice were injected with same amounts of Aβ_{42}-R, BSA, or were injected with PBS only. The levels of NEP protein were high in all injected groups 1 week after the injections (Fig. 2a). However, three weeks after the injections, 3 of 4 Aβ_{42}-injected mice showed a higher NEP protein level than other injected mice (Fig. 2a). At 12 weeks post-injection, all Aβ_{42}-injected mice showed higher NEP concentrations in brain tissue (4 of 4) when compared with their control counterparts, whereas at 20 weeks post-injection, 2 of 4 mice that had been injected with Aβ_{42} exhibited a remarkably higher NEP protein level in the brain (Fig. 2a). NEP protein levels in the brains of PBS- or BSA-injected groups were similar to Aβ_{42}-R group for each time point (data not shown). The persistent elevation of NEP levels was specific to Aβ_{42}-injected mice, because none of the control injected mice showed elevated NEP protein at 3 weeks post-injection or later (Fig. 2, a and b). β-Actin (Fig. 2, a and b) and glyceraldehyde-3-phosphate dehydrogenase (data not shown) Western blotting was performed for all groups as loading controls giving identical results. We next tested the NEP enzymatic activity in brain homogenates of mice 20 weeks after the injections using the ZAAL-pNA peptide as the substrate (21). This assay revealed significantly higher NEP activities in brains that had been injected previously with Aβ_{42} and that had higher brain levels of NEP on Western blots. The NEP enzyme inhibitor thiorphan blocked these increases (Fig. 2c).

To determine whether the increases in brain levels of NEP were paralleled by increased levels of NEP message, we analyzed by real time reverse transcription-PCR the NEP mRNA prepared from same mouse brains. One week after injections, the levels of NEP mRNA were 4–5-fold higher in all injected groups when compared with uninjected mice of the same age. This up-regulation was a consistent feature of the Aβ_{42}-injected mice. At 20 weeks post-injection, the mRNA level of NEP in all other groups had dropped to the level of uninjected mice, whereas Aβ_{42}-injected mice showed a 4.9-fold increase in NEP mRNA levels (Table 1).

**Aβ_{42}-induced Increase in NEP Was Associated with Reduced Amyloid Plaque Formation**—To determine whether the Aβ_{42}-induced increase in neuronal NEP was associated with reduced amyloid plaque formation in transgenic SwAPP mice, we counted the number of brain amyloid plaques stained by immunohistochemistry at 20 weeks after one single injection of Aβ_{42}. Intracranially injected Aβ_{42} suspension generated 4G8-immunoreactive deposits that were detectable up to 20 weeks following the injections (Fig. 3, d and e, open arrow). As expected, all of the control SwAPP mice (4 of 4) tested at the end of the experiment exhibited numerous amyloid plaques that immunoreacted with the monoclonal antibody 4G8 directed against the amino acids 17–24 of human Aβ (Fig. 3, a–c). In particular, amyloid plaques were abundant throughout the cerebral cortex and, to a somewhat lesser extent, in the hippocampus. Other Aβ-specific antibodies including 6E10, 6H1, and 9G10 gave identical results. In striking contrast, 4 of 5...
A sustained overexpression of NEP mRNA was observed after Aβ$_{42}$ injections. When compared to those of uninjected group (set as 100%), the levels of NEP mRNA were significantly higher one week after the injections in all groups. 20 weeks after the injections, however, mRNA levels of the control-injected mice did not differ from those of the uninjected mice, whereas this value was still high in Aβ$_{42}$-injected mice. * p ≤ 0.05 for comparison to uninjected group, n.s., not significant.

| Aβ$_{42}$R (± S.D.) | PBS (± S.D.) | Aβ$_{42}$ (± S.D.) | BSA (± S.D.) | Uninjected (± S.D.) |
|-------------------|-------------|-------------------|-------------|-------------------|
| 1 week 490 (± 190) | 490 (± 180) | 420 (± 110) | 460 (± 150) | 100 (± 160) |
| 20 weeks 57 (± 290) | 58 (± 160) | 490 (± 120) | 110 (± 200) | 100 (± 110) |

![Image](image-url)

**Fig. 3.** Aβ$_{42}$-mediated inhibition of amyloid plaque formation in transgenic SwAPP mice. Age-matched control SwAPP mice (a–c) exhibited numerous 4G8-positive plaques in cortex (b) and hippocampus (c). Twenty weeks after injection of Aβ$_{42}$, aggregates into cortex of SwAPP mice (d–f), a significant reduction of 4G8-positive amyloid plaques was observed throughout the brain (d) and in cortex (e) and hippocampus (f). Twenty weeks after the injections, the injected fibrillar Aβ$_{42}$ was still detectable by 4G8 immunostaining (open arrows). Scale: 1 mm for a and d; 200 μm for b, c, e, and f.

Aβ$_{42}$-injected SwAPP mice were free of amyloid plaques at 31 weeks of age or at 20 weeks after the injection (Fig. 3, d–f). These four mice were evaluated further in this study. In these mice, 4G8-immunoreactive material was observed only around the needle tract; this material most probably represented the previous injected aggregated synthetic peptide (Fig. 3, d–e, open arrow).

To quantify the numbers of amyloid plaques, we stained for the presence of 4G8-positive amyloid plaques reacted with the anti-mouse antibodies. Neither the numbers of 4G8-stained amyloid plaques in Aβ$_{42}$-treated SwAPP mice as compared with control SwAPP littermates (Fig. 4a). To determine whether the reduced amyloid pathology was associated with reduced concentrations of transgenic Aβ, we measured the Aβ concentrations in brain tissue by a sandwich ELISA that specifically recognized intact human Aβ. No human Aβ was detected by ELISA in wild type mice. Aβ levels measured in control SwAPP mice were significantly higher than in Aβ$_{42}$-treated SwAPP mice (Fig. 4b).

In parallel, we measured serum levels of total Aβ and Aβ$_{42}$ in SwAPP mice before and 20 weeks after the injections. We found that neither total Aβ nor Aβ$_{42}$ levels differed among these two time points in any group (data not shown).

**Prevention of Amyloid Plaque Formation Was Accompanied by Reduced Reactive Astrocytosis**—Because brain amyloidosis in SwAPP mice is accompanied by reactive astrocytosis, we analyzed GFAP-reactive astrocytes in response to Aβ$_{42}$ injection (Fig. 5, a–c). Twenty weeks after injections, GFAP staining of reactive astrocytes was significantly lower in brains of Aβ$_{42}$-injected SwAPP mice (Fig. 5c) as compared with control SwAPP littermates (Fig. 5b) but significantly higher than in wild type littermates (Fig. 5a). Moreover, the clusters of GFAP-positive cells commonly found in SwAPP mice were completely absent in response to Aβ$_{42}$ injections, paralleling the absence of amyloid plaques in Aβ$_{42}$-treated mice (Fig. 3, d–f).

**Prevention of Amyloid Formation Was Independent of IgG—**An IgG-mediated immune response was implicated in the prevention of amyloid formation after active immunization of PDAPP mice against Aβ (10). Therefore, we next tested our model for the presence of endogenous immunoglobulin antibodies at the amyloid injection sites and on the few plaques that had developed after Aβ$_{42}$ injections by immunochemistry with mouse-specific anti-immunoglobulin antibodies. Neither the injected Aβ$_{42}$ remaining around the needle tract nor the few 4G8-immunoreactive plaques reacted with the anti-mouse antibodies. This was in sharp contrast to peripherally immunized mice that had abundant IgG-positive plaques (this study and...
Ref. 10). We also determined serum levels of anti-Aβ antibodies in SwAPP mice and non-tg littermates collected before and 20 weeks after the injections. We found no significant changes of anti-Aβ antibody levels because of the Aβ42 injections in any group (Fig. 6).

Although these data do not exclude a role of the immune system in the prevention of amyloid formation in our experiments, they strongly suggest that the mechanisms involved in prevention of amyloid formation differ between the intracerebral injections of Aβ42 fibrils and the peripheral immunization protocols with added adjuvants and repeated boosts.

Role of Other Aβ-degrading Enzymes—To determine whether other Aβ-degrading proteases were involved in preventing amyloid formation in our mice, we also analyzed brain tissue levels of IDE, a 100-kDa Zn-metalloproteinase that is a major Aβ-degrading enzyme in tissue culture (11, 22). 20 weeks after the injections, the IDE-1 antibody (11) revealed very little if any differences in tissue levels of IDE in response to Aβ42 injections (data not shown). Moreover, metalloprotease 24.15, another Aβ-degrading enzyme (12), was also unchanged in response to Aβ injections. Together, these data do not exclude the possible roles of IDE and 24.15 in degrading Aβ in vivo, but they clearly suggest an important role of NEP in preventing amyloid formation in our experimental model. As an additional control experiment, we tested brain tissue levels of ACE, an unrelated neuronal Zn-metalloendopeptidase (23–25) with no known affinity to Aβ (26). The ACE levels also did not differ between treated and untreated following an Aβ42 injection.

DISCUSSION

Our data show that injection of synthetic fibrillar Aβ42 into mouse brains caused sustained increases of NEP over a period of 20 weeks was associated with a dramatic reduction in brain tissue levels of Aβ and with inhibition of amyloid plaque formation in our mice. As claimed by the amyloid cascade hypothesis (3, 27), the overproduction of Aβ or the failure to remove it can lead to the formation of amyloid plaques and subsequently to neuronal damage and is crucial to the development of AD. Aβ42 fibrils are probably formed by initial "seeding" followed by increased precipitation of additional Aβ molecules into amyloid plaques (28). However, our data show clearly that the injection of pure synthetic fibrillar Aβ42 did not accelerate amyloid formation in SwAPP mice.

These data are possibly at variance with reports that found evidence for accelerated amyloid plaque formation after intracerebral infusion of AD brain extracts into SwAPP mice (29). This apparent discrepancy may be related to the fact that in those experiments a complex mixture of plaque-bearing human brain extracts was injected into SwAPP mice, whereas our injected material consisted of chemically pure synthetic Aβ42 fibrils. The possibility of existence of additional amyloidogenic factors in human brain extracts is further supported by the finding that similar seeding effect, caused by human brain extracts also occurred after immunodepletion of >80% Aβ from the extracts. Thus, the effects of injections of brain extracts on amyloid formation may be independent of Aβ and principally different from those described here. Amyloidogenic factors in human brain extract may include the heptapeptide spinorphin, the endogenous brain-specific NEP inhibitor, that was shown to slow Aβ-degradation in vivo (1, 30), thus accelerate amyloid plaque formation.

Intracranially injected Aβ can be cleared from the brain within minutes (31), possibly via receptor-mediated transport through ventricle and choroid plexus cells (32, 33). These data are consistent with two observations we made during this study, a sustained astrogliosis in hippocampus along with high 4G8 immunoreactivity in the fimbria of Aβ42-treated SwAPP mice. Taken together, these data suggest an active role of the hippocampus and the ventricular system in clearing Aβ from the brain.

We found no evidence for an involvement of IgG in mediating Aβ clearance, because in contrast to active immunization by peripheral injections of Aβ, we found no IgG in any injected brain. In addition, serum levels of anti-Aβ antibodies were unchanged before and 20 weeks after the injections. Moreover, serum levels of total Aβ and Aβ42 were identical before and after the injections. Therefore, a decrease in brain Aβ levels in our experiments were not the result of immune responses similar to those found after intramuscular or intraperitoneal immunizations against Aβ42 (10, 34, 44).

These results are in agreement with the interpretation that increased brain levels and proteolytic activities of NEP resulted in the cleavage and removal of Aβ and consequently in prevention of amyloid plaque formation in these mice. Our results are consistent with previous data showing that deleting the NEP gene in mice caused deficient cleavage of either endogenous or exogenous Aβ (35) and that elevating the expression of NEP in neurons reduced both secreted and cell-associated Aβ (36).

The mechanism that links Aβ42 to increased NEP levels is

2 L. Walker, personal communication.
unknown. Our data favor the possibility of a transcriptional activation of NEP gene expression in response to injections and a sustained stimulation of this mechanism by highly insoluble Aβ_{42} aggregates, ensuring a long term elevation of NEP protein levels in the brain. This scenario is supported by the fact that the synthetic Aβ aggregates injected into mouse brains were remarkably stable over 20 weeks following the injection and seem to be resistant to the clearing mechanism triggered here, constantly causing the induction of the unknown signal for the SE. We suggest the possibility that the increase in NEP is related to fibrils in brain tissue.

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