Amyloid β40/42 clearance across the blood-brain barrier following intra-ventricular injections in wild-type, apoE knock-out and human apoE3 or E4 expressing transgenic mice

Yong Ji[a], Bruno Permanne[a], Einar M. Sigurdsson[a,b], David. M. Holtzman[c] and Thomas Wisniewski[a,b,*]

[a]*Department of Neurology, New York University School of Medicine, New York, NY, USA*

[b]*Department of Pathology, New York University School of Medicine, New York, NY, USA*

[c]*Department of Neurology, Molecular Biology & Pharmacology, Center for the Study of Nervous System Injury, Washington University School of Medicine, St. Louis, MO, USA*

An important event in the pathogenesis of Alzheimer's disease (AD) is the deposition of the amyloid β (Aβ)1–40 and 1–42 peptides in a fibrillar form, with Aβ42 typically having a greater propensity to undergo this conformational change. A major risk factor for late-onset AD is the inheritance of the apolipoprotein E (apoE) 4 allele [3,14,31]. We previously proposed that apoE may function as a "pathological chaperone" in the pathogenesis of AD (i.e. modulate the structure of Aβ, promoting or stabilizing a β-sheet conformation), prior to the discovery of this linkage [7,40–42]. Data from apoE knockout / AβPP^{V717F} mice, has shown that the presence of apoE is necessary for cerebral amyloid formation [1, 2], consistent with our hypothesis. However, in AβPP^{V717F} mice expressing human apoE3 or E4 early Aβ deposition at 9 months is suppressed, but by 15 months both human apoE expressing mice had significant fibrillar Aβ deposits with the apoE4 expressing mice having a 10 fold greater amyloid burden [8,9]. This and other data has suggested that apoE, in addition to having a facilitating role in fibril formation, may also influence clearance of Aβ peptides. In order to address if apoE affects the clearance of Aβ peptides across the blood-brain barrier (BBB) and whether there are differences in the clearance of Aβ40 versus Aβ42, we performed stereotactic, intra-ventricular micro-injections of Aβ40, Aβ42 or control peptides in wild-type, apoE knock-out (KO) or human apoE3 or apoE4 expressing transgenic mice. We found that consistent with other studies [5], Aβ40 is rapidly cleared from the brain across the BBB; however, Aβ42 is cleared much less effectively. This clearance of exogenous Aβ peptides across the BBB does not appear to be affected by apoE expression. This data suggests that Aβ42 production may favor amyloid deposition due to a reduced clearance across the BBB, compared to Aβ40. In addition, our experiments support a role of apoE as a pathological chaperone, and do not suggest an isotype specific role of apoE in exogenous Aβ peptide clearance from the CSF across the BBB.

1. Introduction

Amyloid β (Aβ) is a 39 to 44 amino acid long peptide, which is the major component of amyloid deposits found in Alzheimer's disease (AD). Parenchymal amyloid deposits are composed mainly of Aβ peptides extending to residue 42 (Aβ42), while the major component of cerebrovascular amyloid is Aβ1–40 (Aβ40) [6, 18,21,27,38,45]. The normal soluble Aβ (sAβ) peptides that are produced by constitutive proteolytic process of the amyloid β precursor protein consist mainly of Aβ40, although sAβ also contains Aβ42 [37]. The deposition of Aβ peptides in the brain is in part related to local levels of the peptides, which reflect a

*Correspondence to: Department of Neurology, New York University Medical Center, Millhauser Laboratory, HN419, 550 First Avenue, New York, NY 10016, USA. Tel.: +1 212 263 7993; Fax: +1 212 263 6751; E-mail: thomas.wisniewski@med.nyu.edu.

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balance between synthesis and degradative/clearance pathways. Aβ42 peptides typically have a greater tendency to deposit, due to their slightly more hydrophobic nature. Prior studies have shown that Aβ40 peptides are cleared rapidly following intraventricular injections in animal models [5], but clearance of Aβ42 across the blood-brain-barrier (BBB) from the brain has not been examined. Aβ peptide fibrillization is also critically dependent on the presence of potential pathological chaperone proteins, such as apolipoprotein E4 [16,20,30,40,41]. Our work and that of several other groups has shown the involvement of apolipoproteins in the pathogenesis of AD. A major risk factor for late-onset AD is the inheritance of the apoE4 allele. ApoE4 heterozygous individuals have a threefold increased risk, while apoE4 homozygous persons have an approximately eightfold increased risk for developing late-onset AD [3,33]. Prior to discovery of this linkage, we suggested that apoE can directly interact with Aβ to promote a β-sheet conformation [41]. It has been shown that under certain *in vitro* conditions apoE can promote a β-sheet conformation in Aβ peptides [16, 30,32,40]. ApoE-Aβ complexes have been extracted from brain tissue from AD patients suggesting that this is an interaction that can take place *in vivo* [22,25,29, 44]. Data from apoE knockout mice that were crossed with AD models, AβPP$^{\mathrm{V717F}}$ and AβPPsw transgenic mice, has shown that the presence of apoE is needed for fibrillar amyloid formation, consistent with this hypothesis [2,10]. However, in AβPP$^{\mathrm{V717F}}$ mice expressing human apoE3 or E4 early Aβ deposition at 9 months is suppressed [9], but at 15 months both human apoE expressing mice have significant fibrillar Aβ deposits with the apoE4 expressing mice having a 10 fold greater amyloid burden [8]. This and other data has suggested that apoE, in addition to having a facilitating role in fibril formation, may also influence clearance of Aβ peptides. In order to address if apoE affects the clearance of Aβ peptides across the BBB and whether there are differences in the clearance of Aβ40 versus Aβ42, we performed stereotactic, intraventricular micro-injections of Aβ40, Aβ42 or control peptides in wild-type, apoE knock-out (KO) or human apoE3 or apoE4 expressing transgenic mice.

2. Materials and methods

Peptides DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV (sAβ_{1-40}), and DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA (sAβ_{1-42}) were custom synthesized at the W.M. Keck Facility at Yale University using solid-phase N-tert-butyloxycarbonyl chemistry. All-D-enantiomer of Aβ1–40 was kindly provided by Dr. C. Glabe (University of California, Irvine). Scrambled sequence Aβ1–40 was purchased from AnaSpec (San Jose, CA). Polyethylene glycol (PEG) was used as a reference compound for the BBB passage studies since it does not cross the BBB, is not taken up by brain cells and has a molecular weight similar to the Aβ peptides [5]. [^{14}C]PEG was purchased from Amersham (Arlington Heights, IL). Peptides were purified by reverse-phase high pressure liquid chromatography (HPLC) using a C18 column (Vydac, Hesperia, CA) and a linear gradient of 0–80% acetonitrile in 0.05% trifluoroacetic acid (TFA). The final product was lyophilized and characterized by analytical reverse phase HPLC, amino acid analysis, and laser desorption spectrometry. Aβ peptides were labeled with Na[^{125}I] (13.7 mCi/μg; Amersham Corp., Arlington Heights, IL) using Iodobeds (Pierce Chemical Co., Rockford, IL) following the manufacturer's instructions. To revert the potential sAβ_{1-40} oxidation at Met35 due to the radioiodination procedure, the peptide was subjected to reduction with 0.1 M dithiothreitol for 6 h before purification. Labeled peptides were separated from free iodine and oxidation derivatives by reverse-phase HPLC using a C4 narrow bore column (Vydac) and a 45-min linear gradient from 0–70% acetonitrile [17]. The absence of oxidation or other modifications of the Aβ peptides following purification was verified by laser desorption mass spectroscopy (LDMS) at the Skirball Institute at NYU School of Medicine [13,43]. Protein elution was monitored by absorbance at 280 nm and radioactivity evaluated in a gamma counter (Model 4000 Beckman Instruments, Fullerton, CA). The resulting specific activity of the [^{125}I] sAβ peptides was from 5 to 7 μCi/μg, and $>$ 97% TCA precipitable. All measurements were done in triplicate. [^{125}I] and [^{14}C] radioactivities were determined in a gamma counter and a liquid scintillation spectrometer (model LS-7500; Beckman Instruments), respectively. Changes in the secondary structure due to the radioiodination procedure were evaluated by circular dichroism. Spectra in the far ultraviolet range (260–190 nm) were recorded at 24 °C with a spectropolarimeter (model J-720; Jasco Inc., Easton, MD) using a cell path of 0.1 cm at a peptide concentration of 0.15 mg/ml in 20 mM Tris, pH 7.4. The circular dichroism analysis revealed the Aβ1–40 to be 26% α-helical, 25% β-sheet and 49% random coil. The Aβ1–42 was 9% α-helical, 36% β-sheet and 55% random

coil. The scrambled Aβ1–40 was 26% α-helical, 25% β-sheet and 49% random coil.

In order to assess for differences in the degree of aggregation following radiolabeling and to determine the intactness of the labeled peptides after intra-ventricular injection, the peptides were also subjected to SDS-PAGE. A portion of the radiolabeled peptides extracted from the CSF/brain was run in Laemmli sample buffer under reducing conditions on a 16% Tris Tricine SDS-PAGE system. After elecrophoresis, gels were dried and exposed to Kodak XOMAT AR film.

C57BL/6 mice used for these experiments were wild-type, and murine apoE$^{-/-}$. In addition, human apoE3$^{+/-}$ on an murine apoE$^{-/-}$ background and apoE4$^{+/-}$ on a murine apoE$^{-/-}$ background, which had been backcrossed 5 times to apoE$^{-/-}$ mice on a C57BL/6 background, were used [8,9,35]. Six to seven months old mice were anesthetized with a mixture of 80 mg/kg ketamine and 5 mg/kg xylazine delivered intraperitoneally. The mice were placed in Kopf stereotactic equipment. Injection coordinates measured from the bregma and the surface of the skull (anteroposterior -0.5, mediolateral $+1.0$, dorsoventral -2.2) were empirically determined based on the atlas of Franklin and Paxinos [4]. A volume of 1.0 μl was administered over 1 min by a CMA/100 microsyringe pump (Carnegie Medicin AD, Sweden), using a 26-gauge infusion needle. The number of counts of ^{125}I labeled Aβ peptide in each experiment was approximately 150,000. The cannula was left in situ for 1 min following the injection and then slowly withdrawn completely. After intervals ranging from 1 minute to 45 minutes, groups of 8 to 10 animals each were decapitated. The severed head was immediately dropped into isopentane cooled to $-45\,^{\circ}$C. By careful dissection within a chest freezer at $-20\,^{\circ}$C the frozen brain (consisting of meningeal and vascular tissue attached and ventricular and subarachnoid CSF fixed in place) was removed from the rest of the head. The percentage of radioactivity remaining within the brain versus the rest of the body was determined at 1, 5, 10, 25 and 45 minutes following injection. All measurements were done in triplicate. [^{125}I] and [^{14}C] total radioactivities and TCA precipitable counts were determined in a gamma counter (model 4000; Beckman Instruments) and a liquid scintillation spectrometer (model LS-7500; Beckman Instruments).

The data was analyzed by two-way ANOVA (GraphPad Prism version 2.01).

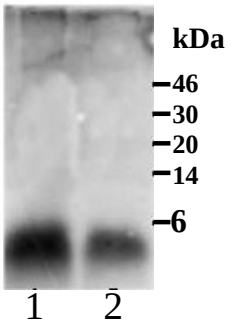

Fig. 1. [^{125}I]Aβ1–42 (lane 1) and [^{125}I]Aβ1–40 (lane 2) were run from a sample of CSF/brain 5 minutes after intra-ventricular injection on 16% Tris Tricine SDS-PAGE, in order to compare the degree of aggregation and the intactness of the [^{125}I] Aβ1–40 and [^{125}I] Aβ1–42. The gel was dried and exposed to Kodak XOMAT AR film. As can be seen from the figure, the majority of both peptides under these conditions was monomeric.

3. Results

The TCA precipitability of the radiolabeled Aβ peptides in the CSF/brain at 10 and 30 min was 95 ± 4% (mean $\pm$ SD) and 90 ± 5% at 45 min, suggesting that there was little or no proteolysis of the ^{125}I-labeled material in this compartment. However, in the plasma there was evidence of significant degradation. The TCA precipitability at 10, 30 and 45 minutes was 75 ± 8%, 54 ± 6% and 41 ± 7%, respectively.

In order to compare the degree of aggregation and the intactness of the [^{125}I] Aβ1–40 and [^{125}I] Aβ1–42, the peptides were in select animals obtained from the CSF/brain and run on SDS-PAGE. As seen in Fig. 1, where [^{125}I]Aβ1–42 (lane 1) and [^{125}I]Aβ1–40 (lane 2) were run from a sample of CSF/brain 5 minutes after injection, the majority of both peptides under these conditions was monomeric. Hence the different clearance properties of these two peptides are not likely to be related significantly to variation in aggregation.

As seen in Fig. 2 the injected [^{125}I]Aβ1-40 was rapidly cleared from the brain, with almost 50% being gone after 5 minutes. Approximately 70% is cleared after 25 minutes. This is in agreement with prior studies done in rats [5]. The clearance of [^{125}I]Aβ1–42 was significantly slower than the [^{125}I]Aβ1–40 ($p<0.001$) and did not differ from the clearance of [^{14}C]PEG. The clearance of the [^{14}C]PEG is related to bulk flow clearance of CSF from the brain. The clearance of the [^{125}I]Aβ1–40 appeared to be specific in that both the [^{125}I]all-D-enantiomer of Aβ1–40 and the [^{125}I]scrambled Aβ1–40 did not clear from the brain (see Fig. 3). Aβ peptide receptor interactions at the BBB have previously been shown to be stereoiso-

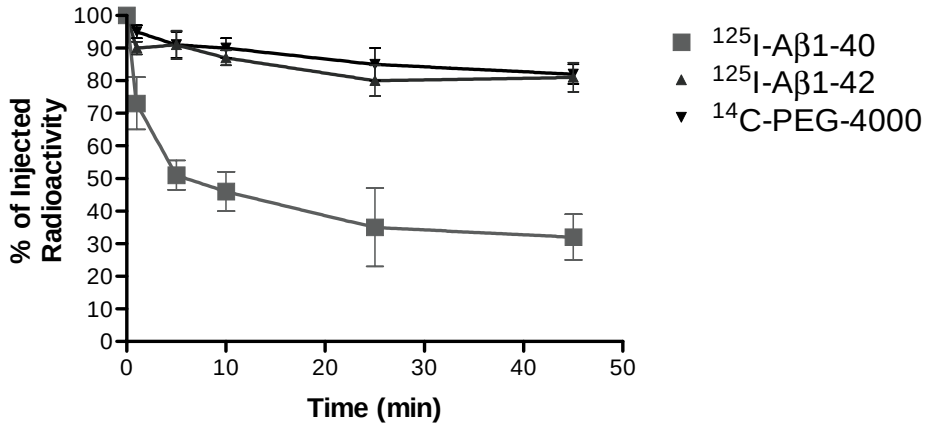

Fig. 2. Shows a plot of the percentage of radioactivity remaining in the brain, following intra-ventricular injections of ^{125}I-Aβ1–40, ^{125}I-Aβ1–42 and 14C-PEG-4000. Each point represents from 8 to 10 animals and the plotted value is the mean $\pm$ SD (bars). There is no statistically significant difference between the ^{125}I-Aβ1–42 and ^{14}C-PEG-4000, while the difference between ^{125}I-Aβ1–40 and ^{125}I-Aβ1–42 is significant ($p < 0.001$) by two-way ANOVA.

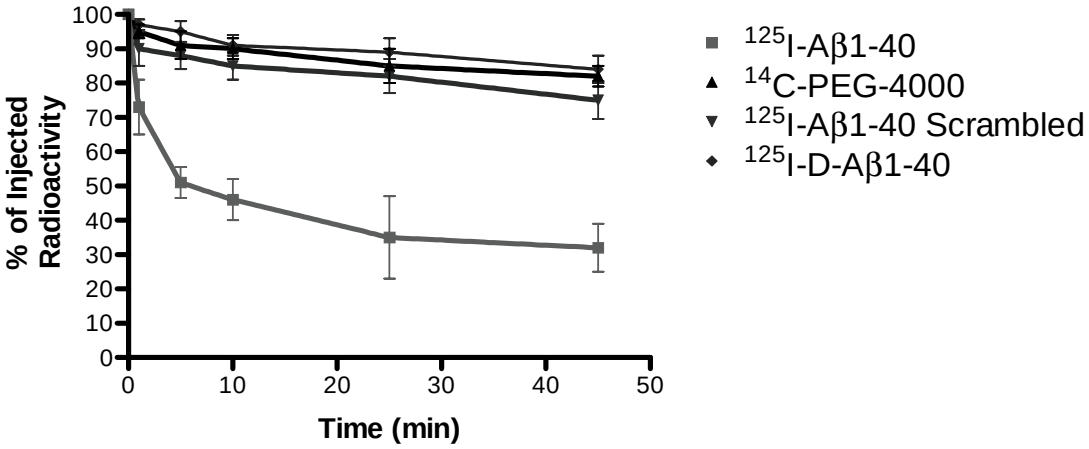

Fig. 3. Shows a plot of the percentage of radioactivity remaining in the brain, following intra-ventricular injections of ^{125}I-Aβ1–40, ^{125}I-scrambled Aβ1–40, ^{125}I-all-D-enantiomer Aβ1–40 and ^{14}C-PEG-4000. Each point represents from 8 to 10 animals and the plotted value is the mean $\pm$ SD (bars). There is no statistically significant difference between the ^{125}I-scrambled Aβ1–40, ^{125}I-all-D-enantiomer Aβ1–40 and ^{14}C-PEG-4000, while the difference between ^{125}I-Aβ1–40 and ^{125}I-scrambled Aβ1–40, ^{125}I-all-D-enantiomer Aβ1–40 and ^{14}C-PEG-4000 is significant ($p < 0.001$) by two-way ANOVA.

mer specific in the systemic circulation to brain direction [26]. The lack of clearance of the [^{125}I]scrambled Aβ1–40, compared to [^{125}I]Aβ1–40 indicates the sequence specificity of the receptor interaction.

As can be seen in Figs 4 and 5, when the [^{125}I]Aβ1–40 and [^{125}I] Aβ1–42 were injected into the apoE KO animals and transgenic mice expressing human apoE3 or E4 on a murine apoE KO background, the clearance did not differ significantly from that seen in wild-type animals. This suggests that under these conditions the expression of apoE or the lack of apoE expression in the CSF does not affect the clearance of Aβ1–40 or Aβ1–42 from the CSF to the systemic circulation.

4. Discussion

Our studies indicate that exogenous Aβ1–40 is normally rapidly cleared from the brain into the systemic circulation. This is in agreement with prior studies that have evaluated Aβ1–40 clearance from the brain in rat [5], as well as many studies that have indicated a receptor mediated transport for Aβ1–40 at the systemic circulation side of the BBB [26,46,47]. The rapid transport of the Aβ1–40 out of the brain is sequence specific in that scrambled sequence Aβ1–40 does not significantly cross the BBB and also appears to be stereoisomer specific in that D-Aβ1–40 also had

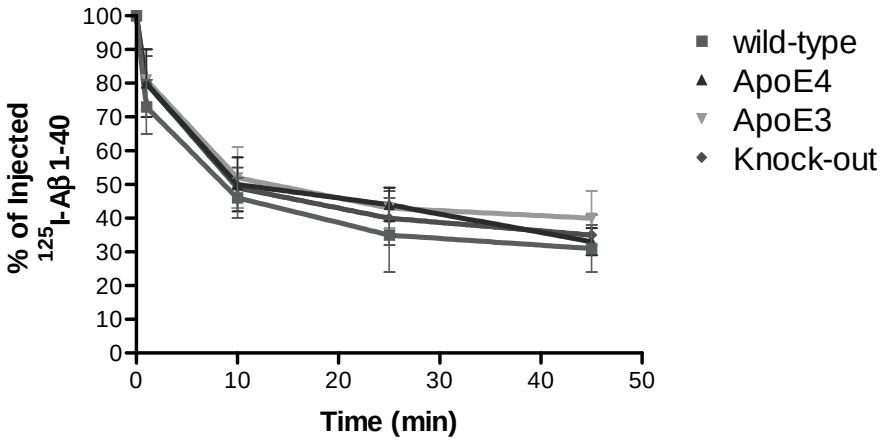

Fig. 4. Shows a plot of the percentage of radioactivity remaining in the brain, following intra-ventricular injections of ^{125}I-Aβ1–40 in wild-type mice, murine apoE knock-out (KO) mice and transgenic mice expressing either human apoE3 or E4 (on a murine apoE KO background). Each point represents from 8 to 10 animals and the plotted value is the mean $\pm$ SD (bars). There is no statistically significant difference between the clearance of the ^{125}I-Aβ1–40 in the different animal groups.

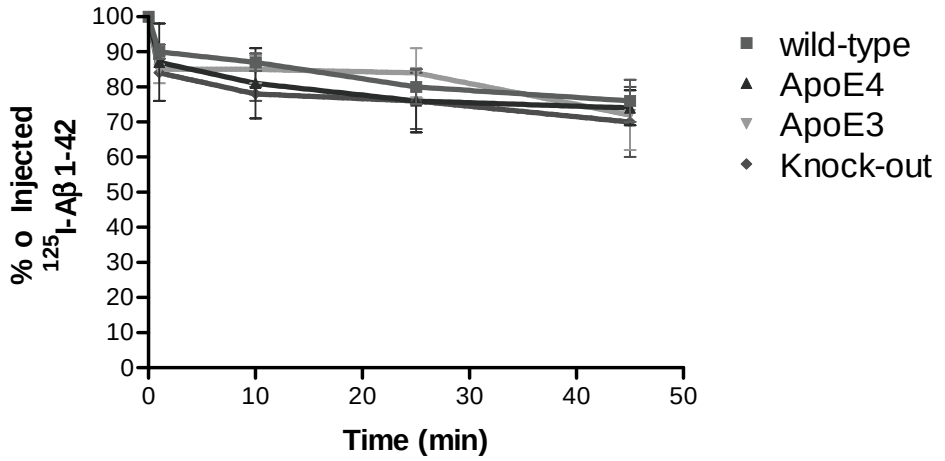

Fig. 5. Shows a plot of the percentage of radioactivity remaining in the brain, following intra-ventricular injections of ^{125}I-Aβ1–42 in wild-type mice, murine apoE knock-out (KO) mice and transgenic mice expressing either human apoE3 or E4 (on a murine apoE KO background). Each point represents from 8 to 10 animals and the plotted value is the mean $\pm$ SD (bars). There is no statistically significant difference between the clearance of the ^{125}I-Aβ1–42 in the different animal groups.

reduced clearance across the BBB. Passage of Aβ1–40 had previously been shown to be stereoisomer-specific at the circulation side of the BBB [26]. The differences in the clearance of the Aβ1–42 versus Aβ1–40 are not likely to be significantly affected by differences in aggregation of these peptides, since under the conditions of the experiment the radiolabeled peptides are mainly monomeric. The peptides are injected into the CSF, which has previously been shown to inhibit Aβ aggregation [19,39]. Therefore the different clearance properties of the Aβ1–40 and Aβ1-42 are likely to be related to the sequence difference between these two peptides and the higher β-sheet content of Aβ1–42. Our results suggest that Aβ1–40 and Aβ1–42 are cleared from the brain by distinct mechanisms. Prior studies have indicated a major catabolic pathway of Aβ1–42 in the brain involving neutral endopeptidase (NEP), which is similar to neprilysin [12]. These authors found that NEP inhibitor infusion resulted in pathological deposition of Aβ1–42 in the brain. Hence, a major clearance pathway of Aβ1–40 from the brain may be via the BBB, while the clearance of Aβ1–42 may be more dependent on catabolic pathways.

How apoE is involved in the pathogenesis of AD remains unclear; several hypotheses have been put forward. For example a differential effect on neurite out-

growth of apoE3 and apoE4 has been documented [23, 24], suggesting apoE3 is more neurotrophic than apoE4 and it has also been suggested that apoE interacts with tau [11,28]. Significant evidence exists for a direct apoE-Aβ interaction. Several studies have demonstrated that Aβ peptides bind in vitro to apoE from CSF and from cell culture medium, as well as to purified apoE [34,42], with K_D values in the low nanomolar range [7,36]. It has also been shown that Aβ-apoE complexes exist in vivo both by biochemical extraction from amyloid [22,44] and in brain fractions containing sAβ [25,29]. In addition, several studies have indicated that purified apoE enhances the formation of Aβ into amyloid fibrils, as well as its neurotoxicity with apoE4 being a greater catalyst than apoE3 [15,16,20,30,40]. The type of fibril formed also appeared to depend on the presence of apoE3 or 4 [30]. The importance of apoE for amyloid formation in vivo has clearly been shown by experiments with apoE knockout mice that were crossed with AD models, AβPP$^{\mathrm{V717F}}$ and AβPPsw transgenic mice, where it was found the formation of fibrillar amyloid deposition is dependent on the presence of apoE [1,2,10]. However, in AβPP$^{\mathrm{V717F}}$ mice expressing human apoE3 or E4 early Aβ deposition at 9 months is suppressed compared to mice expressing murine apoE [9], but by 15 months both human apoE expressing mice had significant fibrillar Aβ deposits with the apoE4 expressing mice having a 10 fold greater amyloid burden [8]. This observation has raised the possibility that in addition to having an effect on Aβ fibrillization apoE may influence Aβ's clearance. Our present experiments suggest that at least in the CSF to systemic circulation direction the expression of apoE in the CSF and brain does not significantly influence the passage of injected free Aβ peptides. However, this does not rule out the possibility that there is an isotype specific apoE role on the clearance of endogenous Aβ across the BBB or on the cell mediated clearance of Aβ. Our past and present data suggest that the role of apoE4 in AD is primarily as a pathological chaperone.

Acknowledgements

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