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Amyloidogenicity of rodent and human βA4 sequences

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Previously we have shown that aggregation of the C-terminal 100 residues (A4CT) of the βA4 amyloid protein precursor (APP) and also of βA4 itself depends on the presence of metal-catalyzed oxidation systems [T. Dyrks et al. (1988) EMBO J. 7, 949–957]. We showed that aggregation of the amyloidogenic peptides induced by radical generation systems requires amino acid oxidation and protein cross-linking. Here we report that aggregation of A4CT and βA4 induced by radical generation systems involves oxidation of histidine, tyrosine and methionine residues. The rodent βA4 sequence lacking the single tyrosine and one of the three histidine residues of human βA4 and a βA4 variant in which the tyrosine and the three histidine residues were replaced showed a reduced tendency for aggregation. Thus our results may explain why βA4 amyloid deposits could so far not been detected in the rodent brain.

A4CT; βA4; Radical; Rodent sequence; Aggregation; Alzheimer's disease

1. INTRODUCTION

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder of the human central nervous system. The neuronal dysfunction is correlated with massive deposition of fibrillar aggregates in the brain in the form of amyloid plaques [2–4]. These extracellular plaques consist mostly of a 42–43 amino acid polypeptide called the βA4 peptide, which is derived from one or several larger transmembrane proteins, i.e. the (APPs) [5]. Similar deposits have been detected in aged monkeys, dogs, and polar bears [6–10], but rarely have they been found in rats and mice [11]. The sequence of APP of mice [12] and rats [13], deduced from cDNA clones, are about 96% similar to the human APP protein [14]. Only three amino acid substitutions are found in the βA4 region of rodents: Arg→Gly, Tyr→Phe and His→Arg at βA4 positions 5, 10 and 13, respectively.

Proteolytic processing of amyloid protein precursors, leading to secretion of the extracellular part, occurs within the amyloid βA4 sequence [15–17]. This suggests that the N-terminus of the amyloid βA4 protein is not produced by the normal cleavage, which leads to secretory APP's. How βA4 is generated, and why its deposition as amyloid is largely restricted to the brain, may involve a combination of altered APP processing and the unusual amyloidogenic properties of the human βA4 sequence [18,19].

Recent reports have shown that β-A4-related peptides, corresponding to βA4 residues 1–40, are normally secreted by cultured cells and can be detected in human cerebrospinal fluid [20–23]. These βA4 related peptides are soluble and show no tendency for plaque formation or neurotoxicity.

Here we show that βA4 and A4CT, an APP fragment which is likely to be an intermediate in the pathway leading to amyloid deposition [24], depend on amino acid oxidation and protein cross-linking to be amyloidogenic. Furthermore, we demonstrate that within aggregated A4CT, histidine, tyrosine and methionine residues are oxidized by the metal-catalyzed oxidation systems. In addition we give evidence for a different aggregational behavior of the rodent βA4 sequence.

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2. EXPERIMENTAL

2.1. *Cloning procedures*

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation and bacterial transformation were carried out as described by Sambrook et al. [25].

2.2. *Plasmid construction*

Construct SP65/A4CT and the prokaryotic expression vector NCO/A4CT were constructed as described previously [26,27].

The constructions of SP65/βA4, SP65/βA4rodent and SP65/βA4H/Y were achieved by polymerase chain reaction (PCR) of the βA4 sequence using APP695 cDNA as template. Oligonucleotides 5′βA4 and 3′βA4 were used to amplify the human βA4 sequence (codons 597–638 of APP695 numbering is according to Kang et al. [5]) starting with the methionine codon 596 as initiation codon. Oligonucleotides βA4rodent and 3′βA4 were used to amplify the rodent βA4 sequence with the following substitutions Arg-601→Gly, Tyr-606→Phe and His-609→Arg. Oligonucleotides βA4H/Y and 3′βA4 were used to amplify the H/Y βA4 sequence with substitutions of all three histidine residues and the tyrosine residue (His-602→Arg, Tyr-606→Phe, His-609→Arg and His-610→Arg). The oligonucleotide sequences are shown below. The amplified cDNAs were digested with *Eco*RI/*Hin*dIII and subcloned into SP65/A4CTII. Plasmid SP65/A4CTII was obtained by cloning an oligonucleotide-linker (CATGCGGATCCATGGATGCAGAATT) into SP65/A4CT. The resulting plasmids

were digested with *Bam*HI/*Hin*dIII and the βA4-sequences were cloned into SP65 digested with the same enzymes. The resulting in vitro expression vectors SP65/βA4, SP65/βA4rodent and SP65/βA4H/Y included methionine codon 596 of APP695 as initiation codon and the human, rodent or H/Y amyloid βA4 sequence (codons 597–638 of APP695) followed by a stop codon.

The oligonucleotides used were:

5′βA4, GCAGAATTCCGACATGACTCAGG;

3′βA4, CGATGAAGCTTACGCTATGACAACACCGCCCACC;

βA4rodent, GCAGAATTCGGACATGACTCAGGATTTGAAGTTCGTCATCAAAAATTGG,

H/YβA4, GCAGAATTCCGACGTGACTCAGGATTTGAAGTTCGTCGTCAAAAATTGG.

2.3. *In vitro transcription [28]*

DNA templates (100 μg/ml) were transcribed in 40 mM Tris, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, RNasin (1 unit/μl), 100 μg/ml BSA, 500 μM each ATP, CTP and UTP, 50 μM GTP, and 500 μM m⁷G(5′)ppp(5′)G (Pharmacia, Freiburg, Germany). Typically, 1 unit of SP6 RNA polymerase (Boehringer, Mannheim, Germany) was added per μg DNA template for a 1-hour synthesis at 40°C. DTT and rNTPs stocks were prepared with diethylpyrocarbonate treated water. The components of the transcription reaction were mixed at room temperature to prevent precipitation of DNA.

Following RNA synthesis, the DNA template was removed by the addition of RNase-free DNase and after a 10 min incubation at 37°C the reaction mixtures were phenol:chloroform extracted after addition of NaOAc (pH 5.2) to 0.3 M. The RNA was precipitated with ethanol and washed with 70% ethanol.

2.4. *In vitro translation*

Translation of mRNA in a cell-free rabbit reticulocyte lysate or a wheat germ extract followed the procedures as described in the supplier's manuals (Promega, Germany). Typically, the reactions were carried out for 60 min at 30°C in the presence of 50 μCi of [^{35}S]methionine and 0.5–1.0 μg mRNA. The translation mixture was diluted with 2 × Laemmli sample buffer. After heating for 5 min at 100°C, labelled proteins were analyzed by SDS-PAGE and fluorography with EN³HANCE (DuPont).

2.5. *Hemin and hemoglobin promoted peroxidation [29]*

The hemin stock solution was always freshly dissolved in 5 mM NaOH and centrifuged for 10 min at 12,000 × *g*. The oxidation reaction was performed in the presence of 20 μM hemin and 1 mM H_2O_2, or 0.5 mg/ml hemoglobin and 1 mM H_2O_2 in PBS at 37°C for 1 h. The reaction was terminated by the addition of 2 × Laemmli sample buffer (10% β-mercaptoethanol; see SDS-PAGE) and heating for 5 min at 100°C.

2.6. *SDS-PAGE*

Analysis of in vitro translated A4CT was by SDS-PAGE and of βA4 peptides was by 16.5% Tris-tricine gel separation [30]. The gels were analyzed by flourography with EN³HANCE (DuPont, Boston).

2.7. *Prokaryotic expression and purification of bacterially expressed A4CT*

Construction and expression of prokaryotic expression vektor NCO/A4CT were performed as described [26].

2.8. *Amino acid analysis*

A4CT was hydrolyzed with 6 N HCl/0.1% phenol for 20h by 110°C under N_2 prior to analysis. Quantitative amino acids analysis was performed on an Applied Biosystems 130A/920A amino acid analyzer. For each analysis calibration was done with a standard mixture of all amino acids (200 pmol).

3. RESULTS

3.1. *Inhibition of metal-catalyzed aggregation of A4CT by specific amino acids*

Previously we showed that translation of A4CT in wheat germ extract results in a soluble non-aggregated protein which could be transformed to an aggregated molecule by incubation with hemoglobin/H_2O_2 or other metal-catalyzed oxidation systems [26]. By the addition of amino acids, vitamin C, and the vitamin E derivative trolox we were able to inhibit the aggregation of A4CT. We suggested that the aggregation of A4CT induced by radical initiation systems may be due to the oxidation of amino acids and subsequent covalent cross-linking. To verify the latter assumption we tested the inhibitory potential of different amino acids on the aggregation process.

For this purpose, A4CT was translated in the WG extract and incubated with hemoglobin/H_2O_2 in the absence or presence of different amino acids. Incubation of monomeric A4CT, translated in the WG extract, with hemoglobin/H_2O_2 resulted in the previously-reported aggregation pattern of A4CT (Fig.1, lane 2) [26]. Fig. 1, lanes 3 to 13, shows that of the amino acids tested, only histidine and tyrosine showed a significant and reproducible inhibition of the aggregation. The same results were obtained if hemin/H_2O_2 were used as the radical generation system (data not shown).

3.2. *Identification of amino acids modified by metal-catalyzed oxidation systems*

To analyze the amino acid composition of the aggregated A4CT obtained in the presence of metal-catalyzed

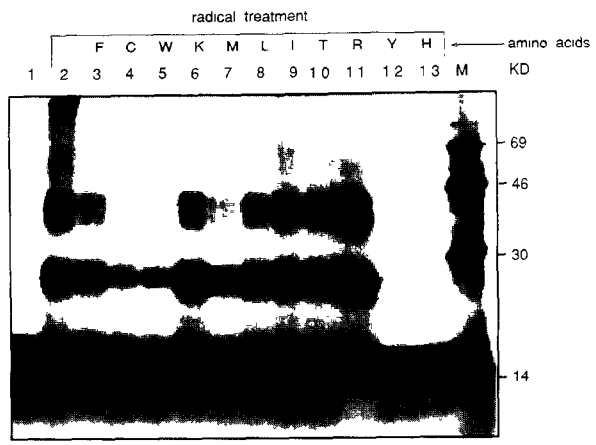


Fig. 1. Inhibition of metal-catalyzed aggregation of A4CT by specific amino acids. A4CT mRNA was transcribed from the corresponding SP6 vector, translated in the WG extract in the presence of [^{35}S]methione, precipitated with TCA and after incubation with or without 0.5 mg/ml Hb/1 mM H_2O_2 and different amino acids (30 mmol), proteins were analyzed by SDS-PAGE (12.5%). Lane 1, A4CT translated in the WG, precipitated with TCA and incubated with PBS (0.2% SDS) for 1 h at 37°C; lane 2, same as lane 1, but in the presence of 0.5 mg/ml Hb/1 mM H_2O_2; lanes 3 to 13 same as lane 2, but in the presence of the indicated amino acids (30 mmol). M, molecular mass marker; KD, kilo dalton.

Table I

Amino acid compositions of A4CT before and after radical treatment

hem/H_2O_2	–	+	A4CT/ A4CT + hem
Amino acid			
D	6,47 ± 1,75	5,73 ± 1,87	1,13
E	15,71 ± 4,16	13,37 ± 2,80	1.18
S	5,81 ± 0,54	7.26 ± 1,08	0,80
G	11.96 ± 3,89	17.13 ± 4.42	0,70
H	**3,61** ± 0,91	**1,46** ± 1,17	**2,47**
R	4,07 ± 0,24	4,67 ± 0,63	0,87
T	5,25 ± 0,38	5,53 ± 0,34	0,95
A	9,06 ± 0,74	9,23 ± 0,54	0,98
P	3,07 ± 0,47	3,50 ± 0,62	0,88
Y	**3,68** ± 0,45	**1,52** ± 0,94	**2,43**
V	8,33 ± 2,10	8,35 ± 1,12	1,00
M	**0,21** ± 0,10	**0,07** ± 0,10	**2,99**
I	4,01 ± 0,93	3,27 ± 1,66	1,23
L	7,65 ± 1,07	8,62 ± 0,97	0,89
F	4,79 ± 0,48	4,60 ± 0,51	1,04
K	6,11 ± 0,60	5,41 ± 0,79	1,13

Data are the means ± S.D. of six experiments in mol% amino acid.

oxidation systems, purified bacterial-expressed A4CT was incubated with and without hemin/H_2O_2 and the amino acid composition was analyzed by amino acid analysis. Table I shows the means ± SD of six experiments in mol% amino acid. After the radical treatment a significant decrease in mol% amino acid is only seen for histidine, tyrosine and methionine. Fig. 2 shows the quotient of mol% amino acid of untreated A4CT and A4CT after radical treatment. It is obvious that only histidine, tyrosine and methione are oxidized by the radical treatment.

3.3. *Aggregation behavior of different βA4 sequences*

Because the inhibition assay and the amino acid analysis of oxidized A4CT strongly suggest that aggregation of A4CT is induced by oxidation of histidine, tyrosine and methionine, we analyzed whether substitution of some or all of these amino acids in the βA4 sequence had any effect on the aggregation properties of βA4. Therefore we constructed SP65/βA4rodent and SP65/H/Y, in addition to SP65/βA4, which codes for the human βA4 sequence. SP65/βA4rodent codes for the rodent βA4 sequence, which shows three amino acid substitutions, two of which change one of the three histidines and the single tyrosine residue of human βA4 respectively. SP65/βA4H/Y codes for a βA4 sequence in which all three histidine residues are substituted with arginine and the single tyrosine with phenylalanine (Scheme 1).

To determine whether the different amino acid substitutions have any effect on the aggregation properties of βA4 induced by metal-catalyzed oxidation systems, we incubated the corresponding peptides, βA4human, βA4rodent, and βA4H/Y translated in the WG extract with the same radical generating systems as described previously [26].

Fig. 3, lanes 1 to 3, shows translations of the different βA4 sequences in the WG extract and analysis by Tris-tricine SDS-PAGE. Incubation of monomeric βA4human, translated in the WG extract, with hemoglobin/H_2O_2 resulted in the previously-reported aggregation pattern including three prominent bands at 4 kDa, 19 kDa and 46 kDa (Fig.4, lane 4). In contrast, the βA4rodent peptide and the βA4H/Y peptide show a clear and reproducible decrease of the aggregational bands at around 19 kDa and 46 kDa. The same results are obtained if hemin/H_2O_2 are used as radical generation system (data not shown).

Densiometric measuring of the major aggregates in Fig. 3, lanes 4, 5 and 6, shows a 4- to 5-fold decrease for βA4H/Y and βA4rodent aggregates in relation to the human sequence (Fig.4). This provides good evidence that the histidine and/or tyrosine residues of the βA4 sequence are responsible for the aggregation behavior of this amyloidogenic peptide.

4. DISCUSSION

Previously, we have presented experimental evidence that A4CT and βA4 are soluble and non amyloidogenic APP fragments. Both peptides, if expressed in the wheat germ translation system and analyzed by SDS-PAGE, resulted in only monomeric forms. Furthermore, we were able to transform monomeric A4CT and βA4 into aggregating amyloidogenic molecules by the addition of metal-catalyzed oxidation systems. This transformation could be inhibited by the addition of radical scavengers such as vitamin C, vitamin E analogue trolox and amino acids. We suggested that the aggregation of A4CT and βA4 requires amino acid oxidation and protein cross-linking induced by radical generation systems.

Rodent					GGA						TTT			CGA		
Human	GAT	GCA	GAA	TTC	CGA	CAT	GAC	TCA	GCA	TAT	GAA	GTT	CAT	CAT	CAA	AAA
Human	D	A	E	F	R	H	D	S	G	Y	E	V	H	H	Q	K
Rodent	–	–	–	–	**G**	–	–	–	–	**F**	–	–	**R**	–	–	–
H/Y	–	–	–	–	–	**R**	–	–	–	**F**	–	–	**R**	**R**	–	–

Scheme 1. Schematic diagram of the different βA4 sequences used in this study. Line 1 shows the rodent βA4-DNA sequence at codons where substitutions occur. Line 2 shows the human βA4 DNA sequence from Asp-597 to Lys-612 (numbering is according to [5]). Lines 3 to 5 represent the amino acid sequences in one letter code of βA4human, the βA4rodent and βA4H/Y form Asp-597 to Lys-612.

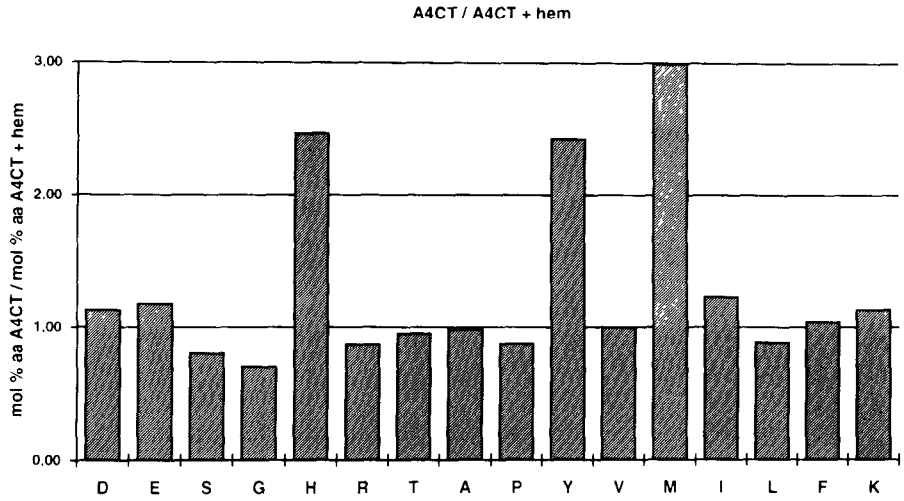


Fig. 2. Modification of the amino acid composition of A4CT by oxygen radicals. 500 pmol of purified bacterial A4CT was incubated with or without 20 μM hemin/1 mM H_2O_2 for 90 min, precipitated with methanol/chloroform and hydrolyzed with 6 N HCl/ 0.1% phenol for 20 h at 110°C under N_2. Amino acid analysis was performed on an Applied Biosystems amino acid analyzer The ordinate presents the quotient of mol% amino acid of untreated A4CT and mol% amino acid of A4CT incubated with hem/H_2O_2.

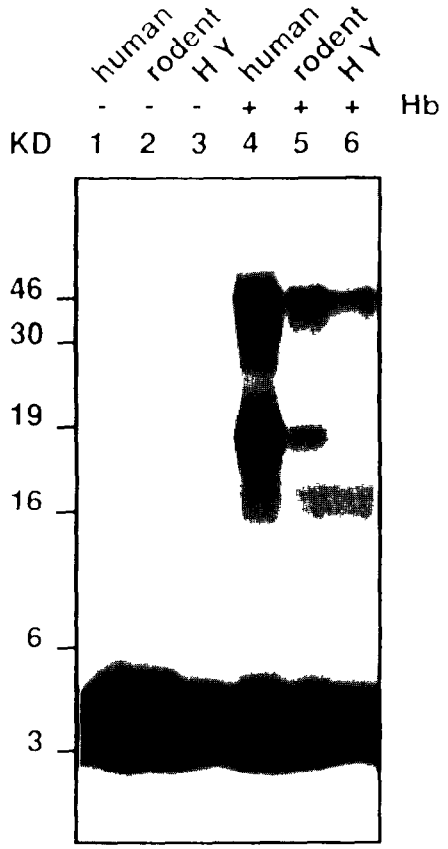


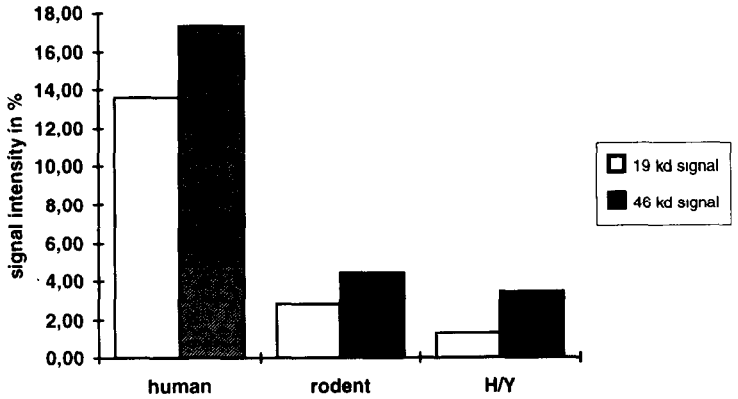
Fig. 3. Aggregation behavior of different βA4-sequences. Translation was as described in Fig 1. The different βA4 peptides were expressed in the WG extract, precipitated with TCA, and after incubation with 0,5 mg/ml Hb/1 mM H_2O_2, peptides were analyzed by Tris-tricine SDS-PAGE (16.5%). Lanes 1, 2 and 3, βA4human, βA4rodent and βA4H/Y incubated with PBS (0.2% SDS) for 1 h at 37°C; lanes 4, 5 and 6, same as lanes 1, 2 and 3, but in the presence of 0,5 mg/ml Hb/1 mM H_2O_2; KD, kilo dalton

To verify this, in the work reported here we have tested the inhibitory potential of different amino acids on the aggregation process. The specific inhibition of the aggregation by histidine and tyrosine strongly supports the idea that amino acid oxidation is involved in the βA4 aggregation process. Both amino acids are known to be particularly sensitive to modification by hydroxyl radicals [31].

Furthermore, direct evidence for the oxidative damage of these amino acids has been obtained by amino acid analysis of modified bacterial A4CT. Only methionine, histidine and tyrosine showed a 2- to 3-fold decrease after radical treatment.

From these results we suggest that those amino acids which are modified by the radical attack should also be necessary for the aggregation process. Substitution of these residues should lead to a peptide which is resistant to free radical-induced transformation into an aggregated molecule.

In the context of the on going discussion of whether the rodent βA4 sequence has an altered conformation and aggregational behavior, we analyzed the rodent βA4 sequence (βA4rodent) which contains three substitutions, two of which change histidine and tyrosine residues, and also a human βA4 sequence (βA4H/Y) without any histidine and tyrosine in our aggregation assay. We show that in the presence of radical generating systems there is a significant difference between the aggregational properties of the wild type human and the rodent βA4 sequences. Under the conditions used, only the human βA4 sequence shows a strong tendency to form higher aggregates. Thus in the presence of biological active radicals, only the human βA4 sequence was highly amyloidogenic. Because in addition the human βA4 sequence without histidine and tyrosine residues



	4 kDa	19 kDa	32 kDa
Human	100	13,61	17,33
Rodent	100	2,82	4,41
H/Y	100	1,35	3,42

Fig. 4. Densiometric measuring of the major aggregates presented in Fig. 3. The autoradiography was analyzed by scanning densitometry (Elsscript 400, Hirschmann) and the area of the signals are calculated (H1D-Calculationprogram, Hirschmann). The data represent the signal-intensity in percent of the 4 kDa signal (100%). The values are normalized to the 4 kDa band.

shows a further decrease in aggregation tendency in the presence of radicals, we suggest that the difference between aggregational behavior of the rodent and human sequence is based on the different content of histidine and tyrosine residues.

Our results are not in conflict with previously published data giving evidence that the human βA4 peptide, but not the rodent homologue, is capable of forming a β-pleated sheet structure at a low peptide concentration. Otvos et al. have already suggested that subtle interspecies amino-acid differences may account for the inability of the rodent peptide to form amyloid fibrils in situ [32]. In contrast, other results showed no difference in solubility and secondary structure of the full- and shorter sized A4 peptides derived from humans and rodents [14]. The latter studies however, did not take into account the effects of varying environmental factors such as radical attack which may contribute to the deposition process of the soluble βA4 peptide into insoluble fibrils. We propose that radical attack cross-links and stabilizes already-preformed βA4 tetramers, which then may be able to aggregate further. We show here that the substitutions in the rodent sequence are responsible for the reduction in protein cross-linking by amino acid oxidation but they also may be responsible for differences in the β-pleated sheet-forming ability of the βA4 peptide at low concentrations [32].

The results presented here provide further evidence for our previous suggestion that amino acid oxidation by metal-catalyzed oxidation systems is required to initiate βA4 aggregation in vitro and in Alzheimer's disease. The aggregation of βA4 or APP-fragments induced by radical attack may therefore be the primary event that leads to amyloid formation. After generation of a core of cross-linked amyloidogenic fragments, further APP-fragments may extend the deposition without involvement of metal-catalyzed oxidation systems. Furthermore we provide important clues as to why humans and animal species expressing the human βA4 sequence, but not rodents, are capable of forming amyloid fibrils in the brain.

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