



# In vivo localization of human acetylcholinesterase-derived species in a $\beta$ -sheet conformation at the core of senile plaques in Alzheimer's disease

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Many neurodegenerative diseases are characterized by amyloid deposition. In Alzheimer's disease (AD),  $\beta$ -amyloid (A $\beta$ ) peptides accumulate extracellularly in senile plaques. The AD amyloid cascade hypothesis proposes that A $\beta$  production or reduced clearance leads to toxicity. In contrast, the cholinergic hypothesis argues for a specific pathology of brain cholinergic pathways. However, neither hypothesis in isolation explains the pattern of AD pathogenesis. Evidence suggests that a connection exists between these two scenarios: the synaptic form of human acetylcholinesterase (hAChE-S) associates with plaques in AD brains; among hAChE variants, only hAChE-S enhances A $\beta$  fibrillization *in vitro* and A $\beta$  deposition and toxicity *in vivo*. Only hAChE-S contains an amphiphilic C-terminal domain (T40, AChE<sub>575–614</sub>), with AChE<sub>586–599</sub> homologous to A $\beta$  and forming amyloid fibrils, which implicates T40 in AD pathology. We previously showed that the amyloid scavenger, insulin-degrading enzyme (IDE), generates T40-derived amyloidogenic species that, as a peptide mixture, seed A $\beta$  fibrillization. Here, we characterized 11 peptides from a T40–IDE digest for  $\beta$ -sheet conformation, surfactant activity, fibrillization, and seeding capability. We identified residues important for amyloidogenicity and raised polyclonal antibodies against the most amyloidogenic peptide. These new antisera, alongside other specific antibodies, labeled sections from control, hAChE-S, hAPP<sup>swe</sup>, and hAChE-S/hAPP<sup>swe</sup> transgenic mice. We observed that hAChE-S  $\beta$ -sheet species co-localized with A $\beta$  in mature plaque cores, surrounded by hAChE-S  $\alpha$ -helical species. This observation provides the first *in vivo* evidence of the conformation of hAChE-S species within plaques. Our results may explain the role of hAChE-S in A $\beta$  deposition and aggregation, as amyloidogenic hAChE-S  $\beta$ -sheet species might seed A $\beta$  aggregation.

A rising number of pathologies of great public health concern (e.g. Alzheimer's disease (AD)<sup>3</sup> and type II diabetes) result

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This article contains Figs. S1–S7 and supporting Experimental procedures.

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<sup>3</sup> The abbreviations used are: AD, Alzheimer's disease; AChE, acetylcholinesterase; A $\beta$ , amyloid- $\beta$  peptide; APP, amyloid precursor protein; a.u., arbitrary

unit; hAChE-S, synaptic form of the human acetylcholinesterase; HHL, hydrophobic–hydrophilic interface; HRP, horseradish peroxidase; IDE, insulin-degrading enzyme; KLH, keyhole limpet hemocyanin; Mab, monoclonal antibody; ThS, thioflavin S; ThT, thioflavin T; DAB, 3,3'-diaminobenzidine; NFT, neurofibrillary tangle; h, human.

from polypeptide misfolding, ordered aggregation, and aggregate deposition, known as amyloids (1). Although amyloids originate from distinct polypeptides and share no sequence homology, they have similar properties (e.g. cross- $\beta$ -sheet structure) (2, 3). Amyloidogenesis is driven by properties of the sequence backbone and specific side-chain interactions (4–7). AD hallmarks are extracellular deposits of  $\beta$ -amyloid peptides (A $\beta$ ) in senile plaques and intracellular deposition of hyperphosphorylated tau in neurofibrillary tangles (NFT) (8). A $\beta$  originates from sequential proteolysis of human amyloid precursor protein (hAPP). Various mutations in hAPP lead to hereditary AD forms (e.g. Swedish mutation (hAPP<sup>swe</sup>)). The amyloid cascade hypothesis of AD proposes that A $\beta$  is ultimately responsible for pathology during which increased A $\beta$  production or reduced clearance leads to toxicity (1). Although elevated plaque burden is the histological criterion for AD diagnosis, only the level of soluble A $\beta$  found in cerebrospinal fluid correlates with disease severity.

Senile plaques in AD brain also contain "secondary" molecules, among which is the synaptic form of human acetylcholinesterase (hAChE-S). The cholinergic hypothesis sees AD as a specific pathology of brain cholinergic pathways: acetylcholine-mediated neurotransmission is selectively and severely impaired or lost, basal forebrain cholinergic neurons are the most affected, normal hAChE-S hippocampal and cortical fiber networks are severely reduced, and hAChE-S inhibition for 1 year with donepezil in suspected prodromal AD patients decreases the rate of hippocampal atrophy by 45% (9–17). Unfortunately, neither the amyloid cascade nor the cholinergic hypothesis alone explains the full extent of AD pathogenesis. Evidence of a connection includes the following: altered hAChE-S (catalytically, oligomerization and glycosylation) is associated with AD plaques and tangles; hAChE-S enhances A $\beta$  fibrillization *in vitro* (leading to stable complexes that alter its catalytic activity) and A $\beta$  toxicity *ex vivo* and *in vivo*, and mice expressing both hAPP<sup>swe</sup> and hAChE-S show earlier disease onset than single transgenic mice (18–25). The spread of senile plaque-containing hAChE-S beyond brain areas rich in cholin-

ary unit; hAChE-S, synaptic form of the human acetylcholinesterase; HHL, hydrophobic–hydrophilic interface; HRP, horseradish peroxidase; IDE, insulin-degrading enzyme; KLH, keyhole limpet hemocyanin; Mab, monoclonal antibody; ThS, thioflavin S; ThT, thioflavin T; DAB, 3,3'-diaminobenzidine; NFT, neurofibrillary tangle; h, human.

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ergic innervation also suggests that hAChE-S role in plaque formation goes beyond cholinergic activity.

Several hAChE variants result from alternative splicing (hAChE-S and hAChE-R, for readthrough) or alternative promoter usage (N-AChE-S and N-AChE-R, with an extended N terminus) (26). In contrast to the synaptic variants, the readthrough variants delay A $\beta$  fibrillization and tau hyperphosphorylation, and inversely affect plaque burden in transgenic mice (hAPP<sup>swe</sup>–hAChE-R *versus* hAPP<sup>swe</sup>–hAChE-S) (21). The two variants only differ in their C-terminal domains, with only hAChE-S containing the amphiphilic 40-residue C-terminal domain (T40, AChE<sub>575–614</sub>), implicating T40 in AD pathology (27). T40's classic role is to mediate oligomerization to form a variety of hAChE-S isoforms (*e.g.* soluble and amphiphilic, membrane-bound or anchored to the extracellular matrix). Intact, T40 is nonamyloidogenic,  $\alpha$ -helical, and does not promote A $\beta$  assembly (28, 29). T40 has a region, AChE<sub>586–599</sub>, with shared homology to A $\beta$ , which as a peptide under physiological conditions adopts  $\beta$ -sheet conformation, is amyloidogenic, and promotes A $\beta$  nucleation (28, 30, 31). *In vitro*, T40-derived amyloid species are generated by insulin-degrading enzyme (IDE), a protease implicated in A $\beta$  processing and genetically linked to AD. IDE–T40 peptides adopt  $\beta$ -structures, are amyloidogenic, and promote A $\beta$  assembly (28). C-terminal T40 proteolysis of hydrophilic monomers and tetramers of bovine AChE-S occurs *in vivo* (32). Also, naturally occurring soluble monomeric G1 AChE from fetal bovine serum, but not the tetrameric G4 form, lacks the T40 domain (33). These studies suggest that *in vivo* T40 is vulnerable to proteolysis even in tetramers, and cleaved T40 from G1 AChE can be further digested to generate amyloidogenic species.

Amyloidogenesis is a nucleation-dependent polymerization with an energetically unfavorable lag phase, an elongation phase (nuclei extension by monomer addition), and a plateau phase when fibril extension ends (3). To promote nucleation, initial triggers may be required. For example, during seeding, homologous or heterologous amyloidogenic species provide a conformationally competent template to accelerate initial misfolding and formation of early assembly intermediates, including toxic oligomers (3). *In vitro* and *in vivo* seeding occurs in various amyloid systems, including A $\beta$  (34–36). Plaque-associated hAChE-S may promote heterologous seeding of A $\beta$ , which would accelerate toxic oligomer formation and might result in stable A $\beta$ –hAChE-S hetero-complexes (19). This seeding hypothesis may represent a previously undescribed pathological trigger for more severe A $\beta$  pathology during AD, in which A $\beta$  abundance is not the only driving factor. Thus, detailed dissections of the protagonists involved, including their recruitment, formation, and participation in amyloidogenesis, should provide invaluable insights for targeting and controlling diseases involving toxic protein aggregation and deposition.

Here, we investigated the *in vivo* implication of hAChE-S in AD pathology, using a double transgenic mouse model for hAChE-S/hAPP<sup>swe</sup> (Tg2576 background, APP<sub>695</sub> isoform) (37). In contrast to hAPP<sup>swe</sup> single transgenic mice, these mice develop more numerous, frequent, and early amyloid plaques (6 months), which contain A $\beta$ <sub>1–40</sub>, A $\beta$ <sub>1–42</sub>, markers of activated

microglia and reactive astrocytes, and hAChE-S, as do natural AD amyloid deposits (37). The extent of amyloid burden was also tightly correlated with memory impairment, more so than in the hAPP<sup>swe</sup> single transgenic mice (18). In contrast to transgenic mice with various forms of hAPP, which only address A $\beta$ -related pathology, the presence of the hAChE-S transgene also confirms changes resulting in an imbalance of cholinergic function and neurotransmission. Indeed, overexpression of hAChE-S in transgenic mice triggers a progressive decline in spatial learning and memory, cessation of dendritic branching, reduced number of spines in cortical neurons, and enhanced high-affinity choline uptake, all of these being hallmarks of cholinergic malfunction and characteristics reported in senile dementia (38, 39). Opposite effects were observed in mice transgenic for hAChE-R (40).

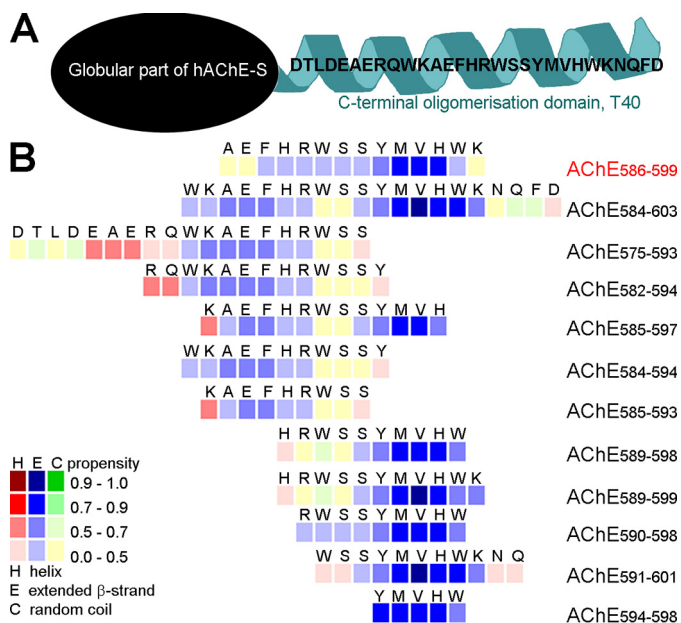
This study used synthetic peptides corresponding to products of IDE proteolysis on hAChE-S T40 to fully characterize their potential in terms of  $\beta$ -sheet propensity and conformation, surfactant activity, fibrillization, co-fibrillization, and seeding of A $\beta$ . We established the importance of sequences or residues underlying plaque assembly, and we selected the most amyloidogenic peptide for raising specific antibodies. The new antisera were characterized for specificity and used with other specific antibodies to immunolabel coronal brain sections from 12-month-old WT, hAChE-S single transgenic, hAPP<sup>swe</sup> single transgenic (Tg2576, APP<sub>695</sub> isoform), and hAChE-S/hAPP<sup>swe</sup> double transgenic mice. Mature plaques in cerebral cortex contained hAChE-S and A $\beta$ , as in AD. Importantly, hAChE-S  $\beta$ -sheet species were detected in plaque cores, surrounded by  $\alpha$ -helical forms. Plaque hAChE-S  $\beta$ -sheet species co-localized with fibrillar amyloid material and A $\beta$ . These findings provide the first *in vivo* evidence of hAChE-S conformation in senile plaques.

## Results

We previously established that IDE proteolysis of T40 generates amyloid species seeding A $\beta$  fibrillization (28). Now we have characterized a set of individual synthetic peptides identified from a T40–IDE digest, rather than as a peptide mixture. Two new antisera were generated by immunization with the 'best' amyloidogenic T40–IDE peptide, to use along with other specific antibodies to label brain sections of control mice, hAChE-S, hAPP<sup>swe</sup>, and hAChE-S/hAPP<sup>swe</sup> transgenic mice.

### Key residues for $\beta$ -sheet propensity and conformation

The  $\beta$ -sheet formation is one driver of amyloidosis in AD. It is important to assess the propensity of T40–IDE peptides for conversion to non-native (hidden)  $\beta$ -strands. We used the algorithm of Yoon and Welsh (31), who predicted minimal amyloidogenic regions for  $\alpha$ -synuclein and A $\beta$  and showed AChE<sub>586–599</sub> as an hAChE-S region with high  $\beta$ -strand propensity. Previously, we identified YMVH as the AChE<sub>586–599</sub> sequence with the strongest propensity for  $\beta$ -strand conversion, and Trp<sup>591</sup>, Tyr<sup>594</sup>, Met<sup>595</sup>, Val<sup>596</sup>, and Trp<sup>598</sup> as crucial to maintain high  $\beta$ -strand propensity along the entire sequence (28). Applied to T40–IDE peptides, the algorithm revealed that  $\beta$ -strand conversion depends on surrounding sequences and



**Figure 1. hAChE-S T40 and propensity of the T40-IDE synthetic peptides for conversion to non-native (hidden)  $\beta$ -strand.** A, schematic of monomeric hAChE-S showing the exposed C-terminal oligomerization domain, T40, in its native  $\alpha$ -helical conformation. B, method developed by Yoon and Welsh (31) was used. Propensities are presented numerically with low values indicating zero to low propensity and high values indicating high propensity to near certainty. Propensity for helices (red squares),  $\beta$ -strands (blue squares), and random coil (green squares) is shown. We have used AChE<sub>586-599</sub> as a benchmark, which is shown in red font.

identified two regions, WKAEFHR and SYMVHWK (Fig. 1). Trp<sup>591</sup> and Ser<sup>592</sup> were predicted as random coils, unlike their configuration in the AChE<sub>586-599</sub> context. It was also evident that T40 N terminus (DTLDEAERQ) and C terminus (NQFD) have propensities for both random coil and  $\alpha$ -helix, and that His<sup>589</sup> has a negative effect on the following RWSS. The two peptides with the strongest predicted propensity for  $\beta$ -strand conversion are AChE<sub>590-598</sub> (RWSSYMVHW) and AChE<sub>594-598</sub> (YMVHW).

We further analyzed the T40-IDE peptides using three other algorithms, which confirmed the findings from the Yoon and Welsh algorithm (Fig. S1-S3). CamSol, which predicts protein solubility and generic aggregation propensity, identified SYMVHWK as being poorly soluble, followed by KA EFHR, the T40 N and C termini being highly soluble, and His<sup>589</sup>-Arg<sup>590</sup> to have negative effects on the following WSS (Fig. S1) (41). TANGO predicts  $\beta$ -sheet aggregation, percentage occupancy of  $\beta$ -sheet aggregation,  $\beta$ -sheet, and  $\alpha$ -helical conformations (42). TANGO identified AEFHR and YMVHW as having  $\beta$ -sheet aggregation propensity; AChE<sub>585-597</sub> and AChE<sub>590-598</sub> (the immunization peptide used later in the study) as having the two highest aggregation propensities, and His<sup>589</sup> along with T40 N terminus as having a negative impact (Fig. S2). WALTZ, which identifies protein motifs that nucleate amyloid fiber formation, recognized RWSSYMV as having amyloid propensity (Fig. S3) (43).

Next, we used far-UV CD to follow conformational changes of T40-IDE peptides before (acidic pH) or during aggregation (neutral pH). In previous studies, AChE<sub>586-599</sub> was random coil

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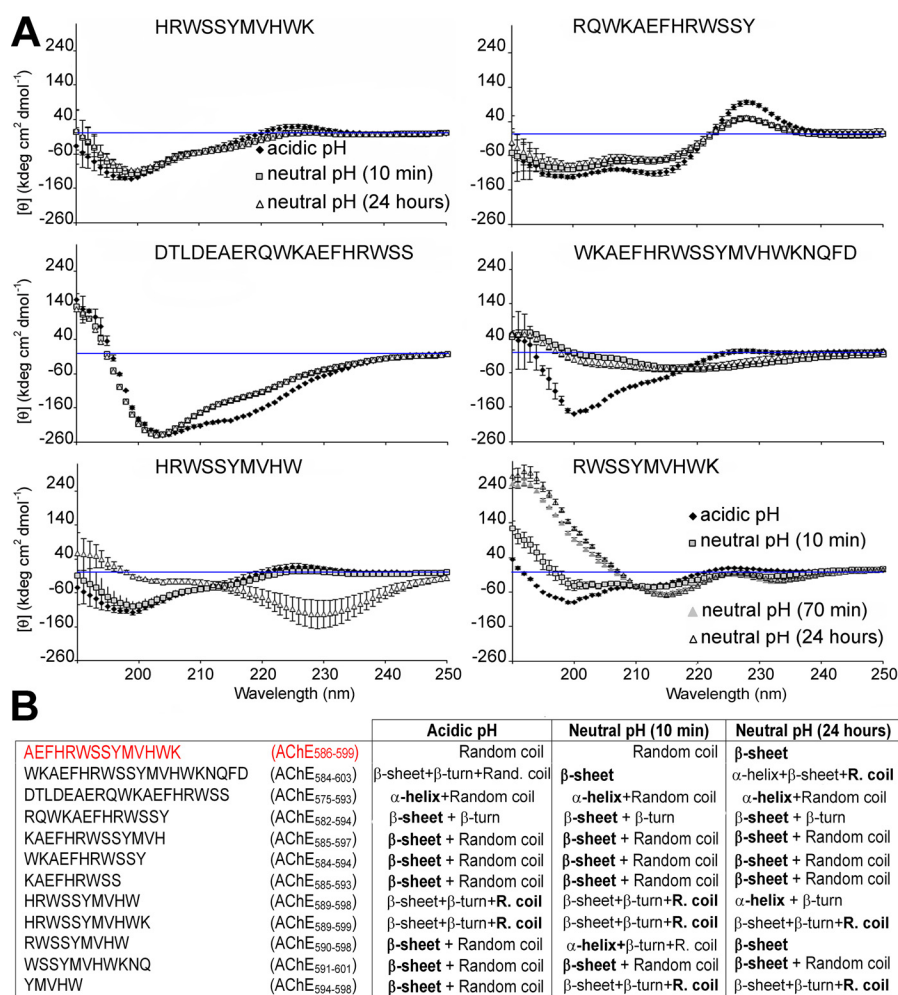
when nonaggregated but  $\beta$ -sheet on neutralization. Phe<sup>588</sup>, Trp<sup>591</sup>, Ser<sup>592</sup>, Ser<sup>593</sup>, Tyr<sup>594</sup>, Met<sup>595</sup>, Val<sup>596</sup>, and Trp<sup>598</sup> were all important in the conformational transition (28, 30). Fig. 2 and Fig. S4 represent and summarize the conformational changes. At acidic pH, all peptides but AChE<sub>582-594</sub> had some random coil conformation (negative ellipticity  $\sim$ 200 nm or less). All peptides but AChE<sub>575-593</sub> ( $\alpha$ -helical, double-negative ellipticity minima  $\sim$ 208 and 220 nm) had partial  $\beta$ -structures, with the majority having a dominant  $\beta$ -sheet conformation (double-negative ellipticity minima at 200 and 215 nm). After 24 h of neutralization, three peptides still showed dominant random coil conformations: AChE<sub>584-603</sub>, AChE<sub>589-599</sub>, and AChE<sub>594-598</sub>. Another AChE<sub>575-593</sub> remained dominantly  $\alpha$ -helical, and AChE<sub>589-598</sub> underwent a conformational change from a dominant random coil to dominantly  $\alpha$ -helical. In contrast, AChE<sub>590-598</sub> only adopted  $\beta$ -sheet conformation, rather than a mixed  $\beta$ -sheet/random coil. In term of adoption and/or conformational change to  $\beta$ -sheet conformation, these results indicate negative effects of the T40 N and C termini (DTLDEAERQ in AChE<sub>575-593</sub> and NQFD in AChE<sub>584-603</sub>) and the detrimental effect of His<sup>589</sup> (AChE<sub>589-598</sub>/AChE<sub>589-599</sub> versus AChE<sub>590-598</sub>). We also found that <sup>582</sup>RQ<sup>583</sup> are important for adoption of a partial  $\beta$ -turn rather than a partial random coil conformation (AChE<sub>582-594</sub> versus AChE<sub>584-594</sub>).

A further question was whether the nondominant  $\beta$ -sheet T40-IDE peptides would adopt a dominant  $\beta$ -sheet conformation at high concentrations or whether membrane-mimicking SDS was present (Fig. 3). For AChE<sub>584-594</sub> (WKA EFHRWSSY), the double concentration caused a transition from weak dual random coil/ $\beta$ -sheet to dominant  $\beta$ -sheet (Fig. 3A). With SDS present, AChE<sub>584-594</sub> remained a mixed random coil/ $\beta$ -sheet (Fig. 3B). For AChE<sub>589-598</sub> (HRWSSYMVHW), which was in  $\beta$ -sheet- $\beta$ -turn-random coil conformation after a 10-min neutralization, double concentration converted it to a strong dominant  $\beta$ -sheet conformation (Fig. 3A). In the presence of SDS, AChE<sub>594-598</sub> (YMVHW) converted from a dominant random coil to a dominant  $\beta$ -sheet (Fig. 3B). These results highlight the importance of local peptide context. Indeed, changing concentrations or the presence of local membranes are likely to influence conformation and in turn amyloidogenesis. Order of  $\beta$ -sheet competence is as follows: AChE<sub>590-598</sub> > AChE<sub>582-594</sub> > AChE<sub>585-593</sub> = AChE<sub>584-594</sub> > AChE<sub>591-601</sub>.

### Key fibrillization residues

To study fibrillization kinetics of T40-IDE peptides, we used a classical amyloid dye, ThT, that intercalates in  $\beta$ -sheet amyloid structures, and we assessed changes in its fluorescence emission (44). Shaking was used to accelerate fibrillization and was necessary for some peptides. AChE<sub>586-599</sub> was a positive control with well-established fibrillization kinetics (28). AChE<sub>586-599</sub> fibrillization showed biphasic behavior: first, rapid assembly (<1 h) followed by a short plateau and decreased ThT signal, and a second slower assembly (>5 h) followed by the same effects (Fig. 4A). This behavior was previously shown to be triggered by assay shaking and thought to arise from susceptibility to breakage by shear forces or increasing surface area and peptide recruitment (28). Also as previously found, the decay of ThT signal after the plateau sug-

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**Figure 2. Secondary structure of T40-IDE peptides.** Far-UV spectra (250 to 190 nm) before and after pH neutralization (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2) of 100 μM T40-IDE peptides. At least three independent assays were performed. *A*, representative spectra of the different structures observed. The blue line indicates 0 M ellipticity. *B*, conformation at acidic pH and conformational changes after pH neutralization for 100 μM T40-IDE peptides. *R. coil*, random coil. The dominant secondary structure, if any, is in bold. We have used AChE<sub>586-599</sub> as a benchmark, which is shown in red font.

gested that AChE<sub>586-599</sub> assembly was not stable under these conditions.

At 100 μM, only six peptides fibrillized, all (except AChE<sub>585-597</sub>) with significantly longer nucleation (lag phase), leading to a much lower plateau than AChE<sub>586-599</sub> (Fig. 4, A–C). For each, except AChE<sub>585-597</sub>, the assembly was stable, and plateau ThT signals did not decay. The order of fibrillization competence was as follows: AChE<sub>585-597</sub> > AChE<sub>590-598</sub> > AChE<sub>589-598</sub> > AChE<sub>591-601</sub> > AChE<sub>584-594</sub> > AChE<sub>584-603</sub>. At 200 μM, the lag phase of the poorest peptide, AChE<sub>584-594</sub>, was significantly reduced (~13.3 to ~2.6 h), and its plateau height more than doubled (1155 to 2699 a.u.) (Fig. 4D). At 200 μM, AChE<sub>589-599</sub> fibrillized but slowly and weakly (lag phase of 16.7 h and plateau height of 333 a.u.) (Fig. 4D).

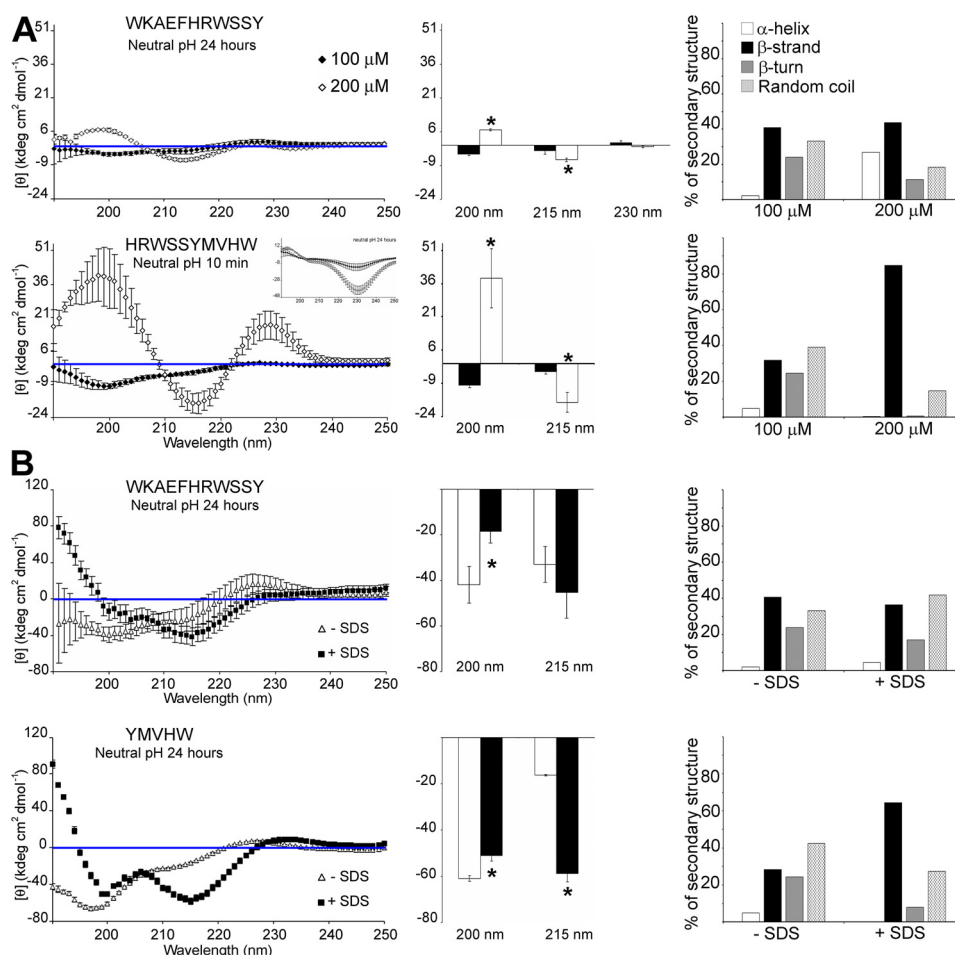
We then examined the aggregation status of the fibrillizing T40/IDE peptides by EM (Fig. 5). All fibrillizing peptides were clearly aggregated into dense meshworks of fibrils (*top panel* for each peptide), with individual typical amyloid fibrils clearly identifiable (*lower panel* for each peptide).

Most fibrillizing peptides adopted a degree of β-sheet conformation after neutralization. The nonfibrillizing AChE<sub>582-594</sub> showed both a β-sheet and β-turn conformation, the latter pos-

sibly hindering amyloid cross-β-sheet formation. Similarly, the nonfibrillizing AChE<sub>589-599</sub> and AChE<sub>594-598</sub> mainly adopted a random coil conformation but also had some β-sheets and β-turns. These results indicate the importance of YMVH as only T40-IDE peptides containing it fibrillized (except AChE<sub>584-594</sub>).

### AChE<sub>590-598</sub> and AChE<sub>585-597</sub> are seeds for fibrillization of other AChE-derived peptides

AChE<sub>585-597</sub> (KAEFHRWSSYMVH) and AChE<sub>590-598</sub> (RWSSYMVHW) were the two best in terms of fibrillization. As these two peptides were unlikely to fibrillize by themselves *in vivo*, we investigated whether the monomers could interact with other T40-IDE peptides and AChE<sub>586-599</sub> monomers to enhance fibrillization. Co-fibrillization of T40-IDE peptide monomers with 50 μM AChE<sub>590-598</sub> or AChE<sub>585-597</sub> monomers triggered fibrillization of T40-IDE peptides that would not fibrillize by themselves at either 50 or 100 μM (AChE<sub>575-593</sub>, AChE<sub>582-594</sub>, AChE<sub>585-593</sub>, AChE<sub>589-599</sub>, and AChE<sub>594-598</sub>), or at 50 μM (AChE<sub>584-594</sub>) (Fig. 6). In contrast to AChE<sub>585-597</sub>, AChE<sub>590-598</sub> also shortened the lag phase and increased the plateau height of AChE<sub>586-599</sub>.



**Figure 3. Secondary structure of T40-IDE peptides.** Far-UV spectra (250 to 190 nm) after pH neutralization (50 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.2) of 100 and 200  $\mu\text{M}$   $\text{AChE}_{584-594}$  and  $\text{AChE}_{589-598}$  (A) or 100  $\mu\text{M}$   $\text{AChE}_{584-594}$  and  $\text{AChE}_{594-598}$  in the absence or presence of 10 mM SDS (B). CD spectra (left panel) quantification of the signals at 200, 215, and 230 nm (middle panel), and percentage of secondary structures (right panel). At least three independent assays were performed. The blue line indicates 0 m ellipticity. \*,  $p < 0.03$  when compared with 100  $\mu\text{M}$  peptide (A) or without SDS (B).

### Key residues for surface activity

Many amyloids are amphiphilic and surface-active, allowing efficient adsorption to hydrophobic-hydrophilic interfaces (HHI) (45–52). HHIs act as fibrillation catalysts due to a concentrating effect and promotion of peptide chain alignments that favor  $\beta$ -sheet formation. This is important because *in vivo* HHIs, cellular membranes (phospholipid bilayers with a hydrophilic outer layer and a hydrophobic core), are structurally and functionally damaged by amyloids (1, 8). Because surface activity plays a major role in amyloid toxicity, it was important to assess surface activity of the T40-IDE peptides. We used a previously described technique based on an off-axial light beam to measure the meniscus lensing effect (47). Normalizing the apparent optical density at the offset using optical density measured on the central axis ( $\Delta\text{OD}$ ) gave values that correlated strongly and inversely with surface tension ( $R > 0.97$ ) (47, 53).

$\text{AChE}_{586-599}$  surfactant activity was previously found highly pH-dependent, with little surface activity at acidic pH, likely due to histidine protonation (47). Hence, we began testing surface activity of the T40-IDE peptides by measuring differential absorbance ( $\Delta\text{OD}$ ) at acidic and neutral pH (Fig. 7, A and C).  $\text{AChE}_{584-603}$ ,  $\text{AChE}_{575-593}$ , and  $\text{AChE}_{585-593}$  had weaker surface activity (smaller  $\Delta\text{OD}$ ) than  $\text{AChE}_{586-599}$ , and  $\text{AChE}_{594-598}$

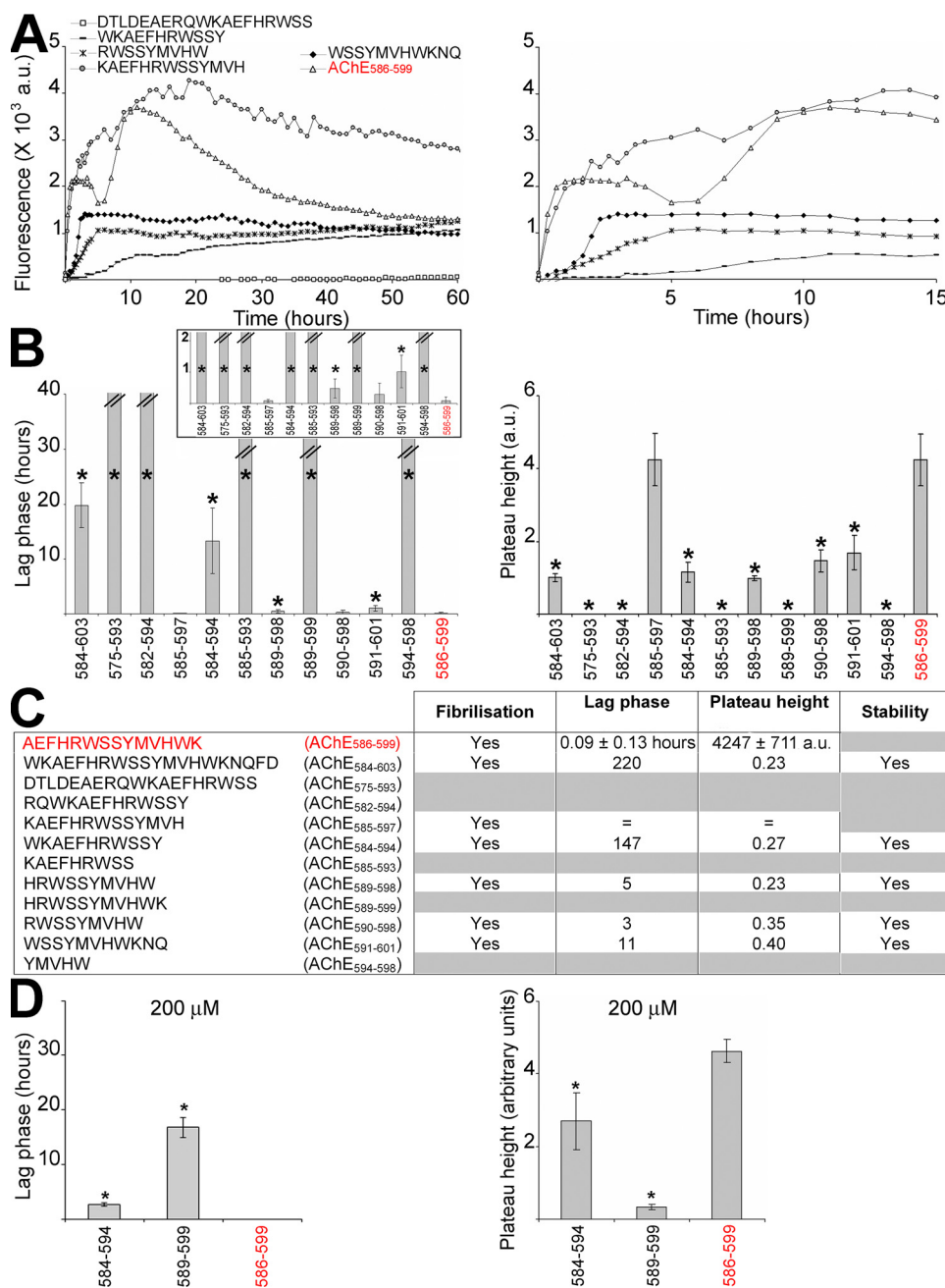
surface activity was similar to that of  $\text{AChE}_{586-599}$ . All other peptides were more surface-active than  $\text{AChE}_{586-599}$  (higher  $\Delta\text{OD}$ ) after 2 min at neutral pH.

Assessing temporal patterns of surfactant activity after neutralization, most peptides were not stably surface-active (Fig. 7, B and C), and their activity dropped readily. This suggests that they quickly adsorbed at the air-water interface after neutralization, but subsequent partial decreases in surface activity may reflect conformational changes at the air-water interface to reduce effects on surface energy. In contrast, surface activities of  $\text{AChE}_{586-599}$ ,  $\text{AChE}_{584-603}$ ,  $\text{AChE}_{590-598}$ , and  $\text{AChE}_{591-601}$  were stable, suggesting these peptides remained associated with the air-water interface all along. Orders of competent surface activity were as follows:  $\text{AChE}_{590-598} > \text{AChE}_{591-601} = \text{AChE}_{582-594} > \text{AChE}_{585-597} > \text{AChE}_{584-594} = \text{AChE}_{589-599} > \text{AChE}_{589-598} > \text{AChE}_{594-598}$ .

### T40-IDE peptides act as heterologous seeds to promote A $\beta$ fibrillization

We examined the ability of the T40-IDE peptides to promote A $\beta$  fibrillization. We used 2  $\mu\text{M}$  peptide seeds, not monomers, to assess heterologous seeding of 15  $\mu\text{M}$  A $\beta$ . These conditions were identical to Jean *et al.* (28) and Diamant *et al.* (29),

## Conformation of hAChE-S species in senile plaques

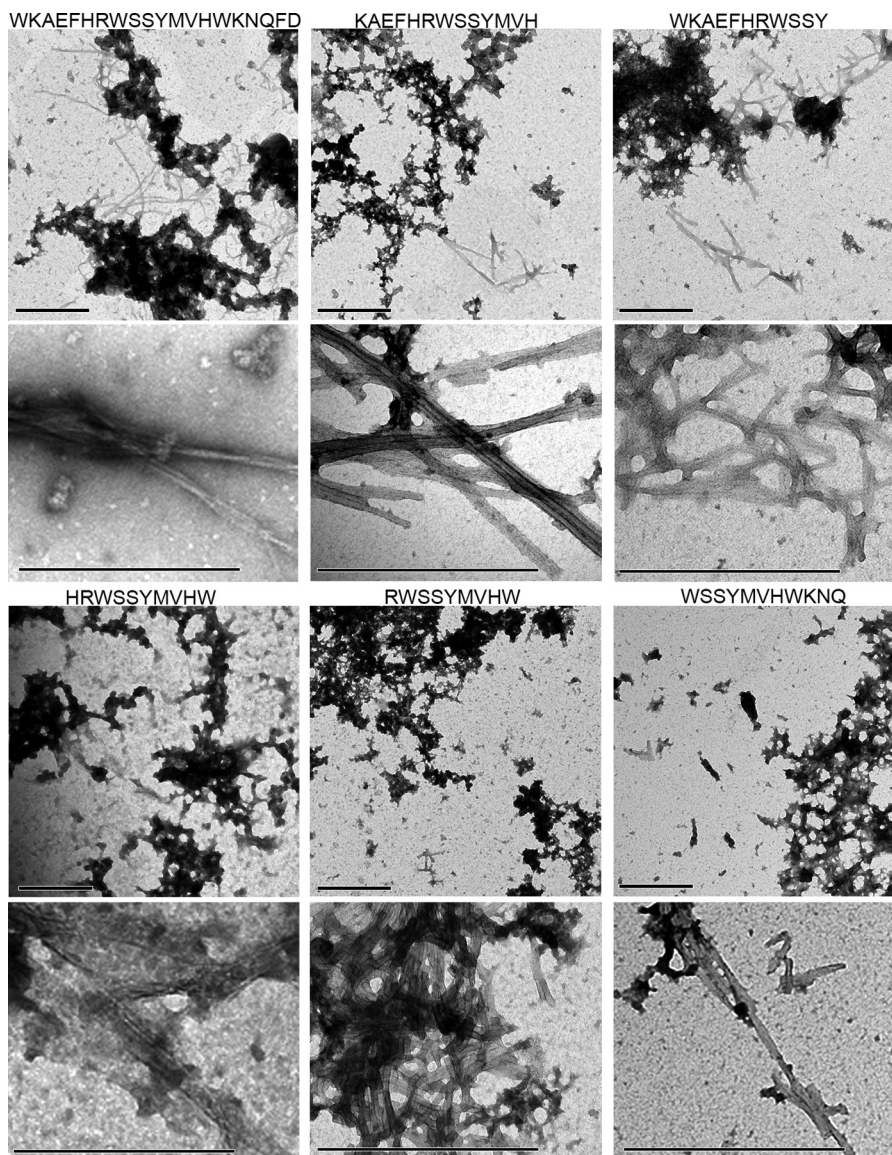


**Figure 4. Fibrillization kinetics of T40-IDE peptides.** 100 or 200  $\mu\text{M}$  peptide was incubated with 165  $\mu\text{M}$  ThT in PBS, and ThT emission was monitored. At least three independent assays were performed. *A*, representatives of the changes in ThT fluorescence observed (the *right panel* is a zoom-in of the time scale to visualize the rapid fibrillization of some peptides). The lag phase of fibrillization (*B*, *left panel*) and plateau height (*B*, *right panel*) are depicted. \*,  $p < 0.03$  when compared with AChE<sub>586-599</sub>. The *inset* in the *left panel* represents a zoom-up of the y axis of the lag phase. The *double bar* in *B* *left panel* indicates the absence of fibrillization (*i.e.* an indeterminably long lag phase). *C*, summary of the fibrillization properties for 100  $\mu\text{M}$  of all T40-IDE peptides: ability to fibrillize, duration of lag phase, height of plateau, and stability of the amyloid products (indicated by stability or decay of the ThT fluorescence after plateau). The peptides that do not fibrillize and/or the peptides in which amyloid products are not stable are indicated by *gray boxes*. The lag phase and plateau height for the AChE peptides are shown as fold ratio of AChE<sub>586-599</sub> (*e.g.* = represents equal value to AChE<sub>586-599</sub> and 100 for the lag phase represents 100 times longer than AChE<sub>586-599</sub>). We have used AChE<sub>586-599</sub> as a benchmark, which is shown in *red font*. *D*, lag phase of fibrillization (*left panel*) and plateau height (*right panel*) of reactions with 200  $\mu\text{M}$  peptides are depicted. \*,  $p < 0.04$  when compared with AChE<sub>586-599</sub>. a.u., arbitrary units.

for direct comparison with their previous studies on hAChE effects on  $\text{A}\beta$  fibrillogenesis.

Seed concentration is the initial monomer concentration used to prepare them. By themselves they did not contribute to ThT signals in test wells, as they did not generate signals over background (data not shown). Moreover, seed-ThT values (no  $\text{A}\beta$ ) were subtracted from all test assays with  $\text{A}\beta$ . Without seeds,  $\text{A}\beta$  showed a plateau height of  $\sim 1095$  a.u. after an

$\sim 43.4$ -h lag and an elongation rate of  $\sim 89.7$  a.u./h (Fig. 8 and Fig. S5). The positive control, 2  $\mu\text{M}$  AChE<sub>586-599</sub> seeds, reduced  $\text{A}\beta$  lag phase 1.4-fold and its plateau height 1.2-fold (Fig. 8, A and C). Similar seeding activity for AChE<sub>586-599</sub> seeds was previously observed (28). With the exception of AChE<sub>584-603</sub> seeds, all other T40-IDE seeds reduced  $\text{A}\beta$  lag phase significantly, with AChE<sub>589-599</sub> being most effective. The seeds of AChE<sub>584-603</sub>, AChE<sub>585-597</sub>, AChE<sub>584-594</sub>, AChE<sub>585-593</sub>,



**Figure 5. Morphology of the fibrillizing T40-IDE peptides.** 100  $\mu\text{M}$  peptide was incubated with 165  $\mu\text{M}$  ThT in PBS until plateau. Shown are electron micrographs of negatively stained reaction at plateau. The scale bar represents 500 nm.

AChE<sub>590-598</sub> and AChE<sub>594-598</sub> accelerated A $\beta$  elongation rate (from 1.1- to 1.3-fold), with AChE<sub>584-603</sub> seeds being the most effective (Fig. 8B). All peptide seeds, except those of AChE<sub>584-603</sub>, increased A $\beta$  plateau height (from 1.1- to 1.2-fold), with AChE<sub>585-593</sub> being the most effective (Fig. 8C).

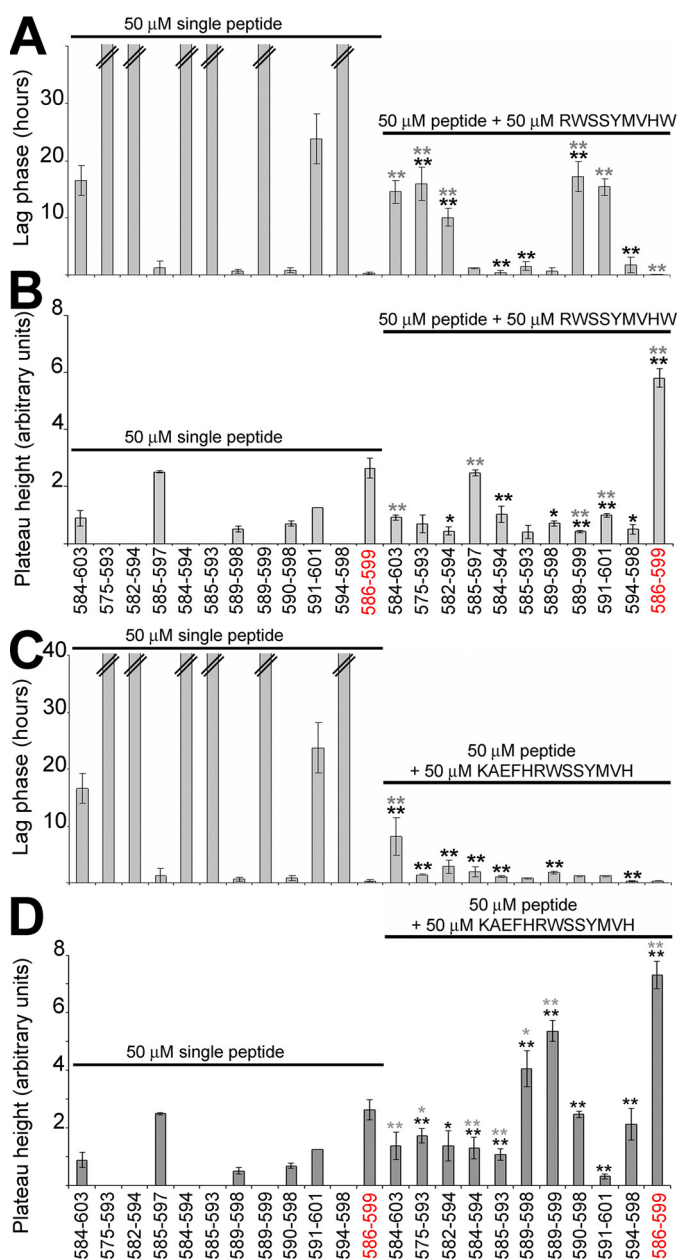
#### Specificity of new antisera against AChE<sub>590-598</sub>

After characterizing T40-IDE peptides, RWSSYMVHW (AChE<sub>590-598</sub>) emerged as the most amyloidogenic. It had the strongest propensity for conversion to  $\beta$ -strand, the strongest surface activity, the best at adopting  $\beta$ -sheet conformation, was second best at fibrillizing, and fair at A $\beta$  seeding. Therefore, two rabbit polyclonal antibodies, 3313 and 3314, were produced using C-Ahx-AChE<sub>590-598</sub> acid coupled at the N terminus to KLH as an antigen. Specificity of these antibodies was tested by immunoblot using T40-IDE peptides (monomers or seeds), T40, hAChE-S, and A $\beta$  (monomers and fibrils) as antigens and were compared with that of Mab 105A, specific to

AChE<sub>586-599</sub> in a  $\beta$ -sheet conformation (Fig. 9). Importantly, neither Mab 105A nor polyclonal 3313 and 3314 cross-reacted with A $\beta$  monomers or fibrils, despite using an amount of A $\beta$  fibrils far above that found *in vivo* (Fig. 9) (54-56). The extremely low reactivity against A $\beta$  fibrils seen in the immunoblots with 3313 and 3314 was a nonspecific cross-reactivity by the polyclonal secondary antibodies anti-rabbit (probably due to the inherent stickiness of fibrils) (Fig. 9B, inset).

For 105A, its previously published specificity, established by scanning alanine mutant recognition, for AChE<sub>586-599</sub> was confirmed in two ways (30). First, 105A epitope was mapped by using 105A as a bait to select, from a large random peptide phage library, clones expressing short seven-residue peptides bearing its epitope (supporting Experimental procedures). With this technique, the previously mapped epitope specificity of 105A was confirmed to be AxFHR (Fig. S6). Second, the recognition of AChE<sub>586-599</sub> was the strongest by slot-blot (Fig. 9). 105A also recognized AChE<sub>584-603</sub> monomers and hAChE-S,

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**Figure 6. Effect of AChE<sub>590-598</sub> or AChE<sub>585-597</sub> on T40-IDE peptides and AChE<sub>586-599</sub> fibrillization.** 50  $\mu$ M T40-IDE peptide monomers were incubated with 165  $\mu$ M ThT in PBS, with or without 50  $\mu$ M AChE<sub>590-598</sub> (A and B) or AChE<sub>585-597</sub> monomers (C and D). At least three independent assays were performed. Changes in ThT fluorescence were monitored with the lag phase of fibrillization (A and C) and the plateau height (B and D) depicted. The black \* or \*\* refers to *p* values when compared with 50  $\mu$ M T40-IDE peptide alone, and the gray \* or \*\* refers to *p* values when compared with 50  $\mu$ M AChE<sub>590-598</sub> or AChE<sub>585-597</sub> alone. \* (black or gray) is for *p* < 0.05, and \*\* (black or gray) is for *p* < 0.03. The double bar in A and C indicates the absence of fibrillization (*i.e.* an indeterminably long lag phase). We have used AChE<sub>586-599</sub> as a benchmark, which is shown in red font.

but the signal was much fainter. Importantly,  $\alpha$ -helical T40 was not recognized, confirming 105A specificity for  $\beta$ -sheet conformations. Polyclonal 3313 reacted strongly against monomers of the immunization peptide and of AChE<sub>584-603</sub>. Significant reactivity was also detected for seeds of the immunization peptide and of AChE<sub>585-597</sub> and T40. Low-level reactivity was observed for AChE<sub>585-597</sub>/AChE<sub>589-598</sub> monomers, seeds of AChE<sub>584-603</sub>/AChE<sub>585-597</sub>/AChE<sub>584-594</sub>, and hAChE-S. Polyclonal 3314 re-

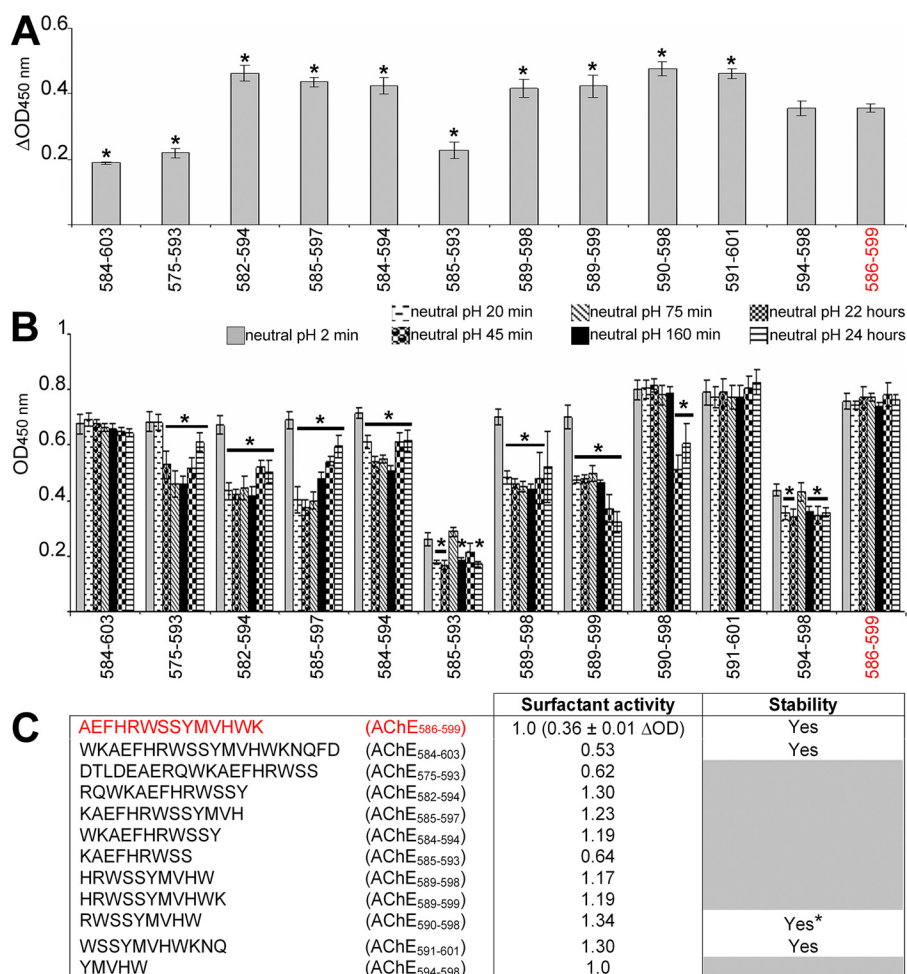
acted strongly against AChE<sub>584-603</sub> monomers and AChE<sub>585-597</sub> seeds. Significant reactivity was also detected for seeds of AChE<sub>584-603</sub>/AChE<sub>589-598</sub>/AChE<sub>590-598</sub>, AChE<sub>586-599</sub>, and hAChE-S. Low-level reactivity was observed for monomers of AChE<sub>575-593</sub>/AChE<sub>585-597</sub>, seeds of AChE<sub>584-594</sub>/AChE<sub>586-599</sub>/immunization peptide, and T40. The specificity of 3313 and 3314 is explained by the fact that most T40-IDE peptides either adopted a  $\beta$ -sheet conformation or fibrillized. Exceptions were fibrillizing AChE<sub>591-601</sub> that is not recognized and nonfibrillizing and random coil AChE<sub>575-593</sub> that is weakly recognized by 3314. To summarize, 3313 and 3314 share some similar specificities for peptides that fibrillize, although the recognition level for individual peptides vary. However, 3313 and 3314 specificities differ from 105A. The strong target all three antibodies share is for AChE<sub>584-603</sub> monomers, which contains both the 105A epitope (FHR) and the full sequence of immunization peptide AChE<sub>590-598</sub> for 3313 and 3314 (30).

### Distribution of A $\beta$ , hAChE-S, hAChE-S-related species, and ThS-positive plaques

We next performed immunolabeling of coronal sections of brain from 12-month-old control, hAChE-S, or hAPPswe single transgenic mice (Tg2576, APP<sub>695</sub> isoform), and hAChE-S/hAPPswe double transgenic mice to probe for presence of hAChE-S-derived peptides. To visualize hAChE-S, we used mouse Mab HR2 anti-cerebellar hAChE-S and rabbit polyclonal KD69 anti- $\alpha$  helical T40 (which recognizes globular intact hAChE-S but also  $\alpha$ -helical T40 in isolation) (30). To visualize A $\beta$  species, we used mouse-specific Mab Bam10 anti-A $\beta$  (first 12 residues). To detect  $\beta$ -sheet amyloid species, ThS was used. To detect hAChE-S species in a  $\beta$ -sheet conformation, we used specific mouse Mab 105A anti-AChE<sub>586-599</sub> (30). To detect hAChE-S species derived from cleavage of T40, we used our two new rabbit polyclonal antibodies 3313 and 3314.

ThS and all antibodies were negative on sections from WT mice (Fig. 10A). The only reactivity in sections from hAChE-S mice was with HR2 and KD69 recognizing globular hAChE-S, as expected because such mice do not develop amyloid plaques (Fig. 10B). In sections of hAPPswe mice, numerous mature plaques could be identified by single antibody labeling (Fig. 11A, left column). Some of these plaques clearly contained A $\beta$  species (labeled with Bam10), but also AChE-S (labeled with HR2) and AChE-S in  $\beta$ -sheet conformation (labeled with 105A, black arrowheads). Some plaques labeled only with anti-AChE-S antibodies, either anti-intact or anti-AChE-S domains (examples marked by blue arrowheads). There was clearly some peroxidase activity on sections incubated with KD69, 3313, and 3314. The brown color was darker and localized in more specific areas than the controls (no first antibody, only biotinylated secondary antibody, avidin-peroxidase, and DAB). But no plaques were obvious. Similarly, numerous mature plaques were identified by single antibody labeling in sections of hAPPswe/hAChE mice (Fig. 11A, right column). Several Bam10-positive plaques were clearly bigger than in hAPPswe mice. ThS confirmed their amyloid nature (Fig. 11B). Labeling with avidin fluorescein reduced nonspecific background but clearly raised the specific signal above background in 3313 and





**Figure 7. Surface activity of the T40-IDE peptides.** The surface activity of 50 μM T40-IDE peptides was measured before and after neutralization (1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2). At least three independent assays were performed. *A*, representative surfactant activity of the T40-IDE peptides and AChE<sub>586-599</sub> 2 min after neutralization. ΔOD = (OD<sub>offset</sub> - OD<sub>central</sub>)<sub>neutral pH 2 min</sub> - (OD<sub>offset</sub> - OD<sub>central</sub>)<sub>acidic pH</sub>. \*, *p* < 0.023 when compared with AChE<sub>586-599</sub>. *B*, temporal pattern of the surface activity for the T40-IDE peptides after neutralization. OD = OD<sub>offset</sub> - OD<sub>central</sub>. \*, *p* < 0.034 when compared with the same peptide after 2 min at neutral pH. *C*, summary of the surfactant properties for the T40-IDE peptides: surface activity dependent on pH (depicted by subtracting the value at acidic pH to the value at neutral pH after 2 min) and stability of the surface activity (indicated by stability or decay of the OD signal). The peptides with unstable surfactant activity are indicated by gray boxes. \* indicates peptides in which the activity remains stable over most of the time course, except two time points. The activity for the T40-IDE peptides is shown as fold ratio of AChE<sub>586-599</sub> activity (e.g. 1 represents equal value to AChE<sub>586-599</sub>). We have used AChE<sub>586-599</sub> as a benchmark, which is shown in red font.

3314. Each antibody highlighted mature plaques in the same brain areas detected by peroxidase/DAB staining.

In single and double transgenic brain sections, plaques were mainly detected in the cerebral and olfactory cortices and a few in the olfactory bulb (e.g. ThS, Bam10, and 3314) (Fig. 11, A-C). In the cerebral cortex section studied (rostral level), all sub-areas (motor, orbital, and prefrontal) showed plaques, with stronger fluorescence in the motor sub-area with some reagent/antibodies (e.g. ThS, KD69, Bam10, and 3314). In the olfactory cortex (anterior olfactory nucleus), plaques were detected in all sub-areas. In the olfactory bulb (main and accessory), the plaques appeared more restricted to the granular layer.

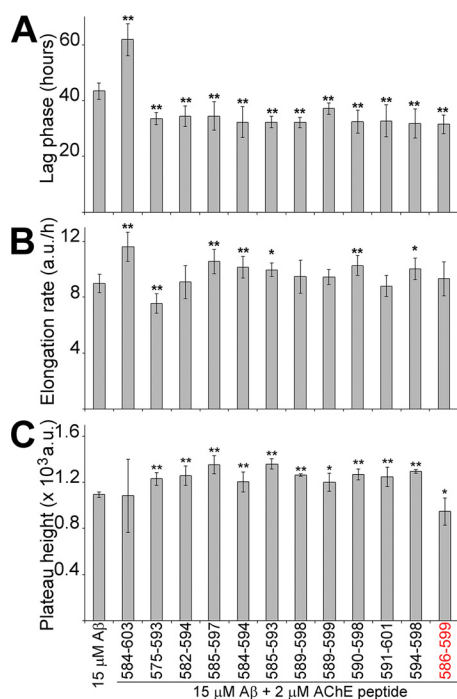
When the area occupied by each fluorescent labeling, in the whole brain section of hAPPswe/hAChE double transgenic, was quantitated, it became evident that each individual antibody labeled only a small portion of the area labeled with ThS (Fig. 11D, top and bottom graphs). Indeed, KD69 labeling occupied an area only ~9.9% that of ThS, Bam10 labeling 13.3%, 105A labeling 1.3%, 3313 labeling 2.8%, and 3314 labeling

25.4%. The same relationship was found when the overall intensity of the fluorescent labeling by each antibody was measured, with KD69 labeling intensity only ~8.9% that of ThS, Bam10 labeling 25%, 105A labeling 3.8%, 3313 labeling 3.8%, and 3314 labeling 20.3% (Fig. 11D, middle and bottom graphs). These results clearly suggest that Aβ and hAChE-S related species only represent a fraction of the overall amyloid burden.

#### **hAChE-S related species in a β-sheet conformation are located in plaques and colocalize with Aβ**

We subsequently addressed by double-fluorescent labeling whether amyloid-positive plaques contained hAChE-S of various kinds, including intact, truncated, α-helical, or β-sheet. In the cerebral cortex of hAPPswe mice, mature amyloid plaques could be detected, which labeled with anti-Aβ Bam10 and ThS (Fig. 12). The Aβ species showed some degree of colocalization with mouse AChE-S (HR2) (68.6% of Bam10 labeling overlapping with that of HR2, and 10.2% of HR2 labeling overlapping with that of Bam10) and mouse AChE-S species with α-helical

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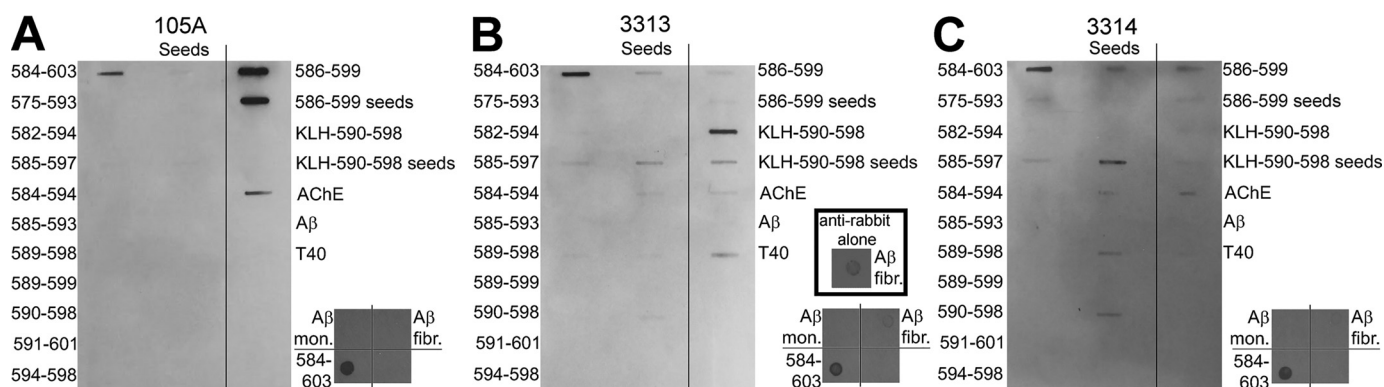


**Figure 8. T40-IDE peptide seeds promote A $\beta$  fibrillization.** 15  $\mu$ M A $\beta$  was incubated with 165  $\mu$ M ThT in PBS, with or without 2  $\mu$ M T40-IDE peptide seeds, and ThT emission was monitored. At least three independent assays were performed. The lag phase of fibrillization (A), elongation rate (B), and plateau height (C) are depicted. \*,  $p < 0.05$ , and \*\*,  $p < 0.03$ , when compared with A $\beta$  without seeds. a.u., arbitrary units. We have used AChE<sub>586-599</sub> as a benchmark, which is shown in red font.

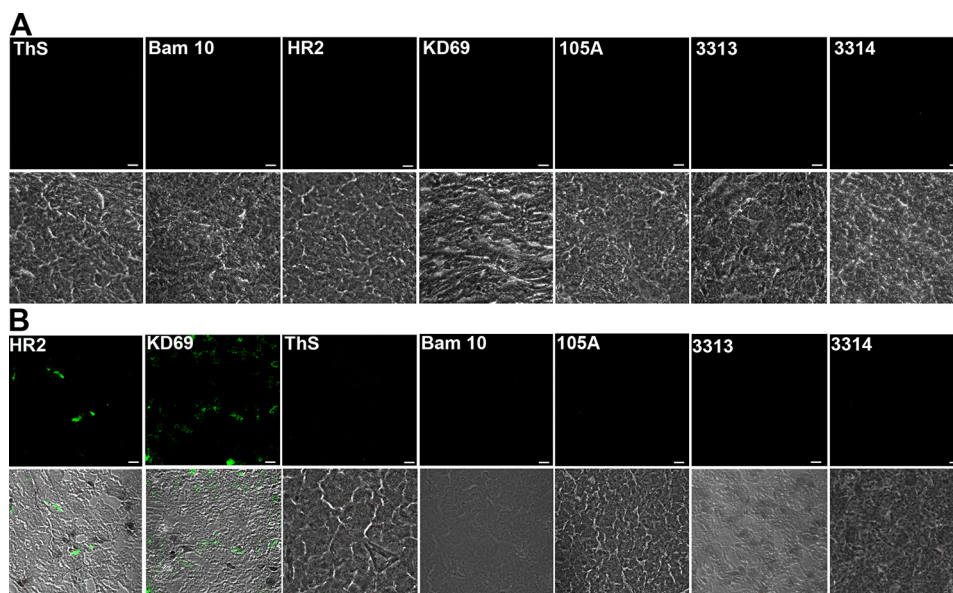
T40 (KD69) (61.8% of Bam10 labeling overlapping with that of KD69, and 36.2% of KD69 labeling overlapping with that of Bam10), with mouse AChE-S surrounding the A $\beta$  species (Fig. 12A). Human AChE T40 and mouse equivalent differ by only one amino acid conservative substitution, Asp<sup>609</sup> in human to Glu<sup>609</sup> in mouse. Therefore, K69 should recognize both human and mouse T40 sequences, as observed. Moreover, the entire hAChE-S and mouse AChE-S amino acid sequences share 88.4% identity, with 71 substitutions among which 43 are conservative, 10 are semi-conservative, and 18 are nonconservative. Moreover, HR2 was also seen to cross-react with mouse AChE-S by immunofluorescence in mouse Neuro-2 neuroblastoma cells (GeneTex and Abcam). Thus, it is not surprising that KD69 and HR2 recognize mouse AChE-S. 105A and 3313/3314 also labeled plaques (Figs. 12B and 13). 105 epitope (Phe<sup>588</sup>–His<sup>589</sup>–Arg<sup>590</sup>) and 3313/3314 immunizing peptide AChE<sub>590-598</sub> are identical between human and mouse. Because the *Ide* gene is conserved between human and mouse, it would be highly likely that mouse T40-IDE peptides would be generated from mouse AChE-S. 105A labeling of mouse AChE-S  $\beta$ -sheet species was found at the core of mature plaques, colocalizing with A $\beta$  (10.4% of Bam10 labeling overlapping with that of 105A, and 68.3% of 105A labeling overlapping with that of Bam10) and  $\beta$ -sheet amyloid species (32.4% of ThS labeling overlapping with that of 105A, and 77.2% of 105A labeling overlapping with that of ThS), and surrounded by  $\alpha$ -helical species of mouse AChE-S (16.3% of HR2 labeling overlapping with that of 105A, and 67.4% of 105A labeling overlapping with that of HR2; 7.7% of KD69 labeling overlapping with that of 105A, and 51.4% of

105A labeling overlapping with that of KD69) (Fig. 12B). Both 3313 and 3314 antisera labeled mouse AChE-S in mature plaques containing an amyloid mass (ThS) and A $\beta$  species (Bam10) (Fig. 13). Indeed, 69 and 31.4% of ThS labeling overlapped with that of 3313 and 3314, respectively; 68.2 and 87.4% of 3313 and 3314 labeling, respectively, overlapped with that of ThS; 55.8 and 81.8% of Bam10 labeling overlapped with that of 3313 and 3314, respectively, and 27 and 23.8% of 3313 and 3314 labeling, respectively, overlapped with that of Bam10. Moreover, 3313 and 3314 labeling colocalized but also surrounded that of mouse AChE-S  $\beta$ -sheet species (65.2 and 61% of 105A labeling overlapped with that of 3313 and 3314 respectively, and 4.9 and 8.2% of 3313 and 3314 labeling, respectively, overlapped with that of 105A).

Mature plaques in the cerebral cortex of hAChE-S/hAPPsw mice contained hAChE-S (KD69 and HR2) and A $\beta$  species (Bam10) (Fig. 14A). The A $\beta$  species showed some degree of colocalization with hAChE-S (73.4% of Bam10 labeling overlapping with that of HR2 and 20.3% of HR2 labeling overlapping with that of Bam10; 65.4% of Bam10 labeling overlapping with that of KD69 and 35.8% of KD69 labeling overlapping with that of Bam10), and both hAChE-S (HR2 and KD69) and hAChE-S species with  $\alpha$ -helical T40 (KD69) surrounded the A $\beta$  species. This labeling pattern is reminiscent of that observed in AD with hAChE-S being associated with senile plaques (24). Importantly, hAChE-S  $\beta$ -sheet species (105A) were detected in mature plaques, representing only a proportion of the hAChE-S present (14.7% of HR2 labeling overlapping with that of 105A, and 73.1% of 105A labeling overlapping with that of HR2), and surrounded by  $\alpha$ -helical forms (6.5% of KD69 labeling overlapping with that of 105A, and 40.2% of 105A labeling overlapping with that of KD69) (Fig. 14B). The plaque hAChE-S  $\beta$ -sheet staining partly co-localized with  $\beta$ -sheet amyloid species (12.1% of ThS labeling overlapping with that of 105A and 47.9% of 105A labeling overlapping with that of ThS) and A $\beta$  species (5.5% of Bam10 labeling overlapping with that of 105A, and 38.6% of 105A labeling overlapping with that of Bam10). In each case, the plaque hAChE-S  $\beta$ -sheet species tended to be centrally located within the mass of amyloids. 3313- and 3314-labeled hAChE-S species associated with fibrillar amyloids (38.7 and 42% of ThS labeling overlapped with that of 3313 and 3314, respectively, and 75 and 79.6% of 3313 and 3314 labeling, respectively, overlapped with that of ThS), A $\beta$  species (49.1 and 86.2% of Bam10 labeling overlapped with that of 3313 and 3314, respectively, and 17.3 and 13.2% of 3313 and 3314 labeling, respectively, overlapped with that of Bam10), and  $\beta$ -sheets hAChE-S species (50.6 and 32.8% of 105A labeling overlapped with that of 3313 and 3314, respectively, and 10.1 and 6.7% of 3313 and 3314 labeling, respectively, overlapped with that of 105A) (Fig. 15). The patterns of colocalization appeared whenever plaques were detected. The species detected by both 3313 and 3314 appeared to represent only a proportion of the fibrillar amyloids (compare, for example, ThS *versus* 3313 or 3314; see above) and to be centrally localized within the amyloid burden. In contrast, when compared with A $\beta$  species (Bam10) or hAChE-S  $\beta$ -sheet species (105A), the hAChE-S species labeled with 3313 not only colocalized with the A $\beta$  and hAChE-S  $\beta$ -sheet species but extended to the surrounding areas as if



**Figure 9. Specificity of Mab 105A (A) and polyclonal antibodies 3313 (B) and 3314 (C).** T40-IDE peptide or peptide seeds, AChE, A $\beta$  monomers (*mon.*), A $\beta$  fibrils (*fibr.*), and T40 were blotted onto a nitrocellulose membrane. Blocking was performed with 0.4% fish skin gelatin before incubation with mouse Mab 105A, or rabbit polyclonal antibody 3313 or 3314, followed by anti-mouse or anti-rabbit IgG conjugated to HRP. Products were visualized by enhanced chemiluminescence. The additional inset in B shows the reactivity of the polyclonal secondary antibodies anti-rabbit (no first antibodies were added) against A $\beta$  fibrils under the same conditions.



**Figure 10. Reactivity of the antibodies in brains of age-matched control and hAChE-S single transgenic mice.** A, absence of reactivity of ThS and the antibodies against A $\beta$ , hAChE-S, and AChE peptides in the brain of age-matched control mice. B, in the brain of hAChE-S single transgenic mice, the only reactivity detected is that of the antibodies recognizing globular hAChE-S. Frozen brain sections, from one animal (12  $\mu$ m), were labeled with the Elite ABC M.O.M. kit or Vectastain Elite ABC rabbit IgG kit using a biotinylated anti-mouse or anti-rabbit secondary antibody and FITC-conjugated avidin (Vector Laboratories). The scale bar represents 10  $\mu$ m. Shown is one of the z slices from a z stack.

encapsulating those in their center. Interestingly, the pattern with 3314 was different. Some colocalization was observed with A $\beta$  and hAChE-S  $\beta$ -sheet species, but 3314 also labeled hAChE-S species in discrete and distinct areas from these (Fig. 15B, arrowheads). Moreover, the labeling of both 3313 and 3314 overlapped to some degree with that of HR2 (globular part of AChE-S) and that of KD69 ( $\alpha$ -helical T40) (Fig. S7). This suggests that 3313 and 3314 also recognize, to some level, AChE-S in an *in vivo* context. These experiments provide the first *in vivo* evidence of the conformation of hAChE-S-related species located in senile mature plaques and colocalizing with A $\beta$  species and fibrillar amyloids.

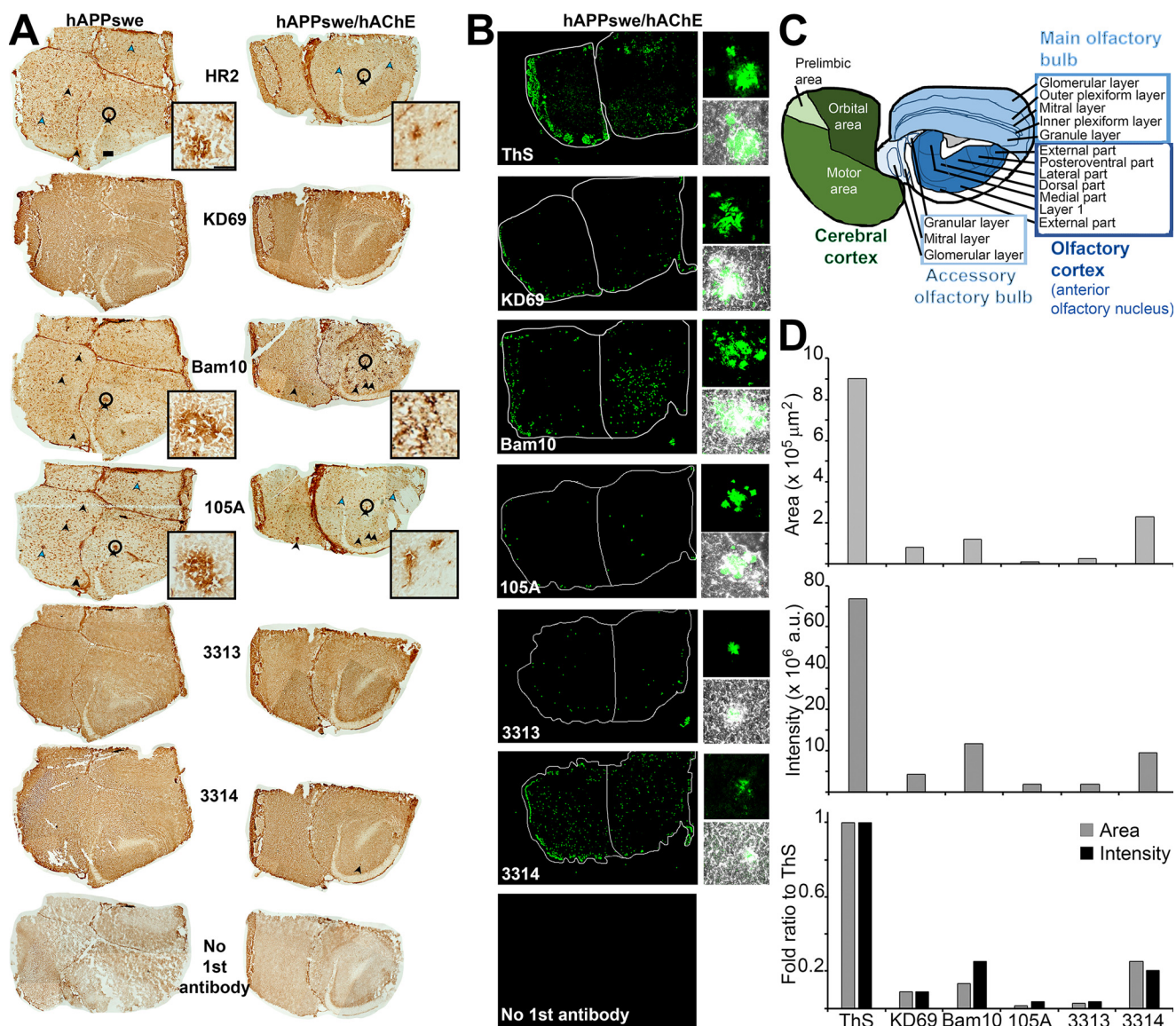
## Discussion

We previously showed that IDE-dependent cleavage of hAChE-S nonamyloidogenic and  $\alpha$ -helical oligomerization domain, T40, generates new peptide species (28). As a mixture,

they form  $\beta$ -sheets, are surface-active, assemble into amyloid protofibrils, and seed A $\beta$  aggregation. In monomeric G1 AChE-S, T40 remains exposed and susceptible to proteolysis, even in bovine AChE-S tetramers (32). Despite clues pointing at T40, the exact nature of the hAChE-S domain involved remains uncertain (18–21). Thus, identifying IDE-derived amyloidogenic hAChE-S species should provide targets to characterize and identify brain fibrillogenic hAChE-S species.

Eleven hAChE-S peptides from IDE-dependent cleavage of T40 were assessed singly rather than mixtures, as amyloidogenic species. Two T40 regions had exceptionally strong  $\beta$ -sheet propensity, WKAEFHR and SYMVHWK. The two peptides with strongest propensity contained the latter region, AChE<sub>590–598</sub> (RWSSYMVHW) and AChE<sub>594–598</sub> (YMVHW). In terms of conformation, we identified negative effects of the T40 N terminus. At the lowest concentration, after neutralization, only AChE<sub>590–598</sub> (RWSSYMVHW) adopted a dominant

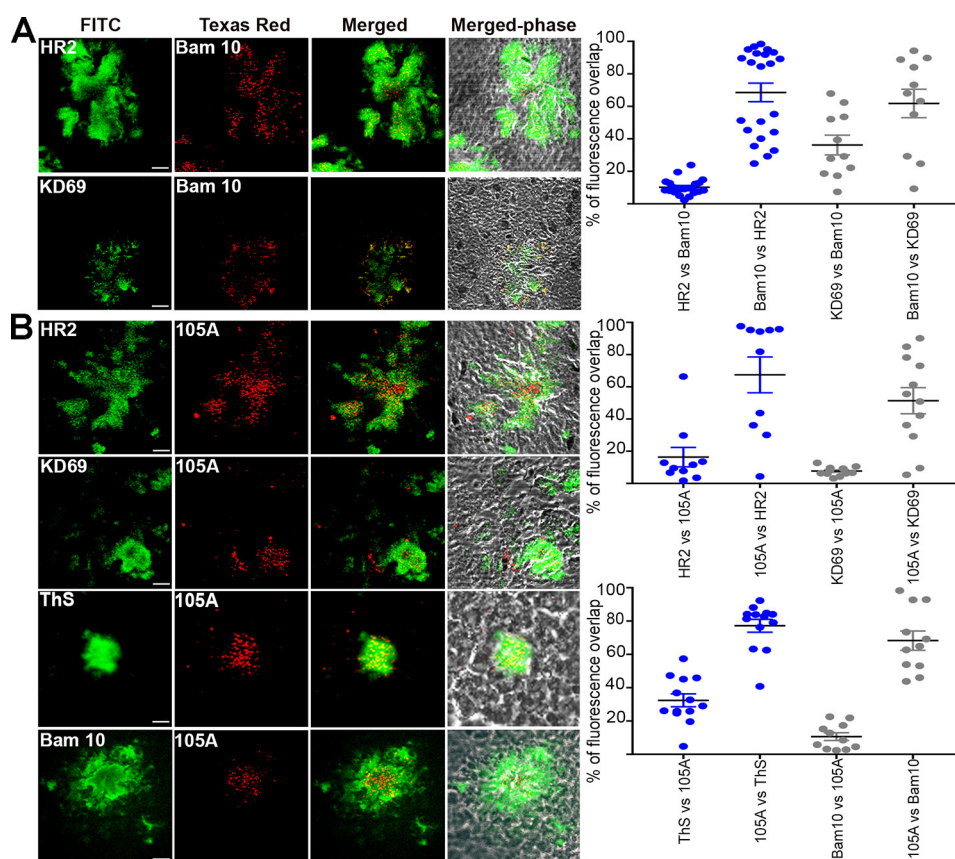
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**Figure 11. Localization and recognition of mature plaques in the brain of hAPPswe single transgenic (A, left column) and hAChE-S/hAPPswe double transgenic mice (A, right column, and B).** A, frozen brain sections (12  $\mu\text{m}$ ) from single and double transgenic mice were single-labeled with the Elite ABC M.O.M. kit or Vectastain Elite ABC rabbit IgG kit using a biotinylated anti-mouse or anti-rabbit secondary antibody, followed by avidin-conjugated peroxidase and then the peroxidase substrate (DAB, Vector Laboratories). The scale bar represents 200  $\mu\text{m}$  (in the top left brain section). Black arrowheads indicate examples of mature plaques labeled with both anti- $\text{A}\beta$  and anti-intact or anti-domains of hAChE-S, and the blue arrowheads indicate examples of mature plaques labeled only with anti-intact or anti-domains of hAChE-S. The insets show z projections of the same mature plaque (identified by a black circle in the full brain section) labeled with each of the following antibodies: HR2, Bam10, and 105A, and the scale bar represents 50  $\mu\text{m}$ . One representative whole brain section per condition was collected as a montage of automatically tiled images. B, frozen brain sections (12  $\mu\text{m}$ ) from double hAChE-S/hAPPswe transgenic mice were single-labeled with the Elite ABC M.O.M. kit or Vectastain Elite ABC rabbit IgG kit using a biotinylated anti-mouse or anti-rabbit secondary antibody and FITC-conjugated avidin (Vector Laboratories). Shown are fluorescent labeling of whole brain sections (left, one representative whole brain section per condition was collected as a montage of automatically tiled images) and z projections of mature plaques (fluorescent labeling and merged phase, right). C, mouse brain map at the rostral level, indicating the main brain regions with their sub-areas. D, shown are quantifications of the area occupied overall by the fluorescent labeling from a specific antibody/reagent in the whole brain section of hAPPswe/hAChE double transgenic (i.e. the whole brain section shown in B) (top graph), the overall intensity of the fluorescent labeling in the whole brain section of hAPPswe/hAChE double transgenic (middle graph), and the fold ratio when compared with ThS of each fluorescent labeling in term of area occupied and intensity (bottom graph).

$\beta$ -sheet conformation. At higher concentrations or in the presence of SDS as a membrane-mimicking agent, other peptides also adopted a  $\beta$ -sheet conformation (e.g. AChE<sub>589–598</sub>, HRWSSYMVHW). These results imply that the *in vivo* environment, in which these peptides are found (e.g. vicinity of cellular membranes), drastically influences their amyloidogenic behavior. AChE<sub>590–598</sub> (RWSSYMVHW) was the most surface-active peptide, a property involved in amyloid toxicity. Only six peptides could fibrillize by themselves, most of them able

to adopt a degree of  $\beta$ -sheet conformation and containing YMVH. Individually, AChE<sub>585–597</sub> (KAEFHRWSSYMVH) and AChE<sub>590–598</sub> (RWSSYMVHW), the best two fibrillizing peptides, allowed fibrillization of T40–IDE peptides that did not fibrillize alone. Importantly, most individual peptide seeds, except AChE<sub>584–603</sub>, significantly reduced  $\text{A}\beta$  lag phase, with AChE<sub>590–598</sub> (RWSSYMVHW) among the most effective. We identified RWSSYMVHW (AChE<sub>590–598</sub>) as the most amyloidogenic peptide and generated two new polyclonal antibody-



**Figure 12. Localization of hAChE-S, T40 (A) and  $\beta$ -sheet derived hAChE peptides (B) in the brain of hAPPSwe single transgenic mice.** Frozen brain sections (12  $\mu$ m) from double transgenic mice were double-labeled with the Elite ABC M.O.M. kit or Vectastain Elite ABC rabbit IgG kit using a biotinylated anti-mouse or anti-rabbit secondary antibody and FITC or Texas red-conjugated avidin (Vector Laboratories). The scale bar represents 10  $\mu$ m. Shown is one of the z slices from a z stack. The right panels show quantitation of the percentage of overlap between the two fluorophores within the plaques (e.g. HR2 labeling overlapping with KD69 labeling), with plaques examined from at least two different sections per condition (with the order of the antibody/reagents having been switched for the staining). The brains from two different mice were examined. Each value is derived from one individual plaque. Also indicated is the mean, and error bars are S.E.

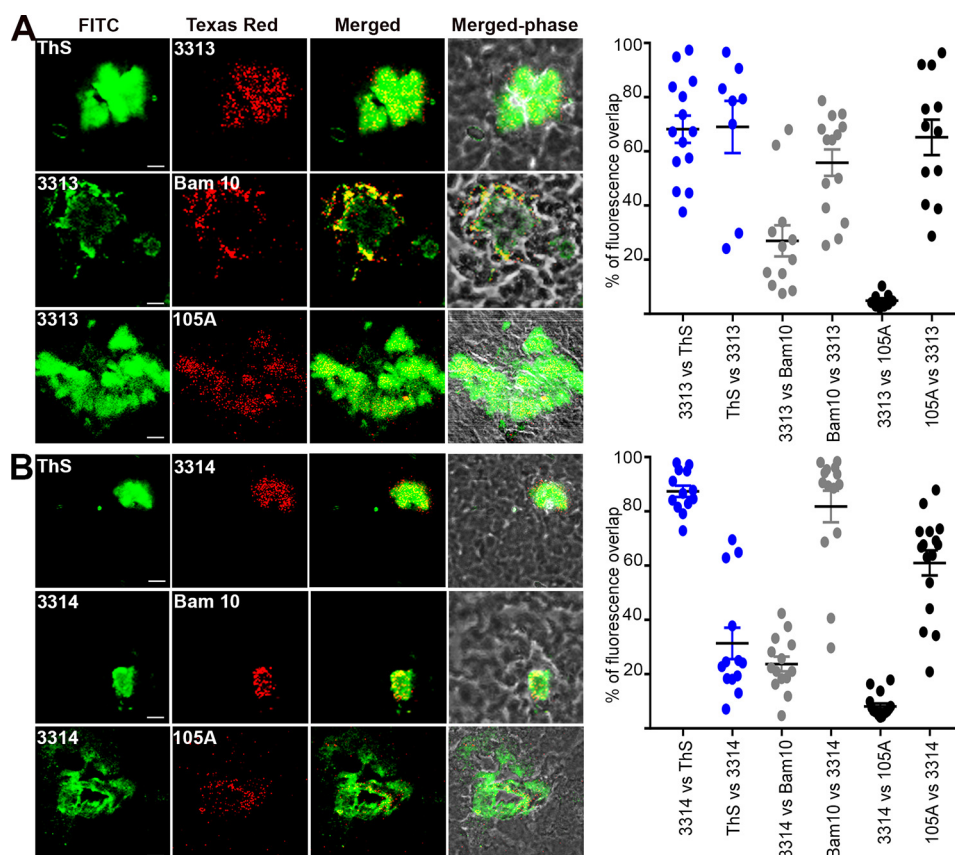
ies against it, 3313 and 3314. These recognized peptides that fibrillize or adopt a  $\beta$ -sheet conformation, although recognition levels varied. They also had a different specificity to Mab 105A, except for recognizing AChE<sub>584–603</sub> (WKAEFHRWSSYMVHWKNQFD). Having created and characterized specific new reagents against hAChE-S T40-derived species of diverse properties, we probed for hAChE-S-derived peptides or hAChE-S with a still attached but proteolytically digested T40 in brains of 12-month-old controls, hAChE-S or hAPPSwe single transgenic mice (Tg2576, APP<sub>695</sub> isoform), and hAChE-S/hAPPSwe double transgenic mice.

In hAChE-S mice, the transgene had been shown to provide greater AChE-S activity in cerebral cortex, doubling the total AChE-S activity but triggering no plaque formation (37). Therefore, it was unsurprising that only antibodies against globular hAChE-S (HR2) and T40 (KD69) achieved labeling.

By 8–9 months, hAPPSwe mice began developing classic amyloid plaques in cerebral cortex, as we saw with ThS and anti- $\beta$  Bam10 in 12-month-old mice (37, 57). The plaques were also rich with HR2 and KD69 labeling.  $\beta$  treatment of cultured mouse neuroblastoma and mouse primary neuronal cultures triggers elevated levels of mouse AChE-S expression and activity (58). Despite an overall decrease of hAChE-S activity in human AD patient brains, it rises in amyloid plaques and

NFT at very early stages of amyloid deposition (15, 59). Also, various hAPP transgenic mice showed selective elevation of mouse AChE activity in brain areas linked to severe AD pathology, in particular cores of amyloid plaques in the neocortex, as we saw with Mab 105A anti-AChE<sub>586–599</sub> (60–63). Some hAPP mouse models also have higher levels of G1 monomeric mouse AChE-S (62). Moreover, in pathologically relevant cortical extracts of Tg2576 mice,  $\beta$  deposition began as early as 4 months before overt pathology began, and the activity of abnormal glycosylated G1 forms of mouse AChE was rising (63, 64). These  $\beta$ -triggered elevations of mouse AChE-S, potentially proteolytically susceptible monomeric G1 forms, combined with high amino acid homology in hAChE-S and its mouse equivalent, explain plaque labeling by HR2, KD69, 105A, 3313, and 3314 in the Tg2576 mice but only in the context of human  $\beta$  plaque deposition. We saw no labeling in WT mice with mouse APP and AChE-S that never develop plaques (65). hAChE-S is also elevated in human brain tissue immediately adjacent to amyloid plaques (66). Therefore, local neurons reacting to increased levels of soluble  $\beta$ ,  $\beta$  deposition, and amyloid burden, in general, could drive AChE-S expression. In turn, proteolytic impact of highly expressed AChE-S could generate amyloidogenic  $\beta$ -sheet species and create a vicious circle of further  $\beta$  seeding or deposition.

## Conformation of hAChE-S species in senile plaques

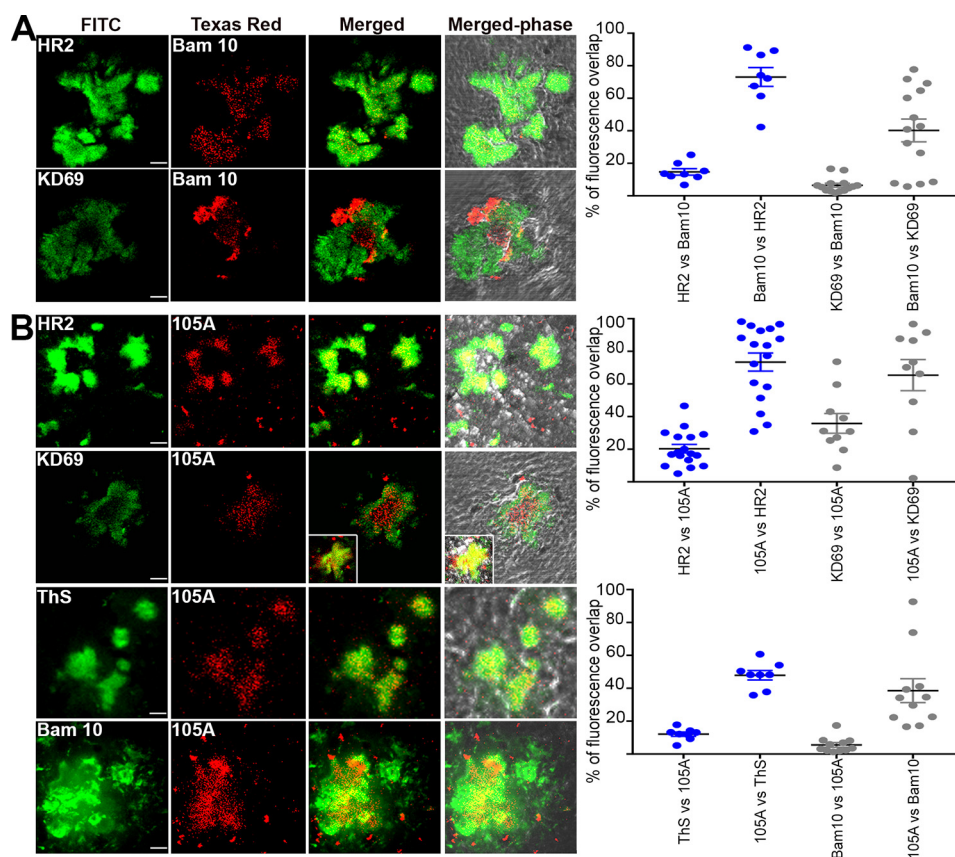


**Figure 13. Localization of AChE-derived peptides, as recognized by antisera 3313 (A) and 3314 (B), in the brain of hAPPswe single transgenic mice.** Frozen brain sections (12  $\mu$ m) from single transgenic mice were double-labeled with the Elite ABC M.O.M. kit or Vectastain Elite ABC rabbit IgG kit using a biotinylated anti-mouse or anti-rabbit secondary antibody and FITC or Texas red-conjugated avidin (Vector Laboratories). The scale bar represents 10  $\mu$ m. Shown is one of the z slices from a z stack. The right panels show quantitation of the percentage of overlap between the two fluorophores within the plaques (e.g. ThS labeling overlapping with 3313 labeling), with plaques examined from at least two different sections per conditions (with the order of the antibody/reagents having been switched for the staining). The brains from two different mice were examined. Each value is derived from one individual plaque. Also indicated is the mean, and error bars are S.E.

In hAPPswe and hAPPswe/hAChE mice, amyloid plaques were detected in brain areas relevant for AD. Indeed, in the cerebral cortex, the motor, orbital, and prelimbic sub-areas all labeled with plaques, as observed in human AD. These cortical sub-areas are also among the most heavily affected by A $\beta$  burden in another mouse model of AD (b6.Cg-Tg(APPswe/PSEN1De9)85Dbo/j, chimeric mouse/human APPswe, and human presenilin-1 gene lacking exon 9) (67). In the orbito-frontal cortex of ~16-month-old Tg2576 transgenic mice, A $\beta$  deposition was modest (68). Likewise, the motor cortex in 12-month-old double transgenic mice tested with all our antibodies/reagents showed the heaviest amyloid burden, with fewer plaques in the orbital area (68). In the olfactory bulb and cortex of Tg2576 transgenic mice, nonfibrillar A $\beta$  deposition and plaque appearance occur earlier than in any other brain areas (6 months), with deposition in the olfactory bulb as one of the earliest pathological changes (68–70). ThS stained mainly the olfactory bulb granule layer, and A $\beta$  deposition occurred across other layers as the mice aged, correlating with progressive olfactory impairments, resembling those in human AD. In the olfactory cortex and bulb of our double transgenic mice, the observed plaque repartition (anterior olfactory nucleus and granular layer of the bulb) was similar to that described in Tg2576 and b6.Cg-Tg mouse models (67). These areas had

among the heaviest amyloid burden. Senile plaques and NFT are commonly found in human olfactory cortex and bulb, and also in brain regions receiving input from it (69, 71). In human dementia, the anterior olfactory nucleus has reduced neuron density, shows NFTs, with number and densities paralleling dementia severity, and contains mature senile plaques when dementia is severe. Thus, in our AD mouse model, amyloid species, A $\beta$ , and AChE-S species accumulate in plaques in various and distinct brain regions that parallel those in other AD mouse models and more importantly in human AD (72, 73).

Mature plaques in the cerebral cortex of our double transgenic mice contained hAChE-S, hAChE-S species,  $\beta$ -sheet amyloid species, and A $\beta$ . Both intact hAChE-S (HR2 and KD69) and hAChE-S species with  $\alpha$ -helical T40 (KD69) surrounded the  $\beta$ -sheet amyloid (ThS) and A $\beta$  (Bam10). In contrast, hAChE-S species (3313 and 3314) and hAChE-S species with a  $\beta$ -sheet conformation (105A) colocalized with  $\beta$ -sheet amyloid (ThS) and A $\beta$  species (Bam10) at a more central location within the amyloid burden. In human AD brains, histochemistry for cholinesterase activity showed that both the central core of plaques and its surrounding rim stained strongly and consistently, which also labeled with ThS and Mab 4G8 anti-A $\beta$  (59). hAChE activity was also found associated with mature amyloid plaques, both at the neuritic core and periph-



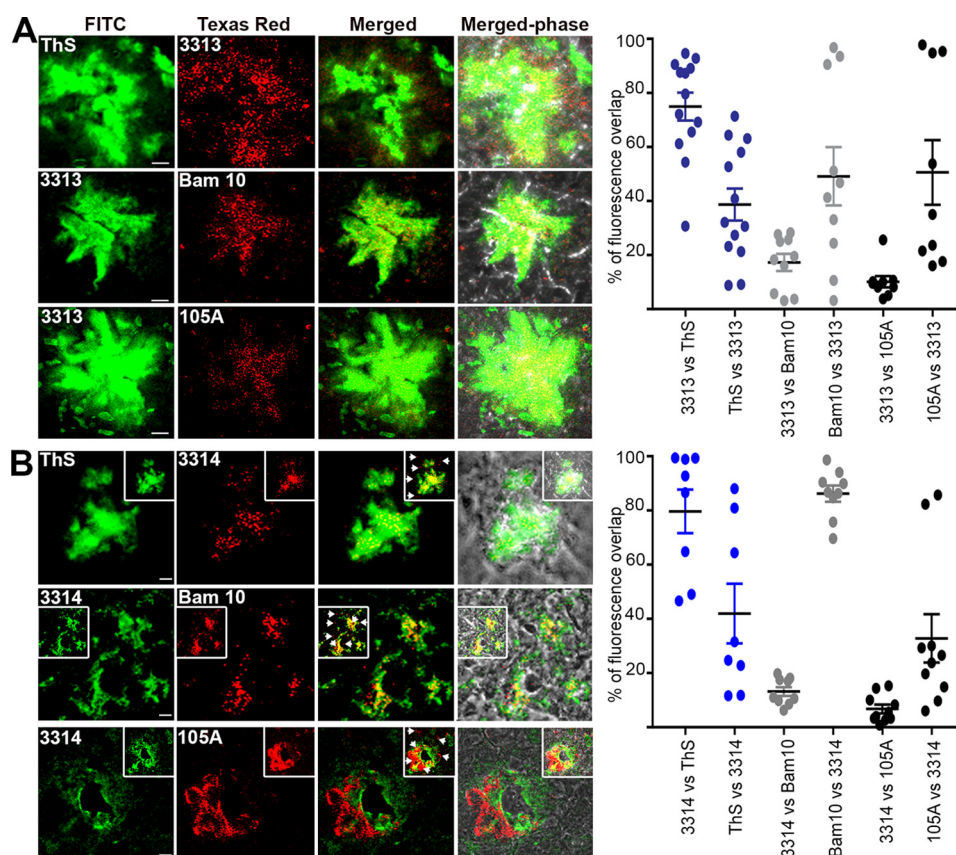
**Figure 14. Localization of hAChE-S, T40 (A), and  $\beta$ -sheet derived hAChE peptides (B) in the brain of hAChE-S/hAPPswe double transgenic mice.** Frozen brain sections (12  $\mu$ m) from double transgenic mice were double-labeled with the Elite ABC M.O.M. kit or Vectastain Elite ABC rabbit IgG kit using a biotinylated anti-mouse or anti-rabbit secondary antibody and FITC or Texas red-conjugated avidin (Vector Laboratories). The scale bar represents 10  $\mu$ m. Shown is one of the z slices from a z stack, with the inset showing a z projection. The right panels show quantitation of the percentage of overlap between the two fluorophores within the plaques (e.g. HR2 labeling overlapping with Bam10 labeling), with plaques examined from at least two different sections per conditions (with the order of the antibody/reagents have been switched for the staining). The brains from two different mice were examined. Each value is derived from one individual plaque. Also indicated is the mean, and error bars are S.E.

ery, by EM of human AD brains (74). Our findings are in perfect agreement with previous reports.

Although linked to AD pathology, and in contrast to APP and A $\beta$ , hAChE-S has been neglected in terms of understanding its role and physiology during AD. Prior research mostly focused on inhibiting cholinesterase activity for short-term symptomatic relief. Clearly, the relationships among cholinergic dysfunction, cognitive deficit, and pathological structures remain poorly understood. To our knowledge, our study is the first to investigate conformations adopted by hAChE-S within plaques and the presence of hAChE-S-related species. Our experiments provide the first *in vivo* evidence that many A $\beta$ -containing amyloid plaques become associated with hAChE-S in  $\alpha$ -helical conformation and related species in  $\beta$ -sheet conformation. The  $\beta$ -sheet species may be the result of proteolytic cleavage generating either T40-derived isolated amyloidogenic peptides and/or shortened and amyloidogenic T40 not released from the hAChE-S globular part. We also show that some individual species generated by proteolytic digestion of hAChE-S T40 domain adopt a  $\beta$ -sheet conformation, are surface active, highly amyloidogenic, and able to seed A $\beta$ . This sets the stage for further studies on pathologically relevant A $\beta$ -hAChE-S interactions (e.g. seeding) in hAChE/hAPPswe mice. Using double transgenic mice for such purposes has previously

proven valid and successful (75). As in prion disease, the spread of AD pathology may be due to template-directed misfolding by progressive seeding of amyloid aggregation, which can spread into adjacent and interconnected neurons (76). This is supported by the following evidence: in mice that do not normally develop AD pathology, induced A $\beta$  deposition occurs *de novo* in brain areas (e.g. cortex) far from the original hippocampal injection site of brain extracts from AD patients; induction of senile plaques in the brains of primates by intracerebral injection of human AD brain homogenates; and seeded A $\beta$  aggregation in an APP transgenic mouse model by intracerebral infusion of AD brain extracts (77–79). In AD, amyloid propagation and dissemination to neighboring neurons or brain areas is conceivable for A $\beta$  as it is naturally present in the extracellular space and can diffuse widely without needing to cross membranes. In mammals, globular hAChE-S is mostly a G1 soluble monomer and a G4 tetrameric membrane-bound form (66, 80). As AD progresses, there is selective reduction or loss of G4 tetramers and a rise in G1 monomers in cerebral cortex and cerebrospinal fluid (66, 81, 82). Furthermore, in cerebral cortex of severely affected AD patients with heavy A $\beta$  loads, G4 hAChE-S activity falls  $\sim$ 50%, and G1 forms rise (66). Rising G1 activity also correlates positively with density of cortical

## Conformation of hAChE-S species in senile plaques



**Figure 15. Localization of AChE-derived peptides, as recognized by antisera 3313 (A) and 3314 (B), in the brain of hAChE-S/hAPPsw double transgenic mice.** Frozen brain sections (12  $\mu\text{m}$ ) from double transgenic mice were double-labeled with the Elite ABC M.O.M. kit or Vectastain Elite ABC rabbit IgG kit using a biotinylated anti-mouse or anti-rabbit secondary antibody and FITC or Texas red-conjugated avidin (Vector Laboratories). The scale bar represents 10  $\mu\text{m}$ . Shown is one of the z slices from a z stack, with the insets showing z projections. B, arrowheads indicate hAChE-S species in discrete and distinct areas from those associated with fibrillar amyloids. The right panels show quantitation of the percentage of overlap between the two fluorophores within the plaques (e.g. ThS labeling overlapping with 3313 labeling), with plaques examined from at least two different sections per conditions (with the order of the antibody/reagents having been switched for the staining). The brains from two different mice were examined. Each value is derived from one individual plaque. Also indicated is the mean, and error bars are S.E.

amyloid deposits. In bovines, AChE T40 is vulnerable to proteolysis even in tetramers and is naturally cleaved or truncated in fetal bovine serum G1 AChE (32, 33). Hence, in AD, it is plausible that increased hAChE-S G1 forms that would be extracellular, soluble, and with a proteolytically susceptible T40 could lead to the generation of hAChE-S-derived amyloidogenic species able to seed  $\text{A}\beta$  aggregation. To date, therapies for diseases like AD do not modify the pathological process but only offer temporary symptomatic alleviation. Template-directed misfolding by seeding of  $\text{A}\beta$  by hAChE-S, but also cellular dissemination of amyloidogenic species, could offer novel therapeutic strategies to delay propagation by stabilizing native protein conformations.

### Experimental procedures

#### Synthetic peptides, antibodies, and mouse brains

Lyophilized T40-IDE-derived peptides and synthetic human  $\text{A}\beta_{1-40}$  (EZBiolab, Carmel, IN) were purchased already purified by reverse-phase HPLC with a C18 column. Before use, the T40-IDE peptides were resuspended at 2 mg/ml in water, and  $\text{A}\beta$  was resuspended in DMSO at 1.6 mM.  $\text{A}\beta$  was then sonicated and centrifuged for 1 h at  $15,000 \times g$  at  $4^\circ\text{C}$  (to remove any pre-aggregated species). DMSO was used to main-

tain  $\text{A}\beta$  in a monomeric pool lacking any  $\beta$ -sheet secondary structures (83).

Specific mouse Mab 105A anti-AChE<sub>586-599</sub> in a  $\beta$ -sheet conformation and rabbit polyclonal KD69 anti- $\alpha$  helical T40 were previously described (30). Two rabbit polyclonal antibodies anti-AChE<sub>590-598</sub>, named 3313 and 3314, were produced by Cambridge Research Biochemicals (Billingham, Cleveland, UK) using C-Ahx-AChE<sub>590-598</sub>-acid coupled at the N terminus to KLH as an antigen, via standard immunization procedures. Other antibodies used were mouse Mab HR2 anti-cerebellar hAChE-S and Mab Bam10 anti- $\text{A}\beta$  (first 12 residues). Brains from 12-month-old WT control, hAChE-S single transgenic, hAPPsw single transgenic (Tg2576, APP<sub>695</sub> isoform), and hAChE-S/hAPPsw double transgenic mice were from the Brimjoin lab (18).

#### Circular dichroism (CD)

Far-UV spectra (250 to 190 nm) before and after pH neutralization (50 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.2 final) of 100 or 200  $\mu\text{M}$  T40-IDE peptides in absence or presence of 10 mM SDS. The mean spectra of multiple scans (speed, 50 nm  $\text{min}^{-1}$ , response time 4 s) were collected at  $20^\circ\text{C}$  in a quartz cuvette (1-mm path length) using a Jasco J-720 spectropolarimeter. The spectra



were blank subtracted and normalized to molar ellipticity. At least three independent assays were performed. Statistical analysis was by two-sample *t* test. To determine the percentage of secondary structures, the CD spectra were analyzed with CONTINLL from the CDPro package (84).

### Fibrillization experiments

100 or 200  $\mu\text{M}$  T40–IDE peptides were incubated with 165  $\mu\text{M}$  ThT in PBS in a 100- $\mu\text{l}$  reaction volume. ThT fluorescence (excitation 450 nm and emission 480 nm) was measured daily in 96-well plates (black wall, clear bottom, Greiner Bio-One, Stonehouse, Gloucestershire, UK), at 37 °C every 6.7 min with 5-min orbital shaking after every measurement and on a BMG Polarstar plate reader (BMG Labtech, Aylesbury, Buckinghamshire, UK) using a bottom–bottom configuration (optical fiber system detecting emission signal from the bottom of the well). Values in control wells (ThT/buffer) were subtracted from those of test wells (ThT/peptide). Kinetic parameters were calculated as follows: elongation rate was calculated from slope at the sigmoidal curve inflection point; plateau height was calculated by averaging the highest curve values (minimum of five); and the lag phase was calculated from the intercept on the time axis of the line tangent to the inflection point. Three or more independent assays were performed and analyzed each with two-sample *t*-tests.

### Co-fibrillization

50  $\mu\text{M}$  T40–IDE peptides were incubated with 50  $\mu\text{M}$  AChE<sub>590–598</sub> or AChE<sub>585–597</sub>, or 15  $\mu\text{M}$  A $\beta$  was incubated with 15  $\mu\text{M}$  T40–IDE peptides, and the fibrillization experiments were carried as described above.

### Heterologous seeding

200  $\mu\text{M}$  T40–IDE peptides in PBS were fibrillized for 2 h at room temperature with continuous shaking to generate seeds, and 15  $\mu\text{M}$  A $\beta$ <sub>1–40</sub> (DMSO stock) was incubated with 165  $\mu\text{M}$  ThT in PBS (100  $\mu\text{l}$  reaction volume), with or without 2  $\mu\text{M}$  T40–IDE peptide seeds. ThT fluorescence was measured as described under “Fibrillization experiments,” but readings were taken every 21 min, with 5 min shaking after each read. Control wells contained ThT, buffers, and 2  $\mu\text{M}$  seeds when required. Values in control wells (ThT/buffer with or without seeds) were subtracted from those in test wells (A $\beta$  with or without seeds), ensuring that the seeds did not affect ThT fluorescence.

### Transmission EM

100  $\mu\text{M}$  T40–IDE peptides were harvested when the plateau phase was reached (as measured by ThT fluorescence emission). The solutions were adsorbed onto Formvar-coated 400 mesh copper grids, air-dried, washed with distilled water, negatively stained with 2% aqueous uranyl acetate, and viewed with a Tecnai electron microscope (Philips).

### Surface activity measurement

Analyses were performed as described previously (47). Stock solutions of T40–IDE peptides were diluted to 50  $\mu\text{M}$  in 200 mM sodium acetate, pH 2 (100  $\mu\text{l}$  final volume), and dispensed in a 96-well plate (black wall, clear bottom; Greiner Bio-One). Sur-

face activity was measured at 37 °C, before and after neutralization with 20  $\mu\text{l}$  of 1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2, at 450 nm on a BMG Polarstar plate reader at the central and offset positions.  $\Delta\text{OD} = (\text{OD}_{\text{offset}} - \text{OD}_{\text{central}})_{\text{neutral pH } 2 \text{ min}} - (\text{OD}_{\text{offset}} - \text{OD}_{\text{central}})_{\text{neutral pH}}$ . Three or more independent assays were performed and analyzed by two-sample *t* test.

### Immunoblot

To generate seeds, T40–IDE peptides were fibrillized (see above). A $\beta$  fibrils were generated by incubating 199  $\mu\text{M}$  A $\beta$  in PBS and leaving it for 1 week at 37 °C under shaking conditions. 2  $\mu\text{g}$  of T40–IDE peptide or peptide seeds, T40, A $\beta$ , A $\beta$  fibrils, and 1  $\mu\text{g}$  of hAChE-S were blotted onto nitrocellulose membrane. This was blocked with 0.4% fish skin gelatin in PBS, 0.5% Tween 20, and incubated with rabbit polyclonal antibody 3313 or 3314, or mouse Mab 105A, followed by anti-rabbit or anti-mouse IgG conjugated to HRP. Products were visualized by enhanced chemiluminescence.

### Immunolabeling and confocal microscopy

Deep-frozen mouse brain material from control, single, and double transgenic mice was derived at the Mayo Clinic (Mayo Clinic study conducted under approval of IACUC study protocol A67914-15-R18).

Mouse brains embedded in Optimal cutting temperature compound generated 12- $\mu\text{m}$  coronal sections kept frozen at –20 °C until used. Sections were fixed in 4% paraformaldehyde on ice for 30 min and permeabilized with 0.4% Triton X-100 for 30 min at room temperature. ABC mouse-on-mouse (M.O.M.) or Vectastain Elite ABC rabbit IgG kits were used, following the manufacturer’s instructions (Vector Laboratories, Burlingame, CA). To block nonspecific binding of components, avidin and biotin (Vector kit) were applied consecutively for 15 min at room temperature. With mouse primary antibody, endogenous mouse Ig sections were treated with Vector M.O.M. IgG blocking reagent for 1 h at room temperature before protein blocking with M.O.M. diluent for 5 min at room temperature. With rabbit primary antibodies, sections were treated with Vectastain blocking serum and labeled with one antibody or two. Single-labeled sections were incubated with primary antibody (30 min), M.O.M. anti-mouse, or Vectastain anti-rabbit biotinylated antisera (10 min), followed by avidin conjugated to peroxidase (5 min) and DAB as chromagen (2 min, Vector Laboratories), or avidin-FITC conjugate, or Texas red (5 min). Double-labeled sections were incubated with first antibody as above. Then, to prevent interactions between first and second labeling sets, Vector avidin and biotin were applied consecutively for 15 min at room temperature. Then, sections were treated 1 h with M.O.M. IgG blocking diluent or Vectastain normal blocking serum, incubated with a second primary antibody (30 min), M.O.M. anti-mouse or Vectastain anti-rabbit biotinylated antisera (10 min), and avidin conjugated to FITC or Texas red (5 min). Finally, section nuclei were counterstained with Harris hematoxylin (10 s) before mounting in Mowiol (4 °C overnight). When required, sections were stained with 1% ThS in water for 5 min at room temperature before mounting. All imaging used an LSM 510 META microscope (Carl Zeiss, Inc.) on an Axio Imager Z1 with a 40  $\times$  0.75 objec-

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tive or a  $63 \times 1.4$  oil immersion lens. Laser lines were 488 and 543 nm to excite FITC and Texas red, respectively. Fluorescence was measured with the following filters: bp 505–530 and bp 625–743. The z stacks were collected with a 16- $\mu$ m interval between slices. Plaques were examined from at least two different sections per conditions per animal (with the order of the antibody/reagents having been switched for the staining). For the control animals, WT, and hAChE-S single transgenic, the brain of one animal was examined. For hAPPswe single transgenic and hAChE-S/hAPPswe double transgenic, the brain of two animals were examined. Tiling functions of the confocal microscope collected a series of images spanning the whole brain section, with one representative section per condition collected. Images were analyzed with Image Browser software (Carl Zeiss, Inc.).

Colocalization (fluorescence overlap) was measured using the FIJI plugin JACoP (85). Labeling area and intensity were measured in FIJI using a Huang threshold, region of interest manager, and a set measurement analysis.

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**Author contributions**—L. J., S. B., and D. J. V. conceptualization; L. J. data curation; L. J. formal analysis; L. J. investigation; L. J. and S. B. methodology; L. J. writing-original draft; L. J., S. B., and D. J. V. writing-review and editing; S. B. and D. J. V. resources; D. J. V. supervision; D. J. V. funding acquisition.

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