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ORIGINAL ARTICLE

De novo induction of amyloid-β deposition *in vivo*

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Alzheimer's disease (AD), the most common type of senile dementia, is associated to the build-up of misfolded amyloid-β (Aβ) in the brain. Although compelling evidences indicate that the misfolding and oligomerization of Aβ is the triggering event in AD, the mechanisms responsible for the initiation of Aβ accumulation are unknown. In this study, we show that Aβ deposition can be induced by injection of AD brain extracts into animals, which, without exposure to this material, will never develop these alterations. The accumulation of Aβ deposits increased progressively with the time after inoculation, and the Aβ lesions were observed in brain areas far from the injection site. Our results suggest that some of the typical brain abnormalities associated with AD can be induced by a prion-like mechanism of disease transmission through propagation of protein misfolding. These findings may have broad implications for understanding the molecular mechanisms responsible for the initiation of AD, and may contribute to the development of new strategies for disease prevention and intervention.
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Introduction

Alzheimer's disease (AD) is the most common type of senile dementia, mainly affecting individuals over 65 years old. Disease manifestation is characterized by progressive impairment of memory and cognition, principally produced by synaptic dysfunction and neuronal loss. The etiology of the disease is currently unknown and it is a matter of great interest, as more than 90% of AD cases arise sporadically.

Cerebral accumulation of misfolded aggregates composed of the amyloid-β (Aβ) protein and hyper-phosphorylated tau have long been associated to the disease.[1,2] Compelling evidence suggest that misfolding and aggregation of Aβ might be the triggering event, which is responsible for inducing the subsequent brain abnormalities.[2–4] However, it is currently unknown why Aβ, which is a naturally produced protein, begin to misfold and aggregate in the brain. Interestingly, brain accumulation of misfolded protein aggregates is a common feature of several neurodegenerative diseases, including, besides AD, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and prion disorders.[4] These diseases can have sporadic or inherited origin, except in case of prion diseases, in which the pathology can be transmitted by infection.[5] Strikingly, the infectious agent responsible for prion diseases is composed exclusively by the misfolded and aggregated form of the prion protein that has the surprising ability to propagate the disease through an infection process, which involve the auto-catalytic conversion of the normal host prion protein.[6]

Extensive *in vitro* studies have shown that disease-associated misfolding and aggregation of proteins follow a seeding-nucleation model in which the formation of oligomeric seeds is a slow and rate-determining event.[7,8] In this model, protein misfolding and aggregation is greatly accelerated by addition of pre-formed seeds. The seeding/nucleation mechanism offers a plausible explanation for the transmissibility of prion diseases by infectious misfolded prion protein and predicts that other misfolded proteins have the potential to transmit the disease through a prion-like infectious process.[8]

Exciting recent studies have reported that Aβ deposition can be accelerated *in vivo* by injecting AD brain homogenates carrying Aβ aggregates.[9–11] These studies have been done in transgenic mice expressing a mutant form of the human amyloid precursor protein (APP), which spontaneously develop AD-like neuropathological alterations. Thus, the

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induction of Aβ deposition observed in these studies only represents an acceleration of few months of the spontaneous process that was set to occur by introduction of the mutant gene. This is drastically different from the *bona fide* infectious process produced by prions in animals, which, without inoculation with the misfolded protein, would not spontaneously develop the disease. The goal of this study is to demonstrate that similar to prion diseases, an AD-like pathology can be induced in animal models that naturally do not develop amyloid aggregates during their lifespan.

Materials and methods

Preparation of human brain homogenates

The AD brain sample used in this study was obtained from the cerebral cortex of a 90-year-old woman with clinical diagnosis of AD, which was confirmed by a post-mortem histopathological analysis. Control sample was obtained from a 163-day-old male, who did not have any detectable amyloid deposits in the brain (data not shown). Because of the existence of misfolded Aβ aggregates in the brain of elderly people, a young control was chosen. Samples were taken within 2 h post-mortem and frozen at −70 °C. Brain samples (cortex) were homogenized at 10% in phosphate-buffered saline plus a protease inhibitor cocktail (Roche, Mannheim, Germany), vortexed for 2 min and sonicated for 3 s at 60 units of amplitude in a Sonics—Vibra Cell manual sonicator (Newtown, CT, USA). Resulting homogenates were vortexed again, and centrifuged at 3000 *g* for 5 min to remove tissue debris. Supernatant was aliquoted and stored at −70 °C until use.

Intracerebral inoculations into mice

For our studies, we used mice that express the human wild-type *APP* gene (*HuAPPwt*).[12] Heterozygous transgenic mice were used in these experiments. Groups of six mice were injected when 165 days old, with 2 μl of 10% brain homogenates. Injections were performed stereotaxically in the hippocampus (both hemispheres), using the following coordinates: anterioposterior (AP) = −1.8 mm; mediolateral (ML) = ± 1.8 mm; dorsoventral (DV) = −1.8 mm. Animals were killed at 30, 90, 285, 450 or 585 days-post injection (dpi).

Immunohistochemistry

Paraffinized tissues were deparaffinized with three 5-min changes of xylene. Samples were later rehydrated and heated for 10 min at 95 °C in citrate buffer. Cold samples were covered with 3% H_2O_2 for 5 min. Tissue sections were covered with M.O.M. blocking reagent working solution (Vector Labs, Burlingame, CA, USA) and 200 μl of 4G8 antibody (1:1000) were used to cover each slide overnight at 4 °C. Slides were treated with biotinylated anti-mouse secondary antibody (1:200) and incubated for 30 min at room temperature. Later, samples were rinsed and incubated with Vectastain ABC Reagent (Vector Labs) for 30 min. Tissue sections were covered with diaminobenzidine at room temperature, and after gentle washing with water, they were covered with Mayer's Hematoxylin. Finally, samples were dehydrated with ethanol and xylene and mounted with Super Mount (Innogenex, San Ramon, CA, USA) and cover slips. Reactive astrocytes were measured by a similar procedure, but using anti-glial fibrillary acidic protein (GFAP) antibody. Samples were analyzed using a Leica DMI6000 B microscope (Wetzlar, Germany) and subjected to image analysis using the ImagePro software (Silver Spring, MD, USA).

Thioflavin S staining

Dewaxed and hydrated tissue slides were placed in 0.1% aqueous Thioflavin S (ThioS) solution. Samples were later dehydrated with 95% ethanol, followed sby two changes of 100% ethanol, and cleared with xylene. Tissue slides were finally covered with resinous mounting medium. Final specimens were stored in a dark place at 4 °C until visualized. Samples were analyzed using a Leica DMI6000 B microscope.

Western blot

Proteins were fractionated by electrophoresis using 4–12% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE; Invitrogen, Carlsbad, CA, USA), electroblotted into Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) and probed with the 4G8 antibody (1:3000) (Covance, Princeton, NJ, USA). The immunoreactive bands were visualized by ECL Plus Western blotting detection system (GE Healthcare, Little Chalfont, UK), using a UVP Bioimaging system EC3 apparatus (UVP, Upland, CA, USA).

Statistical analysis

Results of GFAP burden were analyzed by one-way analysis of variance followed by the Tukey's multiple comparison post-test using the Graph Pad Prism 5.0 software (La Jolla, CA, USA).

Results

To test whether AD can be transmissible by a prion-like mechanism, we used a transgenic mice that expresses human APP without any mutation (here termed HuAPPwt).[12] These animals produce Aβ bearing the human sequence of the protein, but do not develop Aβ deposits during their whole lifespan (Figure 1a). This is significantly different from other AD transgenic mice models such as the widely-used tg2576, which expresses the human APP harboring the Swedish mutation; as a result, these mice show detectable Aβ aggregates (starting at approximately 9 months), which become very prominent by 15 months of age (Figure 1b).[13]

We injected intracerebrally the brain extracts from a clinically and pathologically confirmed AD patient (Figure 2) into the hippocampus (bilaterally) of the

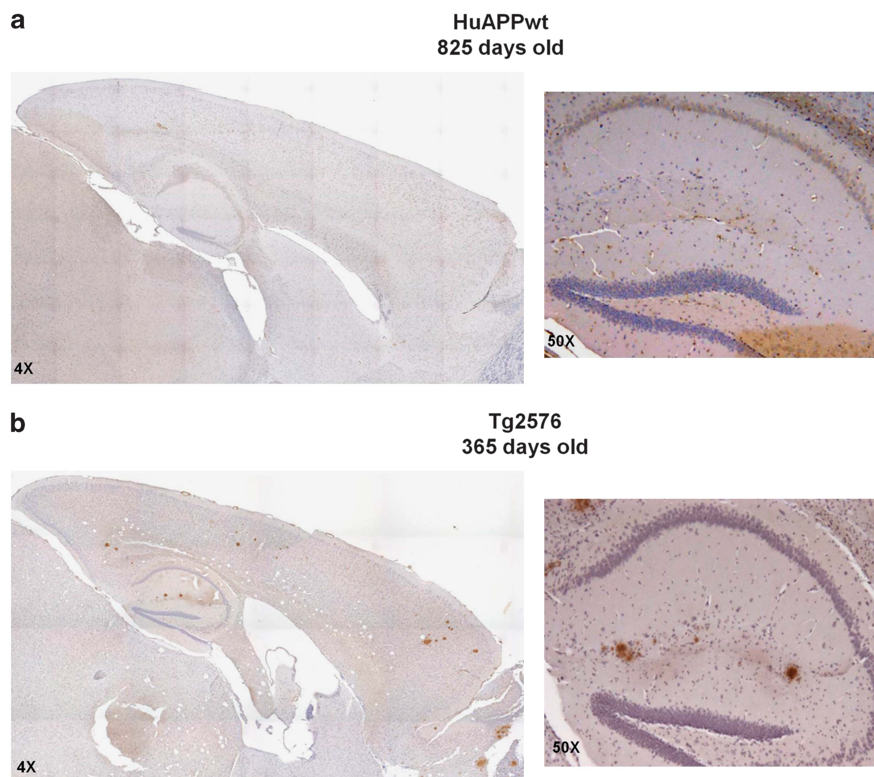

Figure 1 Human wild-type amyloid precursor protein (APP) gene (*HuAPPwt*) mice do not develop amyloid-β (Aβ) deposits spontaneously. (**a**) Brain coronal sections from an 825-day-old HuAPPwt mouse were analyzed by immunohistochemistry using an anti-Aβ antibody. The pictures were taken with two different magnifications (× 4 and × 50) to show the entire slide and a more magnified section of the hippocampal area. (**b**) Equivalent brain sections of a 365-day-old tg2576, harboring substantial amyloid deposits. Image magnification is noted at the bottom-left corner of each picture.

165-day-old HuAPPwt mice. As control, we injected brain preparations of a young individual that did not carry amyloid deposits (data not shown). The amount and aggregation state of Aβ in each of the inoculated samples were considerably different as analyzed by histology and Western blotting (Figure 2). To follow protein deposition over time, we killed HuAPPwt mice injected with AD and control brain extracts at 285, 450 and 585 dpi. Interestingly, only HuAPPwt mice injected with AD brain homogenates showed detectable Aβ aggregates by immunostaining with an anti-Aβ antibody (4G8; Figure 3a). In contrast, none of the mice injected with control brain homogenate presented any Aβ deposit at the same ages and times after injection (Figure 3a, left panels). Staining with ThioS, a dye that specifically binds to amyloid structures,[14] only showed positive reactivity in some of the brains (3/7) from the group inoculated with AD material, which was killed at 585 dpi (Figure 3a, right panels). None of the samples in any other group were ThioS positive. These data suggest that 4G8-positive signals in mice killed at 285 and 450 dpi correspond to diffuse Aβ aggregates, which are considered precursors of mature ThioS-positive AD-senile plaques.[15] To rule out that the aggregates observed do not correspond to the original inocula, but to *de novo* deposition of endogenous Aβ, we injected AD brain extracts into HuAPPwt mice and we killed them at 30 and 90 dpi. Aβ deposits were not detected in any of these mice by immunohistochemical analysis (Figure 3b).

Then, we compared the morphological and staining features of Aβ deposits induced by inoculation of AD brain homogenates into HuAPPwt with those observed spontaneously in the brain of tg2576 mice, which is probably the best characterized and most widely used transgenic mouse model of AD. When comparing the aggregates generated in HuAPPwt mice with those in tg2576, we observed a similar distribution of deposits, mostly located in hippocampus and cortex. Morphologically the Aβ deposits were also similar (Figure 4), albeit a bit smaller and less compact in HuAPPwt mice, suggesting again that they are in the way to grow into large mature plaques. Aggregates were also similar in ThioS staining, and in both cases, they are associated with an extensive astroglial proliferation (Figure 4). Tau staining with AT8 anti-phospho-Tau was negative in all samples (data not shown).

Finally, to assess the progression of Aβ deposition in injected mice, we subjected 4G8-stained slides to image analysis. The percentage of mice harboring detectable Aβ aggregates increased with time, reaching 100% at 585 dpi (Figure 5a). We also measured the

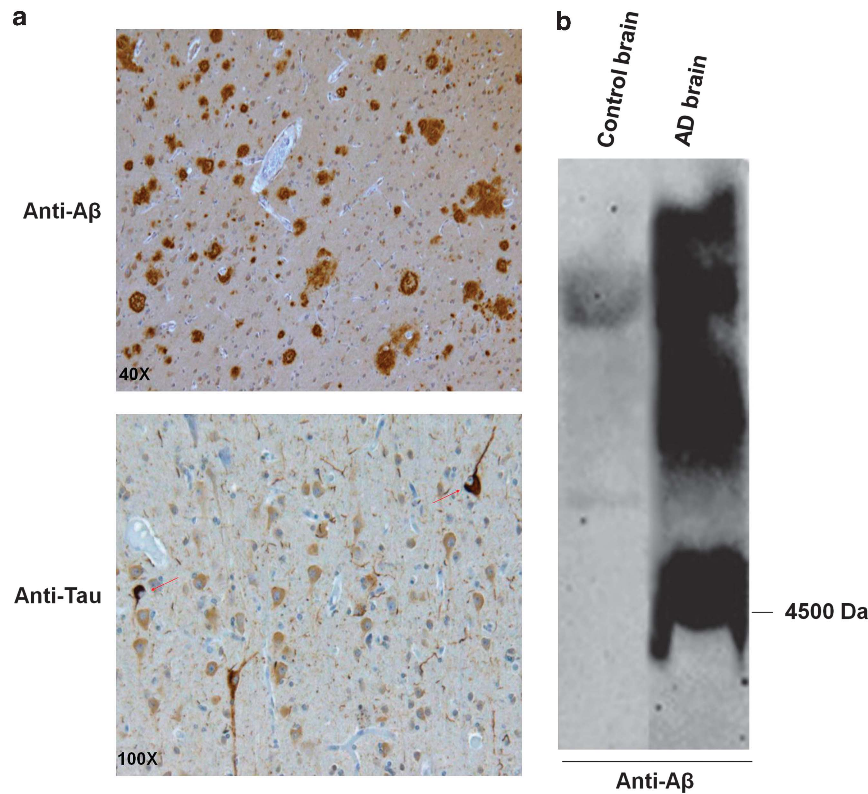

Figure 2 Histological and biochemical characterization of brain used as inocula. (**a**) Sections of the entorhinal cortex of the Alzheimer's disease (AD) brain used to inject human wild-type amyloid precursor protein (APP) gene (*HuAPPwt*) mice was analyzed by immunohistochemistry with 4G8 anti-amyloid-β (Aβ) antibody and 4855 anti-phospho-Tau antibody. Pictures showing Aβ and tau staining were obtained at ×40 and ×100 magnifications, respectively. (**b**) Western blot showing Aβ burden in control and AD brain homogenates used for inoculation in HuAPPwt mice. Each line represents the signal equivalent to 10 μl of a 10% brain homogenate.

amyloid load by counting the number of Aβ aggregates (Figure 5b) and measuring the area occupied by amyloid deposits (Figure 5c) in hippocampus (site of injection) and cortex. Both the number and area of Aβ deposits increased significantly with time in the two brain regions analyzed (Figures 5b and c). The fact that Aβ deposits were also detected far from the injection site (cortex) suggests that the seeding activity can diffuse in the brain. The extent of astrogliosis measured by the area stained by GFAP antibody was also higher in the animals injected with AD brain homogenate (Figure 5d).

Discussion

Recent studies have proposed that other protein-misfolding disorders besides prion diseases could be transmissible *in vivo*, following the principles posited by the heretical prion mechanism.[8,16–20] If this hypothesis proves to be correct, it will open a novel view on the biology of misfolded protein aggregates and the origin of protein-misfolding disorders, which will have broad-ranging implications for understanding the disease mechanisms and development of new strategies for disease prevention and intervention. In this scenario, our results demonstrate that the administration of brain homogenates containing Aβ aggregates can induce some of the neuropathological characteristics of AD in animals, which, without inoculation, will not develop these alterations during their natural lifespan. This experimental paradigm mimic, at least with respect to Aβ aggregation, a situation in which a normal person will live his entire life without developing AD abnormalities, unless the process is induced by exposure to material containing seeds of preformed Aβ aggregates.

Our findings suggest that in an experimental setting, misfolded Aβ aggregates can behave in a similar way as infectious prions. Indeed, as in prion diseases, our data shows that the quantity and degree of maturation of the protein deposits increases with age and that the seeding activity can spread to areas other than the site of injection. At this time, we do not know which of the various Aβ-misfolded species are most efficient in triggering Aβ aggregation, but *in vitro* experiments suggest that soluble, small oligomers may be the key factors in seeding Aβ misfolding and aggregation (Supplementary Figure 1). Long incubation periods are a key feature of infectious prion diseases. In fact, incubation periods in Kuru, a human prion disease, has been reported to reach more than

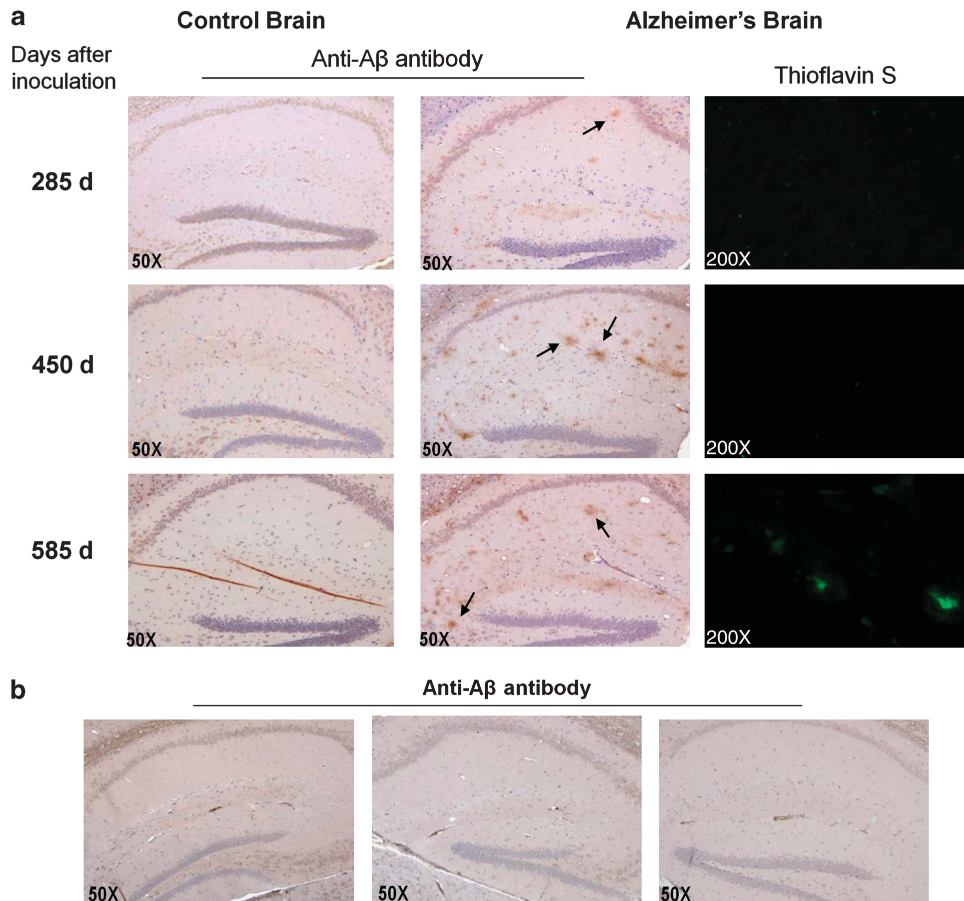

Figure 3 Human wild-type amyloid precursor protein (APP) gene (*HuAPPwt*) mice inoculated with Alzheimer's disease (AD) brain extracts develop cerebral amyloid-β (Aβ) deposits. (**a**) HuAPPwt mice injected with brain homogenates from a healthy individual (left panel) or an AD patient (middle and right panels) were killed at different times after injection. Brain slides were stained with anti-Aβ (4G8) antibody (left and middle panels) or Thioflavin S (ThioS; right panels). Pictures correspond to representative slides of all animals analyzed. Arrows point to Aβ deposits typically observed in the inoculated mice. (**b**) Representative slides of the hippocampus of three different mice killed 30 days-post injection (dpi), where no Aβ deposits were detected by immunohistochemistry (4G8). Similar results were obtained in animals killed 90 dpi (data not shown). Picture magnification is indicated in the bottom-left corner.

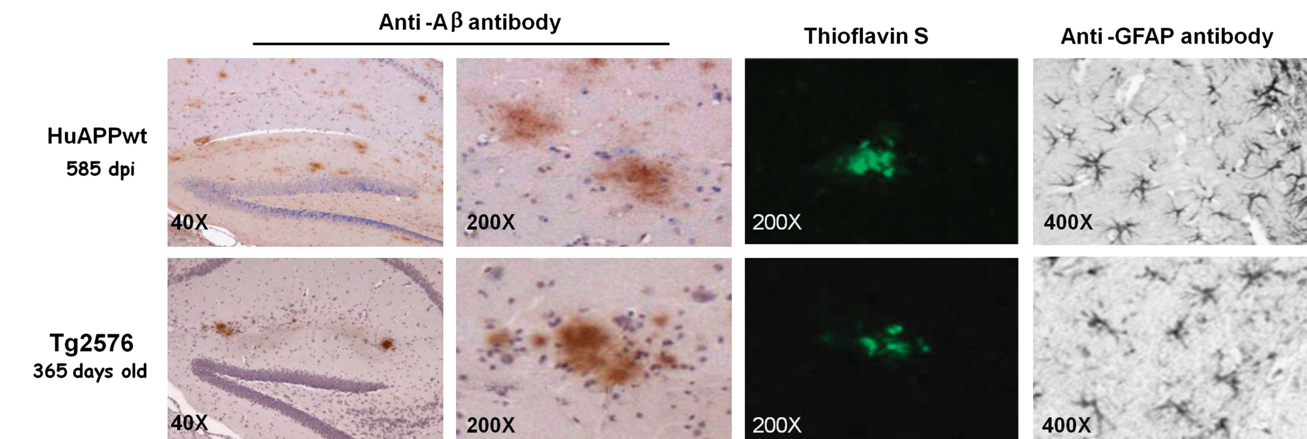

Figure 4 Amyloid-β (Aβ) deposits in human wild-type amyloid precursor protein (APP) gene (*HuAPPwt*; Alzheimer's disease (AD)-injected 585 days post-injection (dpi)) and Tg2576 (365 days old) mice. The figure shows immunohistochemical staining with the 4G8 monoclonal anti-Aβ antibody at two different magnifications ($\times 40$ and $\times 200$), Thioflavin S (ThioS) staining ($\times 200$) and astrogliosis detected by anti-GFAP staining ($\times 400$). The images correspond to representative pictures from the hippocampus (Aβ and ThioS staining) or cortex (GFAP staining) of the HuAPPwt group inoculated 585 days before, with AD brain homogenate and 12-month-old tg2576 mice that spontaneously developed the lesions.

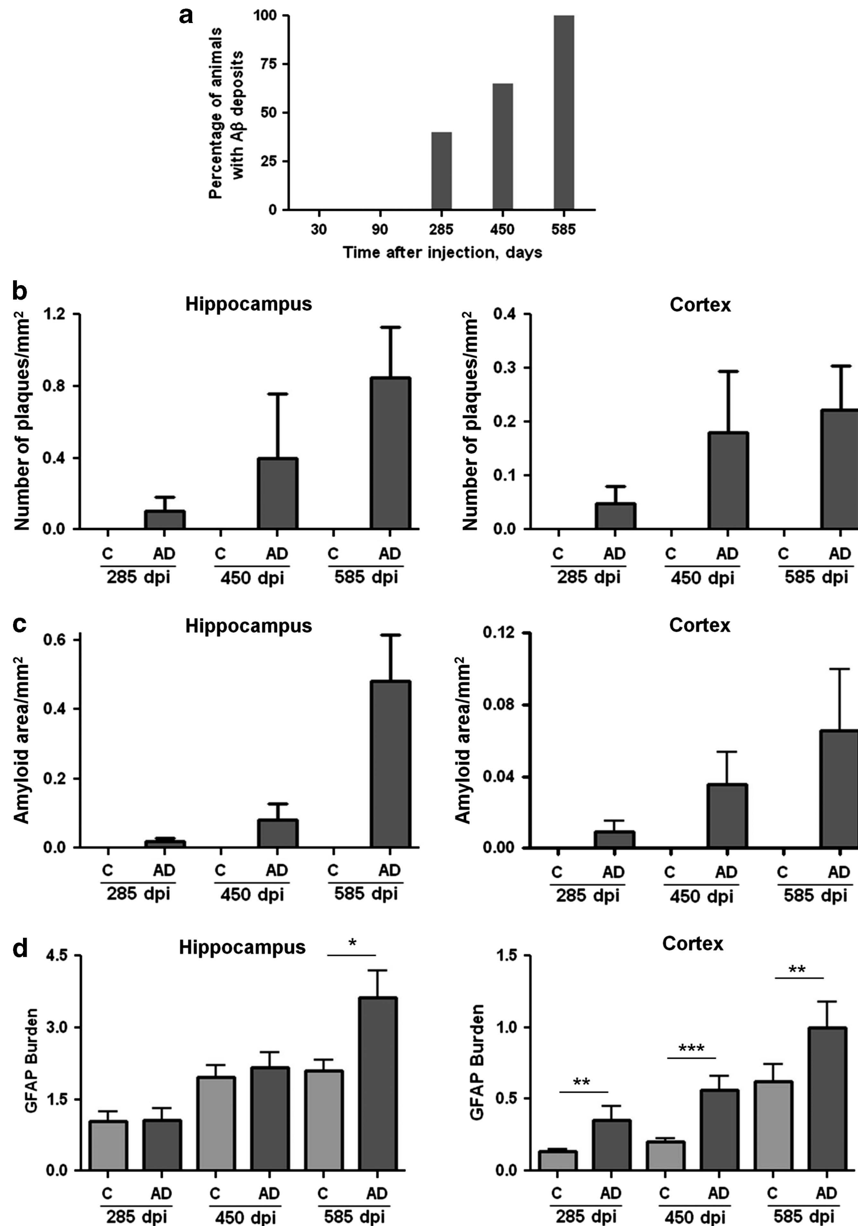

Figure 5 Progressive accumulation of amyloid-β (Aβ) deposits in the brain of HuAPPwt mice. Brain slides from HuAPPwt mice injected with normal (C) or AD brain homogenate (AD) were analyzed for Aβ deposition after 4G8 immunostaining in hippocampus (including the dentate girus, subiculum and hippocampal regions CA1, CA2 and CA3). (**a**) Percentage of HuAPPwt mice exhibiting Aβ deposits in groups killed at different times after injection. (**b**) The number of Aβ aggregates in hippocampus and cortex, was estimated by counting number of 4G8-positive deposits per slide, and is expressed as number of Aβ deposits per mm^2. (**c**) The load of amyloid was estimated by the area stained by 4G8 antibody in relation with the total area analyzed. (**d**) The extent of astrogliosis was estimated by calculating the area stained by GFAP antibody in relation with the total area analyzed. All groups consisted of six animals, except the control in the 285 days-post injection (dpi) in which one animal died from an unrelated problem. Data was analyzed by one way analysis of variance, and in all cases, the differences between animals injected with control or AD brain homogenate were significant with $P<0.001$. For the GFAP burden results, individual differences were evaluated by using the Tukey's multiple comparison post test (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

four decades.[21] These long incubation periods complicate epidemiological tracking of an infectious process. Our findings added to recent experimental evidences suggesting prion-like propagation of neuropathological abnormalities in various common neurodegenerative diseases,[9–11,22–24] may shed new light regarding the etiology of diseases associated with accumulation of misfolded protein aggregates. It remains to be studied whether at least a proportion of AD cases could be initiated through a transmissible prion-like mechanism under natural conditions in humans.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)