nature Vol 440|16 March 2006|doi:10.1038/nature04533

LETTERS

A specific amyloid-β protein assembly in the brain impairs memory

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Memory function often declines with age[1], and is believed to deteriorate initially because of changes in synaptic function rather than loss of neurons[2]. Some individuals then go on to develop Alzheimer's disease with neurodegeneration. Here we use *Tg2576* mice, which express a human amyloid-β precursor protein (APP) variant linked to Alzheimer's disease, to investigate the cause of memory decline in the absence of neurodegeneration or amyloid-β protein amyloidosis. Young *Tg2576* mice (<6 months old) have normal memory and lack neuropathology, middle-aged mice (6–14 months old) develop memory deficits without neuronal loss, and old mice (>14 months old) form abundant neuritic plaques containing amyloid-β (refs 3–6). We found that memory deficits in middle-aged *Tg2576* mice are caused by the extracellular accumulation of a 56-kDa soluble amyloid-β assembly, which we term Aβ*56 (Aβ star 56). Aβ*56 purified from the brains of impaired *Tg2576* mice disrupts memory when administered to young rats. We propose that Aβ*56 impairs memory independently of plaques or neuronal loss, and may contribute to cognitive deficits associated with Alzheimer's disease.

Poor memory function can predict Alzheimer's disease up to 15 years before diagnosis[7], and non-demented individuals at risk genetically for Alzheimer's disease show abnormalities in functional brain imaging tests[8,9]. These and other studies imply that Alzheimer's disease has an insidious onset, which blurs the boundary between age-associated memory impairment and Alzheimer's disease[10]. *Tg(APPSWE)2576Kahs* mice (hereafter *Tg2576* mice) express a human amyloid-β precursor protein (APP) variant linked to Alzheimer's disease, and develop many neuropathological features of Alzheimer's, including amyloid plaques, dystrophic neurites and inflammatory changes[3,4,11]. However, *Tg2576* mice lack neurofibrillary tangles, significant neuronal loss and gross atrophy[4]. They may therefore be a good model to study pre-clinical stages of Alzheimer's disease, before the diagnosis of dementia or the onset of neuronal loss.

In *Tg2576* mice, as in other *APP* transgenic mice, there is strong evidence that amyloid-β (Aβ) is responsible for age-related memory decline[6,12,13]. However, there are several paradoxical findings about the relationship between Aβ and cognitive decline that suggest a complex role for Aβ in cognitive impairment. For example, spatial reference memory in *Tg2576* mice declines modestly but significantly at 6 months of age and then remains stable for 7 to 8 months (Fig. 1a, b). However, no candidate Aβ species measured to date corresponds with the decline in memory observed at 6 months and the cognitive stability observed thereafter (see Supplementary Table 1). Hence, we are faced with the paradox that a rapidly increasing amount of Aβ, the molecule believed to be responsible for memory loss, is associated with no change in memory function. One solution to this conundrum is to posit the existence of soluble Aβ assemblies that disrupt memory[6,14–16], which we designated Aβ* (Aβ star) and sought to identify in *Tg2576* mice.

A challenge in analysing Aβ in the brain lies in reliably separating the specific cellular pools of Aβ (for example, extracellular, intracellular, membrane-associated and insoluble). We overcame this obstacle by developing a high-fidelity extraction procedure that separates proteins in known cellular compartments (Supplementary Fig. 1). Our new extraction method allowed us to quantify and compare four independent pools of transgene-derived Aβ species.

To resolve the problem of a mismatch between Aβ levels and memory deficits, we used our extraction procedure to search for Aβ* in *Tg2576* mice between 4 and 25 months of age. We required candidate Aβ* molecules to satisfy two criteria. First, their appearance should coincide with memory loss at 6 months. Second, their levels should remain stable in middle-aged mice (6–14 months old). By immunoblotting immunoglobulin-depleted forebrain extracts, we found a set of apparent assemblies of Aβ in the soluble, extracellular-enriched fraction from 6-month-old mice (Fig. 1c). In addition to a faint 4-kDa band corresponding to Aβ monomers, 6E10- and 4G8-immunoreactive proteins (see Methods) were detected at molecular masses theoretically corresponding to trimeric (14 kDa), hexameric (27 kDa), nonameric (40 kDa) and dodecameric (56 kDa) $A\beta_{1-42}$ assemblies. These species represent multiples of trimeric Aβ oligomers, with high-molecular-mass assemblies (>20 kDa) appearing in mice older than 6 months. The detection of similar bands using 6E10 and 4G8 antibodies excludes the possibility that they represent degradation products of soluble APP, which lacks the mid-domain Aβ epitope ($A\beta_{17-24}$) recognized by 4G8 (Supplementary Fig. 2a). The bands were not recognized by 22C11 or APPC17-Cter antibodies, indicating they were neither APP nor APP cleavage-end products (data not shown).

Although this result suggests that ageing induces Aβ trimers to associate and form high-molecular-mass assemblies, we also considered the possibility that they might represent Aβ oligomers complexed to binding proteins. However, this is unlikely on the basis of their biochemical properties and immunospecificity. First, we examined their properties in urea, a common denaturant of globular proteins[17]. To our surprise, the Aβ oligomers were unaltered in SDS–polyacrylamide gel electrophoresis (SDS–PAGE) containing 8 M urea (Fig. 2a). However, when exposed to ≥10% hexafluoroisopropanol (HFIP), a solvent with strong hydrogen-bonding properties, the theoretical hexamers, nonamers and dodecamers depolymerized, with a parallel increase in levels of tetramers, trimers and, to a lesser extent, monomers (Fig. 2b). In >20% HFIP, only the

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putative trimers remained; these dissociated into Aβ monomers in >55% HFIP (not shown). These data suggest that the high-molecular-mass Aβ complexes are held together by urea-resistant hydrogen bonds, not by covalent bonds. In addition, the greater resistance of trimers to denaturation supports our conjecture that trimers are the fundamental Aβ assembly unit *in vivo*. Second, A11 antiserum, which specifically detects soluble amyloid assemblies distinct from fibrillar Aβ, detected only 27–56 kDa complexes in extracellular-enriched extracts, confirming previous observations that A11 reacts with Aβ oligomers larger than tetramers[18] (Supplementary Fig. 2b). The immunoreactivity of the high-molecular-mass complexes with 6E10, 4G8 and A11 antibodies indicates that they are soluble Aβ oligomers. Finally, we examined the native size of soluble Aβ assemblies under non-denaturing conditions in order to demonstrate that they were not artificially generated during SDS–PAGE. Extracellular-enriched extracts were fractionated by non-denaturing size-exclusion chromatography (SEC) and subjected to SDS–PAGE (Fig. 2c). High- and low-molecular-mass Aβ assemblies (at expected intervals) were collected in different fractions, arguing against the possibility that SDS or self-oligomerization triggered the formation of the Aβ oligomers. Collectively, our results suggest that endogenous Aβ forms a ladder of stable, soluble, physiological assemblies comprised of trimers and multiples of trimers in *Tg2576* mice older than 6 months.

Trimers and hexamers were excluded as components of Aβ*, because they were present before memory impairment (<6 months). However, a 56-kDa band appeared in extracts at 6 months of age, along with lesser quantities of a 40-kDa species; these correspond to theoretical $Aβ_{1–42}$ dodecamers and nonamers, respectively (Fig. 1c). The mean levels of the soluble Aβ assemblies remained stable after 6 months of age, although there was considerable variability between animals of the same age (Supplementary Fig. 2c–e). As the 56-kDa and 40-kDa species appeared at 6 months of age and remained stable between the ages of 6 and 13 months, they fulfilled the criteria for being designated as Aβ* and thus represented viable candidates for Aβ assemblies that cause memory deficits. We found no further increase in Aβ assemblies in old mice to correspond to the second drop in memory function at 15 months (Fig. 1a, b); by this age it is possible that the abundant plaques with prominent dystrophic neurites disrupt synaptic function sufficiently to cause further memory impairment[19].

The variability in levels of Aβ assemblies between animals of the same age provided an opportunity to examine correlations between the different Aβ oligomers and memory impairment. To do this, we compared spatial memory and levels of soluble Aβ species in two groups of 5- and 6-month-old *Tg2576* mice (Fig. 3). Monomers, trimers and hexamers of Aβ did not correlate significantly with performance in 5- or 6-month-old mice (Fig. 3a–f). However, we observed significant inverse relationships between the levels of 56-kDa and 40-kDa assemblies and memory performance (Fig. 3g, h), with

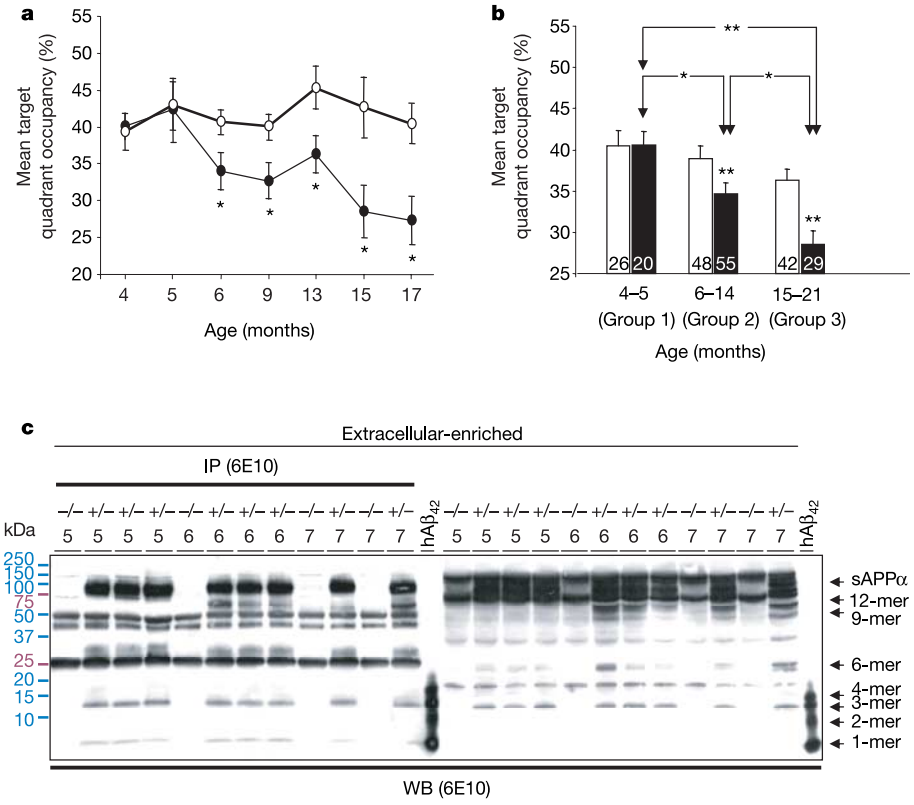

Figure 1 | Temporal patterns of soluble Aβ oligomers and memory decline in *Tg2576* mice. **a**, Memory retention in 4–17-month-old mice shows a progressive but irregular decline with periods of stability. Filled circles, $Tg2576^{+/-}$ mice; open circles, $Tg2576^{-/-}$ mice. $Tg2576^{-/-}$ and $Tg2576^{+/-}$ denote mice harboring no (non-transgenic) or one transgene array, respectively. Asterisk, ANOVA ($P < 0.01$) followed by *t*-test ($P < 0.01$). **b**, Temporal analysis of spatial memory shows three stages of performance. Filled bars, $Tg2576^{+/-}$ mice; open bars $Tg2576^{-/-}$. Numbers inside bars denote numbers of mice. Asterisk, $P < 0.01$; two asterisks, $P < 0.001$ (ANOVA). Target quadrant occupancy scores (±s.e.m.) during probe trials are a measure of spatial reference memory retention. **c**, Identification of Aβ oligomers in soluble, extracellular-enriched extracts of proteins from brains of 5-, 6-, and 7-month-old mice (age indicated above lanes), assessed by western blot (WB) with or without immunoprecipitation (IP). The intensity of the 40-kDa band was 33.92 ± 12.5% (mean ± s.e.m.) ($n = 4$) of the intensity of the 56-kDa band. Synthetic human $Aβ_{1–42}$ peptide ($hAβ_{42}$) was used as a size marker and positive control (right lane). Arrows indicate respective migration positions of monomers (1-mer), dimers (2-mer), trimers (3-mer), tetramers (4-mer), hexamers (6-mer), nonamers (9-mer), dodecamers (12-mer) and sAPPα (secreted form of APP that has been cleaved by α-secretase).

the 56-kDa assembly correlating substantially better than the 40-kDa assembly ($r^2 = 0.66$ and 0.45, respectively). There were no correlations between the levels of any Aβ oligomers and performance in the cued phase of a water maze test (see Methods), arguing against their effects on non-cognitive aspects of behaviour (Supplementary Fig. 3).

Because intracellular Aβ has been proposed to disrupt memory in 3 × Tg-AD transgenic mice (which express APP, presenilin-1 and tau variants)[20], we performed additional analyses to evaluate the potential accumulation of Aβ species within brain cells. Only trimeric and monomeric Aβ species were detected in the soluble, intracellular-enriched fraction, with no modulation between 5 and 6 months of age (Supplementary Fig. 4a). We also examined full-length APP and C-terminal fragments (CTFs) in membrane-associated fractions, and found no change in the levels of APP, CTF-βs or CTF-α (Supplementary Fig. 4b, c). In cytosolic extracts of primary *Tg2576* cortical neurons cultured from 14–15-day-old embryos, monomers and trimers were the only Aβ species detected (Supplementary Fig. 5), supporting our *in vivo* findings in young *Tg2576* mice. Thus, we found no intracellular or membrane-associated Aβ species or CTFs correlating with the onset of memory deficits in 6-month-old *Tg2576* mice.

The observations that the 56-kDa Aβ assembly appears at 6 months of age (when memory declines), that its levels are stable in ageing mice, that it is more abundant than the 40-kDa complex (Fig. 1c), and that it shows the strongest correlation with memory

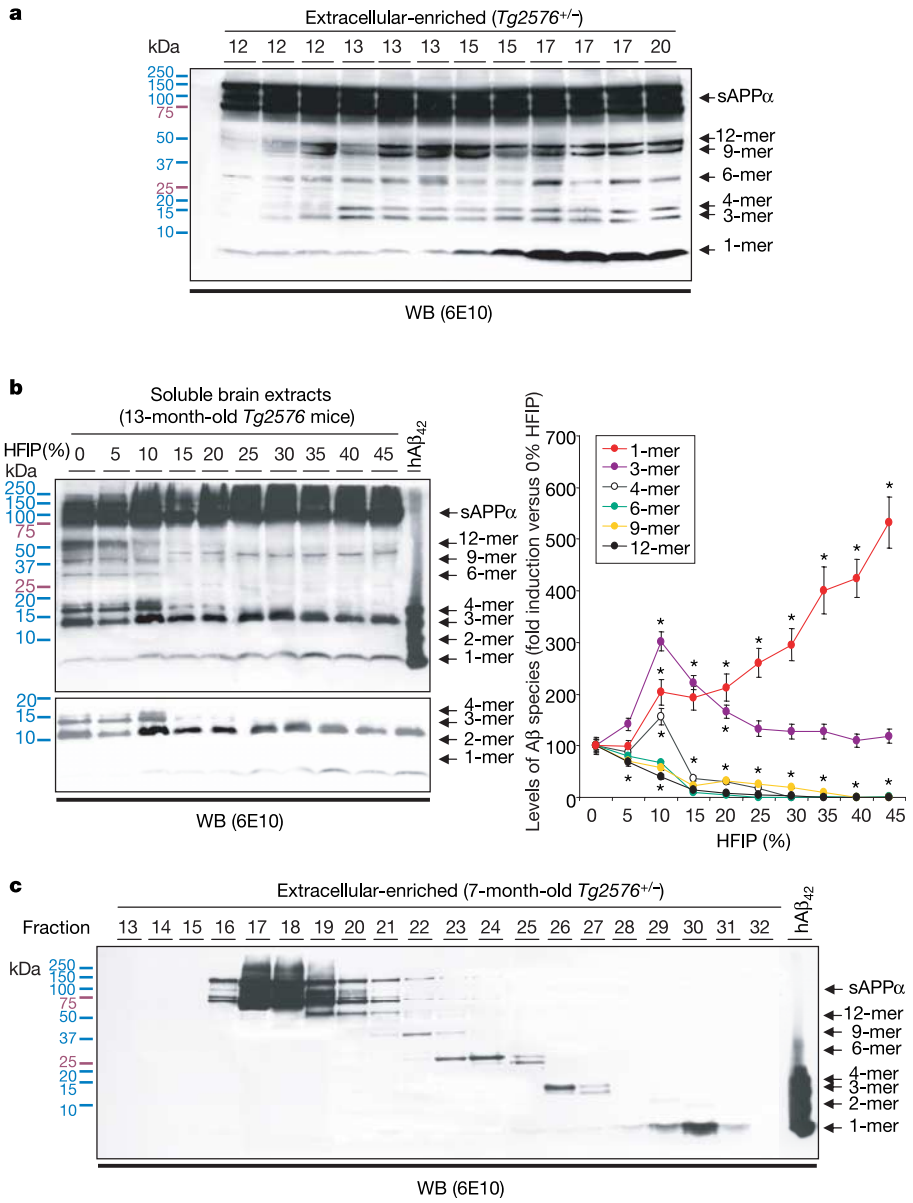

Figure 2 | Biochemical properties of Aβ assemblies in *Tg2576* mice. **a**, The presence of 8 M urea did not alter the electrophoretic pattern of Aβ oligomers in extracellular-enriched extracts from 12- to 20-month-old brains of $Tg2576^{+/-}$ mice that were probed with 6E10 antibodies. **b**, Soluble high-molecular-mass Aβ oligomers depolymerize in ≥15% HFIP, with concomitant enrichment of both monomeric and trimeric Aβ (lower exposure provided for enhanced contrast). The data for fold-change in Aβ species (right panel) is the mean ± s.e.m. of 3 experiments. **c**, SDS–PAGE analysis of brain extracts from 7-month-old $Tg2576^{+/-}$ mice fractionated by SDS-free size-exclusion chromatography (SEC) shows that Aβ oligomers migrate at expected molecular masses. The data confirm that high-molecular-mass Aβ oligomers are not artificially generated from monomeric or trimeric Aβ species during electrophoresis, and that trimers are not degradation products of high-molecular-mass oligomers. Bands revealed at 75 and 150 kDa are non-specific bands caused by the modified avidin used for immunoblotting (also present in blots of extracts from non-transgenic mice).

impairment suggest that it is the most likely candidate for Aβ*, which we thus named Aβ*56. However, so far our data on the relationship between Aβ*56 and memory impairment are correlative rather than causative. To determine whether Aβ*56 has the ability to disrupt memory function directly, we purified it from the brains of impaired *Tg2576* mice, applied it to young rats and assayed its effect using the Morris water maze.

We purified Aβ*56 using immunoaffinity chromatography (with 4G8 antibodies) followed by size-exclusion chromatography. This procedure yielded preparations of Aβ*56 that ran as a single band on silver-stained gels (Fig. 4a), were recognized by 6E10 antibodies (Fig. 4a) and revealed Aβ as a core component when analysed by liquid chromatography tandem mass spectrometry (Supplementary Fig. 7).

To allow observation of the effects of Aβ*56 on spatial memory for an episode of new learning, our behavioural protocol involved pre-training rats in the Morris water maze, implanting a cannula into a lateral ventricle of the brain, and allowing the animals a two-week recovery period (Supplementary Fig. 6a). We used rats for these experiments because chronic cannulae are better tolerated in rats than in mice. The pre-training allowed for the rapid acquisition of new spatial information in an entirely new environment when similar test procedures were used. Infusing 10 μl of 0.85 μM Aβ*56 two hours before testing had no effect on the escape latencies of rats given eight training trials to locate a hidden platform in the new maze environment (Fig. 4b).

After a 24-h retention interval, rats received another injection of Aβ*56 or vehicle, in the same manner as during training, and underwent a probe trial two hours later during which the platform was absent. Spatial memory was shown by the vehicle-injected rats but not by those treated with Aβ*56. There was a significant interaction of group × quadrant ($F_{1,18} = 6.288$, $P = 0.022$, analysis of variance (ANOVA)), but no main effects of group or quadrant (Fig. 4d). Rats in the vehicle group spent significantly more time in a target annulus surrounding the platform than in a control annulus in the opposite quadrant ($t_9 = 2.668$, *t*-test with 9 degrees of freedom; $P = 0.026$). In contrast, rats in the Aβ*56 group spent similar amounts of time in both locations ($P = 0.547$, *t*-test). In addition, rats in the Aβ*56 group spent significantly less time in the target annulus than those in the vehicle group ($t_{18} = 2.533$, $P = 0.021$, *t*-test).

Other measures, including the number of crossings (Fig. 4e) and

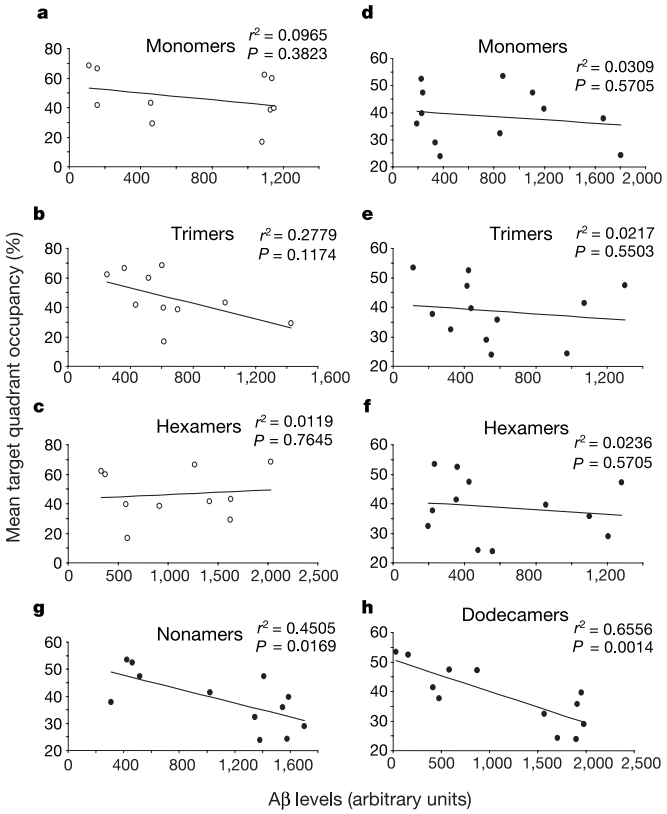

Figure 3 | Levels of the 56-kDa Aβ assembly show the strongest inverse correlation with spatial memory. a–f, Lack of significant correlations between retention of spatial memory and monomeric (4.5 kDa), trimeric (14 kDa) or hexameric (27 kDa) soluble Aβ species detected in extracellular-enriched fractions of 5-month-old (open circles) and 6-month-old (filled circles) $Tg2576^{+/-}$ mice. **g, h**, Levels of Aβ assemblies corresponding to nonameric (40 kDa) and dodecameric (56 kDa) species correlate inversely with memory at 6 months of age (ANOVA).

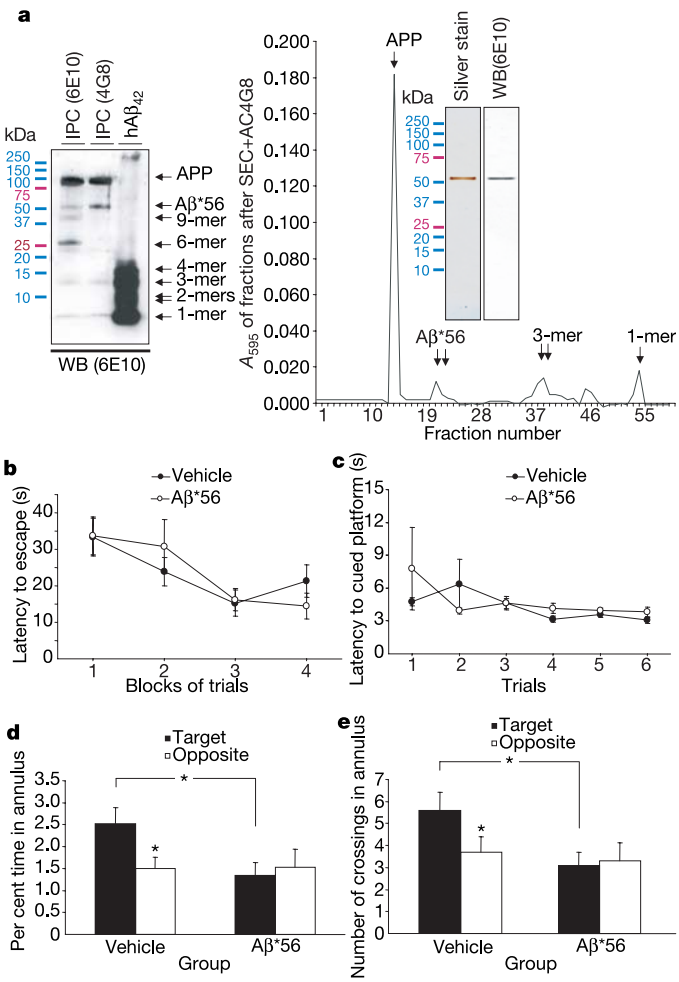

Figure 4 | Purification of Aβ*56 from *Tg2576* brain and effects of purified Aβ*56 on memory in young rats. a, Purification of total (RIPA-buffer-soluble) Aβ species using immunoaffinity purification columns (IPC) packed with 200 μg of 6E10 or 4G8 antibodies (left panel). Right panel shows absorbance at 595 nm (A_{595}) of proteins isolated by affinity chromatography with 4G8 (AC4G8) that were separated by size-exclusion chromatography (SEC) to yield fractions containing Aβ*56. **b**, There is no difference between rats that received Aβ*56 and vehicle (50 mM ammonium acetate, pH 8.5) in terms of latency to locate a hidden platform during training. Data show blocks of two training trials each (mean ± s.e.m.). **c**, There is no difference between rats (Aβ*56 and vehicle treatment groups) in terms of latency to locate a visible platform. **d, e**, Aβ*56 impairs spatial memory. Rats that received vehicle, but not Aβ*56, injections showed a significant spatial bias for the escape location 24 h after training. Target annulus measures are shown in **d**, and number of annulus crossings in **e** (mean ± s.e.m.). Two-way ANOVA ($P < 0.05$) followed by *t*-test (*, $P < 0.05$), $n = 10$ animals per group.

quadrant time, also provided no evidence of spatial bias as an indication of retention in the Aβ*56 group. Thus, Aβ*56 impaired long-term memory, but not acquisition of spatial information (Supplementary Discussion). Ten days later, both groups had equally good acquisition and retention in the same two-day protocol when new spatial information was once again provided but no Aβ*56 was administered, indicating no lasting debilitation as a result of treatment (Supplementary Fig. 6b, c). Notably, the behavioural effects of administering purified Aβ*56 to rats parallel exactly our previous observations in 6- to 11-month-old *Tg2576* mice[6], which show intact performance during the acquisition phase of the water maze training but impairment in probe trials after an overnight retention interval. Furthermore, in the current test, Aβ*56 had no effect on escape latencies to a visible platform (Fig. 4c), consistent with the lack of an Aβ*56 effect on performance of *Tg2576* mice in the cued phase of the water maze training (Supplementary Fig. 3). Our data demonstrate that Aβ*56 impairs memory in healthy, young rats, and strongly support the hypothesis that Aβ*56 is the principal cause of memory decline in middle-aged *Tg2576* mice.

The transient effects of Aβ*56 application in rats imply that Aβ*56 impairs memory by inducing transient physiological, rather than permanent neuropathological, alterations of the brain. The accumulation of insoluble aggregating proteins such as tau, huntingtin, prion protein, ataxin and Aβ occurs in many neurodegenerative disorders. These aggregates often define the disorders neuropathologically, but their relative contribution to disease symptoms compared to other, hypothetical, intermediate protein assemblies is controversial[21–23], and the identity of the theoretical intermediates has been elusive. Our finding that Aβ*56 is responsible for memory loss in plaque-forming *Tg2576* mice, and causes memory deficits when injected directly into healthy rats, sets a precedent for identifying other 'star' proteins inducing brain dysfunction—for example, tau* (ref. 22). That Aβ* is a highly specific form of Aβ offers the potential for developing precise diagnostic methods to detect its correlate in humans with pre-clinical Alzheimer's disease, presenting the possibility of targeting Aβ* and aborting the disease before permanent structural changes have developed.

METHODS

Transgenic animals. *Tg2576* mice[3] were the offspring of mice backcrossed successively to B6SJLF1 breeders.

Behavioural tests. Spatial reference memory was assessed in *Tg2576* mice using a modified version of the Morris water maze[6]. Testing was tailored for *Tg2576* transgene-positive and -negative mice in the B6SJL background strain. *Tg2576* transgene-positive and -negative mice received visible platform training for three days (eight trials per day), followed by hidden platform training for nine days, with four trials per day (trials 60 s each; inter-trial interval (ITI) 20–40 min). Three probe trials (60 s) were performed 20 h after 12, 24 and 36 training trials (after days 3, 6, and 9 of training), and the mean per cent target quadrant occupancy for the three probe trials was calculated.

Long-Evans male rats, approximately 4 months of age (young adults), were used to test the effects of Aβ*56 infusions on memory. For pre-training, rats received three trials (90 s; ITI 60 s) per day for eight days, during which they were allowed to locate a camouflaged platform that remained in the same location 2 cm below the water surface in a 1.83-m diameter maze. Every sixth trial was a probe trial (30 s), which served to assess the development of a spatially localized search for the escape platform. Performances during the probe trials were used to generate a learning index as previously described[24]. Immediately after pre-training, unilateral cannulae were implanted into a lateral ventricle. Coordinates for the cannulae tip were 1.0 mm posterior to bregma, 1.5 mm lateral to midline, and 3.5 mm ventral to the skull surface. Rats were allowed two weeks to recover before being trained to a new platform location in a new water maze environment (with a different physical location and surrounding spatial cues). Ten rats received Aβ*56 and another ten received vehicle (50 mM ammonium acetate buffer, pH 8.5). Rats in the Aβ*56 and vehicle groups were matched for body weight and learning index in pre-training. Mean body weights on the training day for rats in Aβ*56 and vehicle groups were 509.6 ± 24.43 g (mean ± s.e.m.) and 525.1 ± 19.10 g, respectively ($t < 1$). Mean learning indices for rats in the Aβ*56 and vehicle groups were 202.3 ± 10.9 and 214.8 ± 12.4, respectively ($t < 1$). Aβ*56 was injected at a concentration of 0.85 μM. The injection volume was 10 μl (1.0 μl min^{-1}, with one additional minute at the end to allow diffusion away from the cannulae). An equal volume of buffer was injected into rats in the vehicle group. Two hours after injection, rats were trained in the water maze. Rats received eight training trials (90 s each; ITI 8 min) to locate a hidden escape platform. The entire training session was completed within 60 min. The retention interval was 24 h. Two hours before the probe trial, Aβ*56 or vehicle was infused as before training. The percentage of time spent in an annulus that was 1.5× the size of the target platform was used to analyse performance in the probe trial. At the end of the probe trial (120 s), rats received six cued training trials to locate a visible escape platform. The location of this platform varied from trial to trial. Ten days after the test with Aβ*56, both groups were re-tested in the absence of any intraventricular injections before the training session (eight trials) or retention probe trial. The escape platform was in a new location and curtains with new spatial cues surrounded the maze. Both groups improved performance on training and had a similarly accurate spatial search for the correct platform location during the retention test 24 h later (Supplementary Fig. 6b).

Antibodies. The following primary antibodies were used: 6E10 (monoclonal raised against $Aβ_{1–17}$) and 4G8 (monoclonal raised against $Aβ_{17–24}$) (1:100–10,000 dilution; Signet Laboratories), R1282 (polyclonal raised against Aβ; 1:75 dilution)[25], R1736 (polyclonal raised against residues 595–611 of APP695; 1:1,000 dilution)[26], FCA3542 (polyclonal raised against Aβ42)[27], APPCter-C17 (polyclonal raised agaist the carboxy terminus of APP; 1:5,000 dilution)[28], anti-Flotillin-2, anti-ERKs, anti-JNK and anti-c-Jun (all at 1:200 dilution; Santa Cruz), anti-tau-5 (1:1,000 dilution; Biosource International), anti-MAP-2 (1:200 dilution; Sigma and Santa Cruz) and anti-actin (1:250 dilution; Sigma), and anti-tPA (anti-tissue plasminogen activator; American Diagnostica). The A11 anti-oligomer antibody (1:5,000 dilution)[18] was detected with a biotinylated anti-rabbit antibody (1:2,000,000 dilution; Vector Laboratories) and ExtrAvidin (1:5,000 dilution; Sigma).

Protein extractions, protein concentration determination, immunoaffinity chromatography, size-exclusion chromatography, silver staining, liquid chromatography tandem mass spectroscopy, western blotting and immunoprecipitation procedures are described in Supplementary Methods.

Received 23 November; accepted 13 December 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank S. Younkin, D. Walsh, M. Podlisny, D. Selkoe, J. Cleary and S. Prusiner for critical discussions. We are grateful to D. Cooper-Blacketer, J. McQuail and M. Sherman for technical help, and A. Delacourte and N. Sergeant for providing the APPC17-Cter antiserum. This work was supported by grants from the NIH (to K.H.A., M.G. and A.Y.) and a gift from M. and H. Hobbs to M.G.

Author Contributions S.L. and K.H.A. conceived the project. S.L. planned, performed and analysed the biochemistry experiments, including the protein extractions and purification of Aβ*56. K.H.A. wrote most of the paper. M.T.K. and M.G. planned, performed and analysed the rat behavioural experiments, and L.K. planned and analysed the mouse behavioural experiments. A.Y. provided the mass spectrometry analysis of purified Aβ*56, C.G.G. provided biochemistry advice, and C.G.G. and R.K. donated the A11 antiserum.

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