Alzheimer disease is a major cause of cognitive failure, and a pathogenically related but more subtle process accounts for many cases of mild memory symptoms in older humans. Insoluble fibrillar plaques of amyloid β-proteins (Aβ) and neurofibrillary deposits of hyperphosphorylated tau proteins are the diagnostic lesions of AD, but their temporal mechanistic relationship has long been debated. The recent recognition that small, diffusible oligomers may be the principal bioactive form of Aβ raises the key question of whether these are sufficient to initiate cytoskeletal change and neurite degeneration. A few studies have examined the effects of oligomers of synthetic Aβ peptides of one defined length at supra-physiological concentrations, but the existence of such assemblies in the AD brain is not established. Here, we isolated Aβ dimers, the most abundant form of soluble oligomer detectable in the human brain, from the cortices of typical AD subjects and found that at subnanomolar concentrations, they first induced hyperphosphorylation of tau at AD-relevant epitopes in hippocampal neurons and then disrupted the microtubule cytoskeleton and caused neuritic degeneration, all in the absence of amyloid fibrils. Application of pure, synthetic dimers confirmed the effects of the natural AD dimers, although the former were far less potent. Knocking down endogenous tau fully prevented the neuritic changes, whereas overexpressing human tau accelerated them. Coadministering Aβ and soluble Aβ fully prevented the neuritic changes, whereas overexpressing endogenous tau fully prevents this phenotype. Two key advantages of our approach are: (i) it examines natural oligomers of the heterogeneous Aβ peptides that exist at low nanomolar concentrations in AD patients, and (ii) it uses a cell-culture system to apply to a systematic fashion biochemically fractionated and well-defined Aβ species, something not possible in vivo or mouse models, where abundant array of Aβ assembly forms coexist.

Results

Soluble Aβ Oligomers Isolated from the AD Cortex Induce Marked Cytoskeletal Abnormalities at Subnanomolar Concentrations in Primary Hippocampal Neurons. To determine whether soluble Aβ oligomers are sufficient to trigger neurofibrillary degeneration, even in the absence of amyloid plaques, we isolated Aβ dimers, the most abundant detectable form of soluble oligomer, from the AD cerebral cortex as described recently (10). These dimers are principally composed of Aβ42 (10). Soluble (Tris-buffered saline, TBS) extracts from cortices of humans diagnosed with clinically and neuropathologically typical AD (AD-TBS extracts) or age-matched non-AD subjects (Cont-TBS extracts) were immunoprecipitated with 3D6, a monoclonal antibody specific for the free N terminus (Asp-1) of human Aβ, and the immunoprecipitate was eluted with lithium dodecyl sulfate (LDS) and subjected to size exclusion chromatography (a method we refer to as IP-SEC). Aβ monomers and LDS-stable dimers could be separately detected in the SEC fractions of the AD-TBS extracts but not in the corresponding fractions of Cont-TBS extracts, as expected (Fig. L1). Importantly, SDS-stable higher oligomers, including dodecamers, were not detectable by IP-SEC in AD-TBS extracts (Fig. L4); accordingly, we focused on the dimers, cortical levels, of which were recently shown by others to correlate strongly with several features of the AD phenotype (Mini-Mental State Examination score; Blessed cognitive score; Braak stage; synaptic protein levels) (11). We prepared primary cultures of hippocampal neurons from E18 rat embryos. After 18 d in culture, mature hippocampal neurons were exposed to either the monomer-rich or the dimer-rich SEC fractions of the AD-TBS extract.
extracts [or the corresponding fractions of Cont-TBS, which share other cortical proteins with the AD-TBS SEC fractions (Fig. L4)] but contain no Aβ for 3 d, at which time the cultures were fixed for immunochemistry. Application of IP-SEC fractions 8–9 of the AD-TBS extract, which contain soluble, SDS-stable Aβ dimers but few or no monomers (Fig. L4), induced a marked degeneration of neurites after 3 d of exposure, as indicated by extensively beaded, dystrophic neurites and an abnormal microtubule cytoskeleton revealed by Tubulin and tau immunocytochemistry (Fig. 1B and C, and Fig. S1). In contrast, application of the Aβ monomer-rich fractions (fractions 10–11) of AD-TBS from the same SEC run or else either of the corresponding SEC fractions (fractions 8–9, 10–11) of Cont-TBS induced no significant neuritic alteration (Fig. 1B and C). The application of the Aβ dimers to hippocampal neurons for 2 d did not induce significant neuritic degeneration, but at higher magnification we observed some slightly beaded neurites (Fig. S1), suggesting the initiation of a progressive neurite degeneration. Following the 3-d treatment, we were able to recover residual Aβ monomers or dimers by immunoprecipitating the conditioned media (Fig. 1D), thereby verifying the continuous exposure of the neurons to these soluble Aβ species. The concentration of human Aβ dimers in the final culture medium was around 0.5 nM, as determined by ELISA or quantitative Western blot, values similar to those observed for Aβ in the AD cortex (10). Although exposure to the Aβ dimers invariably disrupted the neuritic cytoskeleton, neuronal cell bodies generally survived the 3-d treatment, and there was no significant increase in the very low baseline levels of apoptotic cells in the cultures, as revealed by TUNEL staining (Fig. S2). The collapse of the cytoskeleton was Aβ-dependent, because prior immunodepletion of the dimer-SEC fractions by the Aβ antisera AW7 fully prevented the effects of the dimers, whereas its preimmune serum was without effect (Fig. S3).

Importantly, when hippocampal neurons were cultured for 7 d, application of the soluble Aβ dimers did not induce a collapse of the cytoskeleton or other changes (Fig. S4A), in accord with evidence that the neurotoxicity of synthetic Aβ (at much higher concentrations) requires the maturation of neurons in vitro (12, 13). To explore the basis for this intriguing selectivity, we performed quantitative Western blotting of lysates from hippocampal neurons cultured for 7 or 18 d and found that in the 18-d neurons, the expression of mature tau (~55 kDa) was significantly increased, but the phosphorylation of the tau kinase GSK3β at serine 9, which is a negative regulator of GSK3β activity (14), was decreased (Fig. S4B). These results suggest that the time-dependent maturation of tau and of GSK3β activity (the latter creates the AT8 phosphoepitope on tau; see below) is associated with the ability of Aβ dimers to induce neurite degeneration. However, more work is needed to identify additional changes underlying the selective vulnerability of older cultured neurons to Aβ oligomers.

**Fig. 1.** Cytoskeletal abnormalities induced in primary hippocampal neurons by soluble Aβ oligomers isolated by SEC from the AD cerebral cortex. (A) AD-TBS (Right) or Cont-TBS (Left) was immunoprecipitated with 3D6 (3 μg/mL), eluted with sample buffer containing 4% LDS, and subjected to SEC. SEC of the immunoprecipitation of AD-TBS resolves Aβ dimers (fractions 8–9) from monomers (fractions 10–11), as detected by Western blot with 6E10 + 2G3 + 21F12. IP, 3D6 immunoprecipitates of the starting Cont-TBS or AD-TBS extracts used for this SEC. (B) Confocal images showing the tau (green) and Tubulin (red) immunoreactivities of the cytoskeleton of hippocampal neurons [days in vitro (DIV) 21] after 3-d treatment with Aβ oligomers (SEC fractions 10–11) or Aβ dimers (SEC fractions 8–9) isolated from AD-TBS or the corresponding fractions from Cont-TBS. Scale bar, 50 μm.) (C) Histograms represent the average number of tau-positive beads along 100-μm lengths of Tubulin-positive neurites under different conditions. Asterisk indicates data significantly different from those of neurons without treatment (P < 0.01 by Student t test). Error bars, SEM. (D) After a 3-d incubation on primary neurons, the conditioned media were immunoprecipitated with Aβ antisera AW7. Aβ monomers and dimers were precipitated from medium that contained the reconstituted IP-SEC fractions of AD-TBS but not from that with Cont-TBS.

**Pure Synthetic Aβ Dimers Induce Cytoskeletal Disruption Similar to That Caused by Natural Dimers Isolated from the AD Brain.** In light of several studies describing the neurotoxic effects of synthetic Aβ aggregates or cell-secreted Aβ oligomers (3, 5–8, 15–17), we assessed the effects of such preparations on the neuritic cytoskeleton in an attempt to confirm the changes described above with natural dimers isolated from the AD cortex. We examined a synthetic human Aβ40 peptide in which serine 26 is mutated to cysteine (Aβ40 S26C), enabling the formation of stable, disulfide-bonded dimers under oxidizing conditions (10, 18). This pure dimer was sufficient to induce an increasing alteration of the microtubule cytoskeleton and neuritic architecture in a dose-dependent manner, with a strong effect similar to that of the AD-TBS extracts observed at 500-nM concentration (Fig. S5A and B). A minor degree of neuritic beading could be observed at Aβ40 S26C dimer concentrations as low as 100 nM (Fig. S5B). Importantly, the synthetic dimers always required much higher concentrations (>100-fold) to induce cytoskeletal effects comparable to those of the natural dimers isolated from AD cortex.

Disruption of the Neuritic Cytoskeleton Induced by Soluble Aβ Oligomers Is Dependent on Tau Expression. Neurofilibrillary tangles and dystrophic neurites, the key cytopathological lesions of neurons in AD, contain abnormal filaments of hyperphosphorylated tau proteins (19–21). Numerous studies suggest that the tau protein is a mediator of the neuronal degeneration induced by supraphysiological concentrations of synthetic Aβ fibrils in vitro (8, 16, 22) and of the memory deficits of transgenic mice expressing mutant human amyloid precursor protein (APP) (23). Accordingly, we asked whether the neuritic cytoskeletal disruption caused by natural Aβ oligomers in the absence of amyloid fibrils was dependent on tau expression. Two weeks after neurons were transduced with...
lentivirus encoding siRNA against tau, we detected a highly significant decrease (>85%) of tau expression compared with control siRNA or no transduction (Fig. 2A). When SEC fractions containing soluble Aβ dimers from AD-TBS or else pure synthetic Aβ40 S26C dimers (500 nM) were applied, these each induced neuronal cytoskeletal disruption in control siRNA-treated neurons, but had no significant effect on the tau knock-down neurons (Fig. 2B).

**Acceleration of the Neuritotoxic Effect of Aβ Oligomers by Expressing Human Tau.** A recent analysis of transgenic mice expressing human tau revealed deficits in neural plasticity and memory, suggesting that the presence of human tau is sufficient to impair certain synaptic and cognitive functions (24). Our results above show that the presence of human tau is sufficient to impair certain synaptic and cognitive functions. Our results above show that the presence of human tau is sufficient to impair certain synaptic and cognitive functions. Our results above show that the presence of human tau is sufficient to impair certain synaptic and cognitive functions. Our results above show that the presence of human tau is sufficient to impair certain synaptic and cognitive functions. Our results above show that the presence of human tau is sufficient to impair certain synaptic and cognitive functions. 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**Soluble Aβ Oligomers Alter the Phosphorylation of Tau at AD-Relevant Epitopes.** During AD pathogenesis, tau undergoes abnormal hyperphosphorylation that contributes to neurodegeneration (1, 8, 25, 26). The phosphorylation states of several epitopes within tau are increased in transgenic mice coexpressing mutant human APP, presenilin, and tau (27). Because we found above that tau plays a permissive role in the cytoskeletal alteration induced by soluble Aβ oligomers, it became important to elucidate which phosphoepitopes on tau may be altered by the natural oligomers. We transduced rat hippocampal neurons with EGFP or hTau-EYFP. After being cultured for 18 to 19 d, neurons were exposed to SEC-isolated human Aβ dimers for just 1 d, and the phosphorylation state of both the exogenous human and endogenous rat tau proteins was assayed by quantitative Western blotting with epitope-specific antibodies (the phosphorylated bands were normalized to the respective total tau signal in the...
same neurons) (Fig. 4 and Fig. S6). The cultures were also examined by immunocytochemistry (Fig. S7). Soluble Aβ dimers isolated from AD-TBS and pure Aβ40 S26C dimers each induced substantial increases in tau phosphorylation at Ser202/Ser205 (AT8 epitope) and at Ser262 (12E8 epitope), a moderate increase at Thr181 (AT270 epitope), and no significant changes at Ser231/Thr235 (AT180 epitope) or Ser396 (PHF-1 epitope). Identical application of the Aβ dimers to hippocampal neurons cultured for just 8 d did not induce any increase in tau phosphorylation at the AT8 or 12E8 epitopes (Fig. S6), in accord with the lack of neuritic effects at this age (see above). As an important control, Aβ monomers from the same SEC run of the same AD-TBS extracts did not significantly alter tau phosphorylation. As a positive control for the phosphoepitope quantification, treatment of the neuronal cultures with the phosphatase inhibitor, okadaic acid (200 nM, 2 h), consistently increased phosphorylation levels (Fig. S6). Immunodepleting Aβ from the dimer-rich SEC fractions before their application prevented the hyperphosphorylation of tau at the AT8 and 12E8 epitopes, indicating that Aβ dimers were necessary for the effect (Fig. S8). It is of interest that the oligomer-induced increases in phosphorylation showed some difference between human and rat tau; in the same neurons, human tau phosphorylation was more sensitive to the application of Aβ oligomers, so that the degree of increase at the AT8, 12E8, and AT270 epitopes of human tau (all known to be hyperphosphorylated in AD neurons) was greater than that of rat tau (Fig. 4).

**Specific Immunological Neutralization of the Cytoskeletal Alterations Induced by Human Aβ Dimers.** Immunotherapy against Aβ by both active (vaccination) and passive (antibody infusion) approaches has reached advanced clinical testing in AD patients. In light of all the results above, we asked whether monoclonal antibodies directed at specific Aβ epitopes could modify the cytoskeletal alteration and neuritic degeneration caused by soluble dimers from the human (AD) brain. We tested three monoclonal Aβ antibodies: 3D6 to the free Asp-1 N terminus (a humanized version of which is in Phase 3 human trials); 82E1, another Asp-1 specific N-terminal antibody; and 21F12 to the free Ile-42 C terminus of Aβ42. Each antibody was coadministered (final concentration, 3 μg/mL) with the dimer-rich SEC fraction to mature (≥18 DIV) primary hippocampal cultures. As before, monomer-rich SEC fractions from the same chromatography served as a negative control. We found that when either 3D6 or 82E1 was present with the soluble dimers, no significant alteration of neuritic architecture and tau and Tubulin immunostaining were observed (Fig. 5A and B). In contrast, 21F12 had no significant neutralizing effect (Fig. 5A and B). This relative efficiency of neutralization was further confirmed by pull-down of Aβ dimers by protein G agarose (PGA) beads from the conditioned media of the antibody-treated neurons. Substantially larger amounts of Aβ dimers were pulled down by 3D6 and 82E1 than by 21F12, whereas PGA beads applied in the absence of antibodies pulled down no dimers (Fig. 5C).

**Discussion.**
Understanding the relationship of the two pathognomonic changes of AD, amyloid accumulation and neurofibrillary degeneration, represents an ongoing goal of research on the disease. Here, we show that natural oligomers, principally dimers, isolated directly from the cortex of typical, late-onset AD patients are sufficient to induce tau hyperphosphorylation at AD-relevant epitopes and then disrupt the microtubule cytoskeleton and cause neuritic dystrophy. Soluble dimers from the human brain appear to have a conformation that is highly potent in inducing these neuronal...
changes, as concentrations in the subnanomolar range caused cytoskeletal collapse, whereas levels of pure synthetic dimers at least two orders of magnitude higher were needed to produce otherwise indistinguishable effects in the same experiments.

Numerous controls confirmed that the cytoskeletal changes we report are attributable specifically to human Aβ dimers (the most abundant form of soluble oligomers recoverable from AD cortex (10, 11, 28)); (ii) monomers from the same size-exclusion chromatography at equal or higher amounts had no effect; (ii) corresponding SEC fractions from age-matched control brains lacking Aβ were negative; (iii) the SDS-stable dimers were recoverable from the media at the end of the treatment, proving they were present throughout the exposures that resulted in alteration of the tau cytoskeleton; (iv) pure, synthetic Aβ dimers produced closely similar effects to AD-brain derived dimers (albeit at much higher concentrations); (v) immunodepletion of natural Aβ dimers from the SEC fractions precluded any subsequent neurotropic injury; and (vi) coadministering highly specific monoclonal antibodies to the N terminus but not the C terminus of Aβ prevented the effects.

Intracerebral injection of synthetic Aβ fibrils into mice transgenic for mutant human tau can induce tau hyperphosphorylation and local neurofibrillary changes (29). Moreover, the coexpression of mutant human APP and mutant human tau leads to enhanced neuronal tau accumulation and dystrophic neurites in double-transgenic mice (30). Another mouse line transgenic for mutant human tau can induce tau hyperphosphorylation and local neurofibrillary degeneration (this article) and synapse loss (10). Active immunization with synthetic Aβ induces principally an N-terminal region antibody response in humans (36), and Phase 2 trials of a vaccine comprising an N-terminal Aβ fragment are underway. An earlier vaccine trial using full-length Aβ was halted prematurely because of occurrence of a self-limited meningoencephalitis in 6% of the 300 recipients (36). Nonetheless, those recipients having Aβ antibody responses showed less subsequent decline on some tests of verbal memory and an apparent decrease in CSF phospho-tau levels (36). A postmortem follow-up of a small subset of recipients from the Phase 1 trial of this full-length Aβ peptide vaccine, which suggested that some subjects could undergo marked clearance of Aβ plaques but still die with advanced dementia (37), is inconclusive, as it documented only two such subjects from a trial that originally included 80, and residual levels of Aβ oligomers in the brains were not assessed.

Tau has been found to be phosphorylated at over 30 serine/threonine residues in the human brain (38, 39), and approximately half of these are canonical sites for proline-directed protein kinases, including certain members of the MAP kinase, cyclin-dependent kinase and glycogen synthase kinase 3 (GSK3) families (26, 40). On cultured hippocampal neurons, synthetic Aβ oligomers (sometimes called ADDLs) or AD brain extracts have been shown to induce tau phosphorylation at several epitopes (41). A recent study showed that synthetic ADDLs can induce missorting of tau into dendrites, tau phosphorylation, and disruption of microtubules (8), but this work used 5-μM concentrations of the synthetic ADDLs compared with the subnanomolar levels of natural human brain dimers used here. The mechanism by which diffusible extracellular oligomers of Aβ bind to neurons and lead to increased activity of select kinases that phosphorylate tau at some but not other epitopes remains unclear. Ittner et al. reported that tau protein, in addition to its principally axonal locus, is sorted in small amounts to dendrites and that this helps mediate the postsynaptic targeting of the src kinase Fyn, substrates of which are certain NMDA receptors (32). The authors reason that enhanced targeting of the src kinase substrates could alter this normal function. However, there are numerous different ways in which an interaction between Aβ oligomers and tau could occur in AD brains (42), and clarifying precisely how this occurs is the next major step for the approach we report here. In this regard, we consistently observed that hippocampal neurons cultured for ≤7 d were resistant to the cytoskeletal injury induced by the Aβ dimers, suggesting that signaling programs that develop in more mature neurons are required for expression of this phenotype. In their analyses of APP-only transgenic mice, Roberson et al. (23) saw no change in phosphoepitopes of endogenous murine tau at age 4 to 6 mo but did see phosphotau-positive punctae in periplaque dystrophic neurites at >20 mo. Here, we observed altered phosphorylation of endogenous rat tau (albeit less robustly than transfected human tau). Numerous APP transgenic mouse lines that do not also express human tau show neuritic dystrophy but no AD-type neurofibrillary tangles, suggesting that human tau may be necessary for full-blown tangle formation per se.

Our findings with natural dimers isolated directly from AD patients, coupled with the wide availability of postmortem brain tissue from AD and non-AD subjects, recommends the use of endogenous oligomers isolated from the human cortex (10, 43), as the most biologically relevant approach to learn how Aβ oligomers alter tau phosphorylation and cytoskeletal function. Various aggregated forms of synthetic Aβ designated ADDLs (3) or protofibrils (44, 45) and generated from high concentrations

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of a single, defined Aβ peptide, have not been proven to occur as such in the human brain, whereas heterogeneous dimers, trimers (10, 11, 28), slightly larger low-n oligomers (11), and dodecamers (28) have. Deciphering the mechanisms of these natural oligomers will require purifying them to homogeneity from the AD cortex, labeling them, and exposing primary neurons or brain slices to them in unbiased fashion their molecular targets. We hypothesize that these targets are likely to be plasma membrane lipids (which would be expected to avidly bind the highly hydrophobic oligomers) rather than the hydrophilic ectodomains of protein receptors. Oligomer binding could perturb the fine structure of the lipid bilayer, and this could lead to sec- ondary biophysical effects on the structure and function of various transmembrane receptors (e.g., the NMDA, AMPA, insulin, and α7-nicotinic receptors implicated to date) that may then contribute to the profound changes in the tau cytoskeleton documented here.

Materials and Methods
See SI Materials and Methods for detailed descriptions.

13. Meriney D, Hanisch U, Markram H, Nusser Z (2007) Molecular mechanisms of AMPA receptor dependent biophysical effects on the structure and function of various transmembrane receptors (e.g., the NMDA, AMPA, insulin, and α7-nicotinic receptors implicated to date) that may then contribute to the profound changes in the tau cytoskeleton documented here.

Human Brain Sample Preparation. Frozen human cerebral cortices were provided by C. Lemere (Brigham and Women’s Hospital/Harvard Medical School) or from the Massachusetts General Hospital/Harvard Medical School) under Institutional Review Board–approved human studies protocols and by M. Farrell (Beaumont Hospital, Dublin, Ireland) in accord with local Ethics Committee guidelines and Ethical Review Committee/Institutional Review Board approval. Samples of temporal or frontal cortex containing white and gray matter were weighed. Freshly prepared, ice-cold TBS consisting of 20 mM Tris–HCl, 150 mM NaCl, pH 7.4, was added to the frozen cortex at 4. TBS was removed before adding homogenization with 25 strokes at a rate of 10 on a mechanical Dounce homogenizer. The homogenate was spun at 175,000 × g in a TL100.2 rotor on a Beckman TL 100. The supernatant (called TBS extract) was aliquoted and stored at −80 °C.

Immunoprecipitation/Western Blot Analysis of Aβ. We used an immunoprecipitation/Western blot protocol described previously (10, 15) to detect Aβ in the TBS extracts or neuronal culture medium.

ACKNOWLEDGMENTS. We thank George Bloom for generously providing the pCMV-H human brain Sβ fragment construct, Peter Seubert for the gift of 3D6 and 21F12 antibodies, and Ganesh Shankar and other members of the D.J.S. laboratory for helpful discussions. This work was supported by National Institutes of Health Grants AG027443 and AG006173 (to D.J.S.).

Supporting Information

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SI Materials and Methods

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Immunoprecipitation/Western Blot Analysis of Aβ. We used an immunoprecipitation/Western blot protocol described previously (1, 2) to detect Aβ in the TBS extracts or neuronal culture medium. Samples were immunoprecipitated with either polyclonal Aβ antiserum AW7 (1:50) and Protein A Sepharose (PAS; Sigma) or monoclonal Aβ antibodies 3D6 (3 μg/mL, gift of Elan Corporation, plc) or 82E1 (3 μg/mL, IBL) and Protein G agaroase (PGA; Roche) plus PAS. After the beads were washed, the immunoprecipitates were eluted with 10 μL 4% lithium dodecyl sulfate (LDS) sample buffer, heated at 65 °C for 5 min, and centrifuged at 18,500 × g for 5 min. The supernatant was electrophoresed on a 20-well 4 to 12% polyacrylamide gel (In- vitrogen). Proteins were transferred to 0.2 μm nitrocellulose and Western blotted for Aβ with goat anti-Aβ antibodies AT8, AT180, AT270 (Thermo Scientific), 2G3 + 21F12 (gifts of Elan Corporation, plc) using the LiCor Odyssey Infrared Imaging System.

Immunoprecipitation-Size Exclusion Chromatography. TBS extracts of AD or control cortex were immunoprecipitated with 3D6 (3 μg/mL), 15 μL PAS, and 15 μL PGA. After the beads were washed, the immunoprecipitates were eluted with 10 μL 4% LDS sample buffer, heated at 65 °C for 5 min and centrifuged at 18,500 × g for 5 min. The supernatant was transferred to 500 μL TBS and injected onto a Superdex 75 (10/30 HR) column (Amersham Biosciences) and eluted at a flow rate of 0.8 mL/min into 1-mL size exclusion chromatography (SEC) fractions using 50 mM ammonium acetate, pH 8.5. Next, 750 μL of each fraction were removed and stored at −80 °C. The remaining 250 μL were lyophilized, reconstituted in 15 μL of 2× LDS sample buffer, heated at 65 °C for 5 min, and used for Western blot analysis. Soluble Aβ monomer-rich or dimer-rich SEC fractions from AD-brain TBS extracts (AD-TBS) and the corresponding fractions from control brain extracts (Cont-TBS) were pooled separately and lyophilized before addition to the culture medium of primary cultured hippocampal neurons.

Production and Characterization of Cross-Linked Synthetic Dimers. Aβ40 S26C was synthesized by the Biopolymer Laboratory at the University of California Los Angeles Medical School and the correct sequence and purity confirmed by amino acid analysis, reverse-phase HPLC and mass spectrometry (1). Disulfide-bonded Aβ dimers were generated by atmospheric oxidation of a 20-μM solution of Aβ40 S26C in 20 mM ammonium bicarbonate, pH 8.0, for 4 d at room temperature. To facilitate disassembly of aggregates formed during the oxidation reaction, the peptide solution was lyophilized and subsequently incubated in 5 M GuHCl, 50 mM Tris-HCl, pH 8.0, for 4 h. Disulfide-cross-linked Aβ dimers were separated from unreacted monomer and higher aggregates by SEC. Briefly, two Superdex 75 10/30 HR columns were linked in series and eluted with 50 mM ammonium acetate, pH 8.5, at a flow rate of 0.5 mL/min. Fractions (0.5 mL) were collected, and an aliquot of each was electrophoresed on 16% Tris-tricine polyacrylamide gels and detected by silver staining. Fractions found to contain exclusively dimeric Aβ were pooled and the peptide content determined by comparison with known standards. Samples were stored at −80 °C until use.

Hippocampal Neuronal Cultures. Primary hippocampal cultures were generated from E18 Sprague-Dawley rat embryos. The hippocampus was dissected out in Hank’s Balanced Salt Solution buffered with Hepes and dissociated with 0.125% trypsin (In-vitrogen) for 15 min at 37 °C, followed by trituration. Dissociated cells were plated at a density of 1.5 × 10^5 cells/cm^2 in six-well plates precoated with poly-d lysine (100 μg/mL), or 1 × 10^6 cells/cm^2 on 24-well plates with coverslips coated with poly-d lysine (100 μg/mL). After 4 d culturing in Neurobasal medium with B-27 supplement (Invitrogen) and glutamax, cytosine arabinofuranoside was added to reduce glial proliferation. Half the medium was exchanged every 4 d. The treatment of the hippocampal neurons with Aβ oligomers was begun on various days (7, 14, or 18) after plating, and neurons were fixed for immunostaining and confocal microscopy 1, 2, or 3 d after starting treatment.

DNA Constructs, Lentiviral Generation, and Infection. The pcMV- hTau-EYFP construct was generously provided by George Bloom (University of Virginia). Lentiviral vectors were generated by inserting EFGP or Tau-EYFP cDNAs into pCDH1 (System Biosciences). Lentiviral RNAi constructs were made with the pLenti6/BLOCK-IT-DEST Gateway Vector Kit (Invitrogen). The target sequences were as follows (5’ to 3’): Cont-RNAi: AGGATAGAGTCCAGTCGAA; Tau-RNAi2: AGGATAGAGTCCAGTCGAA; Tau-RNAi1: GGACAGGAAATGAGTCGAA. The pCDH1 lentiviral constructs were cotransfected with ViraPower Packaging Mix (Invitrogen) into 293-FT cells (Invitrogen) for 15 min at 37 °C, followed by trituration. Dissociated hippocampal neurons were transfected with lentiviral conditioned medium overnight, then washed with Neurobasal medium with B-27 supplement.

Western Blotting of Cell Lysates. Various cultures in six-well plates were lysed in 0.2 mL lysis buffer [0.1% SDS, 1% Nonidet P-40, 50 mM Hepes, pH 7.4, 2 mM EDTA, 100 mM NaCl, 5 mM Na2VO4, 40 μM p-nitrophenyl phosphate, and 1% protease inhibitor mixture set I (Calbiochem)]. The lysates were centrifuged at 13,500 × g for 25 min. The supernatants were collected and denatured. The concentration of protein was determined by BCA assay. Twenty micrograms of total protein was loaded in each lane, separated by 4 to 12% SDS/PAGE and blotted onto nitrocellulose membrane. The blot was blocked for 1 h at room temperature, followed by incubation overnight at 4 °C with mouse monoclonal antibodies AT8, AT180, AT270 (Thermo Scientific), 12E8 (gift of E. Mandellkow, Max Planck Unit for Structural Molecular Biology, Hamburg, Germany), Tau1 (Chemicon), GSK3β (Thermo Sci-
entific), GAPDH (Chemicon), or rabbit polyclonal antibodies PHF-1 (Invitrogen), Tau 22690, Tau 39524 (Abcam), Tau K9JA (DAKO), GFP (Molecular Probes), or phospho-GSK3β (Ser-9; Cell Signaling). Membranes were rinsed and incubated for 1 h with fluorescent-conjugated goat anti-rabbit or mouse IgG (1:5,000; Invitrogen). Blots were scanned using the LiCor Odyssey Infrared Imaging System. Intensity of bands was measured by LiCor Odyssey software.

**Immunocytochemistry, TUNEL Staining, and Confocal Microscopy.** Hippocampal neurons cultured for 18 d were treated under various conditions as specified in the text. After treatment, cells were fixed in cold methanol for 15 min. Neurons were rinsed three times with PBS. After blocking in 5% BSA in PBS, neurons were incubated with rabbit antibody Tau K9JA (DAKO; 1:1,000) and mouse antibodies α-Tubulin (Sigma; 1:1,000), AT8 (Thermo Scientific; 1:200) or 12E8 (1:1,000) at 4 °C overnight. After rinsing with PBS three times, cells were incubated with Alexa-fluor 633 goat anti-rabbit IgG and Alexa-fluor 546 goat anti-mouse IgG (Invitrogen; 1:1,000) at 4 °C overnight. After rinsing three times with PBS, coverslips were placed with mounting medium (Southern Biotech). TUNEL staining was performed using Click-IT TUNEL Alexa Fluor488 kit (Invitrogen). Confocal microscopy was performed on a Zeiss LSM510 microscope. We used a 20×/0.75 objective and scanned the samples in a z-stack manner (three stacks, 2-μm interval). Neurons under different conditions were photographically captured in a random manner.

**Quantification of Neuritic Degeneration.** We used Imaris software to process the z-stack images of neurons and measure the total length of Tubulin-positive neurites and the number of tau-positive beads automatically in unbiased fashion. For each condition, at least six microscopic fields from two independent experiments were quantified. The data were expressed as the average density of tau-positive beads along 100-μm lengths of the neurites. Error bars equal SEM. Statistical significance was calculated by Student t test.


**Fig. S1.** Higher-power confocal images showing the tau (green) and Tubulin (red) immunoreactivities of the cytoskeleton of hippocampal neurons [days in vitro (DIV) 20 or 21] after 2- or 3-d treatment with Aβ monomers (M) or dimers (D) isolated by SEC from AD-TBS extracts. Regions in the white boxes are shown immediately below at higher magnification. (Scale bar, 50 μm.)
Fig. S2. Soluble Aβ oligomers did not induce significant neuronal apoptosis after 3-d treatment. (A) Confocal images showing TUNEL (green), propidium iodide (PI) (blue), and the tau-reactive microtubule cytoskeleton (red) of primary hippocampal neurons (DIV21) after 3-d treatment with SEC fraction 10–11 (M, monomers) or fraction 8–9 (D, dimers) from AD-TBS. Neurons without dUTP incorporation served as negative control, and neurons treated with DNase I after fixation served as positive control. (Scale bar, 50 μm.) (B) Histograms of the average numbers of TUNEL-positive cells in each well (≈4 × 10^4 neurons) after the indicated treatments. Means from three independent experiments; error bars, SEM.
Fig. S3. Immunodepletion (ID) of Aβ dimers prevents neuritic disruption of hippocampal neurons. (A) Confocal images showing the tau-reactive (green) and microtubule-reactive (red) cytoskeleton of primary hippocampal neurons (DIV21) after 3-d exposure to Aβ monomers (SEC fractions 10–11, M) or Aβ dimers (SEC fractions 8–9, D) that had been immunodepleted with either AW7 or Preimm. (Scale bar, 50 μm.) (B) Histograms represent the average number of tau-positive beads along 100-μm lengths of Tubulin-positive neurites under different conditions. Asterisk indicates data significantly different from those of neurons without treatment (P < 0.01 by Student t test). Error bars, SEM. (C) SEC fractions enriched in Aβ dimers (D) or monomers (M) were immunodepleted (ID) with Aβ antiserum AW7 or its preimmune serum (Preimm) or not immunodepleted (−). After the immunodepletion, the respective supernates were immunoprecipitated with AW7 to visualize any remaining Aβ species.
Fig. S4. (A) Confocal images showing the tau (green) and tubulin (red) immunoreactivities of the cytoskeleton of hippocampal neurons (DIV10) after 3-d treatment with Aβ monomers (SEC fractions 10–11) or Aβ dimers (SEC fractions 8–9) isolated from AD-TBS, or else with Aβ40 S26C (500 nM). (Scale bar, 50 μm.) (B) Representative Western blots showing the phosphorylation of GSK3β (at ser 9), expression of total GSK3, and tau in primary cultures of hippocampal neurons cultured for 18 d (DIV18, lanes 1–3) or 7 d (DIV7, lanes 4–6). Arrow indicates a mature form of tau (∼55 kDa). Blotting of GAPDH served as a control.
Fig. S5. Disruption of the neuronal cytoskeleton by pure, synthetic dimers of Aβ40. (A) Western blotting of culture media (20 μL) containing increasing concentrations (nM) of crosslinked dimers of Aβ40 S26C. (B) Confocal images showing the tau-reactive (green) and microtubule-reactive (red) cytoskeleton of primary cultured hippocampal neurons (DIV21) after 3-d treatment with increasing concentrations of Aβ40 S26C dimers. (Scale bar, 50 μm.)
Fig. S6. Representative Western blots showing the phosphorylation of human Tau-EYFP (hTau) and endogenous rat tau (rTau) at different epitopes in primary cultures of hippocampal neurons (DIV19 or DIV8) transduced with or without lentivirus encoding EGFP or hTau-EYFP. Neurons were treated under the indicated conditions (see key) for just 1 d. Western blotting for total tau (K9JA, or Tau1) served as a control. O.A., okadaic acid.
**Fig. S7.** Confocal images showing GFP fluorescence (green; first row), phospho-tau [red (second row) and pseudocolor image (third row)], and total tau (blue; fourth row) reactivities of the microtubule cytoskeleton in hippocampal neurons transduced with hTau-EYFP (DIV21) after 1-d (A) or 2-d (B) treatment. Scale bar for the intensity of p-tau staining in the pseudocolored images of the third row is shown at the far right edge of that row. (Scale bars, 50 μm.)

**Fig. S8.** Representative Western blots showing the phosphorylation of human Tau-EYFP (hTau) and endogenous rat tau (rTau) at the AT8 or 12E8 epitopes in primary hippocampal neurons (DIV18) transduced with lentivirus encoding EGFP or hTau-EYFP. Neurons were treated for 1 d under the conditions indicated in the key. Western blotting of total tau (K9JA) served as a control.