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Free Fatty Acids Stimulate the Polymerization of Tau and Amyloid β Peptides

In Vitro *Evidence for a Common Effector of Pathogenesis in Alzheimer's Disease*

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Alzheimer's disease is a degenerative disorder of the central nervous system, characterized by the concomitant deposition of extracellular filaments composed of β-amyloid peptides and intracellular filaments composed of the microtubule-associated protein tau. We have discovered that free fatty acids (FFAs) stimulate the assembly of both amyloid and tau filaments* in vitro. *The minimal concentration of arachidonic acid observed to stimulate tau assembly ranged from 10 to 20 μmol/L, depending on the source of the purified tau. Tau preparations that do not exhibit spontaneous assembly were among those induced to polymerize by arachidonic acid. All long-chain FFAs tested enhanced assembly to some extent, although greater stimulation was usually associated with unsaturated forms. Utilizing fluorescence spectroscopy, unsaturated FFAs were also demonstrated to induce β-amyloid assembly. The minimal concentration of oleic or linoleic acid observed to stimulate the assembly of amyloid was 40 μmol/L. The filamentous nature of these thioflavin-binding amyloid polymers was verified by electron microscopy. These data define a new set of tools for examining the polymerization of amyloid and tau proteins and suggest that cortical elevations of FFAs may constitute a unifying stimulatory event driving the formation of two of the obvious pathogenetic lesions in Alzheimer's disease. (Am J Pathol 1997, 150:2181–2195)

A diagnosis of Alzheimer's disease (AD) is confirmed postmortem by demonstrating the presence of neuritic plaques (NPs) and neurofibrillary tangles (NFTs) in the victim's brain tissues.[1] Both of these lesions are characterized by the accumulation of abnormal polymerization products. NPs contain amyloid fibrils composed of a peptide that is proteolytically cleaved from the amyloid precursor protein,[2–4] whereas NFTs are amassed from paired helical filaments and straight filaments composed of the microtubule-associated protein tau.[5–9] Filamentous tau pathology is also observed within the extensive arrays of dystrophic neurites (neuropil threads) observed in the AD brain and degenerating pre- and postsynaptic elements associated with NPs.[2,10–12] The anatomical distribution of NPs is somewhat variable and does not coincide with the distribution of NFTs.[1] Although considerable progress has been made in the last decade toward defining the physical and biochemical nature of tau and amyloid filaments, a coherent model explaining why these polymers appear concomitantly in this disease has not been elaborated.

In normal neurons, tau proteins in monomeric form bind the surface of microtubules, regulating both microtubule assembly and inter-microtubule spacing.[13–18] In contrast, tau in AD neurons forms the polymeric straight and paired helical filaments. The basis of this abnormal self-association remains unknown. Although considerable attention has been given to experiments implicating excessive protein phosphorylation as a causative agent,[19,20] differences in the phosphate content of soluble and poly-

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meric tau purified from AD brain may be less a reflection of their *in vivo* phosphorylation state than an indication of their relative accessibility to phosphatases during the postmortem interval.[21] Other protein modifications, such as glycation,[22,23] glycosylation,[24,25] carbamylation,[26] or incorporation of D-aspartate,[27] have also been implicated in the process of paired helical filament assembly, but as in the case of phosphorylation, no causal relation has been established between amino acid modification and rates of polymer assembly. NFT formation is not uniquely associated with AD. In Guamanian parkinsonism,[28] progressive supranuclear palsy,[29] and other disease states,[30] the accumulation of tau polymers occurs in the absence of amyloid pathology.

Amyloidic fragments of the amyloid precursor protein are a metabolic product of the normal brain and can be isolated from the cerebrospinal fluid (CSF) of nondiseased individuals.[31,32] Variable cleavage of the precursor protein results in the production of multiple peptides (collectively referred to as Aβ) ranging in length from 39 (Aβ_{1-39}) to 43 (Aβ_{1-43}) amino acids, with Aβ_{1-40} or Aβ_{1-42} being reported as the predominant component of NP amyloid.[33,34] The longer Aβ variants, the carboxy termini of which contain additional residues from the transmembrane domain of the precursor protein, are more prone to aggregation than their shorter counterparts.[35,36] The addition of filamentous amyloid (as opposed to soluble Aβ) to cultured cells induces both neuronal degeneration and the activation of astrocytes,[37–39] suggesting that amyloid polymers may be causally linked to the presence of degenerating neurons and reactive glia in NPs. Mutations in the amyloid precursor protein are linked to the onset of familial AD. These amino acid substitutions increase the rate of Aβ release or the length and subsequent assembly competence of the cleavage product resulting in abnormally high rates of amyloid deposition.[40,41] In the absence of such mutations, however, the impetus for assembly remains unknown. Although amyloid plaques can also be observed in the brains of aged, nondiseased humans and nonhuman species, these brains are largely devoid of NFTs, and the neurites associated with these plaques are devoid of tau filaments.[2,42]

Synthetic amyloid peptides will assemble *in vitro*, forming filaments resembling those isolated from NPs.[35] Polymerization has been demonstrated to proceed as a function of pH and peptide concentration but independently of ionic strength. Tau proteins purified from cycled microtubules (microtubule tau, MTτ) will also assemble *in vitro*, forming 10-nm filaments morphologically similar to the straight filaments observed in AD brain.[43] This assembly reaction was shown to be modulated by temperature, pH, ionic strength, and reducing potential. Employing these previously defined conditions for the *in vitro* assembly of tau and amyloid polymers, we now report that both types of pathological filament can be induced to assemble by a single class of effector molecules, the free fatty acids (FFAs).

Materials and Methods

Protein Isolation

Sprague-Dawley rats were obtained from Charles River, Wilmington, MA, and killed by decapitation at postnatal day 11 (juveniles) or at greater than 6 weeks of age (adults). Fresh porcine brains were obtained from Bryan Meat Packing, Westpoint, MS. Detailed protocols for the isolation of tau from whole brain or twice-cycled brain microtubules have been described.[43] Nonphosphorylated, recombinant human tau (htau40)[44] was the gift of Dr. Jeff Kuret, Northwestern University Medical School, Chicago, IL. Recombinant tau was produced in *Escherichia coli* as a fusion protein with a polyhistidine tag and purified to near homogeneity by nickel-chelate and gel filtration chromatography.[45] After purification, tau isolates were dialyzed against buffer A (20 mmol/L morpholinoethanesulfonic acid, pH 6.8, 80 mmol/L NaCl, 2 mmol/L EGTA, 1 mmol/L $MgCl_2$, 0.1 mmol/L EDTA) and stored at −80°C. Protein concentrations were determined using the method of Lowry[46] after samples in Laemmli sample buffer[47] were precipitated with 10 vol of 10% perchloric acid, 1% phosphotungstic acid. Bovine serum albumin was used as the standard.

Preparation of Fatty Acids

All FFAs were purchased in the *cis* conformation and at maximal available purity from Sigma Chemical Co., St. Louis, MO. Solutions made from crystalline FFAs were prepared fresh before each use. FFAs procured as liquids were repeatedly opened and resealed with Parafilm until oxidation was indicated by discoloration or an increase in viscosity. Qualitatively, no differences in assembly-promoting activity were observed between newly opened products and those stored between intermittent openings. FFAs were diluted into tau and amyloid samples from a 200X ethanolic stock, such that the final ethanol concentration in all samples and controls was 0.5%. Values for the critical micellar concentration (CMC) were obtained based on the phase partitioning of 10

μmol/L phenylnaphthylamine[48] when FFAs were diluted into assembly buffer.

Tau Protein Assembly

For most experiments, tau proteins were diluted to 2X the desired concentration in buffer A and then 0.5X in borate saline (0.1 mol/L H_3BO_3, 25 mmol/L $Na_2B_4O_7$, 75 mmol/L NaCl) supplemented with 20 mmol/L dithiothreitol (DTT) and 2X the required concentration of FFAs (final pH, ~8.4). Assembly was performed at 37°C in siliconized microfuge tubes. For evaluating the dependence of assembly on ionic strength, tau was diluted 1/10 into 111 mmol/L Tris, pH 7.2, 11 mmol/L DTT, supplemented to give the indicated final concentrations of NaCl. At 250 mmol/L NaCl, the measured CMC of arachidonic acid in the Tris buffering system was >2 mmol/L.

Amyloid Peptide Assembly

$A\beta_{1-40}$ and $A\beta_{1-42}$ peptides (Sigma) were resuspended in Aβ assembly buffer (100 mmol/L Tris, pH 7.4, 150 mmol/L NaCl) at a concentration of 0.5 mg/ml ($A\beta_{1-40}$) or 0.05 mg/ml ($A\beta_{1-42}$) and frozen in aliquots at −80°C. Thawed aliquots of $A\beta_{1-40}$ were diluted to 50 μg/ml in assembly buffer and, after a 2-hour preincubation, were centrifuged for 10 minutes at 14,000 rpm in an Eppendorf 5415C desktop centrifuge. The clarified solution was then supplemented with FFAs (final Aβ concentration, ~10 μmol/L). Thawed aliquots of $A\beta_{1-42}$ were not diluted, and the preincubation and centrifugation steps were omitted. Care was taken to perform all procedures at 4°C.

Fluorescence spectroscopy was performed essentially as described.[49] Aliquots were diluted 1/6 into 67 mmol/L glycine, pH 9, 4 μmol/L thioflavin T, vortexed, and placed in a quartz cuvette. Samples were read on a Perkin Elmer LS-50B luminescence spectrometer; excitation = 435 nm, emission = 485 nm, and slit widths = 5 nm. The integrated intensity was obtained from the initial 100-second sampling interval. The signal was stable for several hours. The fluorescence of samples lacking Aβ was averaged and subtracted as background from all readings. FFAs did not contribute to the fluorescence signal over the range of concentrations employed.

Electron Microscopy

Samples were placed in 10-μl aliquots onto 400-mesh nickel grids coated with 0.4% Formvar for 1 minute. Tau samples were rinsed with 4 drops of H_2O and stained with 4 drops of 2% uranyl acetate, the last drop sitting 1 minute before blotting. For staining of amyloid filaments, 4% uranyl acetate was used and the H_2O rinse was omitted. Grids were examined using a JEOL JEM-100CX transmission electron microscope operated at 60 to 80 kV. For filament length measurements, random micrographs obtained at a nominal magnification of ×15,000 were digitized and traced using either software from Universal Imaging Corp. or the public domain NIH Image program (for Macintosh; written by W. Rasband at the National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, Springfield, VA, part number PB93–504868). Only tau filaments measuring at least 50 nm were included in data sets. Because short filaments were sometimes difficult to distinguish from background debris in digitized images, high concentrations of DTT were routinely used to maximize the production of longer filaments. Fields selected at random were chosen at low illumination and without the aid of the 10× binoculars so that Formvar integrity could be assessed without viewing the filaments present.

Results

Fatty Acid Dependence of Tau Assembly

The assembly-promoting activity of FFAs were examined using conditions similar to those previously shown to be useful for studying tau assembly.[43] Arachidonic acid (5,8,11,14-eicosatetraenoic acid), a principal polyunsaturated FFA in mammalian brain,[50] was observed to stimulate the polymerization of all tau preparations examined. Juvenile MTτ (from rats, postnatal day 11), which is uniquely composed of the smallest of the six tau isoforms produced in adult brain,[18] assembled in a dose-dependent manner (Figure 1A). The apparent threshold for stimulation was a function of time and was less than 10 μmol/L at the longest time tested. When the data in Figure 1A are replotted such that the polymer mass is expressed as a function of time (Figure 1B), the curves generated are essentially linear with no evidence of plateauing, indicating that the rate of assembly is relatively constant up to 66 hours (80 μmol/L) or 108 hours (20 μmol/L and 40 μmol/L). Arachidonic acid also stimulated the polymerization of adult rat MTτ (Figure 1C). This preparation exhibited a greater potential for spontaneous assembly than juvenile MTτ, as well as larger absolute increases in polymer formation at arachidonic acid levels of 10 to 40 μmol/L. Juvenile MTτ, however,

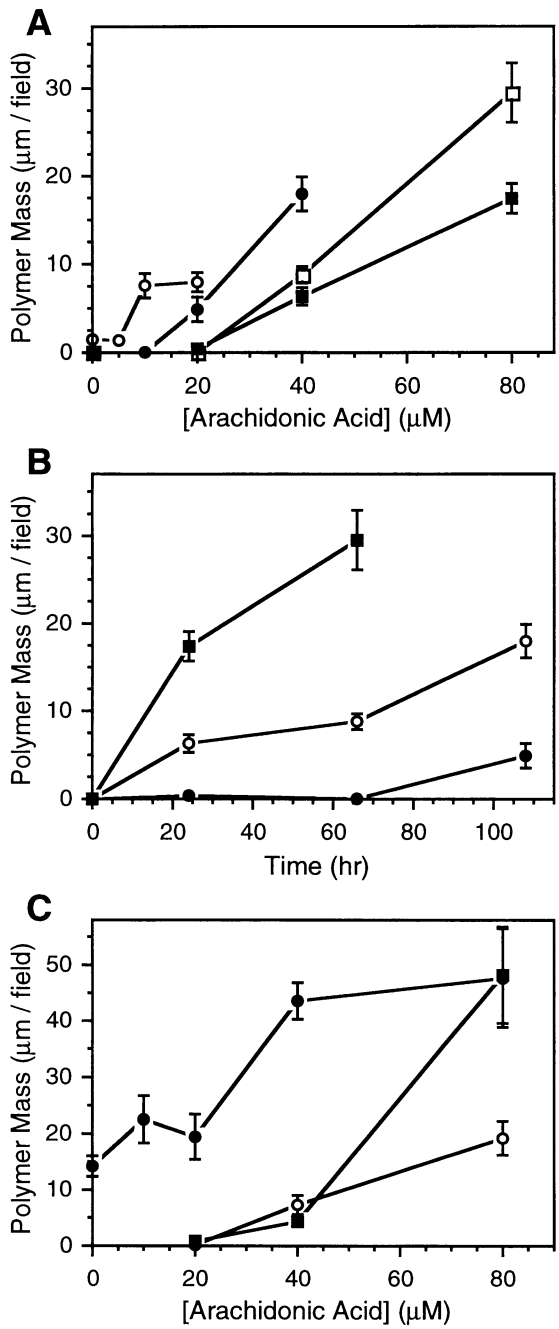

Figure 1. *Arachidonic acid dependence of tau assembly.* A: *Samples of juvenile rat MTτ used at 100 μg/ml (~2.5 μmol/L) were supplemented with the indicated concentrations of arachidonic acid and assembled for 24 hours (■), 66 hours (□), 108 hours (●), or 214 hours (○).* B: *The data from* A, *with polymer mass replotted as a function of time. Samples were incubated in the presence of 20 μmol/L (●), 40 μmol/L (○), or 80 μmol/L arachidonic acid (■).* C: *Tau polymers were assembled from adult rat MTτ (100 μg/ml; ●), tau purified from porcine whole brain (200 μg/ml; ○), or purified human recombinant tau expressed in* E. coli *(200 μg/ml; ■). Samples were incubated for 66 hours. General conclusions cannot be made regarding the relative efficacy of assembly of tau purified by the different methods due to the different species of origin. All samples (*A *to* C*) were negatively stained with 2% uranyl acetate, and electron micrographs of random fields were digitized and traced. Values shown are the average summed polymer length/field ± SEM; n ≥ 12.*

displayed a larger percent increase in assembly when stimulated by 40 to 80 μmol/L arachidonic acid. Under the same conditions, no spontaneous assembly was exhibited by human recombinant tau and tau purified from porcine whole brain. Filaments were observed, however, when these tau preparations were incubated with 20 to 80 μmol/L arachidonic acid (Figure 1C). Although levels of spontaneous and inducible assembly varied between the different tau preparations, the source of this variance could not be determined due to differences in the concentration of protein employed (100 to 200 μg/ml), levels of post-translational modification, and species-specific differences in amino acid sequence.

To determine which FFA could most effectively be used as inducers of tau assembly, the stimulatory effects of FFAs that differed in the length of their carbon chain and extent of saturation were examined. In general, for any given chain length tested, unsaturated FFAs were more potent than saturated FFAs (Table 1). A 20- to 30-fold increase in polymer formation was observed when using 50 μmol/L arachidonic, palmitoleic, or linoleic acid. The stimulatory effects of FFAs were not due to localized concentrations of surface charge produced by fatty acid aggregation, as measurement of the CMC of some representative FFAs indicates that they were effective at concentrations below this value (Table 1). Based on the qualitative examination of grids, tau assembly did not appear to be stimulated by the methyl or ethyl esters of arachidonic acid, which were also used at 50 μmol/L (below their measured CMC values).

Characteristics of Fatty-Acid-Induced Tau Polymers

The addition of FFAs to tau samples appears to modulate the rate of assembly but not the nature of the polymers formed. Filaments assembled from MTτ in the absence of FFAs (Figure 2A) are morphologically indistinguishable from those assembled in the presence of 50 μmol/L arachidonic acid (Figure 2B). Filament morphology did not appear to vary when the source of purified tau was porcine whole brain (Figure 2C) or human recombinant tau (Figure 2D). Arguments for the probable equivalence of these *in vitro* assembled filaments with Alzheimer's straight filaments were provided in an earlier report.[38] We have since found that filaments assembled under these conditions are positive for thioflavin binding (Dr. Jeff Kuret, personal communication) and that their assembly is nucleated by purified paired helical

Table 1. *Tau Polymerization Induced by Different FFAs*

Fatty acid	Polymer mass (μm/field)	% maximum	CMC (mmol/L)
Control (no fatty acid)	0.63 ± 0.17	3	
5,8,11,14,17-Eicosapentaenoic acid (20:5)	11.81 ± 1.51	48	NA
5,8,11,14-Eicosatetraenoic acid (20:4)	24.51 ± 2.58	100	0.16
8,11,14-Eicosatrienoic acid (20:3)	7.99 ± 1.55	33	NA
11,14-Eicosadienoic acid (20:2)	1.20 ± 0.32	5	NA
11-Eicosenoic acid (20:1)	8.23 ± 1.36	34	NA
9,12,15-Linolenic acid (18:3)	4.01 ± 0.72	16	NA
9,12-Linoleic acid (18:2)	14.74 ± 3.31	60	0.21
9-Oleic acid (18:1)	7.36 ± 1.77	30	0.59
Stearic acid (18:0)	3.60 ± 0.54	15	>1
9-Palmitoleic acid (16:1)	23.69 ± 4.11	97	0.44
Palmitic acid (16:0)	7.37 ± 0.92	30	NA
Myristic acid (14:0)	5.50 ± 0.76	22	>1

Tau filaments were assembled using juvenile rat MTτ. Samples were incubated for 72 hours in the presence of 50 μmol/L FFA. Polymer mass is expressed as the average ± SEM (n = 12) or relative to the maximal assembly achieved with arachidonic acid. NA, not available.

filaments (Wilson and Binder, unpublished data). Hybrid filaments formed in these latter experiments suggest that *in vitro* assembled filaments are structurally equivalent to one-half of a paired helical filament.

The polymerization of tau filaments in the presence of 50 μmol/L arachidonic acid was dependent on temperature, ionic strength, and reducing potential, as was previously demonstrated for polymerization in the absence of FFAs,[43] supporting the conclusion that filament structure is not altered by FFAs. Juvenile MTτ exhibited an approximately 500% increase in assembly when the temperature was raised from 4°C to 37°C (Figure 3A). The assembly of adult MTτ was not dependent on temperature (Figure 3A), however, indicating again that adult specific amino acid sequences and/or states of post-translational modification are altering thermodynamic parameters of the assembly process. Concentrations of NaCl near 150 mmol/L appeared to be optimal for the polymerization of adult MTτ, whereas higher concentrations were inhibitory (Figure 3B). This differs somewhat from earlier findings demonstrating inhibition at salt (NaCl plus KCl) concentrations of only 65 mmol/L, suggesting that FFAs shift the ionic strength dependence closer to a physiological optimum. Assembly of adult MTτ was also a function of the reducing potential (Figure 3C). The increase in average filament length produced by increasing the concentration of DTT was accompanied by a decrease in the total number of filaments. Finally, assembly of adult and juvenile MTτ in the presence of 50 μmol/L arachidonic acid was completely inhibited below pH 6, and all filament populations analyzed exhibited an exponential distribution of filament lengths (data not shown), consistent with data generated in the absence of FFAs.[43]

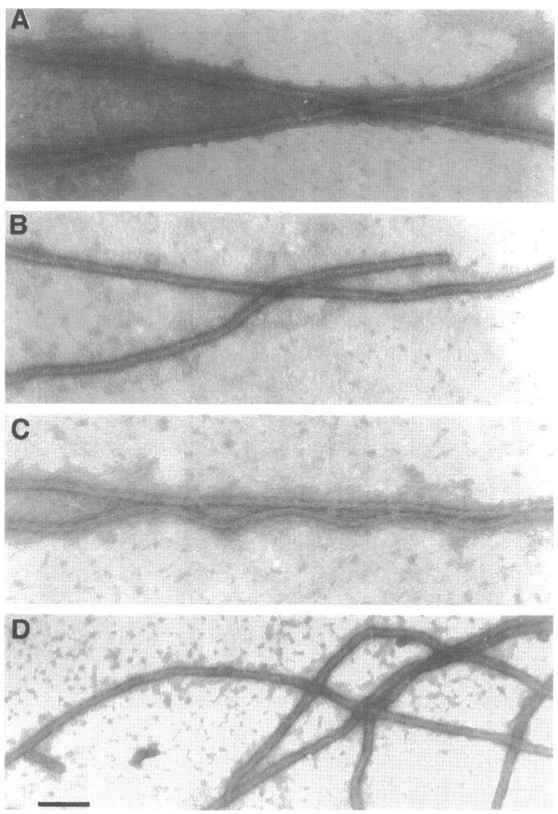

Figure 2. *Morphology of tau filaments formed in the presence or absence of arachidonic acid. Tau polymers were assembled using adult rat MTτ* (A *and* B), *porcine whole brain tau* (C), *or human recombinant tau* (D). *Samples were incubated for 72 hours in the absence* (A) *or presence* (B *to* D) *of 50 μmol/L arachidonic acid. Bar, 100 nm.*

Fatty Acid Dependence of Amyloid Assembly

Given the concomitant appearance of tau and amyloid pathology in the AD brain, we sought to determine whether Aβ assembly could also be stimulated

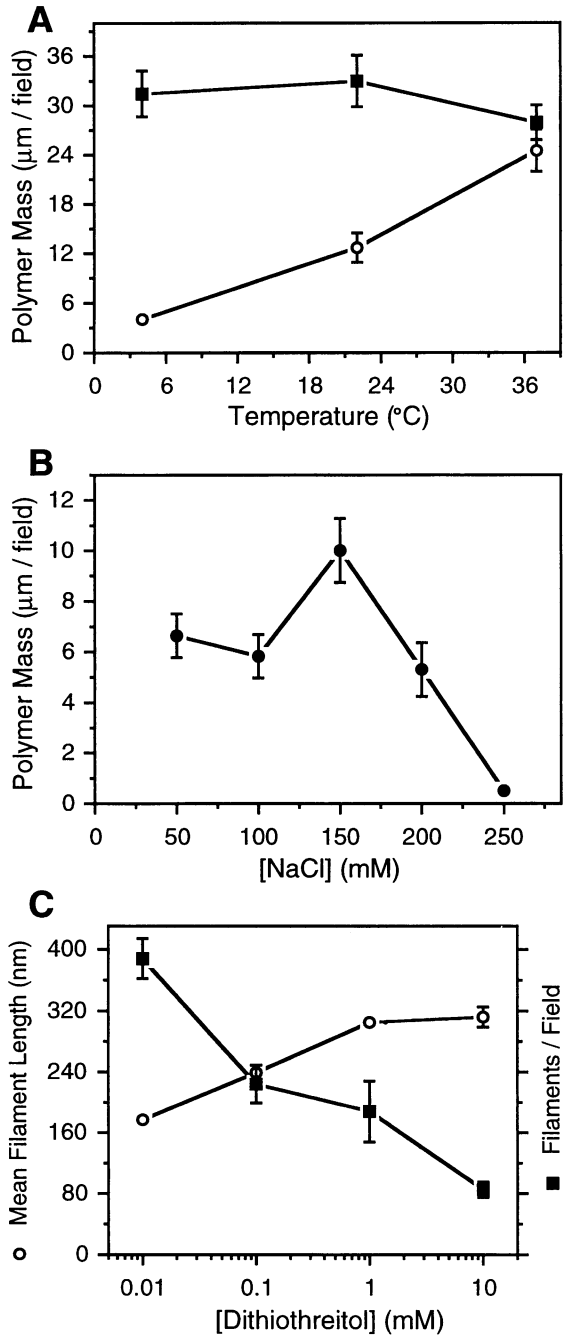

Figure 3. *Modulation of tau polymerization in the presence of arachidonic acid. All samples contained 100 μg/ml rat MTτ and 50 μmol/L arachidonic acid and were incubated for 72 hours. Electron micrographs of negatively stained samples were digitized and traced, and the polymer content was expressed as total mass* (**A** *and* **B**) *or as mean filament length and number of filaments/field* (**C**). *In all cases, values shown are the mean ± SEM; n = 12.* **A**: *Samples of juvenile* (○) *or adult* (●) *tau were incubated at 4, 22, or 37°C.* **B**: *Adult tau was assembled in the presence of the indicated concentration of NaCl (see Materials and Methods for the buffer conditions).* **C**: *Adult tau was assembled in the presence of the indicated concentration of DTT. An increase in mean filament length* (○) *seen with increasing concentrations of DTT was reflected by a decrease in the number of filaments/field* (●).

by FFAs. Oleic acid and linoleic acid, which constitute 45% of the unsaturated fatty acid content of the CSF[51] were initially chosen for these experiments because unsaturated FFAs appeared to induce tau assembly more effectively. Based on an earlier study,[35] conditions were chosen in which spontaneous assembly of the Aβ would be expected to be minimal to optimize our ability to detect FFA-dependent polymerization. After a 2-hour preincubation and a brief clarifying spin, solutions of $A\beta_{1-40}$ (10 μmol/L) examined by electron microscopy were characterized by the presence of relatively short (<0.5 μm) filaments dispersed individually or in small aggregates across the grid surface (Figure 4A). When samples were incubated for 24 hours in the absence of FFAs, filaments were more aggregated and exhibited a moderate increase in length (Figure 4B). In contrast, when samples were incubated with oleic or linoleic acid, a dramatic increase in filament lengths was observed (Figure 4, C and D). Filament widths were on the order of 5 to 10 nm, similar to values reported for other *in vitro* assembled amyloid fibrils.[35,49] When the Aβ concentration was increased from 10 μmol/L to 25 μmol/L, there was a considerable increase in the spontaneous formation of amyloid filaments, making the relative contribution of FFAs difficult to assess. Due to filament aggregation and the non-uniform dispersal of such aggregates on the grid surface, filament densities observed in Figure 4 are not necessarily indicative of filament densities in solution.

Although FFAs appeared to have a pronounced effect on filament elongation, microscopic methods alone could not ascribe this to an induction of subunit incorporation. Filament elongation could result from the lateral or endwise annealing of the short filaments observed in the absence of FFAs. A fluorometric assay was, therefore, employed as a measure of the total polymer mass present in peptide solutions. This assay is based on unique excitation and emission maxima that result from the binding of thioflavin T to the β-sheet structure of proteins.[52,53] Quantitative results obtained by this method were consistent with observations made by electron microscopy. The fluorescence signal indicated low levels of polymer formed in the absence of FFAs and no increase above baseline in the presence of 10 to 20 μmol/L FFAs (Figure 5). Significant increases (2.7- to 3.7-fold) in the polymer content were observed at oleic and linoleic acid concentrations of 40 μmol/L. At higher concentrations of FFAs, linoleic acid was distinguished as the more potent inducer of $A\beta_{1-40}$ assembly. This demonstration of an increase in total polymer mass induced by FFAs indicates that fila-

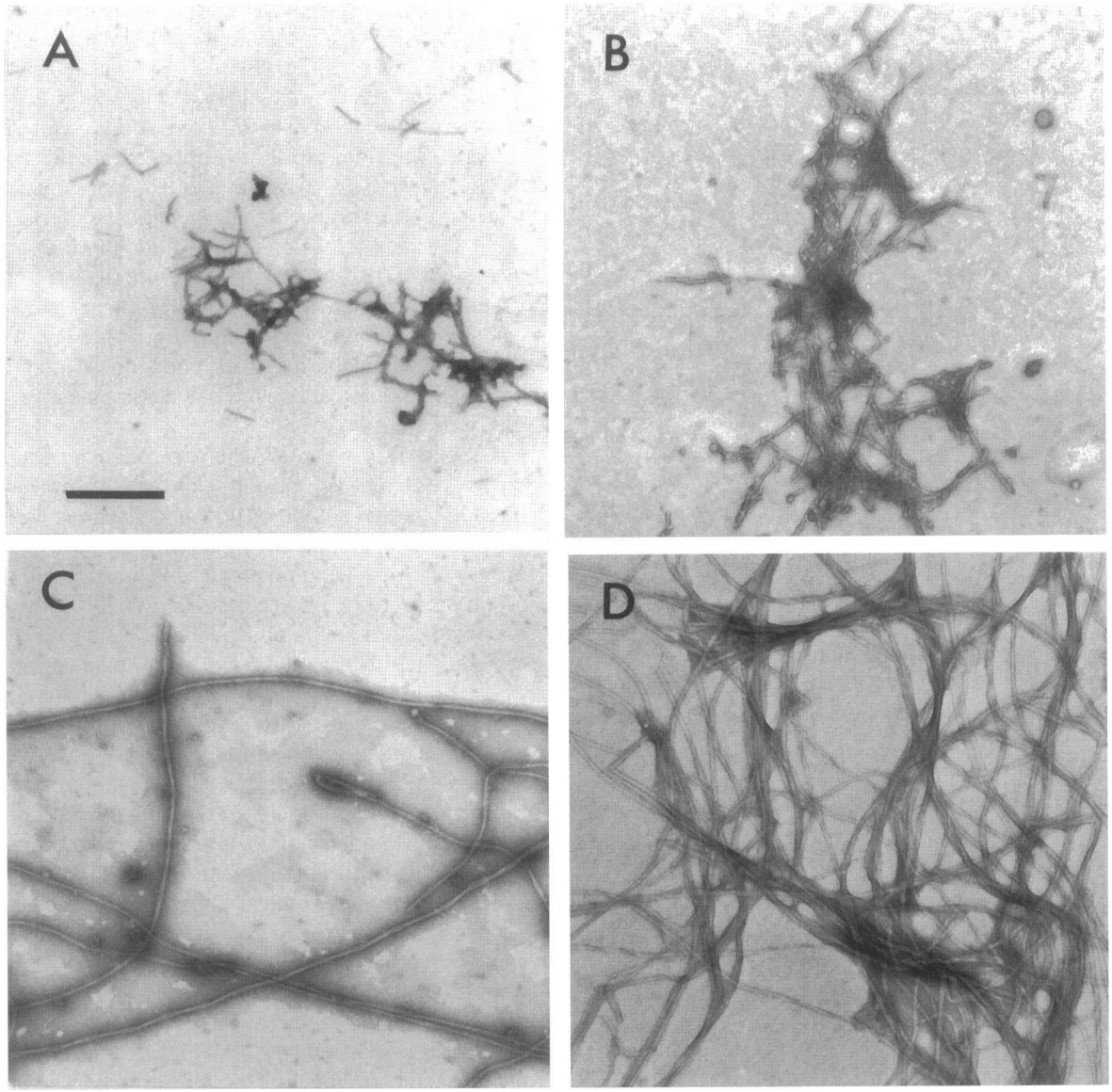

Figure 4. *Fatty-acid-dependent assembly of amyloid: electron microscopy.* A *and* B: *Filamentous aggregates present in solutions of amyloid peptide before* (A) *or after* (B) *a 24-hour incubation in the absence of FFA.* C *and* D: *Long filaments resulting from a 24-h incubation with 40 μmol/L oleic acid* (C) *or 50 μmol/L linoleic acid* (D). *All samples were stained with 4% uranyl acetate and photographed at a nominal magnification of ×30,000. Bar, 275 nm.*

ment elongation cannot be solely attributed to the annealing of pre-existing filaments but rather must involve the further incorporation of peptide subunits.

The potential involvement of FFAs in amyloid formation was further examined using the more highly amyloidogenic peptide $A\beta_{1-42}$. The thioflavin binding assay was used to assess the assembly-promoting effects of palmitic, palmitoleic, oleic, linoleic, and arachidonic acid. These FFAs collectively represent over 50% of the saturated and unsaturated FFAs normally present in the CSF.[51] Each of the FFAs employed at a concentration of 60 μmol/L induced significant increases in $A\beta_{1-42}$ assembly (Figure 6). The least effective was the saturated FFA palmitic acid, which produced a 65% increase in assembly ($P < 0.01$, Student's *t*-test). Polyunsaturated linoleic and arachidonic acid were the most effective inducers of assembly as evidenced by an approximately sevenfold increase in thioflavin binding. Examination of samples by electron microscopy confirmed that increases in thioflavin binding induced by unsaturated FFAs were accompanied in each case by obvious increases in structures possessing a filamentous morphology (data not shown). This could not be confirmed for palmitic acid; smaller increases in the formation of filamentous amyloid were probably ob-

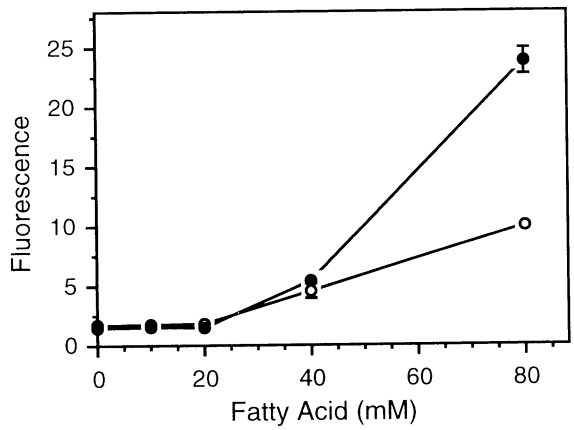

Figure 5. *Fluorescence spectroscopic analysis of $A\beta_{1-40}$ assembly. Amyloid peptide $A\beta_{1-40}$ was incubated for 24 hours with the indicated concentration of oleic (○) or linoleic acid (●). Fluorescence values (arbitrary units) obtained after mixture with thioflavin-T are shown as the mean ± SD; n = 3.*

scured by the presence in both controls and FFA-treated samples of large electron-dense peptide aggregates. It could not be determined whether these aggregates represented nonfilamentous aggregation or closely packed accumulations of short amyloid filaments.

Discussion

A causal relation has been established between FFAs and the *in vitro* assembly of polymers related to those observed in the AD brain. Arachidonic acid was observed to stimulate the polymerization of all tau preparations examined, with assembly induced by as little as 10 to 20 μmol/L FFA at tau concentrations of 2.5 to 5 μmol/L. These threshold levels of

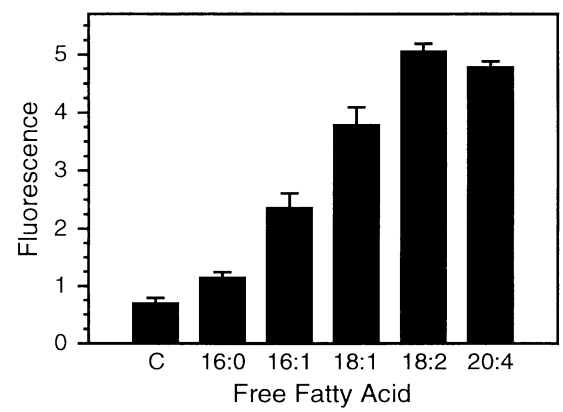

Figure 6. *Fluorescence spectroscopic analysis of $A\beta_{1-42}$ assembly. Amyloid peptide $A\beta_{1-42}$ was incubated for 54 hours with 60 μmol/L of the following FFA: control (C), none; 16:0, palmitic; 16:1, palmitoleic; 18:1, oleic; 18:2, linoleic; and 20:4, arachidonic acid. Fluorescence values (arbitrary units) obtained after mixture with thioflavin-T are shown as the mean ± SD; n = 3.*

arachidonic acid coincide closely with concentrations recently shown to act in concert with tau proteins to activate phospholipase C-γ.[54] This enzyme-modulating activity was attributed to tau proteins after a functional screening of total brain cytosolic proteins. Data describing the coordinate activation of phospholipase C-γ by tau and FFAs also resembled tau polymerization data with respect to the rank order of effectiveness of several of the FFAs examined (20:4 > 16:1 > 18:2 > 18:1 > 18:0). The activation of phospholipase C-γ by tau proteins in conjunction with FFAs underscores the possibility that tau-FFA interactions might be important in normal cellular metabolism as well as pathological assembly reactions.

Measured differences in the spontaneous and inducible assembly of the different tau isolates indicate that sequence and/or phosphorylation is likely to play a role in modulating tau polymerization. The activity of kinases and phosphatases present during tissue processing and microtubule cycling[21,55–57] is expected to produce a phosphorylation state for microtubule tau different from that of tau purified from whole brain, as has been shown for microtubule-associated protein-2.[58] As juvenile and adult brain contain different sets of developmentally regulated kinases and phosphatases, MTτ purified from these two sources will also differ in their phosphate content. Recombinant tau proteins are presumed to contain no phosphorylated residues. It should be stressed that none of these phosphorylation states necessarily occur *in vivo*. A comparison of tau assembled in the presence and absence of FFAs reveals a similar dependence on several physical parameters and the formation of morphologically indistinguishable filaments. As FFAs appear to stimulate assembly without altering the type of filament formed, they should prove to be of general utility in studying the effects of phosphorylation and other factors that might further modulate tau polymerization.

One result that warrants closer examination due to its implications for the mechanism of tau assembly is the reciprocal relation between filament length and filament numbers observed when the reducing potential is varied. If nucleation and elongation events compete for a limited pool of subunits, then the effects of increasing the reducing potential and thereby decreasing disulfide-dependent dimerization could be interpreted in two ways. First, decreasing dimerization may promote elongation at the expense of nucleation. This implies that tau dimers formed in an excess of DTT can inhibit the addition of tau monomers to filament ends, which seems un-

likely given the large excess of monomers expected to be present under these conditions. Second, decreasing dimerization may inhibit nucleation, allowing for greater elongation. Consistent with the latter interpretation is a >50% decrease in total polymer mass (filament number × average length; Figure 3C) observed when assembly at 10 mmol/L DTT is compared with assembly at 0.01 to 1.0 mmol/L DTT, indicating that a larger reducing potential has an overall inhibitory effect on assembly. This interpretation of the data supports previous reports that intermolecular disulfide-based dimerization precedes polymerization of a tau deletion construct.[59,60] We have previously speculated that a hydrophobic domain produced by folding events that precede polymerization might protect a disulfide bond shared by two apposed tau monomers, allowing for the formation of significant numbers of dimers under reducing conditions.[43] The stimulatory effect of FFAs on tau polymerization could be mediated by a stabilization of this hydrophobic domain and the associated folded conformation of tau. As pointed out by other authors,[60,61] the dependence of assembly on sulfhydryl oxidation provides one mechanism by which increases in oxidative stress might contribute to rates of polymer deposition.

The ability of FFAs to stimulate amyloid assembly could also result from the stabilization of an assembly-competent conformation of the Aβ peptide. The thioflavin binding assay indicates that FFAs stimulate an increase in polymer mass, but it does not resolve the relative contributions of filament nucleation and elongation. The paucity of short filaments in samples of $A\beta_{1-40}$ treated with FFAs, however, suggests that nucleation is limited. Because in these samples spontaneous assembly is observed at 25 μmol/L but not 10 μmol/L peptide, in a 10 μmol/L solution lacking FFAs, the concentration of soluble assembly-competent peptide is presumably below the critical concentration (C_c) required for filament nucleation and low enough to produce minimal filament elongation. Stabilization of the assembly-competent conformation of the peptide by FFAs could increase the rate of filament elongation, resulting in measurable growth of the population of short filaments initially present in the peptide solution. Alternatively, the addition of FFAs may lower C_c below 10 μmol/L, resulting in the transient generation of stable nuclei until further subunit incorporation reduces the peptide concentration below the new C_c. Several lines of evidence indicate that the amphipathic nature of Aβ leads to peptide micellation and that filament nuclei are released from the highly aggregated micellar Aβ.[62,63] In this case, C_c is equal to the CMC. Under conditions similar to those employed in the current study, the CMC for $A\beta_{1-40}$ and $A\beta_{1-42}$ was reported to be 25 μmol/L,[62] consistent with the onset of spontaneous assembly we observed at 25 μmol/L. It is possible that the interaction of FFAs with Aβ lowers the CMC and the associated C_c for nucleation. Potential interactions with FFAs are likely to be mediated by the hydrophobic residues of the Aβ peptide. The reported interaction of apolipoprotein E (apoE) with Aβ is also dependent on hydrophobic properties of Aβ and is mediated by the lipid-binding domain of apoE.[64,65] Thus, the assembly-promoting activity of apoE[49,66] and FFAs may be a property shared by a variety of hydrophobic substrates. Given that β-amyloid polymerization appears to be a reversible process,[67] any factors that increase the rate of filament elongation would decrease the net disassembly and normal clearance of amyloid polymers, thereby contributing to amyloid deposition. It is noteworthy that the polymerization of amyloid and tau filaments was stimulated by similar concentrations of FFAs, as would be expected if the dual processes of NP and NFT formation share a common effector molecule.

Recognizing the potential contribution of FFAs to AD pathology, it is of interest to examine whether concentrations of FFAs demonstrated to stimulate polymer assembly *in vitro* might be of physiological relevance *in vivo*. The intracellular concentration of FFAs has not been directly measured, but it is likely to be in the low micromolar range. This is inferred from the dissociation constants (0.2 to 3.0 μmol/L) reported for the binding of FFAs to fatty-acid-binding proteins,[68] a class of proteins believed to facilitate diffusion and act as intracellular buffers of FFAs. The induction of an FFA-dependent pathological effect, therefore, might be expected to occur at FFA concentrations in the low micromolar range (ie, slightly higher than normal physiological concentration), consistent with the apparent threshold of 5 to 10 μmol/L demonstrated for the stimulation of tau assembly. In the CSF, unesterified fatty acid concentrations have also been measured in the low micromolar range (10^{-6} to 10^{-5}) but are reported to rise as high as 30 to 50 μmol/L in response to physical trauma.[69] As the average concentration of albumin in the normal CSF is only 2.2 μmol/L,[70] even with six to eight high-affinity FFA-binding sites,[71–73] the buffering capacity of albumin may be exceeded under some trauma-related conditions. Local elevations of extracellular FFAs could also produce changes in intracellular FFA levels, as FFAs are able to diffuse across cellular membranes. Levels of FFAs sufficient to induce polymer assembly are unlikely to exert

detergent or other toxic effects on neurons; 10 μmol/L oleic or palmitic acid had no effect on the survival of cultured rat hippocampal neurons,[74] and 10 to 100 μmol/L linoleic or linolenic acid actually induced neurite elongation in cultured PC12 cells.[75] Given the substantial release of FFAs observed in response to physical trauma, it is interesting to note the increase in amyloid deposition and the increased prevalence of AD among victims of head trauma.[76,77]

The ability of unsaturated FFAs to stimulate tau and Aβ assembly suggests that enzymes with phospholipase A_2 (PLA_2) activity may be relevant to the generation of AD pathology. Many PLA_2 enzymes are Ca^{2+} activated and coupled directly or indirectly to signal-transducing, heterotrimeric G proteins,[78] which are in turn activated by many factors, including receptor-bound neurotransmitters, hormones, and cytokines, bacterial toxins, and aluminum fluorate.[78–80] Levels of arachidonic acid produced by PLA_2 activity are elevated during long-term potentiation, a phenomenon associated with the process of memory formation.[81,82] Under some conditions, lecithin cholesterol acyl transferase (LCAT) also exhibits a PLA_2 activity. Serum LCAT normally liberates fatty acids from phospholipids and catalyzes their esterification with free cholesterol. In the absence of sufficient free cholesterol, the net effect is the generation of FFAs.[83] This enzyme, which is synthesized in the brain and is a component of lipoproteins in the CSF,[84,85] may be of particular importance given that it is activated by apoE,[86] a recently identified genetic risk factor for AD.[64,87] It is not known whether the risk-defining allelic variations in apoE can modulate its activation of LCAT.

In a multifactorial disease characterized by the appearance of two distinct lesions that occur concomitantly but are nonetheless distributed through the brain in a noncorrelative manner, the diffusable FFAs are attractive candidates as effectors of pathogenesis. The following model (schematized in Figure 7) is proposed as an example of how the spatial and temporal progression of AD could result from FFA disequilibria. Beginning with the earliest stage of NP formation, a toxic effect produced by an initial deposition of filamentous amyloid results in the degeneration of neurites and the activation of glial cells in the surrounding neuropil. This initial polymerization of amyloid occurs when the local concentration of Aβ surpasses the critical concentration for assembly. This would result from an increase in peptide concentration or a decrease in the critical concentration, either of which could potentially be attributed to a number of factors of genetic or environmental origin.

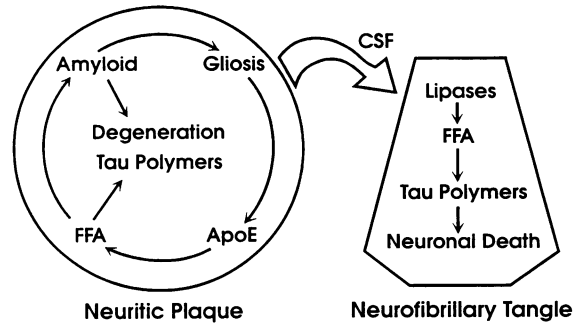

Figure 7. *The FFA model of AD pathogenesis. The relationship between the various elements postulated to contribute to the formation of NPs and NFTs are shown for the case in which apoE is a risk-defining factor. The common effector molecule is the FFA, which can be carried from its point of origin in the NPs to anatomically distant sites of NFT formation by the circulating CSF. See text for details.*

As astrocytes associated with the primordial plaque are activated, either in direct fashion by amyloid filaments[37] or indirectly via cytokines secreted by activated microglia,[88] they exhibit an increase in apoE production as is in fact observed in the AD brain.[89] We would then speculate that the different isoforms of apoE, by virtue of their role in the regulation of lipid trafficking and metabolism, are able to effect an isoform-dependent increase in FFA release. There are several ways in which this might be envisaged, invoking only elements of previously defined metabolic pathways (see below). With the apoE-dependent stimulation of FFA release comes a further induction of amyloid assembly and the establishment of a positive feedback loop that accelerates the further evolution of the plaque. In addition, local increases in FFAs would induce the assembly of tau polymers within degenerating neurites associated with the plaque. With regard to tau polymerization in NFTs and neuropil threads, it should first be noted that tau proteins are constitutively exposed to some baseline concentration of FFAs as a result of the normal activity of intracellular lipases. Vulnerable neurons could be primed for the assembly of tau filaments by any number of conditions that conspire to increase this baseline level of exposure. If intracellular levels of FFAs are supplemented to a sufficient degree by NP-derived FFAs circulating in the CSF, then the process of tau polymerization would be initiated within the population of primed neurons.

As lipid metabolism in the brain is at present poorly understood, the means by which increased levels of apoE could potentially modulate FFA release must in large part be discussed in light of the known roles of this protein in serum lipid metabolism. As mentioned above, apoE is an activator of LCAT. If the rate of cholesterol esterification by LCAT is limited by the availability of unesterified cholesterol in

the CSF, then an increased activation of LCAT resulting from higher concentrations of secreted apoE might cause the deacylating activity of the enzyme to exceed the cholesterol-esterifying activity of the enzyme. This would result in an extracellular release of FFAs from apoE-associated lipids. It is interesting to note in this regard that levels of unesterified cholesterol measured in membranes extracted from temporal cortex are decreased an average of 30% in AD brain relative to nondemented controls.[90] Alternatively, apoE might modulate intracellular release of FFAs through its presentation of lipids to cells. In this regard, an isoform-specific binding of apoE to lipoproteins and the low-density lipoprotein receptor has been demonstrated.[91] The action of lysosomal lipases on internalized lipoproteins results in the generation of FFAs that can diffuse across the lysosomal membrane. Therefore, the release of assembly-inducing FFAs into the cytoplasm could be modulated by the rate at which different apoE isoforms mediate lipoprotein internalization, as well as by the ability of different apoE isoforms to preferentially interact with distinct lipoprotein subpopulations based on their lipid content. One apoE receptor, the very-low-density lipoprotein receptor, is present at increased levels in the AD brain and has been identified as a risk factor for AD in a Japanese population. A twofold increase in the incidence of AD was observed among individuals who were homozygous for a specific allele of this receptor.[92] Regardless of the means of liberation, the FFA model of pathogenesis clearly predicts that CSF levels of FFAs would be increased in AD and that, in the absence of a compensatory mechanism, these levels would be proportional to the total plaque load in the brain.

In the FFA model of pathogenesis, one initiating event is the assembly of amyloid filaments, consistent with the amyloid cascade hypothesis.[93] Pathological rates of amyloid deposition might result from environmental factors or a genetic predisposition, as has been demonstrated to result from mutations in the amyloid precursor protein and presenilin genes.[40,41,94] In contrast to the amyloid cascade hypothesis, however, the FFA model allows that risk factors for AD would not be limited to those that initially increase rates of amyloid polymerization but would also include factors that link NP genesis to mechanisms of FFA release. Thus, apoE is invoked in its originally characterized capacity as a mediator of lipid metabolism. The pivotal role ascribed to gliosis in the FFA model also suggests a means by which chronic inflammatory or acute-phase processes that have been implicated in AD pathogenesis[88,95–97] might initiate or exacerbate some AD cases. The FFA model also implies that the targeting of a neuron for NFT formation is separable from the subsequent death of that neuron and that tau polymerization contributes directly to neuronal dysfunction and the resulting clinical manifestations of the disease. This contrasts with the view that NFTs are merely "tombstones" of the necrotic process. As FFAs derived from sources other than NPs could also potentially induce tau polymerization, NFTs might be expected to occur in other pathological states characterized by intracellular FFA release and the absence of NPs. Similarly, in the absence of risk factors that link the deposition of amyloid to FFA release, moderate levels of amyloid assembly could be tolerated without a concomitant induction of tau pathology.

The demonstration of FFA-stimulated tau and amyloid assembly provides new evidence that formation of these structurally unique lesions in AD could be mediated by a common effector molecule. The relevance of FFAs to biological systems leads to a straightforward model of pathogenesis that effectively incorporates identified risk factors and suggests an explanation for the concomitant appearance of the extracellular amyloid and intracellular tau polymers that is uniquely observed in the AD brain. The implication of various enzymes with lipase activity in AD pathogenesis suggests potential therapeutic targets for the treatment of AD and other diseases characterized by neurofibrillary degeneration.

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