Alzheimer's disease is defined in part by the intraneuronal accumulation of filaments comprised of the microtubule-associated protein tau. In vitro, fibrillization of recombinant tau can be induced by treatment with various agents, including phosphotransferases, polyionic compounds, and fatty acids. Here we characterize the structural features required for the fatty acid class of tau fibrillation inducer using recombinant full-length tau protein, arachidonic acid, and a series of straight chain anionic, cationic, and nonionic detergents. Induction of measurable tau fibrillization required an alkyl chain length of at least 12 carbons and a negative charge consisting of carboxylate, sulfonate, or sulfate moieties. All detergents and fatty acids were micellar at active concentrations, due to a profound, tau-dependent depression of their critical micelle concentrations. Anionic surfaces larger than detergent micelles, such as those supplied by phosphatidyserine vesicles, also induced tau fibrillization with resultant filaments originating from their surface. These data suggest that anionic surfaces presented as micelles or vesicles can serve to nucleate tau fibrillation, that this mechanism underlies the activity of fatty acid inducers, and that anionic membranes may serve this function in vivo.

Alzheimer's disease is a progressive neurodegenerative disease characterized in part by a constellation of intracellular neurofibrillary lesions termed neurofibrillary tangles, neuritic plaques, and neuropil threads (1). Each manifestation of neurofibrillary patholgy is comprised of tau protein polymerized into filaments. Because neurofibrillary lesions appear with a stereotypic spatial distribution (2, 3) and correlate with both neuronal cell loss (4) and cognitive decline (5), they are useful markers of degeneration in Alzheimer's disease and other dementias.

There is much interest, therefore, in identifying cellular components that initiate tau filament formation in disease. Fibrillation of recombinant, full-length tau protein in vitro does not occur spontaneously at physiological concentrations (6). However, tau protein can be induced to fibrillize by changes in its primary structure (7) or its state of posttranslational modification (8, 9); by the addition of polyionic substances such as sulfated glycosaminoglycans (heparin, dextran sulfate, and pentosan polysulfate) (10–12), polyglutamate (11), and RNA pentasaccharides (13); or by addition of fatty acids (14). Of these, fatty acids are especially efficacious in promoting the fibrillation of full-length tau protein at near physiological pH, temperature, reducing environment, ionic strength, and tau protein concentration (14, 15).

Nonetheless, the mechanism by which fatty acids induce tau fibrillization is unknown. Fatty acids resemble detergents in having hydrophobic alkyl chains and charged (anionic) head groups. Above their critical micelle concentrations (CMCs)1 in aqueous solution, fatty acids form micelles in which their hydrophobic moieties are sequestered, and their charged head groups are exposed to solvent. We (6) and others (16) have argued that the behavior of fatty acids such as arachidonic acid in assays of tau aggregation was consistent with it acting in micellar form. Yet fatty acids induce tau fibrillization at concentrations well below their measured CMC values (14). As free monomers, fatty acids have been shown to reversibly bind proteins through specific high-affinity motifs (17). Although such motifs have been suggested to exist in α-synuclein (18), a protein that forms amyloid filaments in Parkinson's disease (19–21), they have not been found in human tau protein.

Here we examine the importance of alkyl chain length, chemical nature of the charged head group, and CMC for induction of tau polymerization using arachidonic acid, a series of ionic and nonionic synthetic detergents, and the anionic lipid phosphatidylserine. The results show that fatty acids induce tau fibrillization in micellar form without stoichiometric incorporation into filaments. The micelles must be negatively charged to promote fibrilization of full-length tau protein and, in the case of alkyl sulfate detergents, must contain at least 38 mol % negatively charged species. Because anionic lipids also induce tau fibrillation, it is proposed that intracellular membranes represent a class of physiologically relevant, intracellular tau polymerization inducers.

**EXPERIMENTAL PROCEDURES**

Materials—Recombinant, His-tagged tau protein (htau40) was purified as described previously (22). Arachidonic acid (AA) and [14C]AA (55 Ci/mol) were obtained from Cayman Chemicals (Ann Arbor, MI) and American Radiolabeled Chemicals (St. Louis, MO), respectively, and stored at −80 °C under argon. Palmitoleic and stearic acids were from Sigma. Alkyl sulfate detergents (12–20 carbons) were obtained from Mallinckrodt (Paris, KY), Acros Organics (Morris Plains, NJ), Lancaster Synthesis (Pelham, NH), and Research Plus (Bayonne, NJ) as sodium salts. Alkyl sulfonate detergents (6–18 carbons) were obtained as sodium salts from Research Plus. Nonionic and cationic detergents (bromide salts) were purchased from Sigma (C10E5, C12E7, C14E8, and C16E10BrN), and Fluka (Milwaukee, WI; C12EO, C18EO, C16H4BrN, and C18H6BrN). Porcine brain 1-α-phosphatidylserine (containing mostly 18:0 and 18:1 fatty acid chains) was from Avanti Polar-Lipids (Alabaster, AL). All detergent stock solutions were prepared in water, MeSO4, or 1:1 water/isopropanol and stored at 20 °C. N-Phenyl-1-naphthylamine was from Sigma.

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Micelle-dependent Tau Fibrillation

TABLE I

| Detergent                        | CMC \( \mu M \) | htau40 | htau40* |
|----------------------------------|----------------|--------|--------|
| Polyoxylethylene nonionic detergents |                |        |        |
| C14E6                             | 586 ± 48       | ND     |        |
| C15E6                             | 609 ± 53       | ND     |        |
| C16E6                             | 627 ± 120      | ND     |        |
| C18E6                             | 49.5 ± 18.6    | 52.2   | 12.8  |
| C19E6                             | 24.7 ± 2.3     | 26.0 ± 3.1 |  |
| TWEEN20                           | 11.3 ± 3.4     | 8.9 ± 1.8 |  |
| Quaternary ammonium cationic detergents |            |        |        |
| C12H2BrN                          | 7,600 ± 100    | ND     |        |
| C12H3BrN                          | 88.1 ± 3.4     | 38.9 ± 7.1 |  |
| C16H4BrN                          | 18.6 ± 4.5     | 19.5 ± 0.9 |  |
| Fatty acids                        |                |        |        |
| Palmitoleic acid                  | 996 ± 26       | 87.3 ± 3.6 |  |
| Stearic acid                      | 393 ± 2        | 50.0 ± 17.1 |  |
| AA                                | 236 ± 12       | 8.1 ± 0.5 |  |

*Measured in the presence of 4 \( \mu M \) htau40.

**ND, not determined.

Results and Discussion

**Anionic but not Nonionic or Cationic Detergents Can Induce Tau Protein Assembly** — The importance of alkyl chain length, head group charge, and CMC for induction of recombinant htau40 polymerization was examined for a selection of ionic and nonionic synthetic detergents. htau40 was used because of ease and yield of preparation and because the presence of the tag does not significantly change the rate or extent of AA-induced tau fibrillation (26). Nonionic detergents tested included the polyoxyethylene solvents Triton X-100 (data not shown), Tween 20, C14E6, C15E6, C16E6, C12E2, and C14E6. These had CMC values ranging from 10 to 600 \( \mu M \) when measured under standard tau assembly conditions in the presence or absence of htau40 (Table I). None of the nonionic detergents were capable of inducing tau fibrillation (using the transmission electron microscopy assay) when tested at concentrations up to 500 \( \mu M \). In addition, tau protein had no observable effect on detergent micellization (Table I). These results suggest that uncharged surface active hydrophobic agents were insufficient to promote tau fibrillation when present in either dispersed or micellar form.

**Extending the analysis to cationic detergents C15H34BrN, C19H42BrN, and C21H4BrN revealed that they too were inactive as fibrillation inducers when assayed above or below their CMC, although the presence of tau protein modestly depressed the CMC for C15H34BrN (Table I).** These data suggest that, like nonionic detergents, positively charged ionic detergents are incapable of inducing tau fibrillation in either dispersed or micellar form.

In contrast to these results, AA, which shares chemical properties with anionic detergents, was a powerful inducer of tau fibrillation at 75 \( \mu M \) concentration (Fig. 1). To determine whether other negatively charged chemical groups could substitute for the carboxylic acid moiety found in AA, the analysis was extended to include synthetic alkylation sulfate and alkylation sulfonate detergents containing 12–20 saturated straight chain carbon atoms. Very few filaments of long length (>500 nm; 1–4 filaments/grid) were observed in the electron microscope when using the 12- or the 14-carbon sulfate or sulfonate series detergents as inducers. In contrast, alkyl sulfate and sulfonate detergents containing 16, 18, and 20 carbons induced significant polymerization. C16H30SO3Na and C19H34SO3Na were the most active inducers among this series; therefore, they were used in the studies described below. They produced abundant straight filaments from recombinant htau40 that were morphologically similar to those induced by AA (Fig. 1). Moreover, the alkyl sulfate detergents were broadly similar to AA in potency, yielding biphasic dose-response curves with maximal filament mass yielded at concentrations between 50 and 150 \( \mu M \) (Fig. 2). Nonetheless, alkyl sulfate inducers differed quantitatively from AA in that they appeared to be weaker nucleating agents, producing a smaller number of filaments that achieved longer length (Fig. 1). This was apparent in filament length distributions, which remained exponential but skewed toward longer lengths when induced by alkyl sulfates (Fig. 3). For example, AA produced >10-fold more filaments than C20H41SO3Na that were on average >5-fold shorter in length. Overall, despite inducing far fewer filaments, the total mass of filaments formed from C20H41SO3Na was typically ~75% of the mass induced by AA. These data suggest that AA and the alkyl sulfate detergents induce tau fibrillation by similar mechanisms and that the minimum structural features responsible for measurable inducer activity are an alkyl chain of at least 16 methylene groups.
carbons in length and a negatively charged head group comprised of at least carboxylate, sulfate, or sulfonate moieties.

**AA-induced Tau Fibrillization Requires Micelle Formation**—CMC values for AA and alkyl sulfate detergents were measured to determine whether micelles were important for tau fibrillization activity. Consistent with earlier observations (14), the CMC for AA in Assembly Buffer alone was measured as \(236 \pm 12 \mu M\), which was well above the concentration required for tau fibrillization. When CMC was measured in Assembly Buffer complete with htau40 at \(4 \mu M\), however, the CMC decreased to \(8.1 \pm 0.5 \mu M\). Tau-mediated CMC depression was observed with other fatty acids as well (palmitoleic and stearic acids; Table I), indicating that the effect was not unique to AA and extended to both saturated and unsaturated fatty acids. These data show that fatty acids aggregate to form micelles at a much lower concentration in the presence of htau40 than in its absence. CMC depression was apparent even at substoichiometric molar ratios of htau40 to fatty acid.

These observations were extended to a series of alkyl sulfate detergents, which follow a log-linear relationship between CMC and alkyl chain length when analyzed in water (Fig. 4; Ref. 25). When measured in the presence of Assembly Buffer (without tau), the relationship between log CMC and alkyl chain length remained linear but was depressed toward lower CMCs (Fig. 4) because of the presence of neutral electrolyte (100 mM NaCl) in the buffer (27). When measured in Assembly Buffer complete with htau40, however, CMC values were depressed still further, so that they were fully 2 orders of magnitude below the values observed in water.
To determine whether anionic lipids could substitute for anionic detergent or fatty acid micelles. Tau fibrillization was induced by incubation of htau40 (4 μM) with mixed micelles (100 μM detergent) prepared from nonionic detergent C14E8 and varying mol % C20H41SO4Na. C14E8 was chosen as carrier because it was incapable of inducing tau fibrillation on its own and because, under assembly conditions, it had a low micromolar CMC, regardless of whether htau40 was present (Table I). Total detergent concentration was held constant at 100 μM to ensure the presence of micelles under all assay conditions, and resultant total filament mass was estimated by assaying tau protein in pellet and supernatant fractions after ultracentrifugation. Tau aggregation was not detectable below 20 mol % C20H41SO4Na but was observed at 40 mol % and ultracentrifugation. Tau aggregation was not detectable below 40 mol % C20H41SO4Na content and tau filament formation and extrapolating to the ordinate intercept, tau fibrillation was supported under standard conditions when 100 μM mixed micelles contained 38.0 ± 6.4 mol % C20H41SO4Na.

Phosphatidylserine Liposomes Induce Tau Polymerization—To determine whether anionic lipids could substitute for anionic detergents as inducers of tau fibrillation, htau40 was incubated (3 h at 37 °C) with 10–400 μM phosphatidylserine vesicles under standard conditions and subjected to electron microscopy assay. Unlike anionic micelles, phosphatidylserine vesicles were readily observable in electron microscopy assays because of their large size (typically >50 nm in diameter compared with <10 nm in diameter for detergents). Moreover, their CMCs in aqueous solution have been estimated in the nanomolar range (29), so that they were almost completely vesicular before incubation with tau protein. Examination of reaction products by electron microscopy showed the presence of vesicles and very long filaments at most phosphatidylserine concentrations tested. Closer inspection revealed that at least 15% of all filaments were associated with phospholipid vesicles through their ends, which appeared to extend from the vesicle surface (Fig. 6). Other vesicles were observed alone or associated with filaments along their length (Fig. 6). These data showed that anionic vesicle-forming lipids were capable of inducing tau filament formation and suggested that the mechanism involved facilitation of tau aggregation at the vesicle surface.

AA-mediated Tau Fibrillation Follows a Ligand-facilitated Mechanism—To test this hypothesis, htau40 was subjected to fibrillation conditions for 3.5 h in the presence and absence of [14C]AA (75 μM), and the amount of labeled AA comigrating with filamentous tau was determined after centrifugation. Under these conditions, ~50% of AA-treated htau40 comigrated with the pellet (filamentous) fraction, whereas most AA (>97%) remained in the soluble fraction (Table II). The pellet fraction contained 0.19 ± 0.05 mol AA/mol tau, confirming that AA remained at least partially associated with tau filaments but was not incorporated with 1:1 molar stoichiometry with respect to tau protomer. These data suggest that AA acted in micellar form to facilitate tau aggregation but did not directly mediate filament extension with concomitant incorporation into growing filaments.

**DISCUSSION**

Here we examined the mechanism of fatty acid-mediated tau fibrillation and showed that it stems from two structural features: (a) an alkyl chain of at least 12 carbons in length, and (b) a negatively charged head group. Although requirement of
Vesicles were observed as bodies (vesicles and then examined by transmission electron microscopy (×22,000 magnification). Vesicles were observed as bodies > 50 nm in diameter (arrows) that were frequently associated with filaments. Approximately 15% of well-resolved filaments were found extending from the surface of vesicles (asterisks). Bar, 100 nm.

![Stimulation of tau fibrillization by anionic lipid](image)

**Fig. 6. Stimulation of tau fibrillization by anionic lipid.** Htau40 (4 μM) was incubated (3 h at 37 °C) with preformed phosphatidylserine vesicles and then examined by transmission electron microscopy. Vesicles were observed as bodies > 50 nm in diameter (arrows) that were frequently associated with filaments. Approximately 15% of well-resolved filaments were found extending from the surface of vesicles (asterisks). Bar, 100 nm.

an alkyl chain has been reported previously (14), its role has been confounding because all fatty acids examined to date have been active well below their CMCs, suggesting that alkyl chains were involved in binding tau protein rather than micellization. Published CMC values were determined in the absence of tau protein, however, and the surprising finding here is that the presence of even micromolar concentrations of tau protein greatly depressed the CMC for AA and other anionic detergents. Protein-mediated depression of alkyl sulfate CMC has been observed previously with micromolar concentrations of peptides derived from uteroglobin (30) and results from electrostatic interactions between positively charged amino acid side chains and negatively charged fatty acids or detergents. Indeed, a single pendent alkylamine (grossly resembling polyethylene glycol) can depress the CMC for fatty acids by an order of magnitude (31). The htau40 construct used here contained a total of 59 Lys and Arg residues and a predicted net charge of +3.51 at assay pH (pH 7.4). Because htau40 had no effect on the CMC of nonionic detergents, it does not appear to depress CMC by modulating solvent surface tension (32). In any event, AA and anionic detergents are mostly micellar at any event, AA and anionic detergents are mostly micellar at

Inducer activity appears to reside with the micelle because incubation of tau with preformed micelles or lipid vesicles results in tau fibrillation. Moreover, tau filament yield initially increases with detergent concentration above the CMC, which corresponds to an increase in micelle but not detergent monomer concentration (28). Therefore, it is concluded that the principal role of the alkyl chain is to support micellization, and micelle formation in the 50–100-μM range in vitro requires an alkyl chain of at least 12 carbons. The alkyl moiety can be saturated (14) or unsaturated (this work and Ref. 14) and can be part of fatty esters such as phospholipids (this work).

Inducer activity also requires an ionizable head group, with sulfate, sulfonate, and carboxylate moieties all supporting tau fibrillation, even when presented as part of a phospholipid head group, as in the case of phosphatidylserine. The resultant negative charges appear to supply more than simple amphiphilic character to promote micelle formation because cationic detergents of similar alkyl chain length and CMC do not support tau fibrillation. Micelle-forming nonionic detergents and uncharged methyl or ethyl esters of AA (14) also are inactive. Therefore, it appears that the key role of ionizable groups is to present a negatively charged surface on the micelle.

Anionic micelles may induce filament formation by concentrating the basic tau protein molecules close to their surface, such that the energy barrier for nucleation is overcome (6, 14). Similar mechanisms have been postulated for the polyanion class of inducers, including heparin, poly-glutamate, nucleic acids, and the microtubule surface (10–13, 33, 34). Alternatively, or in addition, filament formation could stem from micelle-dependent stabilization of assembly-competent protein conformations (35). Indeed, protein conformational alterations have been suggested to underlie observed differences in the ability of protein kinases to phosphorylate tau in the absence or presence of phospholipid liposomes (36). Regardless of its effects, micelle-tau association appears to be reversible because only ~15% of mature filaments were found associated with liposomes and because of the poor recovery of [¹⁴C]AA when tau filaments were isolated by sedimentation.

Although all alkyl sulfate detergents examined form micelles above their CMCs, their efficacy in promoting tau fibrillation under conditions reported here varied widely. For example, 12- and 14-carbon alkyl sulfates are extremely weak inducers and yield insufficient filaments to quantify by current assay methods. Significant quantities of filaments can be observed by electron microscopy using C₁₂H₂₅SO₄Na, with C₁₄H₂₉NaSO₄ and C₁₆H₃₃NaSO₄ inducing large amounts of filaments. Because the degree of ionization of alkyl sulfate micelles is independent of aggregation number and salt concentration (37), it is unlikely that micelle charge varies with increasing alkyl chain length to influence anionic detergent efficacy. In contrast, micelle aggregation number does increase exponentially with alkyl chain length (38) and can lead to major differences in micelle size and shape. Extrapolating this relationship to C₁₄H₂₉SO₄Na predicts an aggregation number of ~250 molecules/micelle near the CMC in solutions containing 100 mM NaCl (38). This corresponds to a radius less than half the hydrodynamic radius of monomeric tau protein (39). Efficient induction of tau fibrillization above this size may be related to micelle curvature, surface area, or volume. Although not demonstrated for tau protein, other amyloid-forming proteins such as Aβ, prion protein, apolipoprotein C-III, and α-synuclein can obliquely insert into lipid membranes in a viral peptide fashion (40–44), and this may contribute to their lipid-dependent aggregation (45–51). Thus, the size of the hydrophobic micelle core may play a role in favoring polymerization-prone conformations of partially inserted proteins. Finally, it is noted that the large differences in aggregation number among alkyl sulfate detergents give rise to vastly different relationships between micelle and total detergent concentrations. This consideration may underlie the higher potency of C₁₆H₃₃NaSO₄ relative to C₂₀H₄₁NaSO₄ (Fig. 2), even though its CMC is greater. It may also limit the apparent efficacy of the smaller alkyl sulfate detergents by greatly narrowing the concentration range over which they are active (see the discussion of biphasic behavior below). Further experimentation will be necessary to determine which of these considerations is most important for differences in alkyl sulfate efficacy.

Although polyanions have emerged as useful tools in vitro, their structures have not pointed toward a clear cellular agent that would serve to promote tau fibrillation in disease. For example, RNA is present at high concentration inside of cells, but it is heavily complexed with protein and presumably not available in free form in sufficient amounts to induce tau filament formation (52). Similarly, heparan sulfate proteoglycans are extracellular and sequestered from the bulk of physiological tau protein. In contrast, the activity of anionic micelles and vesicles points toward cellular membranes as naturally abundant intracellular sources of clustered negative charge. Studies
with Alzheimer’s disease tissue show that hyperphosphorylated tau colocalizes with lipid rafts (53) and that tau filaments appear in association with cytomembranes (54). In fact, membrane association may be a normal function of tau because it has been shown to occur upon heterologous expression in PC12 cells (55) and may be mediated by electrostatic interaction with anionic lipids. Phosphatidylinerine, the most abundant anionic phospholipid, comprises between 10 and 20 mol % of total phospholipid in cell membranes (56). Because phosphatidylserine is distributed primarily on the cytoplasmic face of cellular membranes (57), substantial negative charge is potentially available for binding tau protein. Using purified components, we found that fibrillation of httau40 required at least 38 mol % anionic charge in vitro. These results suggest that whereas phospholipid membranes are potential sites of tau aggregation in vivo, normal levels of anionic phospholipids may not be sufficient to drive fibrillation. Rather, increased levels of anionic lipids (58, 59), increased free tau concentration, or fibril-promoting the cooperative micellization of anionic detergents or phospholipids. The data suggest that anionic tau fibrillization in micellar form and can be replaced by anionic detergents or phospholipids. The data suggest that anionic membranes are candidate nucleation centers in vivo.

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**TABLE II**

| Sample          | Protein | AA (%) | Stoichiometry |
|-----------------|---------|--------|---------------|
| Total           | 218 ± 24| 100 ± 11| 4.54 ± 0.48  |
| Pellet          | 106 ± 6 | 49 ± 3 | 2.21 ± 0.24  |
| Supernatant     | 115 ± 12| 53 ± 6 | 2.40 ± 0.26  |

a Determined by Coomassie Blue binding assay as described under “Experimental Procedures.” Data are reported as average ± S.D. of three independent experiments measured in duplicate.

b Determined using [3H] AA as described under “Experimental Procedures.” Data are reported as average ± S.D. of two independent experiments measured in duplicate.
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