Intracellular aggregation of the microtubule-associated protein tau into filamentous inclusions is a defining characteristic of Alzheimer disease. Because appearance of tau-aggregate bearing lesions correlates with both cognitive decline and neurodegeneration, it has been hypothesized that tau aggregation may be directly toxic to cells that harbor them. Testing this hypothesis in cell culture has been complicated by the resistance of full-length tau isoforms to aggregation over experimentally tractable time periods. To overcome this limitation, a small-molecule agonist of the tau aggregation reaction, Congo red, was used to drive aggregation within HEK-293 cells expressing full-length tau isoforms to aggregation over experimentally tractable time periods. To overcome this limitation, a small-molecule agonist of the tau aggregation reaction, Congo red, was used to drive aggregation within HEK-293 cells expressing full-length tau isoform htau40. Formation of detergent-insoluble aggregates was both time and agonist concentration dependent. At 10 μM Congo red, detergent-insoluble aggregates appeared with pseudo-first order kinetics and a half-life of approximately 5 days. By 7 days in culture, total tau levels increased 2-fold, with ~30% of total tau converted into detergent-insoluble aggregates. Agonist addition also led to rapid losses in the tubulin binding activity of tau, although tau was not hyperphosphorylated as judged by occupancy of phosphorylation sites Ser396/Thr404. Tau aggregation was associated with decreased viability as detected by ToPro-3 uptake. The results, which establish a new approach for analysis of tau aggregation in cells independent of tau hyperphosphorylation, suggest that conformational changes associated with aggregation are incompatible with microtubule binding, and that toxicity associated with intracellular tau aggregation is not acute but develops over a period of days.

Tau is a microtubule-binding protein expressed from a single gene on human chromosome 17 (1). In Alzheimer disease (AD), full-length tau isoforms aggregate in neuronal cell bodies to form neurofibrillary tangles and in neuronal processes to form neuropil threads and dystrophic neurites associated with neuritic plaques (1). Tau aggregates appear as filaments, within which a portion of each tau molecule adopts β-sheet conformation. As in other filamentous aggregates, the resultant β-sheets stack in parallel, with each sheet orthogonal to the axis of the growing tau filament (2). Neurofibrillary lesion formation is accompanied by 4–8-fold increases in total brain tau levels (3, 4). Because expression of total tau does not change at the mRNA level in AD (5, 6), disease-associated increases in tau levels may derive from post-translational events (e.g. fibrillization) that selectively decrease tau turnover (7).

Tau fibrillization correlates spatially and temporally with neurodegeneration and cognitive decline (8, 9). As a result, tau lesion densities are used for postmortem staging and diagnosis of AD (10). These correlations, along with the discovery that mutations in the tau gene cause familial forms of frontotemporal dementia (11–13), suggest that tau misfunction is somehow associated with neurodegeneration. Three general hypotheses have been put forward to rationalize a direct relationship between tau fibrillization and disease. The first argues that tau aggregates and other misformers are directly toxic to cells that harbor them. For example, tau aggregates are capable of inhibiting the ubiquitin-proteasome system (7). Inhibition of this system, which plays important roles in cell homeostasis, has been proposed to be a source of cellular stress (14, 15). The second argues that sequestration of tau from microtubules destabilizes the cytoskeleton leading to a loss-of-function toxicity (16). Although loss of tau is well tolerated over both long (17, 18) and short (19) time periods, simultaneous loss of both tau and other microtubule-associated proteins may lead to more severe phenotypes (20). A third hypothesis is that tau modified post-translationally (e.g. by hyperphosphorylation or truncation) may be directly toxic to cells (21, 22). Under these conditions, aggregation could be protective for cells by sequestering toxic species.

Resolving the role of tau fibrillization in disease is important for assessing the potential of tau-directed therapies. However, directly testing the above hypotheses in biological models has been complicated by the resistance of full-length tau isoforms to spontaneous aggregation over experimentally tractable time periods (23). The problem has been overcome by high level tau overexpression alone (24, 25) or in combination with aggregation-promoting mutations (26). Conditions that drive aggregation-promoting post-translational modifications (e.g. phospho-
rylation (27)) also have been successful. But the efficiency of fibrillization is frequently low, whereas mere overexpression of normal tau in neurons results in a toxic phenotype (28). Because levels of normal tau protein do not increase in AD (4), the significance of overexpression-mediated toxicity found in transgenic systems has been ambiguous.

Recently we found that the kinetic barrier to full-length tau fibrillization can be overcome by small-molecule ligands that bind β-sheet structures such as CR (Congo red), thiazin red, and thioflavin S (23). The ability of CR to populate amyloidogenic protein conformations (29) suggests that these agents drive aggregation by thermodynamic linkage of their binding reaction with the tau self-association reaction (linkage refers to the influence of ligand binding on other equilibria including aggregation reactions and phase transitions (30)). As a result, fibrillization agonists facilitate the study of full-length tau fibrilization in the absence of post-translational modifications, mutations, or macromolecular inducers such as heparin (23).

Here we extend this approach to cell culture as a means of examining the role of full-length tau fibrillization on acute toxicity. The results suggest that large scale aggregation in HEK-293 cells leads to significant losses in cell viability over a period of days.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-tau monoclonal antibodies Tau5 (31) and Tau12 (9) were obtained from Dr. L. I. Binder (Northwestern University Medical School), whereas PHF1 (32) was from Dr. Peter Davies (Einstein College of Medicine). Rat monoclonal anti-α-tubulin antibody MAB1864 (33) was from Chemicon International (Temecula, CA), whereas monoclonal mouse anti-α-tubulin antibody DM-1A (34) along with protease inhibitors (1× complete containing: 1 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride, 0.8 μM aprotinin, 40 μM bestatin, 20 μM leupeptin, 15 μM pepstatin A, 14 μM 1,10-phenanthroline, and 26 μM N-acetyl-leu-leu-norleu-al) and poly-D-lysine were from Sigma. Alexa 546-conjugated goat anti-mouse IgG secondary antibody and Vector Laboratories (Burlingame, CA), respectively.

**Cell Culture**—HEK-293 cells were obtained from ATCC (Manassas, VA), whereas an HEK-293-derived cell line stably expressing human htau40 (stable tau cells) was prepared as described previously (37). A stable tetracycline-inducible htau40 cell line (inducible tau cells) was prepared in Trex-293 cells (Invitrogen) following the manufacturer’s instructions and the same plasmid construct used to create stable tau cells. All cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 250 ng/ml amphotericin B, and 0.1 mg/ml streptomycin (37°C with 5% CO₂). Stable tau cells were grown under selection with 0.5 mg/ml G418, whereas inducible tau cells were grown in 5 μg/ml blasticidin and 0.4 μg/ml zeocin. Tau expression was induced in these cells with tetracycline (1 μg/ml). To assay the effect of CR on tau aggregation and cell viability, all cells were plated and cultured for 24 h, treated with varying concentrations of CR, and harvested after 1–14 days of treatment. CR was refreshed with each change of media.

**Isolation of Soluble and Insoluble Tau**—The solubility of cell-derived tau protein was assayed under microtubule-destabilizing conditions. CR-treated tau cells were suspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃NO₄, 10 mM NaF, 0.5% v/v Nonidet P-40, 1 μM EDTA, and 1× complete protease inhibitors), subjected to three freeze/thaw cycles (38), and then incubated at 4°C for 30 min to induce the disassembly of microtubules. Nuclei were removed from the lysates by centrifugation at 1000 × g for 5 min at 4°C.

The resultant extracts were centrifuged (100,000 × g for 1 h) at 4°C. The resultant pellets were washed three times with lysis buffer, then resuspended in lysis buffer (1/3, v/v, relative to the total volume of supernatant). Equal volumes of pellet and supernatant fractions were boiled and subjected to immunoblot analysis as described below. Aliquots of in vitro aggregation reactions were processed similarly after centrifugation, except that pellets were washed with PBS before being resuspended in SDS-sample buffer.

**Immunoelectron Microscopy**—Tau aggregates were isolated from CR-treated tau cells by a modification of the procedure used to isolate authentic PHF (39). Cells were homogenized in ice-cold buffer H (10 mM Tris-HCl, 1 mM EGTA, 0.8 mM NaCl, 10% sucrose and protease inhibitor mixture, pH 7.4), and centrifuged for 20 min at 20,000 × g. Supernatant fractions (S1) were saved, whereas pellet fractions (P1) were re-homogenized in buffer H and separated into S2 and P2 fractions by centrifugation (20 min at 20,000 × g). Supernatant fractions S1 and S2 were combined and incubated with 1% Sarkosyl for 1 h at room temperature with agitation. After centrifugation of the mixture (1 h at 100,000 × g), the resultant pellets were resuspended in 50 mM Tris-HCl (pH 7.4). Aliquots were absorbed onto Formvar/carbon-coated grids and incubated with the Tau12 monoclonal antibody for 30 min at 37°C. After washing with 0.1% gelatin, grids were incubated with the 10-nm gold-labeled goat anti-mouse IgG + IgM (Amersham Biosciences). Samples were then stained with 2% uranyl acetate for 1 min, and viewed by electron microscopy as described above.

**Tubulin Assays**—To assess tubulin binding activity of tau aggregates prepared in vitro, His₆-htau40 (1 μM) treated with...
A Cellular Model of Tau Aggregation

CR (10 μM) for various lengths of time was incubated with 1 mg/ml purified tubulin at 37 °C for 30 min in binding buffer (80 mM PIPES, pH 6.8, 1 mM MgCl2, 1 mM EGTA, 1 mM GTP) in a final volume of 100 μl. Reactions were incubated with 1 μg of monoclonal anti-α-tubulin antibody for 2 h after which protein G-agarose beads (40 μl of 25% (w/v) slurry) were added and incubation continued for 1 h. Immunoprecipitates were collected by centrifugation (2000 × g for 5 min), washed three times with lysis buffer, and finally subjected to immunoblot analysis as described below. For these experiments, input tubulin was visualized by Coomassie Blue staining after SDS-PAGE.

To assay the influence of aggregation on the ability of tau to bind endogenous tubulin, lysates (100 μg) were prepared from CR-treated or non-treated stable tau cells as described above, after which tubulin was immunoprecipitated with anti-α-tubulin antibody and protein G-agarose as described above. For these experiments, levels of endogenous tubulin cell lysates were estimated by immunoblot analysis with anti-α-tubulin antibody.

To assay tubulin polymerization, 1 mg/ml tubulin was incubated (37 °C) in binding buffer containing 10% glycerol with or without 10 μM CR. The time course of polymerization was followed as classically described (40), except that absorbance was measured at 380 nm (instead of 350 nm) to minimize the absorbance contribution from CR.

**Immunoblot Analysis**—Samples were boiled in SDS-PAGE loading buffer, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membranes as described previously (37). Protein bands were visualized by enhanced chemiluminescence and quantified with a Bio-Rad GS-800 calibrated laser densitometer.

**Cell Death Assay**—Inducible tau cells grown in the presence or absence of 10 μM CR and tetracycline inducer for up to 4 days were isolated by trypsinization, harvested by centrifugation, reuspended in PBS, dispensed in triplicate into 96-well black-walled flat bottom plates (Costar) at initial seeding densities of ≤100,000 cells/well, and incubated with ToPro-3 (5 μM for 30 min), a fluorescent DNA-intercalating dye taken up by dead but not live cells (41, 42). ToPro-3 uptake was then measured directly in a FlexStation plate reader (λ_em = 640 nm; λ_ex = 670 nm) relative to a cell-free blank reaction.

**Immunocytochemistry**—Cells were plated on poly-d-lysine-coated glass coverslips for 24 h, then grown in the presence or absence of 10 μM CR for up to 4 days. Cells were then harvested at various time points, chilled on ice for 30 min, washed three times with PBS, and finally fixed with methanol at −20 °C. Following PBS washes, cells were sequentially labeled with primary monoclonal antibodies Tau5 (1:200) and MAB1864 (1:200) and secondary antibodies Alexa 546-labeled goat anti-mouse IgG (1:400) and/or Cy5-conjugated goat anti-rat IgG (1:400) in PBS containing 3% bovine serum albumin. When present, ToPro-3 was incubated at 5 μM for 1.5 h. Coverslips were then mounted with Vectashield and visualized with a Leica TCS SL laser-scanning confocal system operated at wavelengths optimized for simultaneous detection of Alexa 546 (λ_ex = 546 nm; λ_em = 555–620 nm) and Cy5 (λ_ex = 633 nm; λ_em = 650–750 nm) or ToPro-3 (λ_ex = 640 nm; λ_em = 650–750 nm) in the presence of tau-bound CR (λ_ex = 488 nm; λ_em = 500–535 nm).

**Analytical Methods**—Aggregation progress curves were modeled as a monomolecular growth scheme assuming pseudo-first order kinetics. Filament growth was fit to the function,

$$ y = y_o + y_c(1 - e^{-k_{app}t}) $$

(Eq 1)

where y, y_o, and y_c correspond to filament length or mass at time t, at time 0, and at infinite time, respectively, and k_app is the apparent first-order rate constant. For *in vitro* reactions, y_o was always set equal to zero.

Consistent with this model, time-dependent loss of soluble tau was fit to a simple exponential decay function,

$$ m = m_o e^{-k_{app}t} $$

(Eq 2)

where m and m_o correspond to soluble tau mass at time t and 0, respectively, and k_app is the apparent first-order decay rate constant. Concentration-response curves were fit to log normal distributions as described previously (23).

All linear and non-linear regression fits are reported ± S.E. of the estimate. The probability that an observed difference in the mean of replicates was statistically significant was determined by Student’s t test.

**RESULTS**

**CR Induces Tau Fibrillization in Vitro**—Certain aromatic heterocyes including thiazin red, thiolavin S, and CR are capable of inducing tau fibrillization *in vitro* (23). In preliminary experiments, however, only CR entered HEK-293 cells over a period of days on the basis of color uptake (data not shown). Therefore, CR was used as tau fibrillization inducer in all subsequent experiments. To establish the concentration optimum for tau fibrillization *in vitro*, varying concentrations of CR were incubated (37 °C for up to 96 h) with 1 μM His6-htau40 and assayed for fibrillization using quantitative transmission electron microscopy. No filaments were formed in the absence of CR under these conditions (Fig. 1A). In contrast, the presence of CR-induced filaments that grew as twisted ribbons with maximum widths of 19 ± 4 nm (n = 50), minimum widths of 8 ± 2 nm (n = 50), and half-periodicity of 114 ± 16 nm (n = 32) (Fig. 1B). CR concentration dependence was biphasic, and the data could be fit to a log-normal concentration effect profile to yield an estimate of optimum potency at 19.9 ± 0.5 μM CR (Fig. 1D). These data suggest that CR was ~6-fold more potent than thiazin red as a tau fibrillation inducer (23), and established an optimum CR concentration range of 10–20 μM.

The time course of CR-mediated aggregation (1 μM His6-htau40) was estimated at 10 μM CR. At these reactant concentrations, fibrillation proceeded without a perceptible lag and yielded well resolved filaments for the first 48 h. After that time, however, filaments formed tangled masses that were impossible to quantify by transmission electron microscopy methods (Fig. 1C). When the first 48 h of reaction was modeled as a first-order approach to plateau using the monomolecular equation (1), k_app, the first-order rate constant for growth of the filament population was 0.28 ± 0.03 h⁻¹. Together these data demonstrate that fibrillation of micromolar concentrations of full-length tau protein can be rapidly induced by 10 μM CR under near physiological conditions.
To test the solubility of CR-induced tau filaments in vitro, aliquots from tau assembly mixtures used for the transmission electron microscopy study were centrifuged at 100,000 × g for 1 h and separated into supernatant and pellet fractions. Tau protein was then detected by immunoblot analysis using a monoclonal antibody, Tau5, that binds a continuous, non-phosphorylated epitope common to all human tau species (35). The results showed that tau gradually shifted from the soluble to the pellet fraction over time in the presence of CR (Fig. 2, A and B). The rate of loss of soluble tau was well fit by an exponential decay, consistent with the first-order aggregation model (Fig. 2C). The apparent first-order rate constant for loss of soluble tau, \( k_{\text{app}} \), was 0.10 ± 0.02 h\(^{-1}\). This value was not statistically different from the \( k_{\text{app}} \) determined from appearance of tau in the pellet fraction (0.12 ± 0.07 h\(^{-1}\)), but was significantly lower than \( k_{\text{app}} \) determined from length measurements (Fig. 1). These results indicate that sedimentation is a less sensitive assay method than electron microscopy (e.g. it fails to capture short filaments), or that length and mass share a complex relationship (e.g. because of heterogeneous nucleation occurring along the length of filaments). Overall, however, sedimentation was an adequate method for estimating tau aggregation kinetics in the presence of CR and one that was applicable to cellular models.

Congo Red Induces Tau Aggregation in Cell Culture—To determine whether CR could induce tau aggregation in cell culture, HEK-293 cells stably overexpressing the longest human tau isoform (stable tau cells) were incubated with varying concentrations of CR for 7 days. HEK-293 cells were chosen for this experiment because they tolerate high-level tau overexpression (5.3 ± 0.6 pg/cell, \( n = 4 \) determinations, data not shown) without serious toxicity (Ref. 37 and see below). Cell lysates were then prepared in nonionic detergent Nonidet P-40, separated into soluble and insoluble fractions by ultracentrifugation (1 h at 100,000 × g), and subjected to immunoblot analysis using anti-tau and anti-α-tubulin monoclonal antibodies. Insolubility in both ionic and nonionic detergents is a characteristic of authentic AD-derived tau filaments (43, 44). Results showed that Tau5 immunoreactivity appearing in the pellet fraction had a biphasic dependence on CR concentration (Fig. 3A).
Insoluble tau was not a result of cosedimentation with microtubules, because lysates were prepared under microtubule depolymerizing conditions where most tubulin remained in the soluble fraction (Fig. 3B). The optimum CR concentration for inducing insoluble cellular tau was similar to the optimum determined in vitro (Fig. 3C).

To characterize the time course of tau aggregation, tau cells were treated with 10 μM CR for up to 14 days, then subjected to centrifugal fractionation and immunoblot analysis as described above. Results with monoclonal antibody Tau5 showed that insoluble tau accumulated and approached plateau after 7 days of treatment (Fig. 4A). Microtubule-associated tau did not contribute to the insoluble pool, because assays were conducted under conditions that fostered microtubule disassembly (Fig. 4C). When modeled as a first-order aggregation reaction, the rate constant for growth of the insoluble tau pool, $k_{app}$, was
The appearance of insoluble tau was accompanied by an overall increase in total tau levels, with accumulation of insoluble tau accounting for much of the increase (Fig. 4D). After 7 days treatment, >30% of total tau in the cell was insoluble (Fig. 4E), with the model predicting maximal aggregation asymptotically approaching 38 ± 6% of total tau. These results suggest that CR was capable of driving robust aggregation in this cellular model, and that the increase in total tau levels observed in the presence of CR was related to the degree of intracellular aggregation.

Tau dissociates from microtubules in response to phosphorylation (45, 46). Very high level phosphorylation can drive tau aggregation in vitro (47) and in cell culture (27). To determine whether CR promoted tau hyperphosphorylation, lysate fractions were probed with monoclonal antibody PHF1, which binds tau phosphorylated at Ser396/Ser404 (32). These sites were analyzed because their occupancy modulates the microtubule assembly promoting activity of tau (48) and increases with neuritic lesion maturation (49, 50). In addition, Ser396/Ser404 are substrates for protein kinases thought to contribute to tau hyperphosphorylation including GSK3 (51), Cdk5 (52), and CK1 (37). Results showed that basal levels of PHF1 immunoreactivity in the soluble fraction did not change significantly over 14 days of CR treatment (10 μM CR; Fig. 4B). PHF1 immunoreactivity did increase in the particulate fraction because of tau aggregation, although not in complete unison with Tau5 immunoreactivity (Fig. 4B). These data suggest that CR treatment promoted tau aggregation but not hyperphosphorylation of tau at sites Ser396/Ser404.

To characterize the cellular distribution of tau aggregates, wild-type HEK-293 cells or stable tau cells grown in the presence or absence of 10 μM CR for up to 7 days were subjected to confocal immunocytochemistry using monoclonal antibodies against tau (Tau5) and tubulin (MAB1864). MAB1864 was used in place of DM-1A because its rat isotype facilitated double labeling in the presence of mouse monoclonal IgGs such as Tau5. Immunoreactivity was detected with secondary antibodies linked to fluros Alexa 564 and Cy5 so as to minimize overlap with CR autofluorescence. In the absence of tau expression (HEK-293 cells), tubulin staining predominated at the cell periphery (Fig. 5A). CR treatment of these cells did not alter their microtubule staining pattern, and free intracellular CR did not autofluoresce at visible excitation/emission wavelengths (shown for λex = 488; λem = 500–535 in Fig. 5B). In cells

FIGURE 5. CR-treated tau cells generate intracellular inclusions. Wild-type HEK-293 and tau stable cells were treated with 10 μM CR, fixed with methanol, coimmunostained with primary monoclonal antibodies Tau5 and MAB1864 (anti-tau and anti-tubulin respectively) and secondary Alexa 564- and Cy5-linked secondary antibodies, and sequentially visualized at different excitation and emission wavelengths by confocal microscopy. In wild-type HEK-293 cells grown in the absence (A) or presence (B) of CR, tau was not detectable, whereas tubulin localized primarily at the cell periphery. CR fluorescence was not detectable under these conditions. C, in tau stable cells grown in the absence of CR, tau immunostaining colocalized with tubulin at the cell periphery. D, in contrast, tau stable cells grown in the presence of CR showed a gradual shift in tau distribution away from the cell periphery toward cytoplasmic inclusions (shown for 7 day CR treatment; arrow). The inclusions, which contained CR fluorescence but did not label with anti-tubulin antibody MAB1864, were never seen in HEK-293 cells lacking tau protein regardless of whether CR was absent (A) or present (B) for up to 7 days. Scale bar is for all panels.

FIGURE 6. Cellular CR-induced tau aggregates consist of filaments. Tau aggregates were isolated from CR-treated tau stable cells as described under “Experimental Procedures” and visualized by immunoelectron microscopy using monoclonal antibody Tau12 and gold-labeled secondary antibody. Cellular CR-induced aggregates appeared as clumps of filaments. Scale bar is for all three panels.
expressing htau40 (tau stable cells) in the absence of CR, Tau5 immunostaining colocalized with tubulin (Fig. 5C). After a period of days in the presence of 10 μM CR, however, tau-positive inclusions appeared in the cytoplasm where they colocalized with CR autofluorescence (maximal at λex = 488 under these conditions) but not tubulin (Fig. 5D).

To characterize the ultrastructure of these inclusions, the Sarkosyl-insoluble fraction of lysates prepared from tau cells treated for 7 days with 10 μM CR were subjected to immunogold labeling with anti-tau antibody Tau12 and viewed in an electron microscope. Both isolated and tangled masses of Tau12 positive filaments were observed (Fig. 6). The morphology of the latter was similar to in vitro aggregates incubated for 96 h (Fig. 1C). Together these results suggest that CR is capable of driving tau fibrillation in cells as well as in vitro, and remain associated with the resultant aggregates.

**Tau Aggregation Reduces Its Tubulin-binding Activity**—To determine the effects of CR-induced tau fibrillization on tau function, the ability of His6-htau40 treated with CR in vitro for up to 24 h to bind purified tubulin was examined by immunoprecipitation using α-tubulin antibody DM-1A. In the absence of CR, tau co-immunoprecipitated with tubulin (Fig. 7A). In contrast, the presence of CR led to time-dependent losses in levels of tubulin-bound tau (Fig. 7A), with the initial rate of loss of tubulin-binding activity being faster that the rate of loss of tau from the soluble fraction (Fig. 7B). Loss of tau-binding activity was not the result of direct CR-mediated inhibition of tubulin polymerization, because CR at 10 μM was incapable of antagonizing spontaneous tubulin polymerization in the absence of tau (Fig. 7C). These data indicated that it was the products of CR/tau interaction that were incompatible with tubulin binding.

To confirm these findings, lysates prepared from tau cells grown in the presence or absence of CR for 7 days were subjected to tubulin immunoprecipitation with anti-α-tubulin antibody DM-1A followed by immunoblot analysis with anti-tau antibody Tau5. Over this time period, the presence of CR yielded a doubling of total tau levels relative to cells incubated in its absence (Figs. 4 and 8). Nonetheless, the amount of endogenous tau that immunoprecipitated with endogenous tubulin decreased over 30% (Fig. 8). Together these data suggest that the change in tau conformation or aggregation state induced by CR diminished tubulin binding affinity relative to tau monomer in random coil conformation.

**Tau Aggregation Decreases Cell Viability**—To determine the effects of tau aggregation on cell viability, tetracycline-inducible tau cells were treated with or without 10 μM CR for up to 4 days and then incubated in the presence of ToPro-3, a nuclear stain (38). Inducible cells were used so that the tau dependence expressed as percent control (time 0), whereas the solid line is drawn to aid visualization. For comparison, the amount of soluble tau present at each time point (from Fig. 1) also is shown, with the solid line corresponding to the best fit to an exponential decay (Equation 2). Tubulin binding activity declined rapidly relative to loss of soluble His6-htau40. C, tubulin (1 mg/ml in binding buffer containing 10% glycerol) incubated in the absence (○) or presence (●) of 10 μM CR was assayed for polymerization by absorbance at 380 nm. Each point represents an absorbance measurement at the indicated time normalized to the control reaction (no CR) plateau absorbance at 1 h. Under these conditions, CR did not directly interfere with tubulin polymerization.
copy. In the absence of CR, ToPro-3 selectively labeled cell nuclei and did not colocalize with cytoplasmic tau immunofluorescence (Fig. 9A). In the presence of CR, CR-positive tau inclusions formed, but ToPro-3 staining remained exclusively nuclear (Fig. 9B). These data suggest that the ToPro-3 fluorescence remains dependent on access to nuclei and is not influenced by the presence of CR or tau aggregates.

To assess the effect of CR-mediated tau aggregation on cell viability, inducible cells were treated with and without tetracycline inducer of tau expression in the presence and absence of CR. ToPro-3 fluorescence intensity was linearly related to the number of cells assayed under all conditions (Fig. 10A). However, the relationship between cell number and fluorescence intensity (i.e. the slope) depended on the cell population investigated. Compared with non-tau expressing cells grown in the absence of CR, the presence of either CR or the induction of tau expression in the absence of CR led to a modest 1.6 ± 0.1-fold increase in ToPro-3 fluorescence intensity (p < 0.01; Fig. 10, A and B), suggesting weak toxicity associated with these experimental conditions relative to untreated and uninduced controls. In contrast, tau overexpression in the presence of CR led to a 5.9 ± 0.2-fold increase in ToPro-3 fluorescence intensity compared with non-tau expressing cells grown in the absence of CR (p < 0.001; Fig. 10, A and B). These levels were significantly greater than those for either non-induced cells grown in the presence of CR or induced cells grown in the absence of CR (p < 0.001), and also greater than the effects of CR treatment and tau overexpression combined. Together these data suggest that changes in tau conformation and aggregation state produce a tau-dependent decrease in cell viability.

DISCUSSION

These results suggest that small-molecule agonists of tau fibrillization such as CR can be leveraged to create novel cellular models of tauopathy. The approach has several advantages. First, CR is well tolerated in many cell types, with concentrations as high as 100 μM having limited toxicity over short periods of time (54, 55). It may be possible to minimize nonspecific toxicity over long treatment periods by replacing CR with other cell-permeable small-molecule agonists. Second, the approach facilitates study of full-length tau isoforms without resorting to aggregation-prone truncation (56) or tauopathy mutants (38, 57). Full-length tau is central to AD progression because truncation, whereas appearing early in disease, occurs well after filament deposition is underway (58). Third, aggregation proceeds without the need for tau hyperphosphorylation, and so avoids nonspecific changes in phosphorylation state of cellular proteins that can accompany addition of phosphatase inhibitors (e.g. okadaic acid, (59)) or overexpression of specific protein kinases (27). Finally, agonists such as CR drive the highest levels of aggregation yet achieved in a cellular model, with >30% of cellular tau entering the detergent-insoluble particulate fraction within 7 days. The kinetic treatment used here predicts that the maximal amounts of detergent-insoluble tau at the reaction plateau would attain nearly 40% of total cellular tau. This far exceeds levels achieved using tauopathy mutants (38, 57) and more closely models the high levels of aggregated tau seen in AD neurons (3). CR-mediated increases in total tau
levels correlated with appearance of detergent-insoluble tau, suggesting that the observation results from sequestration of tau rather than increases in tau expression. This mechanism also may drive increased tau levels in disease, because tau expression does not change in AD (5, 6). The shift in tau levels to the new steady state may be an example of the plateau principle (60), which describes the kinetics of accumulations that are cleared by first-order processes (e.g., protein turnover (61)). Assuming that the rate of tau biosynthesis is constant, and that the rate of CR uptake is fast relative to aggregation, then the rate of approach to the new steady state should approximate the turnover rate of the aggregated species. The plateau principle together with the rate data in Fig. 4 predict that tau aggregates have a half-life of ~115 h in HEK-293 cells, which would be ~1–2 orders of magnitude longer than the half-life for non-aggregated tau (62, 63).

In addition to these strengths, the aggregation agonist model also has several limitations. First, CR-induced tau filaments differ from authentic tissue-derived PHFs with respect to morphology. Despite similarities in overall dimensions, CR-induced filaments are more flexible and have a longer periodicity of twist than authentic PHF. Nonetheless, filament morphology varies in different tauopathies (e.g., Refs. 64 and 65), and the significance of morphology for disease progression is poorly understood. Second, the current model employs tau concentrations of ~5.6 pg/cell. Assuming a typical HEK-293 cell volume of ~4 pl (66, 67), these levels correspond to intracellular tau concentrations (~25 µM) well above estimates of physiological tau concentrations in cells (68) and also adult brain homogenates (3). In either case, however, the amount of free tau is unknown. The ability of agonist inducers to support tau aggregation as low as 200–300 nM htau40 (23) suggests that it may be possible to induce tau aggregation at more physiological bulk tau concentrations in the future. Third, CR treatment does not lead to tau hyperphosphorylation as judged by occupancy of sites Ser396/Ser404 and so may not capture potential toxicity of phosphoaggregates. For example, high level accumulation of phospho-tau aggregates have been suggested to sequester PIN1 as part of their mechanism of toxicity (69). Still, there are many sites beyond Ser396/Ser404 that are at least partially occupied in cells and these may change in the presence of CR. Finally, CR appears to drive heterogeneous nucleation of assembly competent species, whereas the association of PHFs with cytomembranes (70) in AD tissue is more consistent with a heterogeneous nucleation mechanism (71). Experience with β-amyloid peptide suggests that it is the location of active fibrillation, rather than accumulation of filaments per se, that influences toxicity (72). If so, then site of nucleation may have crucial effects on cell phenotype. Heterogeneous interactions may contribute to toxicity in part by controlling the location of nucleation and extension reactions, and homogeneous nucleation paradigms may miss this aspect of toxicity.

With these limitations in mind, the results found here in HEK-293 cells are significant in two respects. First, they indicate that changes in full-length tau conformation and aggregation can decrease cell viability. The toxicity is not acute, but develops over a period of days and is statistically significant by 4 days in culture. The results are consistent with the long-term toxicity associated with the tau conformational change and aggregation in some transgenic mouse models (28). Most importantly, toxicity did not depend on direct modulation of signal transduction pathways, suggesting that tau aggregation/conformational change can influence cell viability. It will be important to extend these findings to neurons. In AD, >90% of cortical tau aggregation localizes to neuropil threads and dystrophic neurites associated with neuritic plaques (73). These aggregates may affect end points other than viability that are nonetheless important for disease progression, including retraction and loss of dendritic arbors (74) and synaptic contacts (75). For example, intracellular aggregates formed from polyglutamine repeats can inhibit processes especially important for neuronal homeostasis such as axoplasmic flow (76). The small-molecule agonist method of inducing fibrillation described here should be portable to neuronal cultures for examining these aspects of toxicity.

Second, the results indicate that the initial rate of loss of tubulin binding affinity exceeds the rate of disappearance of soluble tau in the presence of CR. This suggests that small detergent-soluble tau aggregates formed early in the reaction pathway, or perhaps the conformation of tau monomer stabilized by the presence of CR has a lower affinity for tubulin binding than random coil tau monomer irrespective of phosphorylation state. Although it has been proposed that tau fibrillation begins in association with microtubules (77), the observed incompatibility between tau aggregation and microtubule binding suggests that microtubule dissociation precedes aggregation. This...
A Cellular Model of Tau Aggregation

Acknowledgments—We thank Kelly Threm and Lauren Crissman for technical assistance, and Dr. Mike Zhu, OSU Center for Molecular Neurobiology, for generous access to the fluorescence plate reader.

REFERENCES

1. Buee, L., Bussiere, T., Buee-Scherrer, V., Delacourte, A., and Hof, P. R. (2000) Brain Res. Brain Res. Rev. 33, 95–130
2. Margittai, M., and Langen, R. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10278–10283
3. Khatoon, S., Grundke-Iqbal, I., and Iqbal, K. (1992) J. Neurochem. 59, 750–753
4. Khatoon, S., Grundke-Iqbal, I., and Iqbal, K. (1994) FEBS Lett. 351, 80–84
5. Hyman, B. T., Augustinack, J. C., and Ingelsson, M. (2005) Biochim. Biophys. Acta 1739, 150–157
6. Yasojima, K., McGeer, E. G., and McGeer, P. L. (1999) Brain Res. 831, 301–305
7. Keck, S., Nitsch, R., Grune, T., and Ullrich, O. (2003) J. Neurochem. 85, 115–122
8. Arriagada, P. V., Growdon, J. H., Hedley-Whyte, E. T., and Hyman, B. T. (1999) Neurology 42, 631–639
9. Ghoshal, N., Garcia-Sierra, F., Wuu, J., Leurgans, S., Bennett, D. A., Berry, R. W., and Binder, L. I. (2001) Exp. Neurol. 177, 475–493
10. Braak, H., and Braak, E. (1991) Acta Neuropathol. (Berl.) 82, 239–259
11. Hutton, M., Lendon, C. L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., Petersen, R. C., Stevens, M., de Graaff, E., Wauters, E., van Baren, J., Hillebrand, M., Joosse, M., Kwon, J. M., Nowotny, P., Che, L. K., Norton, J., Morris, J. C., Reed, L. A., Trojanowski, J., Basun, H., Lannfelt, L., Neyst, M., Fahn, S., Dark, F., Tannenberg, T., Dodd, P. R., Hayward, N., Kwok, J. B., Schofield, P. R., Andreás, A., Snowden, J., Craufurd, D., Neary, D., Owen, F., Ostrra, B. A., Hardy, J., Goate, A., van Swieten, J., Mann, D., Lynch, T., and Heutink, P. (1998) Nature 393, 702–705
12. Poorkaj, P., Bird, T. D., Wyssen, E., Nemes, E., Garruto, R. M., Anderson, L., Andreás, A., Wiedeholt, W. C., Raskind, M., and Schellenberg, G. D. (1998) Ann. Neurol. 43, 815–825
13. Spillantini, M. G., Murrell, J. R., Goedert, M., Farlow, M. R., Klug, A., and Jakes, R. (1998) FEBS Lett. 427, 179–183
14. Bennett, E. J., Hutton, M., and Feany, M. B. (2001) Science 292, 1552–1555
15. Bennett, E. J., Bence, N. F., Jayakumar, R., and Kopito, R. R. (2005) Mol. Cell 17, 351–365
16. Alonso, A. C., Grundke-Iqbal, I., and Iqbal, K. (1996) Nat. Med. 2, 783–787
17. Harada, A., Oguchi, K., Okabe, S., Kuno, J., Terada, S., Ohshima, T., Sato-Yoshitake, R., Tatei, Y., Noda, T., and Hirokawa, N. (1994) Nature 369, 488–491
18. Iegami, S., Harada, A., and Hirokawa, N. (2000) Neurosci. Lett. 279, 129–132
19. Tint, I., Slaughter, T., Fischer, I., and Black, M. M. (1998) J. Neurosci. 18, 8660–8673
20. DiTella, M. C., Feiguin, F., Carri, N., Kosik, K. S., and Caceres, A. (1996) J. Cell Sci. 109, 467–477
21. Park, S. Y., and Ferreira, A. (2005) J. Neurosci. 25, 5365–5375
22. Shimura, H., Schwartz, D., Gygi, S. P., and Kosik, K. S. (2004) J. Biol. Chem. 279, 4869–4876
23. Chirita, C. N., Congdon, E. E., Yin, H., and Kuret, J. (2005) Biochemistry 44, 5862–5872
24. Andorfer, C., Acker, C. M., Kress, Y., Hof, P. R., Duff, K., and Davies, P. (2005) J. Neurosci. 25, 5446–5454
25. Wittmann, C. W., Wszelek, M. F., Shulman, J. M., Salvaterra, P. M., Lewis, J., Hutton, M., and Feany, M. B. (2001) Science 293, 711–714
26. Santacruz, K., Lewis, J., Spires, T., Paulson, J., Kotilinek, L., Ingelsson, M., Guimaureses, A., DeTure, M., Ramsden, M., McGowan, E., Forster, C., Yue, M., Orne, J., Janus, C., Mariash, A., Kuskowski, M., Hymann, B., Hutton, M., and Ashe, K. H. (2005) Science 309, 476–481
27. Sato, S., Tatebayashi, Y., Akagi, T., Chui, D. H., Murayama, M., Miyasaka,
A Cellular Model of Tau Aggregation

T. Planeel, E. Tanemura, K., Sun, X., Hashikawa, T., Yoshioka, K., Ishiguro, K., and Takashima, A. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15773–15777

28. Terwel, D., Lasrado, R., Snaauwaert, J., Vandeweert, E., Van Haesendonck, C., Borghgraef, P., and Van Leuven, F. (2005) J. Neurosci. Res. 83, 117–127

29. Kim, Y. S., Randolph, T. W., Manning, M. C., Stevens, F. J., and Carpenter, J. F. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5950–5955

30. Wyman, I., and Gill, S. J. (1990) Binding and Linkage: Functional Chemistry of Biological Macromolecules, University Science Books, Mill Valley, CA

31. LoPresti, P., Szuchet, S., Papasozomenos, S. C., Zinkowski, R. P., and Binder, L. I. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10369–10373

32. Otvos, L., Jr., Feiner, L., Lang, E., Szendrei, G. I., Goedert, M., and Lee, V. M. (1994) J. Neurosci. Res. 39, 669–673

33. Kálmán, J. V., Wright, B., and Milstein, C. (1982) J. Cell Biol. 93, 576–582

34. Blose, S. H., Meltzer, D. I., and Feramisco, J. R. (1984) J. Biol. Chem. 259, 24977–24983

35. Carmel, G., Mager, E. M., Binder, L. I., and Kuret, J. (1996) J. Biol. Chem. 271, 32789–32795

36. Necula, M., and Kuret, J. (2004) Neurotox. Res. 17, 177–181

37. Reynold, C. H., Betts, J. C., Blackstock, W. P., Nebreda, A. R., and Anderson, B. H. (2000) J. Neurochem. 74, 1587–1595

38. Lund, E. T., McKenna, R., Evans, D. B., Sharma, S. K., and Mathews, W. R. (2001) J. Neurochem. 76, 1221–1232

39. Greenberg, S. G., and Davies, P. (1990) J. Neurosci. Res. 25, 576–582

40. Mitchison, T., and Kirschner, M. (1984) J. Cell Biol. 98, 847–858

41. Lee-MacAry, A. E., Ross, E. L., Davies, D., Laylor, R., Honeychurch, J., Glennie, M. J., Snary, D., and Wilkinson, R. W. (2001) J. Immunol. Methods 252, 258–35

42. Wilkinson, R. W., Lee-MacAry, A. E., Davies, D., Snary, D., and Ross, E. L. (2001) J. Immunol. Methods 258, 183–191

43. Lee, V. M., Wang, J., and Trojanowski, J. Q. (1999) Methods Enzymol. 309, 81–89

44. Sergeant, N., Wattez, A., Galvan-Velencia, M., Ghestem, A., David, J. P., Lemoine, J., Sautiere, P. E., Dachary, J., Mazat, J. P., Michalski, J. C., Veilours, J., Mena-Lopez, R., and Delacourte, A. (2003) Neuroscience 117, 293–303

45. Biernat, J., Gisburke, G., Drewes, G., Mandelkow, E. M., and Mandelkow, E. (1993) Nature 365, 153–163

46. Bramblette, G. T., Goedert, M., Jakes, R., Merrick, S. E., Trojanowski, J. Q., and Lee, V. M. (1993) Nature 400, 1089–1099

47. Alonso, A., Zaidi, T., Novak, M., Grundke-Iqbal, I., and Iqbal, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6923–6928

48. Evans, D. B., Rank, K. B., Bhattacharya, K., Thomsen, D. R., Guney, M., and Sharma, S. K. (2000) J. Biol. Chem. 275, 24977–24983

49. Augustinack, J. C., Schneider, A., Mandelkow, E. M., and Hyman, B. T. (2002) Acta Neuropathol. (Berl.) 103, 26–35

50. Kimura, T., Ono, T., Takamatsu, J., Yamamoto, H., Ikegami, K., Kondo, A., Iwakawa, M., Hara, Y., Miyamoto, E., and Miyakawa, T. (1996) Dementia 7, 177–181

51. Reynolds, C. H., Betts, J. C., Blackstock, W. P., Nebreda, A. R., and Anderson, B. H. (2000) J. Neurochem. 74, 1587–1595

52. Lund, E. T., McKenna, R., Evans, D. B., Sharma, S. K., and Mathews, W. R. (2001) J. Neurochem. 76, 1221–1232

53. Chirita, C. N., Necula, M., and Kuret, J. (2004) Biochemistry 43, 2879–2887

54. Apostol, B. L., Kazantzsev, A., Raffioni, S., Illes, K., Pallos, J., Bodai, I., Slepko, N., Bear, J. E., Gertler, F. B., Hersch, S., Housman, D. E., Marsh, J. L., and Thompson, L. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5950–5955

55. Bao, Y. P., Sarkar, S., Uyama, E., and Rubinsztein, D. C. (2004) J. Med. Genet. 41, 47–51

56. Chambard, J. M., and Ashmore, J. F. (2003) Methods Enzymol. 367, 667–677

57. Dhruvin, D. G., Feinstein, S. C., Shooter, E. M., and Kirschner, M. W. (1985) J. Cell Biol. 101, 1799–1807

58. Gray, E. G., Paula-Barbosa, M., and Roher, A. (1987) Neuropathol. Appl. Neurol. 13, 91–110

59. Kuret, J., Congdon, E. E., Li, G., Yin, H., Xu, X., and Zhong, Q. (2005) Microsc. Res. Tech. 67, 141–155

60. Wogulis, M., Wright, S., Cunningham, D., Chilcote, T., Powell, K., and Rydel, R. E. (2005) J. Neurosci. 25, 1071–1080

61. Mitchell, T. W., Nissanan, J., Han, L. Y., Mufson, E. J., Schneider, J. A., Cochran, E. J., Bennett, D. A., Lee, V. M., Trojanowski, J. Q., and Arnold, S. E. (2000) J. Histoch. Cytochem. 48, 1627–1638

62. Falke, E., Nissanan, J., Mitchell, T. W., Bennet, D. A., Trojanowski, J. Q., and Arnold, S. E. (2003) Am. J. Pathol. 163, 1615–1621

63. Hama, J. S., and Drachman, D. A. (1989) Neurology 39, 64–70

64. Morfini, G., Pigino, G., and Brady, S. T. (2005) Trends Mol. Med. 11, 47–51

65. Achmann, M., Wiech, H., and Mandelkow, E. (2000) J. Biol. Chem. 275, 30335–30343