β-Amyloid Induces Paired Helical Filament-like Tau Filaments in Tissue Culture*

Alessandra Ferrarị‡§, Frederic Hoernlị, Thomas Baechị§, Roger M. Nitsch‡, and Jürgen Götz‡∥

From the ‡Division of Psychiatry Research, University of Zürich, August Förel Strasse 1, 8008 Zürich, and the ∥Central Laboratory for Electron Microscopy, University of Zürich, Gloristrasse 30, 8028 Zurich, Switzerland

† Present address: Medical Research Council Laboratory for Molecu-lar Cell Biology, University College of London, Gower St., London WC1E 6BT, United Kingdom.

‡ To whom correspondence should be addressed: Division of Psychiatry Research, University of Zürich, August Förel Str 1, 8008 Zürich, Switzerland. Tel: 41-1-634-8873; Fax: 41-1-634-8874; E-mail: goetz@bli.unizh.ch.

§ The abbreviations used are: NFT, neurofibrillary tangles; AD, Alzhe-imer’s disease; PHF, paired helical filaments; PBS, phosphate-buff-ered saline; FA, formic acid; IC, immunocytochemistry; ELISA, enzyme-linked immunosorbent assay.

Paired helical filaments (PHF) are the principal pathologic components of neurofibrillary tangles in Alzheimer’s disease (AD). To reproduce the formation of PHF in tissue culture, we stably expressed human tau with and without pathogenic mutations in human SH-SY5Y cells and exposed them for 5 days to aggregated synthetic β-amyloid peptide (Aβ42). This caused a decreased solubility of tau along with the generation of PHF-like tau-containing filaments. These were 20 nm wide and had periodicities of 130–140 nm in the presence of P301L mutant tau or 150–160 nm in the presence of wild-type tau. Mutagenesis of the phosphoepitope serine 422 of tau prevented both the Aβ42-mediated decrease in solubility and the generation of PHF-like filaments, suggesting a role of serine 422 in the phosphor-ylation in tau filament formation. Together, our data underscore a role of Aβ42 in the formation of PHF-like filaments. Our culture system will be useful to map phosphoepitopes of tau involved in PHF formation and to identify and characterize modifiers of the tau pathol-ogy. Further adaptation of the system may allow the screening and validation of compounds designed to pre-vent PHF formation.

Neurofibrillary tangles (NFT) are abundant in many neu-rodegenerative diseases, including Alzheimer’s disease (AD) and frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) caused by mutations in the tau gene (1–3). NFT are composed of filamentous aggregates that include paired helical filaments (PHF) and narrow twisted ribbons. These are made of the microtuble-associated protein tau (4, 5). Tau in these filaments is hyperphosphorylated both at physiological sites and at additional, pathological sites. Phosphorylation of tau is associated with the dissociation of tau from microtubules and with its relocalization from axons to cell bodies and dendrites. Pathologic increases in this pool of soluble tau, along with conformational changes of the natively unfolded tau, are initial critical steps in the assembly of the more insoluble pathologic tau filaments.

Transgenic mice expressing pathogenic mutations of human tau generate a few low abundant NFT (6, 7), and both intracerebral microinjections of β-amyloid into P301L mutant tau transgenic mice and co-expression of mutant amyloid precursor protein greatly accelerate the rate of NFT formation in mice (8, 9). These data established a mechanistic relationship of β-amyloid toxicity with NFT formation.

To circumvent the inherent limitations of the above in vivo experiments (including poor breeding of doubly transgenic mice as well as stereotactic techniques) and to establish PHF forma-tion in tissue culture, we expressed both wild-type and mutant forms of human tau in human SH-SY5Y cells and exposed them to preparations of aggregated synthetic β-amyloid. We were able to produce bona fide PHF-like tau filaments within 5 days of tissue culture.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The longest human 4-repeat tau isoform, htau40, was cloned into the pBluescript expression vector (InVitrogen). Site-directed mutagenesis (QuikChange kit; Stratagene) was performed to generate the tau mutant P301L and the double mutants, P301L/S422E and P301L/S422A. The sequences of the mutagenized oligonucleotides were as follows: P301L, 5′-GGTTTGTAGACTATTGCA-CAC-3′; P301L/S422E, 5′-GGTTTGTAGACTATTGCAC-3′; P301L/S422A, 5′-GGTTTGTAGACTATTGCAA-3′. All constructs were sequenced to confirm the absence of randomly introduced mutations.

Cell Culture—Human SH-SY5Y neuroblastomas were transfected with either wild-type or mutant htau40 cDNA constructs using LipofectAMINE 2000 (InVitrogen) and selected with 125 μg/ml G418 for 30 days. Pools of selected clones were seeded at a density of 0.3–2.0×10⁴ cells/cm², depending on the type of experiment (low density culture for immunocytochemistry and high density culture for electron micros-copy and Western blot analysis). To normalize for cell numbers, undifferen-tiated cells were seeded at a density which, after Aβ42 incubation for 5 days, was below 50% and thus comparable with numbers of differentiated cells at the time of Aβ42 treatment. To induce neuronal differentiation, cells were seeded on collagen type 1-coated dishes and treated with 20 μM all-trans-retinoic acid (Sigma) for 5 days in standard culture medium and cultivated for an additional 5 days in serum-free medium complemented with 50 ng/ml brain-derived neurotrophic factor (Peprotech, Lucerne, Switzerland) (10).

Neuronal properties were confirmed by the presence of neuronal markers and the absence of glial markers (data not shown). Moreover, the cells expressed synaptophysin when cultured on murine organotypic hippocampal slices. Two independent lots of Aβ42 (Nos. 524548 and 535120) and Aβ42-1 (Lot No. 536763) were synthesized by Bachem (Bubendorf, Switzerland), reconstituted in PBS at calculated concen-trations of 220 μM, shaken at 1000 rpm for 24 h at 37 °C, and analyzed by electron microscopy. This preparation contained β-amyloid fibrils

This paper is available on line at http://www.jbc.org
along with protofibrils and SDS-stable oligomers (11). Suspensions of \( \Delta \beta_{42} \) preparations corresponding to 10 \( \mu \)g soluble peptide were added to each culture for 1–5 days for immunohistochemistry or 2–5 days for sarkosyl extractions and electron microscopy. Once \( \Delta \beta_{42} \) preparations had been added to the cells, the medium was not changed. This, in addition to the stiffness of \( \Delta \beta_{42} \), may explain why \( \Delta \beta_{42} \) formed large clumps on the cells as visualized by immunocytochemistry and electron microscopy. Lactate dehydrogenase assays (Sigma) were performed to measure the toxicity of \( \Delta \beta_{42} \).

**Immunofluorescence Analysis and Antibodies**—For immunocytochemistry, cells were grown on collagen-coated coverslips and fixed with 4% paraformaldehyde in microtubule-stabilizing buffer at room temperature for 30 min, followed by permeabilization with 0.3% Triton X-100. The primary antibodies were anti-human immunofunctional protein (IC), Western blotting (WB), and electron microscopy (EM): HT7 (Innotech Corp., San Leandro, CA). Two independent experiments were performed and 200 cells were counted for each experimental condition.

**Western Blot Analysis, \( \Delta \beta \)-ELISA, and Quantitative RT-PCR**—Tau-expressing cells were grown on 10-cm dishes and extracted in RIPA buffer as described previously (14). Proteins were separated on a 10% NuPAGE gel (Innvitrogen), blotted onto a nitrocellulose membrane, and probed with HT7 and a \( \beta \)-actin-specific antibody (Abcam, Cambridge, UK: 1:5000). The levels and solubility of tau protein were determined by extracting \( \Delta \beta \)-treated and control cells using buffers with increasing ionic strengths. Cells were homogenized in high-salt RAB buffer (RAB) to generate the RAB-insoluble fractions. Following centrifugation, the pellets were homogenized in RIPA buffer and centrifuged. The RIPA-insoluble pellets were extracted with 70% formic acid (FA) to give the FA fractions. Centrifugation was done at 50,000 \( \times \) g for 20 min. Protein concentrations of the samples from each fraction were adjusted to equal levels and the same amounts of protein run on a 10% NuPAGE gel and blotted as described above. As loading control and to measure the toxicity of \( \Delta \beta_{42} \), TRITC-conjugated 4G8 (Signet Laboratories, Dedalre, NC; IC, 1:500; WB, 1:200) and Tau-5A6 (Developmental Studies Hybridoma Bank; EM, 1:50) were used to specifically detect human tau. Antiseria Ser224P (988; Dr. Andre Delacourte; IC, 1:1000) (15), and S422 (Biosource Inc., Nivelles, Belgium; IC, 1:1000) were used to detect tau phosphorylated at Ser224. Antibodies for neuronal markers included NF200 (Sigma; HL, 1:400), NSE (Dako, Glostrup, Denmark; IC, 1:200), and MAP2 (Chemicon, Temecula, CA; IC, 1:400), and as a glial marker GFAP (Sigma; IC, 1:100) was used. Monoclonal antibody 4G8 (Signet Laboratories, Dedalre, MA; IC, 1:500; EM, 1:50) was used to detect \( \Delta \beta \) amyloid peptide. Secondary antibodies for immunofluorescence were obtained from Jackson Laboratories (West Grove, PA). For a quantification, two independent experiments were performed and 200 cells were counted for each experimental condition.

**RESULTS**

**A\( \beta_{42} \) Decreases the Solubility of Tau**—To assess the role of \( \Delta \beta_{42} \) in tau aggregation and filament formation in tissue culture, we added preparations of aggregated synthetic \( \Delta \beta_{42} \) to the media of SH-SYSY cells that had been stably transfected with expression constructs encoding the longest 4-repeat isoform of human tau, with or without the pathogenic FTDP-17 mutation P301L (Fig. 1A). Confoocal analysis confirmed the presence of \( \Delta \beta_{42} \) aggregates deposited on both cell bodies and neurites (Fig. 1B). Neither the size nor the number of these aggregates varied within 1 and 5 days of incubation. Electron-microscopic analyses demonstrated that \( \Delta \beta_{42} \) aggregates contained the expected amorphous fibrous material that was similar to that extracted from brain \( \beta \)-amyloid plaques (Fig. 1, C–E). In this assay system the initial 5 days of treatment with \( \Delta \beta_{42} \) were not accompanied by any obvious increases in cell death, and lactate dehydrogenase (LDH) assays did not reveal increased LDH release, as compared with vehicle controls (data not shown).

Western blots confirmed that cellular levels of human tau were similar in all transfected conditions, both in undifferentiated cells (data not shown) and cells that were sequentially differentiated with retinoic acid and brain-derived neurotrophic factor (Fig. 1A). To determine the role of \( \Delta \beta_{42} \) in the aggregation of tau, we used undifferentiated cells that expressed wild-type human tau. We extracted cellular proteins in high-salt RAB buffer to generate the RAB fraction. We then homogenized the RAB-insoluble pellet in RIPA buffer to obtain RIPA-soluble proteins and extracted the RAB-insoluble pellet with FA to obtain the FA-soluble protein fraction (Fig. 2). Equal amounts of total protein of the treated and untreated fractions were loaded, but the FA fractions contained less protein than the RAB and RIPA fractions. The signals in the three fractions could not be added up to determine the total amount of tau, because tau had been sequentially extracted. After 1 day of \( \Delta \beta_{42} \) treatment, most tau was present in the RAB fraction and only a small fraction was RAB-insoluble and found in the RIPA fraction, as compared with none in the RIPA fraction of the vehicle-treated cells (Fig. 2A). During 5 days of incubation with \( \Delta \beta_{42} \), the solubility of tau decreased significantly because a substantial amount of it appeared in the RIPA and FA fractions. In contrast, in untreated
control cells, tau was absent from the FA fraction after 5 days of incubation with the vehicle (Fig. 2B). In addition to full-length tau, we observed, at variable degrees, truncated tau with a molecular mass of 28 kDa. Probing of the blots (Fig. 2B) with a GAPDH-specific monoclonal antibody confirmed equal amounts of protein in the RAB fraction. Moreover, we could exclude a carryover of soluble proteins into the FA fraction (Fig. 2C). An Aβ42-specific ELISA (Innogenetics) was included for a subset of the samples. This assay showed that substantial amounts of Aβ42 were in the RIPA and FA fraction (data not shown). Therefore, by loading equal amounts of protein of the Aβ42- and PBS-treated FA fractions, the amount of FA-soluble tau is likely to be underestimated in the Aβ42-treated compared with the PBS-treated FA fractions. Next, to monitor the kinetics of tau assembly, combined levels of FA-soluble full-length and truncated tau were quantified after incubation with Aβ42 for 0 h, 6 h, 1 d, 3 d, and 5 d and compared with PBS-treated controls. Three independent experiments revealed an increase of tau in the FA fraction with longer exposure to Aβ42 that was statistically significant at 5 d (Fig. 2D, *), two-tailed Student’s t test; p = 0.03).

To determine whether Aβ42 affects tau transcription, we performed a quantitative RT-PCR analysis. When undifferentiated P301L cells were compared with mock-transfected cells at time point 0, we found that incubation with Aβ42 for 5 days did not alter tau mRNA levels, either in mock- or P301L-transfected cells (Fig. 3).

Aβ42 Induces PHF-like Tau Filaments—To determine whether the decreased solubility of tau in response to exposure to Aβ42 was associated with the formation of PHF, we analyzed sarkosyl protein extracts by negative contrast electron microscopy (Fig. 4). As a control, we included sarkosyl extracts from a human brain with a confirmed NFT pathology. It contained many PHF with expected widths of 20 nm and periodicities of 75–80 nm (Fig. 4A) (16). Immunoelectron microscopy of sarkosyl protein extracts of undifferentiated SH-SY5Y cells expressing wild-type human tau with the monoclonal antibody Tau-5A6 identified many twisted filaments with widths of up to 20 nm, periodicities of 150–160 nm, and lengths of up to 1200 nm (Fig. 4, B and C and Table I). They resembled the PHF extracted from NFT in human neurodegenerative diseases and are best described as narrow twisted ribbons (17).

Because the ultrastructural characteristics of abnormal filaments in human neurodegenerative diseases vary with pathogenic mutations (4, 5), we analyzed the abnormal filaments generated by P301L mutant tau. Again, Aβ42 caused the generation of PHF-like filaments both in differentiated and undifferentiated cells stably transfected with P301L mutant tau (Fig. 4, D and E and Table I). These PHF had shorter periodicities of 130–140 nm with similar widths as compared with the

**Fig. 1.** Aβ42 aggregates are deposited on tau-expressing cells. A, Western blot analysis of neuronally differentiated cells using a human tau-specific antibody reveals comparable levels of transfected 4-repeat tau in wild-type, P301L, P301L/S422E, and P301L/S422A cells (arrow). Undifferentiated cells express comparable levels of 4-repeat tau (data not shown). Levels of 4-repeat tau are more than 10-fold increased when compared with endogenous 3-repeat tau in the mock-transfected control cells (arrow). β-Actin staining is included to confirm equal loading. B, confocal analysis of cells shows that Aβ42 (4G8, green) forms large clumps on the cell bodies and processes of neuronally differentiated tau-expressing cells (HTT, red). Intracellular accumulation of Aβ42 is not detectable. C, electron microscopy of an Aβ42 deposit in contact with a cell (low magnification) illustrates the filamentous structure of Aβ42 in the two boxed areas shown at higher magnification in panels D and E. The inset in panel E shows sarkosyl-extracted Aβ42 fibrils that are 7–10 nm wide and lack regular periodicities. Scale bar, 20 μm (B); 3 μm (C), and 40 nm (panel E, inset).

**Fig. 2.** Solubility of tau in transfected SH-SY5Y cells. A, sequential extraction of both untreated and Aβ42-treated undifferentiated wild-type tau-expressing cells in high-salt RAB buffer, RIPA buffer, and FA already reveals after a 1-day incubation with Aβ42 a small fraction of tau in the RIPA fraction, in contrast to untreated cells. B, after a 5-day incubation with Aβ42, the solubility of tau is significantly decreased as a substantial fraction of tau is present in both the RIPA and FA fractions, whereas in untreated cells after a 5-day culture period, tau is present in the RIPA fraction but absent in the FA fraction. C, as loading control, GAPDH (39 kDa) has been included. D, to monitor the kinetics of tau insolubility, tau in the FA fraction is shown after incubation with PBS (□) or Aβ42 (■) for 0 h, 6 h, 1 d, 3 d, and 5 d. For each experiment, the ratio of the intensities for each time point was determined relative to the value at time point 0. Intensity values are given in percentages. The data represent the mean of three independent experiments for time points t 6 h, 1 d, and 5 d, and two for t 3 d. A two-tailed Student’s t test reveals statistically significant differences (*, p = 0.03) after 5 days of incubation with Aβ42.
wild-type tau filaments, consistent with the fact that mutations can affect the phenotype of the tau filaments.

To exclude that the PHF-like filaments contained β-amyloid fibrils derived from our Aβ42 preparation, we showed that the anti-Aβ42 antibody 4G8 failed to decorate the PHF, whereas it clearly decorated β-amyloid fibrils extracted with the identical sarkosyl protocol (data not shown). Moreover, we found with electron microscopy that our β-amyloid fibrils were 7–10 nm wide and lacked regular periodicities (Fig. 1E, inset) (11). Control experiments showed that neither vehicle controls nor identical preparations of the reverse peptide Aβ42−1 caused the formation of PHF.

Neuronally differentiated SH-SY5Y cells transfected with either wild-type or P301L tau also formed many PHF-like filaments after 5 days of exposure to the Aβ42 preparation (Table I). This may be because of similar cellular levels of human tau in both differentiated and undifferentiated cells, in that cellular levels of tau in the transfected cells exceeded those of endogenous 3-repeat tau (18) by more than 10 times (Fig. 1A).

A quantitative analysis of filament formation in sarkosyl extracts by negative staining electron microscopy is impossible because the adherence of the sample to the grid surface is irregular, due to the irreproducibility of the surface charge of the carbon-coated grids. Thus, filaments may cluster in one field of a grid, whereas another field may be completely empty.

A semi-quantitative analysis can be achieved by immunoelectron microscopy on ultra-thin cryosections of cells. We treated undifferentiated wild-type tau-expressing cells either with Aβ42 or PBS and obtained cryosections that were incubated for 5 days with aggregated Aβ42 preparations, extracted with sarkosyl, and protein extracts were analyzed by immunoelectron microscopy. A, a typical PHF extracted from an AD brain has a width of 20 nm and a periodicity of 80 nm. B and C, tau filaments extracted from wild-type tau-expressing cells have a width of 20 nm and a periodicity of 150–160 nm. To reveal the periodicity, part of the filament in panel C is shown at a higher magnification in (C′). D and E, filaments extracted from undifferentiated P301L tau-expressing cells have a width of 20 nm and a periodicity of 130–140 nm, resembling the narrow, twisted ribbons identified in human P301L carriers. Both types of filaments are labeled with antibody Tau-5A6. Scale bar, 40 nm (A–C, D–E), 20 nm (C′).

To better characterize the role of Ser-422 in tau filament formation, we mutated Ser-422 to alanine or glutamic acid and expressed either P301L/S422A or P301L/S422E double mutants both in undifferentiated and in neuronally differentiated cells at similar levels as compared with the wild-type and P301L single mutant cells (Fig. 1A). The Ser-422 mutations were introduced into the P301L mutant and not into the wild-type tau expression constructs because the P301L cells showed the strongest phospho-Ser-422 staining. Next, we incubated the undifferentiated cells for 5 days with Aβ42 and fractionated tau into the RAB fraction, the RIPA-soluble fraction, and the FA-soluble fraction. In contrast to Aβ42-treated P301L tau-expressing cells, tau was not present in the FA fraction of P301L/S422A and P301L/S422E extracts, indicating that mutantogenesis of Ser-422 blocked the Aβ42-mediated decrease in the solubility of tau (Fig. 6E).
A decrease in the number of filamentous tau aggregates in Aβ/H9252 of tau in the FA fraction indicates a role of Aβ in the presence of the S422A or the S422E mutation. Together, these data suggest an important role of Aβ-induced PHF-like tau filaments in either double mutant cell lines. We did not find any evidence for PHF-like tau filament formation in the presence of the S422A or the S422E mutation (Table I). The data were obtained after 5 days of treatment with Aβ42 preparations.

|                | Undifferentiated | Differentiated |
|----------------|-----------------|----------------|
|                | PBS             | Aβ42 | PBS             | Aβ42             |
| Mock           | ND*             | ND   | No              | No               |
| Wild-type tau  | No              | Yes  | No              | Yes              |
| P301L tau      | No              | Yes  | No              | Yes              |
| P301L/S422E tau| No              | No   | No              | No               |
| P301L/S422A tau| No              | No   | No              | No               |

* ND, not determined

Finally, we determined whether treatments with Aβ42 would induce PHF-like tau filaments in either double mutant cell line. We did not find any evidence for PHF-like tau filament formation in the presence of the S422A or the S422E mutation (Table I). Together, these data suggest an important role of Ser-422 in Aβ42-induced tau filament formation.

**DISCUSSION**

The results of this study show that Aβ42 decreases the solubility of tau and induces PHF-like tau filaments in a human tissue culture system. A 5-day incubation with aggregated synthetic Aβ42 of SH-SY5Y neuroblastoma cells that had been stably transfected with expression constructs encoding the longest human tau isoform, with or without the pathogenic FTDP-17 mutation P301L, caused a substantial amount of tau to appear in the FA fraction. A quantitative TaqMan PCR analysis of Aβ42-treated cells did not reveal increased tau mRNA synthesis, although increases below 1.5-fold would not be detected by this method. Instead, the increased appearance of tau in the FA fraction indicates a role of Aβ42 in the biophysical properties of tau that determine its solubility within cells. However, we cannot exclude that Aβ42 may also affect the turnover rate of tau. The decreased solubility of tau in response to exposure to Aβ42 was also associated with the formation of twisted tau filaments as shown by negative contrast electron microscopy of sarkosyl protein extracts. These resembled the PHF extracted from NFT in human neurodegenerative diseases and are best described as narrow twisted ribbons (17). This difference in periodicity to the PHF extracted from human postmortem brains may be related to the known influence of the ratio of 4-repeat to 3-repeat human tau. In comparison to the PHF extracted from NFT in human neurodegenerative diseases and are best described as narrow twisted ribbons (17), the ultrastructural characteristics of abnormal filaments in human neurodegenerative diseases vary with pathogenic mutations (4, 5). We found that the PHF-like filaments extracted from cells expressing P301L mutant tau had shorter periodi-
ties of 130–140 nm compared with wild-type tau filaments, consistent with the fact that mutations can affect the phenotype of the tau filaments. For comparison, the twisted ribbons in FTDP-17 patients carrying the P301L mutation have periodicities of greater than 130 nm (4), whereas the tau filaments in Drosophila generated by expression of human 4-repeat wild-type tau have periodicities of 45 nm (20).

Our data are consistent with our previous results of Aβ42-induced PHF-like tau filament formation in transgenic mice (9), but in contrast to the transgenic mice Aβ42-induced PHF formation in tissue culture also occurred with wild-type tau. This may be related to the species difference and points to the possibility that human cells in culture may be more susceptible to the formation of abnormal tau filaments as compared with murine cells in vivo. Filibrillar aggregates of tau were previously observed in Chinese hamster ovary cells that had been transfected with a triple mutant tau expression constructs (21). In a related study (22), combined treatments of SH-SY5Y cells with okadaic acid and 4-hydroxymonenal induced 2–3-nm-wide fibrillar tau polymers. The PHF-like filaments in our tissue culture system clearly differed from both of the above in that they were much longer, about 10 times wider, and had readily identifiable twisted structures (Fig. 4). This difference could well be related to the prolonged exposure to Aβ42 in our experiments, because we could not observe any filamentous structures for up to 2 days of exposure to Aβ42 (Table 1).

A semi-quantitative analysis of filament analysis by immunoelectron microscopy on ultra-thin cryosections of cells revealed a significant increase in the number of tau aggregates in Aβ42-treated cells. The albeit-low number of aggregates in the untreated group is not unexpected, because overexpression of tau in itself is likely to cause some aggregation as has been shown in vivo for wild-type or P301L mutant tau-expressing mice in the absence of Aβ42 (6, 7, 23).

The expression of P301L mutant tau in transgenic mice showed that the presence of a pathogenic mutation is required for both early and substantial tau filament and tangle formation; wild-type tau transgenic mice develop only a few tangles at very old age, despite higher expression levels of the transgene (7, 24). Obviously, in mice, the pathogenic mutation is needed to catalyze the pathogenic conversion of tau, which is not the case in our cellular model. Therefore, our cellular model mimics more closely human sporadic AD, where tau filament formation occurs in the absence of tau mutations and tau overexpression. However, it has to be kept in mind that in humans AD needs decades to develop, whereas in vitro pathological changes have to be achieved within a few days or in vivo within a few months, asking for high (and therefore unphysiological) levels of tau expression.

Our data also imply that either the Ser-422 site of tau itself or its phosphorylation are necessary, although not sufficient, for tau filament formation. Despite the presence of the P301L mutation that in humans and in transgenetic mouse models enhances tau filament formation, the S422A and S422E mutants did not form tau filaments by incubating the cells with Aβ42. In transgenic mice expressing P301L tau, Ser-422, but not the AT8 epitope Ser-202/Thr-205, was among the epitopes that were selectively phosphorylated in response to Aβ42 injection (9). Aβ42 treatment did not cause increases in the numbers of AT8-positive cells because all HT7-positive tau-expressing cells were also AT8-positive, even in the absence of Aβ42. These findings are in agreement with those obtained in mice (9). In contrast, incubation of P301L-expressing cells with Aβ42 for only 1 day caused a more than 2-fold increase in the number of pS422-positive cells compared with HT7-positive cells. Mutagenesis of Ser-422 revealed that tau was not present in the FA fraction of Aβ42-treated P301L/S422A or P301L/S422E cells, in contrast to Aβ42-treated wild-type or P301L tau-expressing cells. Similarly, we did not find any evidence for PHF-like tau filament formation in the presence of the S422A or the S422E mutation, suggesting an important role of Ser-422 both in the Aβ42-induced decrease in tau solubility and in tau filament formation (Table 1). The finding that mutating the serine of epitope 422 to glutamic acid also precluded tau filament formation was unexpected because the S422E modification was designed to mimic the negative charge caused by phosphorylation of this site. Thus, the sole addition of a negative charge at this site is not sufficient to induce the formation of PHF-like tau filaments; coordinated phosphorylation at multiple residues, including Ser-422, may be required. Tau adopts distinct conformations during sequential phosphorylation reactions, and the AD-specific phosphopeptide AT100 can only be generated by a sequential phosphorylation of tau, initially by glycosynthetic kinase-3β followed by protein kinase A, indicating that prephosphorylation at particular sites can alter the conformation and prevent phosphorylation at other sites (25). An alternative explanation is provided by the fact that Ser-422 is located next to a putative caspase-3 cleavage site at position 421 and that altered caspase cleavage is involved in the rates of filament formation (26–28).

CONCLUSION

In conclusion, we established a tissue culture system for the generation of bona fide PHF-like filaments that closely resembled those extracted from brains of AD patients. The results provided by this system are compatible with an important role of Aβ42 in the generation of NFT in AD, and with a pivotal, yet not exclusive, role of the Ser-422 epitope of tau in tau fibrillogenesis. Our data imply that either the Ser-422 site itself or its phosphorylation are necessary, but not sufficient, for tau filament formation. PHF-like tau filament formation was achieved within 5 days, much faster than in current transgenic mouse models (8, 9). Therefore, this culture system will be useful to map phosphoepitopes of tau involved in PHF formation and to identify and characterize modifiers of the tau pathology. Further adaptation of the system may allow the screening and validation of compounds designed to prevent PHF formation.

Acknowledgments—We thank Barbara Knecht, Hans-Peter Gautschi, Theres Bruggmann, and Ursula Lüthi from the electron microscopy facility for excellent technical assistance, Eva Moritz for help with histology, Jay Tracy for the AT100-ELISA, and Uwe Konietzko for help with confocal microscopy. The Tau-5A6 antibody developed by G. V. W. Johnson was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

REFERENCES

1. Hutton, M., Lendon, C. L., Rizzu, P., Baker, M., Frolich, 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., Kwan, J. M., Nowotny, P., Heutink, P., et al. (1998) Nature 393, 702–705
2. Poirier, P., Bird, T. D., Wijman, E., Nemens, E., Garruto, R. M., Anderson, L., Andreadis, A., Wiederhold, W. C., Raskind, M., and Schellenberg, G. D. (1998) Ann. Neurol. 43, 815–825
3. Spillantini, M. G., Murrell, R. J., Goedert, M., Farlow, M. R., King, A., and Goedert, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7737–7741
4. Lee, V. M., Goedert, M., and Trojanowski, J. Q. (2001) Annu. Rev. Neurosci. 24, 1121–1159
5. Gotz, J. (2001) Brain Res. Brain Res. Rev. 35, (suppl.) 266–286
6. Lewis, J., McGowan, E., Rockwood, J., Melrose, H., Nacharaju, P., Van Slegtenhorst, M., Gwinn-Hardy, K., Murphy, M. P., Baker, M., Yu, X., Duff, K., Hardy, J., Corral, A., Lin, Y., Yon, S. H., Dickson, D. W., Davies, P., and Hutton, M. (2000) Nature 402, 402–405
7. Gotz, J., Chen, P., Barmettler, R., and Nitsch, R. M. (2001) J. Biol. Chem. 276, 5229–5234
8. Lewis, J., Dickson, D. W., Lin, W.-L., Chisholm, L., Corral, A., Jones, G., Yen, S.-H., Sahara, N., Skipper, L., Yager, D., Eckman, C., Hardy, J., Hutton, M., and McGowan, R. (2001) Science 293, 1487–1491

β-Amyloid-induced PHF-like Tau Filaments

40167
9. Gotz, J., Chen, F., van Dorpe, J., and Nitsch, R. M. (2001) Science 293, 1491–1495
10. Encinas, M., Iglesias, M., Liu, Y., Wang, H., Muhaisen, A., Cena, V., Gallego, C., and Comella, J. X. (2000) J. Neurochem. 75, 991–1003
11. Hartley, D. M., Walsh, D. M., Ye, C. P., Diehl, T., Vasquez, S., Vassilev, P. M., Teplow, D. B., and Selkoe, D. J. (1999) J. Neurosci. 19, 8876–8884
12. Johnson, G. V., Seubert, P., Cux, T. M., Motter, R., Brown, J. P., and Galasko, D. (1997) J. Neurochem. 68, 430–433
13. Bussiere, T., Hof, P. R., Mailliot, C., Brown, C. D., Caillet-Boudin, M. L., Perl, D. P., Buee, L., and Delacourte, A. (1999) Acta Neuropath. 97, 221–230
14. Probst, A., Gotz, J., Wiederhold, K. H., Tolnay, M., Mietl, C., Aton, A. L., Hong, M., Ishihara, T., Lee, V. M., Trojanowski, J. Q., Jakes, R., Crowther, R. A., Spillantini, M. G., Burki, K., and Goedert, M. (2000) Acta Neuropath. 99, 469–481
15. Goedert, M., Spillantini, M. G., Cairns, N. J., and Crowther, R. A. (1992) Neuron 8, 159–168
16. Crowther, R. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2288–2292
17. Spillantini, M. G., Crowther, R. A., Kamphorst, W., Heutink, P., and van Swieten, J. C. (1998) Am. J. Pathol. 153, 1359–1363
18. Uberti, D., Rizzini, C., Spano, P. F., and Memo, M. (1997) Neuronech. Lett. 235, 149–153
19. Haque, N., Tanaka, T., Iqbal, K., and Grundke-Iqbal, I. (1999) Brain Res. 838, 69–77
20. Jackson, G. R., Wiedau-Pazos, M., Sang, T.-K., Wagle, N., Brown, C. A., Massachi, S., and Geschwind, D. H. (2002) Neuron 34, 509–519
21. Vogelsberg-Rapaglia, V., Bruce, J., Richter-Landsberg, C., Zhang, B., Hong, M., Trojanowski, J. Q., and Lee, V. M. (2000) Mol. Biol. Cell 11, 4095–4104
22. Perez, M., Hernandez, F., Gomez-Ramos, A., Smith, M., Perry, G., and Avila, J. (2002) Eur. J. Biochem. 269, 1484–1489
23. Ishihara, T., Zhang, B., Higuchi, M., Yoshiyama, Y., Trojanowski, J. Q., and Lee, V. M. (2001) Am. J. Pathol. 158, 555–562
24. Ishihara, T., Hong, M., Zhang, B., Nakagawa, Y., Lee, M. K., Trojanowski, J. Q., and Lee, V. M. (2001) Am. J. Pathol. 158, 555–562
25. Zheng-Fischli, F., Biernat, J., Mandelkow, E. M., Illenberger, S., Godemann, R., and Mandelkow, E. (1998) Eur. J. Biochem. 252, 542–552
26. Abraha, A., Ghoshal, N., Gamblin, T. C., Cryns, V., Berry, R. W., Kuret, J., and Binder, L. I. (2000) J. Cell Sci. 113, 3737–3745
27. Fasulo, L., Ugolini, G., Visintin, M., Bradbury, A., Brancolini, C., Verzillo, V., Novak, M., and Cattaneo, A. (2000) J. Neurochem. 75, 624–633
28. Berry, R. W., Abraha, A., Lagalwar, S., LaPointe, N., Gamblin, T. C., Cryns, V. L., and Binder, L. I. (2003) Biochemistry 42, 8325–8331