Aggregated and highly phosphorylated tau protein is a pathological hallmark of Alzheimer’s disease (AD) and other tauopathies. We identified motifs of alternating polar and apolar amino acids within the microtubule-binding repeats of tau which were interrupted by small breaking stretches. Minimal mutation of these breaking sequences yielded a unique instantly aggregating tau mutant containing longer stretches of polar/apolar amino acids without losing its microtubule-binding capacity. These modifications produced rapid aggregation and cytotoxicity with accompanying occurrence of pathologic tau phosphoepitopes (AT8, AT180, AT270, AT100, Ser422, and Thr212/Ser214). The third MTBR contains a sequence that aggregates in isolated microtubule bundles (5). In the course of several diseases of the nervous system, tau aggregates like neurofibrillary tangles, a β-strand conformation can be identified (3, 4). The physiological function of tau involves the binding and stabilization of the microtubule cytoskeleton. It exists as several splicing isoforms in the central nervous system. The expression levels of the different isoforms vary during the development and maturation of the central nervous system. This probably serves to modulate neuronal microtubule stability and function.

The tau isoforms contain three or four C-terminal microtubule-binding repeats (MTBRs) and one or two acidic residues at the N terminus. The latter domains are not directly involved in the binding of microtubules, whereas the MTBRs of tau bind to the acidic C terminus of the tubulin monomers. The naturally unfolded nature of tau is probably explained by the fact that an extended conformation allows its interaction with several tubulin dimers (5).

In the course of several diseases of the nervous system, tau molecules aggregate in the form of pathological multimers. Isolated tau protein exhibits a tendency to aggregate in the form of fibrils when incubated in the presence of polyanionic compounds or fatty acid-like molecules (3). It has been shown that the third MTBR contains a sequence that aggregates in isolated form (6), and accordingly, the MTBRs have been shown to be located in the core of fibrils by tryptophan fluorescence scanning spectroscopy (4). However, the relevance of these findings for aggregation of the intact tau protein inside cells remains to be elucidated. Despite the detailed information gained from these in vitro studies, attempts to obtain more mechanistic insights by reconstruction of aggregation-mediated disease models in cell culture and animals have proven to be difficult.

Various models producing an AD-like tau pathology exist in rodents, Drosophila melanogaster, Caenorhabditis elegans, and others (7–9). These animal models take a considerable part of the life span before they develop pathological features and do not readily allow the progression of cellular pathology and toxicity to be followed.

Current cellular model systems based on tau expression fail to reliably model AD-like tau pathology, since they are unable to simultaneously show hyperphosphorylation, aggregation, and tau-related toxicity. Okadaic acid treatment of neuroblastoma cells (10) results in tau hyperphosphorylation, but its possible toxicity effects are masked by the high toxicity of the oka-
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daic acid *per se*. Tau polymerization in neuroblastoma cells occurs upon incubation with preaggregated amyloid-β peptide, but its effects cannot be uncoupled from the high toxicity of the amyloid-β protein itself (11). Expression of an FTDP-17 triple mutant of tau in CHO cells (12) shows morphological tau inclusions but fails to reveal toxicity effects and moreover shows a decreased phosphorylation. A cellular model based on the conditional expression of a tau repeat domain carrying the ΔK280 familial tau mutation was described recently (13). It reveals aggregation, toxicity, and hyperphosphorylation, but it is not clear how such a behavior will be modulated in the presence of the full-length tau sequence. There is therefore a high demand for an experimental model system that unifies prominent tau aggregation and cytotoxicity.

We identified motifs of alternating polar and apolar amino acids in parts of the microtubule binding repeats of tau. These ordered patterns are known to impart α-helical or β-strand structure in selected protein domains (14) and could therefore locally introduce secondary structure inside the otherwise unstructured tau molecule.

We noticed furthermore that these motifs were interrupted by short stretches of three or four amino acids, which we “bridged” by changing only one or two residues. This optimization served to increase the stability of these isolated structured motifs by the correction of “imperfections” in the pattern, leading to large motifs of alternating polar and apolar residues inside the tau protein. This approach carries relevance to the familial mutant of tau in CHO cells (12) shows morphological tau inclusion of an alternating “hydrophilic/hydrophobic” pattern (15). These rational mutations resulted in a tau mutant that exhibits aggregation, pathological modifications, and toxicity in cellular systems within extremely short time frames.

Our mutant tau system therefore represents a novel experimental tool for the study of AD and other tauopathies, which allows the facile introduction of highly aggregated tau species into living cells to aid in the investigation of the molecular mechanisms underlying the cellular responses that lead to cytotoxicity.

EXPERIMENTAL PROCEDURES

**Generation of DNA Vectors**—The 441-amino acid form of human tau (WT-tau), GenBank™ accession number NM_005910; NCBI Protein Database accession number AAC04279; a kind gift from R. Brandt, University of Osnabrück, Germany) was cloned by PCR using the primers agagagagcgccgagctggagccacctggag (3′-tau-BamHI). The cDNA was inserted into the pcDNA3 vector (Invitrogen). N-terminal fusions with fluorescent protein (enhanced GFP, enhanced YFP, or enhanced CFP) were created using the 5′ NotI-Kozak-5′ site-directed mutagenesis (QuikChange kit, Stratagene, Heidelberg, Germany) using the following mutation primer pairs: MTBR2, caggacgagacggagaagtctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctgctctg...
isothiocyanate, Cy3, and Cy5 (Dianova, Hamburg, Germany) were used in a 1:200 dilution. TUNEL staining was performed using the InSitu Cell Death Detection Kit (Roche Applied Science) following the manufacturer’s instructions. A positive control for apoptosis was provided by a 24-h incubation of cells with 10 µM staurosporine (Sigma).

For electron microscopic analysis of Sarkosyl extracts, prepared as described previously (18), samples were absorbed on Formvar carbon-coated grids (PlanoGmbH) and counterstained with 2% (w/v) aqueous uranyl acetate. For electron microscopy on cells, the cells were fixed 24 h after transfection using 2% (w/v) formaldehyde and 0.25% (v/v) glutaraldehyde (EM grade; Sigma) in PBS, pH 7.4, for 30 min at room temperature. Pre-embedding labeling was performed to identify the transfected cells (19). The cells were permeabilized with 0.1% Triton X-100 and blocked with 1% bovine serum albumin fraction V, 0.045% (w/v) cold water fish gelatin (EM grade; Sigma) in PBS. Pre-embedding immunogold labeling was performed with the anti-C-terminal tau antibody (clone T46; 1:400) and 1.4-nm gold- and AlexaFluor488-conjugated goat anti-mouse IgG (Molecular Probes; 1:30) in PBS, 0.05% Triton X-100 for 1 h. Silver enhancement of the nanogold particles was performed using the LI Silver Enhancement Kit from Molecular Probes. Cells were gently scraped in PBS, collected, and post-fixed with 1% (w/v) osmium tetroxide. The fixed cells were dehydrated in a graded series of ethanol containing 0.5% uranylacetate and embedded in Araldit (Serva) by passage through propylene oxide and propylene oxide-Araldit (1:1) solutions followed by polymerization at 60 °C. Ultrathin sections were cut, mounted on Formvar-coated copper grids (PlanoGmbH, Wetzlar, Germany), and counterstained with 0.4% lead citrate. All samples were analyzed on a Zeiss EM 109 electron microscope at an accelerating voltage of 60 kV.

**Imaging and Fluorescence Recovery after Photobleaching (FRAP)**—Confocal imaging was performed on a Leica TCS SP2 confocal system equipped with an AOB5 (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). FRAP was performed on CHO cells 24 h PT grown in a 4-well chamber slide with a German borosilicate glass coverslip bottom (Nunc GmbH & Co. KG) using a heated ×63 oil objective and a thermostatted microscopy chamber. The cells were imaged in 142 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM NaHPO₄, 25 mM Hepes, 5 mM glucose, 0.8 mM MgSO₄, pH 7.4, buffer and for not longer than 120 min. Bleaching was performed by repeated scanning (10 frames) at 488 nm with 100% laser power of a 4 µm size area. Immediately after bleaching, recovery images were acquired at 5-s intervals for 150–250 s with a laser power of 1–4% at 488 nm, and fluorescence was detected in a spectral window of 495–540 nm. WT-tau-YFP was bleached with the 514-nm laser and detected from 520 to 560 nm. 3PO-tau-CFP was bleached with the 458-nm laser and detected from 465 to 500 nm. Experiments were performed with the same laser settings.

**Toxicity and Cell Counting**—Toxicity was analyzed by counting 3PO-tau-GFP- or WT-tau-GFP-expressing SH-SY5Y neuroblastoma cells at 24, 48, and 72 h PT. Cell numbers were normalized to the number of cells at 24 h in the same microscopy fields of view and served as an internal reference for the toxicity evaluation. For immunofluorescence experiments, cell counting was performed as described previously (11). An average of 100 transfected cells were counted per preparation. Cells transfected with non-GFP-fused tau were identified on the basis of increased immunolabeling with an anti-C-terminal tau antibody. All toxicity experiments and other experiments with cell counting were repeated independently five times, and the results were represented as mean ± S.E.

**Protein Biochemistry**—Western blotting was performed as described previously (20). Cells were lysed in a buffer containing 120 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40 (Sigma), and protease inhibitor mix (Roche Applied Science). Samples were centrifuged at 13,000 × g for 20 min at 4 °C to obtain the supernatant and pellet fraction. For the immunodetection of phosphorylation epitopes, the samples were boiled under strong reducing conditions, using 5% (v/v) β-mercaptoethanol (Sigma). Amounts of protein were adjusted to contain equal amounts of tau and were loaded and subjected to 10% (w/v) SDS-polyacrylamide electrophoresis and transferred onto a nitrocellulose membrane by wet blotting (Bio-Rad) at 50 V for 3 h. The blots were blocked with 5% (w/v) skimmed milk powder, 0.1% Tween 20 in PBS for 1 h at room temperature and subsequently incubated with PHF-1 (1:1000), AT8 (1:1000), or anti-C-terminal tau (clone T46; 1:2000) primary antibodies for 1 h. The primary antibodies were detected with the corresponding goat anti-mouse IgG conjugated to horseradish peroxidase (1:5000; Dianova), and the signal was visualized by chemiluminescence (ECL; Amersham Biosciences). Sarkosyl extracts were absorbed onto nitrocellulose membranes using slot blotting and detected by the anti-C-terminal tau antibody (clone T46; 1:2000).

**Statistics**—Statistical analysis was performed using GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA). Densitometric analysis of Western blots was performed using NIH Image 1.62 software (National Institutes of Health, Bethesda, MD).

**RESULTS**

**Modification of MTBRs Producing an Instantly Aggregating Tau Mutant**—We identified motifs of 4 or 5 amino acids consisting of alternating polar/apolar residues in the second, third, and fourth MTBR of the 441-amino acid-long form of human wild-type tau (WT-tau) (Fig. 1, upper panel). Each of these MTBRs contains two such motifs (MTBR2, 274KVQI277 and 281KLDSL285; MTBR3, 305SVQI308 and 313VLDS316; MTBR4, 336QVEVK340 and 343KLDK347) situated after the flexible PGGG repeats. These motifs are separated by a short breaking stretch of 2–4 amino acids that interrupts the optimal polar/apolar pattern. We mutated these small breaking sequences, thereby forming a sequential motif of 11 alternating polar and apolar amino acid residues in each of the second, third, and fourth MTBR (Fig. 1, lower panel). The breaking sequence 278INK280 in MTBR2 was optimized by inversion of Ile278 and Asn279, followed by the deletion of Lys280. The breaking sequence 309VYKP312 in MTBR3 was optimized by inversion of Val309 and Tyr310 and the subsequent deletion of the structure breaking Pro312. The breaking sequence 341SE342 in MTBR4 was optimized by replacing these two polar amino acids with...
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We expressed the WT-tau and 3PO-tau in CHO cells, which lack endogenous tau, to examine the microtubule interactions and behavior of the proteins. Both the WT-tau and 3PO-tau showed normal microtubule localization at early times (within the first 12 h after transfection) (Fig. 2A). During this initial period, 3PO-tau was properly transported within the cytosol without the formation of inclusions. Furthermore, proper microtubule binding was observed, indicating that the microtubule binding function of the proteins bearing modified MTBRs was unperturbed in the early times after transfection. After 24 h of transfection, the 3PO-tau staining revealed morphologically prominent bulky aggregates and amorphous aggregates in the majority of transfected cells (more than 60% of these cells) (Fig. 2, A and C). The aggregation pattern was heterogeneous in the form of small punctate aggregates (~30% of the transfected cells) to large spherical aggregates, consistent with inclusion bodies (~10% of the transfected cells) or amorphous aggregates (~20% of the transfected cells) that occupy a significant portion of the cytosol. The use of GFP fusion constructs of 3PO-tau produced identical morphological results when compared with untagged 3PO-tau as detected by immunofluorescence using tau antibodies.

Western blotting of cell lysates of WT-tau and 3PO-tau-transfected CHO cells showed the presence of higher molecular weight aggregates consisting of di- and oligomeric 3PO-tau complexes (molecular mass ~130 kDa and higher) both in the detergent-soluble and in the detergent-insoluble fractions (Fig. 2B). A monomeric product (~60–65 kDa) was detected in the detergent-soluble fraction of both the WT-tau- and 3PO-tau-expressing cells.

To verify the role of the individual MTBR modifications in tau aggregation, we also constructed several partial mutations, designated M3, M34, and M23, to indicate the modified repeats. The modifications demonstrated a dose-dependent phenotype as aggregation accumulated with increasing modifications, 3PO-tau being most potent (Fig. 2C). However, this also shows that all breaking sequences inside polar/apolar patterns have the same effect on preventing aggregation. In contrast to pathological tau oligomers in human brain tangles and in vitro aggregated tau, we did not detect staining with the fibril-sensitiv​

dy thioflavin-S and -T and thiazin red in cells expressing 3PO-tau. We also did not observe fibrillary structures in Sarkosyl extracts of 3PO-tau cell lysates by immunoelectron microscopy. This was confirmed by the absence of tau immunoreactivity in slot blot analysis of these Sarkosyl extracts (not shown).

We performed EM with pre-embedding nanogold immunolabeling of tau, followed by silver enhancement, to identify transfected cells. Whereas only a few scattered isolated particles could be detected in WT-tau-expressing cells, numerous large clusters of silver-enhanced gold particles were observed in

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**FIGURE 1. Schematic overview of pattern-optimizing tau mutations.**

Shown are regions of the microtubule-binding repeats 2–4 (MTBR2, -3, and -4) of the 441-amino acid form of WT-tau (upper panel) and 3PO-tau (lower panel). The mutations correct the breaking motifs, leading to an extended sequence of 11 alternating polar and apolar residues. The pattern of polar (●) and apolar (○) amino acids is shown under each panel.

**FIGURE 2. Aggregation of 3PO-tau in CHO cells.** A, localization of WT-tau and 3PO-tau at different times after transfection in CHO cells. WT-tau shows proper microtubule localization at 24 h PT, whereas 3PO-tau shows the formation of amorphous or globular aggregates. Cells were methanol-fixed to preserve microtubule binding of tau. Scale bars, 8 μm. B, Western blot analysis of detergent-soluble supernatant (S) and detergent-insoluble pellet (P) fractions of cell lysates (24 h PT). Di-/oligomeric tau complexes (~130 kDa and higher) are detected in both fractions of the 3PO-tau-expressing cells with an anti-C-terminal tau antibody. C, relative number of cells with aggregated tau (all forms of aggregates), expressing WT-tau, 3PO-tau, or partial tau mutations: M3 (mutated third MTBR), M23 (mutated second and third MTBR), M34 (mutated third and fourth MTBR). D, electron microscopic localization of WT-tau and 3PO-tau in CHO cells detected by anti-C-terminal tau antibody/IGG-1.4-nm gold, using pre-embedding labeling followed by silver enhancement. WT-tau-expressing cells reveal sparse silver-enhanced gold particles (arrows) in contrast to numerous large clusters in the cytosol of 3PO-tau-expressing cells. Scale bars, 200 nm.
the cytosol of 3PO-tau-expressing cells. The reduced staining of the WT-tau-expressing cells is attributed to the known solubilization of the microtubule-bound tau under formaldehyde fixation (21) and the extensive permeabilization necessary for pre-embedding labeling. Since fibrils are highly resistant to detergent extraction, they would have coincided with the strong tau immunoreactivity had they been formed. The absence of discernable fibrillar structures indicates that tau aggregates form in a nonfibrillar manner (Fig. 2D).

FRAP Analysis of Aggregation Dynamics—We examined the aggregation properties of GFP-labeled 3PO-tau using FRAP in CHO cells. After 24 h, WT-tau-GFP exhibited an exponential recovery after bleaching with a half-time of ~13 s and a mobile fraction of ~96%. In comparison, 3PO-tau-GFP revealed a nearly complete immobility with a very slow linear course of recovery, typical for aggregating proteins, only reaching a recovery of ~14% at 250 s after bleaching. FRAP dynamics on 3PO-tau-GFP-expressing cells showed two populations with distinct slower dynamics at earlier time points (6–8 h) of expression, at which tau is still bound to the microtubules without obvious signs of aggregation. A substantial immobility of 3PO-tau with a mobile fraction of ~56% and a recovery half-time of ~46 s (Fig. 3A) was found in strongly expressing cells as judged by confocal imaging with standardized settings and intensity thresholding. Weakly expressing cells, on the other hand, exhibited a higher motility and an exponential recovery with a mobile fraction of ~96% and a recovery half-time of ~98 s (Fig. 3A). This indicated that 3PO-tau aggregated into multimeric complexes very soon after the onset of expression and in a time- and expression level-dependent manner. FRAP analysis of the partial forms of 3PO-tau (M3, M34, and M23) after 24 h of expression confirmed the cumulative nature of the individual MTBR mutations (supplemental Fig. S1).

Next, we investigated the possible induction of aggregation of wild-type tau by 3PO-tau. 3PO-tau-CFP and WT-tau-YFP were co-expressed, and the WT-tau-YFP motility was analyzed by FRAP. A pronounced “all-or-none” cellular response with distinct behaviors was observed; 78% of the double-transfected cells showed typical WT-tau-YFP diffusion rates, whereas 22% of the cells showed a reduced recovery with a half-time of ~46 s and a mobile fraction of ~35% (Fig. 3B). These effects were not correlated with the relative expression levels of both constructs as judged by CFP/YFP fluorescence ratio measurements (not shown). Surprisingly, in half of the population of cells that exhibited normal WT-tau-YFP motility, WT-tau-YFP showed an aggregated appearance as its signal co-localized with the majority of cells for unknown reasons. This shows that 3PO-tau aggregates do not simply act as a nonspecific steric trap for proteins.

Hyperphosphorylation and Pathological Conformation of 3PO-tau—A typical feature of aggregated tau in AD and other tauopathies is the increased phosphorylation at specific epitopes. These modifications were also present in our 3PO-tau model. The increase of PHF-1 (simultaneous phosphorylation at Ser396/Ser404) and AT8 (phospho-Ser202/phospho-Thr205)
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**FIGURE 4. 3PO-tau exhibits hyperphosphorylation and pathological conformation.** A, Western blot analysis of lysates of WT-tau (WT)- and 3PO-tau (3PO)-expressing CHO cells revealing increased PHF-1 and AT8 epitope phosphorylation of 3PO-tau at 24 h PT. The total tau protein is detected by the anti-C-terminal tau antibody (T46). B, detection of the number of 3PO-tau phosphopositive SH-SY5Y neuroblastoma cells judged by immunolabeling with AT8, AT270, Ser422, AT100, and AT180 antibodies at 36 h PT (mean ± S.E., n = 5 independent experiments, 100 cells/experiment; ***, p < 0.001, Mann-Whitney U test). C, evaluation of phosphorylation levels judged by immunofluorescence with PHF-1 and Ser422 antibodies (mean ± S.E., n = 5 independent experiments, 100 cells/experiment; ***, p < 0.001; one-way analysis of variance). D, time-dependent immunofluorescence labeling of the AT8 epitope in 3PO-tau-expressing SH-SY5Y cells (mean ± S.E., n = 40 cells; **, p < 0.01; ***, p < 0.001; one-way analysis of variance). E, pathological conformation of 3PO-tau judged by MC1 and Alz50 immunolabeling at 36 h PT (mean ± S.E., n = 5 independent experiments, 100 cells/experiment; *, p < 0.05; **, p < 0.01, Mann-Whitney U test). F, corresponding fluorescence intensity images of GFP-labeled WT-tau or 3PO-tau detected with AT8 and AT180 phosphoantibodies and the MC1 conformation antibody in formaldehyde-fixed cells. Scale bar, 8 μm.

We determined the time course of 3PO-tau phosphorylation using AT8 immunofluorescence, since this was the most sensitive indicator for 3PO-tau phosphomodification. At 12 h, AT8 recognized 3PO-tau associated with deformed microtubule structures, but labeling was absent in approximately half of the expressing cells. At 24 h, all 3PO-tau was detected by AT8, with an additional 3-fold increase that remained unchanged until 36 h (Fig. 4D and supplemental Fig. S3).

Finally, MC1 and Alz50 immunoreactivity was significantly up-regulated in 3PO-tau-expressing cells at 36 h (Fig. 4, E and F). These antibodies recognize a conformation-dependent epitope that includes both the N terminus (amino acids 2–6) and the third MTBR (amino acids 313–322), which is specifically formed in pretangle-aggregated tau and neurofibrillary tangles (22).

**3PO-tau Is Cytotoxic—**SH-SY5Y cells were transfected with equal amounts of either WT-tau-GFP or 3PO-tau-GFP to assess their cytotoxicity by counting GFP-positive cells at different time points after transfection. At 48 and 72 h PT, a significant decrease (p < 0.001) in the number of fluorescent cells was observed in 3PO-tau-expressing cells versus WT-tau-expressing cells (~66% at 48 h and ~53% at 72 h versus ~110% at 48 h and ~114% at 72 h) normalized to the respective fluores-
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FIGURE 5. 3PO-tau induces toxicity in neuroblastoma cells. A, counting of 3PO-tau-GFP- and WT-tau-GFP-expressing SH-SY5Y neuroblastoma cells showing the progression of 3PO-tau cytotoxicity. (mean ± S.E. of five independent experiments; ***, p < 0.001, Mann-Whitney U test). The number of transfected cells at different time points PT was normalized to the initial cell number for the same microscopic fields of view at 24 h PT, serving as internal reference. B, time lapse microscopy of degenerating 3PO-tau-GFP-expressing cells showing shrinkage and rounding during the indicated observation times PT. Bulky inclusion bodies (arrow) persisted during the course of degeneration. Scale bars, 16 μm. C, detection of the apoptotic markers anti-activated caspase-3 immunostaining and TUNEL staining at 50 h PT showing an increase in the number of apoptotic cells in 3PO-tau-GFP-expressing neuroblastomas (mean ± S.E., n = 5 independent experiments, 100 cells/experiment; * p < 0.05, ** p < 0.01, Mann-Whitney U test). D, TUNEL and activated caspase-3 labeling in 3PO-tau-GFP-transfected human neuroblastoma cells. Note the pyknotic nuclear morphology of TUNEL-positive cells, which differs from the fragmented nuclei of staurosporin-treated cells. Scale bar, 10 μm.

detected only in cells with advanced morphological signs of damage and bearing pyknotic nuclei (Fig. 5D).

3PO-tau Induces Cytoskeletal Changes, Hyperphosphorylation, and Cellular Damage in Primary Neurons—We expressed the WT-tau and 3PO-tau mutant in primary cultures of embryonic mouse hippocampal neurons. Neurons expressing GFP-fused WT-tau exhibited normal morphology of the cell body and neurites (Fig. 6A, upper panel), whereas 3PO-tau-GFP transduction caused somatic and neurite abnormalities after 24 h (Fig. 6A, lower panel). Cell bodies were distorted and dilated, and the tubulin cytoskeleton was abnormally displaced, as can be seen by the loss of co-localization of 3PO-tau and microtubules (Fig. 6A, arrows). Wild-type tau is physiologically highly phosphorylated in embryonic neurons. Of all of the phosphoantibodies tested, AT180 staining is absent in cultured embryonic neurons, which makes it a suitable marker for pathological tau phosphorylation. 3PO-tau-expressing neurons showed a significant increase in the number of AT180-labeled cells (~93% versus ~17% for WT-tau, p < 0.01) (Fig. 6B).

Additionally, nuclear morphology was evaluated by 4',6-diamidino-2-phenylindole staining. Chromatin-condensation and marginalization, indicative for neurodegeneration, occurred significantly more frequently (p < 0.05) in 3PO-tau-expressing neurons (Fig. 6C). The experiments with primary embryonic mouse neurons thus recapitulated the pathological phenotype of 3PO-tau established in CHO cells and human neuroblastoma cells.

DISCUSSION

Here we demonstrate a novel aggregation-enhancing tau modification strategy, based on the “bridging” of internally present patterns of alternating polar and apolar residues in the second, third, and fourth MTBR of tau by minimal point mutagenesis of intermittent breaking sequences. The expression of this structurally enforced protein in cells led to an almost instantaneous aggregation of the protein associated with cytotoxicity and pathologic phosphorylation patterns. This detailed pathological phenotype furthermore establishes a cellular model of tau pathology that can be used for the investigation of aggregation-induced toxicity mechanisms.

The possible role of the MTBRs in the pathological aggregation behavior of tau has been debated for some time. The pep-
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**FIGURE 6.** Hyperphosphorylation, cytoskeletal abnormalities, and cell damage in primary neurons expressing 3PO-tau-GFP. A, fluorescence images of neurons expressing WT-tau-GFP and 3PO-tau-GFP. Neurons, expressing WT-tau-GFP, show co-localization of tau and microtubules, whereas neurons, expressing 3PO-tau-GFP, developed cytoskeletal changes, exclusion of microtubules outside tau aggregates, and abnormal somatic shapes (see arrow) 28 h post-transduction. Scale bar, 8 μm. B, the AT180-epitope was strongly up-regulated 28 h post-transduction (mean ± S.E., n = 4 independent experiments, 50 cells/experiment; **, p < 0.01, Mann-Whitney U test) as detected by immunofluorescence. Abnormal neurite configurations can be observed in 3PO-tau-GFP-transduced neurons. Scale bar, 8 μm. C, chromatin marginalization and condensation 26–28 h after 3PO-tau-GFP transduction as detected by 4',6-diamidino-2-phenylindole staining (n = 4 independent experiments, 30 cells/experiment; *, p < 0.05, Mann-Whitney U test).

tide fragment, 306VQIVYK311, derived from the third MTBR has been shown to aggregate spontaneously outside the context of the intact tau protein (6). Furthermore, most of the hereditary pathological tau mutations locate to the MTBRs (3) or are in close proximity inside the C-terminal projection domain of tau (e.g. R406W) (23). Markedly, the ΔK280 mutation improves the alternating polar/apolar sequence in a natural mutant tau form (15). We have therefore compared the behavior of ΔK280-tau with WT-tau and 3PO-tau in SH-SY5Y neuroblastoma cells but failed to discover increased (AT8, PHF-1) phosphorylation, toxicity, or decreased FRAP mobility (supplemental Fig. S4). Apparently, although the ΔK280 mutation increases polar/apolar amino acid order, this does not suffice to produce the effects observed with our 3PO-tau. In contrast, conditional expression of truncated ΔK280-tau (containing only the MTBRs) aggregates and is toxic (13). In this respect, it is interesting to note that deletion of the tau projection domains greatly increases the aggregation capacity of tau and its mutants in vitro (6, 24).

Patterns of ordered polar/apolar amino acid residues are known to determine either a α-helical or β-strand structure in selected protein domains (14). Experiments with de novo designed combinatorial protein libraries containing extended motifs of alternating polar/apolar residues demonstrated the formation of long β-strand-containing fibrils that can form large intermolecular aggregates (25). Moreover, analysis of protein sequence data bases from a wide variety of organisms shows that patterns of alternating polar/apolar residues are strongly underrepresented in nature in comparison with other ordered patterns with the same composition. Obviously, there is an evolutionary selection against such toxic conformations (14).

In the generation of the pattern-optimized tau protein, we aimed for optimal aggregation capacity by modifying all MTBRs that contained relevant stretches of alternating polar and apolar residues. Furthermore, we demonstrate that even modifications of one or two of the second, third, and fourth MTBRs suffice to produce tau aggregation (although not as strong as in 3PO-tau) when the polar/apolar pattern is introduced in the breaking sequences.

It might be speculated that the aggregation-inhibiting effect of the breaking sequences in spontaneous AD is compromised by intermolecular alignment after cysteine oxidation, promoting disulfide bridge formation (26), or by the introduction of charge in the MTBRs (e.g. by phosphorylation of serine residue 262, 324, or 356). Interestingly, analysis of wild-type tau by multidimensional NMR spectroscopy also identified partial β-strand content in the same first 8–10 amino acids of MTBR2, -3, and -4 (27). This study furthermore shows that these sequences correspond to the sites of polyanion binding, which leads to their increased β-strand structure. Polyanion incubation is known to permit the aggregation of purified tau in vitro. Our approach thus resembles this structural stabilization but is achieved in cells, allowing the investigation of the cellular reaction to this situation.

Sequence comparison (GenBank ™ data base, National Institutes of Health) shows that the MTBRs of tau are completely conserved between mammals. The homology in lower species (i.e. D. melanogaster, C. elegans, and the fish Tetraodon nigroviridis) is lower. However, the presence of polar/apolar motifs, separated by breaking motifs, seems to be relatively well maintained (Fig. 7).

Recent data point at the possibility that tau oligomerization could occur on the microtubule surface (28, 29), which is negatively charged like the polyanionic compounds that are known to promote tau aggregation in vitro. These observations are in line with the observed early microtubule interaction of the aggregation-optimized 3PO-tau. The mutations introduced in 3PO-tau do not decrease the microtubule-binding capacity of the MTBRs, since 3PO-tau binds properly to microtubules at early time points of expression (6 h PT). The microtubule-binding capacity was preserved early after expression, and the protein showed no sign of early retention in cellular compartments but was properly diffusing throughout the cytosol to bind its target. We therefore assume that its normal physiologic function is not disturbed as long as aggregation has not advanced.

Moreover, FRAP analysis at these early time points revealed that the entire cellular pool of 3PO-tau was significantly aggregated in both a time- and expression level-dependent manner. The bulky aggregates in ~10% of the cells, at later time points,
probably reflect a rescue response to remove the harmful aggregated proteins by the formation of inclusion bodies (30).

FRAP analysis also demonstrated that 3PO-tau possesses the capacity to recruit WT-tau protein into its aggregates, since WT-tau showed a significantly decreased motility in 22% of the cells. Surprisingly, co-aggregation occurred in an all-or-none fashion. On the other hand, the finding that most cells co-expressing 3PO-tau did not affect the diffusion properties of WT-tau-YFP (and YFP alone), even in cases where WT-tau seemed to co-localize with the aggregated 3PO-tau, furthermore suggest that the inherent diffusion of cytosolic proteins is not largely affected and thus that 3PO-tau aggregates do not act as a steric nonspecific protein trap. These findings point to the usefulness of our approach in the identification of proteins that are specifically recruited to aggregated tau to mediate pathological cellular processes.

A significant increase in the phosphorylation level of 3PO-tau was found in CHO fibroblasts and SH-SY5Y human neuroblastoma cells at sites that constitute known pathological hyperphosphorylation epitopes. Although the phosphoantibodies AT8, AT270, PHF-1, AD2, and Ser422 labeled the total pool of cellular 3PO-tau, AT100 and AT180 preferentially labeled bulky aggregates. In the latter case, the densely packed tau aggregates could be less accessible to phosphatases that are specific for the AT100 and AT180 epitopes, as has been suggested before (31).

Furthermore, 3PO-tau exhibits a pathological aberrant conformation as judged by labeling with MC1 and Alz50 conformation-specific antibodies. Both recognize a nonlinear epitope in tau that includes sequences in the third MTBR and its N terminus when tau undergoes pathological modification (22).

The most prominent feature of 3PO-tau is the presence of substantial cell toxicity. Other existing tau-based cellular models require pharmacological treatment (10) or additional gene expression (32) before toxicity is observed. Although the cells showed an increase in TUNEL and active caspase-3 reactivity, the morphological criteria did not completely fulfill the classical description of apoptosis. Therefore, we cannot exclude the possibility that the up-regulation of the apoptotic markers in our system is related to the rapidly induced cell death rather than a result of the induction of apoptosis.

Contrary to most reports on AD and other tauopathies, our aggregated tau does not stain with thioflavin-S, thioflavin-T, or thiazin red dyes, which recognize fibrillar structures that are rich in β-strand conformation (3). This observation is corroborated by the lack of tau immunoreactivity upon 3PO-tau Sarkosyl extraction and the absence of identifiable fibrillar structures in our immuno-EM investigations. Braak et al. (33) defined five incremental cytological “groups” of pathological neuronal changes in AD that are based on the degree of AT8 staining and silver impregnation labeling. Tau hyperphosphorylation, aggregation, and conformation-specific antibody labeling are already present in mildly affected group 1 neurons, considered to represent the pretangle stage, whereas silver impregnation-positive structures are only sporadically observed in the neuronal soma in group 2 neurons and frequently in group 3 neurons. It is tempting to speculate that our 3PO-tau shares a resemblance with pretangle tau forms in groups 1 and 2. It should, however, be noted that these could also be explained by the unknown effect of the extremely rapid aggregation in our model system. Nevertheless, the concept that tau aggregation but not its exact polymeric fibril structure is of importance for the protein toxicity was recently proposed (34). This is consistent with the current recognition of the high toxic potency of smaller oligomeric forms of other aggregating proteins (i.e. amyloid-β-protein, huntingtin, and others) (35).

It should be noted that the advantageous fast aggregation in our experimental system limits its relevance to the slowly developing pathological processes in AD and the other tauopathies. We devised 3PO-tau as a tool for the study of molecular events...
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downstream of aggregation. Its artificially enhanced aggregation, although providing mechanistic insight into this property, negates its suitability for the investigation of pathological aggregation. The resulting lack of paired helical filament formation is likely to influence the pathological processes that occur in the course of human tau pathology.

Our findings show that modification of small breaking sequences in the MTBRs uncovers the high intrinsic aggregation capacity of MTBR polar/apolar motifs. The resulting 3PO-tau mutant shares numerous features with AD and other tauopathies, thus generating an attractive cellular model system that complements existing models for the investigation of molecular processes in these diseases.

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