Phosphorylation-mimicking glutamate clusters in the proline-rich region are sufficient to simulate the functional deficiencies of hyperphosphorylated tau protein

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The microtubule-associated tau proteins represent a family of closely related phosphoproteins that become enriched in the axons during brain development. In Alzheimer's disease (AD), tau aggregates somatodendritically in paired helical filaments in a hyperphosphorylated form. Most of the sites that are phosphorylated to a high extent in paired helical filament tau are clustered in the proline-rich region (P-region; residues 172–251) and the C-terminal tail region (C-region; residues 368–441) that flank tau's microtubule-binding repeats. This might point to a role of a region-specific phosphorylation cluster for the pathogenesis of AD. To determine the functional consequences of such modifications, mutated tau proteins were produced in which a P- or C-region-specific phosphorylation cluster was simulated by replacement of serine/threonine residues with glutamate. We show that a phosphorylation-mimicking glutamate cluster in the P-region is sufficient to block microtubule assembly and to inhibit tau's interaction with the dominant brain phosphatase protein phosphatase 2A isoform ABαC. P-region-specific mutations also decrease tau aggregation into filaments and decrease tau's process-inducing activity in a cellular transfection model. In contrast, a phosphorylation-mimicking glutamate cluster in the C-region is neutral with regard to these activities. A glutamate cluster in both the P- and C-regions induces the formation of SDS-resistant conformational domains in tau and suppresses tau's interaction with the neural membrane cortex. The results indicate that modifications in the proline-rich region are sufficient to induce the functional deficiencies of tau that have been observed in AD. They suggest that phosphorylation of the proline-rich region has a crucial role in mediating tau-related changes during disease.

Key words: Alzheimer's disease, microtubule, paired helical filaments, protein phosphatase 2A.

INTRODUCTION

The microtubule-associated tau proteins consist of a family of closely related phosphoproteins that are produced by alternative splicing of a single gene [1]. In the brain, tau proteins are enriched in the axonal compartment, where they are thought to contribute to the organization of the microtubule array. During several neurodegenerative disorders, including Alzheimer's disease (AD) as well as frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), tau accumulates in the somatodendritic compartment, where it aggregates into paired helical filaments (PHFs) and/or straight filaments [2]. Filament-associated tau is phosphorylated to a greater extent than normal tau and is unable to bind to microtubules [3]. It is still unclear how the hyperphosphorylation of tau is linked to tau aggregation and to neuronal degeneration observed in these pathologies.

Tau can be phosphorylated by many kinases *in vitro* and in cells [4]. In the fetal brain, tau is phosphorylated to a relatively large extent at many sites. In the adult brain the stoichiometry of tau phosphorylation is decreased; however, during AD the phosphorylation of tau is again increased. Although few, if any, phosphorylation sites are unique to PHF-tau, the proportion of tau phosphorylated at any given site is significantly higher in PHF-tau than in biopsy-derived normal tau protein [5,6]. Thus the phosphorylation abnormality in PHF-tau seems to be a stoichiometric increase in the amount of phosphate at selected sites.

The most striking feature in the primary structure of tau protein is the presence of three or four tandem repeats in the C-terminal half of the protein, which constitute the primary microtubule interaction site [1]. Most of the sites that are phosphorylated in tau isolated from PHFs are clustered in two regions that flank the repeat domain N-terminally and C-terminally [5,7]. Several antibodies that are used to detect PHF-tau specifically recognize phosphorylated residues within these two regions, which might indicate a role of a region-specific phosphorylation cluster for the pathogenesis of AD. The region flanking the repeat domain N-terminally is characterized by a high content of proline residues. Previously it has been demonstrated that the presence of the proline-rich region (P-region) increases tau's interaction with microtubules [8,9] and is required for tau's ability to promote microtubule nucleation *in vitro* [10]. Phosphorylation in the P-region seems to drastically decrease tau's ability to promote microtubule nucleation [11]. This might point to an important role of the P-region and of phosphorylation within this region for tau's functional activities during development and disease.

With regard to the involvement of tau during the disease process, tau's activities other than on microtubules could be important, as could its regulation by phosphorylation. Tau can

Abbreviations used: AD, Alzheimer's disease; DMEM, Dulbecco's modified Eagle's medium; DTAF, 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein; PHF, paired helical filament; PP2A, protein phosphatase 2A; wt, wild-type.

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interact with the dominant brain phosphatase protein phosphatase 2A (PP2A) isoform ABαC [12,13], which might be relevant for the regulation of the phosphorylation state of tau and other proteins during the disease. In addition, tau binds to components of the neural membrane cortex in a phosphorylation-dependent manner, which might contribute to tau's axon-specific localization in the brain and its mislocalization during disease [14,15]. Furthermore, phosphorylation of tau by certain kinases seems to directly influence tau's activity to aggregate [16], which might be an important event during the development of filaments in affected neurons.

We have shown previously that the replacement of individual serine/threonine residues with negatively charged amino acids simulates structural and functional consequences of the phosphorylation of the respective tau residue [17]. In carrying this approach further, we demonstrated that the serine/threonine to glutamate exchange of ten residues that are phosphorylated to a great extent in PHF-tau (tau 352Glu_{10}) induces structural changes and a functional loss of tau's ability to promote microtubule assembly, closely reflecting what has been observed for hyperphosphorylated tau protein [18]. As in PHF-tau, the modified sites were clustered in the P-region and C-region that flank tau's microtubule-binding domain; however, the contribution of each of these clusters to the structural changes and the functional deficiencies are not known.

Using mutated tau proteins in which a phosphorylation cluster in the P-region or the C-region was simulated, we show that a phosphorylation-mimicking glutamate cluster in the P-region is sufficient to block microtubule nucleation, to inhibit the interaction of tau with ABαC and to decrease the aggregation of tau into filaments. In contrast, a phosphorylation-mimicking glutamate cluster in the C-region is neutral with regard to these activities. Both P- and C-region modifications induce the formation of SDS-resistant conformational domains in tau protein and abolish tau's interaction with the neural membrane cortex. The results indicate that P- and C-region-specific modifications differentially affect structural and functional properties of tau protein and suggest an important role for P-region-specific phosphorylation events in the development of the functional deficiencies of tau during tauopathies.

EXPERIMENTAL

Materials

Chemicals were purchased from Sigma (Deisenhofen, Germany), cell culture media and supplements from Life Technologies (Gaithersburg, MD, U.S.A.), and tissue culture flasks and dishes from Nunc (Roskilde, Denmark), unless stated otherwise. Tubulin was isolated from bovine brain by two assembly–disassembly cycles and phosphocellulose chromatography as described previously [10].

Construction of expression plasmids

Eukaryotic expression plasmids for fetal and human tau with the sequence MDKDDDDK (FLAG) fused to the N-terminal end as an epitope tag were constructed in fpRc-cytomegalovirus (CMV) as described previously [14]. Mutated tau with a region-specific glutamate cluster was constructed in which the codons for Ser^{198}, Ser^{199}, Ser^{202}, Thr^{231} and Ser^{235} in the proline-rich region (P-region), or Ser^{396}, Ser^{404}, Ser^{409}, Ser^{413} and Ser^{422} in the C-terminal tail region (C-region) were mutated to GAA (glutamate) by PCR or site-directed mutagenesis (Muta-Gen™ phagemid *in vitro* mutagenesis kit; Bio-Rad, Richmond, CA, U.S.A.) (all numbers refer to the longest low-molecular-mass tau isoform containing 441 residues [19]). Constructs were verified by didoxy sequencing with T7 Sequenase (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.). For prokaryotic expression, the mutated tau sequences were cloned into a pET vector containing the human tau sequence [10] by using the *Bsp*119I site in tau and an *Apa*I site that was introduced into the pET vector by PCR.

Purification of tau and mutated tau proteins

pET plasmids were transformed into *Escherichia coli* [BL21-(DE3)pLysS] cells for expression, then grown, induced and harvested as described previously [10]. Tau was purified from the bacterial pellet by chromatography on DE52 and P11 [10]. For microtubule assembly assays, the eluate from the P11 column was dialysed against BRB80 [80 mM Pipes/KOH (pH 6.8)/1 mM $MgCl_2$/1 mM EGTA] and concentrated with poly(ethylene glycol) (molecular mass 10 kDa). For tau assembly assays, the eluate from the P11 column was dialysed against 50 mM imidazole/HCl (pH 7.4), adjusted to 1 mM dithiothreitol, freeze-dried, resuspended in 30 mM Mops/NaOH (pH 7.4) and precleared by centrifugation for 2 h at 100000 ***g*** and 4 °C. Protein concentrations were determined by densitometry of Coomassie-Blue-stained gels with BSA as a standard.

Microtubule assembly and co-sedimentation assays

Microtubule assembly assays were performed as described previously [10], in the presence of 15 μM tubulin, 1 mM GTP and various tau concentrations adjusted to a final volume of 25 μl in BRB80. After incubation for 10 min at 37 °C, the reaction mixture was fixed with glutaraldehyde and 0.1 % aliquots were sedimented on polylysine-treated coverslips. After post-fixation with methanol, the samples were stained with a mouse monoclonal antibody against α-tubulin (DM1A), Cy3-coupled donkey anti-mouse secondary antibody (Dianova, Hamburg, Germany) and analysed by fluorescence microscopy with a Zeiss Plan-Apochromat 63× lens. The determination of the number and length distribution of the microtubule populations was computer-assisted. Total microtubule mass was calculated as the product of the mean length and microtubule number.

Cell culture, transfection and immunocytochemistry

PC12 cells were cultured in serum-Dulbecco's modified Eagle's medium [DMEM supplemented with 10 % (v/v) fetal bovine serum, 5 % (v/v) horse serum, 292 μg/ml glutamine, 50 i.u./ml penicillin and 50 μg/ml streptomycin] and transfected with Lipofectin as described previously [14]. For the generation of stable lines, cells were selected with Geneticin; individual clones were isolated as described previously [14]. Stable lines were cultured in serum-DMEM supplemented with 250 μg/ml Geneticin on collagen-coated culture dishes. For analysis of the activity of the constructs to induce process formation, PC12 cells were plated at a density of 50000 cells/cm^2 on polylysine-treated coverslips, transfected on the next day, cultured for a further 2 days and treated for 30 min with 20 μM cytochalasin B to disrupt the cortical actin and to allow microtubule-dependent process formation in the absence of nerve growth factor as described previously [17]. Cells were fixed for 20 min at room temperature with 4 % (w/v) paraformaldehyde in PBS containing 4 % (w/v) sucrose. After being washed with PBS, cells were incubated for 20 min with 0.1 M glycine in PBS and then permeabilized for 5 min in 0.2 % (v/v) Triton X-100 in PBS. Immunofluorescence staining employed a monoclonal mouse antibody against the FLAG epitope (M5) and Cy3-coupled donkey anti-mouse anti-

body (Dianova). For total protein staining, cells were washed five times for 2 min each with PBS after the secondary antibody reaction, incubated for 15 min with 10 μg/ml 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein (DTAF), and washed five times for 2 min each with PBS. Coverslips were mounted in anti-fade medium, as described previously [14]. Cells that expressed exogenous tau as judged by an intense anti-FLAG immunofluorescence (see Figure 3A, left panels) were analysed for process extension by visual inspection with 40 × Neofluar lenses on a Zeiss Axiophot microscope. Cells were judged to be positive for process extension when they had established at least one process with a length exceeding the diameter of the cell body. All constructs were expressed to similar levels in the cells as judged from the relative staining intensities for transfected tau compared with DTAF-stained total cellular protein.

Tau assembly assays and electron microscopy

All tau assembly assays were performed with tau441 proteins in 30 mM Mops/NaOH, pH 7.4, containing 200 μg/ml heparin and 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (Applichem, Darmstadt, Germany) at 33 °C and analysed by electron microscopy as described previously [18]. To quantify the amount of filament formation, the mixture was centrifuged for 1 h at 100000 ***g*** and 4 °C after the assembly reaction; 50 % of supernatant and pellet fractions were separated by SDS/PAGE with 10 % (w/v) polyacrylamide and stained with Coomassie Brilliant Blue.

PP2A-tau binding assays

PP2A-tau binding assays were performed with non-denaturing gel electrophoresis, as described previously [13]. Purified bovine brain ABαC (500 nM) was incubated for 20 min on ice with 5 μM wild-type tau or the phosphorylation-mimicking constructs, in a final volume of 5 μl in 25 mM Tris/HCl/1 mM dithiothreitol/1 mM EDTA/50 % (v/v) glycerol (pH 7.5). The samples were separated by native gel electrophoresis on 4–15 % (w/v) polyacrylamide gels, transferred to nitrocellulose and detected with a monoclonal antibody against the C subunit of PP2A (Transduction Laboratories, San Diego, CA, U.S.A.). Similar results were obtained when immunoblotting was performed with an antibody directed against the B subunit of PP2A, as reported previously [13], or when purified bovine brain ABαC was replaced in the binding assays with a commercial preparation of purified PP2A (Upstate Biotechnology, Lake Placid, NY, U.S.A.).

Separation of neural plasma membranes with microspheres

Separation of surface-biotinylated cells was performed with microspheres as described previously [15]. PC12 cells stably transfected with wild-type tau or the phosphorylation-mimicking tau constructs were surface-labelled with sulphosuccinimidyl 2-(biotinamido)ethyl-1,3′-dithiopropionate (Pierce) and incubated with streptavidin-coupled microspheres. A cellular homogenate was prepared by freeze-thawing the scraped-off cells in a separation buffer (0.25 M sucrose containing 1 mM ATP, 1 mM $MgCl_2$, 1 mM EGTA and the protease and phosphatase inhibitors 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM sodium pyrophosphate, 1 mM Na_3VO_4 and 20 mM NaF) and the plasma membrane fraction was separated with a magnetic bead attractor. The unbound material was separated by ultracentrifugation into a crude organelle pellet and a cytosol fraction. Equal quantities of the plasma membrane fraction and the cytosol fraction were separated by SDS/PAGE [10 % (w/v) gel].

Other methods

SDS/PAGE and Coomassie Blue staining of gels were performed as described previously. Immunoblot analysis used monoclonal anti-actin (IgM) (Amersham Pharmacia Biotech), anti-tubulin (DM1A), anti-tau {Tau5 (PharMingen, San Diego, CA, U.S.A.) and Tau1 [20]} and horseradish-peroxidase-coupled goat anti-mouse secondary (Dianova) antibodies. Detection and development with enhanced chemiluminescence (Amersham Pharmacia Biotech) were performed as described previously [15]. Quantification of Coomassie-Blue-stained gels employed an image capture computer (Cybertech) and the program NIH IMAGE 1.61. Protein concentrations for tubulin were determined by the method of Bradford [21], with BSA as a standard.

RESULTS

Phosphorylation-mimicking glutamate clusters in the proline-rich region and the C-terminal tail domain induce distinct structural changes in tau protein

Hyperphosphorylated tau protein that has been isolated from PHFs exhibits a decreased electrophoretic mobility and is unable to promote microtubule assembly *in vitro* [22]. To determine which modifications are responsible for conferring these structural changes and the functional deficiencies, region-specific mutated tau proteins were constructed, in which serine/threonine residues in the proline-rich region (P-region) upstream [$Glu_{5(P)}$] or the C-terminal tail region (C-region) downstream [$Glu_{5(C)}$] of the repeat domain were replaced with glutamate to simulate a phosphorylation cluster in each region (Figure 1A). The constructs were prepared from human fetal tau protein lacking all adult-specific exons (352wt), expressed in *E. coli* and purified from the bacterial pellet by sequential ion-exchange chromatography on DE52 and phosphocellulose (P11). Consistent with the decrease in the calculated pI was the observation that the glutamate mutants were eluted from the phosphocellulose column at a slightly lower salt concentration than wild-type tau [peak elution at 0.3 M KCl for $352Glu_{5(P)}$ and $352Glu_{5(C)}$, and 0.2 M KCl for $352Glu_{10}$]. The purified phosphorylation-mimicking proteins displayed decreased electrophoretic mobilities during SDS/PAGE compared with wild-type tau (Figure 1B, top panel). The shift was more pronounced for $352Glu_{5(C)}$ than for $352Glu_{5(P)}$ and corresponded to apparent molecular mass differences of 2 kDa [$352Glu_{5(P)}$] and 4 kDa [$352Glu_{5(C)}$] respectively. The mobility shifts of the region-specific mutants added up to the electrophoretic shift observed for $352Glu_{10}$ (6 kDa) in which all ten sites were replaced with glutamate, suggesting that P- and C-region-specific modifications independently induced structural changes in tau protein. All mobility shifts were abolished in the presence of urea (Figure 1B, bottom panel), indicating that they resulted from conformational changes that produced unfolded and SDS-resistant domains in tau protein. Such changes were also observed after tau phosphorylation with kinases that phosphorylate tau in regions flanking the repeat domain [11,23].

To test the effect of the glutamate clusters on the immunoreactivity with antibodies against tau protein, we performed immunoblot analysis. All tau constructs were recognized by the phosphorylation-insensitive antibody Tau5 (Figure 1C, top panel). Mutations in the P-region but not in the C-region abolished immunoreactivity for Tau1, which recognizes a dephosphorylated serine residue between residues 192 and 204 [24] (Figure 1C, bottom panel). However, the replacement of Ser^{202} and Thr^{231} with glutamate in the P-region or Ser^{396} and Ser^{404} in the C-region were not sufficient to induce immunoreactivity with the phosphorylation-sensitive antibodies AT8,

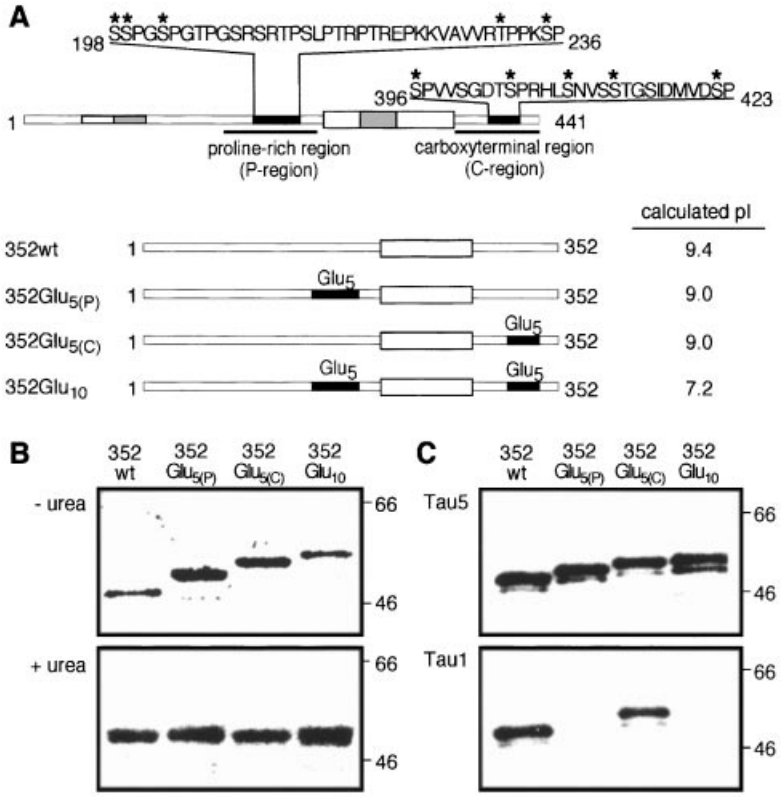

Figure 1 Simulation of phosphorylation by region-specific glutamate clusters

(**A**) Schematic representation of the constructs. Phosphorylation sites that have been mutated to glutamate are indicated above the sequence by asterisks. The numbering refers to the longest low-molecular-mass tau isoform containing 441 residues. The constructs were prepared from the shortest (352 residues) tau isoform in the central nervous system. The repeat domain is indicated by the thick white box. Adult-specific exons are shown shaded. The positions of the glutamate cluster in the proline-rich region (P-region) and the cluster in the C-terminal tail region (C-region) are indicated by black boxes. (**B**) Electrophoretic mobility of the purified constructs during SDS/PAGE. Note that phosphorylation-mimicking glutamate clusters induce a decreased mobility that is abolished in the presence of urea. (**C**) Immunoblot of the purified constructs. The phosphorylation-mimicking glutamate cluster in the P-domain, which contains the Tau-1 epitope [24], induces a loss of immunoreactivity. The constructs were prepared, expressed, purified and analysed by gel electrophoresis as described in the Experimental section. Samples [1 μg (**B**) or 0.1 μg (**C**)] of the proteins were separated by electrophoresis on 15% (w/v) polyacrylamide with or without 6 M urea. The gels were stained with Coomassie Brilliant Blue (**B**) or processed for immunodetection (**C**) with monoclonal Tau5 or Tau1 antibody and horseradish-peroxidase-coupled anti-mouse secondary antibody. Detection was by enhanced chemiluminescence. The positions of molecular mass markers are indicated (in kDa) at the right in (**B**) and (**C**).

AT180 and PHF1 (recognizing phosphorylated Ser^{202}/Thr^{205}, phosphorylated Thr^{231} and phosphorylated Ser^{396}/Ser^{404} respectively [25–28]) (results not shown). This indicates that the glutamate clusters, although inducing structural changes similar to those induced by phosphorylation in the respective regions, do not mimic the phosphorylated epitopes recognized by these phosphorylation-specific antibodies.

Glutamate cluster in tau's proline-rich region is sufficient to block tau's microtubule-nucleating activity and to reduce tau-dependent process formation

We have previously shown that hyperphosphorylation-mimicking tau mutants fail to promote microtubule nucleation in cell-free assembly assays [18]. To determine whether modifications in the proline-rich region and/or the C-terminal tail region are responsible for this deficiency, microtubule assembly assays were performed with the purified constructs and the reaction products were analysed by immunofluorescence microscopy. Tau 352wt and $352Glu_{5(C)}$ both efficiently promoted the polymerization of microtubules in a dose-dependent manner with a minimum tau concentration required for tubulin polymerization of less than 2 μM tau under the conditions used (15 μM tubulin; Figure 2A). In contrast, only very few microtubules were formed in the presence of constructs with a glutamate cluster in the P-region [$352Glu_{5(P)}$, $352Glu_{10}$], indicating that phosphorylation-mimicking modifications in the proline-rich region are sufficient to block tau's activity to promote microtubule assembly.

Microtubule assembly as measured in our polymerization assay reflects the behaviour of a population of microtubules that is the product of microtubule number and length. To compare the efficiency of the constructs to promote microtubule nucleation *de novo*, the number of microtubules in the assay reaction was plotted against the concentration of the different constructs. We observed no striking difference between the activities of 352wt and $352Glu_{5(C)}$ in assembling an increasing microtubule number in a dose-dependent manner (Figure 2B). In contrast, only very few microtubules were polymerized in the presence of $352Glu_{5(P)}$ and $352Glu_{10}$. To compare the activity of the constructs in promoting microtubule elongation, the length distribution of the assembled microtubules was determined. For these experiments, to limit microtubule nucleation a low tau concentration was used in the reaction because it has previously been shown that a high nucleation rate decreases the elongation of individual microtubules [10]. All constructs promoted the formation of microtubules with similar lengths (Figure 2C). This is consistent

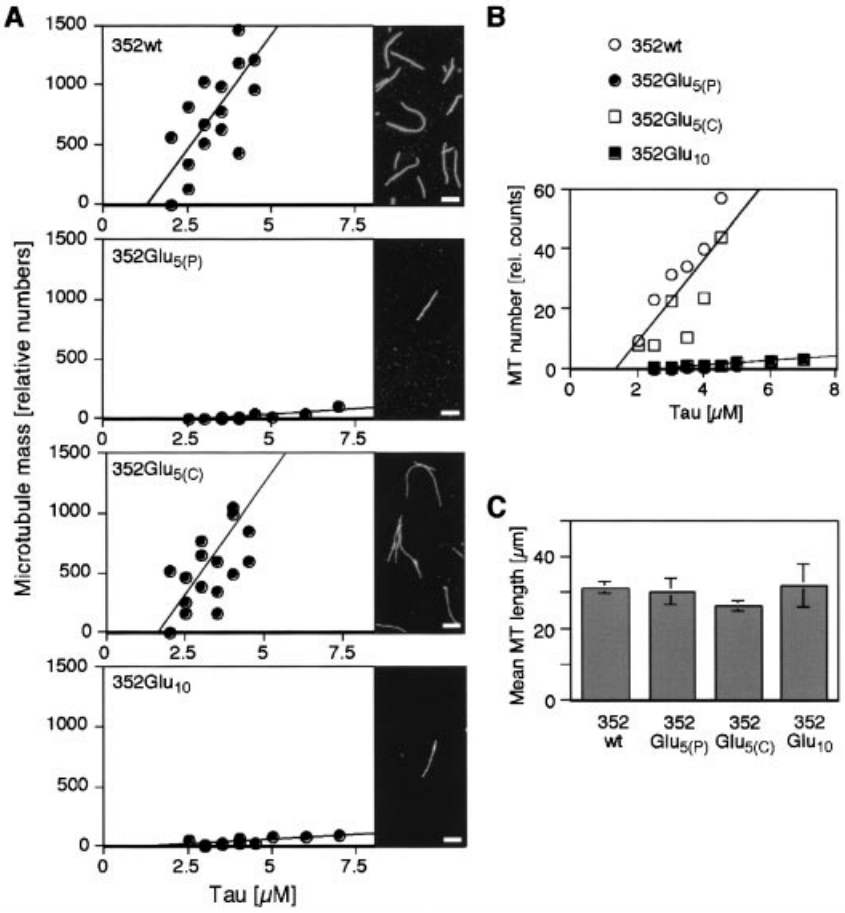

Figure 2 Activity of phosphorylation-mimicking tau constructs to promote microtubule assembly *in vitro*

(**A**) Effect of the constructs on the assembly of total microtubule mass. Typical fluorescence images of assembled microtubules are shown at the right. (**B**) Effect of the tau constructs on the number of assembled microtubules. Note that phosphorylation-mimicking glutamate clusters in the P-region are sufficient to block almost completely tau's activity to nucleate microtubules *de novo*. (**C**) Effect of the constructs on the mean length of the assembled microtubule population. Cell-free microtubule assembly assays were performed in the presence of 15 μM tubulin and purified tau as indicated for 10 min at 37 °C. The assembled microtubules were fixed, collected by centrifugation and processed for immunofluorescence as described in the Experimental section. For (**A**), total microtubule mass was calculated as the product of the number and the mean length of the assembled microtubule population. For (**B**), the number of assembled microtubules was plotted against different concentrations of the tau constructs. For (**C**), the lengths of 14–165 microtubules (MT) from two independent assembly experiments with 3 μM tau were measured. Results are means $\pm$ S.E.M. Immunofluorescence staining employed a monoclonal antibody against tubulin (DM1A) and Cy3-coupled anti-mouse secondary antibody. Scale bars in (**A**), 10 μm.

with the observation that hyperphosphorylation-mimicking tau constructs retain their ability to bind to microtubules as indicated by co-sedimentation assays with Taxol-stabilized microtubules [18]. The results suggest that the tau-dependent formation of a functional nucleation complex is much more sensitive than microtubule elongation to the effect of a phosphorylation-mimicking glutamate cluster in the P-region.

It has previously been shown that the promotion of microtubule assembly by the transfection of exogenous MAPs induces the formation of long processes in undifferentiated cytochalasin-treated PC12 cells [29]. This provides an assay system with which to test the effect of tau on microtubule assembly in a cellular context. To test the effect of the different phosphorylation-mimicking constructs in this model, the cDNA species of the tau mutants were constructed in pRc-CMV, which provides a high cellular expression level of exogenous proteins under the control of the strong cytomegalovirus promoter. The tau constructs were epitope-tagged by fusing a sequence coding for the FLAG epitope at the N-terminal end, which permits the detection of the exogenously expressed protein with commercially available antibodies. The different constructs were transiently expressed in PC12 cells and analysed for their activity to induce process formation 2 days after transfection. We did not observe an obvious difference in the transfection efficiency or the staining intensities of the constructs as judged by visual inspection of the cells, which suggests that all constructs are expressed at comparable levels (Figure 3A, left panel). The expression of tau352wt and 352Glu$_{5(C)}$ induced the formation of long thin cellular processes in approx. 40 % of the transfected cells after application of the F-actin-disrupting drug cytochalasin B. In the absence of exogenous tau expression, only a minority of the cytochalasin-treated cells extended processes. The ratio of tau-transfected cells with processes was 40–50 % lower in cells expressing 352Glu$_{5(P)}$ or 352Glu$_{10}$ than in cells expressing wild-type tau. Thus phosphorylation-mimicking glutamate clusters that block microtubule nucleation in cell-free assembly assays also decrease process formation in this cellular model and vice versa. The results indicate that modifications in the proline-rich region are sufficient to decrease process formation to the state observed for the PHF-tau-like construct (352Glu$_{10}$).

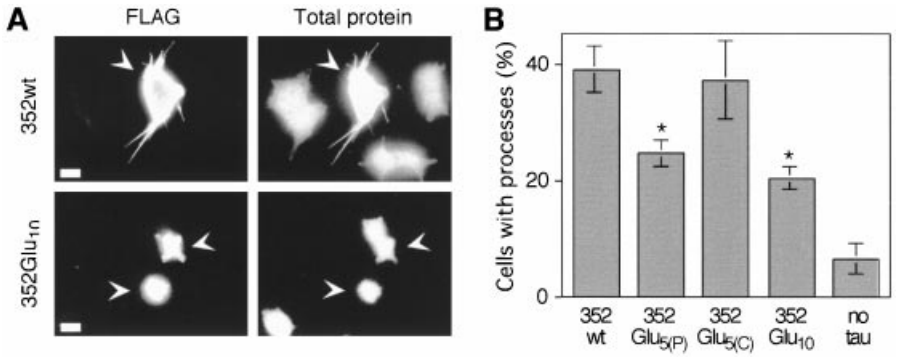

Figure 3 Activity of phosphorylation-mimicking tau constructs in inducing processes in transfected PC12 cells

(**A**) Immunofluorescence micrograph of cytochalasin B-treated undifferentiated PC12 cells that have been transfected with wild-type tau (top panels) or the PHF-tau-like construct ($352Glu_{10}$; bottom panels). The cells were double-stained against the FLAG epitope and total protein. In each frame, cells that expressed exogenous tau protein as judged from intense anti-FLAG immunofluorescence are indicated by arrowheads. A representative cell with multiple processes after tau expression is shown at the top. (**B**) Quantification of the effect of tau expression on the induction of processes. Note that a phosphorylation-mimicking glutamate cluster in the P-region significantly decreased the ratio of transfected cells with processes. The constructs were prepared, expressed and analysed as described in the Experimental section. Immunofluorescence staining employed monoclonal anti-FLAG antibody (M5) and a total protein stain (DTAF). For each construct, between 278 and 1014 tau-expressing cells from four or five independent experiments were evaluated for process formation. Results are means ± S.E.M. *Significant difference ($P < 0.05$) in values from the 352wt result as determined with a paired Student's t test. Scale bars in (**A**), 10 μm.

Phosphorylation-mimicking glutamate clusters that block tau's microtubule-nucleating activity also inhibit tau's association with PP2A and abolish binding to the neural membrane cortex

Tau binds to the dominant brain phosphatase PP2A isoform ABαC, an activity that might also be important for the regulation of tau's phosphorylation state [12]. Interestingly, PHF-tau-like tau constructs were deficient in binding to ABαC [18]. To determine which modifications were responsible for this deficiency, binding assays were performed *in vitro* with purified ABαC and the different tau constructs; the binding of PP2A to tau was monitored by mobility shifts during native gel electrophoresis. Constructs 352wt and $352Glu_{5(C)}$ bound to ABαC, as indicated by the shift of the ABαC band to a tau-containing complex with a higher molecular mass (Figure 4A). In contrast, neither the PHF-like tau construct ($352Glu_{10}$) nor $352Glu_{5(P)}$ interacted with ABαC. The results indicate that phosphorylation-mimicking glutamate clusters that block tau's activity in microtubule assembly also inhibit its interaction with the PP2A isoform ABαC, suggesting a crucial role of phosphorylation in the proline-rich domain also for tau's interaction with this phosphatase.

In neural cells, tau interacts with the membrane cortex through its N-terminal domain in a phosphorylation-dependent manner [14,15]. To determine whether modifications in the proline-rich region and/or the C-terminal tail region affect tau's membrane association, stable PC12 lines were produced that constitutively express the different tau constructs; we used a recently developed protocol to isolate plasma-membrane-associated tau by the separation of surface-biotinylated cells with microspheres [15]. The plasma membrane fraction contained a significant amount of actin as would be expected for a fraction containing plasma membranes and a submembrane cortex (Figure 4B). Tubulin, which behaves as a cytosolic protein under the conditions used for the fractionation, was almost completely absent from the plasma membrane fraction, indicating a very low degree of contamination with cytosolic proteins. A significant portion of tau was present in the plasma membrane fraction isolated from cells stably transfected with wild-type tau (352wt). In contrast, $352Glu_{10}$, $352Glu_{5(P)}$ and $352Glu_{5(C)}$ were completely absent from the plasma membrane fraction. The results indicate that modifications in the proline-rich region or the C-terminal tail region are each sufficient to block tau's association with the plasma membrane. The results suggest that tau's activity in microtubule assembly and its binding to the membrane cortex are differentially affected by tau modifications and open the possibility that, in a neuron, both activities are differentially regulated by phosphorylation in distinct regions.

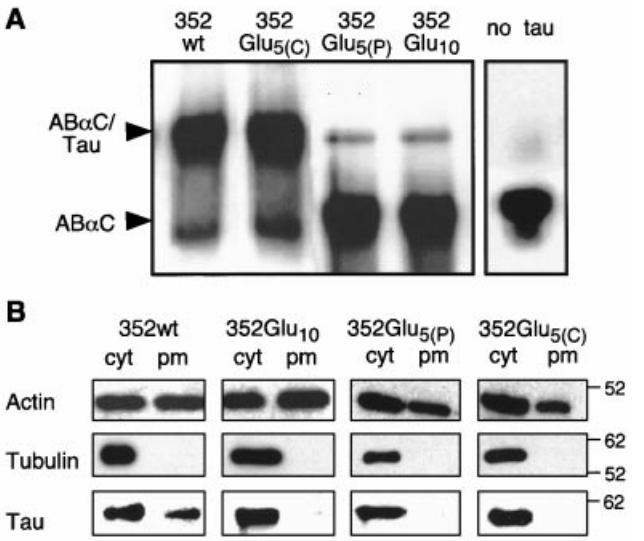

Figure 4 Binding of phosphorylation-mimicking tau constructs to the ABαC PP2A heterotrimer and to the membrane cortex

(**A**) Gel-shift assays for the analysis of tau–ABαC binding. Note that constructs containing a phosphorylation-mimicking glutamate cluster in the P-region failed to interact with PP2A. (**B**) Distribution of actin, tubulin and tau in the cytosolic (cyt) and plasma membrane (pm) fractions after the use of microspheres to separate surface-biotinylated PC12 cells stably transfected with the indicated construct. Note that tau is present in the plasma membrane fraction only in cells expressing wild-type tau. Gel-shift assays in (**A**) were performed and developed with an antibody directed against PP2A as described in the Experimental section at a tau-to-PP2A ratio of 10:1 (left panel) or in the absence of tau (right panel). Separation with microspheres and immunoblot analysis were performed as described in the Experimental section. Samples [2% (v/v) (tubulin and actin) or 30% (v/v) (tau)] of the fractions were separated by SDS/PAGE [10% (w/v) gel], immobilized on PVDF, stained with monoclonal antibodies against actin, tubulin, tau (Tau5) and horseradish-peroxidase-coupled anti-mouse secondary antibody, and detected with enhanced chemiluminescence. An independent experiment gave very similar results.

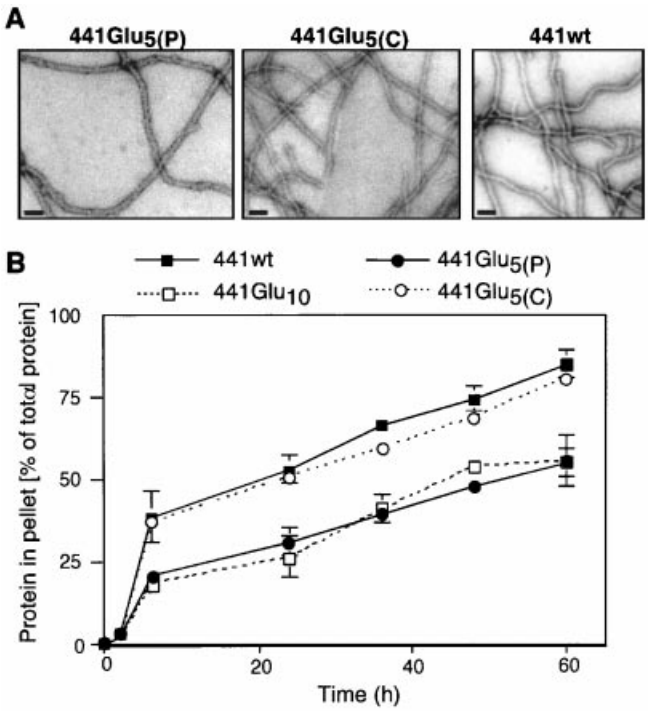

Figure 5 Filament formation of phosphorylation-mimicking tau constructs *in vitro*

(**A**) Electron micrographs of tau filaments that were assembled from phosphorylation-mimicking constructs with a modified P-region (left panel) or C-region (middle panel) and from wild-type tau (right panel). All constructs assembled into straight filaments with similar morphologies. (**B**) Time course of tau aggregation from the different constructs. Note that the constructs with a phosphorylation-mimicking glutamate cluster in the P-region exhibited decreased filament formation. Tau assembly assays, electron microscopy and quantification of filament formation were performed as described in the Experimental section. Tau assemblies were performed with 1 mg/ml tau for 2 days (**A**) or for the durations indicated (**B**). Means ± S.D. for a total of three experiments with two independent tau purifications are shown. Scale bars in (**A**), 100 nm.

Phosphorylation-mimicking glutamate clusters in the P-region, but not in the C-region, decrease tau filament assembly

A characteristic histopathological feature of tauopathies is the presence of intracellular filaments composed of hyperphosphorylated tau protein. We have shown previously that the simulation of a PHF-tau-like hyperphosphorylation decreases tau aggregation by approximately one-third [18]. Adult-specific tau isoforms containing 441 residues were chosen for the experiments because, in our previous experiments, they produced filaments more reproducibly than the fetal tau isoform. To determine the region whose modification was responsible for the decrease in aggregation, $441Glu_{5(P)}$ and $441Glu_{5(C)}$ were prepared, in which phosphorylation-mimicking glutamate clusters were introduced in the P- or C-region similarly to those in the fetal tau constructs. $441Glu_{5(P)}$ and $441Glu_{5(C)}$ assembled into straight filaments with similar morphologies as judged by electron microscopy (Figure 5A). We observed no obvious morphological difference from filaments that were assembled by wild-type tau (441wt) or the PHF-tau-like construct ($441Glu_{10}$). $441Glu_{5(C)}$ aggregated in a time-dependent manner very similar to that of the wild-type tau construct (441wt) (Figure 5B). In contrast, the aggregation of $441Glu_{5(P)}$ was decreased by approximately one-third at all time points; its kinetics was very similar to that of $441Glu_{10}$ aggregation. The results indicate that phosphorylation-mimicking glutamate clusters in the proline-rich region that block tau's activity in microtubule assembly are also responsible for the decreased activity in aggregating into filaments. Glutamate clusters in the C-terminal tail region of tau are neutral with regard to both activities.

DISCUSSION

A stoichiometric increase in the phosphate content of tau protein (hyperphosphorylation) is a pathological hallmark of AD and other tauopathies. It has been shown that most of the sites that are phosphorylated to a high degree in tau isolated from PHFs from patients with AD are clustered in two regions that flank tau's repeat domain N-terminally and C-terminally [5,7]. Many AD-diagnostic antibodies, for example AT8, AT180 and PHF-1, specifically recognize phosphorylated epitopes within these two regions. This could suggest that region-specific phosphorylation clusters have an important role in conferring the structural and functional changes that have been observed for tau in AD.

The functional analysis of the role of individual tau phosphorylation sites or region-specific phosphorylation clusters presents several practical problems: region-specific tau phosphorylations are difficult to produce *in vitro* and usually result in a population of many differentially phosphorylated tau species. In addition, in cells, the effect of a defined phosphorylation pattern cannot be analysed directly because tau phosphorylated *in vitro* would quickly be dephosphorylated after microinjection into the cell. We have previously shown that replacement of serine residues with negatively charged amino acids simulates structural and functional aspects of site-specific tau phosphorylation or hyperphosphorylation [17,18], indicating that phosphorylation-mimicking constructs provide a useful model for the analysis of structural and functional consequences of tau phosphorylation and to identify the region(s) whose phosphorylation critically affects tau's properties. However, it should be noted that the phosphorylation-mimicking constructs were not immunoreactive with the AD-diagnostic antibodies AT8, AT180 and PHF1, which detect phosphorylated epitopes in the mutated regions, indicating that these antibodies require a phosphoserine or phosphothreonine residue for immunoreaction that cannot be simulated by replacement with glutamate.

By using constructs in which we have simulated PHF-tau-like phosphorylation clusters in the two regions that flank tau's microtubule-binding repeats, we demonstrate that modifications in the P-region suffice to block the ability of tau to promote microtubule nucleation *in vitro* and to decrease process induction in cells. In contrast, the same number of mutations in the C-region were neutral with regard to both activities. It is unclear whether tau's activity to promote microtubule nucleation has a role in a postmitotic neuron that has a well-established microtubule network. Previous results have suggested that the formation of a functional nucleation complex is much more sensitive to the effect of tau phosphorylation than microtubule growth [17], an effect that might be important for the activities of tau protein in a cellular context. For example, despite a similar activity in promoting microtubule growth, hyperphosphorylation-mimicking tau protein was displaced almost completely from the microtubule surface by equimolar concentrations of wild-type tau protein [18], indicating a different mode of its interaction with microtubules.

Modifications in the P-domain that decreased tau's activity in microtubule assembly were also sufficient to block tau's binding to the dominant brain phosphatase PP2A isoform ABαC. This is consistent with previous results showing that the sites through which tau interacts with ABαC and with microtubules are mutually indistinguishable [13], and suggests that both activities

are also regulated in parallel. Because the relative affinities of PP2A for tau are correlated with the tau phosphatase activity [12], phosphorylation in the P-region would also inhibit a PP2A-dependent dephosphorylation of tau protein at sites other than those in the P-region. The results suggest that P-domain phosphorylation might serve as a regulator of tau's interaction with microtubules and the PP2A-dependent dephosphorylation of tau. This might indicate an important role of disease-related phosphorylation events within the P-domain in mediating the functional loss and the hyperphosphorylation of tau observed during AD.

It is known that tau filament assembly occurs through the repeat domain, which also constitutes the primary microtubule interaction site [30,31]. Phosphorylation *in vitro* with kinases that led to a detachment of tau from microtubules also inhibited tau aggregation and vice versa [16], suggesting that phosphorylation affects both interactions in parallel. Here we report that phosphorylation-mimicking glutamate clusters in the P-region are sufficient to decrease, but not to block completely, the aggregation of tau protein *in vitro* to a state observed for the PHF-like tau construct. In contrast, a glutamate cluster in the C-terminal tail region was neutral with regard to tau filament assembly. Thus the results indicate that all activities that are mediated through tau's repeat domain (the interaction with microtubules, process formation in transfected cells, binding to PP2A and the aggregation of filaments) are also affected in parallel by a phosphorylation-mimicking glutamate cluster in the P-domain. However, it should be noted that P-region modifications decrease tau aggregation by a moderate 30 %, in contrast with the more drastic effect of this modification on tau's microtubule-nucleating and PP2A-binding activity. Thus the increase in the concentration of free tau as the result of tau's functional loss of activity in promoting microtubule nucleation and in binding PP2A might easily compensate for the relatively moderate decrease in the tendency of hyperphosphorylated tau to form filaments in neurons.

We have shown previously that tau interacts with the neural membrane cortex through its N-terminal non-microtubule-binding projection domain [14]. Thus, tau's interactions with microtubules and the neural membrane cortex are mediated by distinct domains, raising the possibility that tau functions as a linker protein between the microtubule system and the cortical cytoskeleton in the axon. Here we show that phosphorylation-mimicking glutamate clusters in the P-region or the C-region are both sufficient to confer a complete loss of tau's ability to interact with the neural membrane cortex. Because the presence of a glutamate cluster in the C-region was neutral with regard to tau's activity to promote microtubule nucleation and binding to ABαC, C-region phosphorylation might have a role in selectively regulating tau's interaction with the membrane cortex. It seems surprising that a glutamate cluster in the C-terminal tail region abolishes tau's interaction with the membrane cortex, which occurs through a region that is located at the opposite end of the molecule. However, it is conceivable that tau's ability to interact with the membrane cortex is highly sensitive to conformational changes of the whole protein that are induced by modifications in either the P-region or the C-region. In fact, the glutamate cluster in the C-region results in a much more pronounced decrease in the electrophoretic mobility than for a construct with P-region mutations, which might indicate that C-region phosphorylation induces marked conformational changes in tau.

The functional role of tau's association with the neural membrane cortex is unclear. However, because the binding of tau phospho-isoforms to the membrane cortex is correlated with its enrichment in the axon, tau's membrane association might be involved in localizing tau to the distal axon, where it is particularly enriched during axon formation and elongation [15,32,33]. If this is true, a loss of tau's ability to interact with the membrane cortex as a result of phosphorylation in the P-region or the C-region could result in a redistribution of tau towards the somatodendritic compartment, an event that is characteristic of the development of tau pathology during AD [34]. It will be interesting to determine whether disease-related mutations in the C-domain of tau protein such as the exchange of Arg^{406} for tryptophan, which has been observed in cases of the inherited dementia FTDP-17 [35], also affect tau's association with the membrane cortex and could contribute to the mislocalization of tau protein during disease.

In addition, phosphorylation sites outside of the P-region or the C-region that were not addressed in this study might be important in regulating the functional activity of tau protein. For example, the phosphorylation of Ser^{262}, which is located in the repeat region and which abolishes tau's association with microtubules, renders microtubules dynamically unstable and inhibits tau aggregation, might be functionally relevant [16,36]. It will be interesting to analyse the effect of phosphorylation of Ser^{262} in conjunction with a glutamate cluster in the P-domain or the C-domain to assess potential functional interferences that are caused by modifications in different regions of tau protein.

Phosphorylation sites *in vitro* of many kinases have been identified and can be classified with regard to the phosphorylation of specific regions [4]. Proline-directed protein kinase (p34cdc2/p58cyclinA) mediates phosphorylation exclusively in the P-region, whereas calmodulin kinase acts only on sites in the C-region. Phosphorylation in both regions is mediated by tau protein kinase I/glycogen synthase kinase 3β, which has been proposed to have a role in the amyloid β-induced neurotoxicity [37] and by glycogen synthase kinase 3α. Other kinases such as protein kinase A, which phosphorylates tau *in vitro* and is tightly associated with the neurofibrillary pathology [38], mediate phosphorylation in tau's microtubule-binding domain and in both the P-region and the C-region. Recently, evidence has been provided for an important role for the activation of cyclin-dependent kinase 5 (Cdk5) by a truncated fragment (p25) of its regulatory subunit p35 during neurodegenerative processes [39]. Cdk5 phosphorylates tau at four positions in the P-region and at two positions in the C-region; it inhibits tau's activity in promoting microtubule assembly [40]. In addition to the action of kinases, it has been demonstrated that tau's phosphorylation state depends on the activity and distribution of phosphatases [6,12] and might itself have a role in localizing phosphatases to particular subcellular domains [13,41]. Because our results indicate that P-region phosphorylation has a crucial role in mediating the functional deficiencies observed for PHF-tau, kinases and phosphatases acting on this part of the protein should be at the focus of further studies.

It is not known how the tau pathology is linked to the degeneration of neurons, although this would obviously be very important because it could provide a potential target for therapeutic drugs. In particular, it is important to determine whether a functional loss of tau protein, as a result of hyperphosphorylation and/or tau aggregation, results in neuronal degeneration or whether aberrantly phosphorylated tau protein itself gains cytotoxicity. Here we have shown that glutamate clusters that mimic a PHF-like hyperphosphorylation in the P-region or the C-region have distinct effects on tau's functional activities. Analysing the effect of these constructs in a neuronal context might yield important information on how the distinct functional deficiencies that are induced by these modifications contribute to neuronal degeneration and how the modifi-

cations of these regions have a crucial role in the disease process.

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