Mitotic phosphorylation of tau protein in neuronal cell lines resembles phosphorylation in Alzheimer's disease

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Introduction

The organization of microtubules changes markedly depending on their different functions in the cell cycle [56]. In interphase cells microtubules are responsible for the maintenance of cellular architecture and for intracellular transport, whereas during mitosis they form the spindle apparatus and ensure the accurate segregation of the chromosomes. The transition from interphase to mitosis requires the rapid reorganization of the microtubule network [37] and is accompanied by changes in the dynamic properties of the microtubules [5, 67]. Microtubule-associated proteins (MAPs) are factors which control microtubule dynamics. Their functions include the stabilization of microtubules, spacing between microtubules and other cell components, or anchoring of enzymes [32, 55, 68]. At the onset of mitosis, which correlates with a wave of phosphorylation, MAPs and other proteins become phosphorylated [53, 59, 63, 76]. Therefore, it is believed that the phosphorylation of MAPs could be a mechanism by which the binding to microtubules is weakened so that they become more dynamic and ready for the rearrangement of the cytoskeleton [16, 17, 51]. The details of this hypothesis still have to be worked out; at present, the MAPs involved, the phosphorylation sites affected, and the kinases are known only in part or not at all.

Tau is one class of microtubule-associated proteins and occurs predominantly in the axonal compartments of neurons [9, 42]. This protein belongs to the family of structural MAPs comprising tau, MAP2 and MAP4. Although these proteins originate from different genes, they share a comparable domain structure, in particular several repeated motifs of –31 residues near the C-terminus that are involved in microtubule binding [1, 19, 24, 31, 34, 47]. In Alzheimer’s disease tau protein becomes hyperphosphorylated, notably at Ser-Pro and Thr-Pro motifs and forms the basis for the paired helical filaments (PHFs). Thus, it is likely that hyperphosphorylation of tau is a key event for neuronal degeneration which effects, among others, the organization of the cytoskeleton, leading to deposition of hyperphosphorylated tau into PHFs.

Abbreviations: AD Alzheimer’s disease, CHO cells Chinese hamster ovary cells, MAPs Microtubule-associated proteins, MEM Minimal essential medium, NFTs Neurofibrillary tangles, PHFs Paired helical filaments, SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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To elucidate possible mechanisms for the hyperphosphorylation of tau in Alzheimer's disease, it is necessary to understand the phosphorylation of tau under physiological conditions. Hence, we started an approach by transfecting the largest human tau isoform (htau40) into CHO cells which do not contain endogenous tau protein [63]. We found that in transfected interphase cells, tau is in a state of low phosphorylation, whereas in mitotic cells it became hyperphosphorylated and part of it was detached from microtubules. The similarity between the phosphorylation of tau in dividing cells and Alzheimer degenerated neurons prompted us to investigate the phosphorylation of tau in cells of neuronal origin.

In the present study we have stably transfected a murine neuroblastoma cell line (N2a) with htau40, the largest of the six human tau isoforms in the brain. We show that tau protein from N2a cells is hyperphosphorylated during mitosis similar to Alzheimer's disease tau, monitored by gel shift, immunoblotting and immunofluorescence, using the antibodies Tau-1, AT8, AT180, and PHF-1, which are sensitive to the phosphorylation of Ser202, Thr205, Thr231, Ser235, Ser396, and Ser404 and are used in the diagnosis of Alzheimer tau. Furthermore, we demonstrate that this hyperphosphorylation also occurs in mitotic human neuroblastoma cells (LAN-5), which contain endogenous tau protein. We suggest that the analogy between the phosphorylation of tau in dividing cells and Alzheimer degenerated neurons could be the result of an inappropriate attempt of ageing neurons to re-enter the cell cycle leading to cell death instead of rejuvenation.

Materials and methods

Antibodies

Several monoclonal antibodies used in this study were generous gifts by the following colleagues: Tau-1 clone by Dr. L. Binder (Northwestern Univ., Evanston, IL, USA); AT8 and AT180 by Dr. A. Vandervoordt and Dr. E. Vanmechelen (Innogenetics, Ghent, Belgium); PHF-1 by Drs. S. Greenberg and P. Davies (Albert Einstein College of Medicine, Bronx, NY, USA); T46 by Dr. VM.-Y. Lee (University of Pennsylvania, Philadelphia, PA, USA). The monoclonal antibody MMP-2 was obtained from UBI (Lake Placid, USA) and the rat monoclonal anti-tubulin antibody YL1/2 (recognizing the C-terminus of tyrosylated a-tubulin, [39]) from Sera Lab (Sussex, England). The antibody epitopes on tau protein are shown in Fig. 1: The tau antibodies Tau-1 [9] and T46 [41] are directed against unphosphorylated epitopes, AT8 [57], AT180 [28] and PHF-1 [29] recognize phosphorylated epitopes. The sites of Tau-1 and AT8 are nearly complementary to one another around residue 200; Tau-1 reacts when there is no phosphorylation in this region [41], AT8 reacts when there are two phosphorylation sites, Ser202 and Thr205 in full-length tau [7, 27, 72, 81]. AT180 reacts with phosphorylated Thr231 and Ser235 [28, 81] and PHF-1 reacts with phosphorylated Ser396 and Ser404 [44, 61]; in this regard the antibody is similar to SM131 [50]. The monoclonal antibody MMP-2 reacts with a set of phosphorylated proteins present in mitotic cells, presumably at Ser-Pro motifs [20, 77].

Cell culture and transfection

The methods used here were mostly described previously [63]. N2a cells were grown in MEM and LAN-5 cells in RPMI medium supplemented with 1% l-glutamine and 10% fetal calf serum (Biochrom, Berlin, Germany). The full-length coding region of htau40 was sub-cloned into the NdeI/BglII site of the modified multiple cloning site of pRe/CMV (Invitrogen, San Diego, CA, USA). Approximately 70% confluent cells were seeded in 24-well plates for transfection or on coverslips for immunofluorescence analysis and incubated at 37°C with 5% CO2. The cells were transfected with 1µg of DNA and 2µl of Lipofectamine according to the manufacturer’s recommendations (Gibco/BRL, Eggenstein, Germany). Stably transfected cells were selected in the presence of Geneticin (G-418). After incubation for additional 2 to 3 weeks, cells were cloned by limiting dilution and screened for htau40-expressing cells by immunofluorescence.

Immunofluorescence

Cells were washed in a stabilizing buffer (80 mM Pipes, 1 mM MgCl2, 1 mM EGTA, 4% (w/v) polyethylene glycol, pH 6.9) and fixed with methanol at -20°C for 5 min. The cells were treated with 5% non-fat dry milk for 1 h and incubated with the mouse monoclonal antibodies T46, Tau-1, AT8, AT180, PHF-1, and MMP-2 at 1:600, 1:50, 1:200, 1:100, 1:400, and 1:200 dilutions, respectively, and the rat monoclonal anti-tubulin antibody YL1/2 at 1:200 for 1 h at 37°C. For the secondary antibodies fluorescein-conjugated (FITC) goat anti-mouse or rhodamine-conjugated goat anti-rabbit antibodies (Dianova, Hamburg, Germany) were used at 1:300 dilution and incubated at 37°C for 30 min. The cells were examined with an Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a 63X immersion objective.

Cell extracts

Extracts of N2a cells transfected with htau40 and of LAN-5 cells were prepared from mitotically arrested cells following treatment with 0.2 µg/ml nocodazole (Sigma, Deisenhofen, Germany) for 5 h. Cells were detached by mechanical shake-off and lysed on ice in a buffer containing 50mM Tris (pH 7.4), 1% Nonidet P-40, 1 mM MgCl2, 5mM EGTA, 5mM DTT, 500 mM NaCl, 20mM NaF, 1mM vanadate, 0.1mM PMSF, 1µg/ml leupeptin, 1µg/ml aprotinin, 1µg/ml pepstatin and 10mM benzamid. Extracts were boiled for 10 min and centrifuged at 15,000g for 15 min. The soluable fraction containing heat-stable tau was applied to SDS-PAGE [43]. Protein concentrations were determined by the method of Bradford [12].

SDS-PAGE and immunoblotting

Extract samples of N2a and LAN-5 cells were electrophoresed on 10% SDS-polyacrylamide gels (15 µg protein per lane) and electrophoretically transferred to PVDF-membranes (Millipore, Eschborn, Germany). Residual protein-binding sites on the membrane were blocked...
with 5% non-fat dry milk in TBS/Tween following incubation with the monoclonal antibodies T46 (1:6,000), Tau-1 (1:500), AT8 (1:5,000), PHF-1 (1:400) and AT180 (1:1,000). Bound antibody was detected with peroxidase-conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany) using diaminobenzidine as substrate. For immunoblot analysis recombinant htau40 and htau23 from E. coli were isolated on the basis of their heat stability and by FPLC Mono S (Pharmacia) chromatography. Phosphorylation reactions were carried out as described [22].

Results

Phosphorylation of tau in neuroblastoma cells in interphase and mitosis

Murine neuroblastoma cells (N2a) are dividing cells of neuronal origin that contain only a very low level of endogenous tau (a few ng per 10^6 cells, Illenberger, personal communication). These cells are therefore suitable for analyzing transfected tau from other sources against a low endogenous background. N2a cells were stably transfected with the largest tau isoform from human CNS (htau40, 441 residues) which yielded about 200–300 ng of tau per 10^6 cells. They were analysed 42 h after plating on coverslips by immunofluorescence microscopy using several monoclonal tau antibodies and tubulin antibodies. Fig. 2A and B show the result of the double-immunofluorescence staining of tau transfected N2a cells with the antibody T46 and the tubulin antibody YL1/2. The antibody T46 (Fig. 2A), which reacts independently of the phosphorylation state of tau, recognized all transfected cells equally well, as shown by the double-staining with the tubulin antibody YL1/2 (Fig. 2B). This antibody was used in all immunofluorescence experiments to demonstrate the co-localization of tau protein to cellular microtubules. Note that after transfection with tau strong microtubule bundles were formed (e.g. Fig. 2A, B), due to the increased concentration and nucleation capacity of tau in the cytoplasm of the cells. This phenomenon has been observed with several MAPs after transfection [25, 35, 49, 63].

Tau-1, an antibody which binds in a region around residue 200 when it is not phosphorylated [7, 28] stains microtubule-associated tau in a manner similar to T46 in interphase N2a cells, indicating that the epitope is in an unphosphorylated state (data not shown). By contrast, mitotic N2a cells show no discernible Tau-1 staining (Fig. 2C, arrowheads), whereas the tubulin antibody (Fig. 2D) stained cells in all stages of the cell cycle. These results are consistent with our previous findings with tau-transfected CHO cells, indicating that mitotic tau protein is phosphorylated at the Tau-1 site.

To specify the phosphorylation of tau during mitosis, we used antibodies raised against Alzheimer's PHFs with known epitopes that discriminate between "normal" tau and the hyperphosphorylated "pathological" forms, and thus are useful in the diagnosis of Alzheimer's disease [9, 28, 29, 48, 57]. Most of these antibodies recognize epitopes that contain Ser-Pro or Thr-Pro motifs of tau. The result with the phosphorylation-dependent antibody AT180 (directed against phosphorylated Thr231 and Ser235; [28, 81]) is seen in Fig. 2E. AT180 specifically reacts with mitotic cells, but not with non-mitotic cells (compare the tubulin staining of all cells in Fig. 2F). This clearly demonstrated that in mitosis, tau protein is phosphorylated at Thr231 and Ser235. Part of the tau protein phosphorylated at these sites still can bind to microtubules but most of the phosphorylated tau is detached from microtubules (data not shown, compare Fig. 6 in [64]). Non-transfected mitotic N2a cells, which only contained endogenous tau protein showed no reaction with the AT180 antibody, due to the low concentration of endogenous tau in these cells (data not shown).

To provide further evidence for cell cycle-dependent phosphorylation, we tested other monoclonal antibodies, recognizing additional phosphorylated epitopes of tau. The phosphorylation-dependent antibody AT8 recognizes phosphorylated Ser202 and Thr205 [7, 27, 81]). This antibody is of special interest because it reacts in a nearly complementary fashion to the antibody Tau-1 (see above); Tau-1 only reacts if

![Figure 2](image-url)
the region of tau around residue 200 is completely unphosphorylated, whereas AT8 recognizes phosphorylated Ser202 and Thr205. Therefore, the increase of AT8 staining and the decrease of Tau-1 staining can be used to demonstrate the appearance of PHF-like phosphorylation. The antibody AT8 specifically labeled mitotic cells (see detailed Fig. 3), analogously to the antibody AT180 in Fig. 2E, whereas the tubulin antibody showed the typical microtubular staining of all cells. Similar results were observed with the phosphorylation-dependent antibody PHF-1. This antibody reacts when Ser396 and Ser404 are phosphorylated (44, 61). The antibody PHF-1 preferentially stained transfected mitotic cells, but also showed weak reaction with non-mitotic cells (data not shown).

In order to compare the reactivity of the phosphorylation-dependent tau antibodies with a marker for mitotic phosphoproteins, we also performed immunofluorescence analysis with the monoclonal antibody MPM-2. This antibody raised against a mitotic Hela cell extract [20], identifies a phosphopeptide highly conserved in mitotic proteins of all eukaryotic cells [23, 38, 75, 76, 77]. Moreover, MPM-2 selectively stains neurofibrillary lesions of AD brains and reacts with neurofibrillary tangles (NFTs), senile plaque neurites and neuropil threads, but has no detectable reactivity in normal brain [78, 40]. As expected, MPM-2 strongly stained mitotic N2a cells (data not shown), but failed to recognize the neighbouring interphase cells, reminiscent to the reactions of the phosphorylation-dependent tau antibodies.

Summarizing our findings, we conclude that the residues 202, 205, 231, 235, 396, and 404 of tau, all of them within Ser-Pro or Thr-Pro motifs, became preferentially phosphorylated during mitosis. Furthermore, the serines 396 and 404 were phosphorylated to a small extent even in non-mitotic transfected N2a cells.

**Stages of mitosis and tau phosphorylation**

Next we wanted to correlate the phosphorylation state of tau with the individual stages of the cell cycle. Therefore, we examined mitotic N2a cells by immunofluorescence microscopy with the phosphorylation-dependent antibody AT8, because this antibody exclusively reacts with cells undergoing mitosis (Fig. 3, left panels). Progression through mitosis was monitored by the dynamic-phase-specific changes in the microtubular network visualized with the anti-tubulin antibody YL1/2 (Fig. 3, right panels). Immunoreactivity with the antibody AT8 was observed through all stages of mitosis from prophase (B) until telophase (F), but the intensity of the immunoreaction varied. Staining by the antibody AT8 appeared at early prophase and increased during metaphase (B and C, respectively). From anaphase on (D), AT8 staining decreased and remained only detectable in the midbody region of the dividing cells (F). After telophase, at the beginning of cytokinesis, AT8 staining diminished and was not observed in daughter cells (G). In comparison, the tubulin antibody (right panels) showed no differences in immunoreactivity throughout the cell cycle. As expected, the antibody Tau-1, that reacts in a complementary fashion to AT8, showed the opposite immunoreaction during mitosis: no staining from early prophase until early telophase, but Tau-1 staining strongly increased after telophase (data not shown). These data clearly demonstrated that upon entry into and exit from mitosis neuroblastoma cells showed exactly the same cycle of tau phosphorylation and dephosphorylation as tau-transfected CHO cells [63].

**Multiple phosphorylation in tau-transfected N2a cells**

The results obtained by immunofluorescence microscopy indicated that tau protein was highly phosphorylated during mitosis in vivo. To verify this at the biochemical level, we isolated tau from interphase and mitotically arrested cell populations and checked their phosphorylation state by SDS-PAGE and Western blotting, using the same set of antibodies. On SDS gels, phosphorylated tau can shift markedly to higher Mr val-
ues, depending on the phosphorylation sites. This type of Mr shift can also be observed with Alzheimer's disease tau, and has long been recognized as one of the hallmarks of the Alzheimer state of tau [30, 48, 50]. We applied the heat-stable fractions of transfected interphase and mitotic N2a cells onto SDS-gels, immunoblotted the proteins and probed the PVDF membrane with different tau antibodies (Fig. 4). As references, lane 1 and 2 show recombinant htau40, unphosphorylated and phosphorylated with a mouse brain extract. Gained by the phosphorylation-independent antibody T46. The arrows indicate the shift in electrophoretic mobility of tau observed after phosphorylation. Lane 3 and 4 represent tau preparations from interphase and mitotic N2a cells, immunostained with antibody T46. Even though transfection was done with a single tau isoform (giving a single band when expressed in E. coli, lane 1), the phosphorylation-independent antibody T46 stained three distinct tau bands in interphase cells (lane 3) and four tau bands in mitotic cells with a clear shift upward of one tau band due to the phosphorylation of tau in mitosis. Such heterogeneity is characteristic of tau in cells because of its heterogeneous phosphorylation, independently of and in addition to the multiplicity of isoforms (see results on tau-transfected CHO cells [26, 63]).

Although the antibody Tau-1 weakly reacted with the lower protein bands of the mitotic N2a sample (lane 6), no staining of the upper phosphorylated tau band was observed, confirming that in mitosis tau protein was phosphorylated in the region around residue 200. Conversely, the phosphorylation-dependent antibody AT8 only stained the upper band of the mitotic N2a cell extract (lane 8), but totally failed to stain the interphase tau bands in lanes 7, indicating that the mitotic tau protein was almost completely phosphorylated at Ser202 and Thr205. The antibody PHF-1 recognized non-phosphorylated tau protein to some extent (lane 9), but also showed strong reactivity with the upper tau band from mitotic N2a cells (lane 10). The phosphorylation-dependent antibody AT180 reacted similar as the antibody AT8 and only labeled the upper tau band in mitotic cell extracts (lane 12), whereas no reactivity was observed with transfected interphase N2a cells. The antibody MPM-2 showed neither reaction with tau protein from interphase nor from mitotic N2a cells (data not shown), but showed an increase in immunoreactivity with a number of other proteins in the nocodazole-treated N2a cells, similar to the staining of mitotic MSN cells (human neuroblastoma line) as reported previously [78].

**Endogenous neuronal tau protein becomes phosphorylated during mitosis**

Finally we wanted to examine if the cell cycle-dependent hyperphosphorylation also occurs with endogenous tau in neuronal cells. As noted before, N2a cells contain only minute amounts of endogenous tau which is hardly detectable by immunohistochemical methods. This cell line is therefore suitable for identifying transfected tau, but not for studying endogenous tau. We therefore turned to the human neuroblastoma cell line LAN-5 which contains a higher level of endogenous tau, about 20 ng per 10⁶ cells. The protein was analysed biochemically by SDS-PAGE and immunoblotting similar to the transfected N2a cells (see Fig. 4). The LAN-5 cells were arrested with nocodazole, and the heat-stable fractions of interphase and mitotic cells were probed with the different tau antibodies (Fig. 5). Since LAN-5 cells originate from a juvenile tumor [70], we used the shortest of the human tau isoforms (htau23) as a reference to demonstrate the shift in electrophoretic mobility after phosphorylation. Lane 1 shows the unphosphorylated recombinant htau23 and lane 2 the phosphorylated recombinant htau23. Both lanes were stained with the antibody T46. In contrast to the tau-transfected N2a extracts, only a single tau band could be visualized in mitotic and non-mitotic LAN-5 cells after staining with the antibody T46 (lanes 3 and 4), whereby the tau protein from mitotic LAN-5 cells (lane 4) showed a pronounced upward shift in the SDS-gel. The phosphorylation-dependent antibody Tau-1 (lanes 5 and 6) only reacted with tau protein from interphase cells (lane 5) and showed no reactions with mitotic LAN-5 cells (lane 6). The phosphorylation-dependent antibody AT8 reacted complementary to the antibody Tau-1; no reactivity

![Fig. 4](image1.png)  
**Fig. 4.** Phosphorylation of tau in mitotic and nonmitotic transfected N2a cells. The heat-stable fractions of the cell extracts were incubated with different phosphorylation-dependent antibodies. Lanes 1 and 2: recombinant htau40 before and after phosphorylation with mouse brain extract. Lanes 3, 5, 7, 9, 11: interphase extracts of transfected N2a cells; lanes 4, 6, 8, 10, 12 mitotic extracts of transfected N2a cells. Lanes 1-4 were probed with antibody T46; lanes 5-6 with antibody Tau-1; lanes 7-8 with antibody AT8; lanes 9-10 with antibody PHF-1, and lanes 11-12 with antibody AT180. The arrows indicate the shift in electrophoretic mobility of tau protein. I — interphase, M — mitosis.

![Fig. 5](image2.png)  
**Fig. 5.** Immunoblot analysis of mitotic and non-mitotic LAN-5 cells. The heat-stable fractions of LAN-5 cells expressing endogenous tau protein were stained with several tau antibodies: Lanes 1 and 2: recombinant htau23 before and after phosphorylation with mouse brain extract. Lanes 3, 5, 7, 9, 11: interphase extracts of LAN-5 cells; lanes 4, 6, 8, 10, 12 mitotic extracts of LAN-5 cells. Lanes 1-4 were stained with antibody T46; lanes 5-6 with antibody Tau-1; lanes 7-8 with antibody AT8; lanes 9-10 with antibody AT180, and lanes 11-12 with antibody PHF-1. The shift in electrophoretic mobility of tau protein after phosphorylation is indicated by arrows. I — interphase, M — mitosis.
with tau protein from interphase cells (lane 7), whereas the tau band recognized by the antibody AT8 showed the lowest electrophoretic mobility (highest Mr, lane 8). The phosphorylation-dependent antibody AT180, like the antibody AT8, only reacted with mitotic tau protein (lane 10), but failed to recognize tau protein from interphase cells (lane 9). The antibody PHF-1 stained various bands of lower and higher molecular weight besides tau protein (lanes 11 and 12), but the latter still being the most prominent band in interphase and mitotic cell extracts.

These results taken together confirmed the immunofluorescence results and clearly demonstrated that the hyperphosphorylation of tau during mitosis visualized with the phosphorylation-dependent tau antibodies not only takes place in tau-transfected murine neuroblastoma cells but also occurs in human neuroblastoma cells expressing endogenous tau protein.

**Discussion**

The motivation for this study came from the observation that the elevated phosphorylation of tau and its aggregation into paired helical filaments is one of the most reliable indicators of the progression of Alzheimer's disease, an age-related dementia [2, 10]. Hyperphosphorylation appears to be one of the earliest signs of incipient degeneration of a neuron [3, 11, 30]. Like other MAPs, tau is a phosphoprotein even in normal cells and can be phosphorylated by a variety of kinases in vitro [15]. However, a characteristic feature of Alzheimer tau is the increased phosphorylation at Ser-Pro or Thr-Pro motifs which are abundant in tau (17 in the isoform htau40). A number of antibodies raised in different laboratories against Alzheimer tau turned out to recognize specifically such motifs in their phosphorylated state [28, 50] and are therefore suitable for diagnosing the Alzheimer-like state of tau, tau aggregates, or brain tissue (e.g., antibody PHF1 [29]; T3P [48]; AT8 [7, 57]; AT180, [28], AT100, [82] and others). These antibodies are complemented by others which are either independent of phosphorylation and therefore serve as controls (e.g., T46 [41]) or recognize only unphosphorylated epitopes; an example is Tau-1 [9] which is complementary to AT8 so that this pair of antibodies is particularly useful for comparing different states of phosphorylation in the cell, e.g., during interphase and mitosis.

The function of elevated phosphorylation is not well understood at present. There appears to be a consensus that phosphorylation tends to decrease the interaction with microtubules, albeit to different extents, depending on the combination of phosphorylation sites (e.g., 8, 14, 21, 33, 74, 80)). This concept would agree with a scenario whereby tau would become hyperphosphorylated following some extracellular signal (stress, excitotoxicity etc.), detach from microtubules, and then become aggregated into the insoluble PHFs. This model has not yet been proven, in fact, stressing a neuronal cell or exposing it to toxins does not necessarily lead to hyperphosphorylation, e.g., [46], and it has been surprisingly difficult to demonstrate an influence of phosphorylation on the aggregation of tau (in contrast to oxidation which clearly promotes PHF aggregation [69]).

Given this situation we asked whether the "Alzheimer-like" hyperphosphorylation of tau occurred in normal cells, hoping that this would lead to its cellular functions and perhaps to the signals that control the phosphorylation. One unexpected finding came from our earlier studies on CHO cells which were transfected with tau so that biochemical quantitation became possible. In these cells, the phosphorylation became robustly upregulated during mitosis, and in fact, the reaction with some phosphorylation-dependent antibodies such as AT8 was diagnostic for a mitotic state. Thus, tau served as a "flag" for detecting an increased level of phosphorylation during mitosis [63]. Moreover, in the mitotic cells tau became largely detached from microtubules, consistent with the hypothesis mentioned above. Similar results were reported by Vincent et al. [78].

An important criticism of these earlier experiments was that CHO cells were not neuronal cells, and therefore the conclusions could not be applied to neurons and their degeneration. We therefore began to study cells of neuronal origin. In general, differentiated neurons show very little or no staining with antibodies raised against Alzheimer phosphorylated tau [54]. This is largely true even for dividing cells of neuronal origin. However, a more detailed inspection shows that the phosphorylation increases specifically during mitosis. We showed this by using two complementary approaches. In one case we used murine N2a neuroblastoma cells. Their level of endogenous tau is so low that it becomes difficult to analyse tau by biochemical or immunofluorescence methods. However, these cells can be transfected with human tau which becomes amenable to study. The results show that practically all "Alzheimer-like" phospho-epitopes appear during mitosis. In the second approach we used human LAN-5 neuroblastoma cells which contain a low but detectable amount of endogenous tau. Here again the phosphorylation is upregulated specifically during mitosis. We can therefore conclude that the Alzheimer-like phosphorylation occurs in healthy cells, it is not necessarily a sign of degeneration, on the contrary, it indicates a high state of activity. This situation correlates well with the analysis of tau from brain tissues which showed that Alzheimer-like phosphorylation is upregulated in the fetal state, (e.g., [13, 15, 36, 62, 73]). The increase is presumably in part explained by the higher fraction of dividing cells.

What could the elevated phosphorylation mean in functional terms? On one hand it could cause the detachment of tau from microtubules, as in the case of the transfected CHO cells. A counterargument is that phosphorylation at Ser-Pro or Thr-Pro motifs has only a moderate influence on the tau-microtubule interaction, compared to other sites (e.g., Ser262 and Ser214 [74, 33]), and that the cells may have other proteins to destabilize microtubules in the transition to mitosis (e.g., [6]). A second role of the phosphorylation of tau could be to change the anchoring of other proteins, notably kinases and phosphatases. These enzymes are in part localized to the microtubule cytoskeleton, (e.g., PKA [60], MAP kinase [58, 65], GSK-3 [52], cdk5 [4] or phosphatase PP-2A [71]). If anchoring of these enzymes determined their effective activity, as it does for PKA, then a change in activity will be felt foremost in their immediate vicinity, including MAPs such as tau protein.

We may ask what kinases are responsible for the increase of the phosphorylation of tau. Since the sites reacting with the diagnostic antibodies are mainly of the Ser-Pro or Thr-Pro type this points to proline-directed kinases. In the context of mitosis, the kinases cdk2 or one of its relatives would seem most likely [79]. However, the association with other proline-directed kinases such as MAP kinase or GSK-3 makes the situ-
ation complex, especially considering the large number of potential phosphorylation sites. This issue will have to be addressed in separate studies using specific inhibitors.

Finally, returning to our initial question, why should an Alzheimer-like phosphorylation be elevated in dividing neuronal cells? This scenario is attractive; however, the caveat is that the degeneration can cause an appearance similar to mitosis? At present we can only hypothesize that the signals that lead to degeneration may initially lead the cell into an abortive program of cell division. In this context, it has been postulated that the same signals that lead to cell division in cells capable of dividing may lead to apoptosis in cells that can no longer divide [66]. This scenario is attractive; however, the caveat is that the degeneration in Alzheimer's disease does not appear to be apoptotic but rather necrotic [45]. To resolve the issue one will have to analyse the signal transduction pathways affecting the phosphorylation state of tau in neuronal cells as well as other tissues.

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Note added in proof. We have recently found that the site S214 becomes highly phosphorylated in mitotic cells, that phosphorylation at this site can detect tau from microtubules, and that the site can be phosphorylated by PKA [33]. Interestingly, this site is also part of the phosphorylated epitope of antibody AT100, one of the most Alzheimer-specific antibodies known thus far [82]. These observations further strengthen the hypothesis that there is a link between tau phosphorylation in mitotic and in degenerating cells.

References


