

Hyperphosphorylated tau in SY5Y cells: similarities and dissimilarities to abnormally hyperphosphorylated tau from Alzheimer disease brain

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Abstract Unlike normal tau, abnormally hyperphosphorylated tau (AD P-tau) from Alzheimer disease (AD) does not promote but instead inhibits microtubule assembly and disrupts already formed microtubules. Tau in the human neuroblastoma cell line SH-SY5Y is hyperphosphorylated at several of the same sites as AD P-tau, and accumulates in the cell body without any association to the cellular microtubule network. The aim of the present study was to elucidate why the SY5Y tau does not affect the viability of the cells. We found that, like AD P-tau, SY5Y tau because of hyperphosphorylation does not bind to microtubules and inhibits the tau-promoted assembly of microtubules. However, the tau/HMW MAP ratio is about 10 times less in SY5Y cells than in AD brain. These findings suggest that the hyperphosphorylated tau from SY5Y cells has similar biological characteristics as AD P-tau from AD brain, but is not lethal to the SY5Y cells because of its low tau/HMW MAP ratio.

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Key words: Microtubule-associated protein; Microtubule; Alzheimer disease; SY5Y cell

1. Introduction

The abnormally hyperphosphorylated microtubule-associated protein (MAP) tau is the major protein component of the paired helical filaments, a hallmark lesion of Alzheimer disease (AD) [1–3]. In the AD brain, tau protein occurs in three forms: (i) cytosolic normally phosphorylated tau (C-tau), (ii) soluble abnormally hyperphosphorylated tau (AD P-tau), and (iii) abnormally hyperphosphorylated tau polymerized into paired helical filaments (PHF). The C-tau has normal microtubule assembly activity [4]. The hyperphosphorylated tau has not only lost its ability to bind to microtubules and to promote microtubule assembly, but instead it sequesters both normal tau and high molecular weight (HMW) MAPs which result in the disassembly of microtubules in vitro [5,6]. AD P-tau can be dephosphorylated in vitro by protein phosphatase-1 [7], -2A [8], and -2B [9], which restores its microtubule assembly activity [10]. SH-SY5Y human neuroblastoma cells only contain one fetal isoform of tau, tau-352, with three microtubule binding repeats and no N-terminal insert. Previous studies showed that tau in SY5Y cells (SY5Y tau) when cultured in medium with 5% fetal calf serum is hyperphosphorylated at several of the same sites as AD P-tau. The SY5Y tau, like AD P-tau in AD, accumulates in the cell body and does not associate to the cellular microtubule network in situ or bind to taxol-stabilized microtubules

in vitro [11]. However, unlike neurons in the AD brain, SY5Y cells have an intact microtubule network and grow normally in low serum concentration.

The present study was undertaken to elucidate why, in contrast to AD P-tau, SY5Y tau does not disrupt the cellular network. We show that SY5Y tau is hyperphosphorylated at several sites examined to a degree comparable to AD P-tau, and that it inhibits the tau-promoted microtubule assembly. This ability to inhibit microtubule assembly can be reversed by dephosphorylation, but its concentration in SY5Y cells is only one tenth of that of tau in AD brain. In contrast, the levels of the HMW MAPs in SY5Y cells are comparable to those in the AD brain. These data suggest that the hyperphosphorylated tau from living SY5Y cells has similar biological characteristics as AD P-tau from AD brain, but is not lethal to the SY5Y cells because of its low tau to HMW MAPs ratio.

2. Materials and methods

2.1. Antibodies and reagents

The following phosphorylation-dependent anti-tau antibodies were employed: Tau-1, 1:50 000 (Ser-195, 198, 199 or 202) [12,13]; PHF-1, 1:250 (P-Ser-396/404) [14,15] and 12E8 (P-Ser-262/356, 1:500) [16]. Other primary antibodies used were anti-MAP1B (3G5, 1:250), anti-MAP1 (1:1000, Chemicon, Temecula, CA) and anti-MAP2 (1:1000, CalBiochem, La Jolla, CA). Alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG antibodies (1:30 000, Sigma, St. Louis, MO), and ¹²⁵I-conjugated anti-mouse or anti-rabbit IgG (0.1 µg/ml, Amersham, Arlington Heights, IL) were used as secondary antibodies. Recombinant tau, tau₄₁₀ (three microtubule binding domains with two N-terminal inserts) was prepared as previously described [17]. AD P-tau was prepared by the method of Kópke et al. [18]. GTP, taxol and alkaline phosphatase were purchased from Sigma (St. Louis, MO). Phenyl Sepharose high performance hydrophobic column was purchased from Pharmacia Biotech (Uppsala, Sweden).

2.2. Cell culture

SH-SY5Y human neuroblastoma cells were obtained from Dr. June L. Biedler (Sloan Kettering Institute, New York). Cells were cultured up to 70–80% confluence in 100 mm dishes at 5% CO₂ and 37°C in D-MEM/F-12 medium (Gibco BRL, Gaithersburg, MD) with 5% fetal calf serum (FCS Gibco), 100 IU/ml of penicillin and 100 µg/ml of streptomycin. The medium was changed every 2 days. For harvesting, the cells were first washed with warm PBS and then collected by trituration.

2.3. Partial purification of tau from SY5Y cells

Cells were lysed on ice in a Teflon/glass homogenizer (20 strokes) in 100 mM MES, pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 25 mM NaF, 1 mM NaVO₃, 1 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 2 µg/ml pepstatin, 25 µg/ml phosphoramidon, kept on ice for 20 min and centrifuged at 100 000 × g for 30 min at 4°C. The supernatant was heated in a boiling water bath for 5 min and centrifuged again at 100 000 × g for 20 min. The heat-stable supernatant (heat-stable fraction) was adjusted to 2 M NaCl with 5 M NaCl, and was loaded on a phenyl Sepharose hydrophobic column (2 mg protein/ml medium),

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which was pre-equilibrated with MES buffer (100 mM MES, pH 6.8, 1 mM MgCl_2 , 1 mM EGTA) containing 2 M NaCl. The column was washed with about two times the column volume of MES buffer containing 2 M NaCl. The loading and wash fractions were pooled and dialyzed against MES buffer for 24 h, and concentrated by centrifugation in Centricon filter (Amicon, Beverly, MA). This partially purified SY5Y tau is called 'SY5Y tau' in the following. The proteins bound to the column were eluted with MES buffer without NaCl and dialyzed, and are called 'eluate'.

2.4. *In vitro* dephosphorylation

SY5Y tau was dephosphorylated *in vitro* as previously described [4]. Briefly, after dialysis against 0.1 M Tris (pH 8.0), the sample was adjusted to 0.4 mg/ml protein and incubated with alkaline phosphatase (200 U/ml) in a mixture of protease inhibitors (5 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ pepstatin, 25 $\mu\text{g}/\text{ml}$ phosphoramidon, 1 mM PMSF) at 37°C for 16 h. The sample was then boiled for 5 min to inactivate the alkaline phosphatase, centrifuged at $15000\times g$ for 10 min, dialyzed against 100 mM MES (pH 6.8), 1 mM MgCl_2 and 1 mM EGTA (MES buffer) for 15 h, concentrated by Centricon filtration and the tau levels determined by radioimmuno-dot-blot.

2.5. Isolation of tubulin

Bovine brain tubulin was isolated through two temperature-dependent cycles of microtubule polymerization-depolymerization [19] followed by phosphocellulose ion-exchange column chromatography [20]. Taxol-stabilized microtubules were then prepared by incubation of 1 mg tubulin/ml with 20 μM taxol at 37°C for 30 min.

2.6. Microtubule binding assay

SY5Y tau (0.6 μg) with and without pretreatment with alkaline phosphatase was incubated with 50 μg of taxol-stabilized microtubules in 100 μl MES buffer containing 10 μM taxol at 37°C for 30 min. The microtubule pellet was separated by centrifugation over a cushion of 100 μl 10% sucrose in MES buffer containing 10 μM taxol at $60000\times g$ for 30 min. The supernatant containing the unbound proteins and the pellet of stabilized microtubules with microtubule-bound proteins were analyzed by Western blots.

2.7. Microtubule assembly assay

Recombinant tau (100 $\mu\text{g}/\text{ml}$) was added at 4°C to purified bovine tubulin (2 mg/ml) and 1 mM GTP in polymerization buffer (100 mM MES, pH 6.8, 1 mM EGTA, 1 mM MgCl_2). After rapid mixing, the samples were pipetted into quartz microcuvettes and incubated at 37°C in a thermostatically controlled Cary 1 recording spectrophotometer. The turbidity was continuously monitored at 350 nm. For the inhibition assay, AD P-tau or SY5Y tau was added together with recombinant tau. The state of microtubule assembly was further confirmed by negative-stain electron microscopy [21].

2.8. Protein determination, Western blots and radioimmuno-dot-blot

The protein concentration of the samples was determined by the modified Lowry method [22]. Sample preparation and Western blots were performed as previously described [23]. Levels of tau and HMW MAPs in SY5Y cells and AD brain were determined by radioimmuno-dot-blot assay as described previously [11,24]. Briefly, cells and brain tissue homogenates were boiled in 1% SDS and 1% β -mercaptoethanol for 10 min followed by centrifugation at $15000\times g$ for 20 min at 4°C. Triplicate samples containing 1 μg , 2 μg and 4 μg protein each in 5 μl were applied to nitrocellulose membrane (Schleicher and Schuell, Keene, NH). After drying at 37°C for 1 h, the membranes were incubated with primary antibodies at room temperature for 6 h. The blots were then developed with ^{125}I -labeled anti-mouse antibody (Amersham), and the radioactivity of the blots was quantified using Fuji Bio Image Analyzer 1500 (Raytest USA Inc., Wilmington, DE) equipped with the Tina software system.

3. Results

3.1. Separation of tau from SY5Y cells

To study *in vitro* the biological activity of SY5Y tau, it was of high significance to separate tau from other MAPs. After heat treatment of cell extracts, tau was enriched in the heat-

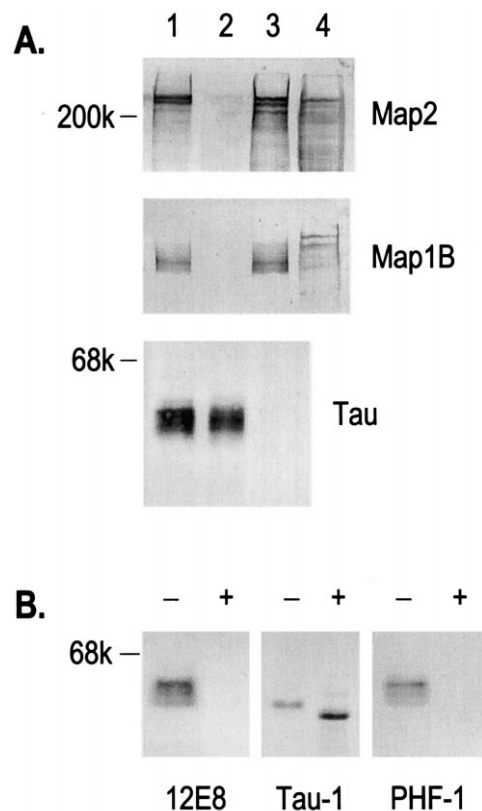


Fig. 1. A: Separation of HMW MAPs and SY5Y tau by phenyl Sepharose chromatography. The heat-stable fraction (2 μg , lane 1) of the cell extract was applied to the column and separated into unbound (1 μg , lane 2) and bound (1 μg , lane 3) fractions. Human brain homogenate (10 μg , lane 4) was used as positive control of HMW MAPs. Western blots were developed with MAP2 (1:1000) and MAP1B (3G5, 1:250) and tau (92e, 1:5000) antibodies. MAP1B and MAP2 bound quantitatively to the column whereas tau was not bound and free of high molecular weight MAPs. B: Western blots of purified SY5Y tau. SY5Y tau isolated by phenyl Sepharose chromatography was electrophoresed on 10% SDS-PAGE 1 $\mu\text{g}/\text{lane}$ with (+) or without (-) *in vitro* dephosphorylation. After electroblotting, the membranes were immunodeveloped with phosphorylation dependent antibodies Tau-1 (1:50 000), PHF-1 (1:250), or 12E8 (1:500). With the conditions used, tau can be fully dephosphorylated *in vitro* at the epitopes shown.

stable fraction. However, Western blot analysis revealed that this fraction contained strong MAP2 and some residual MAP1 immunoreactivity. These MAPs were separated from tau by phenyl Sepharose hydrophobic chromatography where tau was in the unbound and MAP1B and MAP2 in the bound fraction (Fig. 1A). Western blots revealed that SY5Y tau was highly phosphorylated at the Tau-1, PHF-1, and 12E8 sites (Fig. 1B). Radioimmunoassays showed that the phosphorylation at the Tau-1 site was $73 \pm 8\%$ in SY5Y tau compared to $>95\%$ in AD P-tau. Phosphorylation of SY5Y tau at 12E8 and PHF-1 sites was $77 \pm 18\%$ and $44 \pm 11\%$ respectively of that of AD P-tau. After *in vitro* dephosphorylation with alkaline phosphatase, the phosphorylation at these sites was fully eliminated, the Tau-1 immunoactivity increased with a concomitant increase of the electrophoretic mobility of tau, and the PHF-1 and 12E8 immunoactivities were negative (these two antibodies recognize phosphorylated tau).

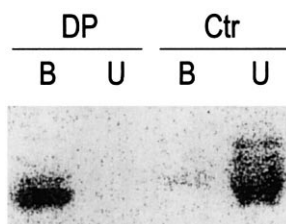


Fig. 2. Microtubule binding activity of SY5Y tau. SY5Y tau (0.6 μ g) with or without pretreatment with alkaline phosphatase was incubated with 50 μ g of taxol-stabilized microtubules in MES buffer containing 10 μ M taxol at 37°C for 30 min. The microtubule pellet was separated by centrifugation over a 10% sucrose cushion at 60 000 $\times g$ for 30 min. The supernatant containing the unbound proteins (U) and the pellet containing the stabilized microtubules with microtubule bound proteins (B) were analyzed by immunoblots probed with anti-tau polyclonal antibody, R134d (1:5000). SY5Y tau bound to microtubules only after dephosphorylation by alkaline phosphatase.

3.2. The binding of SY5Y tau to taxol-stabilized microtubules

It has been previously reported that tau in SY5Y cells is not associated with microtubules *in vitro* and does not bind to taxol-stabilized microtubules [11]. In the present study, we show that the microtubule binding activity of SY5Y tau was fully restored after dephosphorylation (Fig. 2). Only about 10% of untreated SY5Y tau bound to taxol-stabilized microtubules, whereas after dephosphorylation by alkaline phosphatase, more than 95% of dephosphorylated SY5Y tau was bound.

3.3. Inhibition of tau-promoted microtubule assembly by SY5Y tau

Previously, we demonstrated that *in vitro* AD P-tau sequesters normal tau and thus inhibits the tau-promoted microtubule assembly [4]. To investigate whether SY5Y tau, which is hyperphosphorylated at several of the same sites as AD P-tau, has the same characteristics as AD P-tau, SY5Y tau was added together with recombinant tau to promote microtubule assembly. As shown in Fig. 3A, SY5Y tau as well as AD P-tau inhibited the microtubule assembly-promoting activity of recombinant tau. About 20 μ g/ml of SY5Y tau was required to partially inhibit the microtubule assembly and about 60 μ g/ml of SY5Y tau completely inhibited the microtubule assembly promoted by 100 μ g/ml of tau₄₁₀, while for AD P-tau 20 μ g/ml was sufficient to obtain the full inhibition (data not shown). When SY5Y tau was dephosphorylated *in vitro* and

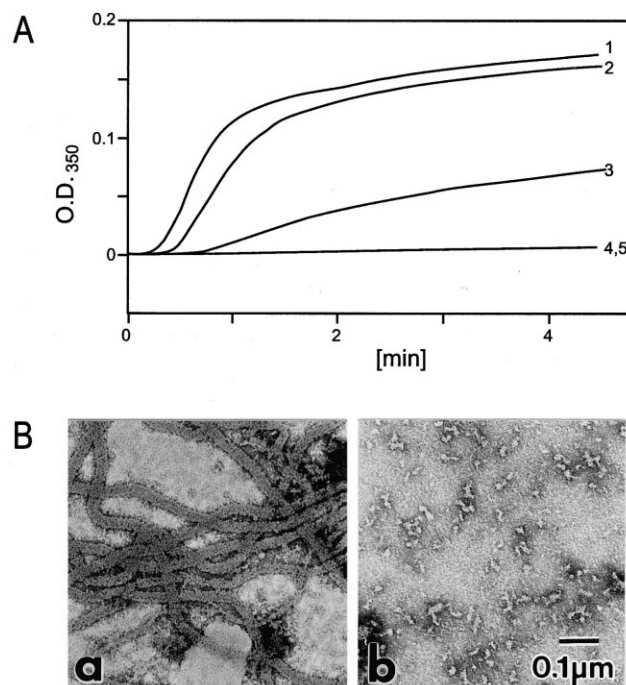


Fig. 3. The effect of SY5Y tau on the tau-promoted microtubule polymerization. A: Turbidimetric assay measuring microtubule assembly using recombinant and SY5Y tau. Bovine brain tubulin (2 mg/ml), recombinant human tau₄₁₀ (100 μ g/ml), GTP (1 mM) in 100 mM MES, 1 mM EGTA, 1 mM MgCl₂, pH 6.8, and SY5Y tau were mixed and the turbidimetric changes at 350 nm recorded in a Cary 1 spectrophotometer at 37°C. Curve 1, tau₄₁₀, 100 μ g/ml (recombinant tau with two N-terminal inserts and three microtubule binding repeats); 2, dephosphorylated SY5Y tau (90 μ g/ml) plus 100 μ g/ml tau₄₁₀; 3, untreated SY5Y tau (20 μ g/ml) plus 100 μ g/ml tau₄₁₀; 4, dephosphorylated SY5Y tau (90 μ g/ml) without tau₄₁₀; 5, bovine brain tubulin control. B: Electron micrographs showing products of microtubule assembly reaction. Assembly from bovine brain tubulin promoted by tau₄₁₀ in the presence of dephosphorylated SY5Y tau (left panel) or untreated (right panel). Samples were taken at steady state of polymerization and were negatively stained with phosphotungstic acid.

then added together with recombinant tau to tubulin, there was no inhibition of the microtubule assembly even when up to 90 μ g/ml dephosphorylated SY5Y tau was added. Almost no microtubules were found by electron microscopy in experiments with recombinant tau and SY5Y tau added together, whereas many microtubules were observed when the assembly was carried out with dephosphorylated SY5Y tau and re-

Table 1
Levels of microtubule-associate proteins in SY5Y cells and in AD brain

	SY5Y cells (n = 3)	AD brain (n = 5)
Tau (PSL/ μ g) (mean $\pm$ S.D.)	37.6 $\pm$ 4.6	418.1 $\pm$ 109.8
MAP1B (PSL/ μ g) (mean $\pm$ S.D.)	96.3 $\pm$ 1.9	51.9 $\pm$ 11.4
MAP1 (PSL/ μ g) (mean $\pm$ S.D.)	17.9 $\pm$ 1.4	18.5 $\pm$ 4.9
MAP2 (PSL/ μ g) (mean $\pm$ S.D.)	25.5 $\pm$ 2.8	31.9 $\pm$ 3.9
Tau/MAP1B	0.39	8.05
(relative percentage)	(4.8%)	(100%)
Tau/MAP1	2.1	22.6
(relative percentage)	(9.3%)	(100%)
Tau/MAP2	1.47	13.1
(relative percentage)	(11.2%)	(100%)

MAPs were quantitated in SY5Y cells and brain homogenates by radioimmunodot-blot assays using Tau-1 (1:50 000, after dephosphorylation on the blot), anti-MAP2 (1:1000), anti-MAP1 (1:1000) and anti-MAP1B (1:250) antibodies. The values are expressed as relative mean immunoreactivities (PSL) per μ g protein $\pm$ S.D. of SY5Y cells of three individual culture dishes and brain samples from five different AD cases.

combinant tau (Fig. 3B). However, in contrast to AD P-tau, when SY5Y tau was dephosphorylated, it even at 90 µg/ml was not able either to increase the polymerization of microtubules when added together with tau₄₁₀ or to promote the polymerization on its own. A second tau preparation from another batch of cells produced almost identical results.

3.4. Comparison of the levels of tau and other MAPs between SY5Y cells and AD brain

Although tau in SY5Y cells is similarly hyperphosphorylated as tau in AD brain, SY5Y cells have an intact microtubule network and proliferate normally. To study why the endogenous hyperphosphorylated tau is not cytotoxic to SY5Y cells, the levels of tau and other MAPs both in SY5Y cells and AD brain were determined by radioimmunodot-blot assays. Table 1 shows that the level of tau protein was about 10 times less in SY5Y cells than in AD brain, whereas the levels of MAP2 and MAP1B in SY5Y cells were similar or higher than in AD brain.

4. Discussion

Tau, a major neuronal MAP, promotes microtubule assembly and stabilizes microtubules *in vitro*, and is believed to maintain the microtubule network of the neuron [25–27]. The abnormal hyperphosphorylation of tau protein is thought to be an important initial event in neurodegeneration in AD brain, because the AD hyperphosphorylated tau is not only unable to promote microtubule assembly but instead it inhibits the assembly and disassembles already assembled microtubules by sequestering normal tau, MAP1 and MAP2 *in vitro* [4,6]. Furthermore, ultrastructural examination of brain biopsies from AD patients has revealed disruption and replacement of the microtubule network with paired helical filaments and condensation in some areas of the mitochondria and liposomes, all reminiscent of neurodegeneration due to inhibition of the axoplasmic transport [28]. Braak et al. [29] have also demonstrated initial stages of retrograde degeneration of neurons with abnormally hyperphosphorylated tau in 60 µm thick sections of entorhinal cortex of very early cases of AD.

Tau in SY5Y cells is hyperphosphorylated at some of the same sites as AD P-tau and, like AD P-tau, does not bind to microtubules [11], but it does not seem to destroy the microtubule network of the cell. One reason for this discrepancy might be insufficient phosphorylation at sites critical for converting tau into a toxic molecule. Phosphorylation of tau at Ser-262, the 12E8 epitope, has been shown to be a strong determinant for the binding of tau to microtubules [30], but phosphorylation at additional sites like Thr-231/Ser-235, Ser-396/404 and Ser-214 seem to be required for the full inactivation of the biological activity of tau [17,31–33]. SY5Y tau not only does not bind to taxol-stabilized microtubules but also fully inhibits tau-promoted microtubule assembly *in vitro*, although about three times more SY5Y tau was required to achieve the same inhibition as AD P-tau. These data show that in comparison to AD P-tau, only about one third of the SY5Y tau is hyperphosphorylated sufficiently to inhibit the microtubule assembly.

The data in the present study show that in the case of SY5Y tau and as well as AD P-tau, both the inhibition of microtubule assembly and loss of microtubule binding activity are

due to hyperphosphorylation of SY5Y tau. However, in contrast to the dephosphorylated AD P-tau, the dephosphorylated SY5Y tau was not capable of inducing nucleation of microtubules. Nucleation, elongation and microtubule binding activities are believed to be distinct aspects of tau function. Mutation or phosphorylation in the amino-terminal flanking region of the microtubule binding domain of tau can selectively reduce the microtubule nucleating ability of tau [34,35]. The exact cause of the inability of the dephosphorylated SY5Y tau to promote microtubule assembly is currently not understood.

Obviously tau's hyperphosphorylation did not seem to affect the maintenance of the microtubule network of SY5Y cells, most likely because of the low concentration of tau as compared to that in the AD brain. SY5Y cells contain only one tenth of tau but almost the same levels of the other HMW MAPs as the AD brain. Thus in SY5Y cells, instead of tau, the other HMW MAPs, especially MAP1B and MAP2 are the dominant microtubule-associated proteins that promote and stabilize the microtubule system. In the light of the low affinity of hyperphosphorylated tau towards HMW MAPs [6], it seems most likely that the concentration of hyperphosphorylated tau in SY5Y cells is insufficient to fully sequester the other MAPs to induce microtubule breakdown. However, the possibility that a modification other than the phosphorylation of tau in SY5Y cells might prevent it from causing degeneration of these cells cannot be eliminated.

Abnormal phosphorylation of tau is believed to be an early step and a main cause of the breakdown of the neuronal microtubule network and neurofibrillary degeneration in AD. Our study indicates that the breakdown of the microtubule network induced by abnormally hyperphosphorylated tau is not only dependent on the phosphorylation of tau, but also on the ratio of hyperphosphorylated tau protein to other HMW MAPs. SY5Y cells have been widely used as a model to study Alzheimer-like tau phosphorylation. The present study illustrates, as an experimental cell model for Alzheimer neurofibrillary degeneration, the limitations of this cell line, and probably also other cell lines derived from cancerous tissue, most of which have low tau content.

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