

Abnormally phosphorylated tau in SY5Y human neuroblastoma cells

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Abstract In Alzheimer disease (AD) the microtubule associated protein (MAP) tau is hyperphosphorylated at several sites. In the present study, like AD tau, tau in the human neuroblastoma SH-SY5Y was found to be hyperphosphorylated, at Ser-199/202, Thr-231, Ser-396 and Ser-404. However, in contrast to AD, the tau in SY5Y cells was not hyperphosphorylated at Ser-235 and there was only one tau isoform. Quantitative analysis revealed that approximately 80% of the SY5Y-tau was phosphorylated at Ser-199/202. The phosphorylated tau was deposited in perikarya and processes of the cells whereas most of the unphosphorylated (at Ser-199/202) tau was localized in the nucleus. Tau from the cell lysates did not bind to taxol-stabilized microtubules. In contrast, MAP1b and MAP2 from cell lysates bound to stabilized microtubules *in vitro* and were associated to the microtubule network *in situ*. Phosphorylation of tau at high levels, its inactivity with microtubules and its accumulation in SY5Y cells provide for the first time a cell model of cytoskeletal changes seen in AD.

Key words: Alzheimer disease; Microtubule associated protein; Microtubule; Neuroblastoma cell; Protein phosphorylation; Tau; Tubulin

1. Introduction

A major biochemical abnormality in the Alzheimer disease (AD) brain is the abnormally hyperphosphorylated microtubule associated protein (MAP) tau [1–3]. The abnormal tau is the major protein subunit of the paired helical filament (PHF) [1,3–5] and it is in this form that tau accumulates as neurofibrillary tangles in the perikaryon, in the neuropil threads [6] of the neuropil and in the dystrophic neurites of neuritic (senile) plaques. In addition, the abnormal tau also accumulates in normal appearing neurons in amorphous aggregates intermixed with a few short PHF, forming the so-called ‘stage 0’ tangles [7,8]. Unlike the mature neurofibrillary tangles, the stage 0 tangles are only poorly argentophilic and are not stained by thioflavin-S or Congo red. Recently, hyperphosphorylated tau (AD P-tau) was isolated from AD brain that, although not ubiquitinated and not polymerized into PHF, sediments at 27,000 to 200,000 × g, is soluble in buffers after denaturation with urea and has phosphate levels comparable to those of the PHF [9]. Furthermore, like PHF, the AD P-tau is phosphorylated at several sites and does not promote *in vitro* microtubule assembly unless dephosphorylated with alkaline phosphatase [10,11].

Previously it was not known whether the altered solubility characteristics of AD-P tau and its negative effect on microtubule assembly observed *in vitro* also occur in the living cell. In

the present study we show that approximately 80% of tau in the human neuroblastoma cell line SH-SY5Y is hyperphosphorylated at the Tau-1 epitope, and, like the AD P-tau is hyperphosphorylated at least at three other sites. Furthermore, the abnormal tau, like in AD, accumulates in the cell body and does not seem to be biologically active since it is neither associated to the cellular microtubule network *in situ* nor *in vitro* binds to taxol-stabilized microtubules. However, unlike the abnormal tau in AD, the SY5Y-tau is not phosphorylated at Ser-235. The microtubule network itself seems to be intact in the SY5Y cells and is supported by MAP2 and MAP1b.

2. Materials and methods

2.1. Cells and proteins

SH-SY5Y human neuroblastoma cells were obtained from Dr. June L. Biedler (Sloan Kettering Institute, NY). Cells were cultured up to about 70% confluence in 35-mm diameter dishes employing D-MEM/F-12 medium (Gibco BRL, Gaithersburg, MD) (5% CO₂; 37°C) supplemented with 5% fetal calf serum, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin. The cells were fed every three days. In some cases the cells were deprived of nutrient by keeping them in the same medium without feeding for two to four additional days. For Western blots and immunoassays, the cells were collected from each dish by pipetting up and down, were lysed in 100 µl of 2% SDS/µ-mercaptoethanol, heated in a boiling water bath for 5 min and sonicated in a water bath for 10 min. The protein concentrations were assayed by the modified Lowry method of Bensadoun and Weinstein [12]. In some cases tau was immunoprecipitated from the cell lysates with polyclonal anti-tau antibody, 92e [13] bound to agarose linked protein G (Pierce, Rockford, IL).

Tau protein from normal human brain and soluble abnormally phosphorylated tau from Alzheimer disease (AD P-tau) brain were prepared as previously described [9]. Sources and dilutions of anti-tau antibodies employed are shown in Table 1. Other antibodies employed were DM1A (anti-α tubulin, 1:1000, Sigma, St. Louis, MO), YL1/2 (anti-tyrosinated tubulin, 1:500, Sera Lab, Westbury, NY), anti-MAP1b (1:400, Amersham, Arlington Heights, IL), and anti-MAP2 [14] (1:1000; a gift from Dr. R.B. Vallee, Shrewsbury, MA). Immunoblots were performed as previously described [11].

To determine the isoforms of tau protein in SY5Y cells, the cell lysates were first dialysed against 100 mM Tris, pH 8.0, 1 mM EGTA, 0.1 mM EDTA, 1 mM MgCl₂, and protease inhibitors (5 µg/ml aprotinin, 5 µg/ml leupeptin, 2 µg/ml pepstatin and 1 mM PMSF) and then treated with 100 U/ml alkaline phosphatase (Boehringer-Mannheim, Indianapolis, IL) at 37°C for 8 h. Recombinant tau 23 and 24 [15] expressed in *E. coli* were employed as M_r markers.

2.2. Radioimmuno-dot-blot assay

Triplicate samples of cell lysates containing 1 µg and 3 µg of total protein in 5 µl were applied to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and dried at 37°C for 30 min. The tau contents were determined using Tau-1 and ¹²⁵I-labeled anti-mouse IgG (Amersham, Arlington Heights, IL) with or without prior dephosphorylation of the protein on the membrane with alkaline phosphatase as previously described [16]. Purified recombinant tau 39 was employed for the standard curve in the immunoassay.

2.3. Indirect immunofluorescence microscopy

SH-SY5Y cells were cultured in Lab-Tec slides (Nunc, Naperville,

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IL) and, fixed in 4% formaldehyde in 100 mM HEPES, pH 6.8, 0.2% Triton X-100, 5 mM EGTA and 10 mM MgCl₂ [17]. The cells were post-fixed with -20°C methanol for 5 min, washed with phosphate buffered saline, blocked in 2% BSA/ Tris buffered saline and incubated with primary antibodies followed by FITC-labeled secondary antibodies (Cappel, Durham, NC). The slides were mounted in Vector-Shield mounting media (Vector Lab. Inc., Burlingame, CA). Fixation of the cells in -20°C methanol instead of formalin resulted in immunostaining of comparable intensity.

2.4. Taxol stabilized microtubules

Twice cycled microtubules from rat brain [18] were cold-disassembled and employed to prepare MAP-free tubulin by phosphocellulose chromatography [19]. Taxol stabilized microtubules were then prepared by incubating 1 mg tubulin/ml with 20 μM Taxol at 37°C for 30 min.

2.5. Microtubule binding assay

The cells ($\sim 1 \times 10^7$) were lysed in 100 mM PIPES, pH 6.8, 0.1% Triton-X 100, 2 mM MgCl₂, 1 mM EGTA, 0.1 mM EDTA, 10 mM sodium fluoride, 1 mM sodium vanadate and protease inhibitors (see above), and centrifuged at $200,000 \times g$ for 30 min. The supernatant (900 μg protein) was incubated with 100 μg of taxol-stabilized microtubules and 10 μM taxol at 37°C for 30 min and centrifuged at $70,000 \times g$ for 45 min. The supernatant containing the unbound proteins and the pellet of stabilized microtubules and microtubule bound proteins were analyzed by immunoblots.

3. Results

3.1. Tau in SY5Y cells is hyperphosphorylated at several sites

Western blots developed with anti-tau antibodies demonstrated the expression of tau protein in SH-SY5Y human neuroblastoma cells (Fig. 1). AT8 (P Ser-199/202), PHF-1 (P Ser-396), SMI-31 (P Ser-396 and P Ser-404) and M4 (P Thr-231) antibodies all of which react with phosphorylated epitopes of tau, but not with normal tau, reacted with tau in SY5Y cell lysate. Tau-1 antibody that only recognizes its epitope if it is not phosphorylated (Ser-199/Ser-202), stained only one faint band on the Western blot but strongly reacted with a series of polypeptides in the 51–63 kDa range after pretreatment of the blots with alkaline phosphatase. In contrast, antibody SMI33 (Ser-235) stained the tau polypeptides, and the pretreatment of the blots with alkaline phosphatase had no apparent effect on the intensity, indicating that SY5Y-tau is not phosphorylated at Ser-235 (data not shown). SY5Y-tau dephosphorylated in vitro was stained as a single band and co-migrated with the recombinant tau 23, the human tau isoform with three repeats of tubulin binding domains and no N-terminal inserts (same as fetal tau) [15].

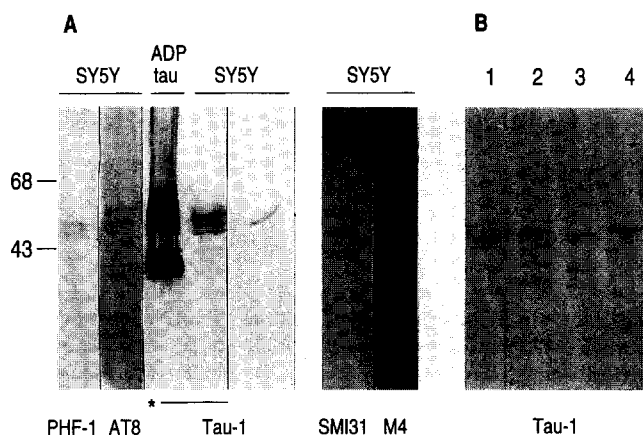


Fig. 1. Phosphorylation sites and M_r of SH-SY5Y tau. (A) 100 μg of cell lysates were applied to each lane; AD P-tau was used at 2 $\mu\text{g}/\text{lane}$. *Indicates alkaline phosphatase treatment of the Western blot prior to the application of the antibodies. In the case of SMI31 and M4, samples immunoprecipitated with tau antibody 92e were applied to avoid non-specific staining. Phosphorylation of SY5Y-tau at Ser-199/202 is indicated by the reaction with AT8 as well as by a marked increase in tau staining with Tau-1 after dephosphorylation of the blot. The reaction of PHF-1 and SMI 31 with SY5Y tau indicates phosphorylation at Ser-396/404. The reaction of M4 with SY5Y-tau indicates phosphorylation of tau at Thr-231. Not shown in this figure, SY5Y tau is not significantly phosphorylated at Ser-235 since there was no increase of tau staining with antibodies SMI33 after dephosphorylation of the blots. (B) 50 μg of cell lysates dephosphorylated in vitro were applied to lane 1 and 0.2 $\mu\text{g}/\text{each}$ of recombinant tau 23 and 24 were applied to lanes 2 and 3 respectively. Apparent M_r weight of tau 23 and 24 are 48 kDa and 52 kDa, respectively [15]. Dephosphorylated SY5Y-tau (lane 1) was visualized as a single band that corresponded to tau 23, the shortest human tau isoform. SY5Y-tau in the untreated cell lysate (100 μg) in lane 4 has slower mobility than dephosphorylated SY5Y tau in lane 1.

3.2. Most of the tau is phosphorylated at Ser-199/202

To determine what percentage of tau was hyperphosphorylated in the SY5Y cells, levels of tau in cell lysate were determined by radioimmuno-dot-blot assay using Tau-1 with and without pretreatment of the blots with alkaline phosphatase. A 5–6-fold increase in the tau levels after dephosphorylation was observed (Fig. 2), while the levels of tau isolated from control brains were not affected by the phosphatase treatment (data not shown). From three independent experiments the percentage of tau abnormally phosphorylated at the

Table 1
Source and dilution of the tau antibody

Ab	Type	Epitope	Phosphorylated (P)/Not-Phosphorylated (N)	Dilution	References
Tau antibodies					
Tau-1	mono	Ser 199/202	N	1:50,000	34
AT8	mono	Ser 199/202	P	1:50	35
M4	mono	Thr 231	P	1:2,000	36
SM133	mono	Ser 235	N	1:500	37
SM131	mono	Ser 269/404	P	1:100	37
PHF1	mono	Ser 396	P	1:100	38
92e	poly	—	—	1:5*	13

*Dilution used for immunoprecipitation.

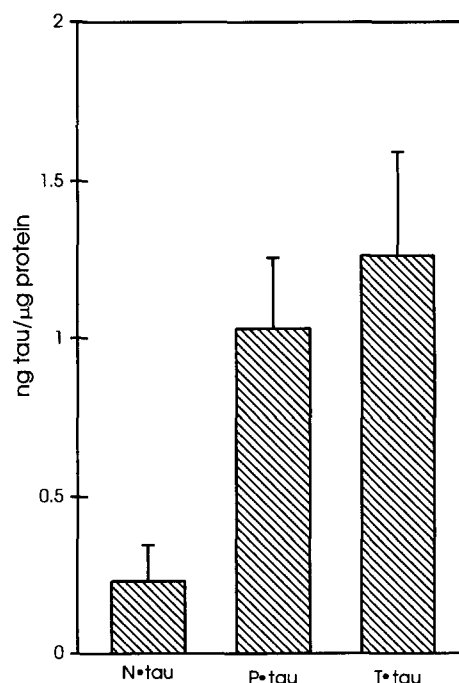


Fig. 2. Levels of tau hyperphosphorylated at Ser-199/202. Levels of tau in SY5Y lysates were estimated by comparison with defined amounts of recombinant tau 39. Blots treated (T-tau = total tau) or untreated with alkaline phosphatase (N-tau = tau not phosphorylated at the Tau-1 epitope) were exposed to Tau-1 antibody [125 I]anti-mouse IgG. The value for P-tau (phosphorylated at the Tau-1 epitope) was determined by subtraction of the value obtained for N-tau from that of T-tau. The data were obtained from three independent experiments.

Tau-1 epitope was determined to be $82\% \pm 10$ of the total tau level of 1.3 ± 0.2 ng/ μ g lysate protein.

3.3. Phosphorylated tau accumulates in the cytoplasm in SY5Y cells

The association of tau to the microtubule network in SY5Y neuroblastoma cells was examined by indirect immunofluorescence (Figs. 3, 4). A dense microtubule network was seen with anti-tubulin antibody in cell bodies and neurites, (Fig. 3d) whereas no fibrillar staining by Tau-1, AT8 or PHF-1 antibodies (Fig. 3a–c) was observed. Tau-1 labeling was seen as several bright dots in the nucleus while in the cytosol only a faint background staining was observed (Fig. 3a). In contrast, strong immunoreactivity of AT8 (P Ser-199/202) was found in the cytoplasm and in the several neuronal processes (Fig. 3b). No binding to fibrous structures was apparent. PHF-1 (P Ser-396) stained brightly fluorescent deposits along the cytoplasmic seam around the unstained nucleus (Fig. 3c). Mitotic cells were most intensely labeled with PHF-1. In contrast to tau, anti-MAP1b and anti-MAP2 antibodies stained the intracellular fibrous network, and in addition several neuronal processes of the SY5Y cells (Fig. 3e).

When cells were cultured at suboptimal conditions, i.e. without changing the medium for 5 days to 7 days instead of 3 days, large accumulations of phosphorylated tau were observed in the cytoplasm, that were strongly immunoreactive with PHF-1 and AT8 (Fig. 4a,b). Apparently, the microtubule network of these cells was intact since it was labeled both with mAb DMIA to α tubulin and with YL1/2 antibody to the labile microtubule population (Fig. 4c,d).

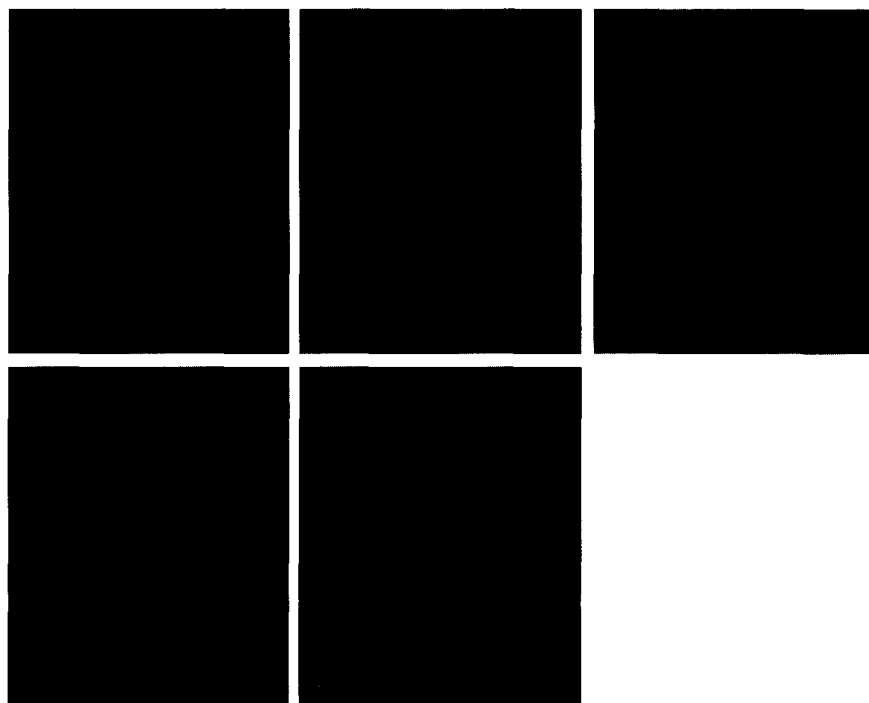


Fig. 3. Immunofluorescent staining of formalin fixed SH-SY5Y cells with antibodies to (a–c) tau: (a) Tau-1, (b) AT-8, and (c) PHF-1, (d) tubulin and (e) MAP1b. The microtubule network was stained with antibodies to tubulin, MAP1b and, not shown here, MAP2. No microtubule staining was observed with any of the tau antibodies.

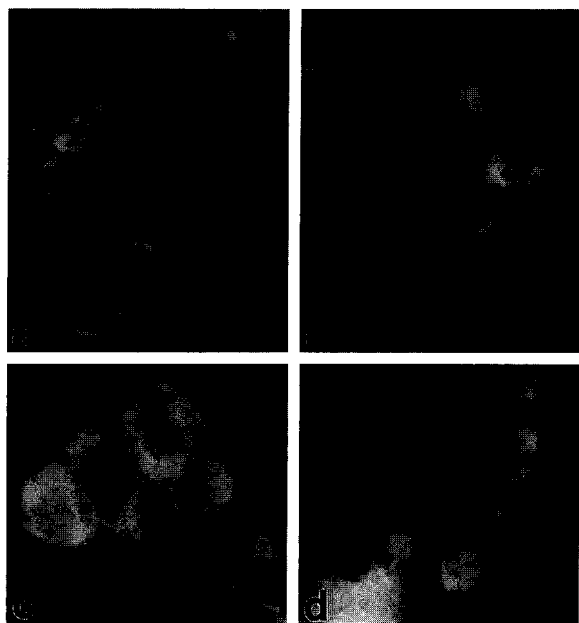


Fig. 4. Immunofluorescent staining of SH-SY5Y cells that were for 5 days in culture without medium change. Antibodies to hyperphosphorylated tau: AT-8 (a), PHF-1 (b), and to tubulin: DM1A (c), YL1/2 (d) were employed. The microtubule network appeared slightly less dense than in Fig. 3 while tau aggregation appeared more prominent. Not shown in this figure, a similar pattern was observed after 7 days of nutrient deprivation.

3.4. Most SY5Y tau does not bind to microtubules

To determine whether tau was biologically active in binding to microtubules, the $200,000 \times g$ extract of the SY5Y cells was incubated with taxol-polymerized microtubules, and the microtubule bound and unbound proteins were separated into pellet and supernatant, respectively. The immunoblots of these fractions revealed that almost all of MAP1b and MAP2 bound but most of the SY5Y-tau did not bind to the microtubules (Fig. 5).

4. Discussion

In Alzheimer disease 75–85% of the MAP tau, including all six isoforms, is altered both in its degree of phosphorylation and its solubility-characteristics [1,3,9,13,16]. In vitro studies have shown that the abnormal hyperphosphorylation of tau renders it biologically inactive [10,11,20]. In the present study we show for the first time that tau in SY5Y cells is phosphorylated at five of six of the abnormal phosphorylation sites examined, and also does not bind to microtubules. These findings suggest that like in AD, tau in SY5Y cells is hyperphosphorylated, has minimal biological activity and accumulates in the cell cytoplasm. Hyperphosphorylation of tau similar to that in AD has also been observed in fetal rat and human brains [21,22]. However, immunocytochemical studies indicate that in contrast to AD, tau in the fetal brain is immobilized only in a relatively small number of neurons [23].

Of the total SY5Y-tau, about 80% is hyperphosphorylated at the Tau-1 epitope. Similarly high phosphorylation levels of the Tau-1 epitope have been previously demonstrated both in the soluble abnormally phosphorylated tau and the PHF-tau in AD [1,9]. Whether the degree of phosphorylation at Thr-231,

Ser-396 and Ser-404 is as high as at the Tau-1 epitope, cannot be determined with the antibodies available to date. Furthermore, it is not as yet known at what additional sites, if any, tau in SY5Y cells is phosphorylated. However, it appears that the hyperphosphorylation of SY5Y tau is sufficient to make it not bind to microtubules and accumulate in the living cell.

Generally, endogenous tau in cells as well as tau in cells transfected with tau cDNA have been shown to decorate a fibrous network, presumably microtubules [24–26]. In contrast, in the present study most of the hyperphosphorylated tau is deposited in the perikarya of SY5Y cells and is not bound to the microtubule network. These findings suggest that the hyperphosphorylation of tau might be the cause for both its accumulation and biological inactivity. The greater deposition of tau in SY5Y cells in nutrient deprived medium might be due to a decrease in pH. In vitro self-assembly of tau or its fragments has been reported at pH 4.5–5.5 [27–29].

Although most of the cytoplasmic tau was phosphorylated at the Tau-1 epitope, tau in the nucleus was not hyperphosphorylated. Tau has been shown previously to colocalize with the nucleolus of several neuroblastoma cells [30]. There might be unknown regulatory mechanism that affect the localization of tau by phosphorylation. For example, certain proteins such as the nuclear factor of activated T-cells (NF-AT) and MAP kinase are known to be translocated to the nucleus depending upon their phosphorylation state [31,32].

The lack of microtubules in neurons with neurofibrillary tangles [33] as well as the in vitro inhibition of tau-mediated microtubule assembly by Alzheimer tau [11] suggests a potential role of the hyperphosphorylated tau in the microtubule defect in AD. In SY5Y cells, on the other hand, the microtubule system is apparently functional despite the presence of mostly hyperphosphorylated tau. A reason for this discrepancy might be that MAP1b, which is most probably expressed at higher levels in the readily dividing SY5Y cells than in the non-divid-

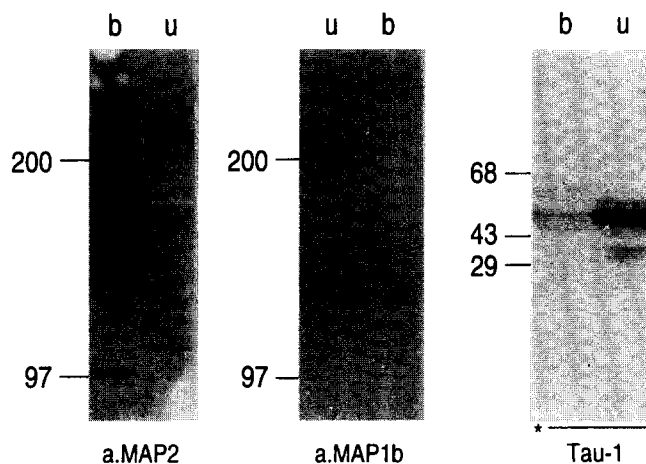


Fig. 5. Microtubule binding of MAPs from SY5Y cell extract. The $200,000 \times g$ extract of the SY5Y cells was incubated with taxol-stabilized microtubules and the MAPs bound or unbound to microtubules were examined by Western blots as described in section 2. For blots 10% (20 μ g) of the pellet containing the microtubules and the bound proteins and 10% (100 μ g) of the supernatant representing the unbound proteins were applied per lane, and the MAPs that were bound (b) or unbound (u) to the microtubules were studied with the appropriate antibodies; *Indicates alkaline phosphatase treatment of the immunoblot before antibody application. Although MAP1b and MAP2 bound to the microtubules, most of tau did not bind.

ing neurons of the brain, together with MAP2 compensates for the adverse effect of the hyperphosphorylated tau. Alternatively, SY5Y tau is probably not as hyperphosphorylated as AD tau to cause the breakdown of the microtubule system. The differences in the degree of tau phosphorylation between SY5Y cells and AD brain, are already indicated from its lack of phosphorylation at Ser-235 in the cultured cells. Nevertheless the present study shows the inactivity of hyperphosphorylated tau with microtubules and its accumulation in cultured cells i.e. outside of the human brain.

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