

Shift from fetal-type to Alzheimer-type phosphorylated Tau proteins in SKNSH-SY 5Y cells treated with okadaic acid

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Abstract Tau proteins are abnormally phosphorylated in Alzheimer's disease. Pathological Tau proteins named PHF-Tau 55, PHF-Tau 64, and PHF-Tau 69, are the main constituents of the paired helical filaments (PHF). When treating SKNSH-SY 5Y cells with okadaic acid (OA), Tau 55 protein was clearly induced whereas Tau 64 protein was only faintly induced. Here, we show that the absence of Tau 69 could be explained by the fact that adult isoforms containing N-terminal inserts are not detected. Phosphorylation is similar for untreated cellular Tau proteins and fetal Tau proteins, while OA cell treatment transformed fetal-type into Alzheimer-type phosphorylated proteins.

Key words: Tau protein; Okadaic acid; Phosphorylation; Alzheimer's disease; SKNSH-SY cell

1. Introduction

Tau proteins are microtubule-associated proteins of 50,000–64,000 Da, mainly found in axons of neurons. In human, six isoforms arise by alternative splicing of a primary transcript originating from a single gene [1]. Exons 2, 3 and 10 (nomenclature according to Andreadis's paper [2]) are under developmental regulation and they are only expressed in some adult isoforms. The fetal isoform (FF) (which expresses no exon among the alternatively expressed exons) is found in both fetal and adult brains. The other five isoforms found in adult brain correspond to the fetal isoform modified by insertions corresponding to the expression of 1, 2 or 3 alternative exons, i.e. the exons 2, 3, and 10 [1]. In this paper, these isoforms are named FF-10; FF-2; FF-2,10; FF-2,3 and FF-2,3,10 according to the expressed alternative exon (as described in Fig. 1).

In Alzheimer's disease, abnormal phosphorylation of Tau proteins leads to their aggregation in paired helical filaments (PHF) [3,4]. All six isoforms are abnormally phosphorylated and then migrate in SDS-polyacrylamide gels as a triplet named PHF-Tau 55, PHF-Tau 64 and PHF-Tau 69. Both Goedert et al. [5] and Brion et al. [6] have identified the lower PHF-Tau band (here named PHF-Tau 55) as the abnormally phosphorylated Tau molecules containing neither exon 2 nor 3 (i.e. FF and FF-10). The upper band (here named PHF-Tau 69) was identified as the phosphorylated products of Tau proteins containing exon 2 and exon 3 (i.e. FF-2,3 and FF-2,3,10) [5]. The middle band (here named PHF-Tau 64) would correspond to the abnormally phosphorylated isoforms containing exon 2 (i.e. FF-2 and FF-2,10). The distribution of the different

isoforms in the triplet, as proposed by Goedert et al. [5], is shown in Fig. 1.

The mechanisms leading to abnormal phosphorylation are unknown. Some purified members of the proline-directed protein kinase family (MAP kinase, cyclin-dependent kinase, GSK3 kinase) were successfully tested for their capacity to phosphorylate in vitro Tau proteins and to generate Alzheimer-type epitopes [7–12]. The molecular weight of in vitro phosphorylated Tau protein and PHF-Tau triplet was only compared in the two following systems. First, in human brain slices treated by okadaic acid (OA), an inhibitor of protein phosphatases-1 and -2A [13], PHF-Tau triplet was induced: Tau 64 band was mainly detected at low OA concentrations whereas Tau 55 and Tau 69 only appeared at higher OA concentrations [14]. Second, in NGF-differentiated SKNSH-SY 5Y cells treated by OA, only Tau 55 and Tau 64 were detected, Tau 55 being the major band and Tau 64 the minor one [15,16]. The differences between the two systems could be due to a different ratio between the Tau isoforms present. According to Goedert et al.'s results [1] (Fig. 1), in OA treated cells, the absence of Tau 69 might be due to the lack of isoforms containing exons 2 and 3 (i.e. FF-2,3, FF-2,3,10 isoforms). In a similar way, the low quantity of Tau 64 might reflect a low quantity of Tau isoform(s) with exon 2 (FF-2 and FF-2,10). To verify this assumption, we characterized the Tau isoforms present in our cell model by biochemical and molecular biology methods. Here, we show that cellular Tau proteins are similar to fetal Tau proteins and that adult isoforms containing N-terminal inserts are not detected in SKNSH-SY 5Y. Moreover, the treatment of the neuroblastoma cells by OA allowed a shift from fetal-type to Alzheimer-type phosphorylated proteins.

2. Materials and methods

2.1. Cell cultures, cell- and Alzheimer brain extracts

SKNSH-SY 5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Boehringer, Mannheim) supplemented with 10% fetal calf serum. Differentiation, OA treatment, and cell extraction were performed as described in [16]. Briefly, NGF-differentiated cells were treated with 0.25 μ M OA for 6 h over 4 days. Cells and brain tissues were homogenized in Laemmli's buffer with 0.25% dithiothreitol, and heat treated before loading onto polyacrylamide gels.

2.2. PAGE and Western blotting

Electrophoresis, transfer, Ponceau red staining and Western blotting were performed as described in [16]. The antibodies used were: the absorbed anti-PHF serum (abs PHF) (specific for PHF-Tau [4]), the polyclonal amino-terminal Tau serum (N-term) (specific for both Alzheimer and normal Tau proteins [16]), Tau 1 antibodies (specific for normal Tau [17,18]), AT8 (from Innogenetics, specific for Alzheimer-type Tau proteins [19]) and AD2 (monoclonal antibody specific for an abnormal site of phosphorylation in Alzheimer Tau proteins (V. Buée

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et al., manuscript in preparation)). Tau 1 and AT8 are located in the same region (amino acids 198-202) but AD2 is located in the C-terminal part of the Tau molecule. After incubation with the anti-rabbit or anti-mouse antibodies conjugated with peroxidase (Diagnostic Pasteur), visualization was performed using the ECL (Enhanced chemiluminescence) detection kit from Amersham.

2.3. Alkaline phosphatase treatment

OA cell extracts were dialysed against a buffer containing 50 mM Tris, pH 8.3, 50 mM NaCl, 1 mM MgCl₂ and 0.2 mM DTT, overnight at 4°C, and were dephosphorylated using calf intestine alkaline phosphatase (Boehringer, Mannheim) at 100 U/ml as described by Flament and Delacourte [20].

2.4. mRNA isolation and reverse transcriptase-polymerase chain reaction

Total cellular RNA was extracted by the RNAzol B method (Cinna/Biotech) according to the manufacturer's instructions. For first-strand cDNA synthesis, 10 µl reaction mixture contained the provided enzyme buffer, 1.5 µg of total RNA, 20 pM of reverse primer, 100 U of reverse transcriptase Mu-MLV, 2.5 mM deoxynucleotide triphosphates and 1 µg serum albumin. The reaction mixture was first incubated at 80°C for 5 min, then at 37°C for 90 min. For the 100 µl PCR mixture, 1 U

of *TaqI* polymerase, 20 pM of forward primer and the provided *TaqI* buffer were added to the 10 µl of reverse transcription reaction mixture. Forward and reverse primers were located, respectively, in exon 1 and in exon 4, i.e. on each side of the alternatively expressed exons 2 and 3 and corresponded, respectively, to bases 5' TACGGGTTGGGG-ACAGGAAAGAT 3' and to bases 5'GGGGTGTCTCCAATGCCT-GCTTCT 3'. PCR was carried out in a Perkin Elmer thermal cycler using cycles consisting of denaturation at 94°C for 1 min, followed by annealing at 65°C for 1 min and DNA extension at 72°C for 2 min for 30 cycles. The obtained DNA was characterized by gel agarose or acrylamide electrophoresis, digestion with endonuclease restriction and cDNA sequencing.

2.5. cDNA sequencing

The amplicon was first cloned into pBluescript II SK (Stratagene), then sequenced with the Sequenase Kit (USB) following the manufacturer's protocol.

3. Results and discussion

The electrophoretic pattern of SKNSH-SY 5Y cell Tau proteins was compared with that of fetal, adult and Alzheimer

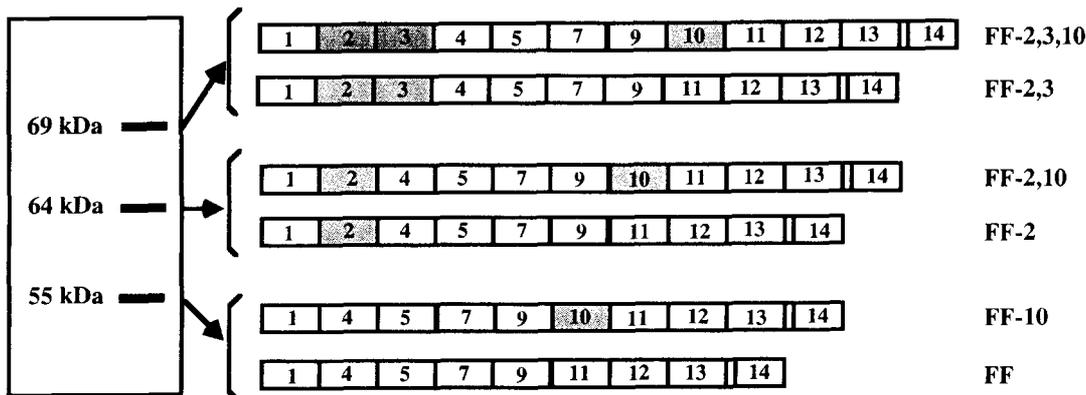


Fig. 1. Correspondance between pathological Tau triplet and different Tau isoforms according to Goedert et al. [1,6]. The exon numbers are indicated according to Andreadis et al.'s nomenclature [2].

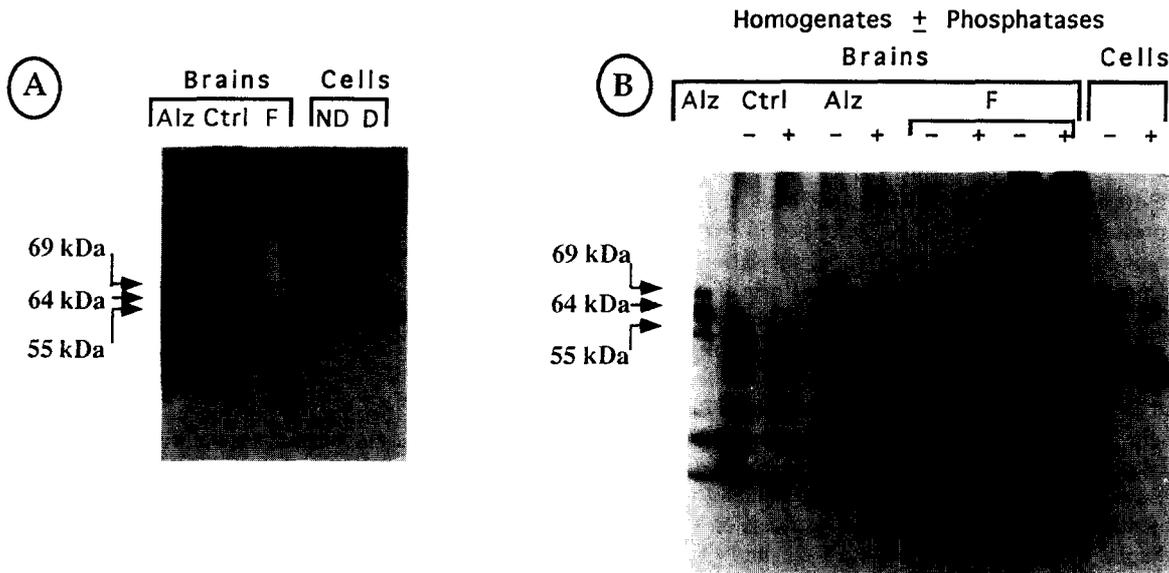


Fig. 2. Western blot analysis of homogenates from brains and SKNSH-SY 5Y cell cultures. (A) Electrophoretic pattern of Tau proteins from Alzheimer (Alz), control (Ctrl) and fetal (F) brain homogenates and from 4 days NGF-differentiated (D) or not differentiated (ND) cell homogenates. SKNSH-SY 5Y Tau proteins migrated as fetal Tau proteins. (B) Migration of Tau proteins from brain (Alz, Ctrl, F) and differentiated cell (Cell) homogenates before (-) or after phosphatase treatment (+). Two fetal Tau proteins were revealed after two different exposure times. Tau proteins in A and B were revealed by N-term serum.

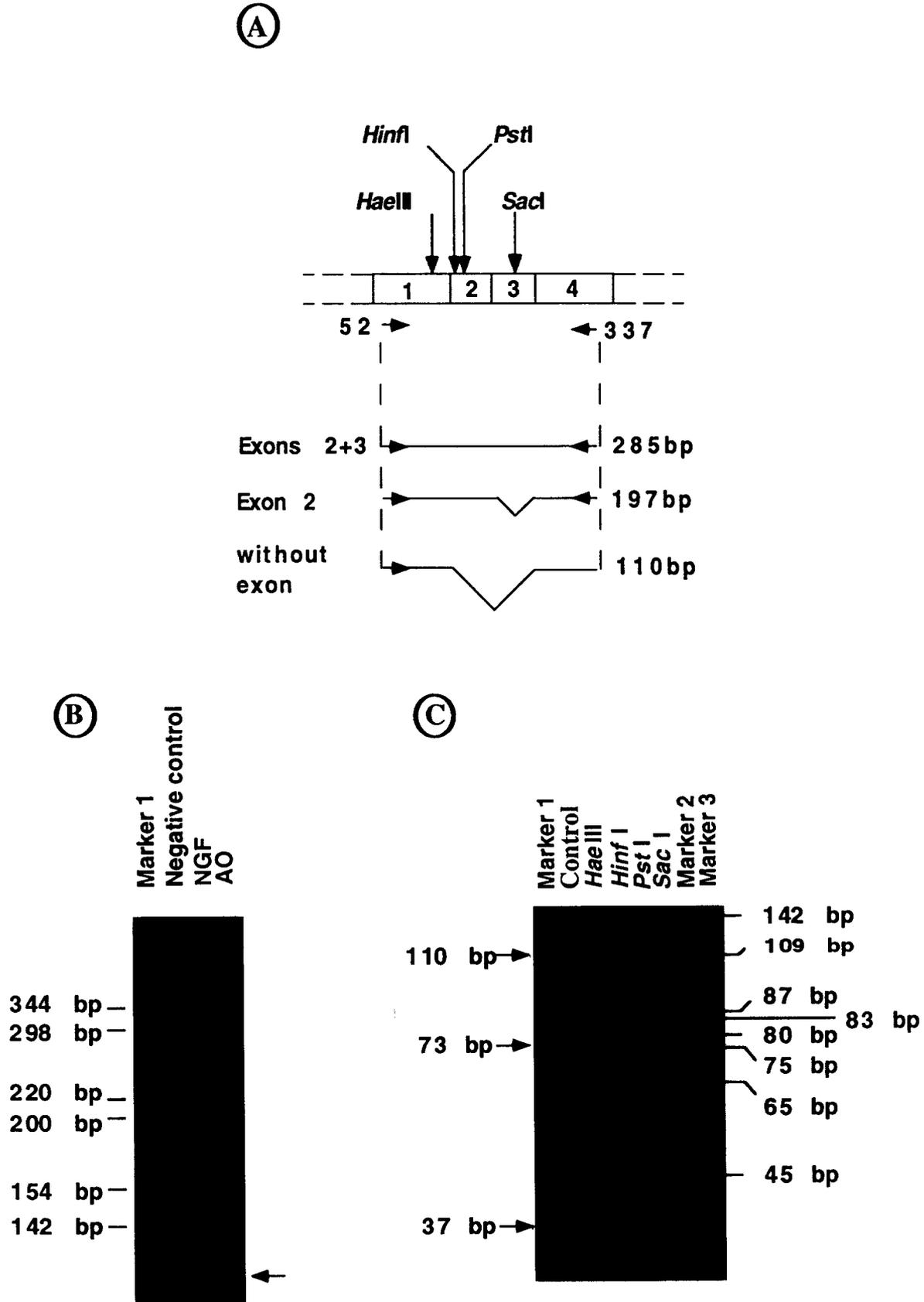


Fig. 3. Analysis of the exons 2 and 3 presence in SKNSH-SY 5Y Tau mRNA. (A) Drawing of the studied region indicating the restriction enzyme sites and the sizes of PCR products corresponding to the different possibilities of splicing. (B) RT-PCR product of Ctrl and OA treated-cells. Marker 1, DNA ladder from BRL. (C) Enzyme restriction analysis of the 110 bp band. Plasmid pblcat5 cut by *HaeIII* or *HinfI* enzymes was used as the size marker (respectively marker 2 and marker 3).

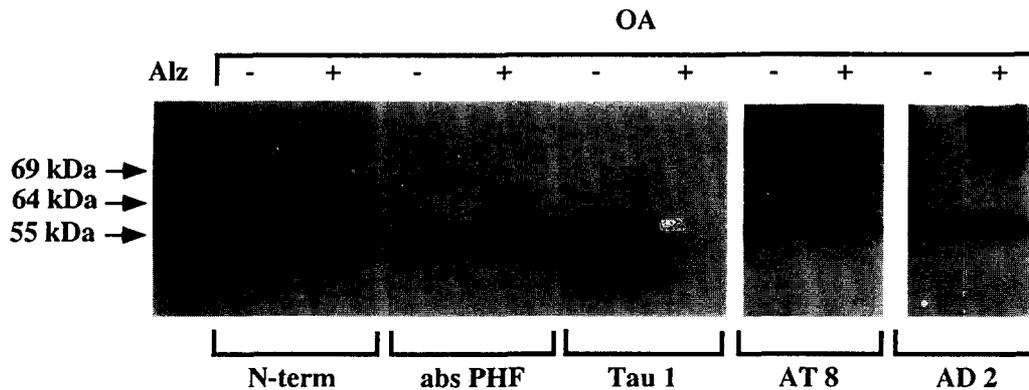


Fig. 4. Alzheimer specific antibodies' immunoreactivity on non-treated (-) and OA-treated (+) cells. NGF-differentiated cells were treated with 0.25 μ M OA for 6 h over 4 days. Tau 64 was only detected with the N-term and abs PHF polyclonal antibodies whereas Tau 55, the major band, was also detected by AT8 and AD2 monoclonal antibodies.

brains. As seen in Fig. 2A, fetal and cell Tau proteins migrated in a similar way. By comparison of Tau patterns of fetal and cellular samples after alkaline phosphatase, we confirm a similar migration for both dephosphorylated fetal and cellular Tau proteins (Fig. 2B). Therefore, the result of alkaline phosphatase treatment demonstrates a similar phosphorylation degree and the presence of the same isoforms in cellular and fetal Tau proteins. Cellular Tau protein did not then contain the N-terminal inserts.

To ascertain the absence of isoforms containing exons 2 and 3 in SKNSH-SY 5Y cells, we examined the 5' extremity of cellular mRNA by RT-PCR. Using primers on each side of exon 2 and 3, only one amplified product of about 110 bp was obtained (Fig. 3B) and corresponded to the size expected in the absence of exons 2 and 3 (Fig. 3A). The restriction enzyme digestion profile effectively corresponded to this isoform (Fig. 3C). This result was confirmed by cloning and sequencing this band of 110 bp. The sequence was identical to the one published by Goedert et al. [21] and confirmed the absence of exons 2 and 3 in this DNA and then in the cellular mRNA (not shown). Nevertheless, isoforms containing exons 2 and 3 could be newly synthesized during OA treatment. To check this point, RT-PCR analysis was performed on mRNA prepared from OA treated cells. The absence of exons 2 and 3 was confirmed since no new band was detected (Fig. 1B).

In SKNSH-SY 5Y cells, only the isoform without exons 2 and 3 was detected unless both Tau 55 and Tau 64 (corresponding to isoforms without exon 2 and to isoforms with exon 2, according to Goedert et al.'s results [5]) were induced when treating cells with OA ([16] and Fig. 3). Tau 55 was the major band, Tau 64 the minor one. Thus, in OA treated cells, the detection of Tau 64 did not seem to be due to the phosphorylation of the exon 2-containing isoform as reported for PHF-Tau 64 by Goedert et al. [5]. Then, Tau 64 detection might then be due to a hyperphosphorylation of the 55 kDa band. This would support the idea of the existence of different states of phosphorylation of the same isoform. Such a hypothesis is compatible with (i) the observation reported by Ksiezak-Reding et al. [22] that phosphatase treatment of PHF-Tau proteins induced a relative increase in the immunoreactivity of Tau 64 polypeptide and a decrease in the immunoreactivity of the

Tau 69 band; and (ii) a multiple step phosphorylation of Tau proteins by *in vitro* kinase assays [7,10].

Some common phosphorylation sites were described between fetal and Alzheimer Tau molecules [23–27]. Fetal Tau phosphorylation is highly heterogeneous and only a proportion of fetal Tau molecules is phosphorylated in the majority of the Alzheimer common sites. Since in SKNSH-SY 5Y cells, fetal-type Tau proteins were mainly detected (this paper), it was important to know if the Tau phosphorylation induced by OA treatment [16] was more related to a fetal stage or to a pathological phosphorylation. In untreated SKNSH-SY 5Y cells, Tau proteins are already phosphorylated, as shown by the action of phosphatase on Tau migration (Fig. 2B). Tau protein phosphorylation degree seemed similar to fetal phosphorylation since we observed a similar migration of both proteins before and after phosphatase treatment (Fig. 2B). Indeed, detection by Tau 1 was increased by phosphatase treatment, (not shown) then some of the Tau 1 sites must already be phosphorylated before OA treatment. This was confirmed by AT8 binding to control cellular Tau proteins (Fig. 4). In the same way, a weak AD2 immunoreactivity is seen on control Tau proteins (Fig. 4). Thus, like fetal Tau proteins, cellular Tau proteins are partially phosphorylated in some Alzheimer specific sites. Yet OA treatment induced a hyperphosphorylation and the OA-modified Tau protein electrophoretic migration was very similar to Tau 55 and Tau 64 migration of the Alzheimer triplet as described in [16]. After treatment, all the Tau 1 sites were phosphorylated since Tau proteins were no longer detected by Tau 1 antibodies. We also observed a clear increase of Tau 55 phosphorylation in Alzheimer-specific AT8 and AD2 sites after OA treatment on Tau 55 (Fig. 4). We did not succeed in obtaining the same strong immunodetection of Tau 55 with the AT8 and AD2 monoclonal antibodies as with the N-term and abs PHF polyclonal sera (perhaps because of the antibodies' concentration). This might explain why the minor Tau 64 band was only detected by using polyclonal sera.

In the OA treated cells, the apparent molecular weight change, the loss of Tau 1 immunoreactivity and the increased detection of Alzheimer epitopes on Tau molecules are in favour of a shift from fetal-type into Alzheimer-type phosphorylated Tau proteins. These results further reinforce the value of our

SKNSH-SY 5Y model for the in vitro study of Alzheimer-type Tau phosphorylation mechanisms.

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