

Abnormal Tau phosphorylation of the Alzheimer-type also occurs during mitosis

Patrice Delobel,^{*1} Stéphane Flament,^{†‡1} Malika Hamdane,^{*} Christel Mailliot,^{*} Anne-Véronique Sambo,^{*} Séverine Bégard,^{*} Nicolas Sergeant,^{*} André Delacourte,^{*} Jean-Pierre Vilain[†] and Luc Buée^{*}

^{*}INSERM U422, Institut de Médecine Prédictive et Recherche Thérapeutique, Lille, France

[†]UPRES EA 1033, University Lille 1, Villeneuve d'Ascq, France

[‡]UPRES EA 3442, Université de Nancy I, Vandœuvre les Nancy, France

Abstract

In Alzheimer's disease, neurofibrillary degeneration results from the aggregation of abnormally phosphorylated Tau proteins into filaments and it may be related to the reactivation of mitotic mechanisms. In order to investigate the link between Tau phosphorylation and mitosis, *Xenopus laevis* oocytes in which most of the M-phase regulators have been discovered were used as a cell model. The human Tau isoform ht412 (2+3–10+) was microinjected into prophase I oocytes that were then stimulated by progesterone that activate cyclin-dependent kinase pathways. Hyperphosphorylation of the Tau isoform, which is characterized by a decrease of its electrophoretic mobility and its labelling by a number of phosphorylation-dependent antibodies, was observed at the time of germinal vesicle breakdown. Surprisingly, Tau immunoreactivity, considered as typical of Alzheimer's pathology

(AT100 and phospho-Ser422), was observed in meiosis II. Because meiosis II is considered as a mitosis-like phase, we investigated if our observation was also relevant to a neuron-like model. Abnormal Tau phosphorylation was detected in mitotic human neuroblastoma SY5Y cells overexpressing Tau. Regarding AT100-immunoreactivity and phospho-Ser422, we suggest that phosphatase 2A inhibition and a phosphorylation combination of mitotic kinases may lead to this Alzheimer-type phosphorylation. Our results not only demonstrate the involvement of mitotic kinases in Alzheimer-type Tau phosphorylation but also indicate that *Xenopus* oocyte could be a useful model to identify the kinases involved in this process.

Keywords: Alzheimer's disease, microtubule-associated Tau proteins, mitosis, oocyte maturation, phosphorylation.

J. Neurochem. (2002) **83**, 412–420.

The mechanisms leading to neurodegenerative disorders referred to as tauopathies, such as Alzheimer's disease (AD) are still unknown but recent evidences had shown that neurones affected in these diseases and undergoing neurofibrillary degeneration may re-express some cell cycle genes notably implicated in the G₂/M transition (Vincent *et al.* 1998; Husseman *et al.* 2000).

Neurofibrillary degeneration is one of the neuropathological hallmarks of AD. It results from the aggregation of abnormally phosphorylated Tau proteins into paired helical filaments (PHFs). Tau proteins are mainly found in neurones. They play important roles in the polymerization and stability of microtubules (MTs). Moreover, phosphorylation is a key post-translational modification involved in their regulation. At least 30 phosphorylation sites have been described in Tau proteins, most of which occur on Ser/Pro and Thr/Pro motifs.

Despite the fact that many phosphorylation sites are common between Tau aggregated into filaments in AD and normal Tau from biopsy-derived material, some differences exist and support the idea of an abnormal phosphorylation of Tau proteins in AD (for review see Buée *et al.* 2000). This latter is also a common feature among tauopathies. Despite the lack of evidence, a possible link may exist between abnormal Tau

Received April 3, 2002; revised manuscript received June 13, 2002; accepted July 11, 2002.

Address correspondence and reprint requests to Luc Buée, INSERM U422, Institut de Médecine Prédictive et Recherche Thérapeutique, Place de Verdun, 59045 Lille, France. E-mail: buee@lille.inserm.fr

¹These authors equally contributed to the work.

Abbreviations used: AD, Alzheimer's disease; Cdks, cyclin-dependent kinases; GVBD, germinal vesicle breakdown; MTs, microtubules; PHFs, paired helical filaments.

phosphorylation leading to neurodegeneration and reactivation of kinases and/or phosphatases involved in the cell cycle.

A few phosphorylation-dependent antibodies such as AP422/988, AT100 and PHF27 that recognize conformation-dependent epitopes can visualize phosphorylated Tau aggregated into filaments (for review see Buée *et al.* 2000). Concerning this abnormal phosphorylation, there is actually no evidence that these epitopes can be generated in the mitotic phase. In fact, Tau hyperphosphorylation was already reported in mitotic cells but none of these Alzheimer-type epitopes were analysed (Pope *et al.* 1994; Preuss and Mandelkow 1998). Only okadaic acid treatment, which inhibits PP2A and PP1, allows for the genesis of these Alzheimer-type epitopes in cell culture (Mailliot *et al.* 1998; Ksiezak-Reding *et al.* 2000). One can ask if many of the key proteins involved in mitosis and found within neurofibrillary tangles, including Cdc25, cyclin-dependent kinases (Cdk5)/cyclins and polo-like kinase (Vincent *et al.* 1998; Husseman *et al.* 2000), are related to this abnormal Tau phosphorylation. In this regard, re-expression or de-regulation of the genes involved in G₂/M transition control may be some of the mechanisms that facilitate neurofibrillary degeneration in AD and other tauopathies. In order to test this hypothesis, we used the oocyte of the amphibian *Xenopus laevis* in which most of the regulators of M-phase entry have been discovered, including MPF, the famous maturation promoting factor (p34cdc2/cyclin B; Masui 2001). Following progesterone stimulation, immature oocytes undergo meiosis resumption, i.e. maturation. The oocytes arrested at prophase of the first division of meiosis, characterized by an intact nucleus called germinal vesicle, are released from this block. The first visible sign of maturation is germinal vesicle breakdown (GVBD) that releases the nucleoplasm within the cytoplasm. Microtubule polymerization allows the migration of the nuclear material towards the apex of the cell. There, spindle forms and replicated homologous chromosomes are segregated into two

batches. The end of the first division of meiosis is attested by extrusion of the first polar body. The second division of meiosis occurs without further DNA replication and without any significant interphase period. However, the cell cycle is naturally arrested at metaphase. This metaphase II arrest is maintained up to fertilization that allows sister chromatids separation as in normal mitosis. So, microtubules play a key role in meiosis and we have shown that the oocyte could be a useful model for the study of Tau function. Indeed, injection of recombinant human Tau proteins in immature oocytes modified the maturation process (Delobel *et al.* 2002a). Very recently, we also demonstrated that the AT100 epitope is generated during *Xenopus laevis* oocyte maturation without GSK3 β and pKA contribution (Delobel *et al.* 2002b). In the present study, the same model was used to show that the abnormal Tau phosphorylation including AT100 epitope occurs in mitosis (the second division of meiosis) and these data were confirmed in a human neuroblastoma cell line. These results suggest that there is probably a direct or indirect link between mitosis and abnormal phosphorylation of Tau protein and that, in neurones, as mitosis is not a physiological condition, it may lead to neurodegeneration.

Materials and methods

Antibodies

p42MAPK was detected using the anti-Erk2 antibody D-2 (Santa Cruz Biotechnology, Heidelberg, Germany). Tau antibodies used in the present study are summarized in Fig. 1, and numbering of the epitopes is given according to the longest human tau 441 isoform. AT antibodies were obtained from Innogenetics (Innogenetics, Gent, Belgium). Phosphorylation-dependent monoclonal antibodies included AD2 directed against phosphorylated Ser396–404 (Buée-Scherrer *et al.* 1996), AT180 labelling phosphorylated Thr231, AT270 recognizing phosphorylated Thr181, AT8 directed against phosphorylated Ser202/Thr205 (Goedert *et al.* 1994, 1995)

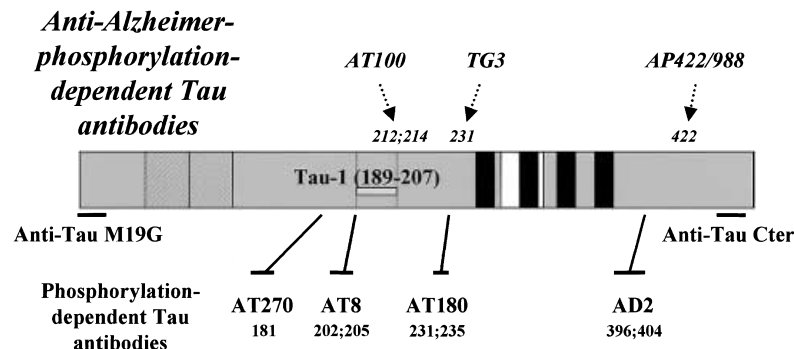


Fig. 1 Epitopes of anti-Tau antibodies. Schematic representation of the longest human Tau brain isoform (2+3+10+), which differs from the htau412 isoform by the addition of the sequence encoded by exon 3 (29 amino-acids; right hatched box). The microtubule-binding domains are black boxes. M19G and Tau-Cter recognizes Tau proteins

independently of their phosphorylation state. Tau-1 binds to its epitope when it is not phosphorylated. All other phosphorylation-dependent monoclonal antibodies recognize their epitopes when they are phosphorylated. Phosphorylation-dependent anti-Tau antibodies shown in italics are Alzheimer's pathology-specific.

and Tau-1 that binds amino-acids 189–207 only when they are dephosphorylated (Szendrei *et al.* 1993). All phosphorylation-dependent monoclonal antibodies (AD2, AT8, AT180, AT270) recognize Ser/Thr-Pro sites. Well characterized antibodies against PHF-Tau, including 988, AP422, AT100 and TG3, were also used (Hasegawa *et al.* 1996; Vincent *et al.* 1996; Zheng-Fischhöfer *et al.* 1998; Bussi re *et al.* 1999). Finally, M19G and Tau-Cter are well-characterized antisera, directed against the first 19 amino acids of the Tau sequence encoded by exon 1 (Bu e-Scherrer *et al.* 1996) and the carboxy-terminal fragment of Tau (last 15 amino acids), respectively (Sergeant *et al.* 2001). They both recognize their epitope independently of the Tau phosphorylation state.

Cell culture and stable Tau transfections

SY5Y human neuroblastoma cells were grown in 25-cm² flasks in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 1 mM non-essential amino-acids and penicillin/streptomycin (Invitrogen SARL, Cergy Pontoise, France) in a 5% CO₂ humidified incubator at 37°C. cDNA of 2 + 3–10– (3R, htau381) Tau isoform cloned in pSG5 vector (Stratagene, La Jolla, CA, USA) was a kind gift of Dr Michel Goedert (Cambridge, UK). It was subcloned into stably eucaryotic expression vector pcDNA3.1Neo (Invitrogen SARL), allowing for a G418 (Invitrogen SARL) selection of stable clones (Mailliot *et al.* 2000). This 3R tau cDNA was independently stably transfected into SY5Y cells using the ethyleneimine polymer ExGen500 (Euromedex, Mundolsheim, France) according to manufacturer's instructions. A mock cell line was stably transfected with the pcDNA3.1Neo vector alone, without any tau cDNA insert (Mailliot *et al.* 2000).

Poison-arrested G₂/M cells were obtained by treating transfected SY5Y cells (50% confluent) with either 0.4 mg/mL nocodazole (Calbiochem, San Diego, CA, USA) or 10 µM Taxotere (Aventis Pharma, Vitry, France) for 24 h. To generate abnormal Tau phosphorylation by inhibiting phosphatases, SY5Y cells were treated by 125 nM okadaic acid for 6 h as previously described (Mailliot *et al.* 1998, 2000).

Cell lysates and immunoprecipitation

To prepare cell lysates for biochemical analyses, cells were centrifuged after EDTA treatment and recovered in ice lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA) with protease inhibitors (Complete Mini, Roche Molecular Biochemicals, Meylan, France), sonicated and rocked overnight at 4°C. After 13 000 g centrifugation, one volume of cell supernatant was then mixed to one volume of 2× Laemmli sample buffer and heat-treated for 5 min at 100°C.

For immunoprecipitation studies, cell pellets were lysed in ice Tris-buffered saline (10 mM Tris pH 7.4, 150 mM NaCl), sonicated and treated 10 min by DNase I (Invitrogen). After 10 000 g centrifugation at 4°C for 10 min, the supernatant was treated with 5 µL of UltralinkTM Immobilized Protein A/G Plus (Pierce, Perbio Science, Bezons, France) during 30 min at 4°C. Supernatant was clarified by 2000 g centrifugation at 4°C for 5 min, incubated with 10 µL of Tau-Cter or AP422 antibodies and mixed overnight at 4°C. Then, 10 µL of A/G Protein were added to the solution and mixed 30 min at 4°C. After centrifugation, supernatant was removed and immunoprecipitated complexes were washed three times with Tris-buffered saline. Finally, complexes were treated with 50 µL

Laemmli sample buffer and heated for 5 min at 60°C. All Laemmli samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting.

Handling of oocytes and Tau injections

Adult *Xenopus laevis* females were purchased from Centre de Recherches de Biochimie Macromol culaire (C.R.B.M.) (CNRS, Montpellier, France). They were not primed with any gonadotropins. After anaesthesia with 1 g/L MS222, ovarian lobes were surgically removed and placed in ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES/NaOH, pH 7.5). After defolliculation, full-grown stage VI oocytes were stored at 12°C in ND96 medium. Recombinant Tau proteins (2+3–10+; htau412) or buffer alone were microinjected in oocytes at a constant volume of 50 nL per stage VI oocyte by the use of a positive displacement digital micropipette (Nichiryo, Tokyo, Japan) as previously described (Delobel *et al.* 2002a,b).

Maturation was induced in ND96 medium by progesterone addition (10 µM; Flament *et al.* 1996). With the exception of post-GVBD Tau injection, the hormone was always added 1 h after the injection of htau412 or drug application to allow diffusion in the oocyte due to its size (1.2 mm in diameter). Maturation was determined either by white spot detection or by microscopic examination after dissection of heat-treated oocytes (100°C, 3 min).

Protein synthesis was inhibited using cycloheximide (100 (g/mL) and oocytes were maintained in this medium from 2 h before the microinjection of htau412 until the end of the experiment.

The fusion protein GST-Cdc25A was prepared as previously described (Borgne and Meijer 1996) and microinjected at 30 nL/oocyte 1 h after htau412 microinjection.

Electrophoresis and western blotting

Depending on the experiment, 5–20 oocytes were homogenized in homogenization buffer [MOPS 25 mM pH 7.2 with protease and phosphatase inhibitors (Azzi *et al.* 1994) – 10 µL/oocyte] and centrifuged for 5 min at 13 000 g (4°C) to eliminate yolk platelets. One volume of 2× Laemmli sample buffer was added to 1 volume of supernatant. Pellet was resuspended in a similar volume of the same mixture. Proteins were denatured by heating the mixture (100°C–5 min) and then separated by 10% SDS–PAGE. In the particular case of p42MAPK immunodetection, proteins were resolved by 15% SDS–PAGE (prepared from a stock solution containing 29.82% acrylamide/0.18% bisacrylamide). Such gels allowed a good discrimination between active and inactive MAPK (Bodart *et al.* 1999). Separated proteins were transferred using a semidry apparatus onto either Hybond nitrocellulose sheets or polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia, Orsay, France). After blocking, membranes were then washed in Tris-buffered saline pH 8 with 0.05% Tween-20 (TBST) and incubated for 90 min with primary antibody. After three washes in TBST, horseradish peroxidase (HRP)-coupled secondary antibody was added for 1 h (Sigma, St Louis, MO, USA). After washing, HRP activity was detected with ECL system (Amersham Pharmacia).

Immunofluorescence studies

Transfected SY5Y cells were subcultured on poly L-Lys-treated two-well cell chamber slide (Laboratory-Tek, Nalge Nunc International,

VWR, Fontenay, France) for 48 h. Cells were then fixed for 30 min in 4% paraformaldehyde, and permeabilized with 0.25% Triton X-100. After blocking, fixed materials were incubated for 2 h at room temperature with primary antibodies. After washing, TRITC-conjugated secondary antibody was used. DAPI staining was performed after the immunocytochemistry to discriminate non-proliferating from mitotic cells. Samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA).

Cells were mitotically arrested either by 0.4 mg/mL nocodazole (Calbiochem) or 10 μ M Taxotere (Aventis Pharma) treatments for 24 h. Similar conditions were used for immunofluorescence studies. All data were analysed using the Leica image analysis.

Results

Hyperphosphorylation of injected Tau during *Xenopus* oocytes maturation

As previously described (Delobel *et al.* 2002a), 0.6 μ M httau412 did not interfere with oocyte maturation exhibiting GVBD, white spot appearance, extrusion of the first polar body and meiotic spindle (Fig. 2a, data not shown). This concentration was kept for all further experiments.

Recombinant httau412 was injected into prophase I oocytes. At that time (0) of induced maturation, it exhibited an apparent molecular weight (M_r) at about 61 kDa and was detected with a polyclonal anti-Tau antibody (M19G) (Fig. 2b). Then, slight changes were already observed after 1 h of progesterone stimulation. At that time, the immunodetection profile was made of two bands at 61 and 65 kDa. A 69-kDa shifted form of httau412, detected with the M19G antibody, occurred 4 h after hormone stimulation (Fig. 2b). Several antibodies were then used to analyse the phosphorylation state of Tau proteins during oocyte maturation. First, the staining by the Tau-1 monoclonal antibody was observed at the beginning of the time course experiment and disappeared 4 h later. Then, the staining with antibodies raised against phosphorylation-dependent epitopes, such as AT180, AT270 or AD2 only fully appeared after 4 h of incubation in progesterone-containing medium. These antibodies recognized the 69 kDa band of httau412. Moreover, always 4 h after progesterone addition, TG3, specific of a mitotic Tau epitope, exhibited a similar labelling. Finally, other immunological tools were also studied: AT100, AP422 and 988 that recognize specifically PHF-Tau. These latter ones also detected the upper form of httau412 and did not stain the protein during the first 4 h of the experiment (Fig. 2b). These data indicate that, in this model, it is possible to follow the time-course of phosphorylation of Tau proteins, and particularly that leading to AD-specific epitopes. It should be noted that the appearance of AT100-immunoreactivity was also seen with an oocyte maturation using insulin stimulation (Delobel *et al.* 2002b). However, in

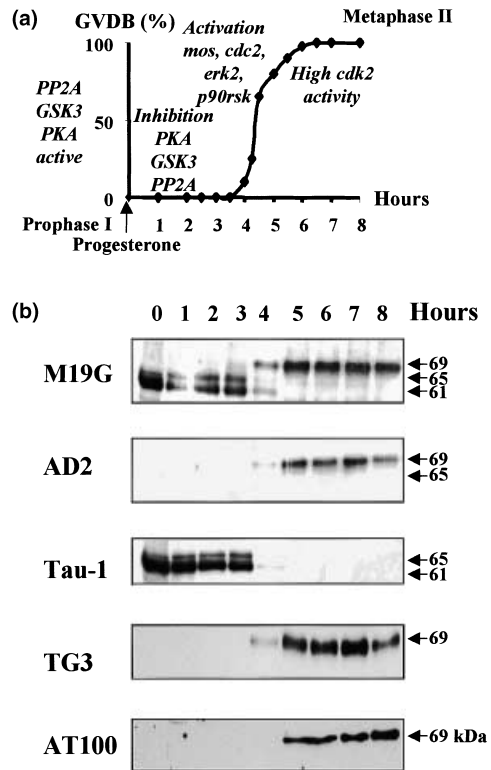


Fig. 2 Tau microinjection and oocyte maturation. Effect of Tau injection on *Xenopus* oocyte maturation. (a) Time-course experiment after progesterone stimulation shows that Tau injection (50 ng) does not affect oocyte maturation compared to buffer injection alone (control). GSK3 β and pKA are active in prophase I and inactivated after progesterone stimulation. In meiosis I, a number of kinases are activated including *mos*, *erk2*, *cdc2* and *p90rsk*. Other kinases may be activated during GVBD such as *cdk2*. (b) Immunoblotting analysis with several anti-Tau antibodies of the phosphorylation state of httau412 during oocyte maturation. M19G labels Tau proteins independently of their phosphorylation state. A 61-kDa Tau isoform is observed at t0. Two Tau variants at 61 and 65 kDa are visualized between 1 and 3 h after progesterone stimulation. After 4 h, a main variant is seen at 69 kDa. These different electrophoretic mobilities of Tau variants reflect their state of phosphorylation. It is possible to confirm that using phosphorylation-dependent antibodies. AD2 recognizes phosphorylation sites on Tau. It strongly labels the high molecular weight variant at 69 kDa after 4 h. A weak signal poorly detectable is observed at 3 h for the 65 kDa variant. No AD2-immunoreactivity is observed for the 61 kDa variant. Similar data were obtained with AT8, AT180 and AT270 antibodies. Conversely, Tau-1 recognizes a dephosphorylated Tau epitope and never labels the 69 kDa variant. It only labels the 61 and 65 kDa variants. TG3 and AT100 are two Alzheimer-specific phosphorylation-dependent antibodies and only label the 69 kDa Tau variant after 4 and 5 h, respectively. Similar data were observed with 988 antibody.

these conditions, maturation requires more time. All further experiments on oocyte maturation were also done using progesterone stimulation.

Requirement of oocyte maturation for Alzheimer-type epitopes formation

Because these last experiments and our last work (Delobel *et al.* 2002b) indicated that maturation process is absolutely required for the appearance of Alzheimer epitopes, different experiments were then performed to determine what part of the maturation process was needed. In fact, meiosis I is a particular division whereas meiosis II is considered as a mitosis-like stage.

Analysis of Tau phosphorylation in meiosis I

First, microinjection of *cdc25A* phosphatase was used to induce entry into meiosis without progesterone stimulation. In this case, the maturation is not complete. Indeed, the oocytes undergo GVBD, chromosomes condense but the spindle is not organized and the oocytes stay in meiosis I (Rime *et al.* 1994). At the biochemical level, p34cdc2 is activated as a result of the dephosphorylation of its Thr 14 and Tyr 15 residues by *cdc25*. We also observed that the kinase p39mos is synthesized and that MAP kinase is activated (data not shown). Following *cdc25A* microinjection, htau412 was phosphorylated and its electrophoretic mobility decreased to an apparent M_r lower (65 kDa) than those observed with progesterone alone (69 kDa). Both AD2 and TG3 antibodies recognized the shifted band as soon as 1 h after *cdc25A* microinjection and for at least 6 h (Fig. 3; Cdc25). However, no staining was observed with AT100 (Fig. 3).

Secondly, the same immunodetection profile was observed when *cdc25A* was injected in the presence of cycloheximide (Fig. 3; Cdc25 + CHX). However, in the latter case, even if p34cdc2 is also activated, p39mos was not synthesized and MAP kinase remained inactive (data not shown). Similar results to those obtained with Cdc25 injection alone were

obtained (Fig. 3). Thus, these data suggested that TG3-immunoreactivity in oocytes is related to the initial activation of p34cdc2 and not to MAPK.

Analysis of Tau phosphorylation in meiosis II

Altogether, these experiments suggested that kinases involved in meiosis I are not sufficient to generate either AP422/988 or AT100 epitopes. Nevertheless, the phosphorylation events that occur at the beginning of the maturation process may be a prerequisite for the appearance of 988 and AT100 stainings at later stages. To show if meiosis II entry is sufficient for inducing Alzheimer-type Tau immunoreactivity, injection of htau412 was done in progesterone-stimulated stage VI oocytes after GVBD. Oocytes at GVBD stage were identified by the appearance of a white spot at their animal pole 4–5 h after hormone addition. After Tau injection, oocytes were frozen every hour during 8 h. The results of the western blot analysis shown in Fig. 4 revealed that the phosphorylation that decreased the electrophoretic mobility of htau412 and led to its recognition by AT100, AP422 and 988 has occurred in these oocytes as soon as 2 h after microinjection, whereas AD2 and TG3 were obtained 1 h after the Tau injection. All of these data indicate that meiosis II is required and sufficient for Alzheimer-type epitopes and that meiosis I is neither essential nor a prerequisite for the appearance of AP422/988 and AT100 epitopes.

Abnormal Tau phosphorylation in mitotic neuroblastoma cells

To ascertain that Alzheimer Tau epitopes are really generated during mitosis and that the present data are not particular to the *Xenopus* oocyte maturation, the presence of these epitopes was investigated in neuroblastoma cells stably transfected with Tau. Immunofluorescent studies using

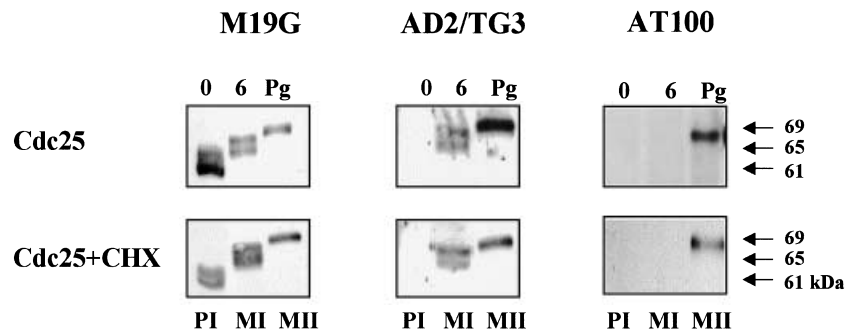


Fig. 3 Effect of the *cdc25* phosphatase microinjection. The p34cdc2-activating phosphatase was microinjected in ND96 medium (Cdc25) or in cycloheximide-containing medium (Cdc25 + CHX). At prophase I (PI), after Cdc25 injection (0), Tau is weakly phosphorylated and migrates at 61 kDa as indicated by M19G labeling. No AD2 or TG3-immunoreactivity is observed. One hour after Cdc25 injection (1), the oocyte enters meiosis I (MI). Tau exhibits a 65-kDa molecular weight

and is labelled by not only M19G but also both AD2 and TG3 antibodies (AD2/TG3). No AT100 labelling was observed. Conversely, after progesterone treatment (Pg), in metaphase II (MII), Tau migrates at 69 kDa as a highly phosphorylated variant and is labelled by all antibodies including AT100. Similar data are observed in presence of cycloheximide (Cdc25 + CHX).

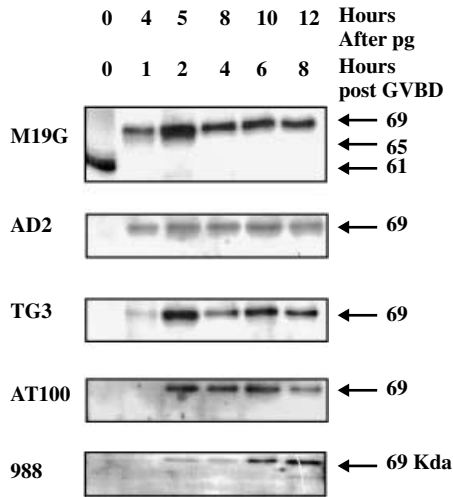


Fig. 4 Study of htau412 injected after GVBD into progesterone-stimulated oocytes. The 61 kDa recombinant htau412 (0) is injected into oocyte when GVBD is observed. A time-course experiment is then performed and Tau is analysed after 1, 2, 4, 6 and 8 h after Tau injection (post-GVBD). Only one Tau variant is observed at 69 kDa from 1 to 8 h, and is labelled by all antibodies including M19G, AD2 and TG3. Only AT100 and 988 epitopes occur 2 h after Tau injection (post-GVBD).

anti-Tau antibodies indicated that Tau proteins were detected in all cells. There was no increase in Tau-immunoreactivity in mitotic cells (Fig. 5d). Conversely, using phosphorylation-dependent anti-Tau antibodies (AD2 and AT8), a stronger

immunoreactivity was observed in mitotic cells compared to other cells (Fig. 5c). Thus, Tau proteins are hyperphosphorylated during mitosis. Moreover, Alzheimer-type epitopes were also visualized in these mitotic cells (Figs 5a and b). Furthermore, nocodazole or taxotere-treated cells confirmed these data. Indeed, in these conditions (24-h exposure), between 40 and 60% of the cells are mitotically arrested at G_2/M transition. Nocodazole or taxotere-arrested G_2/M cells were effectively labelled not only with phosphorylation-dependent antibodies such as AT8 (Figs 5g and k), but also with Alzheimer-dependent antibodies (Figs 5e, f, i and j) whereas non-mitotic cells were only labelled by AT8 (Figs 5g and k). Alzheimer-type Tau-immunolabelling was slightly observed at the cell periphery and did not localize with microtubule structures (data not shown). As the two drugs act in an opposite way on microtubules (either stabilization or depolymerization), abnormal Tau phosphorylation was likely to be related to the G_2/M arrest and not to microtubule stability. Finally, it should be noted that in apoptotic cells with or without treatment by these drugs, we were not able to detect any Tau phosphorylation (AD2, AT8, 988 or AT100).

By immunoblotting, Tau proteins were highly phosphorylated, as visualized by phosphorylation-dependent antibodies such as AD2, but no Alzheimer-type Tau phosphorylation was detected in non-synchronized SY5Y neuroblastoma cells (Fig. 6). However, in poison-arrested cells, most of Tau proteins exhibited low M_r suggesting that their degree of phosphorylation was low. However, one thin Tau band

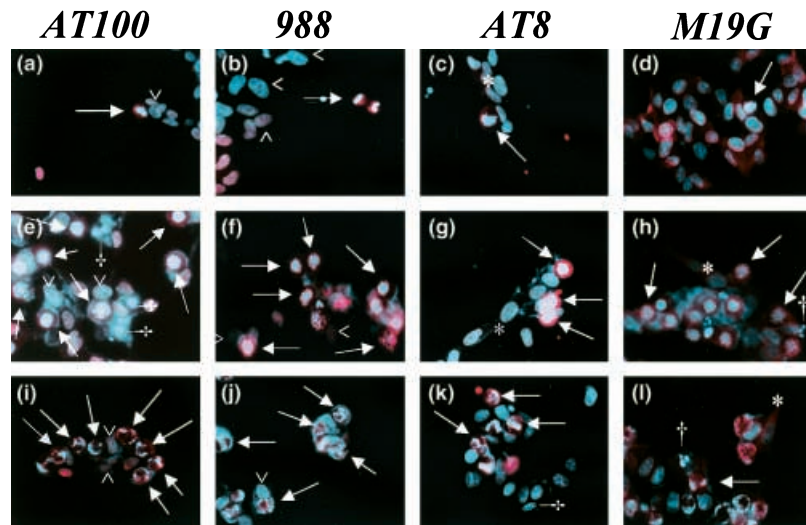


Fig. 5 Tau phosphorylation in Tau stably transfected neuroblastoma cells: immunocytochemistry. Cells were 50% confluent and then treated or not for 24 h (a–d) with either nocodazole (0.4 mg/mL; e–h) or taxotere 10 μ M (i–l). Immunofluorescence was performed with AT100 (a, e, i), 988 (b, f, j), AT8 (c, g, k) and M19G (d, h, l). DAPI staining was also performed to discriminate non-proliferating, mitotic and apoptotic cells. Some examples of the different stainings were

illustrated using specific signs. Arrows designate mitotic cells. †Arrows label apoptotic cells. Arrowheads indicate lack of staining. Asterisks indicate labelling of non-dividing cells (asterisks were not included in (d) as all cells, with the exception of the mitotic one (arrow), should have an asterisk. Note that treatment by taxotere or nocodazole provide 40–60% G_2/M cell arrest.

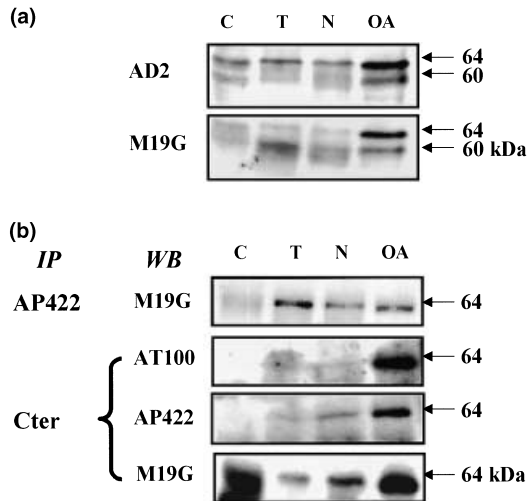


Fig. 6 Tau phosphorylation in Tau stably transfected neuroblastoma cells: immunoblotting. Cells were 50% confluent and then treated or not for 24 h (C) with taxotere (T), nocodazole (N) or okadaic acid (OA). (a) Immunoblotting analysis with anti-Tau antibodies AD2 and M19G. The antibodies label both Tau transgene and endogenous Tau at 60 and 64 kDa, respectively. It should be noted that in poison-arrested cells (lanes N and T), most of the Tau variants showed low apparent molecular weight (around 60 kDa) and poor AD2-immunoreactivity with the exception of a high M_r Tau variant at 64–65 kDa. (b) As it was not possible to detect Alzheimer-type phosphorylation in total cell lysates, immunoprecipitation of Tau proteins was performed. Tau proteins were immunoprecipitated (IP) using either the carboxy-terminal anti-Tau antibody or 988. Immunoblotting (WB) was performed using antibodies against Alzheimer-type epitopes (AT100 and AP422) and M19G. Only the 64 kDa Tau variant was clearly observed in OA-treated cells whereas no labelling was visualized in control cells (C) although large amounts of Tau proteins were immunoprecipitated (see M19G-immunoreactivity). The endogenous Tau was visualized with longer exposure. Interestingly, the high M_r variant (64 kDa) in nocodazole- and taxotere-treated cells was also detected confirming immunocytochemistry and indicating that poison-arrested G_2/M cells exhibited an Alzheimer-type abnormal phosphorylation.

exhibited the same high M_r than Tau isolated from okadaic acid-treated cells and it was recognized by phosphorylation-dependent antibodies such as AD2 (Fig. 6a). To facilitate the detection of this high M_r Tau variant in poison-arrested G_2/M cells, immunoprecipitation of Tau proteins was performed and an Alzheimer-type Tau phosphorylation using AT100 and AP422 was observed (Fig. 6b).

Altogether, these data indicated that as observed in *Xenopus* oocytes, the so-called abnormal phosphorylation of Tau proteins is related to molecular mechanisms similar to those encountered during mitosis.

Discussion

Neuropathologically, Alzheimer's disease is characterized by amyloid deposition and neurofibrillary degeneration. This

latter results from the intraneuronal accumulation of paired helical filaments constituted mainly of abnormally phosphorylated Tau proteins. Aggregated Tau variants are specifically recognized with a few antibodies including AP422/988 (Hasegawa *et al.* 1996; Bussi re *et al.* 1999), AT100 (Zheng-Fishh fer *et al.* 1998) and TG3 (Vincent *et al.* 1996).

The first aim of this study was to demonstrate that mitotic mechanisms could induce the abnormal phosphorylation of Tau proteins in Alzheimer's disease. Indeed, it has been shown by immunofluorescence that there is a TG3-immunoreactivity in mitotic cells of the human neuroblastoma line MSN, but not in quiescent ones (Vincent *et al.* 1996). Nevertheless, it should be noted that TG3 also cross reacts with nucleolin, a mitotic phospho-protein (Peter *et al.* 1990; Husseman *et al.* 2000). However, indirect evidence based on the observation of aberrant expression of the *cdc2/cyclin B1* mitotic kinase and other cell cycle modulators in degenerating neurones of AD brains also supported this mitotic hypothesis (Vincent *et al.* 1997; Arendt *et al.* 2000; Ding *et al.* 2000; Harris *et al.* 2000).

The results obtained in *Xenopus* oocyte as a model not only support this hypothesis but also first demonstrate a direct link between M-phase and the appearance of all phosphorylation-dependent Alzheimer epitopes (AT100, TG3 and AP422/988). They are not particular to the *Xenopus* oocyte as similar data were obtained in the human neuroblastoma cell line SKN-SH SY5Y.

In fact, an interesting finding of our study is that following progesterone stimulation, all PHF-Tau-specific antibodies AP422/988, AT100 and TG3, recognized htau412. These immunological tools appear to be the most specific to neurofibrillary degeneration and are the only ones that specifically recognize abnormally phosphorylated Tau proteins. Furthermore, it is well known that *cdc25A* injection alone does not lead to a normal maturation: oocytes undergo GVBD but they arrest their cell cycle at the first division of meiosis as demonstrated by the absence of polar body extrusion (Rime *et al.* 1994). So, as no AP422/988 or AT100 Tau-immunoreactivity was found in these conditions, these first data suggested that the second division of meiosis is likely to be essential for the genesis of Alzheimer-type epitopes. It was confirmed when Tau proteins were injected post-GVBD. Two hours after Tau injection, both AP422/988 and AT100 epitopes were detected indicating that molecular events of the second division of meiosis, which is considered as mitosis, are sufficient to produce the Alzheimer-type phosphorylation.

The fact that we observed these epitopes after GVBD suggests that M-phase mechanisms are involved in their genesis. These phosphorylated epitopes include Thr212/Ser214, Thr231 and Ser422. It has been shown recently that Ser214 was a prominent phosphorylation site in cells mitotically arrested with nocodazole (Illenberger *et al.* 1998). However, the detection of Alzheimer epitopes such

as AT100 was not reported and this treatment may lead to activation of numerous kinases and apoptosis (Wang *et al.* 1998; Shtil *et al.* 1999). Regarding Thr231, it is clear that p34cdc2 is responsible for the immunoreactivity recognized by TG3 antibody as experiments done in meiosis I stage allowed discrimination between this kinase and classical MAPK pathway. Moreover, Cdc2 is still activated in meiosis II and may be responsible for TG3 epitope genesis. However, other phenomena may also be implicated. For instance, phospho-Thr231 is a binding site of Pin1, an enzyme involved in G₂/M transition (Lu *et al.* 1999). Regarding AP422/988 epitope, Ser422 is also a proline-directed protein kinase site and phosphorylated by PK40erk2 and stress-activated protein kinases (Hasegawa *et al.* 1996; Roder *et al.* 1997; Buée-Scherrer and Goedert 2002). Furthermore, it was suggested that PP2A might be inactivated during oocyte maturation (Karaiskou *et al.* 1999). These data are consistent with fact that Tau is hyperphosphorylated in cell models using the PP2A inhibitor, okadaic acid (Mailliot *et al.* 1998; Ksiezak-Reding *et al.* 2000). Thus, Alzheimer-type epitopes may result from the activation of a combination of kinases (Akt, Cdks, PK40erk2 and SAPKs) and a PP2A inhibition.

The results obtained with oocyte maturation were also confirmed using stable transfected neuroblastoma cells. In these cells, abnormal Tau phosphorylation was detected in mitotic cells, but the signal was weak compared to Tau hyperphosphorylation (AT8). This result may be explained by the fact that hyperphosphorylation of Tau proteins is increased by 18 times in mitotic cells compared to non-proliferating cells (Pope *et al.* 1994). Moreover, these epitopes implicate certainly other kinases or events than those involved in abnormal phosphorylation of Tau proteins. Finally, treatments by nocodazole or taxotere ascertain these results. Indeed, these drugs provide G₂/M arrest by polymerization or depolymerisation of microtubules. In both cases, G₂/M arrested cells exhibited AP422/988 and AT100 weak labellings. This poor immunoreactivity may be explained by an arrest in G₂/M transition and not really in mitosis as it occurs in metaphase II in *Xenopus* oocytes. Finally, it should also be noted that no abnormal phosphorylation of Tau proteins was observed when the cells undergo apoptosis and especially after taxotere or nocodazole treatment.

Our results show that *Xenopus* oocyte appears to be a very interesting model in which the kinases involved in the abnormal phosphorylation of Tau could be characterized. Indeed, it is one of the rare cells in which the phosphorylation of Tau is very close to those occurring in AD. For instance, the Alzheimer-like modification of Tau in *Xenopus* oocyte does not require any drug or inhibitor unlike transfected cell lines that should be treated with the phosphatases inhibitor okadaic acid (Mailliot *et al.* 1998; Ksiezak-Reding *et al.* 2000), nocodazole or taxotere (Illenberger *et al.* 1998; present study). Moreover, *Xenopus* oocyte appears as a physiological model. Indeed, even if

there is no endogenous Tau protein in this cell, the phosphorylation of injected Tau proteins (50 ng) occurred following stimulation with progesterone, the natural inducer of meiosis, and does not affect the maturation process.

In conclusion, our study demonstrates that the abnormal Tau phosphorylation of the Alzheimer-type also occurs during mitosis.

Acknowledgements

We would like to thank Drs Michel Goedert and Ross Jakes (MRC Cambridge UK) for providing recombinant htau412 protein, Prof. Leslie Binder (Chicago, IL, USA) for Tau-1 antibody, Prof. Peter Davies (AECOM, New York, NY, USA) for TG3 antibody, Dr Hanno Roder (NADAG, Munich, Germany) for AP422 antibody, M. Garnier (CNRS, Roscoff, France) for GST-Cdc25A. PD is a recipient of a MRT (French Research ministry) Fellowship. These studies were supported by Aventis Pharma, Centre National de la Recherche Scientifique (CNRS), Institut National de la Santé Et de la Recherche Médicale (INSERM), grants from the Institute for the Study of Aging (New York, NY, USA), the French 'Ministère de l'Éducation Nationale' (EA1033), the 'Région Nord-Pas-de-Calais' (Centre de Biologie Cellulaire and Génopole de Lille), the 'Region Guadeloupe' and the FEDER.

References

- Arendt T., Holzer M., Stobe A., Gartner U., Luth H. J., Bruckner M. K. and Ueberham U. (2000) Activated mitogenic signaling induces a process of dedifferentiation in Alzheimer's disease that eventually results in cell death. *Ann. NY Acad. Sci.* **920**, 249–255.
- Azzi L., Meijer L., Ostvold A. C., Lew J. and Wang J. H. (1994) Purification of a 15-kDa cdk4- and cdk5-binding protein. *J. Biol. Chem.* **269**, 13279–13288.
- Bodart J. F., Bechard D., Bertout M., Gannon J., Rousseau A., Vilain J. P. and Flament S. (1999) Activation of *Xenopus* eggs by the kinase inhibitor 6-DMAP suggests a differential regulation of cyclin B and p39 (mos) proteolysis. *Exp. Cell Res.* **253**, 413–421.
- Borgne A. and Meijer L. (1996) Sequential dephosphorylation of p34 (cdc2) on Thr-14 and Tyr-15 at the prophase/metaphase transition. *J. Biol. Chem.* **271**, 27847–27854.
- Buée L., Bussièrè T., Buée-Scherrer V., Delacourte A. and Hof P. R. (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res. Rev.* **33**, 95–130.
- Buée-Scherrer V. and Goedert M. (2002) Phosphorylation of microtubule-associated protein Tau by stress-activated protein kinases in intact cells. *FEBS Lett.* **515**, 151–154.
- Buée-Scherrer V., Condaminès O., Mourton-Gilles C., Jakes R., Goedert M., Pau B. and Delacourte A. (1996) AD 2, a phosphorylation-dependent monoclonal antibody directed against Tau proteins found in Alzheimer's disease. *Mol. Brain Res.* **39**, 79–88.
- Bussièrè T., Hof P. R., Mailliot C., Brown C. D., Caillet-Boudin M. L., Perl D. P., Buée L. and Delacourte A. (1999) Phosphorylated serine422 on Tau proteins is a pathological epitope found in several diseases with neurofibrillary degeneration. *Acta Neuropathol.* **97**, 221–230.
- Delobel P., Flament S., Hamdane M., Jakes R., Rousseau A., Delacourte A., Goedert M. and Buée L. (2002a) Functional characterization of FTDP-17 tau gene mutations through their effects on *Xenopus* oocyte maturation. *J. Biol. Chem.* **277**, 9199–9205.

- Delobel P., Flament S., Hamdane M., Delacourte A., Vilain J. P. and Buée L. (2002b) Modelling Alzheimer-specific Tau phosphorylation independently of GSK3 β and pKA kinase activities. *FEBS Lett.* **516**, 151–155.
- Ding X. L., Husseman J., Tomashevski A., Nochlin D., Jin L. W. and Vincent I. (2000) The cell cycle Cdc25A tyrosine phosphatase is activated in degenerating postmitotic neurons in Alzheimer's disease. *Am. J. Pathol.* **157**, 1983–1990.
- Flament S., Browaeys E., Rodeau J. L., Bertout M. and Vilain J. P. (1996) *Xenopus* oocyte maturation: cytoplasm alkalization is involved in germinal vesicle migration. *Int. J. Dev. Biol.* **40**, 471–476.
- Goedert M., Jakes R., Crowther R. A., Cohen P., Vanmechelen E., Vandermeeren M. and Cras P. (1994) Epitope mapping of monoclonal antibodies to the paired helical filaments of Alzheimer's disease: identification of phosphorylation sites in Tau protein. *Biochem. J.* **301**, 871–877.
- Goedert M., Jakes R. and Vanmechelen E. (1995) Monoclonal antibody AT8 recognises Tau protein phosphorylated at both serine 202 and threonine 205. *Neurosci. Lett.* **189**, 167–169.
- Harris P. L., Zhu X., Pames C., Rottkamp C. A., Ghanbari H. A., McShea A., Feng Y., Ferris D. K. and Smith M. A. (2000) Neuronal polo-like kinase in Alzheimer's disease indicates cell cycle changes. *Neurobiol. Aging* **21**, 837–841.
- Hasegawa M., Jakes R., Crowther R. A., Lee V. M., Ihara Y. and Goedert M. (1996) Characterization of mAb AP422, a novel phosphorylation-dependent monoclonal antibody against Tau protein. *FEBS Lett.* **384**, 25–30.
- Husseman J. W., Nochlin D. and Vincent I. (2000) Mitotic activation: a convergent mechanism for a cohort of neurodegenerative diseases. *Neurobiol. Aging* **21**, 815–828.
- Illenberger S., Zheng-Fischhofer Q., Preuss U., Stamer K., Baumann K., Trinczek B., Biernat J., Godemann R., Mandelkow E. M. and Mandelkow E. (1998) The endogenous and cell cycle-dependent phosphorylation of Tau protein in living cells: implications for Alzheimer's disease. *Mol. Biol. Cell* **9**, 1495–1512.
- Karaïskou A., Jessus C., Brassac T. and Ozon R. (1999) Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification. *J. Cell Sci.* **112**, 3747–3756.
- Ksiezak-Reding H., He D., Gordon-Krajcer W., Kress Y., Lee S. and Dickson D. W. (2000) Induction of Alzheimer-specific Tau epitope AT100 in apoptotic human fetal astrocytes. *Cell Motil. Cytoskeleton* **47**, 236–252.
- Lu P. J., Wulf G., Zhou X. Z., Davies P. and Lu K. P. (1999) The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated Tau protein. *Nature* **399**, 784–788.
- Mailliot C., Bussière T., Caillet-Boudin M. L., Delacourte A. and Buée L. (1998) Alzheimer-specific epitope of AT100 in transfected cell lines with Tau: toward an efficient cell model of Tau abnormal phosphorylation. *Neurosci. Lett.* **255**, 13–16.
- Mailliot C., Bussière T., Hamdane M., Sergeant N., Caillet-Boudin M. L., Delacourte A. and Buée L. (2000) Pathological Tau phenotypes. The weight of mutations, polymorphisms, and differential neuronal vulnerabilities. *Ann. NY Acad. Sci.* **920**, 107–114.
- Masui Y. (2001) From oocyte maturation to the *in vitro* cell cycle: the history of discoveries of maturation-promoting factor (MPF) and cytosstatic factor (CSF). *Differentiation* **69**, 1–17.
- Peter M., Nakagawa J., Doree M., Labbe J. C. and Nigg E. A. (1990) Identification of major nucleolar proteins as candidate mitotic substrates of cdc2 kinase. *Cell* **60**, 791–801.
- Pope W. B., Lambert M. P., Leybold B., Seupaul R., Sletten L., Krafft G. and Klein W. L. (1994) Microtubule-associated protein Tau is hyperphosphorylated during mitosis in the human neuroblastoma cell line SH-SY5Y. *Exp. Neurol.* **126**, 185–194.
- Preuss U. and Mandelkow E. M. (1998) Mitotic phosphorylation of Tau protein in neuronal cell lines resembles phosphorylation in Alzheimer's disease. *Eur. J. Cell Biol.* **76**, 176–184.
- Rime H., Huchon D., De Smedt V., Thibier C., Galaktionov K., Jessus C. and Ozon R. (1994) Microinjection of Cdc25 protein phosphatase into *Xenopus* prophase oocyte activates MPF and arrests meiosis at metaphase I. *Biol. Cell* **82**, 11–22.
- Roder H. M., Fracasso R. P., Hoffman F. J., Witowsky J. A., Davis G. and Pellegrino C. B. (1997) Phosphorylation-dependent monoclonal Tau antibodies do not reliably report phosphorylation by extracellular signal-regulated kinase 2 at specific sites. *J. Biol. Chem.* **272**, 4509–4515.
- Sergeant N., Sablonniere B., Schraen-Maschke S., Ghestem A., Maurage C. A., Watzet A., Vermersch P. and Delacourte A. (2001) Dysregulation of human brain microtubule-associated tau mRNA maturation in myotonic dystrophy type 1. *Hum. Mol. Genet.* **10**, 2143–2155.
- Shtil A. A., Mandelkar S., Yu R., Walter R. J., Hagen K., Tan T. H., Roninson I. B. and Kong A. N. (1999) Differential regulation of mitogen-activated protein kinases by microtubule-binding agents in human breast cancer cells. *Oncogene* **18**, 377–384.
- Szendrei G. I., Lee V. M. and Otvos L. Jr (1993) Recognition of the minimal epitope of monoclonal antibody Tau-1 depends upon the presence of a phosphate group but not its location. *J. Neurosci. Res.* **34**, 243–249.
- Vincent I., Jicha G., Rosado M. and Dickson D. W. (1997) Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain. *J. Neurosci.* **17**, 3588–3598.
- Vincent I., Rosado M. and Davies P. (1996) Mitotic mechanisms in Alzheimer's disease? *J. Cell Biol.* **132**, 413–425.
- Vincent I., Zheng J. H., Dickson D. W., Kress Y. and Davies P. (1998) Mitotic phosphoepitopes precede paired helical filaments in Alzheimer's disease. *Neurobiol. Aging* **19**, 287–296.
- Wang T. H., Wang H. S., Ichijo H., Giannakakou P., Foster J. S., Fojo T. and Wimalasena J. (1998) Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. *J. Biol. Chem.* **273**, 4928–4936.
- Zheng-Fischhöfer Q., Biernat J., Mandelkow E. M., Illenberger S., Godemann R. and Mandelkow E. (1998) Sequential phosphorylation of Tau by glycogen synthase kinase-3 β and protein kinase A at Thr212 and Ser214 generates the Alzheimer-specific epitope of antibody AT100 and requires a paired-helical-filament-like conformation. *Eur. J. Biochem.* **252**, 542–552.