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Research Report

Microtubule Depolymerization and Tau Phosphorylation

Dedicated to Inge Grundke-Iqbal

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Abstract. Inge Grundke-Iqbal and Khalid Iqbal found a connection between microtubule associated tau and Alzheimer's disease. They described that abnormally phosphorylated tau is a component of the paired helical filaments found in the disease. Afterwards they described that tau hyperphosphorylation prevents microtubule assembly. Now trying to complement the relationship between microtubules and tau phosphorylation, we have commented on the effect of microtubule disassembly on tau phosphorylation. In this study, we investigated the role of microtubule depolymerization induced by nocodazole on tau phosphorylation in human neuroblastoma SH-SY5Y cells. Our results indicate that nocodazole provokes tau phosphorylation mediated by GSK3, as determined by using AT-8 or Tau-1 antibodies. Interestingly, total GSK3β and GSK3β phosphorylation on Ser-9 are not altered during nocodazole treatment. In addition, microtubule stabilization with taxol had similar effects, likely because taxol and tau compete for the same binding sites on microtubules, and in the presence of taxol, tau could be detached from microtubules. Thus, unbound tau from microtubles can be phosphorylated by GSK3, even if the activity of GSK3 is not altered, probably because tau unbound to microtubules could be a better substrate for the kinase than microtubule-associated tau. These findings suggest that microtubule depolymerization can be a primary event in neurodegenerative disorders like Alzheimer's disease and that tau phosphorylation takes place afterwards.

Keywords: Disassembly, GSK3, microtubules, phosphorylation, tau

INTRODUCTION

Inge Grundke-Iqbal and Khalid Iqbal formed an excellent team. Thanks to that team we know that words like microtubules, tau, or phosphorylation are related to Alzheimer's disease (AD). In 1986, Grundke-Iqbal et al. published two seminal works that made wider the view of AD pathology, indicating that tau protein was very important to understanding the disease. Sometimes, to publish seminal papers is not an easy task, and Inge and Khalid wrote in a review [1] how difficult it was to publish that microtubule-associated tau was a component of paired helical filaments. Two top journals rejected the paper that finally was published in the *Journal of Biological Chemistry* [2] and those top journals lost the 936 citations to that paper. Prior to publication, the manuscript (in revision) was stolen, along with other things, from their car and they started the writing work again [1]. Finally, the work was published, and some months later they also published a second paper indicating that tau present in paired helical filaments was abnormally phosphorylated [3].

In this review, dedicated to Inge Grundke-Iqbal, we will discuss the relationships between microtubules (disassembly) and tau (phosphorylation).

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In AD, there are microtubule cytoskeleton changes in the damaged neurons [4, 5]. A marker for these changes is the microtubule-associated protein tau, in phosphorylated form, a protein that is mainly modified by GSK3 [6]. The consequences of those cytoskeletal changes could be a net microtubule depolymerization. Those microtubule changes could be induced by presenilin-1 mutations [7] or by the increase in amyloid-β peptide levels [8] that could activate downstream a protein kinase like GSK3.

There are two hypotheses to link microtubule depolymerization and tau phosphorylation. One of these hypotheses suggests that tau phosphorylation (based on the GSK3 over-activation) could be the first event [9, 10]. Phospho-tau is unable to bind to microtubules that lose their stability and could depolymerize. The hypothesis is that such a process could take place in AD. Indeed, it has been reported that tau hyperphosphorylation results in the disassembly of microtubules [11, 12].

The other hypothesis suggests that microtubule depolymerization is the first step [13] and that the resulting free tau (unbound to microtubules) is a more suitable substrate to be phosphorylated by GSK3 (or other protein kinases) than tau bound to microtubules. By looking at microtubule network reduction in samples from different stages of AD, it was determined that microtubule depolymerization is probably the earliest event and that tau phosphorylation occurs afterwards [13].

In this work, we have tested that hypothesis in a human neuroblastoma cell model. Our results indicate that microtubule depolymerization is sufficient for the promotion of tau phosphorylation without an increase in GSK3 activity. It suggests that the increase in tau phosphorylation is due to an increase in the amount of an available and suitable substrate and not due to a previous increase in kinase activity that could favor phosphorylation of tau and its detachment from microtubules.

METHODS

Reagents and antibodies

Nocodazole, paclitaxel (taxol), and AR-A014418 were purchased from Sigma. For immunoblot analysis, the following anti-tau antibodies were used: 7.51 (1/1000; a kind gift from Dr. C. Wischik, University of Aberdeen), AT-8 (1/200; Innogenetics, Ghent, Belgium,) and tau-1 (1/2000; Chemicon, Temecula, CA). Antibody AT-8 recognizes tau when serine 202 is phosphorylated [14], whereas antibody tau-1 recognizes tau when it is dephosphorylated at serines 198, 199, and 202, and the 7.51 antibody recognizes total tau protein. The numbering of the tau epitopes is given according to the longest human tau isoform of 441 amino acids. The other antibodies used were anti-phospho-GSK3β (Ser-9) and anti-GSK3β from Cell Signaling (1/1000, Beverly, MA). The relevant secondary antibodies were used (1/2000; DAKO).

For immunofluorescence, we used anti-β-tubulin (1/500; Sigma), and the secondary antibody (Molecular Probes) was used at 1/1000. Biochemical reagents were from Sigma, unless otherwise indicated. All other chemicals were of analytical grade.

Culture of SH-SY5Y neuroblastoma cells

Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (Invitrogen-Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen-Gibco), 2 mM glutamine, and 50 μg/ml gentamycin. On the day before the experiment, the cells were plated in multiwell plates with neurobasal serum-free medium (Invitrogen-Gibco). Data was presented as mean values ± SEM, of two or three different experiments.

Immunofluorescence analysis

For immunofluorescence studies, SH-SY5Y cells were plated on glass cover slips coated with 1 mg/ml polyL-lysine. After treatments, SH-SY5Y cells were fixed with cooled methanol (−20°C) for 20 min at 4°C and then washed with buffer A (0.1 M MES; 2 mM EGTA; 0.5 mM $MgCl_2$). Fixed cells were incubated with 1 M glycine for 30 min and then permeabilized with 0.2% Triton X-100 in buffer A for 5 min at room temperature. The cover slips were blocked with 1% BSA/ Buffer A for 1 h at room temperature and incubated with primary antibodies in 1% BSA, in buffer A, for 1 h at room temperature. After washing three times with buffer A, the secondary antibody was incubated for 1 h, at room temperature. Finally, the coverslips were washed three times with PBS or with buffer A and once with H_2O, and mounted with FluorSave Reagent (Calbiochem, San Diego, CA).

Gel electrophoresis and western blots

Cells were homogenized at 4°C in 50 mM HEPES, pH 7.4, 10 mM EDTA, 0.1% Triton X-100, including the phosphatase inhibitors 20 mM NaF, 0.1 mM

sodium orthovanadate, and with the following protease inhibitors: 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml pepstatin, and 10 μg/ml aprotinin. The cell lysates were centrifuged at 10,000 g for 30 min at 4°C and then heated at 100ºC for 5 min in electrophoresis sample buffer. The protein concentration was determined by using the BCA protein assay (Pierce, Rockford, IL). Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience, Dassel, Germany). After blocking of non-specific protein binding to the membranes with 0.05% Tween 20 and 5% nonfat dry milk in PBS, the membranes were incubated overnight with the primary antibodies in blocking buffer at 4ºC. The proteins recognized by the antibodies were visualized by using enhanced chemiluminiscence (Perkin Elmer Life Sciences, Boston, MA) after incubation with horseradish peroxidase (HRP)-linked secondary antibodies (Dako A/S, Glostrup, Denmark). Densitometric analysis of labelled protein bands was performed with GS-710 software (Bio-Rad, Hercules, CA).

RESULTS

Nocodazole treatment results in microtubule depolymerization and tau phosphorylation, in a GSK3 dependent manner, in human neuroblastoma cells

Nocodazole is a potent microtubule polymerization inhibitor [15] and we have used this compound to induce the disruption of microtubule network in a culture of human neuroblastoma cells. Figure 1 shows an increase in tau phosphorylation at the residues

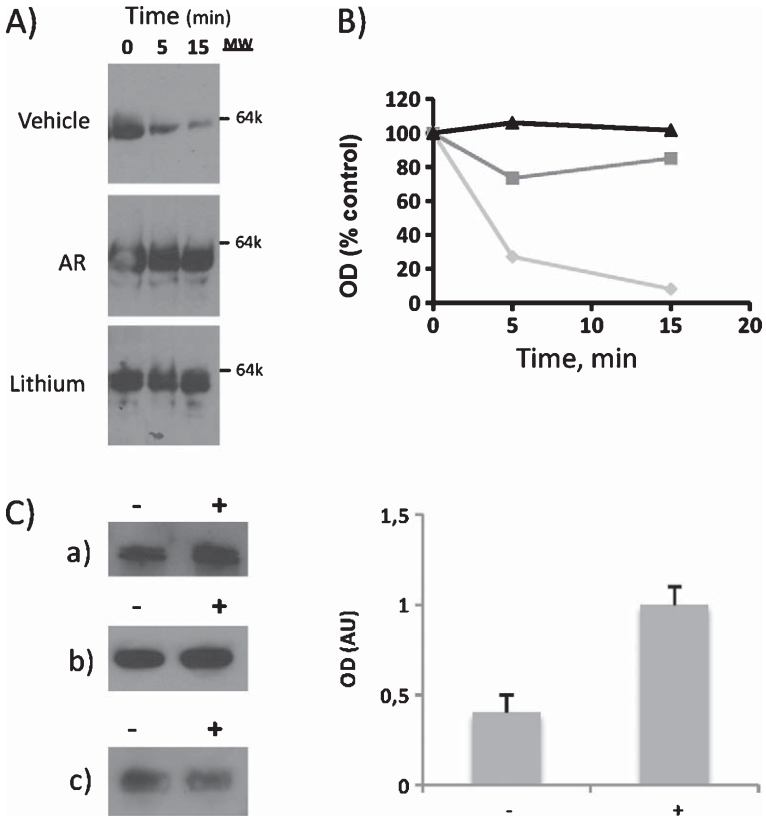

Fig. 1. Nocodazole treatment results in an increase of tau phosphorylation at the sites recognized by abTau-1 or abAT8. A) Representative immunoblots of SH-SY5Y cells pretreated with vehicle, AR-A014418 (AR-18, 33 μM) or lithium (20 mM) for 1 h and then with nocodazole (10 μM) for 0, 5, or 15 min. The samples were immunoblotted with the Tau-1 antibody. B) Quantified data of Tau-1 levels are expressed in terms of the percentage of the signal present at before nocodazole adding (♦ - vehicle, ▲ - AR18, ■ - Lithium). C) Reaction of tau with abAT8 (a) (that recognizes phospho tau) in the absence (−) or presence (+) of 10 μM nocadazole, after 15 min of treatment. At the left, bottom of the figure (b), the reaction with an antibody against GAPDH used as protein loading marker or (c) with ab7.51, a tau antibody that recognizes the protein independently of its phosphorylation status is shown. Quantified data are expressed in terms of arbitrary units (AU) of optical density (OD). The electrophoretic mobility of a protein marker (MW) is also shown.

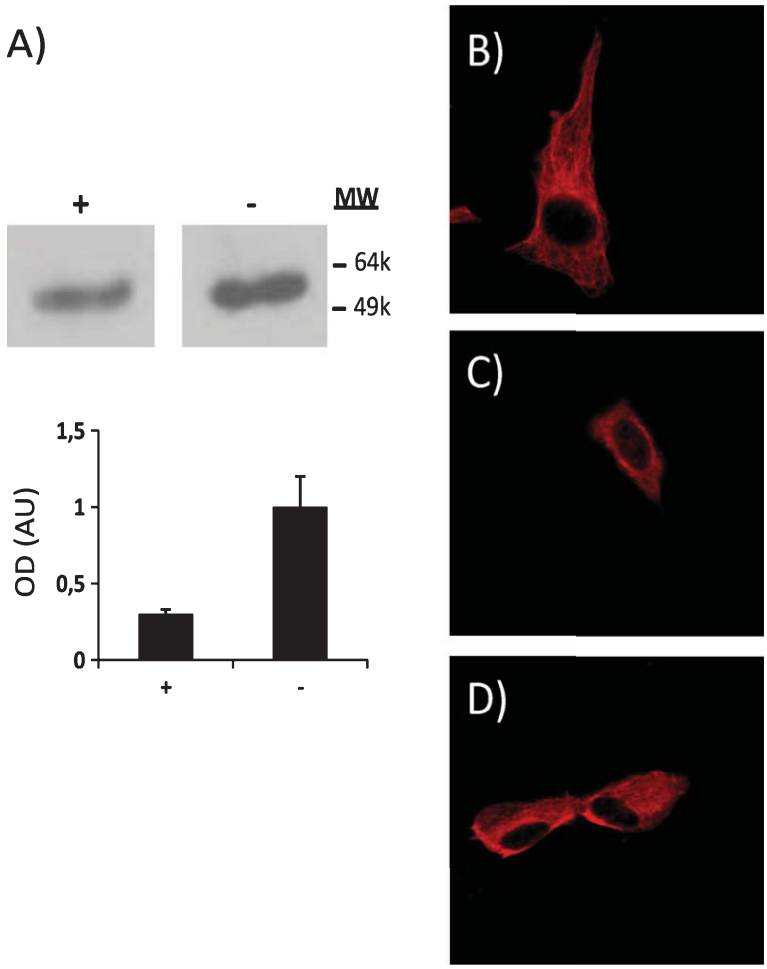

Fig. 2. Effect of nocodazole on microtubule network of SH-SY5Y cells. A) Nocodazole (10 μM) treated SH-SY5Y cells (+) were washed to remove the drug and tau phosphorylation was measured (using tau-1 antibody) 15 min after nocodazole removal (–). SH-SY5Y human neuroblastoma cells were cultured, fixed, and stained with tubulin antibody in the absence B) or presence of nocodazole C) for 15 min. D) After incubation, nocodazole was removed and, after 15 min, the cultured cells were fixed and stained with tubulin antibody to test microtubule network recovery. Quantified data are expressed in terms of arbitrary units (AU) of optical density (OD). The electrophoretic mobility of protein markers (MW) are also shown.

196-202 in the presence of nocodazole. This analysis was carried out by using two complementary antibodies, Tau-1 (Fig. 1A-B, that reacts with those residues only if they are in dephosphorylated form) and with its complementary antibody, AT8 (Fig. 1C), that only reacts if the residue 202 is phosphorylated. As shown in Fig. 1, upon addition of nocodazole an increase in the reaction with AT8 and a decrease in the reaction with Tau-1 was found.

To test if GSK3 is indeed the kinase involved in the phosphorylation recognized by Tau-1 antibody, as previously suggested [16, 17], we have added two inhibitors of GSK3 (lithium and AR-A014418) in the presence of nocodazole treatment. Figure 1A, B shows that in the presence of both GSK3 inhibitors the increase in tau phosphorylation, induced by nocodazole, was prevented, suggesting that GSK-3 was the kinase involved in the phosphorylation of that site on tau molecule. As indicated by other groups [18] we cannot rule out that other kinases may also modify tau at the AT8 site. However, it has also been shown that under certain conditions, tau is mainly phosphorylated at that site by GSK3 [18], a result that agrees well with the data shown in Fig. 1.

Taking into account that nocodazole is a reversible microtubule polymerization inhibitor [15], we have analyzed if these effects on tau phosphorylation revert after removing the compound. Figure 2A shows a decrease in tau phosphorylation at Tau-1 epitope (an increase in antibody labeling) after removing

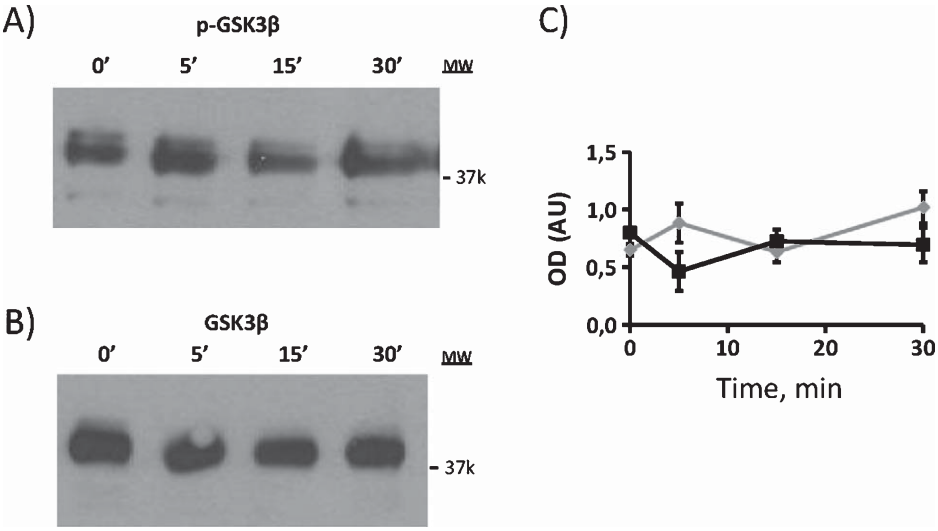

Fig. 3. There is no increase in active GSK3β upon microtubule depolymerization. SH-SY5Y cultured cells were incubated in the absence or presence of 10 μM nocodazole (0, 5, 15, 30 min) and the levels of A) phosphorylated (at serine 9) GSK3β, and B) total GSK3β, were measured by using specific antibodies. C) Quantified data of GSK3β (■) and p-GSK3β (♦) levels are expressed in terms of arbitrary units (AU) of optical density (OD). The electrophoretic mobility of a protein marker (MW) is also shown.

nocadazole from the culture. To analyze the effect of these treatments on microtubule network, we performed immunofluorescence analysis of SH-SY5Y cells with an anti-tubulin antibody. Figure 2B-D shows the presence of the microtubule lattice in neuroblastoma cells in the absence of nocodazole (Fig. 2B); while in the presence of nocodazole (panel 2C), a loss of the network was found. Microtubule assembly was recovery after removing the nocodazole from the culture (Fig. 2D).

There is no increase in GSK3 activity upon microtubule depolymerization

A routinely used method to test for GSK3β activity is to identify levels of phosphorylation at serine 9. Figure 3 shows that upon addition of nocodazol, differences in the level of GSK3β phosphorylation were not found. This result suggests that the increase in tau phosphorylation by GSK3β is not due to an increase in the kinase activity.

Taxol treatment results in microtubule stabilization and tau phosphorylation in human neuroblastoma cells

Taxol binds to β-tubulin and stabilizes microtubules. Interestingly, taxol could bind to a site on microtubules where tau also binds [19]. We have used this compound to stabilize microtubules (Fig. 4C-D) and to analyze its effects on tau phosphorylation, since in the presence of taxol, the amount of tau unbound to microtubules increases. Figure 4A-B shows an increase in tau phosphorylation at the residues 196-202 in the presence of taxol as it is demonstrated by the increase observed with the AT-8 antibody and the decrease obtained with the Tau-1 antibody.

DISCUSSION

Our results support the possibility that microtubule depolymerization (in our case induced with nocodazole) could be an early event inducing a subsequent tau phosphorylation similar to that found in neurodegenerative disorders like AD. It agrees with the previous observations done on human samples [13] and with a recent study suggesting that rotenone (that could act like an inhibitor of microtubule polymerization [20]) facilitates tau phosphorylation [21]. Interestingly, stathmin, a microtubule-disrupting protein, has similar effects [22]. In good agreement with these data, taxol also induces an increase in tau phosphorylation. Tau binding to microtubules is sensitive to taxol [19] and we have observed an increase in tau phosphorylation probably because tau competes with taxol provoking an increase in unbound tau. This unbound tau is likely a suitable substrate for phosphorylation.

Recently, NAP (davunetide), a peptide that protects microtubules from nocodazol depolymerization

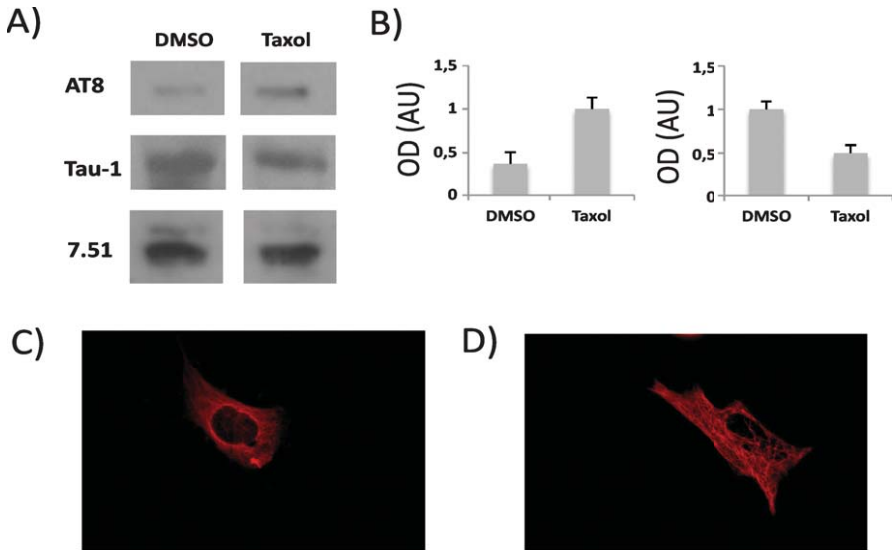

Fig. 4. Taxol treatment results in an increase of tau phosphorylation at the sites recognized by AT8 or Tau-1 antibodies. A) Representative immunoblots of SH-SY5Y cells pretreated with DMSO or taxol (10 μM) for 15 min. The samples were immunoblotted with the AT-8, Tau-1, or 7.51 antibodies; ab7.51 is an antibody that reacts with total tau. B) Quantitation of the data shown in (A) for AT8 (left) and for Tau-1 (right) antibodies. C, D) SH-SY5Y human neuroblastoma cells were cultured, fixed, and stained with tubulin antibody to test microtubule network in the absence (C) or presence (D) of taxol (10 μM) for 15 min.

[23], and unlike taxol, was found to enlist tau to the microtubule network [24], preventing tau hyperphosphorylation [25]. This strengthens the current data indicating that microtubule breakdown precedes tau hyperphosphorylation.

Our results demonstrate that tau phosphorylation is reversible since removing nocodazole from culture induces microtubule repolymerization as well as tau dephosphorylation (Fig. 2).

Although factors like amyloid-β peptide could facilitate the increase in GSK3 activity through different pathways [26–28], our results indicate that such activation is not necessary for an increase in tau phosphorylation (Fig. 3). We suggest that upon microtubule depolymerization, tau that is not bound to microtubules is available for modification by the kinase and that it could be the way to explain the increase in tau phosphorylation observed in some neurodegenerative diseases.

Our results emphasize the importance of microtubule dynamics on tau phosphorylation suggesting that microtubule depolymerization can be a primary event in neurodegenerative disorders like AD.

DISCLOSURE STATEMENT

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1796).

REFERENCES

[1] Iqbal K, Grundke-Iqbal I (2006) Discoveries of tau, abnormally hyperphosphorylated tau and others of neurofibrillary degeneration: A personal historical perspective. *J Alzheimers Dis* **9**, 219-242.

[2] Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM (1986) Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *J Biol Chem* **261**, 6084-6089.

[3] Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI (1986) Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc Natl Acad Sci U S A* **83**, 4913-4917.

[4] Braak E, Braak H, Mandelkow EM (1994) A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. *Acta Neuropathol* **87**, 554-567.

[5] Kao PF, Davis DA, Banigan MG, Vanderburg CR, Seshadri S, Delalle I (2010) Modulators of cytoskeletal reorganization in CA1 hippocampal neurons show increased expression in patients at mid-stage Alzheimer's disease. *PLoS One* **5**, e13337.

[6] Avila J, Lucas JJ, Perez M, Hernandez F (2004) Role of tau protein in both physiological and pathological conditions. *Physiol Rev* **84**, 361-384.

[7] Pigino G, Pelsman A, Mori H, Busciglio J (2001) Presenilin-1 mutations reduce cytoskeletal association, deregulate neurite growth, and potentiate neuronal dystrophy and tau phosphorylation. *J Neurosci* **21**, 834-842.

[8] Henriques AG, Vieira SI, da Cruz ESEF, da Cruz ESOA (2010) Abeta promotes Alzheimer's disease-like cytoskeleton abnormalities with consequences to APP processing in neurons. *J Neurochem* **113**, 761-771.

[9] Leroy K, Boutajangout A, Authelet M, Woodgett JR, Anderton BH, Brion JP (2002) The active form of glycogen synthase kinase-3beta is associated with granulovacuolar degeneration in neurons in Alzheimer's disease. *Acta Neuropathol* **103**, 91-99.

[10] Jope RS, Johnson GV (2004) The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* **29**, 95-102.

[11] Alonso AC, Grundke-Iqbal I, Iqbal K (1996) Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. *Nat Med* **2**, 783-787.

[12] Alonso AD, Grundke-Iqbal I, Barra HS, Iqbal K (1997) Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration: Sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau. *Proc Natl Acad Sci U S A* **94**, 298-303.

[13] Cash AD, Aliev G, Siedlak SL, Nunomura A, Fujioka H, Zhu X, Raina AK, Vinters HV, Tabaton M, Johnson AB, Paula-Barbosa M, Avila J, Jones PK, Castellani RJ, Smith MA, Perry G (2003) Microtubule reduction in Alzheimer's disease and aging is independent of tau filament formation. *Am J Pathol* **162**, 1623-1627.

[14] Porzig R, Singer D, Hoffmann R (2007) Epitope mapping of mAbs AT8 and Tau5 directed against hyperphosphorylated regions of the human tau protein. *Biochem Biophys Res Commun* **358**, 644-649.

[15] De Brabander MJ, Van de Veire RM, Aerts FE, Borgers M, Janssen PA (1976) The effects of methyl (5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl) carbamate, (R 17934; NSC 238159), a new synthetic antitumoral drug interfering with microtubules, on mammalian cells cultured *in vitro*. *Cancer Res* **36**, 905-916.

[16] Illenberger S, Zheng-Fischhofer Q, Preuss U, Stamer K, Baumann K, Trinczek B, Biernat J, Godemann R, Mandelkow EM, 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.

[17] Godemann R, Biernat J, Mandelkow E, Mandelkow EM (1999) Phosphorylation of tau protein by recombinant GSK-3beta: Pronounced phosphorylation at select Ser/Thr-Pro motifs but no phosphorylation at Ser262 in the repeat domain. *FEBS Lett* **454**, 157-164.

[18] Liu SJ, Zhang JY, Li HL, Fang ZY, Wang Q, Deng HM, Gong CX, Grundke-Iqbal I, Iqbal K, Wang JZ (2004) Tau becomes a more favorable substrate for GSK-3 when it is prephosphorylated by PKA in rat brain. *J Biol Chem* **279**, 50078-50088.

[19] Kar S, Fan J, Smith MJ, Goedert M, Amos LA (2003) Repeat motifs of tau bind to the insides of microtubules in the absence of taxol. *EMBO J* **22**, 70-77.

[20] Marshall LE, Himes RH (1978) Rotenone inhibition of tubulin self-assembly. *Biochim Biophys Acta* **543**, 590-594.

[21] Hongo H, Kihara T, Kume T, Izumi Y, Niidome T, Sugimoto H, Akaike A (2012) Glycogen synthase kinase-3beta activation mediates rotenone-induced cytotoxicity with the involvement of microtubule destabilization. *Biochem Biophys Res Commun* **426**, 94-99.

[22] Miyasaka T, Sato S, Tatebayashi Y, Takashima A (2010) Microtubule destruction induces tau liberation and its subsequent phosphorylation. *FEBS Lett* **584**, 3227-3232.

[23] Gozes I, Divinski I (2007) NAP, a neuroprotective drug candidate in clinical trials, stimulates microtubule assembly in the living cell. *Curr Alzheimer Res* **4**, 507-509.

[24] Oz S, Ivashko-Pachima Y, Gozes I (2012) The ADNP derived peptide, NAP modulates the tubulin pool: Implication for neurotrophic and neuroprotective activities. *PLoS One* **7**, e51458.

[25] Idan-Feldman A, Ostritsky R, Gozes I (2012) Tau and caspase 3 as targets for neuroprotection. *Int J Alzheimers Di* **2012**, 493-670.

[26] Townsend M, Mehta T, Selkoe DJ (2007) Soluble Abeta inhibits specific signal transduction cascades common to the insulin receptor pathway. *J Biol Chem* **282**, 33305-33312.

[27] Tackenberg C, Brandt R (2009) Divergent pathways mediate spine alterations and cell death induced by amyloid-beta, wild-type tau, and R406W tau. *J Neurosci* **29**, 14439-14450.

[28] Magdesian MH, Carvalho MM, Mendes FA, Saraiva LM, Juliano MA, Juliano L, Garcia-Abreu J, Ferreira ST (2008) Amyloid-beta binds to the extracellular cysteine-rich domain of Frizzled and inhibits Wnt/beta-catenin signaling. *J Biol Chem* **283**, 9359-9368.