Role of tau phosphorylation by glycogen synthase kinase-3β in the regulation of organelle transport

Yoshitaka Tatebayashi*, Niloufar Haque, Yunn-Chyn Tung, Khalid Iqbal and Inge Grundke-Iqbal§

New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, New York, NY 10314, USA

*Present address: Laboratory for Alzheimer's Disease, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-shi, Saitama, 351-0198, Japan

§Author for correspondence (e-mail: i_g_iqbal@yahoo.com)

Accepted 27 November 2003
Journal of Cell Science 117, 1653-1663 Published by The Company of Biologists 2004
doi:10.1242/jcs.01018

Summary

Anterograde organelle transport is known to be inhibited by overexpression of the microtubule-associated protein tau in cultured cells. However, the molecular mechanism regulating this function of tau protein has not previously been understood. We found that in PC12 cells treated with NGF or fibroblast growth factor-2, glycogen synthase kinase-3β and tau were upregulated simultaneously from around day 2 of differentiation, with increasing glycogen synthase kinase-3-mediated tau phosphorylation. This phosphorylation did not alter tau's ability to bind to microtubules but appeared to be required for the maintenance of the anterograde organelle transport in differentiated cells. Lithium, alsterpaullone or valproate, three independent glycogen synthase kinase-3 inhibitors, but not butyrolactone 1, an inhibitor of cyclin-dependent protein kinases, induced mitochondrial clustering in association with tau dephosphorylation. In CHO cells transfected with human tau$_{441}$, mitochondrial clustering was found in cells in which tau was unphosphorylated. These findings raise the possibility that the phosphorylation of tau by glycogen synthase kinase-3 might be involved in the regulation of organelle transport.

Key words: Axonal transport, Differentiation, GSK-3, Mitochondria, Tau, Phosphorylation

Introduction

Tau is a major neuronal microtubule associated protein (MAP). One of its established functions is the promotion of assembly and maintenance of microtubule structure (Weingarten et al., 1975; Drubin et al., 1986). One of the most important post-translational modifications of tau is phosphorylation, because the degree of phosphorylation regulates its microtubule-binding and tubulin-polymerizing activities (Lindwall and Cole, 1984). Normal adult tau, which binds to microtubules and promotes microtubule assembly in vitro, contains at least two to three phosphate groups per molecule (Köpke et al., 1993). Glycogen synthase kinase-3 (GSK-3), a microtubule-binding protein, which is expressed abundantly in neurons of adult brain (Woodgett, 1990), is one of the kinases that may be involved in such physiological tau phosphorylation. For example, in most embryonic or neonatal neuronal cultures, endogenous tau is already phosphorylated by GSK-3 to some extent and can be downregulated by the GSK-3 inhibitors, lithium (Klein and Melton, 1996; Muñoz-Moñtano et al., 1995; Hong et al., 1997) and valproic acid (Chen et al., 1999). We have previously shown that in adult rat hippocampal progenitor cells (AHPs) GSK-3β phosphorylates adult tau isoforms mostly at the Tau-1 site (Ser 195/198/199/202) and that fibroblast growth factor-2 (FGF-2) upregulates the expression of both the substrate and the kinase dose dependently with increasing phosphorylation at the Tau-1 site but without decreasing tau's microtubule-binding ability (Tatebayashi et al., 1999) (Y.T. and I.G.-I., unpublished data). We have also observed that phosphorylation of tau at the Tau-1 and other sites during differentiation of SY5Y cells does not affect its microtubule-binding activity (Haque et al., 1999). However, the physiological role of the tau phosphorylation by GSK-3β has been elusive, but of major interest and importance because abnormal hyperphosphorylation of this protein is believed to cause neurodegeneration in Alzheimer disease (AD) and related tauopathies (Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b; Iqbal et al., 1986; Alonso et al., 1996).

In the present study, we show that in PC12 cells differentiated by nerve growth factor (NGF) or FGF-2, the expression of GSK-3β and tau and the phosphorylation of tau are upregulated. Inhibition of GSK-3β by lithium (LiCl), sodium valproate (VA) or alsterpaullone, three structurally independent GSK-3β inhibitors induced reversible inhibition of anterograde transport of mitochondria in association with dephosphorylation of tau but without any change in the ratio between microtubule-bound and -unbound tau. Similar association between anterograde transport and the phosphorylation state of tau was found in transfected CHO cells overexpressing tau. These data raise the intriguing possibility that the phosphorylation of tau by GSK-3β might be involved in the regulation of anterograde organelle transport.

Materials and Methods

Materials

The following phosphorylation-dependent site specific monoclonal antibodies to tau were employed: Tau-1 (to unphosphorylated Ser 195/198/199/202; numbers according to the longest human brain tau isoform tau$_{441}$; 1:50,000) (Binder et al., 1985; Grundke-Iqbal et al.,

1986b; Szendrei et al., 1993); PHF-1 (to phosphorylated Ser 396/404; 1:1000) (Greenberg et al., 1992; Liu et al., 1993; Otvos et al., 1994); 12E8 (to phosphorylated Ser 262/356; 1:500) (Seubert et al., 1995); polyclonal phosphorylation-dependent antibodies to tau phosphorylated at T212, S396, or S404 (all 1:1000, BioSource International, Hopkinton, MA, USA). The phosphorylation-independent antibodies to recombinant human tau_{410}, R134d (1:5000) (Tatebayashi et al., 1999) were raised in a rabbit. Polyclonal antibodies to GSK-3 were 127d (to C-terminal residue 353-364 of rat GSK-3β; 1:500) (Pei et al., 1997) and GSK-3β Tyr 214/216 phosphospecific antibody to PTyr 214/216 (1:500, Biosource International, Hopkinton, MA, USA). Other primary antibodies employed were monoclonal antibody DM1A to α-tubulin (1:2000, Sigma, St. Louis, MO, USA) and monoclonal antibody 3G5 to MAP1B (1:1000) (Tucker et al., 1988). NGF and FGF-2 were purchased from Alamone Lab. (Israel) and Sigma, respectively. PD 98059, butyrolactone I, LiCl, valproic acid (VA), alsterpaullone and myo-inositol were purchased from Calbiochem (La Jolla, CA), Biomol (Plymouth Meeting, PA), Alexis Biochemicals (San Diego, CA) and Sigma, respectively.

Cell culture and transfection

PC12 cells were grown in 10 cm culture dishes (Corning, NY) which had been precoated with 0.01% poly-D-lysine (Sigma, St Louis, MO) and maintained in RPMI medium 1640 with L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 5% horse serum and 10% fetal bovine serum. For differentiation, cells were transferred to BIOCOAT® poly-D-lysine 60 mm culture dishes or slides (Becton Dickinson, Bedford, MA) and grown overnight in regular medium. Cells were differentiated either with 50 ng/ml NGF or 20 ng/ml FGF-2 in medium containing 2% horse serum (differentiation medium). The medium was changed every 2 days.

CHO cells were maintained in RPMI medium 1640 with L-glutamine, supplemented with 10% fetal bovine serum, 100 μg/ml penicillin G and 100 μg/ml streptomycin. Cells were plated on Lab Tek™ slides (Nunc, Naperville, IL, USA) coated with poly-D-lysine 24-48 hours prior to use. For the experiments, either cell lines stably expressing tau (Haque et al., 1995) or cells transiently transfected with tau were employed. For transient transfection, 5-10 μg of pcDNA3-T_{10} (tau_{441} cDNA) generated as described previously (Haque et al., 1995), was complexed with 20 μg of Lipofectamine (Invitrogen) and overlaid on cells containing medium without serum and antibiotics. After 15 hours the cells were replenished with fresh, regular culture medium. Studies were carried out 24-48 hours post-transfection.

Neural progenitor cells from adult (3-month old) rat hippocampus (AHPs) were isolated and cultured as described previously (Tatebayashi et al., 1999) with some modifications. Briefly, cells isolated from tissue after enzymatic dissociation were further separated using a 4-ml step density gradient [Optiprep, at 7, 9.4, 11.7 and 16.4%, 1 ml each, in Hibernate A/B27 (Invitrogen), centrifuged at 800 ***g*** at room temperature for 15 minutes]. The resulting lowest 3 ml supernatant, including a dense band of cells and the pellet, were collected, resuspended in Neurobasal A (12.5 mM NaCl + Neurobasal, Life Technologies) containing 2% B27 supplement and 0.5 mM glutamine and plated on 60 mm culture dishes that had been precoated with 0.01% poly-D-lysine (135 kDa, Sigma). After 30 minutes, cells were washed and further cultured with Neurobasal A/B27 containing 0.5 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 ng/ml FGF-2 (Life Technologies). Medium was changed every 2-3 days. Proliferating cultures, maintained for about 6 months through more than 15 passages, were used for this study. Tau expression and phosphorylation and its upregulation by FGF-2 (Tatebayashi et al., 1999) were monitored throughout this period.

To study the effect of GSK-3 and cyclin-dependent kinase 5 (cdk5) on different phosphorylation sites of tau, PC12 cells differentiated with FGF-2 for 7 days were incubated for 3 hours with medium alone or with 20 mM LiCl (GSK-3 inhibitor) or 10 μM Bu1 (cdk5 inhibitor). The phosphorylation of tau was determined by ^{125}I western blots developed with phosphodependent and site-specific tau antibodies.

Western blots

For harvesting, the culture medium was first replaced by cold (4°C) PBS and the cells were then detached from the plate with a cell-scraper and centrifuged at 800 ***g*** at 4°C for 5 minutes. The cell pellets were lysed in 0.4% sodium dodecyl sulfate (SDS) and 0.4% β-mercaptoethanol, probe sonicated and boiled for 5 minutes. The resulting cell lysates were aliquoted and stored at –85°C until used. The protein concentrations were determined by the modified Lowry method (Bensadoun and Weinstein, 1986). Indicated amounts of protein samples were electrophoresed at least in triplicate on 10% SDS-polyacrylamide gels, transferred to Immobilon membrane (Millipore, Bedford, MA) and probed with primary antibodies. To assay the degree of phosphorylation at the Tau-1 site, the membrane was untreated or treated with alkaline phosphatase (196 U/ml) at 37°C for 8 hours in dephosphorylation buffer (50 mM Tris, pH 8.2, 2 mM $MgCl_2$, 1 mM PMSF, 5 μg/ml leupeptin and aprotinin, 2 μg/ml pepstatin A, 50 μg/ml phosphoramidon) prior to application of Tau-1 antibody (Tatebayashi et al., 1999). This procedure fully dephosphorylates even the hyperphosphorylated tau from Alzheimer brain (Khatoon et al., 1992). Bound antibodies were probed with ^{125}I-conjugated anti-mouse or anti-rabbit IgG (0.1 μg/ml; Amersham, Arlington Heights, IL, USA). The radio-immunoblots were scanned with a Fuji BAS 1500 Bio Image analyzer (Raytest USA Inc., Wilmington, DE). Images were processed using the Tina software and the strength of immunostaining was expressed as pixels per square length (PSL). Phosphorylation at the Tau-1 epitope (% phosphorylation) was calculated by the following formula: (PSL of staining by Tau-1 in alkaline phosphatase treated blot, i.e. total tau minus PSL of staining by Tau-1 in the untreated blot/PSL of staining by Tau-1 in alkaline phosphatase treated blot) × 100.

GSK-3β assay

GSK-3β activity was assayed according to the method of Tanaka et al. (Tanaka et al., 1998) with some modifications. Briefly, cells were lysed on ice for 30 minutes in lysis buffer containing 50 mM Tris (pH 7.4), 0.1% Triton X-100, 20 mM NaCl, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 μg/ml pepstatin A, 50 μg/ml phosphoramidon and centrifuged at 200,000 ***g*** for 30 minutes. The resulting cell extract protein (50 μg) was diluted to 250 μl with lysis buffer, followed by addition of 250 μl of immunoprecipitation buffer (Tris-buffered saline (TBS) with 300 mM NaCl and 1 mM PMSF) and then half of this solution was mixed with 2 μl of 127d antibody to GSK-3β and the other half served as the control. After incubation at 4°C overnight, 20 μl of immobilized protein G (Pierce, Rockford, IL) was added to the above mixture, incubated at 4°C for 2 hours and then centrifuged. The pellets were washed three times, suspended in 50 μl of 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM PMSF, and employed for assaying GSK-3 activity towards its specific substrate phosphoglycogen synthase peptide 2 (Upstate Biotechnology, Lake Placid, NY, USA) for 30 minutes at 30°C as described previously (Tatebayashi et al., 1999). The immunoprecipitation reaction in which the antibody to GSK-3β was omitted was used as a negative/background control.

The effect of GSK-3 inhibitors on the GSK-3 enzyme activity was measured in undifferentiated PC-12 cells. Cells were plated on poly-D-lysine-coated dishes and after 5 days in culture treated with the GSK-3 inhibitors LiCl (20 mM) or alsterpaullone (20 μM) for 1.5 hours. As controls the cells were treated with medium alone or with 10 μM Bu1, an inhibitor of cyclin-dependent kinases (cdk), including cdk5. Cells were then lysed and the GSK-3 activity determined as

above. Three independent experiments were carried out with each inhibitor and GSK-3 activity assayed in duplicate.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes, permeabilized with 0.5% Triton X-100 in PBS for 5 minutes, blocked in 5% bovine serum albumin in TBS (BSA/TBS) for 10 minutes and then incubated with primary antibodies in BSA/TBS at 4°C overnight. With this fixative the intensity of tau staining is optimal. However, tau binding to microtubules is eliminated and cannot be observed. Bound antibodies were probed with a combination of secondary antibodies (Molecular Probes, Eugene, OR): Oregon Green™ 488-conjugated goat anti-mouse antibody (1:1000) with or without Texas Red®-conjugated goat anti-rabbit antibody (1:2000), or Oregon Green™ 488-conjugated goat anti-rabbit antibody (1:1000) together with biotin-XX goat anti-mouse antibody (1:250) followed by avidin NeutrAvidin™-Cascade Blue® conjugate (1:1000). Images were captured either with a Nikon PCM 2000 epifluorescent microscope or a Zeiss Axiophot fluorescent microscope. Both were equipped with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Mitochondrial staining

For observation of mitochondria in live PC12 cells the fluorescent dyes Rhodamine 123 or Mito Tracker Red CMXRos (both from Molecular Probes) were employed. Since Mito Tracker Red is stable after fixation with paraformaldehyde this dye was used when the mitochondrial staining was followed by immunocytochemistry. Both dyes were stored as 1 mg/ml DMSO stock solutions at 4°C. To study the effect of GSK-3 inhibitors on the mitochondrial movement, PC12 cells differentiated by NGF or FGF-2, or tau-transfected CHO cells were treated with the GSK-3 inhibitors LiCl (20 mM), VA (0.6 mM) and alsterpaullone (20 μM). As controls, cells were treated with medium alone or with Bu1 (10 μM), an inhibitor of cyclin-dependent protein kinases for 1.5 to 3 hours. Cells were then further incubated in addition to the inhibitors with Mito Tracker Red (1 μg/ml) for 30 minutes, washed in PBS three times, fixed and immunostained. In PC12 cells, both Rhodamine 123 and Mito Tracker Red usually stained cell bodies more intensely than the cell periphery and processes, probably because of their small (10-20 μm) and round cell bodies. To study the reversal of the lithium effect on mitochondria, PC12 cells differentiated with NGF or FGF-2 were treated with 20 mM LiCl for 30 minutes, or left untreated (control), and then Rhodamine 123 was added in the medium to a final concentration of 10 μg/ml. After a further 30 minutes incubation at 37°C, the cells were washed gently three times in differentiation medium (with or without lithium). Images were captured with either a Nikon PCM 2000 epifluorescence microscope or a Zeiss Axiophot fluorescence microscope using a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

For reversal of the LiCl effect, cells that had been incubated with LiCl were washed three times in differentiation medium without LiCl, followed by incubation for 1 hour at 37°C. Accumulated dye was observed with a 20× objective of a Nikon inverted fluorescence microscope and the same field observed before and after the washout period.

Determination of Tau binding to microtubules

PC12 cells differentiated with NGF for 4 days or FGF-2 for 7 days were treated with or without 20 mM LiCl for 3 hours, and tau binding to microtubules determined as described by Hong et al. (Hong et al., 1997). Briefly, cells were washed once with PBS at 37°C, and lysed at 37°C in RAB buffer (0.1 M 4-morpholineethanesulfonic acid, 0.5 mM $MgSO_4$, 1 mM EGTA and 2 mM dithiothreitol, pH 6.8) supplemented with 0.1% Triton X-100, 20 μM taxol, 2 mM GTP, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 μg/ml pepstatin A and 50 μg/ml phosphoramidon. Cell lysates were homogenized with 20 strokes in a Dounce homogenizer and centrifuged for 20 minutes at 50,000 ***g***, 25°C. The supernatants from treated and untreated cells were adjusted to the same protein concentrations as determined by the Bradford method (Pierce, Rockford, IL). The pellets containing the cytoskeleton were resuspended in ice-cold RAB buffer to the same volume as the corresponding supernatants. Equal volumes of pellets and supernatants were then subjected to 10% SDS-PAGE and analyzed by ^{125}I western blots.

Results

NGF and FGF-2 upregulate the expression of tau and GSK-3β and phosphorylation of tau during differentiation of PC12 cells

NGF and FGF-2 are known to induce neuronal differentiation of PC12 cells probably by sustained activation of the mitogen-activated protein kinase (MAPK) pathway (Cowley et al., 1994; Young et al., 1994; Maher, 1999). We found that PC12 cells differentiated with NGF had a larger proportion of thicker neurites than those differentiated with FGF-2 (Fig. 1A). During differentiation with NGF both tau and MAP1B levels increased gradually and were, at 10 days, about five- and nine-times higher, respectively, than in the untreated cells. In contrast, upon differentiation with FGF-2, only tau expression was increased in a time-dependent manner, whereas MAP1B levels remained low (Fig. 1B,C). Neither MAP1A nor MAP2 were detected in FGF-2 or NGF-differentiated cells (data not shown). Thus, of the four major MAPs, only tau seems to be upregulated upon differentiation of PC12 cells.

Western blots of the PC12 cells differentiated with NGF or FGF-2 and developed with monoclonal antibody Tau-1, which recognizes unphosphorylated tau, showed maximal staining when the blots were treated with alkaline phosphatase (Fig. 2A, Tau-1 dp and Tau-1). These findings indicate that during differentiation tau is phosphorylated at the Tau-1 epitope. The Tau-1 epitope is known to be a preferred site for GSK-3β. Like tau, the expression of GSK-3β was also found to be increased in NGF- and FGF-2-differentiated PC12 cells (Fig. 2A, 127d). The phosphorylation of GSK-3β at tyrosine 216, which activates this kinase, increased in parallel with the total level of the enzyme in both NGF- and FGF2-differentiated PC12 cells (Fig. 2A, Y216).

The activity of GSK-3β in NGF- and FGF-2-differentiated PC12 cells was assayed by immunoprecipitating the enzyme from the cell extracts and using phosphoglycogen synthase peptide 2 as a substrate. Both growth factors decreased GSK-3β activity to about 60 – 80% of that of the control during the first 20 minutes after the stimulation (Fig. 2B). This decrease in GSK-3β activity was probably due to transient activation of the phosphoinositide 3-kinase pathway (Raffioni and Bradshaw, 1992; Kleijn et al., 1998). The GSK-3β activity in the treated cells remained low for about 2 days and then increased almost simultaneously with the increase in the expression of tau and GSK-3β. In NGF-differentiated PC12 cells, the activity peaked at day 4 of the differentiation, and decreased at days 7 and 12. Since the phosphorylation of GSK-3β at Tyr 216 (Fig. 2A) was not decreased in total cell lysates at day 7, one of the reasons for the decrease of immunoprecipitated GSK-3β activity might be a change in

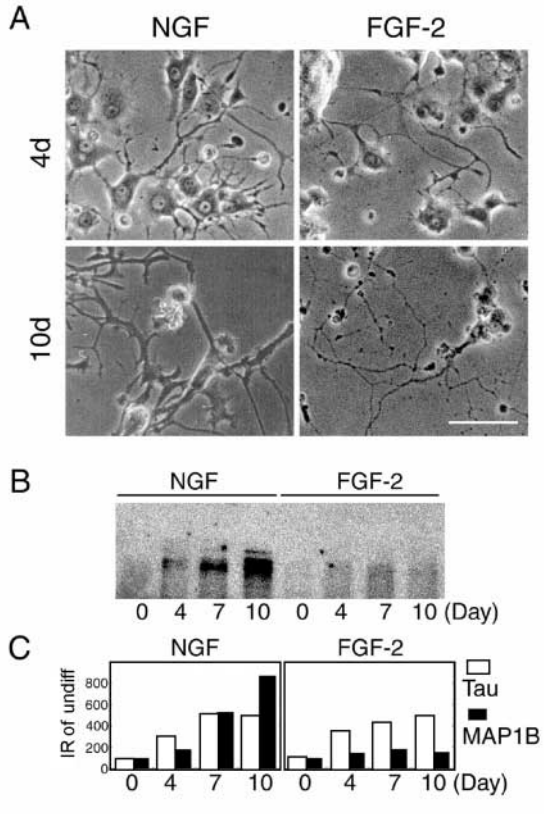

Fig. 1. Effect of FGF-2 and NGF on the differentiation of PC12 cells with respect to morphology and levels of MAP1B and tau. (A) Representative cell morphologies during differentiation (4 and 10 days) of PC12 cells in the presence of NGF or FGF-2. Note that PC12 cells after differentiation for 10 days with NGF have a larger number of thicker neurites than those with FGF-2. Bar, 50 μm. (B) Western blot showing MAP1B in PC12 cells differentiated with NGF or FGF-2 from 0 to 10 days. (C) Quantitative analysis of total tau and MAP1B levels in PC12 cells differentiated with NGF or FGF-2. Immunoreactivities (IR) of tau or MAP1B were converted based on the value (PSL) of these proteins from undifferentiated cells as 100%. Tau was analyzed on ^{125}I western blots with Tau-1 after 8 hours dephosphorylation on the membrane with alkaline phosphatase. The increase of tau expression after 7 days of differentiation was confirmed with the phospho-independent tau antibody R134d and was 3.5 and 4 times higher (data not shown). MAP1B was analyzed with mAb 3G5.

distribution of the enzyme from the cytosol to the membrane pool. In contrast, in FGF-2-differentiated PC12 cells, the increased activity was sustained as long as 10 days (Fig. 2B).

The increased GSK-3β activity was associated with increased phosphorylation of tau at the Tau-1 site (Fig. 2A,C). The degree of phosphorylation at the Tau-1 site was about 35% in undifferentiated PC12 cells at the start and increased to 65-70% at day 4 of differentiation (Fig. 2C). This increased phosphorylation was followed by a slight decrease in NGF-differentiated cells and by sustained hyperphosphorylation in FGF-2-differentiated PC12 cells.

Besides GSK-3β, cdk-5 and mitogen-activated protein kinase (MAPK) have been shown in vitro to phosphorylate tau at the Tau-1 site. To identify which one(s) of these kinases might phosphorylate tau in vivo at this site, NGF- and FGF-2-differentiated PC12 cells were incubated with different specific

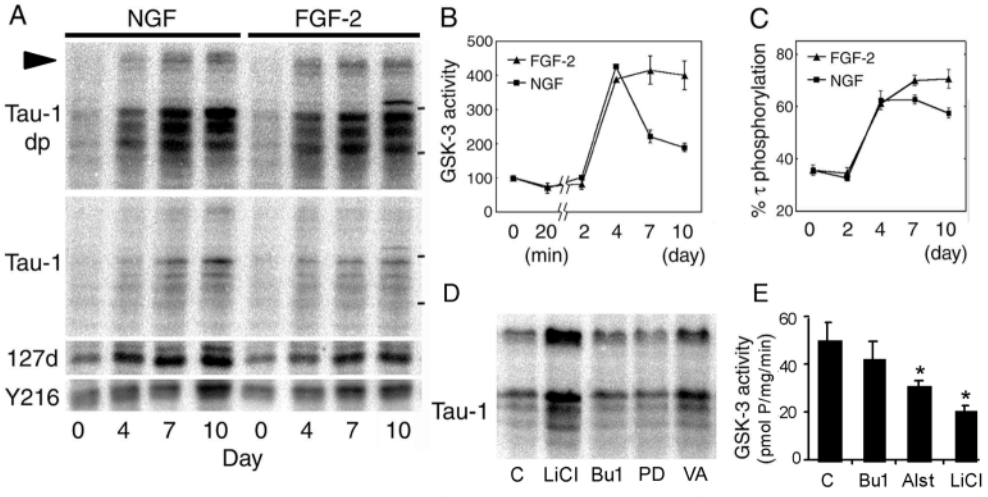

Fig. 2. Differentiation of PC12 cells with NGF or FGF-2 upregulates the expression of tau and GSK-3β and phosphorylation of tau at the Tau-1 site. (A) ^{125}I western blots of tau and GSK-3β in PC12 cells differentiated with NGF or FGF-2; 12 μg of total cell lysate was applied per lane for SDS-PAGE. For immunostaining of tau with Tau-1 antibody, the blots were untreated (Tau-1) or pretreated with alkaline phosphatase (Tau-1 dp). Arrowhead indicates the position of high molecular mass tau; bars on the right indicate the positions of 70 kDa and 50 kDa molecular mass markers (from top). For GSK-3β immunostaining, the blots were developed with antibody 127d to total GSK-3β and with antibody Y216 to GSK-3β phosphorylated at Tyr 216. (B) NGF and FGF-2 upregulate GSK-3β activity in differentiated PC12 cells. GSK-3β activities were measured by using 127d-immunoprecipitates of the extracts of cells cultured in NGF or FGF-2 for the indicated time periods. The activities (%) were converted based on the value of unstimulated cells as 100% (mean±s.e.m.). (C) NGF and FGF-2 increase tau phosphorylation at the Tau-1 site. The percentage of phosphorylation at the Tau-1 site was determined as described in Materials and Methods (mean±s.e.m.). (D) Lithium and valproate (VA) inhibit tau phosphorylation at the Tau-1 site in differentiated PC12 cells. PC12 cells differentiated by NGF for 4 days were further incubated without (control, C) or with LiCl (20 mM, 3 hours, LiCl), butyrolactone 1 (10 μM, 6 hours, Bu1), PD98059 (50 μg/ml, 1 hour, PD) or VA (0.6 mM, 3 hours, VA), lysed and analyzed by western blots developed with Tau-1 (20 μg of cell lysate per lane). Not shown in this figure, these treatments did not affect total tau levels significantly as determined by immunolabeling with Tau-1 after dephosphorylation. Only the GSK-3 inhibitors, lithium and VA increased Tau-1 staining. (E) GSK-3 inhibitors alsterpaullone and LiCl inhibit the GSK-3 enzyme activity in PC12 cells. PC12 cells were treated for 1.5 hours with medium (C), 10 μM butyrolactone I (Bu), 20 μM alsterpaullone (Alst) or 20 mM lithium chloride (LiCl). Cells were lysed and the GSK-3 enzyme activity determined. *P<0.001.

kinase inhibitors. Western blot analysis demonstrated that the phosphorylation of tau at the Tau-1 site was inhibited by the GSK-3β inhibitors LiCl and VA, but not by the cdk inhibitor Bu1, or by the MAPK kinase inhibitor, PD98059, indicating that GSK-3β is most probably the major kinase that phosphorylates tau at the Tau-1 site in differentiated PC12 cells (NGF; Fig. 2D, FGF-2; data not shown).

To confirm the involvement of GSK-3 activity in the phosphorylation of tau at the Tau-1 site, we determined in PC12 cells the activity of this enzyme in cells treated with LiCl, a GSK-3 inhibitor, alsterpaullone, a GSK-3/cdk5 inhibitor (Leost et al., 2000) and as a control Bu1, a cdk5 inhibitor. Since a reversal of LiCl-mediated inhibition of tau phosphorylation in cultured differentiated neurons has been observed previously after withdrawal of the inhibitor (Hong et al., 1997; Takahashi et al., 1999) (this study), care was taken to minimize any potential reversal of the inhibition by performing all washes, lysing of the cells and immunoprecipitations at 4°C. Only the phosphorylation of the substrates was carried out at 30°C for 30 minutes. Despite these precautions the degree of inhibition of GSK-3 in situ might be higher than measured here in immunoprecipitates. Treatment of the cells for 1.5 hours with LiCl or alsterpaullone, but not with butyrolactone, decreased the GSK-3 activity significantly (Fig. 2E). These findings suggested that the phosphorylation of tau at the Tau-1 site observed in the PC12 cells was most likely catalyzed by GSK-3.

In vitro tau can be phosphorylated by the proline-dependent kinases GSK-3 and cdk5 at a number of sites. To elucidate which of these sites are phosphorylated by these two kinases in differentiated PC12 cells, we determined the degree of inhibition of phosphorylation of tau at specific sites in cells treated with LiCl and Bu1. PC12 cells that had been differentiated for 7 days with FGF-2 and then incubated for 2 hours with the inhibitors were analyzed by ^{125}I western blots using site-specific phosphodependent tau antibodies. We found that inhibition of GSK-3 led to a decrease of phosphorylation in addition to the proline-dependent Tau-1 epitope (Fig. 2D), at T212, S396 and S404 sites (Fig. 3). Like LiCl, Bu1 also inhibited the phosphorylation of tau at S404 (Fig. 3). These results indicate that in differentiated PC12 cells the Tau-1 epitope (see Fig. 2D) as well as the T212 and S396 sites are phosphorylated preferentially by GSK-3, whereas at S404 tau is phosphorylated both by cdk5 and GSK-3. The proline-independent site 12E8 was not affected by either GSK-3 or cdk5.

The effect of phosphorylation on the binding of tau to microtubules was studied in differentiated PC12 cells incubated without or with 20 mM LiCl for 3 hours. Although in both NGF- (Fig. 3C) and FGF-2- (data not shown) differentiated PC12 cells LiCl decreased the phosphorylation at the Tau-1 epitope by ~70%, tau bound to endogenous taxol-stabilized microtubules with the same efficacy regardless of its phosphorylation state (Fig. 3C).

Inhibition of GSK-3β induces dephosphorylation of tau and clustering of mitochondria around the nucleus

Since tau has been shown to affect organelle transport, especially the kinesin-mediated anterograde transport (Ebneth et al., 1998) and its levels during differentiation were increased

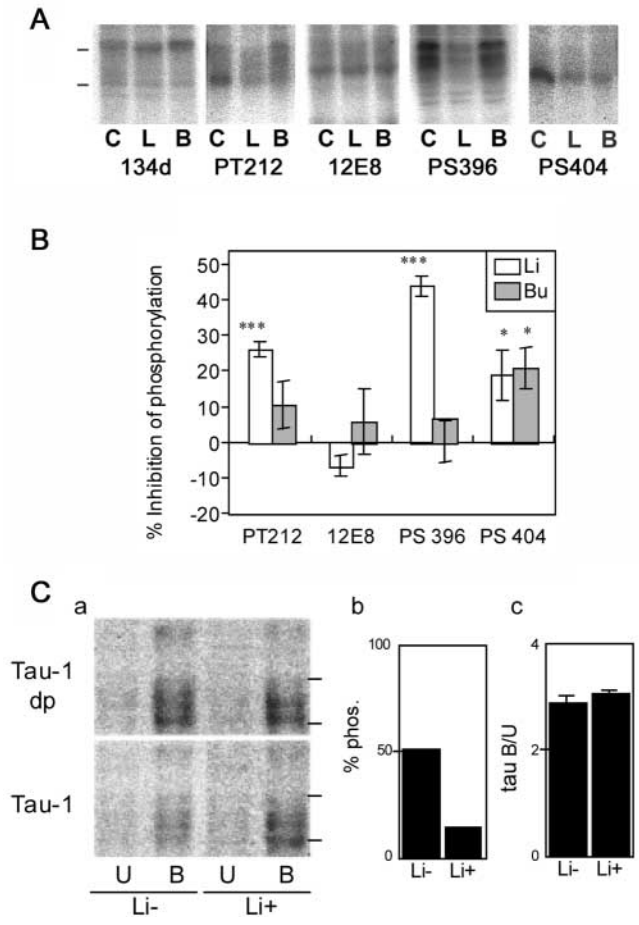

Fig. 3. Site-specific effect of LiCl and Bu1 on tau phosphorylation in differentiated PC12 cells. PC12 cells were differentiated with FGF-2 for 7 days and then treated with medium alone (C), or medium containing 20 mM LiCl (L) or 10 μM Bu1 (B). (A) The cell lysates (20 μg/lane) were analyzed by ^{125}I western blotting with phospho-independent antibody R134d to tau and antibodies to different phosphorylation sites on tau (indicated at the bottom of each individual panel). Bars on the left indicate the positions of 70 kDa and 50 kDa molecular mass markers. (B) Quantitative evaluation of the effect of the inhibition of GSK-3 with LiCl (white bars) and of cdk5 with Bu1 (hatched bars) on the phosphorylation of tau at different sites. The values represent the degrees of inhibition of site specific phosphorylation calculated after normalization of each individual value with the corresponding values for total tau (R134d). Values are means of three to nine individual western blots. Bars represent s.e.m.; **P*<0.05, ***P*<0.01, ****P*<0.001, Student's *t*-test. (C,a) Western blot analysis of tau bound (B) or unbound (U) to microtubules in differentiated PC12 cells (NGF, for 4 days) pretreated with (Li+) or without (Li–) 20 mM LiCl for 3 hours. Blots were untreated (Tau-1) or treated with alkaline phosphatase (Tau-1 dp) and stained with Tau-1 or directly stained with DM1A for α-tubulin (data not shown). Lines on the right side of the western blots indicate the positions of the 70 kDa and 50 kDa molecular mass markers. (b) Quantitative analysis of the degree of phosphorylation of tau at the Tau-1 site from a in the bound fraction. Lithium (Li+) markedly decreased tau phosphorylation at the Tau-1 site. (c) Quantitative analysis of the ratio of bound tau to unbound tau (B/U) from a (mean±s.e.m.). Lithium (Li+) did not affect tau's ability to bind to microtubules (compare Li- with Li+).

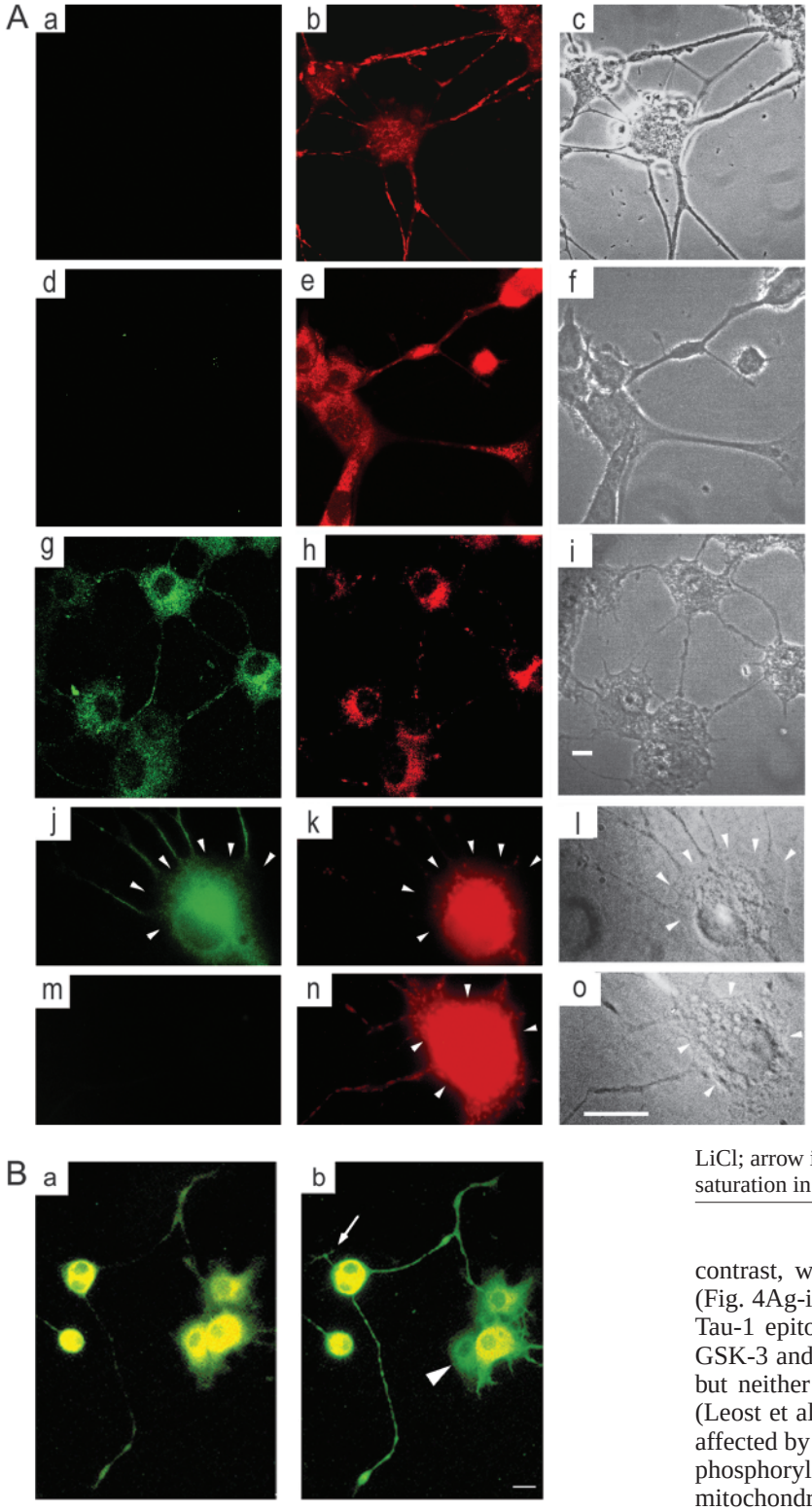

Fig. 4. Inhibition of GSK-3β but not of cdk5 induces reversible mitochondrial clustering in differentiated PC12 cells in association with dephosphorylation of tau at the Tau-1 epitope. (A) PC12 cells differentiated with FGF-2 for 5 days were treated with medium alone (a-c,m-o), or medium with GSK-3 inhibitors LiCl (g-i), VA (j-l) and alsterpaullone (images not shown) or the cdk5 inhibitor butyrolactone 1 (d-f) for 1.5 hours. The cells were then fixed and immunostained with Tau-1 (a,d,g,j,m) and the distribution of mitochondria was visualized with Mito Tracker Red® which had been added after 1 hour of incubation with kinase inhibitors (b,e,h,k,n). The size of the cells and their processes can be seen by phase contrast microscopy (c,f,i,l,o). Inhibition of GSK-3 induced mitochondrial clustering associated with increased staining at the Tau-1 epitope (dephosphorylation) as well as loss of mitochondria from the neurites (g-i,j-l) and the periphery of the cell body (compare l with o). Inhibition of cdk5 (d-f) resulted in flattening of cells and processes but neither mitochondrial movement nor Tau-1 phosphorylation were affected. Images in a, b, d, e, g, h are maximum projections of four confocal captured sections (j,k,m,n) are fluorescence micrographs. Arrowheads in j-o indicate the position of the cell membrane as seen in the corresponding phase contrast images (l,o). Scale bars: 10 μm. (B) Partial reversal of mitochondrial clustering. PC12 cells differentiated with NGF for 4 days were treated with 20 mM LiCl for 1 hour (a). Cells were then washed and incubated in medium without LiCl for another 1 hour (b). The distribution of mitochondria was visualized with Rhodamine 123 through FITC filter using fluorescence microscopy. More intense signals were observed in the periphery of the cells and the neurites after washing out lithium (b). Since it is unlikely that mitochondria sequester additional dye during the wash-out period of LiCl in the conditions used, the increased signals in the periphery and the neurites in b most probably reflect mitochondria transported anterogradely during the wash-out period. Arrowhead indicates restoration of mitochondrial distribution following washing out of LiCl; arrow indicates newly appeared neurites; identical color saturation in a and b. Scale bars: 10 μm.

dramatically (Fig. 2A), we studied the distribution of mitochondria in the differentiated cells. Both in the control (Fig. 4Aa-c,m-o) and the Bu1-treated (Fig. 4Ad-f) differentiated cells we found an almost complete blockage of the Tau-1 epitope associated with an apparently normal distribution of mitochondria in cell bodies and processes. In contrast, when GSK-3β was inhibited with either LiCl, VA (Fig. 4Ag-i,j-l), or alsterpaullone (not shown in the figure) the Tau-1 epitope was dephosphorylated. Alsterpaullone inhibits GSK-3 and, to a lesser degree, cyclin-dependent kinases/cdk5 but neither kinases of the ERK family nor protein kinase C (Leost et al., 2000). ERKs and PKC have been reported to be affected by LiCl and VA (Manji and Chen, 2002). The reduced phosphorylation of tau was associated with a reduction of mitochondria in the neurites and clustering around the nucleus. Higher magnification phase-contrast images revealed that the inhibition of GSK-3 not only affected the transport of mitochondria but also of most cellular organelles (Fig. 4Al,o). While the effect of GSK-3 inhibition was observed in PC12 cells that had been differentiated with FGF-2 at all stages of differentiation up to the 10 days studied, in the case of NGF the mitochondrial clustering was only observed in cells differentiated for up to 4 days. In cells differentiated for 7 days

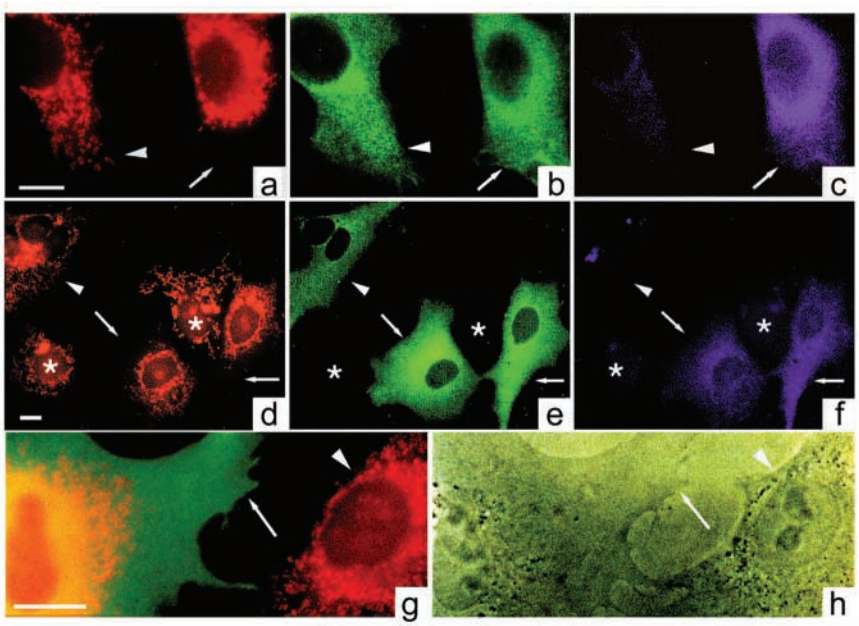

Fig. 5. Mitochondrial clustering in tau transfected CHO cells in which tau is not phosphorylated at the Tau-1 site. (a-f) CHO cells transfected with tau were triple stained with Mito Tracker Red® (mitochondria) and antibodies R134d to total tau (green) and Tau-1 to unphosphorylated tau (blue). Arrows indicate the cells with mitochondrial clustering, arrowheads indicate the cells with normal mitochondrial distribution. Both arrows and arrowheads show the position of the cell membrane seen by phase microscopy (not shown). In cells with unphosphorylated tau (blue) clustering of mitochondria was observed (a,d; arrows). In contrast, the distribution of mitochondria was almost normal in cells containing phosphorylated tau (a,d; arrowheads). Asterisks indicate non-tau-expressing cells (e,f) with normal distribution of mitochondria (d). Tau-transfected CHO cell (g,h; left) with mitochondria clustered around the nucleus, double stained (g) for tau (R134d, green) and mitochondria (Mito Tracker Red®). The cell on the right in g, h is not transfected. Orange in g indicates overlapping areas of tau and mitochondrial stains. (h) Phase micrograph of g showing that the periphery of the transfected cell (g,h; left) is practically free of organelles. Scale bars: 10 μm.

or longer, concomitant with the increased expression of MAP1B (see Fig. 1B), no mitochondrial clustering was observed (data not shown).

It has been shown previously that the LiCl-induced inhibition of tau phosphorylation is reversible in situ upon removal of LiCl (Hong et al., 1997; Takahashi et al., 1999). Similarly, in the present study, the effect of LiCl on the mitochondrial aggregation was reversible, because the deletion of lithium from the medium reversed the distribution of the Rhodamine 123-positive mitochondria into the neurites and the periphery of the cells (Fig. 4B). These data raise the possibility that GSK-3β might regulate the anterograde organelle transport including that of mitochondria in differentiated PC12 cells concomitant with the alteration of the phosphorylation state of tau on microtubules.

Mitochondrial clustering in CHO cells induced by overexpression of tau is restored by its phosphorylation at the Tau-1 site

To confirm the possible role of the phosphorylation of tau in mitochondrial transport, we studied CHO cells transfected with the longest human brain tau isoform, tau_{441}, with respect to tau phosphorylation and mitochondrial distribution (Fig. 5). Immunocytochemical staining with phospho-independent polyclonal antibody R134d to total tau (green in Fig. 5) and the phospho-dependent antibody Tau-1 to unphosphorylated tau (blue) was employed for this study. In CHO cells stably or transiently transfected with tau we observed cells in which tau was phosphorylated (Fig. 5a,d, arrowheads) and others in which it was not (Fig. 5a,d, arrows). In most cells with phosphorylated tau (Tau-1 negative, arrowheads) the distribution of mitochondria (red) was similar to that in untransfected cells (asterisk), while in the cells with unphosphorylated tau (Tau-1 positive, arrows), the mitochondria were clustered around the nucleus. In the latter cells we also observed, by phase contrast microscopy, loss of organelles from the periphery (Fig. 5g,h, arrow); a similar phenomenon was also observed in PC12 cells treated with GSK-3 inhibitors (Fig. 4Al). These data, showing the association between the phosphorylation state of tau and change in distribution of mitochondria, are consistent with the possibility that the phosphorylation of tau might be involved in the regulation of the mitochondrial transport in the cell.

Lithium inhibits initial neurite outgrowth of PC12 cells and of neural progenitor cells from adult rat hippocampus.

The effect of LiCl on the initial neurite outgrowth and the distribution of mitochondria was examined in PC12 cells in the presence of NGF or FGF-2. LiCl (20 mM) or VA (0.6 mM) induced mitochondrial clustering (after 1 day in NGF or FGF-2) and almost completely inhibited initial neurite outgrowth (up to 6 days examined) of both NGF- and FGF-2-treated PC12 cells (data not shown). Another prominent known effect of LiCl and VA is their inhibition of inositol monophosphatase, which might result in inositol depletion (Klein and Melton, 1996; Hallcher and Sherman, 1980). However, excess myo-inositol administration failed to rescue the inhibition of neurite outgrowth by LiCl (Fig. 6A) or VA (data not shown), suggesting that inositol depletion might not be involved in this phenomenon.

To learn whether the regulation of the neurite outgrowth through mitochondrial transport by the tau-GSK-3β phosphorylation system is similar in immature cells, we carried out the above studies on neural progenitor cells derived from adult rat hippocampus (AHPs). In these cells, FGF-2 is known to upregulate the expression of adult isoforms of tau and GSK-3β-mediated phosphorylation at the Tau-1 site (Tatebayashi et

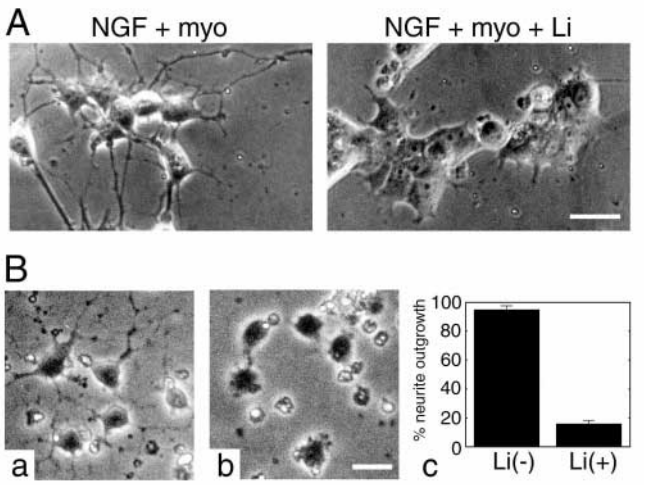

Fig. 6. Lithium inhibits the initial neurite outgrowth of PC12 cells and neural progenitor cells derived from adult rat hippocampus. (A) Excess myo-inositol administration failed to rescue the inhibition of neurite outgrowth of PC12 cells by LiCl. PC12 cells induced to differentiate by NGF in the presence of excess myo-inositol (20 mM) were left untreated (NGF + myo) or simultaneously treated with lithium (NGF + myo + LiCl) for 3 days. Scale bar: 25 μm. (B) AHPs expanded by FGF-2 (10 ng/ml) were replated on culture slides with 40 ng/ml FGF-2 with or without 20 mM LiCl for 1 day. (a,b) Cell morphologies of AHPs without (a) or with (b) lithium. Scale bar: 10 μm. (c) Quantitative data showing the percentage of neurite-bearing cells. PC12 cells possessing one or more neurites of a length more than 1.5-fold the diameter of the cell body were scored as positive. Lithium almost completely blocked initial neurite outgrowth of AHPs (mean±s.e.m.). $P<0.001$.

al., 1999). We found that AHPs do not express MAP1B (data not shown). AHPs were plated with FGF-2 in the presence or absence of LiCl for 1 day. Lithium caused mitochondrial accumulation (data not shown) and blocked initial neurite outgrowth of AHPs almost completely (Fig. 6Ba-c). These data are consistent with the possibility that the tau-GSK-3β phosphorylation system might be involved in the neurite generation of PC12 cells and of neural progenitor cells derived from the adult central nervous system.

Discussion

Tau is a major MAP of neurons. To date the most established function of tau is its promotion of assembly and maintenance of microtubules (Weingarten et al., 1975; Drubin and Kirschner, 1986). That tau might also play a role in the regulation of organelle transport was shown by Ebneth et al. (Ebneth et al., 1998) in CHO and differentiated Neuro 2A cells where upon transfection with tau, the anterograde kinesin-mediated mitochondrial/organelle movement was disrupted. The present study shows that the phosphorylation of tau by GSK-3β during differentiation might be involved in the regulation of the intracellular anterograde mitochondrial/organelle transport in small caliber neurites where tau represents a large proportion of total MAPs.

In the present study in PC12 cells differentiated by NGF or FGF2, the levels of tau and GSK-3β were upregulated simultaneously and the GSK-3β-mediated tau phosphorylation was increased at several sites canonic for proline-dependent kinases including the Tau-1 site. Previously most of these sites had been shown in vitro to be phosphorylated by ERK 1/2, cdc2, GSK-3 and cdk5 (for a review, see Johnson and Hartigan, 1998). In situ, in differentiated neurons, GSK-3 clearly seems to have a role in tau phosphorylation as was shown by inhibition of GSK-3 with LiCl (Hong et al., 1997; Muñoz-Montaño et al., 1997). Of the cyclin-dependent kinases in differentiated neurons, cdc2, which is activated in dividing cells (Meyerson et al., 1992) is most probably of less importance than cdk5, the activity of which is increased in differentiating neurons (Fu et al., 2002). However, relatively little is known about tau phosphorylation by cdk5. That this kinase might play a role in tau phosphorylation in situ is indicated by the finding that transfection of primary neurons with its activator p25 induced phosphorylation of tau at the AT8 (PS202/T205) epitope (Patrick et al., 1999). In the present study, in differentiated PC12 cells, treatment with LiCl and Bu1 revealed that the Tau-1 epitope, T212 and S396 were almost exclusively phosphorylated by GSK-3, whereas both GSK-3 and cdk5 phosphorylated tau at S404. As expected, the proline-independent 12E8 site, which is phosphorylated by PKA, MARK and CaMKII (Drewes et al., 1997; Sironi et al., 1998) was not affected by inhibition of either GSK-3 or cdk5.

The inhibition of GSK-3 by LiCl for 3 hours and the concomitant decrease in phosphorylation of tau at various sites in PC12 cells did not significantly affect the binding of tau to microtubules. This finding is consistent with a previous study in which LiCl treatment of differentiated SY5Y cells (for 24 hours) did not change microtubule binding (Xie et al., 1998). In contrast, in differentiated NT2 neurons treated overnight with LiCl, the binding of tau to microtubules and the degree of polymerization of tubulin was found to be enhanced (Hong et al., 1997). The exact cause of this discrepancy between different neuronal cell lines is, at present, not understood.

The present study indicated that in differentiated PC12 cells the GSK-3-mediated increase in phosphorylation of tau at defined sites might be required for organelle/mitochondrial transport. When the GSK-3β activity was inhibited, tau was dephosphorylated at both the proline-rich N-terminal and the C-terminal domain and the anterograde organelle transport was inhibited. In contrast, inhibition of the cyclin-dependent kinases/cdk5, which also phosphorylated tau at Ser-404, but not at the GSK-3-preferred sites studied, resulted in flattening of the cells but no apparent disturbance of the mitochondrial transport. Similarly, in tau-transfected CHO cells, the mitochondrial/organelle transport was disrupted in those cells in which tau was not phosphorylated, but was largely intact in cells containing phosphorylated tau. Also it has been shown previously that phosphorylated tau can affect the mitochondrial transport. In this case conditions favoring the phosphorylation of tau at sites inhibitory to its microtubule binding, such as phosphorylation with A-kinase led to enhanced and less staggered transport of organelles in the axons of primary sensory neurons (Sato-Harada et al., 1996). The present study shows that in differentiated PC12 cells, endogenous tau, when physiologically phosphorylated by GSK-3, is not detached from the microtubules but seems to support kinesin-mediated mitochondrial anterograde transport. Inhibition of GSK-3 resulted in dephosphorylation of tau and inhibition of the anterograde transport, possibly because of phosphorylation-

dependent conformational changes of tau or its effect on the dynamics of microtubule assembly. It has been shown previously that the microtubule nucleation activity of tau is reduced by dephosphorylation (Morita-Fujimura et al., 1996). The present study supports the notion that a balance between the levels of GSK-3 and tau phosphorylation is required to maintain the anterograde transport. As was previously shown, overexpression of tau in cultured cells led to disturbance of the axonal transport (Ebneth et al., 1998; Trinczek et al., 1999), and to axonopathy and motor deficiencies in tau transgenic mice (Spittaels et al., 1999). The deficient anterograde transport together with the axonopathy was restored to almost normal when GSK-3 levels were increased by crossing these mice with GSK-3 transgenic animals (Spittaels et al., 2000; Nuydens et al., 2002). In addition to tau, GSK-3 has also been shown, in vitro, to phosphorylate kinesin light chains, resulting in a release of cargo organelles and thus disturbance of the axonal transport (Morfini et al., 2001). It is not known whether tau and kinesin may be connected functionally and what is the effect of GSK-3 inhibition on kinesin.

The role of phosphorylated tau in organelle transport was observed when tau was the major MAP. At 4 days of NGF- but not FGF-2-induced differentiation, coincident with the expression of MAP1B, this role of tau was eliminated. A similar functional relationship between tau and MAP1A has been speculated in the normally tau-rich small-caliber axons of adult tau-knockout mice (Harada et al., 1994). In these structures decreased microtubule stability and altered microtubule organization was observed. However, it is not known whether the expression of MAP1B in NGF-differentiated PC12 cells observed in the present study causally or incidentally affected the Li-mediated disturbance of the axonal transport.

That GSK-3 might play a role in regulating the neurite outgrowth has been shown previously (Burstein et al., 1985) and is also indicated by the findings in the present study that exposure to lithium for 3 days and 1 day, respectively, inhibited almost completely the initial neurite outgrowth of differentiating PC12 cells (Fig. 6A) and AHPs (Fig. 6B). This is in agreement with the data of Goold et al. (Goold et al., 2001) who observed inhibition of neurite formation and MAP1B expression during prolonged lithium treatment. Exposure to lithium for several days has also been reported to result in the formation of shorter than normal axons in cultures of hippocampal and cerebellar neurons (Lucas et al., 1998; Takahashi et al., 1999). However, it should be kept in mind that besides tau, GSK-3 phosphorylates a number of other proteins such as MAP1B (Goold et al., 2001), and several transcription factors (Welsh et al., 1996). Especially upon chronic administration of lithium, besides GSK-3, other factors and signaling cascades such as the PKC and MAPK cascades (Manji and Chen, 2002) are affected that might also modulate the regulation of neurite outgrowth and retraction.

Expression of MAP1B is upregulated during embryogenesis where it peaks at 2-3 days and decreases within 2-3 weeks after birth (Riederer et al., 1986; Schoenfeld et al., 1989). Thus, the effect of the phosphorylation of tau by GSK-3 on neuritic extension might be only minor during development, and major during adulthood, especially in axons. This role of GSK-3 might be one of the critical functions lost in adult tau-knockout mice, which were reported to exhibit some neurological defect (Ikegami et al., 2000), and in the human, in certain cases with frontotemporal dementia where tau protein is not expressed (Zhukareva et al., 2001).

Activated GSK-3β has been shown to be localized to paired helical filaments/neurofibrillary tangles in AD brain (Yamaguchi et al., 1996; Pei et al., 1997; Pei et al., 1999) and is believed to be a major protein kinase involved in the abnormal hyperphosphorylation of tau (Mandelkow et al., 1992; Ishiguro et al., 1993; Lovestone et al., 1994; Lucas et al., 2001). Moreover, FGF-2 levels are elevated in AD brains (Stopa et al., 1990) and strong immunoreactivities of FGF-2 also have been found in the tau lesions (Cummings et al., 1993). The FGF-2-induced upregulation of both the expression of tau and GSK-3β and the GSK-3β-mediated tau phosphorylation in PC12 cells in the present study and in AHPs shown previously (Tatebayashi et al., 1999) raise the possibility that this growth factor might be involved in the process of neurofibrillary degeneration in an environment favorable to phosphorylation, such as the AD brain in which the phosphatase activity is compromised (Gong et al., 1993).

In conclusion, in differentiated PC12 cells, GSK-3β through phosphorylation of tau at a number of sites seems to regulate anterograde transport of mitochondria in tau-dominated small caliber neurites. Since in situ the GSK-3β activity is regulated by several kinases such as protein kinase B via the receptor-mediated phosphoinositide 3-kinase pathway (Proud and Denton, 1997), it is possible that the GSK-3β-tau phosphorylation pathway might play a role in the regulation of neurite outgrowth and the plasticity of tau-dominated thin neurites. Although the role of these neurites in the central nervous system is still unclear, their abnormal upregulation can be involved, via upregulation of hyperphosphorylated tau, in the pathophysiology of AD and other tauopathies.

We are grateful to Lester I. Binder (University of Alabama, Birmingham, AL, USA) for the monoclonal antibody Tau-1, Peter Davies (Albert Einstein College of Medicine, Bronx, NY, USA) for PHF-1, and Dale Schenk (Athena Neurosciences, San Francisco, CA, USA) for 12E8 antibody. We also acknowledge Janet Biegelson and Sonia Warren for secretarial assistance. These studies were supported in part by funds from the New York State Office of Mental Retardation and Developmental Disabilities and NIH grants NS18105, AG05892 and AG19158.

References

Alonso, A. del C., Grundke-Iqbal, I. and Iqbal, K. (1996). Alzheimer disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. *Nature Medicine* **2**, 783-787.

Bensadoun, A. and Weinstein, D. (1986). Assay of protein in the presence of interfering materials. *Anal. Biochem.* **70**, 241-250.

Binder, L. I., Frankfurter, A. and Rebhun, L. I. (1985). The distribution of tau in the mammalian central nervous system. *J. Cell Biol.* **101**, 1371-1378.

Burstein, D. E., Seeley, P. J. and Greene, L. A. (1985). Lithium ion inhibits nerve growth factor-induced neurite outgrowth and phosphorylation of nerve growth factor-mediated microtubule-associated proteins. *J. Cell Biol.* **101**, 862-870.

Chen, G., Huang, L. D., Jiang, Y. M. and Manji, H. K. (1999). The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. *J. Neurochem.* **72**, 1327-1330.

Cowley, S., Paterson, H., Kemp, P. and Marshall, C. J. (1994). Activation of MAP kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77**, 841-852.

Cummings, B. J., Su, B. J. and Cotman, C. W. (1993). Neuritic involvement

within bFGF immunopositive plaques of Alzheimer's disease. *Exp. Neurol.* **124**, 315-325.

Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E. M. and Mandelkow, E. (1997). MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. *Cell* **89**, 297-308.

Drubin, D. G. and Kirschner, M. W. (1986). Tau protein function in living cells. *J. Cell Biol.* **103**, 2739-2746.

Ebneth, A., Godemann, R., Stamer, K., Illenberger, S., Trinczek, B. and Mandelkow, E. (1998). Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria and endoplasmic reticulum: implications for Alzheimer's disease. *J. Cell Biol.* **143**, 777-794.

Fu, W.-Y., Wang, J. H. and Ip, N. Y. (2002). Expression of cdk5 and its activators in NT2 cells during neuronal differentiation. *J. Neurochem.* **81**, 646-654.

Gong, C. X., Singh, T. J., Grundke-Iqbal, I. and Iqbal, K. (1993). Phosphoprotein phosphatase activities in Alzheimer disease brain. *J. Neurochem.* **61**, 921-927.

Goold, R. G. and Gordon-Weeks, P. R. (2001). Microtubule-associated protein 1B phosphorylation by glycogen synthase kinase 3β is induced during PC12 cell differentiation. *J. Cell. Sci.* **114**, 4273-4284.

Greenberg, S. G., Davies, P., Schein, J. D. and Binder, L. I. (1992). Hydrofluoric acid-treated tau PHF proteins display the same biochemical properties as normal tau. *J. Biol. Chem.* **26**, 564-569.

Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y.-C., Zaidi, M. S. and Wisniewski, H. M. (1986a). Microtubule-associated protein tau: A component of Alzheimer paired helical filaments. *J. Biol. Chem.* **261,** 6084-6089.

Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. W. and Binder, L. I. (1986b). Abnormal phosphorylation of the microtubule-associated protein (tau). in Alzheimer cytoskeletal pathology. *Proc. Natl. Acad. Sci. USA* **83**, 4913-4917.

Hallcher, L. M. and Sherman, W. R. (1980). The effects of lithium ion and other agents on the activity of myo-inositol-2-phosphatase from bovine brain. *J. Biol. Chem.* **255**, 10896-10901.

Haque, N., Denman, R. B., Merz, G., Grundke-Iqbal, I. and Iqbal, K. (1995). Phosphorylation and accumulation of tau without any concomitant increase in tubulin levels in Chinese hamster ovary cells stably transfected with human tau441. *FEBS Lett.* **360**, 132-136.

Haque, N., Tanaka, T., Iqbal, K. and Grundke-Iqbal, I. (1999). Regulation, phosphorylation and biological activity of tau during differentiation in SY5Y cells. *Brain Res.* **838**, 69-77.

Harada, A., Oguchi, K., Okabe, S., Kuno, J., Terada, S., Ohshima, T., Sato-Yoshitake, R, Takei, Y., Noda, T. and Hirokawa, N. (1994). Altered microtubule organization in small-caliber axons of mice lacking tau protein. *Nature* **369**, 488-491.

Hong, M., Chen, D. C. R., Klein, P. S. and Lee, V. M. Y. (1997). Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3. *J. Biol. Chem.* **272**, 25326-25332.

Ikegami, S., Harada, A. and Hirokawa, N. (2000). Muscle weakness, hyperactivity and impairment in fear conditioning in tau-deficient mice. *Neurosci. Lett.* **279**, 129-132.

Iqbal, K., Grundke-Iqbal, I., Zaidi, T., Merz, P. A., Wen, G. Y., Shaikh, S. S., Wisniewski, H. M., Alafuzoff, I. and Winblad, B. (1986). Defective brain microtubule assembly in Alzheimer's disease. *Lancet* **2**, 421-426.

Ishiguro, K., Shiratsuchi, A., Sato, S., Omori, A., Arioka, M., Kobayashi, S., Uchida, T. and Imahori, K. (1993). Glycogen synthase kinase 3β is identical to tau protein kinase I generating several epitopes of paired helical filaments. *FEBS Lett.* **325**, 167-172.

Johnson, G. V. W. and Hartigan, J. A. (1998). Tau protein in normal and Alzheimer's disease brain: An update. *Alzheimer's Disease Rev.* **3**, 125-141.

Khatoon, S., Grundke-Iqbal, I. and Iqbal, K. (1992). Brain levels of microtubule associated protein tau are elevated in Alzheimer's disease brain: a radioimmunoslot-blot assay for nanograms of the protein. *J. Neurochem.* **59**, 750-753.

Kleijn, M., Welsh, G. I., Scheper, G. C., Voorma, H. O., Proud, C. G. and Thomas, A. A. (1998). Nerve and epidermal growth factor induce protein synthesis and eIF2B activation in PC12 cells. *J. Biol. Chem.* **273**, 5536-5541.

Klein, P. S. and Melton, D. A. (1996). A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. USA* **93**, 8455-8459.

Köpke, E., Tung, Y. C., Shaikh, S., Iqbal, K. and Grundke-Iqbal, I. (1993). Microtubule associated protein tau: abnormal phosphorylation of non-paired helical filament pool in Alzheimer disease. *J. Biol. Chem.* **268**, 24374-24384.

Leost, M., Schultz, C., Link, A., Wu, Y. Z., Biernat, J., Mandelkow, E. M., Bibb, J. A., Snyder, G. L., Greengard, P., Zaharevitz, D. W., Gussio, R., Senderowicz, A. M., Sausville, E. A., Kunick, C. and Meijer, L. (2000). Paullones are potent inhibitors of glycogen synthase kinase-3β and cyclin-dependent kinase 5/p25. *Eur. J. Biochem.* **267**, 5983-5994.

Lindwall, G. and Cole, R. D. (1984). Phosphorylation affects the ability of tau protein to promote microtubule assembly. *J. Biol. Chem.* **259**, 5301-5305.

Liu, W.-K., Moore, W. T., Williams, R. T., Hall, F. L. and Yen, S.-H. (1993). Application of synthetic phospho- and unphospho-peptides to identify phosphorylation sites in a subregion of the tau molecule, which is modified in Alzheimer's disease. *J. Neurosci. Res.* **34**, 371-376.

Lovestone, S., Reynolds C. H., Latimer, D., Davis, D. R., Anderton, B. H., Gallo, J. M., Hanger, D., Mulot, S., Marquardt, B., Stabel, S. et al. (1994). Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau by glycogen synthase kinase-3 in transfected mammalian cells. *Curr. Biol.* **4**, 1077-1086.

Lucas, F. R., Goold, R. G., Gordon-Weeks, P. R. and Salinas, P. C. (1998). Inhibition of GSK-3β leading to the loss of phosphorylated MAP-1B is an early event in axonal remodeling induced by WNT-7A or lithium. *J. Cell Sci.* **111**, 1351-1361.

Lucas, J. L., Hernandez, F., Gomez-Ramos, P., Moran, M. A., Hen, R. and Avila, J. (2001). Decreased nuclear β-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3β conditional transgenic mice. *EMBO J.* **20**, 27-39.

Maher, P. (1999). p38 mitogen-activated protein kinase activation is required for fibroblast growth factor-2-stimulated cell proliferation but not differentiation. *J. Biol. Chem.* **274**, 17491-17498.

Mandelkow, E.-M., Drewes, G., Biernat, J., Gustke, N., Lint, J. V., Vandenheede, J. R. and Mandelkow, E. (1992). Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau. *FEBS Lett.* **314**, 315-321.

Manji, H. K. and Chen, G. (2002). PKC, MAP kinases and the bcl-2 family of proteins as long term targets for mood stabilizers. *Mol. Psychiatry* **7**, 546-556.

Meyerson, M., Enders, G. H., Wu, C.-L., Su, L.-K., Gorka, C., Nelson, C., Harlow, E. and Tsai, L.-H. (1992). A family of human cdc2-related protein kinases. *EMBO J.* **11**, 2909-2917.

Morfini, G., Szebenyi, G., Richards, B. and Brady, S. T. (2001). Regulation of kinesin: implications for neuronal development. *Dev. Neurosci.* **23**, 364-376.

Morita-Fujimura, Y., Kurachi, M., Tashiro, H., Komiya, Y. and Tashiro, T. (1996). Reduced microtubule-nucleation activity of tau after dephosphorylation. *Biochem. Biophys. Res. Comm.* **225**, 462-468.

Muñoz-Montaño, J. R., Moreno, F. J., Avila, J. and Diaz-Nido, J. (1997). Lithium inhibits Alzheimer's disease-like tau protein phosphorylation in neurons. *FEBS. Lett.* **411**, 183-188.

Nuydens, R., Van Den Kieboom, G., Nolten, C., Verhulst, C., Van Osta, P., Spittaels, K., Van den Haute, C., De Feyter, E., Geerts, H. and Van Leuven, F. (2002). Coexpression of GSK-3β corrects phenotypic aberrations of dorsal root ganglion cells, cultured from adult transgenic mice overexpressing human protein tau. *Neurobiol. Dis.* **9**, 38-48.

Otvos Jr, L., Feiner, L., Lang, E., Szendrei, G. I., Goedert, M. and Lee, V. M. Y. (1994). Monoclonal antibody PHF-1 recognizes tau protein phosphorylated at serine residues 396 and 404. *J. Neurosci. Res.* **39**, 669-673.

Patrick, G. N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P. and Tsai, L.-H. (1999). Conversion of p35 to p25 deregulates cdk5 activity and promotes neurodegeneration. *Nature* **402**, 615-622.

Pei, J. J., Tanaka, T., Tung, Y. C., Braak, E., Iqbal, K. and Grundke-Iqbal, I. (1997). Distribution, levels and activity of glycogen synthase kinase-3 in the Alzheimer disease brain. *J. Neuropathol. Exp. Neurol.* **56**, 70-78.

Pei, J., Braak, E., Braak, H., Grundke-Iqbal, I., Iqbal, K., Winblad, B. and Cowburn, R. F. (1999). Distribution of active Glycogen Synthase Kinase 3β (GSK-3β). in brains staged for Alzheimer's disease neurofibrillary changes. *J. Neuropathol. Exper. Neurol.* **58**, 1010-1019.

Proud, C. G. and Denton, R. M. (1997). Molecular mechanisms for the control of translation by insulin. *Biochem. J.* **328**, 329-341.

Raffioni, S. and Bradshaw, R. A. (1992). Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basic fibroblast growth factor and nerve growth factor in PC12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA* **89**, 9121-9125.

Riederer, B., Cohen, R. and Matus, A. (1986). MAP5: a novel brain microtubule-associated protein under strong developmental regulation. *J. Neurocytol.* **15**, 763-775.

Sato-Harada, R., Okabe, S., Umeyama, T., Kanai, Y. and Hirokawa, N. (1996). Microtubule-associated proteins regulate microtubule function as the tract for intracellular membrane organelle transport. *Cell Struct. Funct.* **21**, 283-295.

Schoenfeld, T. A., McKerracher, L., Obar, R. and Vallee, R. B. (1989). MAP1A and MAP1B are structurally related microtubule-associated proteins with distinct developmental patterns in the CNS. *J. Neurosci.* **9**, 1712-1730.

Seubert, P., Mawal-Dewan, M., Barbour, R., Jakes, R., Goedert, M., Johnson, G. V. W., Litersky, J. M., Schenk, D., Leiberburg, I., Trojanowski, J. Q. and Lee, V. M. Y. (1995). Detection of phosphorylated Ser262 in fetal tau, adult tau and paired helical filament tau. *J. Biol. Chem.* **270**, 18917-18922.

Sironi, J. J., Yen, S. H., Gondal, J. A., Wu, Q., Grundke-Iqbal, I. and Iqbal, K. (1998). Ser-262 in human recombinant tau protein is a markedly more favorable site for phosphorylation by CaMKII than PKA or PhK. *FEBS Lett.* **436**, 471-475.

Spittaels, K., Van den Haute, C., Van Dorpe, J., Bruynseels, K., Vandezande, K., Laenen, I., Geerts, H., Mercken, M., Sciot, R., Van Lommel, A., Loos, R. and Van Leuven, F. (1999). Prominent axonopathy in the brain and spinal cord of transgenic mice overexpressing four-repeat human tau protein. *Am. J. Pathol.* **155**, 2153-2165.

Spittaels, K., Van den Haute, C., Van Dorpe, J., Geerts, H., Mercken, M., Bruynseels, K., Lasrado, R., Vandezande, K., Laenen, I., Boont, T., Van Lint, J., Vandenheede, J., Moechars, D., Loos, R. and Van Leuven, F. (2000). Glycogen synthase kinase-3β phosphorylates protein tau and rescues the axonopathy in the central nervous system of human four-repeat tau transgenic mice. *J. Biol. Chem.* **275**, 41340-41349.

Stopa, E. G., Gonzalez, A. M., Chorsky, R., Corona, R. J., Alvarez, J., Bird, E. D. and Baird, A. (1990). Basic fibroblast growth factor in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* **171**, 690-696.

Szendrei, G. I., Lee, V. M. Y. 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.

Takahashi, M., Yasutake, K. and Tomizawa, K. (1999). Lithium inhibits neurite growth and tau protein kinase I/Glycogen synthase kinase-3β-dependent phosphorylation of juvenile tau in cultured hippocampal neurons. *J. Neurochem.* **73**, 2073-2083.

Tanaka, T., Zhong, J., Iqbal, K., Trenkner, E. and Grundke-Iqbal, I. (1998). The regulation of phosphorylation of tau in SY5Y neuroblastoma cells: the role of protein phosphatases. *FEBS Lett.* **426**, 248-254.

Tatebayashi, Y., Iqbal, K. and Grundke-Iqbal, I. (1999). Dynamic regulation of expression and phosphorylation of tau by fibroblast growth factor-2 in neural progenitor cells from adult rat hippocampus. *J. Neurosci.* **19**, 5245-5254.

Trinczek, B., Ebneth, A., Mandelkow, E. M. and Mandelkow, E. (1999). Tau regulates the attachment/detachment but not the speed of motors in microtubule-dependent transport of single vesicles and organelles. *J. Cell. Sci.* **112**, 2355-2367.

Tucker, R., Binder, L. I. and Matus, A. (1988). Neuronal microtubule associated proteins in the embryonic avian spinal cord. *J. Comp. Neurol.* **271**, 44-55.

Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y. and Kirschner, M. W. (1975). A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA* **72**, 1858-1862.

Welsh, G. I., Wilson, C. and Proud, C. G. (1996). GSK-3: SHAGGY frog story. *Trends Cell Biol.* **6**, 274-279.

Woodgett, J. R. (1990). Molecular cloning and expression of glycogen synthase kinase-3/factorA. *EMBO J.* **9**, 2431-2438.

Xie, H. Q., Litersky, J. H., Hartigan, J. A., Jope, R. S. and Johnson, G. V. W. (1998). The interrelationship between selective tau phosphorylation and microtubule association. *Brain Res.* **798**, 173-183.

Yamaguchi, H., Ishguro, K., Uchida, T., Takashima, A., Lemere, C. A. and Imahori, K. (1996). Preferential labeling of Alzheimer neurofibrillary tangles with antisera for tau protein kinase (TPK), I/glycogen synthase kinase-3β and cyclin-dependent kinase 5, a component of TPK II. *Acta Neuropathol.* **92**, 232-241.

Young, S. W., Dickens, M. and Tavarè, J. M. (1994). Differentiation of PC12 cells in response to a cAMP analogue is accompanied by sustained activation of mitogen-activated protein kinase. Comparison with the effects of insulin, growth factors and phorbol esters. *FEBS. Lett.* **338**, 212-216.

Zhukareva, V., Gobelsberg-Ragaglia, V., Van Deerlin, V. M., Bruce, J., Shuck, T., Grossman, M., Clark, C. M., Arnold, S. E., Masliah, E., Galasko, D., Trojanowski, J. Q. and Lee, V. M. (2001). Loss of brain tau defines novel sporadic and familial tauopathies with frontotemporal dementia. *Ann. Neurol.* **49**, 165-175.