Microtubule-associated protein 1B phosphorylation by glycogen synthase kinase 3β is induced during PC12 cell differentiation

Robert G. Goold and Phillip R. Gordon-Weeks*

The MRC Centre for Developmental Neurobiology, New Hunts House, Guy's Campus, King's College London, London SE1 1UL, UK
*Author for correspondence (e-mail: phillip.gordon-weeks@kcl.ac.uk)

Accepted 24 August 2001
Journal of Cell Science 114, 4273-4284 (2001) © The Company of Biologists Ltd

SUMMARY

In recent studies we have demonstrated that glycogen synthase kinase 3β (GSK3β) and its substrate microtubule-associated protein 1B (MAP1B) regulate the microtubule cytoskeleton during axon outgrowth. To further examine the role GSK3β plays in axon outgrowth we investigated the expression of GSK3β and its activity towards MAP1B during nerve growth factor (NGF)-stimulated PC12 cell differentiation. Levels of GSK3β expression increase relatively little during the course of differentiation. However, the expression of a novel GSK3β isoform characterised by a reduced mobility on SDS gels is induced by NGF. Expression of this isoform and the GSK3β-phosphorylated isoform of MAP1B (MAP1B-P) are induced in parallel in response to NGF. This increase lags behind initial neurite formation and the expression of MAP1B in these cells by about two days and coincides with a period when the majority of cells are extending existing neurites. MAP1B and GSK3β are expressed throughout the PC12 cell but MAP1B-P expression is restricted to the growth cones and neurites. Consistent with these observations, we find that neurite extension is more sensitive to the GSK3 inhibitor Li^+ than neurite formation and that this correlates with an inhibition of MAP1B phosphorylation. Additionally, GSK3β from PC12 cells not exposed to NGF can not phosphorylate MAP1B in vitro. However, a soluble factor in differentiated PC12 cell extracts depleted of GSK3β can activate MAP1B phosphorylation from undifferentiated cell extracts otherwise devoid of kinase activity. These experiments provide evidence for an NGF-mediated regulation of MAP1B phosphorylation in growing neurites by the induction of a novel isoform of GSK3β.

Key words: MAP1B, GSK3β, PC12 cell, NGF

INTRODUCTION

Glycogen synthase kinase 3 (GSK3) is a highly conserved serine/threonine kinase originally identified as a negative regulator of glycogen synthase but more recently implicated in signalling pathways influencing cell fate determination during embryogenesis (Welsh et al., 1996). In mammals there are two isoforms, GSK3α and GSK3β, encoded by separate genes (Woodgett, 1990). Genetic (Siegfried et al., 1992) and biochemical (Ruel et al., 1999) studies in *Drosophila* have shown that the GSK3β orthologue, shaggy/zeste-white 3, is an essential component in the wingless signalling pathway, which is important for establishing segment polarity during embryogenesis. In mouse, GSK3β is a component of signalling pathways important for early CNS patterning and in neuronal differentiation (Williams and Harwood, 2000).

In mammals, although GSK3α is the dominant form in most tissues, GSK3β is particularly abundant in neural tissue (Woodgett, 1990), where it is neuron specific (Takahashi et al., 1994; Leroy and Brion, 1999). In the CNS, GSK3β is developmentally regulated, with peak levels of expression occurring during axonogenesis. It is present in growing axons but completely excluded from them at the end of axonogenesis, being restricted in the adult to neuronal cell bodies and dendrites (Leroy and Brion, 1999). This suggests that GSK3β has a role in axonogenesis, an idea supported by pharmacological studies. GSK3 is inhibited by lithium ions (Klein and Melton, 1996) and, although other enzymes are also inhibited by lithium, in immature neurons GSK3 appears to be the primary pharmacological target (Lucas et al., 1998). Lithium has profound morphological effects on developing neurons in culture (Lucas et al., 1998; Goold et al., 1999; Takahashi et al., 1999). It reversibly inhibits neurite growth, enlarges growth cones and induces spread areas along neurite shafts. Significantly, Wnt proteins, the vertebrate orthologues of wingless, produce a similar phenotype, corroborating the involvement of GSK3 (Lucas et al., 1998; Hall et al., 2000).

Glycogen synthase kinase 3β activity is under both positive and negative regulation by upstream kinases. Tyrosine-216 phosphorylation in the kinase domain stimulates activity and may be essential, whereas serine-9 phosphorylation is inhibitory (Wang et al., 1994). Inhibitory phosphorylation occurs in three signal transduction pathways: insulin signalling through the phosphatidylinositol 3-kinase pathway, epidermal growth factor signalling through the mitogen-activated protein kinase (MAPK) pathway and wingless signalling through a protein kinase C-like pathway (Welsh et al., 1996). Activation pathways are less well defined but include the cAMP receptor pathway in *Dictyostelium* through the ZAK1 tyrosine kinase (Kim et al., 1999), and possibly the Fyn tyrosine kinase in SH-

SY5Y neuroblastoma cells (Lesort et al., 1999; Sayas et al., 1999).

We have identified a new, physiological substrate for GSK3β in developing neurons; the microtubule-associated protein 1B (MAP1B) (Lucas et al., 1998; Goold et al., 1999). Microtubule-associated protein 1B is a developmentally regulated phosphoprotein that is expressed at high levels in growing neurons and in regions of the adult nervous system that show neuronal plasticity or regenerate after injury (Müller et al., 1994). It is the first structural MAP to be expressed in developing neurons and its expression is particularly high in growing axons and their growth cones (Bush et al., 1996). Inhibition of expression with antisense oligonucleotides reversibly blocks neurite growth in PC12 cells (Brugg et al., 1993) and cerebellar macroneurons (DiTella et al., 1996). Four MAP1B-knockout mice have been produced (Edelmann et al., 1996; Takei et al., 1997; González-Billault et al., 2000; Meixner et al., 2000). The first three are not true knockouts as they express truncated forms of MAP1B, as predicted from analysis of the MAP1B gene and the nature of the targeting vector (González-Billault and Avila, 2000). The homozygotes of the fourth knockout do not express MAP1B and display various striking neural defects including absence of the corpus callosum (Meixner et al., 2000). Genetic analysis of Futsch, the *Drosophila* orthologue of MAP1B, has recently shown that Futsch is necessary for the extension of axons and dendrites and motoneuron synapse plasticity in fly embryos, probably through the regulation of microtubule dynamics (Hummel et al., 2000; Roos et al., 2000). These findings show that MAP1B plays an important role in neurite growth (Gordon-Weeks and Fischer, 2000). The phosphorylation of MAP1B is developmentally and spatially regulated and GSK3β-phosphorylated MAP1B is only expressed in growing axons (Gordon-Weeks and Fischer, 2000). Our recent work suggests that the function of GSK3β-phosphorylated MAP1B is to regulate microtubule dynamic instability in growing axons and growth cones (Goold et al., 1999). This is an important requirement for axon growth and growth cone turning during pathfinding (Tanaka and Kirschner, 1991; Williamson et al., 1996). Our evidence for this derives from cell transfection studies that show that GSK3β phosphorylated MAP1B maintains the unstable population of microtubules in cells at the expense of the stable microtubules (Goold et al., 1999). In further support of this role, the inhibition of neurite outgrowth and enlargement of growth cones seen when GSK3β is inhibited by lithium or Wnt proteins correlates with an increase in stable microtubules and a decrease in unstable microtubules (Goold et al., 1999; Hall et al., 2000). The enlarged growth cones are completely filled with stable microtubules, which probably contributes to their reduced growth rate.

Here we show that NGF, which differentiates PC12 cells into a neuron-like phenotype (Greene and Tischler, 1976), is a positive regulator of GSK3β phosphorylation of MAP1B in PC12 cells. We have found that the expression of the GSK3β-phosphorylated isoform of MAP1B (MAP1B-P) is induced in parallel with GSK3β activity, assayed using MAP1B as a substrate, in response to NGF. This increase lags behind process formation and the expression of MAP1B in these cells by about two days and coincides with a period when the majority of cells are extending existing neurites that show strong MAP1B-P and GSK3β expression. In addition, we have shown that the recognised post-translational modifications to GSK3β known to regulate kinase activity (i.e. phosphorylation of serine-9 and tyrosine-216) are not likely to modulate MAP1B phosphorylation in response to NGF. However, we demonstrate that a novel GSK3β isoform characterised by a decreased electrophoretic mobility on SDS-gels is induced by NGF and that its expression correlates with both neurite extension and MAP1B phosphorylation. Finally, we provide evidence indicating that a soluble factor present in differentiated PC12 cell extracts is capable of activating MAP1B phosphorylation by GSK3β.

MATERIALS AND METHODS

Cell culture

PC12 cells were maintained on collagen-coated flasks (Gibco) in Dulbecco's minimal essential medium (DMEM, Gibco) containing 10% horse serum and 5% foetal bovine serum (Gibco) supplemented with 2 mM glutamine, 100 I.U./ml penicillin and 100 I.U./ml streptomycin (complete medium). For differentiation, cells were plated at $2.5\times10^4/cm^2$ in complete medium onto collagen-coated 13 mm glass coverslips in 35 mm petri dishes for immunofluorescence microscopy or directly onto collagen-coated petri dishes for biochemical analysis. Cells analysed for transient NGF effects (0-3 hours) were incubated overnight in low serum medium (DMEM containing 1% horse serum supplemented with 2 mM glutamine, 100 I.U./ml penicillin, 100 I.U./ml streptomycin) prior to the addition of NGF (Promega – 7S) to 50 ng/ml. Alternatively, the complete medium was replaced directly with low serum medium containing NGF. This medium was replaced every other day during the course of cell differentiation. Cells were analysed daily after NGF addition and the proportion bearing one or more processes greater in length than one cell diameter was determined. Cells from cultures exposed to NGF for zero to five days were prepared for immunoblot analysis as described below. Cells from cultures exposed to NGF for zero, one, three or five days were imaged in a temperature-controlled environmental box attached to an Olympus IX70 inverted microscope using a 20× phase contrast objective and a Sony F-view 12 bit (1280×1024) CCD monochrome camera. The average neurite length in the field was determined using AnalySIS software. At least 100 cells were analysed for each condition from each of four independent experiments**.** In some experiments, PC12 cells were treated with NGF in the presence of 2.5 mM, 5 mM or 10 mM LiCl or 10 mM NaCl. For each experiment five randomly chosen cell fields were imaged after three days exposure to NGF. The proportion of cells bearing one or more processes greater in length than one cell diameter and the average neurite length in the field were determined using AnalySIS software. At least 100 cells were analysed for each condition from each of four independent experiments. After imaging the cells were harvested and prepared for immunoblot analysis.

Immunoblotting

Cells from cultures exposed to NGF from zero to five days were washed once with phosphate-buffered saline (PBS) and scraped into hot sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Protein samples were subjected to acrylamide gradient (4-15%) gel electrophoresis using Laemmli buffers and western blotted onto nitrocellulose membrane according to Towbin et al. (Laemmli, 1970; Towbin et al., 1979). Immunoblotting was done as described previously (Goold et al., 1999) with the following antibodies: AA6, diluted 1:500 (Sigma); SMI-31, diluted 1:500; anti-GSK3β, diluted 1:2000 (Affiniti); anti-phospho-GSK3 (specific for tyrosine-phosphorylated GSK3α and GSK3β) diluted 1:250 (Upstate) and anti-GSK3β serine-9 phosphospecific antibody diluted 1:1000 (Biosource). To ensure equal protein loading,

protein samples were assayed by densitometry of Coomassie blue-stained gels and adjusted accordingly for blotting. Immunoblots were quantified as described previously using Phoretix 1D Plus software (Goold et al., 1999). The relative affinity of mAb SMI-31 and AA6 for their respective MAP1B epitopes was tested by immunoblotting a dilution series (1-20 μg protein) of a PC12 cell lysate from a culture exposed to NGF for five days in vitro (DIV). Using the dilutions described above mAb SMI-31 was found to detect MAP1B at least as effectively as mAb AA6 (not shown).

Immunofluorescence

Cells were washed once with PBS at 37°C and then fixed either in methanol at –20°C for 5 minutes or in 3% formaldehyde and 0.2% glutaraldehyde in PBS for 10 minutes at 37°C. Immunofluorescence staining was carried out as described previously (Goold et al., 1999) using monoclonal antibodies against MAP1B phosphorylated by GSK3β (SMI-31, Affiniti, diluted 1:100); GSK3β (Affiniti, diluted 1:20); tyrosinated tubulin (Sera Lab, YL12, diluted 1:10) and a polyclonal anti-MAP1B antibody (Johnstone et al., 1997b) diluted 1:100. Cultures were viewed using a Leica TCS confocal microscope equipped with Argon, Krypton and HeNe lasers. Cells were imaged with a 63×/1.32 PLANAPO oil-immersion objective and recorded at 1024×1024 pixels per image.

Measurement of GSK3β activity

PC12 cells were harvested in ice-cold lysis buffer (40 mM HEPES, pH 7.4, 5 mM EGTA, 2 mM $MgCl_2$, 0.1% Triton X-100, 2 mM DTT, 50 mM NaF, 1 mM okadaic acid and protease inhibitors: 1 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Extracts were centrifuged at 100,000 $\boldsymbol{g}_{max}$ for 30 minutes at 4°C and the supernatant (S1) was collected, frozen in liquid nitrogen and stored at –80°C prior to use. Equal amounts of protein (50 μg) from cells exposed to NGF for different times were incubated with a recombinant glutathione-S-transferase (GST) fusion protein, 1B750, comprising amino acids 1109 to 1360 of the mouse MAP1B sequence bound to GSH-agarose beads at 37°C for 16 hours. Monoclonal Glutahione SM antibody SMI-31 was used to detect phosphorylation of 1B750 as described previously (Johnstone et al., 1997a; Lucas et al., 1998). LiCl (20 mM) was included in some assays. Samples of the input material from each kinase assay were prepared for immunoblot analysis to determine their relative GSK3β content using specific antibodies. The linearity of the kinase assay was tested with respect to kinase input (using a range of S1 concentrations from cells exposed to NGF for 5 DIV) and time. A linear response was observed between 12.5 and 100 μg input of S1 and between 0 and 24 hours (not shown).

Immunodepletion and kinase activation assays

Equal amounts of protein from cells exposed to NGF for zero (0 DIV) or five days (5 DIV) were depleted of GSK3β using specific antibodies (Lucas et al., 1998). Anti-GSK3β antibodies (5 μg) were added to S1 fractions (50 μg) and incubated with rotation at 4°C for 1 hour. Protein G-agarose (25 μg, Sigma) was added and the incubation was continued for a further hour. The protein G-agarose and bound material were removed by centrifugation at 10,000 $\boldsymbol{g}_{max}$ for 2 minutes. The supernatants (IPS) were collected and used immediately in kinase assays alone or in conjunction with undepleted (S1) fractions. In some experiments, 1B750 bound to agarose beads was incubated at 37°C for 16 hours with S1 (5 DIV) in the presence of 20 mM LiCl or with IPS (5 DIV) and washed extensively in kinase buffer (at least 20 times the bead volume). The washed 1B750 was then put into the kinase assay for 16 hours at 37°C as described above.

Phosphatase treatment of GSK3β

Soluble extracts (S1-50 μg) from PC12 cells differentiated for 5 DIV with NGF were brought to 1% SDS final concentration by addition from a 10% stock solution and incubated at 100°C for five minutes. The extracts were diluted 1:10 with TBS and GSK3β was immunoprecipitated as described above. The immunoprecipitate was washed twice with TBS and once with 100 mM Mes 2-(N-Morpholino)ethanesulfonic acid pH 6.0 containing protease inhibitors. Acid phosphatase (type III from potato; Sigma) was added to the equivalent of 6.2 units/ml and the extracts were incubated at 37°C for 24 hours and then prepared for SDS-PAGE. Control experiments included no phosphatase addition and the addition of boiled phosphatase. The samples were separated on 7-15% SDS gels and immunoblotted with anti-GSK3β antibodies as described previously.

RESULTS

MAP1B phosphorylation by GSK3β occurs relatively late during PC12 cell differentiation

We have recently identified MAP1B as a substrate for GSK3β and shown that the phosphorylated isoform generated, MAP1B-P, plays an important role in axonogenesis in cultured cerebellar granule cells (Lucas et al., 1998). To extend these findings we have studied MAP1B-P expression during PC12 cell differentiation. In agreement with previous studies (Aletta et al., 1988; Brugg and Matus, 1988; Harper Keating and Asai, 1994) we found low levels of MAP1B expression in unstimulated cells and that expression is upregulated by NGF treatment (Fig. 1A,D). Expression levels peak after three to four days of NGF treatment. Interestingly, MAP1B expression was closely related to cellular differentiation, defined as the appearance of processes equal or greater in length than one cell diameter (Fig. 1B,D). By contrast, MAP1B-P was not detectable in unstimulated cells; its level increased slowly over the first three days of NGF treatment and was still increasing after five days of NGF treatment (Fig. 1A,E). In fact, MAP1B phosphorylation by GSK3β correlated closely with neurite length (Fig. 1C,E). Note that under the conditions used, mAb SMI-31 was found to detect its epitope on MAP1B at least as effectively as mAb AA6 detected total MAP1B (not shown; see Materials and Methods), indicating that the failure to detect phosphorylated MAP1B prior to NGF addition cannot be explained by trivial differences in antibody affinities.

Immunofluorescence analysis of PC12 cell cultures exposed to NGF for five DIV confirmed these findings (Fig. 2A). MAP1B-P was found to be concentrated in the growth cones and neurites of these cells, particularly in the longer processes (Fig. 2A, arrowheads). Indeed, MAP1B-P is undetectable in the shortest processes (Fig. 2A, arrows). By contrast, MAP1B is expressed in the cell soma and in all neurites (Fig. 2A). These data indicate that MAP1B-P expression is not required for initial process formation, but may be necessary for neurite elongation. Note that the mAb SMI-31 also recognises a nuclear epitope unrelated to MAP1B (Goold et al., 1999; Lucas et al., 1998). Interestingly, the levels of GSK3β change relatively little during differentiation, in contrast to the expression of MAP1B-P (Fig. 1A,F). GSK3β is strongly expressed in the soma of PC12 cells and can be detected at a lower level in all of the neurites and growth cones at all stages of differentiation (Fig. 2B).

MAP1B-P is associated with neurite extension

The expression pattern of MAP1B-P suggests that it may play a role in neurite extension but that it is not necessary for

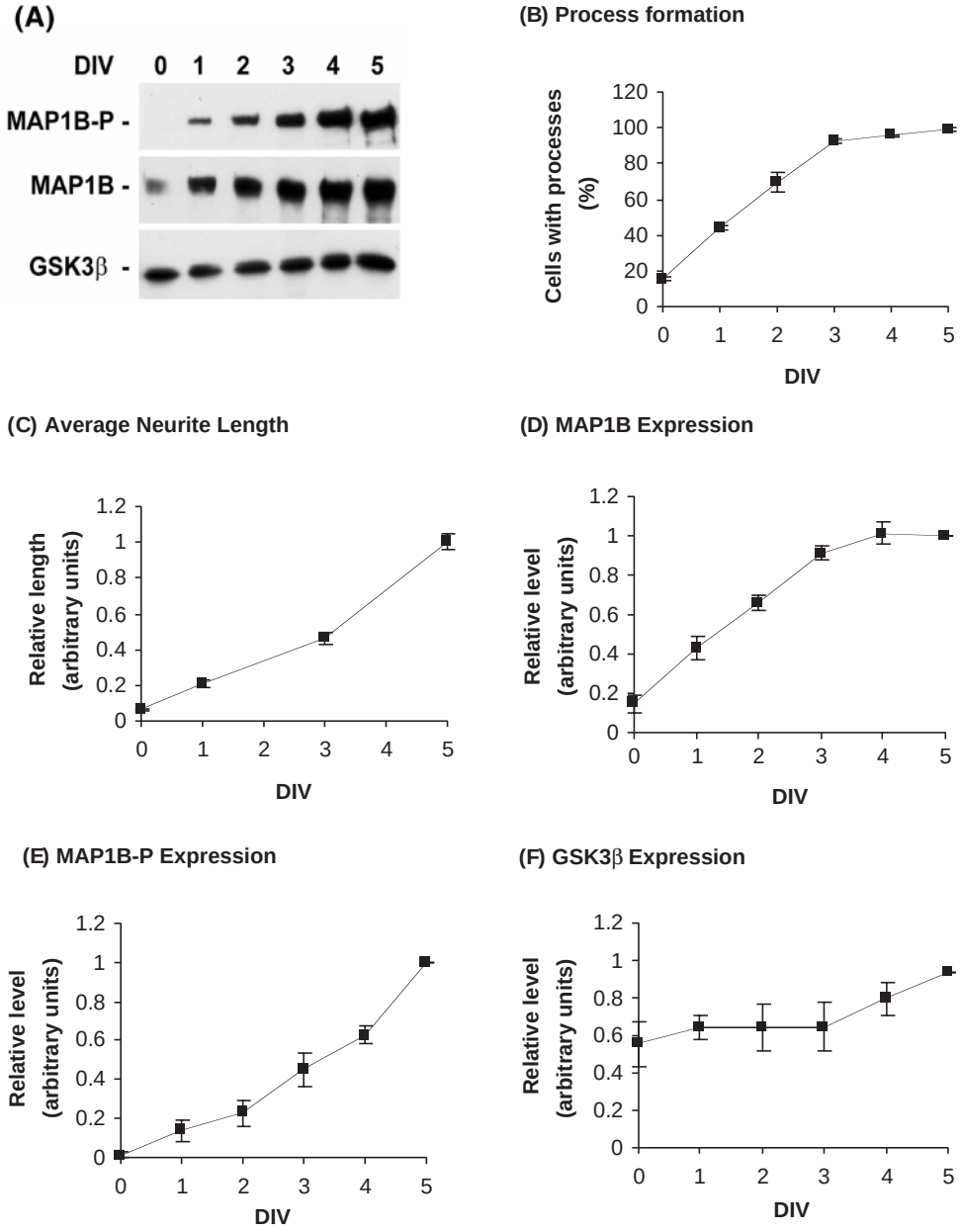

Fig. 1. MAP1B, MAP1B-P and GSK3β levels during PC12 cell differentiation. (A) Immunoblot analysis of whole-cell extracts from PC12 cells treated with NGF from 0 to 5 days, showing the levels of MAP1B, MAP1B-P and GSK3β during cell differentiation. Note MAP1B is not phosphorylated by GSK3β prior to NGF treatment. (B) Time course of cell differentiation for PC12 cells treated with NGF from 0 to 5 days (defined as the percentage of cells bearing processes equal or greater in length than one cell diameter). (C) Average PC12 neurite length measured after zero, one, three and five DIV following NGF addition (B and C are means±s.e.m. from four independent experiments). (D-F) Quantitative immunoblot analysis of the levels of MAP1B (D), MAP1B-P (E) and GSK3β (F) from PC12 cells treated with NGF from 0 to 5 days. Results are expressed as a proportion of the protein levels after five DIV and are means±s.e.m. from four independent experiments. MAP1B expression is closely related to cellular differentiation, whereas MAP1B-P expression is delayed by one to two days. GSK3β levels change relatively little during differentiation, in contrast to the levels of MAP1B phosphorylation (i.e. MAP1B-P expression).

neurite initiation or sprouting. It was therefore interesting to compare the effects of Li^+ treatment on these functions and MAP1B phosphorylation. PC12 cells were grown in the presence of various LiCl concentrations and the effects on differentiation (the proportion of cells bearing one or more processes greater in length than one cell diameter) and on neurite extension (the average neurite length) were compared with the levels of MAP1B phosphorylation. Cells were examined after three days of NGF treatment as most cells have differentiated by this time under control conditions (Fig. 1; Fig. 2). Significantly, neurite extension was far more sensitive to LiCl treatment than neurite initiation. A clear and dose-dependent inhibition of neurite length was observed at 2.5 mM and 5 mM LiCl when compared to control or NaCl-treated cultures (~50% and 60%, respectively). By contrast, these concentrations of LiCl had no detectable effect on the numbers of neurites formed (Fig. 3A,B). At 10 mM LiCl there was a reduction in the proportion of neurite-bearing cells, possibly indicating another site of action for LiCl in process formation or a toxic effect of this relatively high LiCl concentration (although overall cell numbers were not significantly reduced in the fields examined). The levels of MAP1B and MAP1B-P in these cultures were determined by immunoblot analysis (Fig. 3D). Immunoblots were quantified and protein levels were normalised to controls. The proportion of phosphorylated MAP1B was then calculated by dividing the normalised levels of MAP1B-P by those of MAP1B (Fig. 3C). The proportion of phosphorylated MAP1B was reduced in these cultures, indicating that GSK3β is inhibited by these Li^+ concentrations (Fig. 3C). No correlation was found between MAP1B phosphorylation and the number of neurites formed. However, the degree of inhibition of MAP1B phosphorylation correlated closely with the extent of the reduction in average neurite length, suggesting that MAP1B-P expression may be

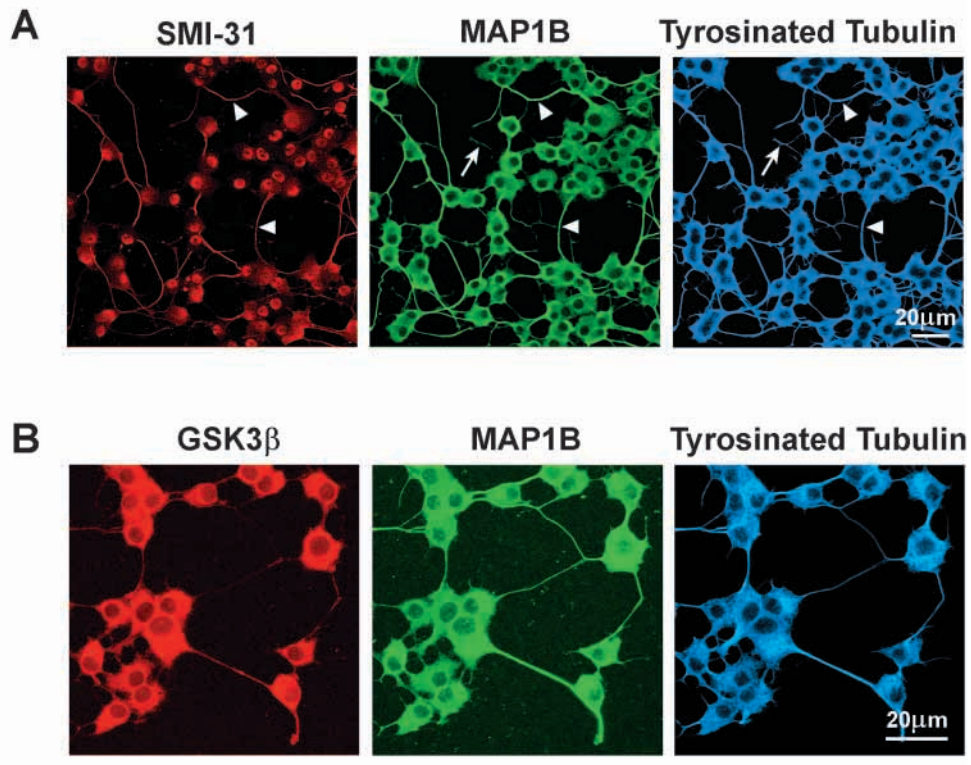

Fig. 2. MAP1B-P expression is concentrated in extending neurites. (A) PC12 cells exposed to NGF for five days were fixed and stained with mAb SMI-31, which recognises MAP1B-P and a nuclear epitope unrelated to MAP1B (red), a MAP1B pAb (green) and YL1/2, a mAb specific for tyrosinated tubulin (blue), which stains all neurites and growth cones. MAP1B-P expression is concentrated in the longer neurites of the PC12 cells (arrowheads) and is undetectable in many shorter processes (arrows). By contrast, MAP1B is distributed throughout the cell, including the cell soma. (B) PC12 cells exposed to NGF for three days were fixed and stained with anti-GSK3β antibodies (red), a MAP1B pAb (green) and YL1/2, a mAb specific for tyrosinated tubulin (blue). GSK3β is expressed throughout the cell soma, neurites and growth cones.

necessary for neurite elongation, but that it is not required for neurite initiation.

MAP1B phosphorylation by GSK3β is induced during PC12 cell differentiation

The GSK3β activity towards MAP1B in PC12 cell extracts exposed to NGF from zero to five days was determined using a recombinant fragment of MAP1B (1B750) as a substrate (Johnstone et al., 1997a; Lucas et al., 1998). This assay utilises the phosphospecific mAb SMI-31 to recognise MAP1B at an epitope generated by GSK3β phosphorylation (Lucas et al., 1998), allowing the activity of GSK3β from a heterogeneous cell extract to be assayed without further purification. However, the presence of other proteins in the assay may affect the phosphorylation of the substrate, meaning that the total activity of the extract is measured. This could be different to the activity of GSK3β purified from these extracts.

Activity was virtually undetectable in unstimulated cells and increased gradually to a maximum level five days after NGF addition (Fig. 4A,B). Inhibition of this activity by LiCl confirms that GSK3 is the kinase phosphorylating MAP1B in these cell extracts (Fig. 4A). This increase in activity is not related to an increase in GSK3β expression, which shows a modest increase over the same time period (Fig. 1; Fig. 4); this is demonstrated by dividing the level of phosphorylated 1B750 by the level of kinase, each determined by immunoblotting and normalised to five DIV, giving a measure of the activity of GSK3β relative to its abundance during differentiation (Fig. 4B). Significantly, GSK3β activity measured in vitro reflects closely the cellular MAP1B-P levels measured by immunoblotting and immunofluorescence (Fig. 1; Fig. 2). Therefore, the lack of endogenous MAP1B-P in PC12 cells prior to NGF stimulation cannot be explained by the low levels of kinase substrate (MAP1B): MAP1B-P is generated only after MAP1B expression has been upregulated in the PC12 cells (Fig. 1), and cellular extracts from unstimulated cells cannot phosphorylate recombinant MAP1B in vitro (Fig. 4).

Activation of GSK3β

The experiments reported here demonstrate a biochemical difference between PC12 cells exposed to NGF and unstimulated cells in their ability to phosphorylate MAP1B in living cells and in vitro. PC12 cell extracts showing the greatest difference in kinase activity (i.e. S1 from cells exposed to NGF for 0 and 5 DIV) were used to examine the reasons for this difference. Equal quantities of protein (50 μg) from these extracts were used in kinase assays either complete (S1, 0 and 5 DIV) or immunodepleted using anti-GSK3β antibodies (IPS, 0 and 5 DIV). GSK3β levels were determined by immunoblotting samples of the input fractions. Kinase activity was determined as described above. Most, if not all, kinase activity in differentiated PC12 cell extracts is associated with GSK3β – immunodepletion with specific antibodies reduces GSK3β levels and kinase activity by approximately 90% (Fig. 5A: compare S1 (5 DIV) + and IPS (5 DIV) +). As expected, no activity is detectable in complete or immunodepleted undifferentiated extracts (Fig. 5A: S1 (0 DIV) + and IPS (0 DIV) +). However, adding immunodepleted differentiated cell extracts, essentially devoid of GSK3β and kinase activity, to undifferentiated extracts containing otherwise inactive GSK3β, stimulates MAP1B phosphorylation almost to the levels present in complete differentiated cell extracts (Fig. 5 A: IPS (5 DIV) +/S1 (0 DIV) +). This increase in kinase activity is not related purely to the increase in protein concentration brought about by adding depleted extracts because immunodepleted undifferentiated cell extracts can not activate phosphorylation in the same way (Fig. 5A: IPS (0 DIV) +/S1 (0 DIV) +).

These data indicate that a soluble factor present in

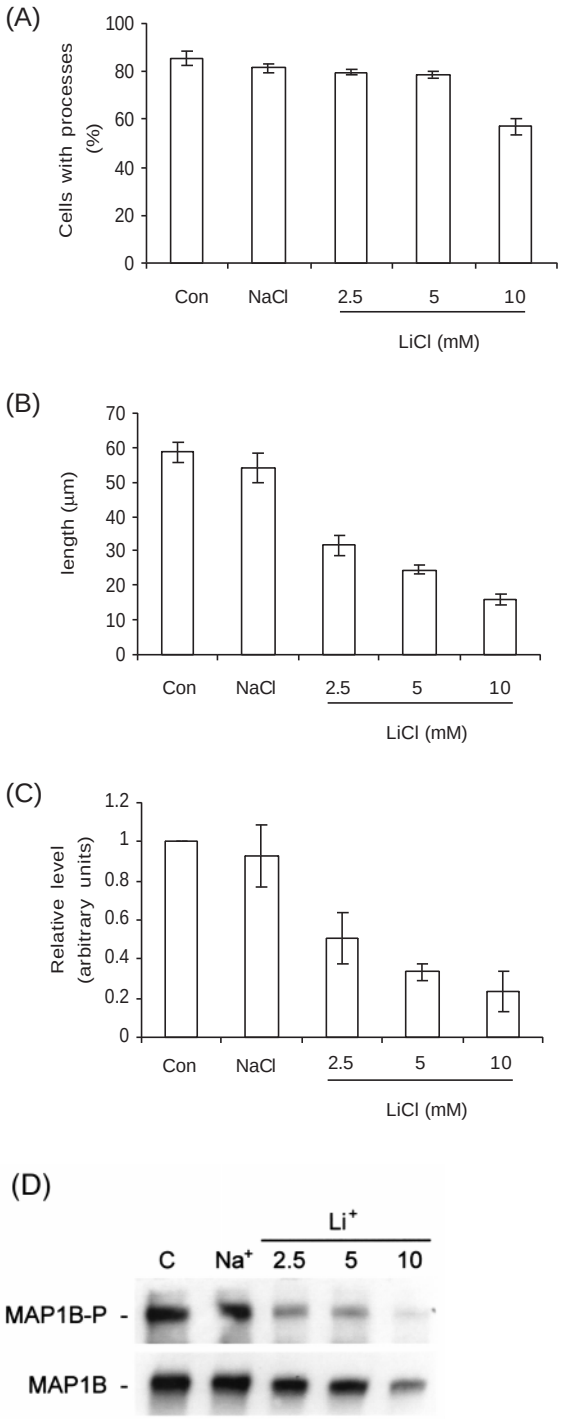

Fig. 3. Lithium inhibits neurite extension more effectively than differentiation. PC12 cells were treated with NGF in the presence of LiCl (2.5-10 mM) or NaCl (10 mM) or in control conditions. After three DIV, cultures were examined and the proportion of cells bearing one or more processes greater in length than one cell diameter (A) and the average neurite length in the field (B) were determined. At least 100 cells were analysed for each condition from each of four independent experiments. The levels of MAP1B and MAP1B-P in these cultures were determined by immunoblot analysis (D). Immunoblots were quantified and protein levels were normalised to controls. The levels of MAP1B-P are expressed as a proportion of MAP1B levels (C). Results are means±s.e.m. from four independent experiments. Note the close correlation between the Li^+-induced inhibition of MAP1B phosphorylation and neurite extension.

differentiated PC12 cell extracts – other than GSK3β itself – can activate the phosphorylation of MAP1B by GSK3β. Phosphorylation could be stimulated by activation of the substrate (priming) or by activation of GSK3β. To distinguish between these possibilities the substrate (1B750) was incubated with immunodepleted differentiated cell extracts or with complete differentiated cell extracts in the presence of Li^+ (20 mM) – conditions that inhibit phosphorylation of MAP1B by GSK3β (Fig. 5B: IPS (5 DIV) and S1 (5 DIV)/Li^+, respectively). Following an extensive wash, the 1B750 was incubated with kinase buffer alone or supplemented with S1 (0 or 5 DIV). Despite exposure of the substrate to potential priming kinases present in differentiated extracts, undifferentiated extracts can not phosphorylate the 1B750 (Fig. 5B: S1 (0 DIV) +). As expected, addition of differentiated extracts results in robust 1B750 phosphorylation (Fig. 5B: S1 (5 DIV) +). These observations suggest changes to the substrate cannot explain the induction of MAP1B phosphorylation by GSK3β following NGF addition.

Increased phosphorylation of MAP1B could be mediated by an increase in the intrinsic kinase activity of GSK3β. One prediction from this is that immunoprecipitated GSK3β from differentiated PC12 cell extracts should phosphorylate 1B750. Although GSK3β immunoprecipitated with this antibody has been used to phosphorylate peptide substrates previously (Pap and Cooper, 1998) we have been unable to phosphorylate 1B750 with immunoprecipitated GSK3β from differentiated cell extracts or from developing rat brain lysates (which were previously shown to contain high kinase activity) (Lucas et al., 1998). The nature of the kinase assay used here – the substrate is phosphorylated attached to agarose beads (Johnstone et al., 1997a) – may be unsuitable for assaying the activity of immunoprecipitated GSK3β, also attached to protein-G agarose beads.

Post-translational modification of GSK3β

Previous studies have shown that phosphorylation of GSK3β regulates kinase activity. Phosphorylation of an N-terminal serine residue (serine-9) inhibits activity, and phosphorylation at tyrosine-216 in the catalytic domain (a conserved domain in GSK3α and GSK3β) may be necessary for kinase activity (Cross et al., 1995; Hughes et al., 1993). Serine phosphorylation can be stimulated by a variety of growth factors in different cell types, and tyrosine phosphorylation of GSK3β in response to insulin in SH-SY5Y neuroblastoma cells has been reported (Lesort et al., 1999). We used antibodies specific for the phosphorylated isoforms of GSK3β to determine the changes in phosphorylation that occur following NGF addition to PC12 cells over both short- (0-3 hours) and longer-term time courses (0-5 DIV) to determine if these changes may be responsible for the observed changes in kinase activity (Fig. 6). Short-term effects (0-3 hours) were analysed by treating quiescent PC12 cells with NGF and collecting cell lysates for immunoblot analysis. This procedure demonstrated that the levels of tyrosine-phosphorylated GSK3β undergo a transient reduction following NGF treatment. Reduced phosphorylation is detectable by 15 minutes and has returned

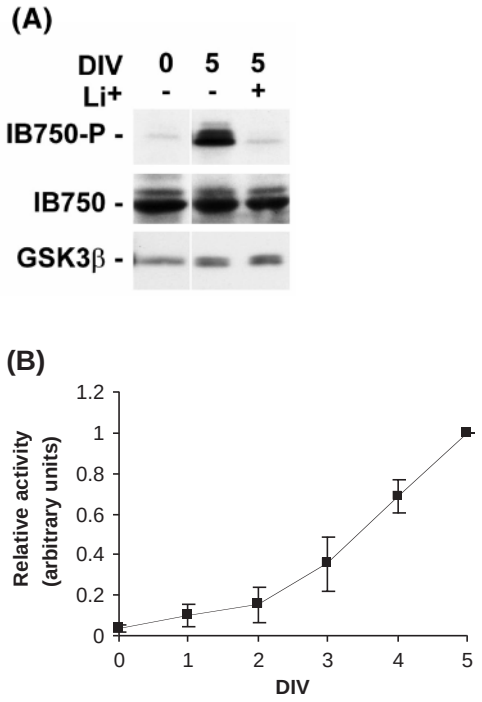

Fig. 4. GSK3β activity increases during PC12 cell differentiation. (A) The GSK3β activity in extracts from untreated PC12 cells and from cells exposed to NGF for five days was determined using a recombinant fragment of MAP1B (1B750) as a substrate. Phosphorylation was assayed by immunoblotting with mAb SMI-31 (1B750-P); recombinant protein was detected with anti-GST serum (1B750). Activity was virtually undetectable in unstimulated cells (DIV 0), but was readily detected five days after NGF addition (DIV 5). Inhibition of this activity by LiCl confirms that GSK3 is the kinase phosphorylating MAP1B in these cell extracts (DIV 5, + Li^+). This increase in activity is not related to an increase in GSK3β expression, which shows only a modest increase over the same period (GSK3β). (B) GSK3β activity towards recombinant MAP1B (1B750) in extracts from PC12 cells exposed to NGF from zero to five days was assayed as above. The level of phosphorylated 1B750 was divided by the level of GSK3β, each determined by immunoblotting and normalised to five DIV, to give a measure of the activity of GSK3β relative to its abundance during differentiation. Results are means±s.e.m. from four independent experiments. The increase in kinase activity induced by NGF reflects closely the level of endogenous MAP1B-P (Fig. 1).

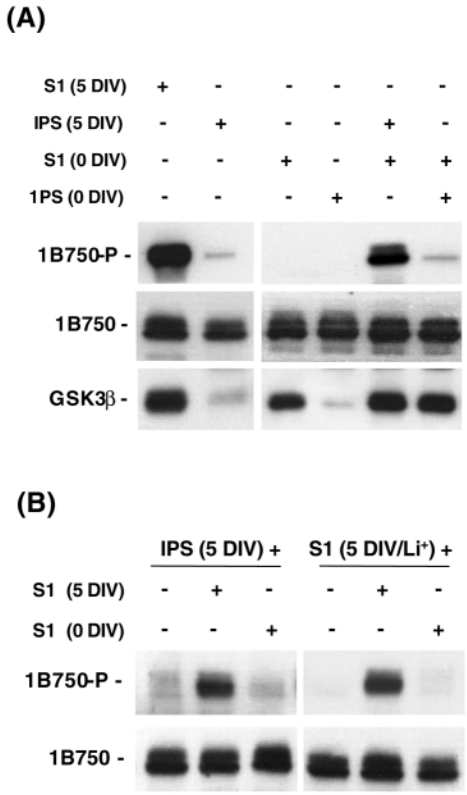

Fig. 5. Activation of GSK3β by a soluble factor from differentiated PC12 cell extracts. (A) Equal amounts of protein from cells exposed to NGF for zero or five days were depleted of GSK3β using specific antibodies (IPS, 0 DIV or 5 DIV). The extracts were collected and used in kinase assays alone or in conjunction with undepleted fractions from cells exposed to NGF for zero or five days (S1, 0 or 5 DIV). The in vitro kinase activity towards recombinant MAP1B present in the resultant samples was determined by immunoblotting with mAb SMI-31 (1B750-P); recombinant protein was detected with anti-GST serum (1B750). GSK3β levels were determined by immunoblotting samples of the input fractions. Note the absence of kinase activity in differentiated cell extracts immunodepleted of GSK3β (IPS (5 DIV)) and undifferentiated cell extracts (S1 (0 DIV)). Undifferentiated cell extracts mixed with immunodepleted differentiated cell extracts show high activity, but no significant increase in GSK3β level. By contrast, immunodepleted undifferentiated cell extracts (IPS (0 DIV)) do not activate phosphorylation of MAP1B. (B) Prior phosphorylation of substrate (priming) does not activate MAP1B phosphorylation by GSK3β. 1B750 was incubated with differentiated cell extracts immunodepleted of GSK3β (IPS (5 DIV)) or with complete differentiated cell extracts in the presence of 20 mM LiCl for 16 hours at 37°C (S1 (5 DIV)/Li^+) – conditions that inhibit phosphorylation of MAP1B by GSK3β – and washed extensively in kinase buffer (at least 20 times the bead volume). The washed 1B750 was incubated with control buffer or with differentiated cell extracts (S1 (5 DIV)) or undifferentiated cell extracts (S1 (0 DIV)) for a further 16 hours at 37°C and kinase activity was then determined as in (A). Note that undifferentiated cell extracts show no kinase activity.

to unstimulated levels by three hours (Fig. 6A). The changes in tyrosine phosphorylation were reflected by an increase in the electrophoretic mobility of a portion of the GSK3β, detected using an antibody independent of phosphorylation (Fig. 6A, GSK3β). Interestingly, the equivalent tyrosine phosphorylation site on GSK3α shows a similar pattern of dephosphorylation following NGF addition. Serine phosphorylation of GSK3β is relatively low prior to NGF addition but shows a rapid increase within five minutes of stimulation. Levels of phosphorylation decline slowly up to three hours following NGF addition, but do not return to basal levels.

Longer-term (0-5 DIV) changes in GSK3β post-translational modifications were also examined by immunoblotting. Note that these cultures had not been incubated in low serum medium prior to NGF addition. As shown earlier, overall levels of GSK3β show a shallow rise following NGF addition (Fig. 1; Fig. 6B). This expression pattern is similar to that shown by tyrosine-phosphorylated GSK3β. Indeed, the relative level of tyrosine-phosphorylated GSK3β changes little when compared to total kinase over the five days of NGF treatment (Fig. 6D). NGF induces the expression of a band that migrates

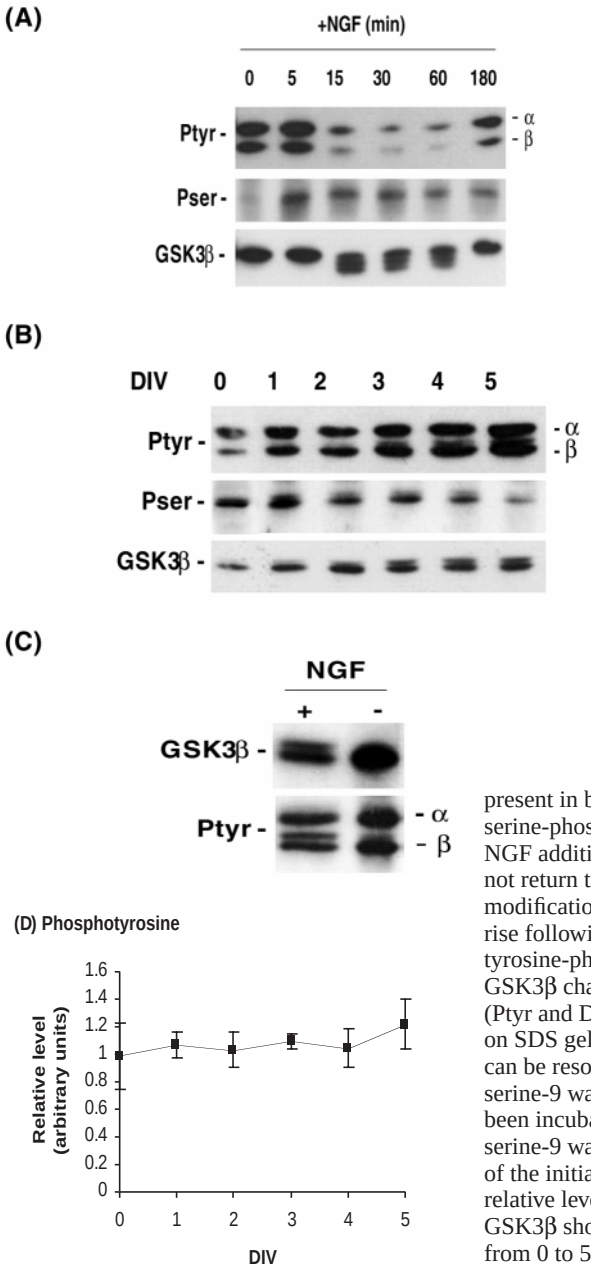

Fig. 6. Post-translational modifications of GSK3β during PC12 cell differentiation. Whole-cell extracts from PC12 cell cultures treated with NGF for the indicated times were immunoblotted with phosphospecific antibodies. (A) The levels of tyrosine-phosphorylated GSK3β show a transient reduction following NGF treatment (Ptyr); changes in tyrosine phosphorylation correlate with the appearance of faster migrating bands reactive with a GSK3β specific antibody (GSK3β). Reduced phosphorylation is detectable by 15 minutes and has returned to unstimulated levels by three hours (Ptyr, lower band: note, the antibody used detects a conserved epitope present in both GSK3α and β, hence the visualisation of a doublet on this blot). The levels of serine-phosphorylated GSK3β (Pser) show a rapid increase within five minutes following NGF addition. Subsequently, phosphorylation gradually declines up to three hours, but does not return to baseline levels. (B) Longer-term changes in GSK3β post-translational modifications were examined by immunoblotting. Overall levels of GSK3β show a shallow rise following NGF addition (GSK3β). This expression pattern is similar to that shown by tyrosine-phosphorylated GSK3β. Indeed, the relative level of tyrosine-phosphorylated GSK3β changes little when compared to total kinase over the five days of NGF treatment (Ptyr and D). NGF induces the expression of a band that migrates significantly more slowly on SDS gels than the GSK3β present prior to NGF addition (GSK3β and F). These two bands can be resolved more clearly on a 10% acrylamide gel (C). GSK3β phosphorylation on serine-9 was readily detected in cells prior to NGF addition; note that these cultures had not been incubated in low serum medium prior to NGF addition. GSK3β phosphorylation on serine-9 was maintained for two to three days after which it decreased to approximately half of the initial levels after five DIV (Pser and E). (D-F) quantitative immunoblot analysis of the relative levels of tyrosine- (D) or serine- (E) phosphorylated GSK3β and the proportion of GSK3β showing reduced electrophoretic mobility (F) from PC12 cells treated with NGF from 0 to 5 days. Results are means±s.e.m. from three independent experiments.

significantly slower on SDS gels than the GSK3β present prior to NGF addition (Fig. 6B). These two bands can be resolved more clearly on a 10% acrylamide gel (Fig. 6C). This isoform of GSK3β has been reported previously in rat brain where its expression is developmentally regulated (Takahashi et al., 1994; Leroy and Brion, 1999). On the basis of phosphatase treatment Takahashi et al. (Takahashi et al., 1994) identified this slower migrating species as a phosphorylated form of GSK3β, but it was not characterised further. Fig. 6B,C shows this 'upper band' and the original lower band recognised by GSK3β antibodies are also visualised by anti-phosphoserine-9 and phosphotyrosine-216 antibodies, indicating that phosphorylation at these sites does not generate this isoform. Phosphatase treatment of denatured GSK3β immunoprecipitated from differentiated PC12 cell extracts resulted in an apparent decrease in gel mobility of the doublet from a relative molecular mass of approximately 42.5/40.5 kDa to 38/36 kDa as determined by immunoblotting with anti-GSK3β antibodies (not shown). Immunoblotting with anti-GSK3β phosphoserine-9 antibodies indicated that this phosphate was completely removed. It should be noted that a GSK3β doublet was still detectable following the extensive phosphatase treatment employed (i.e. the gel mobility of the upper band could not be transformed into that of the lower band). Unlike the transient changes to tyrosine phosphorylation that occur immediately after NGF addition (Fig. 6A) this modification seems to be specific for GSK3β in that no slower migrating band above GSK3α was visualised using anti-phosphotyrosine GSK3 antibodies (Fig. 6C). The proportion of the upper band also increases during PC12 cell differentiation, rising to about 40% of the total after five DIV (Fig. 6F). In fact, the absence of the upper band from undifferentiated cells and

its gradual build up following NGF addition are similar to the time course of MAP1B-P expression and GSK3β kinase activity measured in vitro (Fig. 1D; Fig. 4B). This time course correlates with the period of rapid neurite growth (Fig. 1B).

GSK3β phosphorylation on serine-9 was readily detected in cells prior to NGF addition. This difference in phosphorylation from the cultures treated with NGF for short time periods was probably because these cells were not incubated in low serum media prior to stimulation with NGF. GSK3β phosphorylation was maintained for two to three days after which it decreased to approximately half of the initial levels after five DIV (Fig. 6E).

DISCUSSION

In this study we have investigated the role played by GSK3β-catalysed phosphorylation of MAP1B during PC12 cell differentiation. GSK3β activity toward MAP1B increases following NGF treatment of PC12 cells. Increased kinase activity assayed in vitro reflected increases in the level of endogenous MAP1B-P and expression coincided with neurite extension rather than initial process formation. Consistent with this, PC12 cell neurite initiation and neurite extension differ in sensitivity to Li^+ such that extension is inhibited by more than 60% at concentrations that have no effect on initiation. Significantly, there is a strong correlation between the reduction in neurite length and the inhibition in MAP1B phosphorylation by GSK3β. These data indicate MAP1B-P may play an important role in neurite extension. We have also provided evidence indicating that a soluble factor present in differentiated PC12 cell extracts is capable of activating MAP1B phosphorylation by GSK3β. Finally, we have shown that the increases in GSK3β activity were not related to overall changes in GSK3β phosphorylation known to regulate kinase activity. However, we demonstrate that a novel GSK3β isoform characterised by a decreased electrophoretic mobility on SDS-gels is induced by NGF and that its expression correlates with both neurite extension and MAP1B phosphorylation.

MAP1B is phosphorylated by GSK3β in extending neurites

In a previous report we have provided evidence indicating that MAP1B is a substrate for GSK3β. The kinase assays performed indicate that most, if not all, of the kinase activity toward MAP1B in neonatal rat brain extracts and PC12 cell lysates is catalysed by GSK3β, at least at the site recognised by mAb SMI-31 (Lucas et al., 1998) (Fig. 5A). This is significant as it allows GSK3β activity toward MAP1B at this site to be assayed without immunoprecipitation and also provides a means of determining the level of endogenous GSK3β-phosphorylated MAP1B. It is also consistent with our previous findings indicating that MAP1B phosphorylation can be regulated through Wnt signalling (Lucas et al., 1998). In this report we have used these characteristics of the SMI-31 epitope on MAP1B to examine the phosphorylation of MAP1B during PC12 cell differentiation.

In agreement with previously published data, we have found that MAP1B was present in unstimulated PC12 cells and that its expression was positively regulated by NGF treatment (Aletta et al., 1988; Brugg and Matus, 1988). Expression of MAP1B correlated closely with the proportion of PC12 cells bearing processes of at least one cell diameter in length, saturating after four days (Fig. 1). By contrast, the expression of MAP1B phosphorylated by GSK3β at the site recognised by mAb SMI-31 (MAP1B-P) followed a different time course; prior to NGF treatment MAP1B is not phosphorylated by GSK3β. Phosphorylation levels rise slowly over the first two days of NGF treatment. MAP1B phosphorylation then increases more rapidly up to the maximum levels detected after five DIV (Fig. 1). It should be noted that this increase in endogenous MAP1B phosphorylated by GSK3β is mirrored by an increase in kinase activity measured in vitro (Fig. 4). In these assays an equal quantity of substrate (1B750) is exposed to the same quantity of PC12 cell extracts from cultures treated with NGF from zero to five DIV. Extracts from cultures not treated with NGF are incapable of phosphorylating 1B750 despite containing approximately equal levels of GSK3β (Fig. 4). Therefore the failure to detect endogenous MAP1B-P in cultures not treated with NGF is not likely to reflect a lack in sensitivity of the phospho-dependent antibody (SMI-31) relative to the antibody used to detect total MAP1B levels (AA6) – a possibility given that the levels of MAP1B rise sharply following NGF addition. Immunoblots of a dilution series of a PC12 cell lysate from a culture exposed to NGF for five DIV established that mAb SMI-31 detects MAP1B slightly more effectively than mAb AA6 (not shown). These data indicate that the increased endogenous expression of MAP1B-P and increased in vitro phosphorylation of 1B750 following NGF exposure reflect a real and specific increase in GSK3β phosphorylation of MAP1B. Interestingly, a comparison of MAP1B-P levels with the average neurite length shows that MAP1B-P is expressed during periods of rapid neurite elongation (Fig. 1). High expression in the longer neurites and growth cones of PC12 cells supports the idea that MAP1B-P plays an important role in neurite elongation (Fig. 2).

MAP1B phosphorylation by GSK3β is associated with neurite extension

The differential sensitivity of process formation and neurite length to LiCl treatment also suggests that MAP1B-P is necessary for neurite elongation (Fig. 3). LiCl is known to inhibit GSK3 in the low millimolar concentration range (Klein and Melton, 1996; Stambolic et al., 1996) and this is reflected by the dose-dependent decrease in MAP1B-P levels observed after PC12 cells have been treated for three DIV with LiCl concentrations ranging from 2.5 to 10 mM (Fig. 3C). Concentrations of LiCl up to 5 mM had no detectable effect on differentiation (defined as the proportion of cells with a process greater or equal in length to one cell diameter). By contrast, a dramatic and dose-dependent inhibition on average neurite length is observed at 2.5 and 5 mM LiCl when compared to control cultures or cultures exposed to 10 mM NaCl. Significantly, lithium caused a dose-dependent inhibition of MAP1B phosphorylation, which correlated exactly with the dose-dependency of neurite growth inhibition (Fig. 3). Thus there is a precise correlation between GSK3β phosphorylation of MAP1B and neurite elongation.

This may be of functional significance as recent evidence shows that MAP1B-P maintains a population of unstable microtubules when it is expressed in fibroblast cells cotransfected with MAP1B and GSK3β (Goold et al., 1999),

and it is thought that unstable microtubules are required for axon outgrowth and pathfinding (Williamson et al., 1996; Bamburg et al., 1986; Tanaka and Kirschner, 1991). The physiological relevance of these observations has been demonstrated by inhibiting GSK3β (and hence MAP1B phosphorylation) in cultured dorsal root ganglion cells using Li^+ (Goold et al., 1999). Inhibition of axon outgrowth by Li^+ is well established for many neuronal cell types, and recent evidence suggests that the mechanism is related, at least in part, to the inhibition of MAP1B phosphorylation by GSK3β (Lucas et al., 1998). Dorsal root ganglion neurons cultured in the presence of LiCl for 24 hours bear greatly enlarged growth cones at the tips of shortened axons. In these cultures MAP1B-P expression is reduced by approximately 90% when compared to controls. By contrast, the expression of detyrosinated tubulin (a marker for stable microtubules) is enhanced, particularly in the giant growth cones. Similar events occur in differentiated PC12 cells treated with LiCl (Fig. 3) (R. G. Goold and P. R. Gordon-Weeks, unpublished). Overall, these data indicate that MAP1B-P is important in maintaining a population of unstable, tyrosinated microtubules in growing neurites, an idea consistent with the observed pattern of expression during PC12 cell differentiation reported here. The identification of a *Drosophila* protein (futsch) that is homologous to MAP1B at its N and C termini is therefore significant. Genetic evidence suggests that this protein is necessary for axonal and dendritic outgrowth and for the correct organisation of the microtubule skeleton during synaptic growth at the neuromuscular junction (Roos et al., 2000; Hummel et al., 2000). Collectively, these observations suggest that GSK3β is a key regulator of neurite outgrowth through MAP1B – one of its major substrates in neurites and growth cones – and that phosphorylation of MAP1B by GSK3β affects the control that MAP1B exerts on microtubule dynamics in growing axons.

GSK3β is activated by NGF

The delay in MAP1B phosphorylation relative to its expression is surprising given the ubiquitous expression of GSK3β. Several mechanisms can be imagined to explain the temporal and local restriction of MAP1B phosphorylation: (1) priming of the substrate by another kinase whose expression and/or activity are restricted; (2) high phosphatase activity or inhibition of GSK3β kinase activity prior to the onset of MAP1B phosphorylation; (3) selective activation of GSK3β phosphorylation of MAP1B in the extending neurite – either through an increase in GSK3β activity or through the restricted expression of scaffold molecules capable of facilitating GSK3β-MAP1B interactions, and hence phosphorylation. Priming is a known requirement of some GSK3β substrates (Welsh et al., 1996) and examples of protein inhibitors and activators of kinase activity are well established (Ikeda et al., 1998; Wagner et al., 1997). However, results from the in vitro experiments reported here favour mechanism (3).

We were unable to find evidence for endogenous priming activity in PC12 cells. Moreover, GSK3β depleted extracts from differentiated cells can induce phosphorylation of 1B750 by undifferentiated extracts otherwise devoid of activity (Fig. 5A). This indicates that a dominant inhibitory or phosphatase activity capable of preventing MAP1B phosphorylation is not present in undifferentiated cell extracts and, more significantly, suggests that a soluble factor present in differentiated cell extracts can induce GSK3β activity. What is the nature of this factor? Immunoprecipitation of GSK3β from differentiated cell extracts removes virtually all of the kinase and presumably the majority of its stable binding partners, including potential scaffold proteins. Therefore, unless they are present in large excess, the involvement of a scaffold molecule is unlikely. This leaves the possibility of a direct activation of GSK3β, possibly by some form of post-translational modification.

NGF induces the expression of a novel GSK3β isoform

GSK3 functions in many cellular processes and its activity is regulated through a variety of mechanisms. Post-translational regulation of GSK3β activity is well documented. Phosphorylation of serine-9 (inhibitory) and tyrosine-216 (stimulatory) regulate kinase activity in various systems (Lesort et al., 1999; Welsh et al., 1996). The transient decrease in GSK3 activity following NGF treatment of PC12 cells reported elsewhere (Kleijn et al., 1998; Pap and Cooper, 1998) correlates well with the overall increase in serine phosphorylation and decrease in tyrosine phosphorylation of GSK3β noted above (Fig. 6). This observation suggests that these two modulatory systems work in tandem to decrease GSK3β activity in PC12 cells following NGF treatment. Murai et al. (Murai et al., 1996) have reported similar changes to GSK3β phosphorylation in response to extracellular signals. However, the mechanism that regulates the increase in GSK3β activity toward MAP1B following NGF treatment and how MAP1B-P expression is restricted to the longer neurites remains unclear. We have been unable to detect changes in the known regulatory post-translational modifications to GSK3β consistent with the observed increases in activity towards MAP1B reported here. The proportion of tyrosine-phosphorylated GSK3β does not change and the decreased serine phosphorylation detected occurs two to three days after the initial accumulation of phosphorylated MAP1B (Fig. 6). However, a novel post-translational modification characterised by the expression of a band that migrates significantly more slowly on SDS gels than the GSK3β present prior to NGF addition was observed (Fig. 6B). The expression of this isoform of GSK3β was induced by NGF and increased throughout the period of differentiation, correlating quite closely with the observed increase in MAP1B phosphorylation and neurite outgrowth (Fig. 1; Fig. 6E). This isoform of GSK3β is also present in rat brain where its expression is developmentally regulated, showing highest levels of expression during axonogenesis (Takahashi et al., 1994; Leroy and Brion, 1999). The authors identified this slower migrating species as a phosphorylated form of GSK3β on the basis of phosphatase treatment, but it was not characterised further**.** Antibodies to both phosphoserine-9 and phosphotyrosine-216 recognise the upper band, indicating that these modifications do not cause this shift in SDS gel mobility (Fig. 6B,C). After extensive phosphatase treatment a GSK3β doublet of reduced apparent M_r was still detectable, suggesting that an additional phosphate group may not cause the shift in mobility. However, it is feasible that GSK3β upper band contains a phosphate group resistant to dephosphorylation; therefore the exact nature of this post-translational modification to GSK3β remains to be determined.

In summary, in this report we have provided further evidence

that phosphorylated MAP1B plays an important role in neurite outgrowth. We have found that the expression of MAP1B-P is induced in parallel with GSK3β specific activity, assayed using MAP1B as a substrate, in response to NGF. This increase lags behind process formation and the expression of MAP1B in these cells by about two days and coincides with a period when the majority of cells are extending existing neurites that express high levels of MAP1B-P. Furthermore, we found that lithium, an inhibitor of GSK3, caused a dose-dependent inhibition of MAP1B phosphorylation, which correlated exactly with the dose-dependency of neurite growth inhibition, suggesting that GSK3β is a key regulator of neurite outgrowth through MAP1B. In addition, we have shown that the recognised post-translational modifications to GSK3β known to regulate kinase activity (i.e. phosphorylation of serine-9 and tyrosine-216) are not likely to modulate MAP1B phosphorylation in response to NGF. However, we demonstrate that a novel GSK3β isoform characterised by a decreased electrophoretic mobility on SDS-gels is induced by NGF and that its expression correlates with both neurite extension and MAP1B phosphorylation. Finally, we provide evidence indicating that a soluble factor present in differentiated PC12 cell extracts is capable of activating MAP1B phosphorylation by GSK3β.

This work was supported by the Wellcome Trust and the Central Research Fund of London University.

REFERENCES

Aletta, J. M., Lewis, S. A., Cowan, N. J. and Greene, L. A. (1988). Nerve growth factor regulates both the phosphorylation and steady-state levels of microtubule-associated protein 1.2 (MAP1.2). *J. Cell Biol.* **106**, 1573-1581.

Bamburg, J. R., Bray, D. and Chapman, K. (1986). Assembly of microtubules at the tip of growing axons. *Nature* **321**, 788-790.

Brugg, B. and Matus, A. (1988). PC12 cells express juvenile microtubule-associated proteins during nerve growth factor-induced neurite outgrowth. *J. Cell Biol.* **107**, 643-650.

Brugg, B., Reddy, D. and Matus, A. (1993). Attenuation of microtubule-associated protein 1B expression by antisense oligodeoxynucleotides inhibits initiation of neurite outgrowth. *Neurosci.* **52**, 489-496.

Bush, M. S., Goold, R. G., Moya, F. and Gordon-Weeks, P. R. (1996). An analysis of an axonal gradient of phosphorylated MAP 1B in cultured sensory neurons. *Eur. J. Neurosci.* **8**, 235-248.

Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. (1995). Inhibition of glycogen synthase kinase 3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789.

DiTella, M. C., Feiguin, F., Carri, N., Kosik, K. S. and Caceres, A. (1996). MAP-1B/TAU functional redundancy during laminin-enhanced axonal growth. *J. Cell Sci.* **109**, 467-477.

Edelmann, W., Zervas, M., Costello, P., Roback, L., Fischer, I., Hammarback, J. A., Cowan, J., Davies, P., Wainer, B. and Kucherlapati, R. (1996). Neuronal abnormalities in microtubule-associated protein 1B mutant mice. *Proc. Natl. Acad. Sci. USA* **93**, 1270-1275.

Gonzalez-Billault, C. and Avila, J. (2000). Molecular genetic approaches to microtubule-associated protein function. *Histol. Histopathol.* **15**, 1177-1183.

Gonzalez-Billault, C., Demandt, E., Wandosell, F., Torres, M., Bonaldo, P., Stoykova, A., Chowdhury, K., Gruss, P., Avila, J. and Sanchez, M. P. (2000). Perinatal lethality of microtubule-associated protein 1B-deficient mice expressing alternative isoforms of the protein at low levels. *Mol. Cell Neurosci.* **16**, 408-421.

Goold, R. G., Owen, R. and Gordon-Weeks, P. R. (1999). Glycogen synthase kinase 3β phosphorylation of microtubule- associated protein 1B regulates the stability of microtubules in growth cones. *J. Cell Sci.* **112**, 3373-3384.

Gordon-Weeks, P. R. and Fischer, I. (2000). MAP1B expression and microtubule stability in growing and regenerating axons. *Microsc. Res. Tech.* **48**, 63-74.

Greene, L. A. and Tischler, A. S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**, 2424-2428.

Hall, A. C., Lucas, F. R. and Salinas, P. C. (2000). Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. *Cell* **100**, 525-535.

Harper Keating, H. and Asai, D. J. (1994). Distribution of phosphorylated microtubule associated protein 1B during neurite outgrowth in PC12 cells. *Dev. Biol.* **162**, 143-153.

Hughes, K., Nikolakaki, E., Plyte, S. E., Totty, N. F. and Woodgett, J. R. (1993). Modulation of the glycogen synthase kinase 3 family by tyrosine phosphorylation. *EMBO J.* **12**, 803-808.

Hummel, T., Krukkert, K., Roos, J., Davis, G. and Klambt, C. (2000). Drosophila Futsch/22C10 is a MAP1B-like protein required for dendritic and axonal development. *Neuron* **26**, 357-370.

Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S. and Kikuchi, A. (1998). Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK3β and β-catenin and promotes GSK3β-dependent phosphorylation of β-catenin. *EMBO J.* **17**, 1371-1384.

Johnstone, M., Goold, R. G., Bei, D., Fischer, I. and Gordon-Weeks, P. R. (1997a). Localisation of microtubule-associated protein 1B phosphorylation sites recognised by monoclonal antibody SMI-31. *J. Neurochem.* **69**, 1417-1424.

Johnstone, M., Goold, R. G., Fischer, I. and Gordon-Weeks, P. R. (1997b). The neurofilament antibody RT97 recognises a developmentally regulated phosphorylation epitope on microtubule-associated protein 1B. *J. Anat.* **191**, 229-244.

Kim, L., Liu, J. and Kimmel, A. R. (1999). The novel tyrosine kinase ZAK1 activates GSK3 to direct cell fate specification. *Cell* **99**, 399-408.

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.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Leroy, K. and Brion, J. P. (1999). Developmental expression and localization of glycogen synthase kinase-3beta in rat brain. *J. Chem. Neuroanat.* **16**, 279-293.

Lesort, M., Jope, R. S. and Johnson, G. V. (1999). Insulin transiently increases tau phosphorylation: involvement of glycogen synthase kinase 3β and Fyn tyrosine kinase. *J. Neurochem.* **72**, 576-584.

Lucas, F. R., Goold, R. G., Gordon-Weeks, P. R. and Salinas, P. C. (1998). Inhibition of GSK3β leading to the loss of phosphorylated MAP-1B is an early event in axonal remodelling induced by WNT-7a or lithium. *J. Cell Sci.* **111**, 1351-1361.

Meixner, A., Haverkamp, S., Wassle, H., Fuhrer, S., Thalhammer, J., Kropf, N., Bittner, R. E., Lassmann, H., Wiche, G. and Propst, F. (2000). MAP1B is required for axon guidance and Is involved in the development of the central and peripheral nervous system. *J. Cell Biol.* **151**, 1169-1178.

Müller, R., Kindler, S. and Garner, C. C. (1994). The MAP1 family. In *Microtubules* (ed. J. S. Hyams and C. W. Lloyd), pp. 141-154. New York: Wiley-Liss.

Murai, H., Okazaki, M. and Kukuchi, A. (1996). Tyrosine dephosphorylation of glycogen synthase kinase 3 is involved in extracellular signal-dependent inactivation. *FEBS Lett.* **392**, 153-160.

Pap, M. and Cooper, G. M. (1998). Role of glycogen synthase kinase 3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *J. Biol. Chem.* **273**, 19929-19932.

Roos, J., Hummel, T., Ng, N., Klambt, C. and Davis, G. W. (2000). Drosophila Futsch regulates synaptic microtubule organization and is necessary for synaptic growth. *Neuron* **26**, 371-382.

Ruel, L., Stambolic, V., Ali, A., Manoukian, A. S. and Woodgett, J. R. (1999). Regulation of the protein kinase activity of Shaggy (Zeste-white3) by components of the wingless pathway in Drosophila cells and embryos. *J. Biol. Chem.* **274**, 21790-21796.

Sayas, C. L., Moreno-Flores, M. T., Avila, J. and Wandosell, F. (1999). The neurite retraction induced by lysophosphatidic acid increases Alzheimer's disease-like tau phosphorylation. *J. Biol. Chem.* **274**, 37046-37052.

Siegfried, E., Chou, T. B. and Perrimon, N. (1992). wingless signaling acts through zeste-white 3, the Drosophila homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. *Cell* **71**, 1167-1179.

Takahashi, M., Tomizawa, K., Kato, R., Sato, K., Uchida, T., Fujita, S. C.

and Imahori, K. (1994). Localization and developmental changes of tau protein kinase I/glycogen synthase kinase 3β in rat brain. *J. Neurochem.* **63**, 245-255.

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

Takei, Y., Kondo, S., Harada, A., Inomata, S., Noda, T. and Hirokawa, N. (1997). Delayed development of nervous system in mice homozygous for disrupted microtubule-associated protein 1B (MAP1B) gene. *J. Cell Biol.* **137**, 1615-1626.

Tanaka, E. M. and Kirschner, M. W. (1991). Microtubule behavior in the growth cones of living neurons during axon elongation. *J. Cell Biol.* **115**, 345-363.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Wagner, U., Brownlees, J., Irving, N. G., Lucas, F. R., Salinas, P. C. and Miller, C. C. (1997). Overexpression of the mouse dishevelled-1 protein inhibits GSK3β-mediated phosphorylation of tau in transfected mammalian cells. *FEBS Lett.* **411**, 369-372.

Wang, Q. M., Fiol, C. J., DePaoli-Roach, A. A. and Roach, P. J. (1994). Glycogen synthase kinase-3 beta is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. *J. Biol. Chem.* **269**, 14566-14574.

Welsh, G. I., Wilson, C. and Proud, C. G. (1996). GSK3: a SHAGGY Frog Story. *Trends Cell Biol.* **6**, 274-279.

Williams, R. S. and Harwood, A. J. (2000). Lithium therapy and signal transduction. *Trends Pharmacol. Sci.* **21**, 61-64.

Williamson, T. W., Gordon-Weeks, P. R., Schachner, M. and Taylor, J. (1996). Microtubules are obligatory for growth cone turning. *Proc. Natl. Acad. Sci. USA* **93**, 15221-15226.

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