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The function of the microtubule-associated protein tau is variably modulated by graded changes in glycogen synthase kinase-3β activity

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Abstract The microtubule-associated protein tau favors microtubule nucleation and stabilization and plays a role in the elongation of axons. We have investigated the ability of glycogen synthase kinase-3β (GSK-3β) to control tau-induced processes outgrowth. Tau-transfected Chinese hamster ovary (CHO) cells developed processes containing microtubule bundles after cytochalasin treatment, but a significant reduction in the number of cells harboring processes was observed in tau/GSK-3β-co-transfected cells. Lithium, an inhibitor of GSK-3β, counteracted in a dose-dependent manner this inhibitory effect of GSK-3β. These findings suggest that GSK-3β modulates in a graded manner the ability of tau to control the microtubule-dependent induction of cell processes.

Key words: Tau; Glycogen synthase kinase-3β; Microtubule; Cell process; Phosphorylation; Lithium

1. Introduction

Tau proteins are microtubule-associated proteins, abundant in neurons, that modulate several aspects of microtubule behavior: they favor the nucleation of new microtubules, reduce their dynamic instability, induce a bundling of microtubules in transfected cells and stabilize microtubules against depolymerizing agents (for review, see [1]). The expression of tau in some transfected cells [2,3] and in insect Sf9 cells with a baculoviral vector [4] induces the formation of cell processes containing bundles of ordered microtubules. This property might be related to a role for tau in establishing an axonal morphology: for instance, the development of axons of cultured neurons is inhibited by treatment with tau anti-sense oligonucleotides [5].

The control of cell processes outgrowth also requires intracellular signaling mechanisms that act on cytoskeletal proteins, such as the control of the phosphorylation of MAPs. For instance, highly phosphorylated tau proteins are less efficient in their ability to promote microtubule assembly. Tau is a substrate for many protein kinases in cell-free systems, but less data are available on kinases regulating tau function in cells. Glycogen synthase kinase-3β (GSK-3β), a kinase abundant in brain [6] and widely distributed in neurons in vivo [7], is a candidate physiological kinase for tau phosphorylating tau in transfected cells [8], in cultured neurons [9–11] and in the rat brain [9]. To investigate the ability of GSK-3β to control tau function in a cellular system, we have studied the consequence of GSK-3β expression on tau-dependent processes formation. We have found that the gradual inhibition of GSK-3β results in a graded effect on process growth, demonstrating that changes in GSK-3β activity could be a mechanism for subtle changes in processes properties.

2. Materials and methods

2.1. Cell transfection and experimental treatments

Chinese hamster ovary (CHO) cells were routinely cultured in HAM F12 medium supplemented with 10% fetal bovine serum, 100 IU penicillin and 100 μg/ml streptomycin. CHO cells were transfected using lipofectamine (Gibco BRL) with a tau (one N-terminal insert, four repeats) cDNA cloned into pSG5 [12], and a GSK-3β cDNA cloned into pMT-2 [8]. Transfected cells were treated with cytochalasin B at a final concentration of 20 μM for 1 h and with LiCl at a range of concentrations of 0.1, 0.5, 1, 5, 10 and 25 mM for 6 h before adding cytochalasin B. Cell survival was estimated after incubation with propidium iodide (0.6 μg/ml).

2.2. Antibodies and immunocytochemistry

The B19 rabbit polyclonal antibody [13] recognizes tau in a phosphorylation-independent manner. The mouse monoclonal antibodies AT8 and AT180 (Innogenetics, Belgium), AD2 and 12E8 are specific for tau phosphorylated on Ser-202/Thr-205 (AT8), Thr-231 (AT180) [14], Ser-396/404 (AD2) [15], Ser-262/356 (12E8) [16]. The mouse monoclonal antibodies TPKI (Transduction Laboratories) and DM1A (Sigma) are specific for GSK-3β and α-tubulin, respectively. Cells were rinsed in a stabilization buffer (PIPES 80 mM, $MgCl_2$ 1 mM, EGTA 1 mM, polyethyleneglycol 4% (w/v); pH 6.9) and fixed for 15 min in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.6. For double immunolabelling, cells were treated for 5 min with 0.01 M Tris, 0.15 M NaCl, pH 7.6, containing 0.3% (w/v) Triton X-100, then incubated sequentially with the primary antibodies, with a horse anti-mouse antibody conjugated to biotin (Vector) and a goat anti-rabbit antibody conjugated to Alexa^{488} (Molecular probes), and with streptavidin conjugated to Alexa^{594} (Molecular probes).

The number of transfected cells with processes (defined as an outgrowth greater than 10 μm) was expressed as a percentage of the total number of transfected cells (100 transfected cells examined). The length of processes was measured on digital images using the public domain NIH Image program (version 1.61). Statistical comparisons were performed using the chi-squared test and the Student *t* test. Cells were also fixed for 1 h in 4% (v/v) glutaraldehyde, embedded in Epon and ultrathin sections were examined in electron microscopy.

2.3. Western blotting

For Western blot analysis, cultured cells were homogenized in a

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Abbreviations: GSK-3β, glycogen synthase kinase-3β; CHO, Chinese hamster ovary

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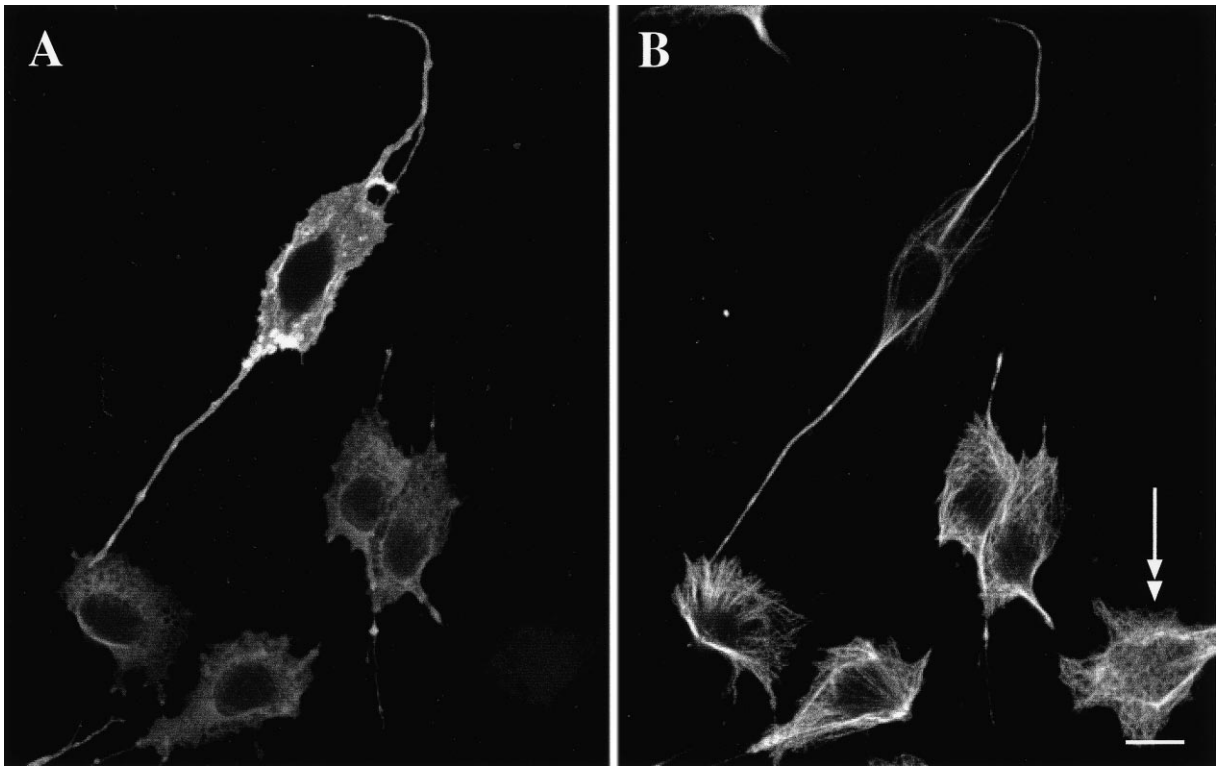

Fig. 1. Double immunolabelling with the B19 antibody to tau (A) and the DM1A antibody to tubulin (B), on tau-transfected CHO cells treated with cytochalasin B. Tau-transfected cells exhibit processes that contain microtubule bundles. These bundles are absent in a non-transfected cell (double arrow in B). Scale bar: 10 μm.

buffer containing proteases and phosphatases inhibitors (Tris 50 mM, EDTA 10 mM, NaCl 100 mM, pH 7.4, PMSF 1 mM, leupeptin 25 μg/ml, pepstatin 25 μg/ml, Na_3VO_4 1 mM, $Na_4P_2O_7{\cdot}10H_2O$ 10 mM, NaF 20 mM) and kept frozen at −20°C. The amounts of proteins in homogenates were estimated with the Bradford method (Bio-Rad reagent). Homogenates were run on 10% (w/v) polyacrylamide gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and proteins were electrophoretically transferred to nitrocellulose membrane. Nitrocellulose membranes were blocked in non-fat milk (10% (w/v) in TBS) for 1 h at room temperature and incubated with the primary antibody overnight followed by anti-mouse immunoglobulins conjugated to biotin followed by streptavidin conjugated to alkaline phosphatase. Membranes were finally incubated in the developing buffer (Tris 0.1 M, NaCl 0.1 M, $MgCl_2$ 0.05 M, pH 9.5) containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The amount of tau in every sample was normalized to give a similar immunoreactivity with the B19 phosphorylation-independent tau antibody.

3. Results

3.1. Cytochalasin B-induced processes in tau-transfected cells

Only after treatment with cytochalasin did many tau-transfected CHO cells harbor one or several thin and straight

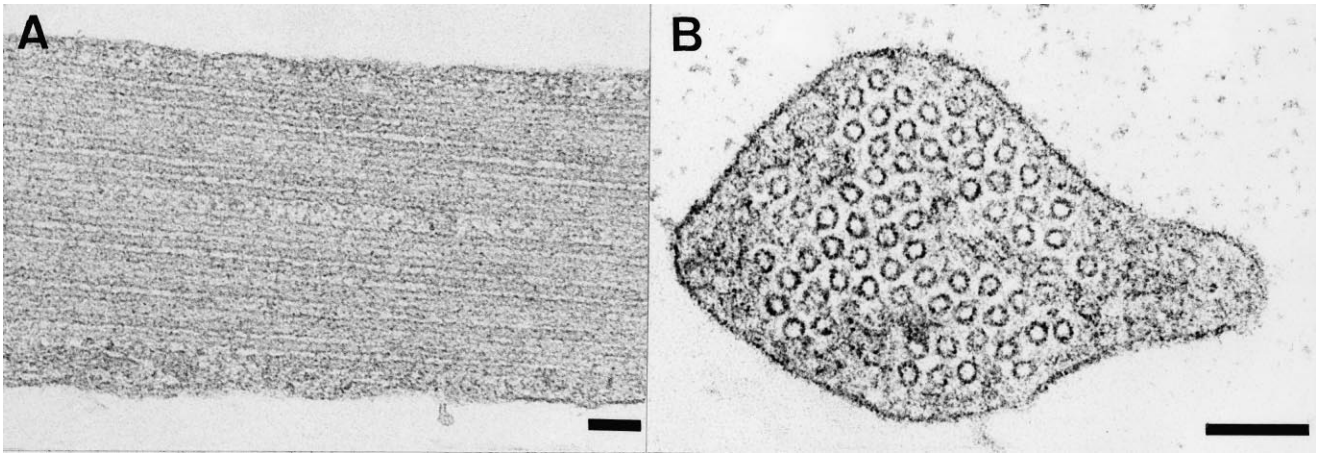

Fig. 2. Transmission electron microscopy. High magnification showing the density of microtubules and their regular spacing in ultrathin sections parallel (A) or perpendicular (B) to the long axis of processes in tau-transfected cells. Scale bar: 100 nm.

processes with a length of 25.2 μm ± 1.2 μm (mean ± S.E.M.) (Fig. 1A,B). These processes contained bundles of microtubules running parallel to their long axis (Fig. 2) and separated from each other by a regular spacing. 54% ± 0.8% (mean ± S.E.M.) of tau-transfected cells and only 1.2% of non-transfected cells showed processes (Fig. 2).

3.2. Inhibition of process induction in cells co-transfected with tau and GSK-3β

The proportion of tau/GSK-3β-co-transfected cells exhibiting a process was less than for tau-transfected cells (54% ± 0.8% versus 30% ± 1.3% (mean ± S.E.M.) (Figs. 3 and 5A,B). In co-transfected cells, the processes had a length of 25.1 μm ± 1.3 μm (mean ± S.E.M.). By immunocytochemistry, most tau-transfected cells (detected by the B19 antibody) were also 12E8- (96%), AD2- (80%) and AT180- (59%) positive but only 10% of them were AT8-positive. This observation was confirmed by immunoblotting analysis: several tau immunoreactive species (corresponding to differentially phosphorylated species of a single transfected tau isoform) were detected both with the B19 phosphorylation-independent antibody and the AD2 phosphorylation-dependent tau antibody in tau-transfected cells (Fig. 4A,B, lanes 2). No tau species were detected in non-transfected cells (Fig. 4A,B, lanes 1). Comparison of immunoblots of tau-transfected cells and tau/GSK-3β-co-transfected cells indicated a decrease in the electrophoretic mobility of tau bands (Fig. 4A, lane 3) and an increase in the immunoreactivity of tau bands with the AD2 phosphorylation-dependent tau antibody species in tau/GSK-3β-co-transfected cells (Fig. 4B, lane 3). The same shift in electrophoretic mobility was observed with the 12E8 and the AT180 antibodies (not shown).

3.3. Lithium reverses the inhibitory effect of GSK-3β on process induction

The LiCl treatment of tau/GSK-3β-co-transfected cells (Fig. 5C,D) increased the percentage of cells exhibiting processes. A significant effect was already observed at a concentration of 0.1 mM of LiCl and the percentage of tau/GSK-3β-co-transfected cells exhibiting processes increased in a dose-dependent manner (Fig. 6). A minor increase in the percentage of dead cells was observed in lithium-treated cultures (1.8%, versus 0.6% in untreated cultures). Comparison of immunoblots of tau/GSK-3β-co-transfected cells treated or not with lithium showed an increase in the electrophoretic mobility of several tau bands after lithium treatment (Fig. 4A, lane 4) and a decrease in the immunoreactivity of the slower migrating tau bands with the AD2 phosphorylation-dependent tau antibody (Fig. 4B, lane 4).

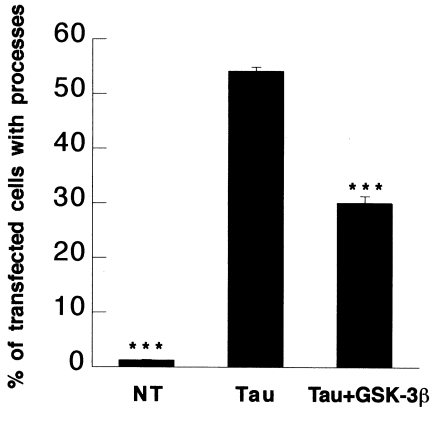

Fig. 3. Processes formation in non-transfected (NT), tau-transfected and tau/GSK-3β-co-transfected cells treated with cytochalasin B, expressed as a percentage of transfected cells with processes (mean ± S.E.M.). In non-transfected cells, data are expressed as a percentage of total cell number. *: $P < 0.001$ compared with tau-transfected cells by chi-squared test.

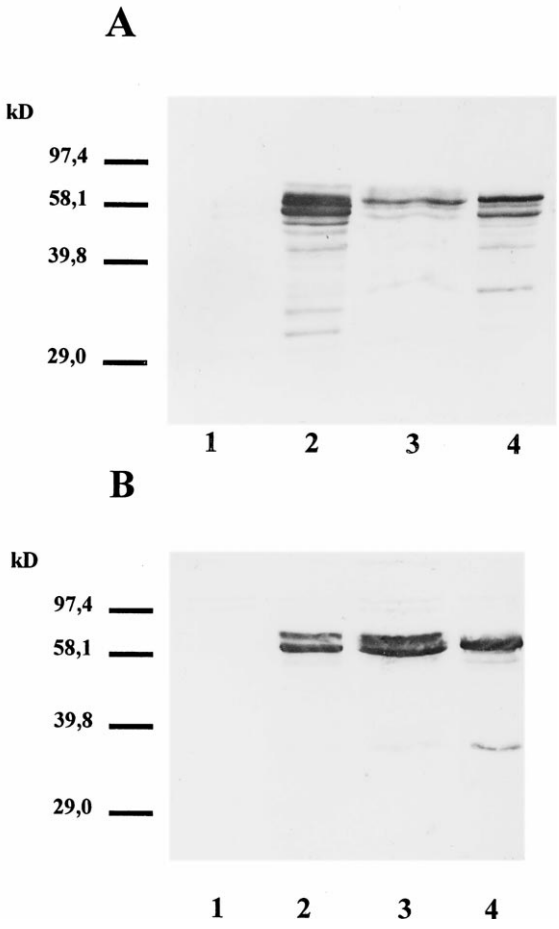

Fig. 4. Immunoblotting analysis of transfected and non-transfected CHO cells with (A) the B19 polyclonal antibody to tau, and (B) the AD2 phosphorylation-dependent anti-tau monoclonal antibody. Lanes 1: non-transfected cells; lanes 2: tau-transfected cells; lanes 3: tau- and GSK-3β double-transfected cells; lanes 4: tau- and GSK-3β double-transfected cells treated with 25 mM LiCl for 7 h. Identical lanes in (A) and (B) have similar loading of protein. Bars on the left indicate the position of molecular weight markers: 97.4 kDa (phosphorylase b), 58.1 kDa (catalase), 39.8 kDa (alcohol dehydrogenase), 29.0 kDa (carbonic anhydrase).

4. Discussion

The induction of cell processes containing microtubule bundles was reported to be enhanced after reorganization of the actin network by cytochalasin treatment in MAP2c-transfected hepatoma cells [17] and in tau-expressing Sf9 cells [18]. We similarly observed that cytochalasin treatment of tau-transfected CHO cells was necessary to induce the formation of processes. We observed a highly significant decrease in the percentage of cells with processes among tau/GSK-3β-co-transfected cells, indicating that the expression of GSK-3β

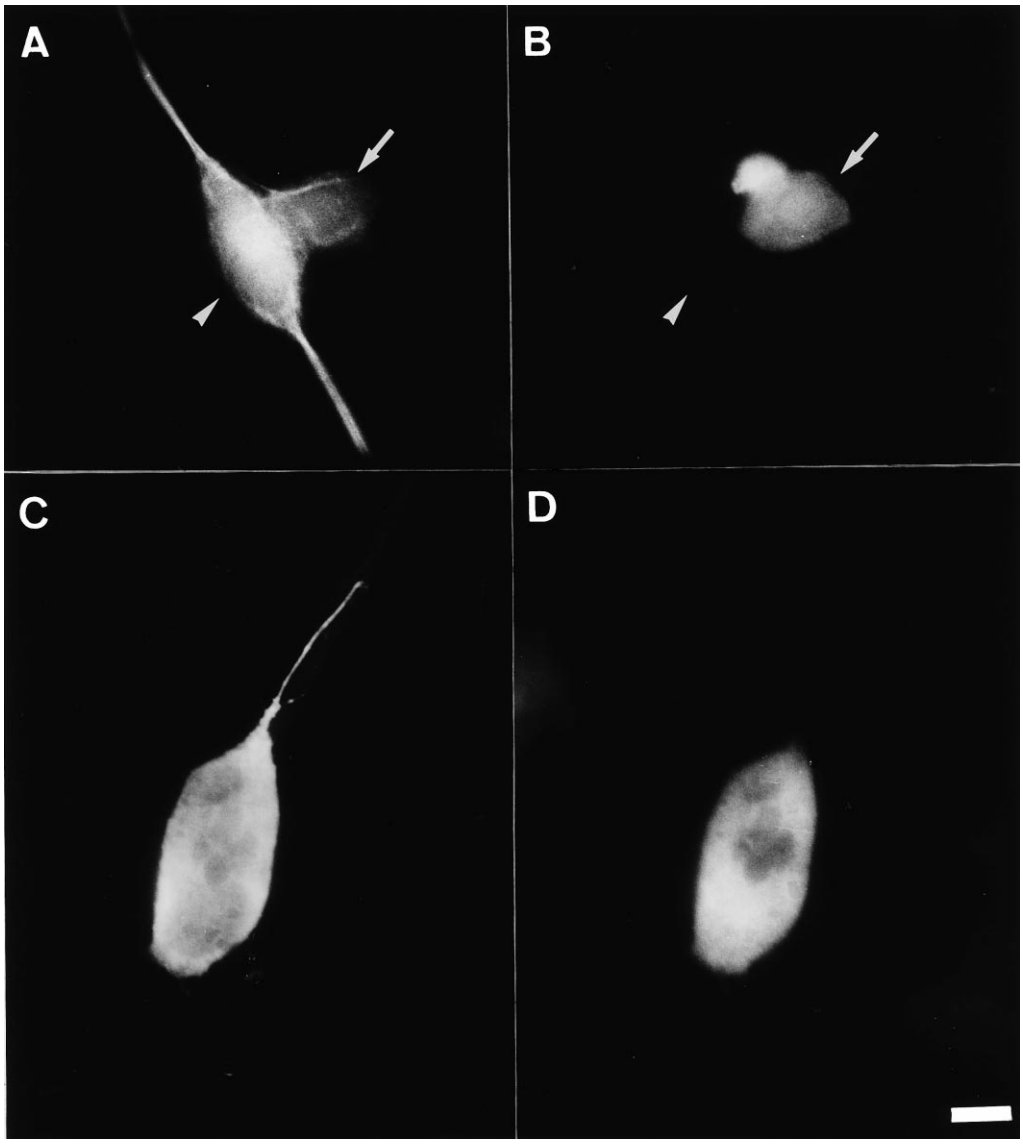

Fig. 5. Tau/GSK-3β-co-transfected cultures were treated with cytochalasin B alone (A and B) or with lithium followed by cytochalasin B (C and D) and double-immunolabelled with the B19 anti-tau (A and C) and anti-GSK-3β (B and D) antibodies. A and B: A tau/GSK-3β-co-transfected cell (arrow in A and B) does not show process whereas an adjacent cell transfected only with tau (arrowhead in A and B) shows two processes. C and D: In the presence of lithium, a tau/GSK-3β-co-transfected cell shows a process. Scale bar: 10 μm.

interferes with process formation. A role for GSK-3β as a negative regulator of processes outgrowth was further suggested by the observation that lithium enabled co-transfected cells to regain an ability to develop cell processes. Lithium acts as a specific inhibitor of GSK-3β in vitro and in intact cells [9–11,19]. The inhibition of GSK-3β activity by lithium is dose-dependent, with a half-maximal effect occurring at 1–2 mM in vitro [19]. We also observed a dose-dependent effect of lithium on process formation, with a half-maximal effect occurring around a concentration of 1 mM. This cellular test system relying on a morphological change was relatively sensitive, since a 10 times lower lithium concentration (i.e. 0.1 mM) still significantly affected process formation.

The mean length of cell processes was not different between tau-transfected and tau/GSK-3β-co-transfected cells. This observation is consistent with GSK-3β acting by reducing the ability of tau to nucleate microtubule assembly, as reported in a previous study [20]. A reduction of microtubule nucleation in tau/GSK-3β-co-transfected cells would be expected to lead to a decrease in the frequency of cell process formation, but not to an important reduction of their length.

GSK-3β could also negatively modulates process induction by decreasing microtubule assembly, since GSK-3β was found to decrease the pool of tau associated to microtubules and to reduce microtubule bundling [11,21,22]. A massive change in tau phosphorylation in cultures co-transfected with tau and GSK-3β could not be demonstrated since many cells transfected with tau only already contained a pool of phosphorylated tau proteins, as observed previously in CHO cells [12,23,24] and confirmed by our immunoblotting analysis. Interestingly, the transfected tau was phosphorylated at Ser-262/356 (as observed with the antibody 12E8), a phosphorylation reported to be associated to a decrease of tau binding to microtubules and necessary for extension of cell processes in

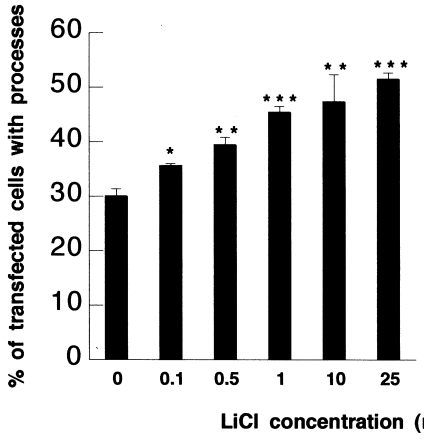

Fig. 6. Effect of different concentrations of lithium on processes formation in tau/GSK-3β-co-transfected cells. Results are expressed as a percentage of tau/GSK-3β-co-transfected cells with processes (mean ± S.E.M.). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ compared with cells not treated with lithium, by Student t test.

Sf9 cells [25]. This suggests that phosphorylation of tau at some sites in CHO cells might actually favor the extension of cell processes in tau-transfected CHO cells.

We however observed a decrease in the electrophoretic mobility of tau in tau/GSK-3β-co-transfected cells and an increase of its immunoreactivity with the AD2 phosphorylation-dependent antibody, suggesting that GSK-3β was able to increase further the phosphorylation of tau in these transfected cells, in agreement with previous studies on transfected cells [8,22,24]. Lithium treatment of tau/GSK-3β-co-transfected cells increased the electrophoretic mobility of tau in tau/GSK-3β-co-transfected cells and decreased its immunoreactivity with a phosphorylation-dependent antibody, as reported previously [9–11]. Altogether, these results support the conclusion that the cellular effects induced by GSK-3β, i.e. a reduction of cell processes outgrowth, were linked to an increased tau phosphorylation. Further studies will however be necessary to establish that the phenotypic changes induced by GSK-3β are only due to a direct action on tau. However, our observations indicate that the microtubule-dependent, tau-induced process outgrowth can be negatively regulated by GSK-3β activity.

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