Lithium inhibits Alzheimer's disease-like tau protein phosphorylation in neurons

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Abstract In Alzheimer's disease, tau protein becomes hyperphosporylated, which can contribute to neuronal degeneration. However, the implicated protein kinases are still unknown. Now we report that lithium (an inhibitor of glycogen synthase kinase-3) causes tau dephosphorylation at the sites recognized by antibodies Tau-1 and PHF-1 both in cultured neurons and in vivo in rat brain. This is consistent with a major role for glycogen synthase kinase-3 in modifying proline-directed sites on tau protein within living neurons under physiological conditions. Lithium also blocks the Alzheimer's disease-like proline-directed hyperphosphorylation of tau protein which is observed in neurons treated with a phosphatase inhibitor. These data raise the possibility of using lithium to prevent tau hyperphosphorylation in Alzheimer's disease.

Key words: Alzheimer's disease; Glycogen synthase kinase-3; Lithium; Okadaic acid; Tau protein; Rat cerebellar granule neuron

1. Introduction

Alzheimer's disease (AD) comprises a group of dementias characterized by certain histopathological hallmarks which include the presence of amyloid plaques, neurofibrillary tangles and dystrophic neurites, as well as the loss of neurons and synapses in patients' brains. At an ultrastructural level, microtubules disappear and aggregates of paired helical filaments (PHF), which are mainly constituted by a modified form of the microtubule-associated protein tau [1–3], accumulate within dystrophic neurites and neurofibrillary tangles. Hyperphosphorylation of tau is an early event in the course of AD and may precede the disruption of the microtubule cytoskeleton [4] and the formation of PHF-tau [5]. Thus, the study of the regulation of tau protein phosphorylation in neurons is important to understand the neurofibrillary degeneration in AD.

Current evidence suggests that tau protein has a key role in the stabilization of microtubules within neurites, particularly within axons where it is most abundant [6]. In vitro analyses have demonstrated that phosphorylation affects the ability of tau to stabilize microtubules [7]. Tau phosphorylation is therefore hypothesized to control microtubule dynamics during neurite growth and maturation. Indeed, embryonic and neonatal tau protein is much more heavily phosphorylated than adult tau [8–11]. Decreased tau phosphorylation after neuronal maturation may be due to down-regulation of protein kinases and up-regulation of phosphatases, and could favor axonal stabilization. Aberrant tau hyperphosphorylation in AD may result from phosphatase down-regulation [10,12] and lead to microtubule disassembly [4].

The sites on tau protein which become phosphorylated in vivo have been identified [13]. Half of them correspond to serine or threonine adjacent to proline residues, which are targeted in vitro by a variety of proline-directed protein kinases such as cyclin-dependent kinases (CDK), mitogen-activated protein kinases (MAPK) and glycogen synthase kinases 3α and 3β (GSK-3α,β). However, the kinases that actually phosphorylate tau within living neurons have not been determined yet. This issue is crucial, as a specific kinase inhibitor might be useful to block tau hyperphosphorylation in AD.

In this report we describe that lithium inhibits site-specific AD-like tau phosphorylation in living neurons. The state of tau phosphorylation was inferred from its electrophoretic mobility and its immunoreactivity toward antibodies that recognize phosphorylation-sensitive epitopes. These results are consistent with an involvement of GSK-3 in neuronal tau phosphorylation.

2. Materials and methods

2.1. Materials

Tau monoclonal antibodies: 7.51 [14] (a kind gift of Dr. C. Wischik, MRC, Cambridge, UK), Tau-1 [2,15] (a kind gift of Dr. L. Binder, MGC, IL, USA), 12E8 [16] (a kind gift of Dr. P. Seubert, Athena, San Francisco, CA, USA) and PHF-1[3,17] (a kind gift of Dr. P. Davies, Albert Einstein Coll., Bronx, NY, USA) were used. According to the residue numbering of the longest human tau isoform of 441 amino acids [18], antibody Tau-1 recognizes tau protein only when serines 195, 198, 199 and 202 are dephosphorylated [15] Antibody 12E8 reacts with tau when serine 262 is phosphorylated [16]. Antibody PHF-1 recognizes tau when serines 396 and 404 are phosphorylated [17]. PD 098059 (2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one) [19] was purchased from RBI. Olomoucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine) [20] was purchased from Calbiochem. All other reagents were from Sigma.

2.2. Neuronal culture

Primary cultures of cerebellar granule neurons were prepared from 7-day-old rat pups following the procedure of Levi and colleagues [21] with slight modifications. Briefly, trypsin-dissociated cells were plated onto poly-L-lysine-coated plastic dishes and cultured in Neurobasal-B27 medium (Gibco) [22] supplemented with 10% fetal bovine serum, 25 mM KCl and 1 mM glutamine. Cytosine arabinoside (10 μM) was added to cultures 24 h after seeding to prevent non-neuronal cell proliferation. Experiments were performed after 6–8 days in vitro. At this stage, purity of cultures was evaluated by immunofluorescence with antibodies to MAP2 and GFAP. The number of glial cells was about 2% as determined by counting cells positive for GFAP. Thus, the cultures were close to 98% pure granule neurons.

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Abbreviations: AD, Alzheimer's disease; CDK, cyclin-dependent kinase; GSK-3, glycogen synthase kinase-3; MAPK, mitogen-activated protein kinase; PDPK, proline-directed protein kinase; PHF-tau, tau from paired helical filaments

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2.3. Animal treatment with lithium

Seven-day-old rat pups (weighing 22–25 g) were intraperitoneally injected with 400 μl of either 1 M LiCl or NaCl. 8 h after injection, rats were killed by decapitation. Then, cerebral cortices and cerebella were dissected and immediately processed as described below.

2.4. Protein preparation

Cells were harvested, washed in chilled PBS (phosphate-buffered saline), resuspended and homogenized in a buffer consisting of 20 mM HEPES, pH 7.4, 100 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 5 mM EDTA, and protease inhibitors (2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin). Brain tissue was also homogenized in the same buffer supplemented with 1 μM okadaic acid (which is included to block any post-mortem tau dephosphorylation). Lysates were centrifuged at $10\,000 \times g$ for 10 min at 4°C. The resulting supernatants were collected and their protein contents determined by the BCA assay.

2.5. Immunoblot analysis

Samples were mixed with Laemmli's buffer, boiled for 3 min, separated by SDS-PAGE on 12% resolving gels and transferred to nitrocellulose according to standard procedures. A blocking buffer of 5% non-fat powdered milk in PBS with 0.1% Tween-20 was used for all incubations. Immunoreactive proteins were visualized by the use of a peroxidase-conjugated anti-mouse antibody and enhanced chemiluminescence detection (Amersham). Quantification of immunoreactivity was performed by densitometric scanning. Individual tau bands may include multiple distinct tau proteins (arising from alternative splicing and differential phosphorylation) that comigrate and were therefore not quantitated separately. Thus, total immunoreactivity for all tau bands was quantitated. The densitometry values obtained for blots corresponding to the antibodies recognized phosphorylation-sensitive epitopes (Tau-1, 12E8 and PHF-1) were normalized with respect to the values obtained for antibody 7.51 in order to correct any deviation in loaded amounts of protein.

3. Results

3.1. Effect of lithium on tau phosphorylation in cultured neurons

Previous studies in tau-transfected non-neuronal cells have pointed to GSK-3 as a good candidate to be responsible for most tau phosphorylation at Ser/Thr-Pro sites [23]. Lithium has recently been described to act as an uncompetitive and specific inhibitor of GSK-3 with an IC_{50} near 2 mM in in vitro assays [24,25]. For this reason we focused on the possible role of this enzyme and examined the effects of lithium on tau phosphorylation in cultured neurons.

Treatment of cultured rat cerebellar granule neurons with LiCl for 6 h results in dose-dependent site-specific tau dephosphorylation (see Fig. 1). The great increase in Tau-1 immunoreactivity and the decrease in PHF-1 immunoreactivity demonstrate that tau proteins are substantially dephosphorylated at these epitopes after LiCl treatment. In contrast, no significant change in 12E8 immunoreactivity is found, which suggests that phosphorylation at this site is unaltered by LiCl. The fact that the electrophoretic mobility of tau proteins is notably augmented after lithium treatment (as observed with antibody 7.51 that recognizes a phosphorylation-independent epitope on tau [14]) is consistent with a net dephosphorylation of tau protein at most proline-directed sites.

Interestingly, the half maximal dephosphorylation of tau protein in cultured neurons is observed at a lithium concentration close to 2 mM, which coincides with the reported IC_{50} for the inhibition of purified GSK-3 in vitro [24]. These results

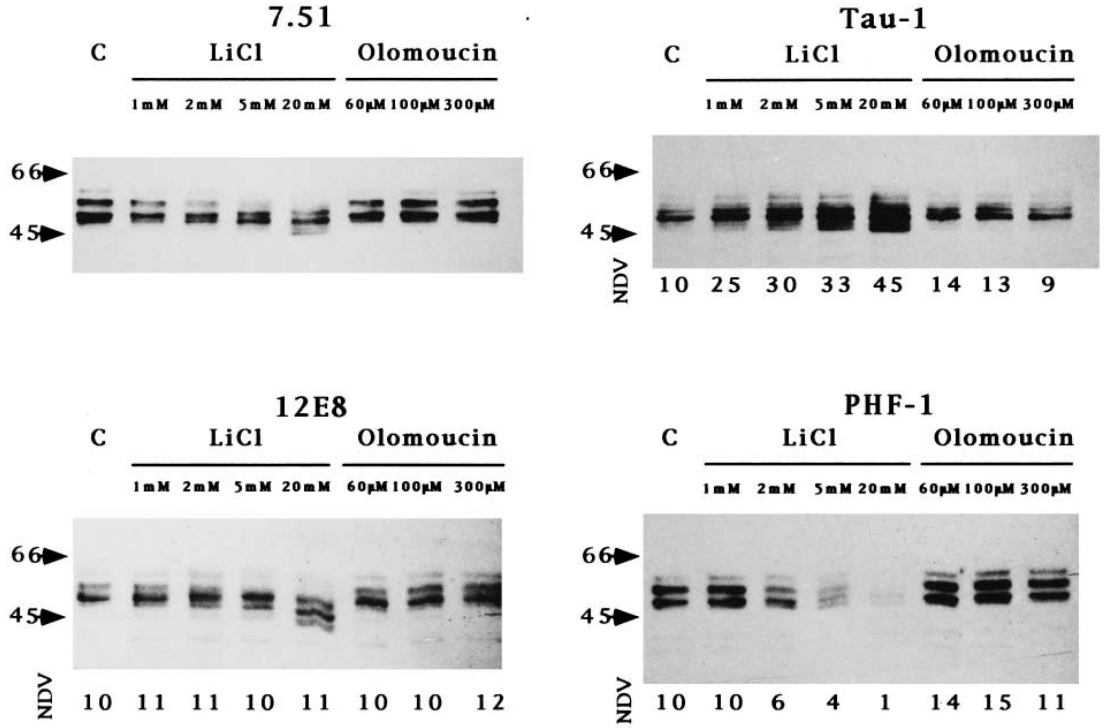

Fig. 1. Comparative effects of lithium and olomoucine on tau protein phosphorylation in cultured neurons. Cells were treated with the indicated concentrations of LiCl or olomoucin for 6 h. Cytoplasmic extracts were prepared, electrophoresed, blotted, and probed with antibodies 7.51, Tau-1, 12E8 and PHF-1. 'C' refers to untreated (control) cells. Immunoreactivities were quantitated as described in Section 2 and showed as normalized densitometry values (NDV) below the lanes. Numbers to the left indicate the position of molecular weight markers in kDa.

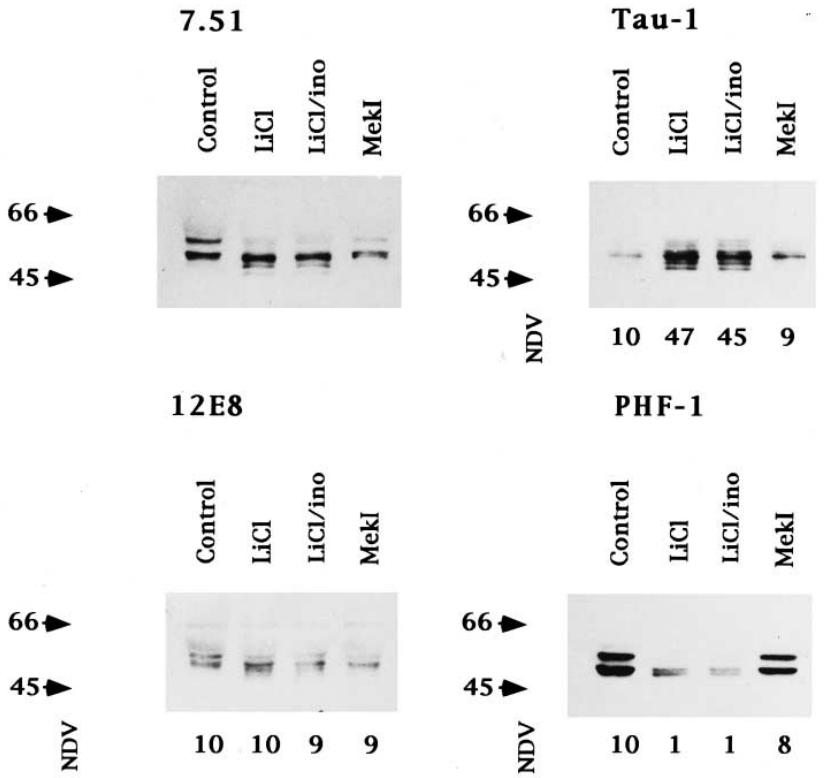

Fig. 2. Comparative effects of lithium, lithium supplemented with myo-inositol and MEK inhibitor (PD 098059) on tau protein phosphorylation in cultured neurons. Cells were treated with 20 mM LiCl for 6 h (LiCl), with 20 mM LiCl in the presence of 50 mM myo-inositol for 6 h (LiCl/Ino) or with 100 μM PD 098059 for 6 h (MekI). Cytoplasmic extracts were prepared, electrophoresed, blotted and probed with antibodies 7.51, Tau-1, 12E8 and PHF-1. 'Control' refers to untreated cells. Immunoreactivities were quantitated as described in Section 2 and showed as normalized densitometry values (NDV) below the lanes. Numbers to the left indicate the position of molecular weight markers in kDa.

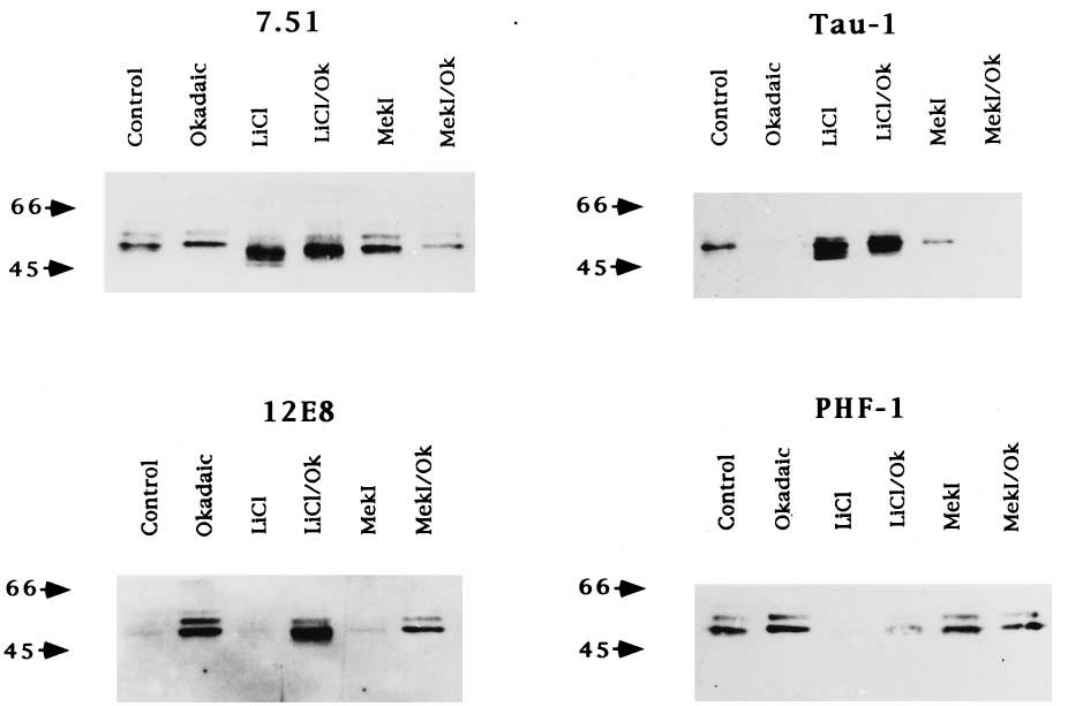

Fig. 3. Comparative effects of lithium and MEK inhibitor (PD 098059) on the okadaic acid-induced tau phosphorylation in cultured neurons. Cells were treated with 1 μM okadaic acid for 1 h (okadaic), with 20 mM LiCl for 7 h (LiCl), with 20 mM LiCl for 6 h followed by a treatment with 1 μM okadaic acid for 1 h in the presence of 20 mM LiCl (LiCl/Ok), with 100 μM PD 098059 for 7 h (MekI) or with 100 μM PD 098059 for 6 h followed by a treatment with 1 μM okadaic acid for 1 h in the presence of 100 μM PD 098059 (MekI/Ok). Cytoplasmic extracts were prepared, electrophoresed, blotted and probed with antibodies 7.51, Tau-1, 12E8 and PHF-1. 'Control' refers to untreated cells.

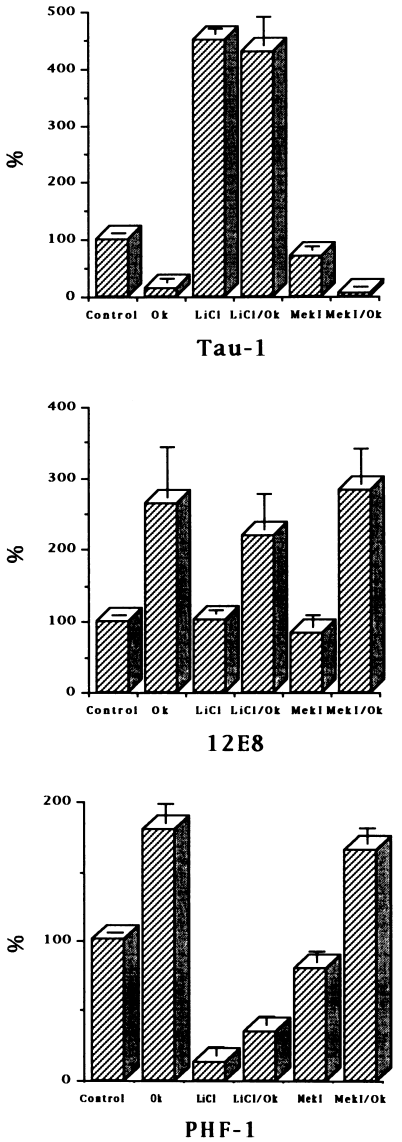

Fig. 4. Quantification of the inhibition of the okadaic acid-induced tau hyperphosphorylation in cultured neurons by lithium. Cytoplasmic extracts from treated cells were probed with antibodies 7.51, Tau-1, 12E8 and PHF-1 as indicated in the legend to Fig. 3. Quantification of immunoreactivities to antibodies Tau-1, 12E8 and PHF-1 were performed by densitometry and normalized with respect to the values corresponding to antibody 7.51. Shown are the mean normalized densitometry values and the corresponding standard deviations from four independent experiments.

suggest that tau protein becomes dephosphorylated in lithium-treated neurons as a consequence of ongoing protein phosphatase activity when GSK-3 is inhibited by lithium. However, the direct assay of GSK-3 inhibition by lithium in cultured neurons cannot be performed with any procedure depending on cell homogenization and GSK-3 purification since the effect of lithium is reversible [24,25].

To test for the specificity of the lithium effect on tau phosphorylation, other protein kinase inhibitors were used. Fig. 1 shows that olomoucin, a potent inhibitor of cyclin-dependent kinases [20], does not affect either the electrophoretic mobility of tau protein or its immunoreactivity to antibodies reacting with phosphorylation-sensitive epitopes. PD 098059, an inhibitor of the MAPK-activating enzyme MEK that blocks stimulation of MAPK and subsequent phosphorylation of MAPK substrates [19], also fails to modify tau phosphorylation in cultured neurons (see Fig. 2). Thus, among the protein kinase inhibitors tested, lithium appears unique in blocking neuronal tau phosphorylation. Lithium may also inhibit inositol monophosphatase, which might cause inositol depletion [24]. To rule out the possibility that lithium effect on tau phosphorylation was due to inositol depletion, neurons were treated with lithium in the presence of a high level of extracellular myo-inositol (50 mM). As shown in Fig. 2, myo-inositol fails to revert the lithium-induced tau dephosphorylation. Furthermore, lithium is not toxic to cultured neurons during the time periods examined (data not shown).

These data suggest that tau phosphorylation at the Tau-1 and PHF-1 epitopes is largely due to the activity of GSK-3. Previous observations on the co-localization of GSK-3 and tau protein in situ in rat brain [26,27] support this view, although the existence of another proline-directed protein kinase acting on tau protein cannot be completely ruled out. Phosphorylation of tau at the 12E8 epitope seems to be independent of GSK-3 in neurons. It is important to note that both Tau-1 and PHF-1 epitopes contain Ser-Pro motifs and are targets for GSK-3 whereas serine at the 12E8 epitope is not adjacent to any Pro residue.

3.2. *Effect of lithium on okadaic acid-induced tau hyperphosphorylation in cultured neurons*

The hyperphosphorylated state of tau protein in Alzheimer's disease may be due to defective dephosphorylation [10,12]. Thus, okadaic acid, a potent phosphatase inhibitor, has been used to induce the appearance of Alzheimer's disease-like hyperphosphorylated tau both in cultured neurons [28] and in vivo in rat brain [29]. Given the efficacy of lithium in inhibiting tau phosphorylation in cultured neurons under physiological conditions, we checked whether okadaic acid-induced tau hyperphosphorylation can also be prevented by lithium.

Treatment of cultured rat cerebellar neurons with 1 μM okadaic for 1 h leads to an Alzheimer's disease-like phosphorylation state of tau protein as judged from its increased immunoreactivity to antibodies 12E8 and PHF-1 and loss of immunoreactivity to antibody Tau-1 (see Fig. 3). Pre-treatment of cultured neurons with LiCl blocked the changes in immunoreactivity to antibodies Tau-1 and PHF-1, without affecting the immunoreactivity to antibody 12E8. Thus, lithium can effectively prevent the increased phosphorylation of tau protein which is observed at the sites recognized by antibodies Tau-1 and PHF-1 when neurons are treated with okadaic acid. In contrast, phosphorylation at the site recognized by antibody 12E8 is enhanced by okadaic acid irrespective of the presence of lithium. A quantification of the observed changes is presented in Fig. 4 to take into account the variations between experiments. It is clearly apparent that the magnitude of okadaic acid-induced change in immunoreactivity is different for the Tau-1 and PHF-1 epitopes. This may be due to different sensitivities of these phosphorylation sites to distinct protein phosphatases. Notwithstanding this fact, lithium is quite effective in blocking hyperphosphorylation at these epitopes.

Another kinase inhibitor (PD 098059) was used for comparison and failed to significantly and consistently modify the augmented phosphorylation of tau at the studied epitopes.

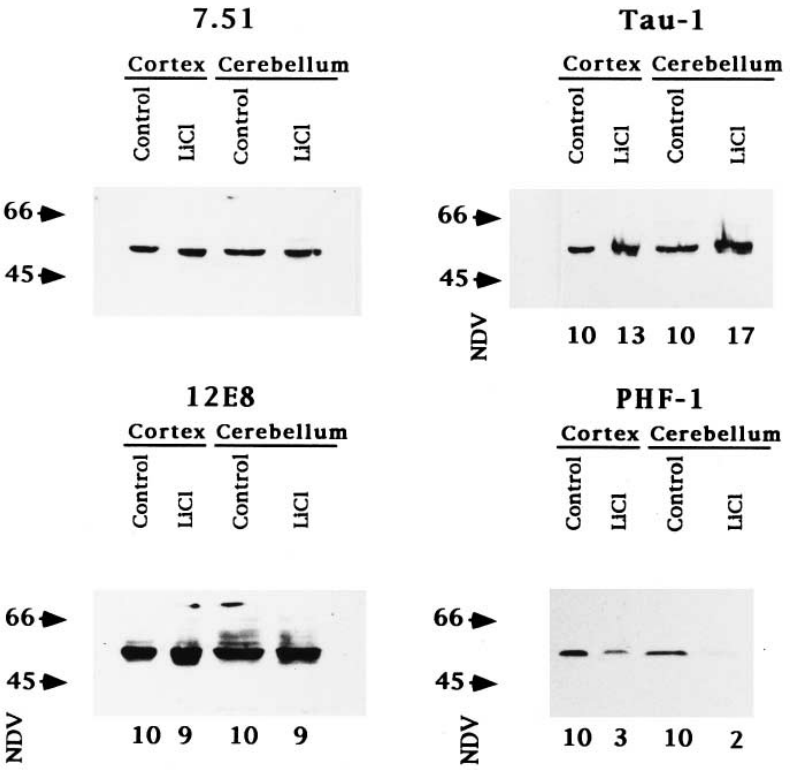

Fig. 5. Effect of lithium on the in vivo tau phosphorylation. Seven-day-old rat pups were treated with either lithium chloride (LiCl) or saline (control) for 8 h as described in Section 2. Extracts from cerebral cortices and cerebella were prepared, electrophoresed, blotted and probed with antibodies 7.51, Tau-1, 12E8 and PHF-1. Tau immunoreactivities from cerebellar and cortical samples were quantitated separately as described in Section 2 and showed as normalized densitometry values (NDV) below the lanes. Numbers to the left indicate the position of molecular weight markers in kDa.

These data agree well with a recent report demonstrating that okadaic acid induces tau hyperphosphorylation independently of MAPK activation [30]. Our results indicate that this tau hyperphosphorylation depends on GSK-3 (for the Tau-1 and PHF-1 epitopes) and additional kinase(s) (for the 12E8 epitope).

3.3. Effect of lithium on tau phosphorylation in vivo in rat brain

In view of the tau dephosphorylation which is observed in lithium-treated cultured neurons, we examined if this also occurs in vivo. Fig. 5 shows that intraperitoneal administration of lithium into 7-day-old rat pups results in the dephosphorylation of cerebellar tau protein at the Tau-1 and PHF-1 epitopes without affecting the phosphorylation at the 12E8 epitopes. These data are qualitatively similar to those found in cultured neurons, although a less marked increase in the electrophoretic mobility of tau protein is observed. Further comparison between the results obtained in vivo and those obtained with cultured neurons is difficult since the intraneuronal concentration of lithium which is attained in vivo after intraperitoneal administration is unknown.

A more modest lithium-induced dephosphorylation is observed in tau protein from neocortical tissue, particularly at the Tau-1 epitope (see Fig. 5). This may be due to the different timing of neuronal development in these two brain regions. Whereas there is a prominent axonal growth in cerebellar granule neurons, most cortical neurons have reached a comparatively more mature stage in 7-day-old rats. As the phosphate turnover on tau protein diminishes after neuronal maturation [11], the inhibition of tau phosphorylation by lithium may not be immediately followed by dephosphorylation in neocortical neurons during the short treatment employed in this experiment. Indeed, we have observed a significant decrease in the lithium-induced dephosphorylation of tau protein after maturation of cultured cerebellar neurons (data not shown). This interpretation agrees well with the block of neurofilament protein phosphorylation without net dephosphorylation which has been found in lithium-treated sensory neurons [31]. Interestingly, dephosphorylation at the PHF-1 epitope is much more apparent than dephosphorylation at the Tau-1 epitope in tau from rat neocortex, which again suggests a different sensitivity of these phosphorylation sites to distinct protein phosphatases.

4. Discussion

Tau protein hyperphosphorylation is an early and characteristic feature of Alzheimer's disease that seems to be correlated with the cognitive impairment of the patients [32]. The first consequence of tau hyperphosphorylation in AD may be the destabilization of the microtubule cytoskeleton [4]. Supportive of this view is the fact that tau phosphorylation at proline-directed sites by GSK-3 (upon co-transfection in non-neural cells) abolishes the microtubule-stabilizing effect which is observed in tau-transfected cells [33,34]. In immature neurons, tau phosphorylation may favor the microtubule dynamics which is probably required for neurite growth. A decreased tau phosphorylation in mature neurons might help to attain and maintain neurite stability. The aberrant hyperphosphorylation of tau in AD may shift the balance toward ex-

cessive microtubule depolymerization [4]. This would lead to defective axonal transport of organelles and impaired retrograde axonal transport of neurotrophic factors, as well as to alterations in neurite morphology.

Another consequence of tau hyperphosphorylation is a diminished susceptibility to protease digestion which results in a decreased tau protein turnover, as it has been observed in neuroblastoma cells treated with okadaic acid [35]. Indeed, PHF-tau obtained from AD patient brains is quite resistant to proteolytic cleavage unless it is previously dephosphorylated [36]. A reduction in the turnover of tau in patient brains may favor the accumulation of other modifications (ubiquination, oxidation, glycation, glycosylation, proteoglycan binding), which could eventually lead to the aggregation of tau into PHF and be therefore responsible for the formation of neurofibrillary tangles.

Thus, the prevention of tau hyperphosphorylation is likely to block the disruption of the cytoskeleton and the neurofibrillary degeneration occurring in AD. Here we have demonstrated that lithium, both in cultured neurons and in vivo in rat brain, inhibits tau phosphorylation at certain proline-directed sites which become hyperphosphorylated in AD. This suggests that GSK-3, the putative target for lithium [24,25], is indeed implicated in tau phosphorylation in living neurons, which extends and confirms all the data obtained from co-transfection studies in non-neuronal cell lines [23,25,33,34]. Whether GSK-3 is also implicated in pathological tau hyperphosphorylation in AD remains to be proven. The significant co-localization of GSK-3 with neurofibrillary lesions in AD patient brains [37,38] is consistent with a role of this enzyme in tau modification in AD. If this is the case, a new window for therapeutic intervention into AD may be opened up, since a chronic lithium treatment similar to that prescribed for manic-depressive patients might slow down tau hyperphosphorylation in the brains of patients with AD.

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