Modulation of the phosphorylation state of tau *in situ*: the roles of calcium and cyclic AMP

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Alterations *in situ* in the phosphorylation state of the microtubule-associated protein tau were examined in response to increasing intracellular levels of Ca^{2+} through *N*-methyl-D-aspartate (NMDA)-receptor activation, or activating cyclic AMP (cAMP)-dependent protein kinase (cAMP-PK), in rat cerebral-cortical slices. Increasing intracellular concentrations of Ca^{2+} by treatment of the brain slices with the glutamate analogue NMDA in depolarizing conditions (55 mM KCl) resulted in dephosphorylation of tau. Addition of KCl+NMDA to the slices resulted in a 40 % decrease in ^{32}P incorporation into tau, whereas addition of KCl or NMDA alone had no effect on tau phosphorylation. The KCl+NMDA-induced dephosphorylation of tau was blocked by the non-competitive NMDA-receptor antagonist MK801. To determine the involvement of the Ca^{2+} /calmodulin-dependent phosphatase, calcineurin, in the KCl+NMDA-induced dephosphorylation of tau, slices were pretreated with the calcineurin inhibitor Cyclosporin A. Pretreat-

ment of the rat brain slices with Cyclosporin A completely abolished the dephosphorylation of tau induced by the addition of KCl+NMDA. The dephosphorylation of tau *in situ* was site-selective, as indicated by the loss of ^{32}P label from only a few select peptides. Activation of cAMP-PK by stimulating adenylate cyclase in rat cerebral-cortical slices with forskolin resulted in a 73 % increase over control levels in ^{32}P incorporation into immunoprecipitated tau. Two-dimensional phosphopeptide mapping revealed that most of the sites on tau phosphorylated in brain slices in response to increased cAMP levels were the same as those phosphorylated on isolated tau by purified cAMP-PK. Although the state of tau phosphorylation is certainly regulated by many protein phosphatases and kinases *in vivo*, to our knowledge this study provides the first direct evidence of a specific protein phosphatase and kinase that modulate the phosphorylation state of tau *in situ*.

INTRODUCTION

Tau is a family of closely related microtubule-associated proteins (MAPs) with predicted molecular masses between 37 and 46 kDa, which are the products of alternatively spliced transcripts from a single gene [1,2]. Tau was the first MAP shown to promote microtubule assembly *in vitro* [3]. In cultured cells, tau co-localizes with microtubules [4] and appears to play a role in determining the asymmetry of neurons [5] and in the development of neuritic processes [6].

Tau is a phosphoprotein, and previous reports have demonstrated that, *in vitro*, tau can be phosphorylated by many protein kinases, including Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) [7,8], cyclic AMP (cAMP)-dependent protein kinase (cAMP-PK) [9], casein kinase II [10] and p42 MAP kinase (also known as ERK2) [11]. *In vitro*, phosphorylation of tau by MAP kinase decreases its binding to taxol-stabilized microtubules [12]. In addition, phosphorylation of tau by cAMP-PK inhibits its hydrolysis by the Ca^{2+} -activated protease calpain [9], and decreases the binding of bovine tau and the individual human tau isoforms to tubulin [8,13]. Phosphorylation of tau by CaMKII does not alter these parameters [8,13]; however, the intrinsic structure of tau is apparently altered by CaMKII phosphorylation, as determined by paracrystal analysis [14].

Although less numerous than the studies of phosphorylation *in vitro*, the dephosphorylation of tau *in vitro* has also been examined. Tau that had been phosphorylated *in vitro* by either cAMP-PK or calmodulin-dependent protein kinase was dephosphorylated by the Ca^{2+} -dependent phosphatase calci-

neurin (also known as phosphatase-2B) [15]. Yamamoto et al. [16] showed that tau phosphorylated by cAMP-PK and calmodulin-dependent kinase could be dephosphorylated by protein phosphatases-1 and -2A, resulting in increased microtubule assembly. In addition, tau phosphorylated by MAP kinase was preferentially dephosphorylated by protein phosphatase-2A [17].

Tau is the primary protein of paired helical filaments (PHFs), which are the major structural components of the neurofibrillary tangles in Alzheimer's disease [1,18,19]. PHF-tau is abnormally phosphorylated [20,21], and protein-sequence and mass-spectrometric analysis have revealed that several sites are phosphorylated to a greater extent in PHF-tau compared with normal tau [22,23]. Gong et al. [24] demonstrated that calcineurin dephosphorylated several of the abnormally phosphorylated sites on tau isolated from Alzheimer-disease brain by measuring loss of immunoreactivity with specific phosphorylation-dependent antibodies to PHF-tau. Finally, a recent report provided indirect evidence that calcineurin may play an important role in modulating the normal phosphorylation state of tau in cultured cerebellar macroneurons during development [25]. However, the kinases and phosphatases responsible for regulating the phosphorylation state of tau *in vivo* have yet to be clearly delineated.

In this study we examined the modulation of the phosphorylation state of tau *in situ* in rat cerebral-cortical slices. We provide direct evidence that the phosphorylation state of tau can be modulated by agents which increase Ca^{2+} and cAMP levels. Our data clearly indicate that tau is a substrate for both calcineurin and cAMP-PK *in situ*.

Abbreviations used: cAMP, cyclic AMP; cAMP-PK, cAMP-dependent protein kinase; CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II; PHFs, paired helical filaments; NMDA, *N*-methyl-D-aspartate; MAP, microtubule-associated protein 2.

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MATERIALS AND METHODS

Slice preparation and labelling

Male Sprague–Dawley rats (220–250 g) were killed; the brains were removed and placed in ice-cold 0.32 M unbuffered sucrose. Cerebral cortices were dissected, cut into $300\ \mu\text{m} \times 300\ \mu\text{m}$ slices with a McIlwain tissue chopper, and rinsed in 0.9% NaCl. Slices were preincubated for 90 min at 37 °C in a phosphate- and Ca^{2+} -free incubation buffer [containing 122 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO_4 , 3.6 mM NaHCO_3 , 11 mM glucose and 30 mM Hepes/ Na^+ , which was bubbled with O_2/CO_2 (19:1) before the pH was adjusted to 7.2] and aerated with O_2/CO_2 (19:1) every 30 min to prevent hypoxia. After rinsing with prewarmed incubation buffer, the slices were incubated in the same buffer as in the preincubation step, with the following exceptions: the concentration of Mg^{2+} was decreased to 0.12 mM, the concentration of Ca^{2+} was increased to 1.3 mM, and 10 mCi of [^{32}P]P_i (ICN) was added. After 90 min the slices were rinsed in incubation buffer (to remove unincorporated [^{32}P]P_i), resuspended in a small volume of incubation buffer and placed on ice. Samples (100 μl) were added to prewarmed assay tubes containing appropriate drug additions in a final volume of 500 μl . Reactions were terminated by aspirating the liquid and immediately freezing the slices in a solid- CO_2 /methanol bath. Frozen slices were stored at $-70\ ^\circ\text{C}$ until use.

Phosphorylation of tau *in vitro*

Tau was purified from heat-stable protein fractions from rat brain as described for isolation of tau from bovine brain by Johnson et al. [26], and the catalytic subunit of cAMP-PK was isolated from bovine heart as previously described [27].

[^{32}P]Phosphorylation of tau by cAMP-PK was carried out as described previously [8]. Briefly, tau protein (200 ng/ μl) was incubated with cAMP-PK (5 ng/ μl) and 50 μM [γ - ^{32}P]ATP (25 Ci/mmol; ICN) for 2 h at 30 °C in a buffer containing 1 mM dithiothreitol, 10 mM MgCl_2 and 20 mM Tris/HCl, pH 7.2. Phosphorylation reactions were terminated by addition of 2 $\times$ Laemmli buffer and subsequent boiling. Samples were electrophoresed on SDS/polyacrylamide gels and prepared for two-dimensional phosphopeptide mapping as described below.

Immunoprecipitation

Frozen slices were solubilized by adding 200 μl of heated (80–90 °C) 1% SDS in precipitation buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris/HCl, pH 7.2), followed immediately by sonication and boiling for 5 min. Samples were centrifuged at 16000 g for 5 min, after which the supernatants were transferred to clean tubes and the pellets discarded. Protein content was determined by the method of Lowry et al. [28] after acid precipitation of proteins. Samples were diluted to 1 mg/ml with precipitation buffer, and Triton X-100 was added to a final concentration 10-fold greater than the SDS concentration present in the samples. Samples (250 μg of protein) were incubated with Tau-1 antibody [29] for 18 h at 4 °C on a rotating platform. Goat anti-mouse IgG (Cappel) was added, and the samples were incubated for an additional 2 h at 4 °C. Washed Pansorbin (Calbiochem) was added to each tube (50 μl /tube), and, after incubation for 1 h at 4 °C, the immune complexes bound to the Pansorbin were pelleted, washed twice with a high-salt buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 1.1 M NaCl, 1 mM EDTA, 10 mM Tris/HCl, pH 7.2) and once with borate saline (100 mM boric acid, 25 mM sodium borate, 75 mM NaCl). In some experiments, Tau-1, T46.1 [30], or the phosphate-independent tau antibody 8C11 [31], was conjugated to Actigel

ALD (Sterogene) as per manufacturer's instructions, washed once with precipitation buffer containing 0.05% Nonidet P-40 and 1 mg/ml ovalbumin, and resuspended as a 1:1 slurry with precipitation buffer. Portions (30 μl) of antibody–Actigel slurry were added to 100 μg of sample and incubated at 4 °C overnight. Samples were centrifuged, and the immune complexes were collected and washed as described above. In both methods the immunoprecipitation pellets were resuspended in 30 μl of 2 $\times$ Laemmli sample buffer, and boiled for 5 min. The Pansorbin or Actigel was removed by centrifugation, and supernatants were run on 8% or 6.5–12% gradient SDS/polyacrylamide gels. Dried gels were exposed to Kodak X-OMAT film for various times. Immunoprecipitations with Pansorbin or conjugated Actigel gave equivalent results. Resulting autoradiographs were quantified by using a Bio-Rad model GS-670 imaging densitometer. Data were analysed by Student's unpaired *t* test or analysis of variance (ANOVA). Values were considered significantly different when $P < 0.05$.

Two-dimensional phosphopeptide mapping

Frozen slices were solubilized by sonication in heated (80–90 °C) Mes buffer (10 mM dithiothreitol, 1.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM Mes, pH 6.9, 0.7 $\mu\text{g}/\text{ml}$ pepstatin A, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 mM sodium pyrophosphate, 0.1 mM phenylmethanesulphonyl fluoride) and boiled for 5 min. Samples were centrifuged at 8000 g for 10 min, the supernatants transferred to clean tubes, and HClO_4 was added to a final concentration of 2.5% (v/v). The supernatants were then mixed and centrifuged at 4 °C. (Tau is a heat-stable protein and is soluble in 2.5% HClO_4 [32].) The supernatants were transferred again to clean tubes, sodium deoxycholate was added to a final concentration of 125 $\mu\text{g}/\text{ml}$, and the samples were incubated for 15 min on ice. Trichloroacetic acid was then added to a final concentration of 6% to precipitate the protein. Samples were centrifuged at 4 °C, and the resulting pellets rinsed with aq. 80% acetone to remove excess acid, and resuspended in 20 μl of 2 $\times$ Laemmli sample buffer before electrophoresis on 8% or 6.5–12% gradient SDS/polyacrylamide gels.

Peptide mapping was carried out as described by Sihag and Nixon [33]. Briefly, the tau bands were excised from SDS/polyacrylamide gels after being stained with Coomassie Brilliant Blue R-250, destained, and rinsed extensively in 20% methanol to remove residual acetic acid. Gel pieces were frozen, freeze-dried, and then resuspended in 1 ml of 50 mM NH_4HCO_3 (pH 8.0) containing 20 μg of tosyl-lysylchloromethane-treated α -chymotrypsin and incubated at 37 °C for 18 h. Tosylphenylalanylchloromethane-treated trypsin (20 μg) was added and the incubation continued for 6 h. The supernatants were removed, frozen, and freeze-dried. The products were subjected to two-dimensional phosphopeptide mapping and autoradiography as described previously [33].

RESULTS

Effects of activating *N*-methyl-D-aspartate (NMDA) receptors on the phosphorylation of tau *in situ*

To evaluate the effects of increasing intracellular levels of Ca^{2+} on the phosphorylation state of tau, rat brain cortical slices were labelled with [^{32}P]P_i and then incubated for 9 min in the presence of 55 mM KCl before a 5 min incubation with 100 μM NMDA. Since the NMDA-receptor ion channel is blocked by Mg^{2+} , 55 mM KCl was included to depolarize the slices and relieve this blockade [34]. A representative autoradiograph of immunoprecipitated tau in Figure 1 shows the effect of these treatments.

Figure 1 Autoradiograph of the effect of NMDA-receptor activation on tau phosphorylation and its reversal by MK801

Slices were ^{32}P -labelled before addition of 55 mM KCl, 100 μM NMDA or 500 μM MK801. Tau was immunoprecipitated from untreated slices (lane 1), or slices treated with KCl + NMDA (lane 2), KCl, NMDA + MK801 (lane 3), or MK801 alone (lane 4).

Table 1 Effect of NMDA-receptor activation on tau phosphorylation and its blockade by treatment with MK801 or Cyclosporin A

Slices were ^{32}P -labelled and then incubated with either no further additions or 55 mM KCl + 100 μM NMDA, in the absence or presence of 500 μM MK801 (a selective NMDA-receptor antagonist) or 100 μM Cyclosporin A (a selective inhibitor of calcineurin). Tau was immunoprecipitated from control and treated slices as described in the text. The NMDA + KCl-induced dephosphorylation of tau was significantly inhibited by either MK801 or Cyclosporin A. Results are given as means $\pm$ S.E.M. from three separate experiments: * $P < 0.05$.

Treatment	Optical density units
NMDA-receptor antagonist	
Control	27.10 $\pm$ 1.27
NMDA + KCl	15.39 $\pm$ 1.11*
NMDA + KCl + MK801	25.71 $\pm$ 1.18
MK801	28.57 $\pm$ 2.19
Calcineurin inhibitor	
Control	24.93 $\pm$ 0.53
NMDA + KCl	16.15 $\pm$ 0.18*
NMDA + KCl + Cyclosporin A	25.31 $\pm$ 3.97
Cyclosporin A	24.45 $\pm$ 1.66

Lane 1 (Figure 1) shows control conditions where ^{32}P -labelled slices were incubated for 14 min without additions. Lane 2 (Figure 1) shows treated conditions where slices were incubated in the presence of 55 mM KCl for 9 min before addition of 100 μM NMDA for 5 min. Incubation of slices with either 55 mM KCl or 100 μM NMDA alone had no effect on tau phosphorylation (KCl alone = $100 \pm 3\%$ of control; NMDA alone = $103 \pm 7\%$ of control; $n = 3$ separate experiments). However, incubation of slices in the presence of KCl + NMDA resulted in a $43 \pm 2\%$ decrease in tau phosphorylation (Table 1). Immunoblot analysis of immunoprecipitated tau revealed that equivalent amounts of protein were precipitated in all cases (results not shown). In addition, identical results (not shown) were obtained when tau was immunoprecipitated with a phosphate-independent tau antibody, T46.1 [30]. To determine if this decrease was specifically mediated by Ca^{2+} entry through the NMDA receptor ion-channel complex, the NMDA-receptor

Figure 2 Autoradiograph of the Cyclosporin A-mediated inhibition of the dephosphorylation of tau by calcineurin in response to treatment with KCl + NMDA

Slices were ^{32}P -labelled before addition of 55 mM KCl, 100 μM NMDA or 100 μM Cyclosporin A. Tau was immunoprecipitated from untreated slices (lane 1), or slices treated with KCl + NMDA (lane 2), KCl, NMDA + Cyclosporin A (lane 3), or Cyclosporin A alone (lane 4).

antagonist MK801 [35] was used. Slices were preincubated in the presence of 55 mM KCl for 7 min before a 2 min incubation with 500 μM MK801. NMDA was then added to the slices for an additional 5 min incubation. In this paradigm, MK801 completely blocked the decrease in tau phosphorylation induced by incubation of slices with KCl + NMDA (Figure 1, lane 3; Table 1). Incubation of slices with MK801 alone did not alter the phosphorylation state of tau (Figure 1, lane 4; Table 1).

The possible role of calcineurin in the Ca^{2+} -mediated dephosphorylation of tau in response to treatment of slices with KCl + NMDA was examined. Cyclosporin A has been shown *in vitro* to complex with cyclophilin and to bind specifically to and inhibit calcineurin [36–39]. Figure 2 shows a representative autoradiograph of tau immunoprecipitated from control and treated slices. Control slices were incubated for 17 min in the absence of additions (lane 1). Treated slices were either preincubated for 10 min before addition of 55 mM KCl + 100 μM NMDA for 7 min (lane 2), or preincubated with 100 μM Cyclosporin A for 10 min before a 7 min incubation with 55 mM KCl + 100 μM NMDA (lane 3), or incubated for 17 min with 100 μM Cyclosporin A (lane 4). Cyclosporin A completely abolished the decrease in tau phosphorylation caused by incubation of ^{32}P -labelled slices with KCl + NMDA, but did not affect tau phosphorylation under control conditions. In this paradigm, treatment of slices with KCl + NMDA resulted in a $35 \pm 1\%$ decrease in tau phosphorylation, which was completely blocked by treatment with Cyclosporin A (Table 1).

To determine which phosphopeptides were dephosphorylated by calcineurin, phosphopeptide maps of control and KCl + NMDA-treated slices were compared. Figure 3(a) shows the basal *in situ* phosphopeptide map generated after incubation of slices for 7 min without additions. Figure 3(b) shows the phosphopeptide map generated after slices were treated for 7 min with 55 mM KCl + 100 μM NMDA. For purposes of comparison, equivalent amounts (d.p.m.) of ^{32}P were spotted on each cellulose-TLC plate, and the phosphopeptide numbered 1 was chosen as a point of reference, as its relative optical density is

Figure 3 Autoradiographs of phosphopeptide maps from control (a) and KCl + NMDA-treated (b) slices

Slices were incubated in the absence or presence of 55 mM KCl + 100 μ M NMDA, and subjected to two-dimensional peptide mapping. Equivalent amounts (d.p.m.) of 32 P-tau were spotted on to each cellulose TLC plate. Peptides numbered 3, 4 and 5 from KCl + NMDA-treated slices were phosphorylated to a lesser extent than controls.

Figure 4 Autoradiograph of tau phosphorylated *in situ* by elevating cAMP levels

Slices were [32 P]phosphorylated *in situ* and incubated in the absence (lane 1) or presence (lane 2) of 30 μ M rolipram and 10 μ M forskolin. Tau was immunoprecipitated from 250 μ g of sample. In the presence of rolipram plus forskolin, tau phosphorylation increased by $73 \pm 20\%$ (mean $\pm$ S.E.M., $n = 3$; $^*P < 0.05$).

similar between treatments (control = 53.7; KCl + NMDA = 52.5). This reference phosphopeptide allows for comparison with other phosphopeptides within the same map. Hence, with respect to phosphopeptide 1, phosphopeptides 3, 4 and 5 show decreased phosphorylation in response to KCl + NMDA treatment compared with the control (Figure 3). These data clearly indicate that tau is dephosphorylated *in situ* by calcineurin in a site-selective manner.

Effect of cAMP on the phosphorylation of tau *in situ*

To determine whether tau was phosphorylated by cAMP-PK *in situ*, 32 P-labelled rat brain slices were incubated with 30 μ M rolipram, a selective inhibitor of cAMP phosphodiesterase [40], and 10 μ M forskolin, which directly activates adenylate cyclase [41]. In this paradigm, 32 P-labelled slices were incubated for 20 min with rolipram before addition of forskolin for another 10 min of incubation. Control slices were incubated for 30 min without additions. Figure 4 shows a representative auto-

radiograph of immunoprecipitated tau from control and treated slices. Treatment of slices with rolipram + forskolin resulted in a $73 \pm 20\%$ ($n = 3$) increase over control in 32 P incorporation into tau. Identical results were obtained when tau was immunoprecipitated with the phosphate-independent antibody to tau, 8C11 (results not shown).

To determine whether the increased phosphorylation state of tau was due to activation of cAMP-PK, comparisons were made between phosphopeptide maps of tau from control and forskolin + rolipram-treated slices, and of isolated tau phosphorylated *in vitro* by cAMP-PK. In Figure 5(a), a phosphopeptide map of tau phosphorylated *in situ* under basal conditions is shown. In the presence of rolipram plus forskolin (Figure 5b) a marked increase in the phosphorylation of several phosphopeptides was observed. Some of these peptides migrated to the same positions as the phosphopeptides generated from tau phosphorylated *in vitro* by cAMP-PK (Figure 5c). To confirm that several of the peptides were identical, tau was [32 P]phosphorylated *in vitro* by cAMP-PK, or *in situ* in the presence of forskolin + rolipram, digested separately with the proteases, and the samples were combined and run together on the same cellulose-coated TLC plate. The resulting phosphopeptide map is shown in Figure 5(d). Phosphopeptides generated from tau which was phosphorylated *in situ* are designated by numbers, and phosphopeptides from tau phosphorylated *in vitro* by cAMP-PK are designated by letters (Figure 5). In this combination experiment, four phosphopeptides, which have been designated 3a, 4b, 5c, and 6e, were found to overlap directly (Figure 5d). These results indicate that the site(s) on tau represented by these four phosphopeptides are phosphorylated *in vitro* by cAMP-PK and *in situ* in response to elevated levels of cAMP. Finally, it should be noted that the control phosphopeptide maps shown in Figures 3(a) and 5(a) cannot be compared, due to differences in the incubation times of the slices, d.p.m. of 32 P loaded and film exposure times.

DISCUSSION

The role of phosphatases in modulating the phosphorylation state of tau *in vitro* has been previously examined by other investigators. For example, tau has been found to be dephosphorylated *in vitro* by protein phosphatase-C [16], protein

Figure 5 Autoradiographs of two-dimensional phosphopeptide mapping of tau resulting from basal phosphorylation *in situ* (a), rolipram + forskolin-stimulated phosphorylation (b), tau phosphorylated *in vitro* by cAMP-PK (c), or a combination of (b) and (c) (d)

Slices were treated as indicated, and tau was excised from polyacrylamide gels, digested with proteases and subjected to electrophoresis on cellulose TLC plates, followed by ascending chromatography. Peptides phosphorylated *in situ* are indicated by numbers (a, b and d), and peptides phosphorylated *in vitro* by letters (c and f).

phosphatase-2A [17] and calcineurin [15]. More recently, PHF-tau was found to be dephosphorylated by protein phosphatase-1, protein phosphatase-2A and calcineurin, but not by protein phosphatase-2C [42]. However, it is not known which phosphatases have access to tau *in vivo* and modulate its phosphorylation state.

Numerous studies have examined the role of protein kinases in modulating the phosphorylation state of tau and PHF-tau *in vitro* (e.g. [7,9–11]). Although these studies have identified many kinases capable of phosphorylating isolated tau *in vitro*, none have directly addressed the possible role that these kinases play *in vivo*. Early studies examining the role of phosphorylation of another MAP (MAP-2) showed that phosphorylation sites *in vivo* (which were concentrated on the projection domain of the protein) largely differed from the sites observed to be phosphorylated *in vitro* (which were concentrated in the binding domain) [43]. This comparison highlights the importance of determining the relevance of phosphorylation sites identified by studies *in vitro* to sites that are actually phosphorylated *in vivo*, in which compartmentalization and other constraints may result in differential phosphorylation events.

In this study, the role of calcineurin and cAMP-PK in modulating the phosphorylation of tau *in situ* was examined. Alterations in intracellular Ca^{2+} levels appear to modulate the phosphorylation state of several cytoskeletal proteins. Previous studies have demonstrated that K^+ depolarization caused increases in MAP-2 phosphorylation in rat brain slices [44], whereas activation of the excitatory amino acid receptor NMDA resulted in a dephosphorylation of MAP-2 [45]. Therefore the effect of Ca^{2+} influx as a result of excitatory amino acid receptor

activation on the phosphorylation state of tau in rat brain slices was examined. Neither depolarization of slices with 55 mM KCl, nor incubation with NMDA alone, altered the phosphorylation of tau. However, activation of NMDA receptors under depolarizing conditions to relieve the Mg^{2+} blockade of the NMDA-receptor-channel complex [34] resulted in decreased ^{32}P incorporation into tau (Figure 3). MK801, an open channel blocker at the NMDA-receptor-channel complex [46], completely blocked the dephosphorylation of tau induced by KCl + NMDA, confirming that this decrease was specifically mediated through the NMDA-receptor-channel complex. This decrease in the phosphorylation state of tau after NMDA-receptor activation probably accompanies other responses that have been reported, such as long-term potentiation [47,48], mechanisms of excitotoxic cell death [49], and neuronal development [50]. Since the phosphorylation state of tau modulates its ability to promote polymerization and stability of microtubules [32], it may also be a contributing mechanism to these NMDA-mediated responses. For example, NMDA-mediated long-term potentiation [51,52] results in changes in synaptic structure [53], an event which most certainly involves reorganization of the microtubule network.

The Ca^{2+} /calmodulin-dependent protein phosphatase calcineurin has been implicated in the dephosphorylation of tau [15,25]. Calcineurin is the principal calmodulin-binding protein in brain [54] and is developmentally up-regulated [55]. Calcineurin, which is present in many cell types, has been immunocytochemically localized to the processes and cell soma of neurons [56,57]. Ferreira et al. [25] demonstrated that this phosphatase is localized in the growth cones of cerebellar macroneurons, that

this localization was dependent on the presence of microtubules and actin filaments, and that inhibition of calcineurin prevented axonal elongation. In the present study, the KCl+NMDA-induced dephosphorylation of tau was completely blocked by Cyclosporin A, which selectively inhibits calcineurin [36]. To our knowledge this is the first study to show *in situ* that tau is dephosphorylated by calcineurin.

cAMP-PK was activated by stimulating adenylate cyclase activity with forskolin [41] and by decreasing the hydrolysis of cAMP with the cAMP phosphodiesterase inhibitor rolipram [40]. Treatment of slices with rolipram + forskolin led to a 73 % increase in ^{32}P incorporation into tau, without any alterations in the amount of tau immunoprecipitated. Comparison of two-dimensional phosphopeptide maps of tau phosphorylated *in situ* with tau phosphorylated *in vitro* by cAMP-PK revealed that four [^{32}P]phosphopeptides were common to the maps for phosphorylation both *in vitro* and *in situ*. This result confirms that the increased phosphorylation of tau induced by elevating cAMP levels *in situ* is due to cAMP-PK-dependent phosphorylation of tau. Phosphopeptides generated after the phosphorylation of tau *in vitro* that were not observed *in situ* after activation of cAMP-PK are likely to represent phosphopeptides containing sites that are inaccessible to the kinase *in situ*. However, there were also two peptides (Figure 5a, phosphopeptides 1 and 2) which were phosphorylated in response to elevated cAMP levels *in situ*, but were absent from the phosphopeptide map of tau phosphorylated *in vitro* by cAMP-PK. These results indicate that cAMP-PK is able to phosphorylate tau *in vitro* and *in situ* at several common sites; however, there are sites on tau which are differentially accessible to cAMP-PK *in situ*. Studies *in vitro* have shown that phosphorylation of tau by cAMP-PK, but not by CaMKII, decrease tubulin binding and increase tau's resistance to calpain hydrolysis [8]. Additionally, recombinant human tau phosphorylated by cAMP-PK was shown to have a decreased ability to promote microtubule assembly [58]. Since tau is apparently phosphorylated by cAMP-PK *in situ*, these phosphorylation-induced metabolic and functional alterations may also be physiologically relevant.

The purpose of this present study was to determine whether the phosphorylation state of tau could be modulated *in situ* by agents which activate calcineurin and cAMP-PK, and to determine whether the pattern of tau phosphorylation *in situ* was comparable with those seen in studies *in vitro* using purified proteins. In summary, we have shown that tau is dephosphorylated by calcineurin in rat brain slices in response to depolarizing conditions in the presence of NMDA in a site-selective manner. Additionally, we have demonstrated that tau is phosphorylated *in situ* in response to increases in intracellular levels of cAMP at the majority of sites which are phosphorylated *in vitro* by cAMP-PK. These findings strongly indicate that tau is a substrate for cAMP-PK *in vivo*. Examining the modulation of the phosphorylation state of tau by specific protein phosphatases and kinases in a model *in situ* is of critical importance if the role *in vivo* of tau in the normal functioning and development of neurons, as well as its role in neurodegenerative diseases, is to be determined. Although this study has identified calcineurin and cAMP-PK as potential modulators *in situ* of the phosphorylation state of tau, the ability of other protein phosphatases and kinases to modulate tau's phosphorylation state *in situ* needs to be examined.

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