The phosphorilation State of Tau in the Developing Rat Brain Is Regulated by Phosphoprotein Phosphatases*

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The paired helical filaments (PHFs) in Alzheimer’s disease neurofibrillary tangles are composed of PHF-tau, which is thought to be hyperphosphorylated because several residues in postmortem samples of PHF-tau and human fetal tau are phosphorylated while the corresponding sites are not phosphorylated in autopsy-derived normal adult human brain tau. To determine how the phosphorylation of these sites is regulated, we isolated tau from rat brains at different embryonic and postnatal ages in the presence of okadaic acid to obtain tau in its most native in vivo phosphorylation state. Fetal tau was highly phosphorylated from embryonic day 18 (E18) until postnatal day 11 (P11). Thereafter, the levels of fetal tau diminished as did its phosphorylation state concomitant with the appearance of the five adult tau isoforms. Several phosphorylation-dependent antibodies (i.e. AT270, AT8, AT180, T3P, and PHF1) that recognize PHF-tau also recognize these tau isoforms, albeit at reduced levels in the mature rat brain. This suggests that Thr212, Ser239, Thr262, Ser267, and Ser356 are normal sites of phosphorylation in rat brain tau. The inclusion of OK in the microtubule assembly buffers did not alter the ability of tau to bind microtubules at any age. However, phosphatases were activated and kinases were down-regulated in the rat brain after P12 since adult tau proteins were partially dephosphorylated at and beyond this time in the absence of OK. Protein phosphatase 2A (PP2A) and 2B (PP2B) activities in the adult rat brain extracts dephosphorylated tau efficiently, but protein phosphatases in extracts of the P6 rat brain did not have a similar effect. This suggests that the sensitivity of tau to OK after P12 may be regulated by the de novo induction of adult brain phosphatases. Finally, PP2A and/or PP2B in adult rat brain extracts dephosphorylated tau in a site-specific manner. Thus, PP2A and PP2B (or closely related phosphatases) may regulate the phosphorylation state of adult tau isoforms in vivo, and the generation of PHF-tau in the AD brain may result from the abnormal inactivation of similar phosphatases.

Paired helical filaments (PHFs) are composed of altered CNS tau proteins (PHF-tau) that form neurofibrillary tangles

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1 The abbreviations used are: PHF, paired helical filament; AD, Alzheimer’s disease; PAGE, polyacrylamide gel electrophoresis; MEF, 3-morpholinosulfonic acid; OK, okadaic acid; TFP, trifluoperazine; PP, protein phosphatase; MT, microtubule; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; BC, binding competent; BI, binding incompetent; AC, assembly competent; AI, assembly incompetent.
of binding to MTs unless it is dephosphorylated (19, 37), a better understanding of how the dynamic phosphorylation and dephosphorylation of tau is regulated will provide important insights into the pathogenesis of PHFs. For these reasons, we monitored the developmental regulation of the phosphorylation state of tau rapidly isolated from fetal, juvenile, and adult rat brains in the presence of okadaic acid (OK).

**EXPERIMENTAL PROCEDURES**

**Materials**

Sprague-Dawley rats at embryonic day 18 (E18), P7, P11, P12, P13, P17, P19, P30, and adult ages (P30) were lethally anesthetized, and the rapid isolation of tau from the brains of these animals (see below) commenced within 2 min after sacrifice. SDS-molecular weight standards, GTP, dithiothreitol, Sephadex G-50 superfine, trifluoperazine (TFP), and MES were from Sigma; 125I-labeled goat anti-mouse IgG and 125I-labeled protein A-agarose and OK were from ICN; cantharidin and protein phosphatase assay kits were from Life Technologies, Inc.

**Methods**

**Isolation of Tau from Brain Tissue—**All isolation steps were carried out at 2°C unless stated otherwise. Rat brains were homogenized in 1.5 volumes of cold RAB buffer (0.1 M MES buffer, pH 6.8, 0.75 mM NaCl, 0.5 mM MgSO4, 1 mM EGTA, 2 mM dithiothreitol) and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin, pepstatin, soybean trypsin inhibitor, N-p-tosyl-L-lysine chloromethyl ketone (TPCK) and N-α-tosyl-L-lysine chloromethyl ketone (TLCK)), and 5 μM OK. The homogenate was centrifuged at 50,000 × g for 40 min, and the supernatant was re-centrifuged at 150,000 × g for 70 min. The high speed supernatant was heated at 85°C for 5 min and centrifuged at 150,000 × g for 30 min at 25°C. This final supernatant (the “heat stable fraction”) was used in immunoblot studies conducted with different phosphorylation-dependent and independent anti-tau antibodies (see below). We also isolated binding competent (BC) and binding incompetent (BI) tau as well as assembly competent (AC) and assembly stable fraction (“BC-tadAC”) was used in immunoblot studies conducted with different phosphorylation-dependent and independent anti-tau antibodies (see below).

**Phosphatase Preparations—**The crude phosphatase extract was isolated from brains as previously described (38) with modifications. Briefly, the brains of juvenile (P6) and adult (3-month-old) rats were homogenized in 3 volumes of cold Tris-HCl buffer, pH 7.0, containing 0.1 mM EDTA, 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol, 0.25 mM sucrose with a mixture of protease inhibitors. The homogenates were centrifuged at 12,000 × g for 30 min at 25°C. The supernatant was used in the protein phosphatase assays (e.g. AMP, inorganic phosphate, and GTP, dithiothreitol, Sephadex G-50 superfine, trifluoperazine (TFP), and MES were from Sigma; 125I-labeled goat anti-mouse IgG and 125I-labeled protein A-agarose and OK were from ICN; cantharidin and protein phosphatase assay kits were from Life Technologies, Inc.

**RESULTS**

**Adult Tau Proteins Are Phosphorylated at Sites Similar to Fetal Tau—**Fig. 2 shows the immunodetection of fetal tau (lane 1), as well as juvenile and adult (lanes 2–6) tau isoforms by several well characterized anti-tau mAbs. Note that juvenile and adult tau isoforms (including five of the six alternatively spliced CNS products of the tau gene) migrate at about 55–62 kDa.
Developmental Regulation of Phosphorylation of Tau in Rats

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![Western blot analysis of fetal, postnatal and adult rat tau.](image)

Nitrocellulose gel replicas were prepared from 7.5% SDS-polyacrylamide gels and representative Western blots are shown in Panels A-I to illustrate immuno-labeled tau bands. Lane 1, embryonic day 18 (E18, 15 µg); lane 2, post-natal day 7 (P7, 15 µg); lane 3, post-natal day 11 (P11, 15 µg); lane 4, post-natal day 19 (P19, 25 µg); lane 5, post-natal day 25 (P25, 40 µg); lane 6, adult tau (Ad, 3-month-old, 40 µg). The antibodies used here are labeled above each blot. The amount of protein loaded for the antibodies T46, T49, 304, and T1 are those mentioned above, while the amount of protein loaded for AT270, PHF1, and T3P was twice the amount used for T46, and four times this was loaded for the mAbs AT8 and AT180. Panels J-Q show quantitative data from PhosphorImager analysis of the immunoblots shown in Panels A-I except for 304 which is a phosphate-independent antiserum that recognizes the first amino-terminal insert in adult tau isoforms (Fig. 1).

The brain has a molecular mass of about 48 kDa on SDS-PAGE gels (Fig. 2, A and B). A polyclonal antibody to the first amino-terminal insert (antisera 304) (15) confirmed that tau proteins containing amino-terminal inserts (i.e. all but one of five adult tau proteins) are induced between P11 and P19 (Fig. 2C). Next,
we probed the phosphorylation state of tau during development (Fig. 2, D-I and L-Q). Fetal tau from E18 rat brains was heavily phosphorylated at all of the phosphorylation sites examined here, and there was a progressive decline in the extent of phosphorylation as the animal matured. For example, AT270 (which binds to rat tau phosphorylated at Thr\(^{172}\)) (50, 51) strongly labeled tau from E18 through P11, and the labeling intensity decreased from P19 into adulthood (Fig. 2, D and L). By contrast, AT8 (which binds to rat tau phosphorylated at Ser\(^{395}\)) (21) strongly recognized a tau band at E18, but AT8 labeling began to decline by P7, and only weak AT8 labeling was seen at P25 and in the adult rat brain (Fig. 2, E and M). Concomitant with the decrease in the extent of phosphorylation at the Ser\(^{180}\) site, there was an increase in the labeling of the tau band by the mAb T1 as the development of the CNS progressed. This signifies a decrease in the phosphorylation state of tau since T1 recognizes an epitope containing amino acid residues 180–198 in rat tau when no Ser in this region is phosphorylated (53) (Fig. 2, F and N). Taken together, the AT8 and T1 data indicate that, during embryogenesis and in the early postnatal period, tau is strongly phosphorylated at Ser\(^{180}\), but phosphates are removed progressively as the animal ages. A similar decrease in the extent of phosphorylation at Ser\(^{397}\) also was detected in adult tau by the antibody T3P (8, 19) (Fig. 2, G and O). However, the phosphorylation of Ser\(^{287}/Ser\(^{280}\) did not decrease as precipitously as Ser\(^{397}\) alone as demonstrated by the binding of tau with the mAb PHF1 (19, 48, 49) (Fig. 2, H and P) suggesting that Ser\(^{280}\) remains more phosphorylated in adult tau than Ser\(^{397}\). Finally, the phosphorylation of Thr\(^{212}\) appeared to echo that of Ser\(^{280}\) and Ser\(^{397}\) as demonstrated with the mAb AT180 (which recognizes tau phosphorylated at Thr\(^{212}\)) (51). Thus, our data clearly indicate that adult rat tau proteins are phosphorylated at the same sites (i.e. Thr\(^{172}\), Ser\(^{395}\), Thr\(^{212}\), Ser\(^{395}\), and Ser\(^{280}\)) as fetal tau although at reduced levels (Fig. 2, L-Q). Furthermore, the time course and the extent of dephosphorylation at specific sites appeared to be differentially regulated. Notably, Thr\(^{172}\) and Ser\(^{395}\) remained more phosphorylated in adult rat tau than did the other sites.

**Effects of Okadaic Acid on the Microtubule Binding Competency of Tau**—To determine the effects of the developmentally regulated phosphorylation state of tau on the binding affinity of tau phosphoisoforms for endogenous MTs at different stages, we performed MT binding assays (as shown schematically in Fig. 1A) (54) in the presence or absence of OK. Fig. 3A shows that OK only has a transient effect on the binding of tau to MTs at around P11 and P12 and this was most evident for the adult tau isoforms containing the first amino-terminal insert (Fig. 3C) which are detected initially at low levels around P11. Interestingly, the newly synthesized adult tau isoforms did not bind to MTs in the presence of OK suggesting that they may be
phosphorylated at sites that inhibit binding to MTs. Indeed, in the absence of OK, the adult tau isoforms were recovered in the MT pellets (Fig. 3C). Similar differences in the binding of adult tau to MTs were observed at P12. At this age, most of the adult tau isoforms did not bind to MTs in the presence of OK, whereas in the absence of OK, almost all of the adult tau proteins were bound to MTs.

Although OK only had a transient effect on the binding of tau to MTs, the amount of unbound tau progressively declined during development until about P17 when almost all of the tau isoforms were bound to MTs (Fig. 3D). In contrast, the amount of MTs recovered as polymers from brain extracts of different age animals was independent of the age of the animals and the presence or absence of OK since the same amount (about 60%) of MT subunits were recovered as MTs in the pellet fraction (Fig. 4). We next examined the regulation of tau phosphorylation during development. Phosphatases appeared to be turned on by P13 since the mAb PHF1 (which is specific for phosphorylated Ser396 and Ser404 in rat tau) showed little change in immunoreactivity before P13 whereas in the absence of OK, almost all of the adult tau isoforms were bound to MTs (Fig. 4). We next examined the regulation of tau phosphorylation by crude phosphatase extracts from adult rat brain. The dephosphorylation reaction was monitored by immunoblotting with mAb T49 indicating a down-shift in electrophoretic mobility of tau as revealed by Western blot analysis of AI-tubulin and AC-tubulin. AI-tubulin in the supernatant (S) and AC-tubulin recovered in the pellet (P) were extracted and electrophoresed onto 7.5% SDS-PAGE for immunoblot analysis using anti-β-tubulin antibodies. The amount of protein loaded for the supernatant (S) fraction (approximately 30 μg for S) was normally three times that loaded for the pellet (P) fraction, but the ratio of the proteins in these fractions was maintained. Key: + = tubulin isolated in presence of OK; - = tubulin isolated in absence of OK (see Fig. 1A).

**Phosphatase Activities in Developing and Adult Rat Brain Extracts**—To probe the regulation of tau phosphorylation in rat brain, we determined the activities of protein phosphatases in fresh brains from P6 and adult rats using the smallest or fetal isoform of tau isolated from P7 rat brains as substrate. Fig. 5 shows the time course of dephosphorylation of P7 rat brain tau using crude protein phosphatases from the adult rat brain. The activity of the crude protein phosphatases (at various dilutions) was first tested using [32P]phosphorylase as substrate, and at higher dilutions there was almost complete dephosphorylation as measured from the counts/min released (data not shown). Fig. 5A shows immunoblot data using mAb T49 indicating a time dependent dephosphorylation of tau as evidenced by a down-shift in electrophoretic mobility (9, 15, 19). Also, PHF1 reactivity declined by 10 min with negligible reactivity after 60 min (Fig. 5B). Quantitative studies confirmed these observations (Fig. 5C).

To identify the protein phosphatases involved in these events, we performed similar assays in the presence or absence of various phosphatase inhibitors. Fig. 6 shows the effect of various phosphatase inhibitors on the dephosphorylation of P7 rat tau by protein phosphatases from P6 and adult rat brain. Panels A and B show T49 and PHF1 immunoreactivity, respectively, using adult rat brain extract. Note that after 60 min of incubation with phosphatases in the brain extract (lane 2) there is significant down-shift in the mobility of tau as revealed with mAb T49 and a loss in PHF1 immunoreactivity. These data are consistent with the data shown in Fig. 5 (lane 5). Highly diluted extracts of brain and other tissues, when incubated with [32P]phosphorylase as substrate, showed that PP1 and PP2A were completely inhibited by 10 nM OK, whereas PP2B was unaffected at this concentration (55). Conversely, PP2B was inhibited specifically by TFP as well as by higher concentrations of OK (5 μM) (39). At a concentration of 10 nM, OK partially restored PHF1 immunoreactivity, while 5 μM OK completely inhibited the dephosphorylation of tau at this site, suggesting that both PP2A and PP2B are involved in the dephosphorylation of tau at Ser396 (Fig. 6, A and B, lanes 3 and 4). This interpretation was supported by studies using cantharidin (for PP2A) and TFP (for PP2B). As seen in Fig. 6, A and B (lanes 5 and 6) 100 and 500 nM cantharidin (but not concen-
We probed dynamic changes in the phosphorylation state of tau in the developing rat brain, and we showed that fetal tau is strongly phosphorylated at Thr$^{172}$, Ser$^{193}$, Thr$^{212}$, Ser$^{287}$, and Ser$^{397}$, and that the phosphorylation of Ser$^{397}$, Thr$^{212}$, and Ser$^{287}$ persisted until P19 after which time these sites were dephosphorylated with a concomitant increase in the levels of the other five mature tau isoforms. However, phosphorylation at Ser$^{397}$ and Thr$^{172}$ persisted through to the adult rat brain (Fig. 2) consistent with previous mass spectroscopy analyses (23). In contrast, Ser$^{397}$, Ser$^{397}$, and Ser$^{404}$ are phosphorylated in tau at 20, 23, and 39 weeks of human gestation, but not in the neonatal or adult brain (19, 21). This loss in AT8, PHF1, and T3P immunoreactivity in the mature brain is due to the dephosphorylation of tau at these sites by phosphatases during the isolation of tau from post-mortem brains (24). Our studies in the rat indicate that when tau is extracted with a post-mortem delay in the presence of OK, phosphorylation of these sites is retained (Fig. 2), just like biopsy-derived human tau (24). However, the present study also demonstrates the extent to which different phosphorylation sites are developmentally regulated since specific sites are preferentially down-regulated as the rat brain matures. For example, Ser$^{193}$ is almost completely dephosphorylated whereas Thr$^{172}$ remains partially phosphorylated in the adult rat brain.

Previous studies have shown that binding of tau to MTs is modulated by phosphorylation as well as by the developmentally regulated increase in the number of MT-binding repeats. Fetal forms of rat and human tau have only three repeats with no amino-terminal inserts, and they do not bind to MTs as well as tau isoforms containing four repeats (13, 56). The shift from the immature doublet at approximately 50 kDa to the complex series of 55–62-kDa bands occurs between P10 and P15. This coincides with our data showing that tau isoforms with the first amino-terminal insert initially appear around P11 and continue to be expressed into adulthood. The time around P8 to P11 is a developmental epoch in the biochemical maturity of the brain that also affects the expression of kinases/phosphatases. Our studies demonstrate that around this time 1) kinase activities begin to decrease (as measured by the decrease in immunoreactivity for the antibodies AT8, AT180, PHF1, and T3P); 2) phosphatase activities (as measured by the inhibition of dephosphorylation by OK) increase; and 3) the binding of tau to MTs also increases concomitant with the increased expression of the adult tau isoforms. Before P11/P12, about 60–70% of the fetal tau proteins are recovered with MT polymers, whereas after that time, almost all of the tau isoforms are recovered with the MT polymers. These data imply that phosphorylation at specific sites in tau may not regulate the binding of tau to MTs. However, it should be pointed out that the amount of tubulin and MT polymers in the total brain extracts that are available to be bound by tau far exceeds that found within neurons alone since the total tubulin, and MTs in brain extracts are derived from all of the different cell types in the brain (e.g., neurons as well as astrocytes and oligodendrocytes). Thus, it may not be possible to evaluate the more subtle regulation of the binding of tau to brain-derived MTs by phosphorylation in neurons due to the greater abundance of MTs in many different cell types in the adult brains.

Previous studies have demonstrated that the Ser/Thr protein phosphatases PP2A1 and PP2B (calcineurin) can dephospho-
rlylate tau in vitro (20, 25, 36). In this study, we determined the phosphatase activities of the brain extracts from P6 and adult rat using fetal tau as a substrate for these phosphorylations. With these brain extracts, the phosphates on Ser181/182 were removed as early as 10 min after sacrifice with complete dephosphorylation after 60 min using brain extracts from adult rats. In contrast, brain extracts from P6 rats were incapable of dephosphorylation toward different phosphorylation sites. For example, complete dephosphorylation was observed for Ser181/182, Ser391 and Thr212 (as indicated by mAbs PHF1 and AT180), whereas Ser193 and Thr172 were only partially dephosphorylated (as detected by mAbs AT8 and AT270, respectively). This lack of complete dephosphorylation of Ser193 and Thr172 suggests that the specific phosphatase activities that could completely dephosphorylate tau at these sites are either inactivated during processing of our brain extracts or that the conditions that we used for assaying the phosphatases are not favorable for probing the activation of this phosphatase. However, we did observe that PP2A is involved in regulating the phosphorylation of tau at Ser193, Ser181/182, Ser391 and Thr212 since dephosphorylation at these sites can be inhibited by OK (10 nm) and cantharidin, both of which are potent inhibitors of PP2A. Although TFP (an inhibitor of PP2B) did not restore the phosphorylation of tau at Ser193, it did partially restore the phosphorylation at Ser181/182 and Thr212 and Thr222. Thus, it appears that PP2A and PP2B (and possibly a third unidentified phosphatase) are involved in the selective dephosphorylation of tau. Our data also point to PP2A as the major phosphatase activity in our brain extracts that dephosphorylate tau.

Until very recently, studies on the abnormal phosphorylation of PHF-tau focused on the identification of the kinases that might be involved in the generation of PHFs in AD while little attention was paid to the possible involvement of phosphatase(s) in the pathogenesis of neurofibrillary tangles in AD. Although we cannot specify the precise mechanisms leading to the generation of PHF-tau in the AD brain, a decrease in the expression of phosphatases or an increase in the levels of phosphatase inhibitors in the AD brain also may contribute to the generation of PHF-tau, the in vivo deposition of PHFs in neurons, and possibly other AD-associated neuropathological changes. Accordingly, the present results suggest that the developing brain may be a useful system in which to study the kinases and phosphatases involved in regulating the dynamic phosphorylation and dephosphorylation of tau. The elucidation of the mechanisms underlying these phenomena could lead to insights into the pathological events involved in the development of the neurofibrillary pathology in the AD brain.

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REFERENCES