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The abnormal phosphorylation of tau protein at Ser-202 in Alzheimer disease recapitulates phosphorylation during development

(fetal tau/adult tau/paired helical filament/neuropathology)

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ABSTRACT **Tau is a neuronal phosphoprotein whose expression is developmentally regulated. A single tau isoform is expressed in fetal human brain but six isoforms are expressed in adult brain, with the fetal isoform corresponding to the shortest of the adult isoforms. Phosphorylation of tau is also developmentally regulated, as fetal tau is phosphorylated at more sites than adult tau. In Alzheimer disease, the six adult tau isoforms become abnormally phosphorylated and form the paired helical filament, the major fibrous component of the characteristic neurofibrillary lesions. We show here that Ser-202 (in the numbering of the longest human brain tau isoform) is a phosphorylation site that distinguishes fetal from adult tau and we identify it as one of the abnormal phosphorylation sites in Alzheimer disease. The abnormal phosphorylation of tau at Ser-202 in Alzheimer disease thus recapitulates normal phosphorylation during development.**

Microtubule-associated protein tau promotes the assembly and stabilization of neuronal microtubules (for review, see ref. 1). The pattern of tau expression is developmentally regulated; in fetal human brain there is a single isoform but in adult brain there are six isoforms. The different isoforms are produced from a single gene by alternative mRNA splicing (2–8). The fetal isoform corresponds to the smallest of the adult isoforms (4, 7). Tau is a phosphoprotein, as shown by its increased gel mobility after dephosphorylation. Developmental regulation extends also to the level of phosphorylation, since the change in mobility is greater for fetal tau than it is for adult tau (for review, see ref. 1). We here identify one of the sites of phosphorylation that distinguishes fetal from normal adult tau and show that it corresponds to one of the sites that becomes abnormally phosphorylated in Alzheimer disease.

The most striking feature of the primary structure of tau is stretches of 31 or 32 amino acids, repeated three or four times in the C-terminal half of the molecule, that constitute microtubule binding units (2–10). Additional isoforms contain 29- or 58-amino acid insertions in the N-terminal region in conjunction with three or four repeats, giving rise in adult human brain to a total of six isoforms (11, 12). The shortest isoform is 352 amino acids long and contains three repeats, whereas the largest isoform is 441 amino acids long and contains four repeats and the 58-amino acid insertion.

In Alzheimer disease, tau becomes abnormally phosphorylated and self-associates through the microtubule-binding domain to form the paired helical filament (PHF) (13–15), the principal fibrous component of the neurofibrillary pathology. Neurofibrillary changes consist of neurofibrillary tangles, neuropil threads, and senile plaque neurites; they appear within the vast majority of nerve cells that degenerate during the course of the disease, where their presence is indicative of dementia (for review, see ref. 16). PHF tau is unable to bind to microtubules, but after dephosphorylation PHF tau does bind to microtubules, indicating the consequences of abnormal phosphorylation (17). By SDS/PAGE, PHF tau migrates as three bands of 60, 64, and 68 kDa (apparent molecular mass) (13, 18, 19) that do not align with native or recombinant tau (20). Alignment is achieved after dephosphorylation with alkaline phosphatase, indicating that PHF tau is abnormally phosphorylated on all six isoforms (20).

Antibodies have been produced that recognize PHF tau in a phosphorylation-dependent manner but fail to recognize normal adult or recombinant tau, permitting the identification of some of the abnormally phosphorylated sites. Thus, antiserum T3P and antibody PHF1 recognize Ser-396 in a phosphorylated state (13, 21, 22), whereas antibody AT8 requires Ser-199 and/or Ser-202 to be phosphorylated (23, 24) [in the numbering of the longest human brain tau isoform (11)].

We show here that AT8 recognizes tau from immature brain in a phosphorylation-dependent manner and that in human brain this recognition ceases abruptly around birth. Using site-directed mutants, we show that the AT8 epitope requires only Ser-202 to be phosphorylated. In preparations from Alzheimer disease brain, AT8 recognizes the abnormal PHF tau bands on immunoblots and decorates PHFs in a phosphorylation-dependent manner. In histological sections of human fetal spinal cord, AT8 stains nerve cell axons. The abnormal phosphorylation of Ser-202 in Alzheimer disease thus recapitulates a normal developmental stage.

MATERIALS AND METHODS

Expression and Purification of Recombinant Tau Proteins. A full-length cDNA clone (htau24) corresponding to a four-repeat isoform of tau and with an *Nde* I site in the context of the initiator codon (12) was subcloned into the *Eco*RI site of M13mp18. Site-directed mutagenesis was used to change codon 199 (Ser → Ala; S199A tau) or codon 202 (Ser → Ala; S202A tau) [using the numbering of the longest human brain tau isoform (11)]; a third construct was made, where both codons 199 and 202 were changed to Ala (S199A/S202A tau). After cleavage with *Nde* I and *Eco*RI, the resulting fragments were subcloned downstream of the T7 RNA polymerase

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Abbreviation: PHF, paired helical filament.
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promoter in the expression plasmid pRK172 and the recombinant plasmids were transformed into *Escherichia coli* BL21(DE3) cells. The bacterial cultures were grown and induced, and tau proteins were purified as described (12). The six human brain tau proteins were expressed as described (12).

Phosphorylation of Recombinant Tau by Brain Protein Kinase Activity. Adult rat brain was homogenized (1 g/2.5 ml) in 10 mM Tris·HCl, pH 7.4/5 mM EGTA/2 mM dithiothreitol/1 μM okadaic acid/1 mM phenylmethylsulfonyl fluoride/leupeptin (20 μg/ml)/aprotinin (20 μg/ml)/pepstatin (20 μg/ml) and centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was used directly for phosphorylation. Incubations (0.05 ml) were carried out at 37°C in 40 mM Hepes, pH 7.2/2 mM ATP/2 mM $MgCl_2$/1 μM tau protein/rat brain extract (1 μl)/5 mM EGTA/2 mM dithiothreitol/1 μM okadaic acid/1 mM phenylmethylsulfonyl fluoride/leupeptin (20 μg/ml)/aprotinin (20 μg/ml)/pepstatin (20 μg/ml). Reactions were initiated by addition of the brain extract and incubated for 24 h, and aliquots of reaction products were used for SDS/PAGE. Controls were incubated under the same conditions, except that the brain extract was omitted.

Extraction of Tau Protein. Rat brains were homogenized in 2.5% (vol/vol) perchloric acid (0.5 g/ml), allowed to stand on ice for 20 min, and centrifuged for 10 min at 10,000 × *g*. The supernatant was dialyzed overnight at 4°C against 5% (vol/vol) formic acid and recentrifuged. The resulting supernatant was concentrated for immunoblot analysis by using a speed vacuum apparatus. Human brain cerebral cortex was homogenized in 0.1 M Mes/0.5 mM $MgSO_4$/1 mM EGTA/2 mM dithiothreitol/750 mM NaCl, boiled for 10 min, and centrifuged for 10 min at 10,000 × *g*. The heat-stable supernatant was treated with 2.5% perchloric acid, dialyzed against distilled water, and concentrated by ammonium sulfate precipitation before immunoblot analysis. PHF tau proteins were extracted from the cerebral cortex of Alzheimer disease patients, as described (20). Alkaline phosphatase treatment was done for 1 h at 67°C with *E. coli* alkaline phosphatase (type III-N; Sigma; 18 units/ml).

Immunoblots. SDS/PAGE was carried out using 10% or 10–20% gradient minigels. For immunoblot analysis, the proteins were transferred to a poly(vinylidene difluoride) membrane and residual protein binding sites were blocked with 1% gelatin in phosphate-buffered saline. The blots were then incubated for 5 h at room temperature with unfractionated anti-tau antiserum 133 or 134 (dilution 1:250) (11) or with monoclonal antibody AT8 (dilution 1:500). Bound antibody was detected by the biotin/peroxidase system (Vectastain; Vector, Peterborough, U.K.).

Electron Microscopy. Dispersed PHFs were extracted from an Alzheimer disease brain as described (20). Methods for electron microscopy and immunolabeling were as described (20). AT8 was used at 1:90 dilution, whereas the monoclonal antibody Tau1 (Boehringer Mannheim) was used at a dilution of 1:100. Filaments were dephosphorylated by treatment with *E. coli* alkaline phosphatase (type III-N, Sigma) at 13.5 units/ml for 12 h at 67°C. Micrographs were recorded on a Philips EM301, at an operating voltage of 80 kV and at a nominal magnification of ×25,000.

Immunohistochemistry. Immunohistochemistry was performed using the monoclonal antibody AT8 on several different regions of fetal and adult human central nervous system. Spinal cord samples were obtained from fetuses at gestational ages of 8, 11, 17, 20, 23, 38, and 40 weeks and fixed in 70% ethanol/150 mM NaCl. A block of hippocampus from a patient with Alzheimer disease fixed in 70% ethanol/150 mM NaCl was also examined. All tissues were fixed within a postmortem interval of 1–23 h. (These studies were approved by the Committee on Studies involving Human Beings at the University of Pennsylvania School of Medicine.)

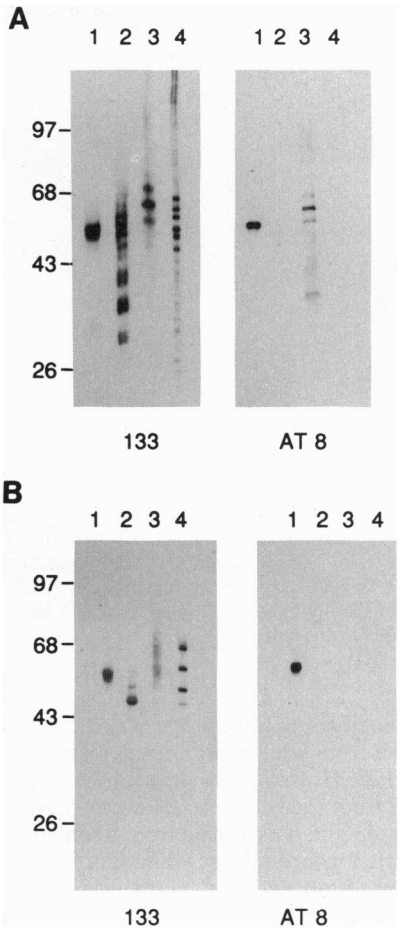

FIG. 1. Immunoblots of fetal and adult brain tau and of PHF tau with anti-tau antiserum 133 and monoclonal antibody AT8. (*A*) Lanes: 1, fetal human brain (20 weeks of gestation; 0.5 μg); 2, adult human brain (1 μg); 3, PHF tau (1 μg); 4, recombinant human brain tau isoforms (1 μg). (*B*) Lanes: 1, newborn rat brain (2 μg); 2, newborn rat brain, alkaline phosphatase-treated (2 μg); 3, adult rat brain (2 μg); 4, adult rat brain, alkaline phosphatase-treated (2 μg).

The processing of the tissue samples into paraffin blocks and the immunohistochemical procedures used have been described (25, 26).

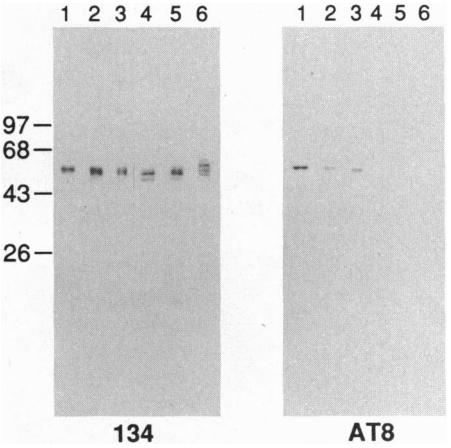

FIG. 2. Immunoblots of fetal, postnatal, and adult human brain tau with anti-tau antiserum 134 and monoclonal antibody AT8. Lanes: 1, 20 weeks of gestation (0.5 μg); 2, 23 weeks of gestation (1.5 μg); 3, 39 weeks of gestation (2 μg); 4, postnatal day 2 (3.5 μg); 5, postnatal day 5 (1 μg); 6, adult (1 μg).

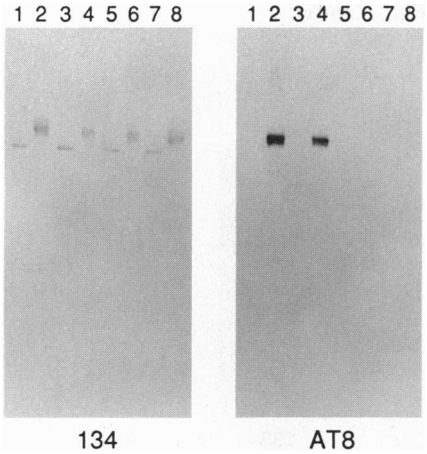

FIG. 3. Phosphorylation of wild-type and mutated recombinant tau (expressed from clone htau24) with the protein kinase activity from rat brain. Immunoblots with anti-tau antiserum 134 and monoclonal antibody AT8. Lanes: 1, tau24; 2, tau24 plus brain extract; 3, S199A tau24; 4, S199A tau24 plus brain extract; 5, S202A tau24; 6, S202A tau24 plus brain extract; 7, S199A/S202A tau24; 8, S199A/S202A tau24 plus brain extract.

RESULTS

AT8 is a monoclonal antibody that recognizes PHF tau but not normal tau from adult brain or recombinant tau (refs. 23 and 24; Fig. 1*A*). However, as shown here, AT8 recognized tau extracted from the brain of a 20-week human fetus (Fig. 1*A*). It also stained tau extracted from neonatal rat brain but not tau extracted from adult rat brain; moreover, the staining by AT8 of tau from neonatal brain was phosphorylation-dependent, as shown by its absence after alkaline phosphatase treatment (Fig. 1*B*). When human brain was used at various developmental stages, AT8 stained tau from fetal brains at 20, 23, and 39 weeks of gestation, but not tau from neonatal or adult brain (Fig. 2). When stained with a tau antiserum that is not phosphorylation-dependent, fetal tau appears as one broad band. AT8 stained the slowest migrating species, indicating that only a proportion of the fetal tau molecules is phosphorylated at the AT8 epitope (Figs. 1 and 2).

A previous study had shown that AT8 requires Ser-199 and/or Ser-202 of tau to be phosphorylated (23). We expressed the 383-amino acid human tau isoform in *E. coli* [from cDNA clone htau24, (4)] and used site-directed mutagenesis to express recombinant S199A tau, S202A tau, and S199A/S202A tau. The recombinant proteins were then phosphorylated by a protein kinase activity from rat brain, separated by SDS/PAGE, and analyzed on an immunoblot using AT8 or a tau antiserum that is not phosphorylation-dependent. As shown (17, 23), phosphorylation with brain extract resulted in a marked reduction in the gel mobility of recombinant tau (Fig. 3). AT8 did not stain wild-type or mutant tau proteins prior to brain extract phosphorylation. However, after a 24-h incubation with brain extract, AT8 recognized wild-type and S199A tau, but not S202A tau or S199A/S202A tau. This establishes that staining by AT8 requires only Ser-202 to be phosphorylated.

By immunoelectron microscopy AT8 decorated ≈60% of dispersed PHFs (Fig. 4*A*). The labeling was phosphorylation-dependent, since it was absent from PHFs treated with alkaline phosphatase (Fig. 4*B*). This contrasts with the labeling by Tau1, a monoclonal antibody that requires tau to be unphosphorylated in the region of the AT8 epitope (27–29). Tau1 decorated very few dispersed PHFs (Fig. 4*C*), but their number was greatly increased after alkaline phosphatase treatment (Fig. 4*D*).

Immunohistochemistry was used to investigate the distribution of AT8 staining in the developing human nervous system. In human spinal cord from an 8-week embryo, AT8 staining was restricted to the white matter of the marginal zone, where it was mostly present in ventral white matter (Fig. 5*A*). An identical staining pattern has been observed (26) with the anti-tau antisera 133 and 134, which are not phosphorylation-dependent. No immunoreactivity was observed with AT8 at subsequent gestational ages. In Alzheimer disease, AT8 intensely labeled neurofibrillary tangles, senile plaque neurites, and a dense network of neuropil threads in the hippocampus (Fig. 5*B*).

DISCUSSION

Phosphorylation of tau protein occurs at certain sites both in normal development and in the pathological setting of Alzheimer disease. These sites are phosphorylated in tau from fetal human brain, become dephosphorylated around birth, and remain nonphosphorylated in normal life. The switch in phosphorylation state presumably serves some presently

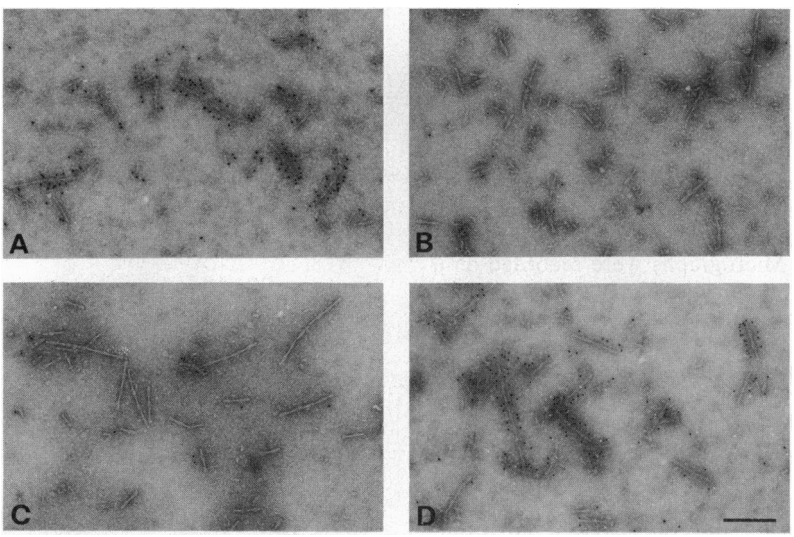

FIG. 4. Immunoelectron microscopy of dispersed PHF preparations. (*A* and *C*) Native material. (*B* and *D*) Grids were treated with alkaline phosphatase prior to immunodecoration. The monoclonal antibodies were AT8 (*A* and *B*) or Tau1 (*C* and *D*). Note immunodecoration of filaments with AT8 in *A* but not in *B* and with Tau1 in *D* but not in *C*. (Bar = 300 nm.)

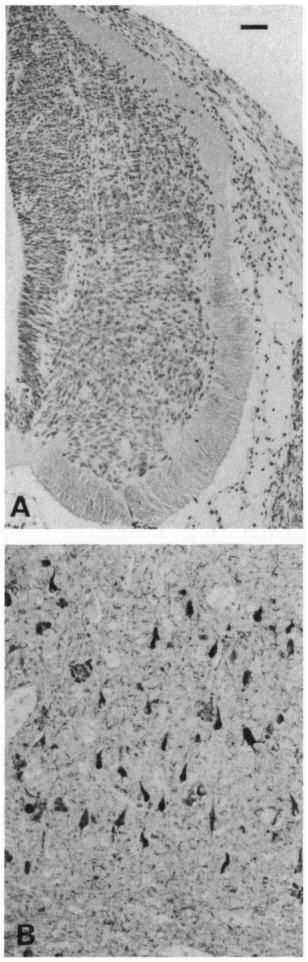

FIG. 5. Immunostaining of human nervous system tissues with monoclonal antibody AT8. A section of 8-week (gestational age) spinal cord is shown in *A* and a section through the subiculum of the hippocampus from an Alzheimer disease patient is shown in *B*. Note the white matter staining in *A* and the staining of neurofibrillary tangles, neuropil threads, and senile plaque neurites in *B*. The sections were counterstained with hematoxylin and enlarged to the same extent. (Bar in *A* = 5 μm.)

unknown control function, possibly related to microtubule binding. In Alzheimer disease, these sites become abnormally phosphorylated, thereby upsetting the normal functioning of cells in which the change occurs. To understand these processes, both normal and pathological, it is important to discover which sites are phosphorylated and which protein kinases and protein phosphatases are involved.

One way to identify amino acids that are abnormally phosphorylated in PHF tau is to use antibodies that label PHF tau in a phosphorylation-dependent manner but do not label normal adult brain tau. Such antibodies can then be used to see whether the corresponding site is phosphorylated in fetal tau. Thus, antiserum T3P (13) and antibody PHF1 (21, 22) permitted identification of Ser-396 as one site in PHF tau that is abnormally phosphorylated. A recent study used antibody AT8 to show that Ser-199 and/or Ser-202 are abnormally phosphorylated (23). In the present study, we expressed recombinant tau proteins with S199A, S202A, and S199A/S202A mutations, phosphorylated these proteins *in vitro* using an adult rat brain extract, and analyzed them on an immunoblot with AT8. As shown (23), AT8 did not recognize wild-type or mutant tau proteins before phosphorylation by the brain extract. However, after phosphorylation, AT8 recognized wild-type tau and S199A tau but not S202A tau or S199A/S202A tau. This demonstrates that AT8 requires only Ser-202 to be phosphorylated and identifies Ser-202 as a site that is abnormally phosphorylated in PHF tau.

The above results also show that in adult rat brain extract there exists a kinase activity that phosphorylates recombinant tau at Ser-202, despite the fact that tau extracted from adult rat brain is not phosphorylated at this site. The phosphorylation state of a protein results from a balance between protein kinase and protein phosphatase activities. The brain extract experiments were carried out for a prolonged time in the presence of okadaic acid, to maximize phosphorylation. This could explain the fact that phosphorylation of recombinant tau at Ser-202 was obtained with the adult rat brain extract. Alternatively, the difference in phosphorylation between brain tau and recombinant tau incubated with brain extract could simply reflect a different compartmentalization of tau and the protein kinase activity.

By immunoelectron microscopy AT8 decorated PHFs in a phosphorylation-dependent manner, as shown by the absence of specific labeling after alkaline phosphatase treatment. This contrasted with the labeling by Tau1, an antibody whose epitope is in the same region of tau as the AT8 epitope (27–29). Tau1 only labeled PHFs after dephosphorylation, in agreement with previous observations showing that it recognizes tau only in a nonphosphorylated state (27–29). Thus, PHFs can be dephosphorylated at least at some sites without losing their characteristic morphology. This is in keeping with the finding that recombinant microtubule-binding domains of tau assemble into PHF-like filaments (14, 15), although it is probably the abnormal phosphorylation of tau that leads to PHF assembly in Alzheimer disease.

In the present study we observed that immature tau is labeled by AT8 in a phosphorylation-dependent manner, implying that it is normally phosphorylated at Ser-202. Thus, on immunoblots of human brain, AT8 stained immature tau at 20, 23, and 39 weeks of gestation, but not at postnatal days 2 and 5. The switch from the phosphorylated to the nonphosphorylated state occurs rapidly around birth. When using tau antibodies that are independent of phosphorylation, immature tau appears as one broad band that shifts to the position of the shortest recombinant tau isoform upon dephosphorylation with alkaline phosphatase (11). AT8 recognized the upper part of the immature tau band before dephosphorylation, indicating that the slowest migrating immature tau species are phosphorylated at Ser-202. Immature tau is less efficient than adult tau in promoting microtubule assembly (30). This results in part from the fact that the immature three-repeat-containing tau isoform is less effective at binding to microtubules (10) and at promoting microtubule assembly (11) than adult four-repeat-containing tau isoforms. This difference is likely to be increased by the specific phosphorylation of immature tau. We have demonstrated (17) that PHF tau is severely impaired in its ability to bind to microtubules and that it regains this ability upon dephosphorylation. Moreover, we have shown that the phosphorylation of Ser-396 contributes to the reduced microtubule binding. Like Ser-202 we found Ser-396 to be phosphorylated in immature tau, as judged by staining with antiserum T3P and antibody PHF1 (17). In addition, antibodies isolated from two antisera raised against PHF preparations were recently reported to stain fetal tau (31).

By immunohistochemistry on fetal human spinal cord, AT8 stained axons. Tau in adult central nervous system is also largely confined to axons (27). This contrasts with Alzheimer disease, where abnormally phosphorylated tau is found as PHFs in cell bodies, dendrites, and abnormal neurites (for review, see ref. 16). Thus, although during development tau is phosphorylated at some of the same sites as in PHF tau, it is nonetheless confined to its characteristic axonal location.

Recently, mass spectrometry was used to identify Thr-231, Ser-235, and Ser-262 as additional abnormal phosphorylation sites in PHF tau (32). With the exception of Ser-262, all the known abnormal phosphorylation sites in tau are SP or TP motifs. Thus, protein kinases or protein phosphatases with specificity for phosphoserylproline and phosphothreonylproline may be involved. Two brain protein kinases (PK36 and PK40) phosphorylate tau at Ser-396 and other Ser/Thr-Pro sites (33) and a kinase called tau protein kinase I also phosphorylates tau at Ser-396 and other Ser/Thr-Pro sites (34). Moreover, p42 mitogen-activated protein (MAP) kinase phosphorylates recombinant tau at most SP and TP sites, including the AT8 and T3P epitopes (35–37). Tau phosphorylated in this manner is severely impaired in its ability to bind to microtubules (38). Of the major brain protein phosphatases, tau phosphorylated by p42 MAP kinase can be dephosphorylated only by protein phosphatase 2A, with phosphatase $2A_1$ as the most effective form of the enzyme (37).

The present results suggest that at least some of the mechanisms that are active during development and are progressively switched off during normal brain maturation may be reactivated in Alzheimer disease. One difference between fetal and Alzheimer disease brain is that in the former only the smallest tau isoform is present to be phosphorylated, whereas in the latter all six brain isoforms are present and become abnormally phosphorylated. This difference could contribute to the pathology. The developing brain may constitute a good system for investigating the regulation of tau phosphorylation. Elucidation of the mechanisms underlying these phenomena could lead to the development of strategies aimed at preventing or retarding the appearance of the neurofibrillary pathology of Alzheimer disease.

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