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Aluminum Modifies the Properties of Alzheimer's Disease PHFτ Proteins *in vivo* and *in vitro*

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Hyperphosphorylated adult human CNS τ (PHFτ or A68) forms paired helical filaments (PHFs) in neurofibrillary tangles (NFTs), neuropil threads, and dystrophic neurites associated with senile plaques (SPs) during the progression of Alzheimer's disease (AD). While amyloid fibrils in SPs are composed of β-amyloid (Aβ), NFTs and SPs contain similar associated components such as ubiquitin, α_1-antichymotrypsin (ACT), apolipoprotein E (ApoE), heparan sulfate proteoglycans (HSPGs), and aluminum salts. Thus, SPs and NFTs may result from specific interactions between PHFτ, Aβ, and these other components. In fact, intracerebral injections of PHFτ induce co-deposits of Aβ, ACT, and ubiquitin (Shin et al., 1993). To examine this issue further, we probed interactions between PHFτ, aluminum salts, and other plaque and tangle components.

We investigated *in vivo* interactions of PHFτ and aluminum chloride ($AlCl_3$) with other plaque and tangle components by injecting PHFτ with and without $AlCl_3$ into the rodent brain. PHFτ co-injected with $AlCl_3$ formed aggregates that persisted much longer in the rat brain, and induced longer-lived co-deposits of Aβ, ubiquitin, ACT, and ApoE than PHFτ alone. Injections of PHFτ with $AlCl_3$ also induced neurons near the injection site to acquire PHFτ-like properties as monitored with antibodies (AT8, T3P, PHF1) that recognize defined PHFτ epitopes containing phosphoserine residues (Ser^{202}, Ser^{396}, Ser^{404}). Injections of $AlCl_3$ alone as well as injections of normal adult and fetal CNS τ, several different synthetic peptides, neurofilament proteins, ACT, HSPGs, or ApoE with and without $AlCl_3$ failed to induce co-deposits of Aβ or alter the immunoreactivity of τ in rodent neurons.

To determine if aluminum salts interact directly and specifically with PHFτ *in situ*, we pretreated sections of AD hippocampus with 10 mM $AlCl_3$ and then probed these sections by immunohistochemistry with antibodies to PHFτ as well as to a number of other plaque and tangle components. Preincubation of these sections with $AlCl_3$ diminished PHFτ immunoreactivity in NFTs and SPs using the PHFτ-specific antibodies AT8, T3P, and PHF1, while the immunoreactivity of other plaque and tangle proteins (Aβ, ubiquitin, ACT, HSPGs, ApoE) was not abolished. We also examined the effects of $AlCl_3$ on PHFτ and normal adult human CNS τ *in vitro*. $AlCl_3$ had no effect on normal adult human CNS τ, while increasing concentrations of $AlCl_3$ (from 0.1 to 1.0 mM) induced PHFτ to aggregate at the top of the stacking gel, and at high concentrations (0.3 and 1.0 mM) of $AlCl_3$, PHFτ completely failed to enter the gel.

These studies suggest that aluminum binds to PHFτ, induces these proteins to aggregate, and retards their proteolysis. Further, since intracerebral injections of PHFτ with and without $AlCl_3$ in rats appear uniquely capable of inducing co-deposits of a number of proteins found in authentic AD SPs and NFTs (including Aβ, ubiquitin, ACT, and ApoE), we speculate that the contributions of PHFτ to plaque and tangle formation in AD may be modulated by aluminum.

[Key words: aluminum, tau proteins, PHFs, β-amyloid, Alzheimer's disease, neurofibrillary tangles]

The major histopathologic abnormalities that characterize the brains of patients with sporadic and familial Alzheimer's disease (AD) as well as elderly individuals with Down's syndrome include an excess of neurofibrillary lesions, amyloid-rich senile plaques (SPs), and the massive loss of telencephalic neurons (for recent reviews, especially to literature prior to 1990, see Kosik, 1992; Lee and Trojanowski, 1992; Goedert, 1993; Hardy, 1993; Mullan and Crawford, 1993; Selkoe, 1993; Trojanowski et al., 1993b, 1994). The neurofibrillary lesions in AD are composed of paired helical filaments (PHFs) that are biochemically and structurally distinct from the amyloid fibrils in SPs. PHFs accumulate with straight filaments and amorphous material in perikaryal neurofibrillary tangles (NFTs), dystrophic neurites associated with SPs, and in neuropil threads (NTs) throughout the neuropil. PHFs and the straight filaments that dominate ultrastructural images of neurofibrillary lesions are formed from hyperphosphorylated adult CNS τ proteins known as PHFτ or A68 (for reviews, see Goedert et al., 1993; Trojanowski et al., 1993b, 1994). In contrast, the subunit proteins of amyloid fibrils are 39–43 amino acid (aa) long β-amyloid peptides (Aβ) derived from one or more larger, alternatively spliced amyloid precursor proteins (APPs) encoded by a gene on chromosome 21 (for detailed reviews, see Hardy, 1993; Mullan and Crawford, 1993; Selkoe, 1993). SPs are sites at which abundant accumulations of both PHFs and amyloid fibrils converge (Trojanowski et al.,

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Table 1. Summary of injections and animals

Sample injected	Postinjection survivals 1 hr	1–2 days	1 week	2/4 weeks	8 weeks
PHF τ	1	4*	3		
PHF τ + $AlCl_3$		4*	4	4/4	2
Aβ		2*			
Aβ + $AlCl_3$		2*			
ACT	2	3			
ACT + $AlCl_3$		2			
APOE		2*			
APOE + $AlCl_3$		2*			
APOE3		2*			
APOE3 + $AlCl_3$		2*			
APOE4		2*			
APOE4 + $AlCl_3$		2*			
HSPG		2*			
HSPG + $AlCl_3$		2*			
A τ		2*			
A τ + $AlCl_3$		4*			
F τ		4*			
F τ + $AlCl_3$		2*	2		
Syn τ		5			
NF-H		2			
NF-H + $AlCl_3$			2	2/0	
$AlCl_3$	2	2	2	2/2	2

This table summarizes the injected samples and the postinjection survival times of the animals studied here. The first column lists the component that was injected, while the subsequent columns indicate the postinjection survival times. The numbers in each column indicate the number of animals studied at each time point. The 2 and 4 week survival times (2/4 weeks) are grouped together in the same column. In at least two of the rats among the groups of animals identified by an asterisk, bilateral injections were performed in which the protein sample alone was injected into one hemisphere, and the same protein sample plus $AlCl_3$ was injected in the opposite hemisphere. A τ, adult CNS τ; F τ, fetal CNS τ; Syn τ, synthetic τ peptides; UB, ubiquitin. Other abbreviations are the same as those used in the text.

1993b, 1994). Despite the presence of APP mutations in rare cases of familial AD, the abundance of NFTs correlates better with the dementia in AD than the density of amyloid plaques (Dickson et al., 1991; Arriagada et al., 1992; Goedert, 1993).

Although PHFs and Aβ amyloid fibrils are the most distinct structural elements in NFTs and SPs, respectively, a number of other components selectively accumulate in SPs and NFTs, including ubiquitin (Mori et al., 1987; Perry et al., 1987; Shaw and Chau, 1988), α_1-antichymotrypsin (ACT) (Abraham et al., 1988; Gollin et al., 1992), heparan sulfate proteoglycans (HSPGs) (Snow et al., 1988; Perlmutter et al., 1991; Perry et al., 1991), and apolipoprotein E (ApoE), a risk factor for sporadic and familial AD (Namba et al., 1991; Wisniewski and Frangione, 1992; Saunders et al., 1993; Schmechel et al., 1993). In addition, aluminum has been detected in NFTs and/or tangle-bearing neurons (Perl and Brody, 1980; Garruto et al., 1984; Picardo et al., 1988; Good et al., 1992; Perl and Good, 1992), as well as in some SPs (Candy et al., 1986, 1992; but see also Stern et al., 1986, and Landsberg et al., 1992, for negative studies). Despite the presence of aluminum and other proteins in NFTs and SPs, the contribution of these components to the pathogenesis of plaques and tangles is not known. However, it has been speculated that components in SPs other than Aβ might contribute to the formation of amyloid plaques in AD (Snow et al., 1988, 1994; Greenberg et al., 1991; Perry, 1993; Perry et al., 1993; Shin et al., 1993; Trojanowski et al., 1993b, 1994).

To develop model systems for studying interactions between different plaque and tangle components, we showed that injections of purified PHFτ into the rodent brain induced long-lived co-deposits of Aβ, ubiquitin, and ACT at the injection site (Shin et al., 1993). Thus, the convergence of PHFτ and Aβ in authentic cortical SPs and NFTs (Hyman et al., 1989; Shin et al., 1989; Arai et al., 1990; Spillantini et al., 1990; Perry et al., 1992) may not reflect the stochastic outcome of separate pathological processes. Instead, it is possible that interactions of PHFτ with other tangle components, including Aβ and other Aβ precursor protein domains (Arai et al., 1990; Gollin et al., 1992; Perry et al., 1993), induce the aggregation of PHFs into perikaryal NFTs. Further, the release of PHFτ from degenerating neurons in the AD brain may contribute to the formation of SPs by interacting with Aβ secreted by neural cells (Shin et al., 1993). To explore the *in vivo* interactions between PHFτ, Aβ, aluminum, and other proteins in SPs and NFTs, we injected PHFτ with aluminum chloride ($AlCl_3$) into the rat brain and examined the injection sites for the presence of several proteins found in authentic SPs and NFTs. In complementary studies, we also probed interactions of PHFτ with $AlCl_3$ *in situ* and *in vitro* using AD brain samples.

Materials and Methods

Intracerebral injections of PHFτ and other proteins with and without aluminum. Three different preparations of PHFτ were purified from the brains of three different AD patients as described earlier (Bramblett et al., 1993; Shin et al., 1993) for intracerebral injection. Briefly, PHFτ was purified from Sarkosyl-insoluble material and rendered water soluble by extraction with 2 M guanidine isothiocyanate followed by dialysis against water. Normal adult, human CNS τ and fetal τ from late gestational age normal fetuses (Tohyama et al., 1991; Lee et al., 1993; Yachnis et al., 1993) were prepared as described (Bramblett et al., 1992, 1993). The normal adult and fetal brains as well as the AD brains were evaluated histopathologically and assigned to the control or AD group using consensus criteria for the diagnosis of AD (Khachaturian, 1985) as previously described (Kosik et al., 1988; Arai et al., 1990; Schmidt et al., 1990, 1991; Tohyama et al., 1991; Bramblett et al., 1992; Lee et al., 1993; Yachnis et al., 1993). Human high-molecular-weight neurofilament proteins (NF-H) were purified as described (Balin et al., 1991), and recombinant human ACT was a generous gift of H. Rubin and B. Cooperman, while purified HSPGs and human ApoE (representing a mixture of ApoE2, ApoE3, and ApoE4) were obtained from Becton Dickinson and Chemicon, respectively. Recombinant human ApoE3 and ApoE4 were obtained from Panvera. Peptides based on $A\beta_{1-42}$, as well as on several domains in CNS τ were synthesized and characterized as described (Stern et al., 1989; Lee et al., 1991; Lang et al., 1992), and samples of these peptides also were used for injection into rat brains. These synthetic τ peptides included sequences extending from aa 389 to 402 (according to the numbering system for the longest CNS τ isoform of Goedert et al., 1989) containing Ser^{396} in a phosphorylated state (GAEIVYKSPVVSGD), a microtubule (MT)-binding repeat (aa 256–273; VKSKIGSTENLKHQPGGG), and a carboxy-terminal τ domain containing phosphorylated Ser^{416} (aa 405–421; PRHLSNVSSTGSIDMVD). $AlCl_3$ (20 mM) in 0.5 M Tris pH 6.5 was made as a stock solution and kept at 4°C. The protein samples used for injection were made up with and without added $AlCl_3$. For co-injection of proteins with $AlCl_3$, the protein samples were mixed at 4°C with the stock solution of $AlCl_3$ (resulting in a final concentration of 10 mM $AlCl_3$/0.25 M Tris pH 6.5) just prior to injection. All of these freshly prepared solutions of protein and aluminum were injected within 2 hr after preparation. Approximately 3 μg of PHFτ, normal adult CNS τ, fetal τ, HSPGs, synthetic Aβ, ApoE, and NF-H or 9 μg of ACT all in 3 μl of PBS or in 3 μl of 10 mM $AlCl_3$/0.25 M Tris buffer pH 6.5 was injected separately at each site. Further, 10 μg of the synthetic $\tau_{389-402}$ peptide, 80 μg of the synthetic $\tau_{256-2731}$ peptide, and 45 μg of the synthetic $\tau_{405-421}$ peptide each in 3 μl volume of vehicle also were injected at separate sites in different rats.

Table 2. Summary of antibodies

Antibody	Specificity	Dilution	Reference
ALZ50	τ_{2-10}	1:10	Wolozin et al., 1988; Goedert et al., 1991
T14	$\tau_{141-178}$	1:500	Kosik et al., 1988; Trojanowski et al., 1989
T46	$\tau_{404-414}$	1:500	Kosik et al., 1988; Trojanowski et al., 1989
Taul	τnpSer^{202}	1:500d	Binder et al., 1985; Kosik et al., 1989; Goedert et al., 1993
AT8	PHFτpSer^{202}	1:500	Goedert et al., 1993
T3P*	PHFτpSer^{396}	1:100	Lee et al., 1991; Bramblett et al., 1993
PHF1	PHFτpSer$^{396\&404}$	1:2000	Greenberg et al., 1992; Lang et al., 1992; Bramblett et al., 1993
2332*	Aβ_{1-42}	1:4000	Shin et al., 1993
2134*	Aβ_{1-40}	1:100	Estus et al., 1992
1510	Ubiquitin	1:10000	Chemicon; Shaw et al., 1988
Anti-ACT*	ACT	1:1000	ICN; Abraham et al., 1988
Anti-ACT*	ACT	1:200	DAKO; Abraham et al., 1988
Anti-HSPG*	HSPG	1:100	Narindrasorasak et al., 1991
Anti-Apoe*	APoe	1:4000	Chemicon; Wisniewski et al., 1992
1062	APoe	1:100	Chemicon
RMO24	NF-H	1:10	Lee et al., 1987; Schmidt et al., 1987

This table summarizes the properties of the antibodies used in this study. The name of the antibody, its specificity, the dilution of the antibody used for immunohistochemistry, and the source and/or references on the properties of these antibodies are shown in the columns from left to right, respectively. All of the antibodies are mouse MAbs except for those identified by an asterisk, which are polyclonal antibodies. ALZ50, T14, and T46 are phosphate-independent antibodies to τ that recognize fetal and adult human CNS τ as well as PHFτ, but T14 does not recognize rat τ proteins. Taul recognizes normal CNS τ when Ser^{202} is not phosphorylated (npSer^{202}), but it only binds to PHFτ after enzymatic dephosphorylation. AT8, T3P, and PHF1 recognize fetal human CNS τ and PHFτ, but not adult human CNS τ. These MAbs bind to PHFτ when the serine residues identified in the table by the superscripts are phosphorylated (as indicated by pSer^{202}, pSer^{396}, pSer$^{396\&404}$). The numbering system for the aa sequences of the epitopes in τ recognized by the anti-τ antibodies listed here (as indicated by the subscript numbers following the τ symbol in the second column) is based on the largest human CNS τ isoform (Goedert et al., 1989).

In control rats, the same volume of 10 mM $AlCl_3$/0.25 M Tris buffer pH 6.5 alone was injected.

The injections of the different proteins into the rodent CNS were performed under anesthesia using procedures similar to those described previously (Trojanowski, 1983; Shin et al., 1993). In some experiments, unilateral injections of the protein samples with or without aluminum were performed at two separate sites in the right hemisphere. In other experiments, bilateral injections of matched protein samples with and without aluminum were performed at two separate sites in the right (protein sample with aluminum) and left (protein sample without aluminum) hemispheres. Following specified postinjection survival intervals, the rats were lethally anesthetized and perfused with PBS prior to removal and immersion fixation of the brains in 70% ethanol with 150 mM NaCl as described (Shin et al., 1993). The number of rats injected with different proteins as well as the postinjection survival times are summarized in Table 1. Finally, sections from a number of the rat brains (n = 36) that had received injections of PHFτ or normal adult human CNS τ in a previous study (Shin et al., 1993) were examined further by immunohistochemistry in the experiments described here.

Light microscopic immunohistochemical studies. The ethanol-fixed rat brains were embedded in paraffin according to previously described infiltration schedules (Trojanowski et al., 1989), and serial 6-μm-thick sections through the injection sites were prepared and examined by immunohistochemistry as outlined in several earlier publications (Schmidt et al., 1991; Tohyama et al., 1991; Bramblett et al., 1992; Shin et al., 1993). The panel of antibodies to normal adult and fetal CNS τ, PHFτ, ubiquitin, ACT, Aβ, HSPGs, ApoE, and NF-H that were used in this study is summarized in Table 2.

Since some PHFτ epitopes (e.g., the amino-terminal τ epitope recognized by the T46 MAb) were not detected at all, or they were weakly immunoreactive in the aggregates that formed following injections of PHFτ plus $AlCl_3$ (see Results), we sought to determine if aluminum might bind to these epitopes and "mask" them. To do this, we treated rat brain sections at 37°C for 1 hr with chelating agents including 0.1 M EDTA in 0.1 M Tris pH 7.6, or 0.1 M desferrioxamine mesylate (DFO; Sigma) in 0.1 M Tris pH 7.6 prior to probing the sections with primary antibodies for immunohistochemical analysis. The rat brain sections were analyzed with and without pretreatment with chelating solutions using antibodies to PHFτ as well as with antibodies to all of the other proteins examined in this study (see Table 2). In studies performed with the Taul MAb, rat brain sections were pretreated with DFO followed by enzymatic dephosphorylation of the sections using *Escherichia coli* alkaline phosphatase (type III-N, Sigma) as described earlier (Lee et al., 1986; Schmidt et al., 1987). Finally, absorption experiments were performed for several of the antibodies as described (Shin et al., 1991).

Immunoelectron microscopic studies of PHFτ injection sites. To monitor the ability of the guanidine isothiocyanate–treated, water-soluble PHFτ proteins to reassemble into filaments in the rat brain, immunoelectron microscopical studies were performed as reported elsewhere (Springall et al., 1984; Balin et al., 1991; Hill et al., 1991; V. M.-Y. Lee et al., 1991; J. H. Lee et al., 1993; Shin et al., 1993). These studies were carried out on sections through PHFτ accumulations produced in three additional rats that received injections of PHFτ alone. These rats survived 1 d postinjection, and they were perfused with buffer as described above. The brains were removed immediately thereafter and small pieces of tissue from the injection sites were excised and immersion fixed in 1% glutaraldehyde/2% paraformaldehyde in phosphate buffer for 4 hr, embedded in paraffin, and then sectioned as described above. Sequential sections were probed with the antibodies to PHFτ and normal CNS τ summarized in Table 2 using the same immunohistochemical methods outlined earlier except that the peroxidase–antiperoxidase complex was replaced with an immunogold reporter system with and without silver intensification of the gold (5 nm) particles (Springall et al., 1984; Shin et al., 1993). Portions of the rat brain adjacent to the injection sites were excised, embedded in araldite, sectioned, and examined using a Hitachi 600 electron microscope.

Assessment of interactions between aluminum salts and AD pathology in situ. In complementary studies of the ability of aluminum salts to interact with proteins found in SPs and NFTs of the AD brain *in situ*, we prepared sections of hippocampus from four different AD brains and treated these sections with 10 mM $AlCl_3$ in 0.25 M Tris pH 6.5 at 37°C overnight prior to immunohistochemical analysis using the same antibodies described in Table 2. The diagnostic evaluation of these AD brains was performed as outlined above.

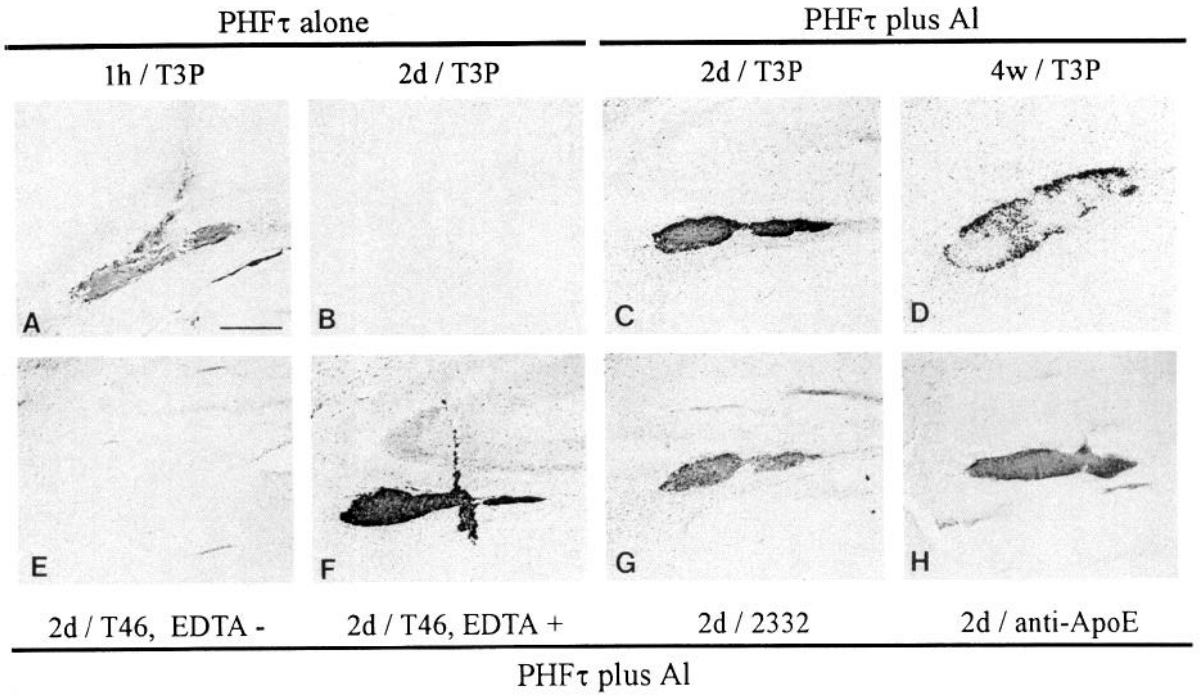

Figure 1. Photomicrographs of rat brains injected with PHFτ alone (*A* and *B*) or with PHFτ plus aluminum (*C–H*) at postinjection survival times of 1 hr (*A*), 2 d (*B, C,* and *E–H*), and 4 weeks (*D*). Some of the sections were not pretreated with chelators (*A, B,* and *E*), while others were pretreated with EDTA (*F*) or DFO (*C, D, G,* and *H*) for 1 hr prior to immunostaining with antibodies specific for PHFτ (*T3P* in *A–D*), normal adult, and fetal CNS τ as well as PHFτ (*T46* in *E* and *F*), Aβ (*2332* in *G*), or ApoE (*H*). The sections were counterstained with hematoxylin. All of the photomicrographs are at the same magnification. Scale bar (in *A*), 300 μm.

In vitro *assessment of interactions between PHFτ and aluminum.* Samples from tangle-rich regions of the brains of three AD patients as well as brain samples from three neurologically and neuropathologically normal subjects were homogenized in reassembly buffer (0.1 M MES, 0.5 mM $MgSO_4$, 1 mM EGTA, 2 mM dithiothreitol pH 6.8) containing 0.75 M NaCl, and a cocktail of protease inhibitors (0.5 M phenylmethylsulfonyl fluoride and TPCK, TLCK, leupeptin, pepstatin, and soybean trypsin inhibitor, each at 1 gm/ml) and centrifuged at 90,000 × *g* for 30 min. The resulting supernatants were boiled for 10 min, followed by centrifugation at 90,000 × *g* for 60 min, and then once again at 350,000 × *g* for 60 min. The final 350,000 × *g* supernatants from the AD brains (which contained an aqueous-soluble fraction of PHFτ as well as aqueous-soluble normal adult CNS τ; see Results for further details) and from the normal brains (which only contained aqueous-soluble normal adult CNS τ) were incubated with different concentrations of $AlCl_3$ (ranging from 0 to 1 mM) in 0.25 M Tris pH 6.5 at 37°C for 1 hr, and centrifuged at 90,000 × *g* for 30 min. The resultant supernatants and pellets were analyzed by 8.5% SDS-PAGE followed by immunoblot analysis with MAbs specific for PHFτ (i.e., PHF1, T3P, AT8) and normal adult CNS τ (i.e., Tau1) as described (Lee et al., 1991; Bramblett et al., 1992, 1993; Shin et al., 1993). The supernatants from the AD brains also were applied to nickel grids, negatively stained, and monitored by electron microscopy using a Hitachi 600 electron microscope for the presence of particulate material, straight filaments, or PHFs as outlined in previous reports (Balin et al., 1991; Lee et al., 1991).

Results

Co-injection of aluminum salts with PHFτ enhances the resistance of PHFτ to in vivo *proteolysis*

To investigate the effects of aluminum on the *in vivo* biological activities of PHFτ, we used a rodent model in which intracerebral injections of PHFτ have been shown to produce clustered aggregates of PHFτ that resist rapid proteolysis relative to normal adult CNS τ and fetal human (Shin et al., 1993). Accordingly, several different preparations of PHFτ from different AD brains ($n = 6$) were screened for their relative resistance to *in vivo* proteolysis by injecting aliquots of each of the preparations into the rat CNS. We confirmed that all of these PHFτ isolates were more resistant to *in vivo* proteolysis than normal adult CNS τ, which is eliminated almost entirely within 1 hr following intracerebral injection. However, the different PHFτ preparations resisted proteolysis to varying degrees; that is, different isolates remained in the rat brain injection site for periods of time ranging from <1 week to >4 weeks. The reasons for this variation are uncertain, but similar variations have been noted earlier (Shin et al., unpublished observations), and we took advantage of this variation to assess rapidly the effects of $AlCl_3$ on the *in vivo* proteolysis of PHFτ. Specifically, in the studies summarized below, we used one of the more rapidly proteolyzed PHFτ preparations that was eliminated routinely from the injection site within 1 week.

Injections of aliquots from this preparation of PHFτ without aluminum produced large aggregates of immunoreactive PHFτ at 1 hr postinjection (Fig. 1*A*), but most of these aggregates were removed from the injection site by 1 d postinjection, and only trace amounts of PHFτ were detected at 2 d to 1 week postinjection (Fig. 1*B*). As described earlier (Shin et al., 1993), codeposits of Aβ appeared in these aggregates at 1 hr postinjection, and this Aβ immunoreactivity disappeared over the ensuing week (data not shown). When the same amount of the same PHFτ preparation (i.e., 3 μg/3 μl PBS) was co-injected with 10 mM $AlCl_3$, aggregates of immunoreactive PHFτ also formed rapidly (within 1 hr of injection) in the rat brain. However, these PHFτ aggregates persisted for up to at 8 weeks postinjection (Fig. 1*C,D*), after which time they were eliminated from the injection site. When sections containing the injected PHFτ plus aluminum were pretreated with metal chelators (i.e., EDTA or DFO), the pretreatment enhanced the immunodetection of the

PHFτ aggregates with antibodies that recognize both normal τ and PHFτ (e.g., compare Fig. 1*E,F*), or only PHFτ (see Fig. 1*C,D*), and this effect was most evident at the postinjection survival times of <1 week.

The effect of co-injecting aluminum with PHFτ and the effect of pretreating sections with chelators were replicated at each postinjection survival time (i.e., two or four different rats per time point) mentioned above (data not shown). These observations also were confirmed by injecting two different PHFτ samples with and without aluminum in opposite sides of the same rat brains ($n = 2$) and examining these brains at 1 week postinjection survival times (data not shown). Thus, co-injection of aluminum with PHFτ dramatically enhanced the resistance of PHFτ to *in vivo* proteolysis, and this resulted in the prolonged persistence of PHFτ aggregates in the rat brain relative to the injections of aliquots of the same preparation of PHFτ without aluminum. Further, the "unmasking" of PHFτ epitopes in sections through the injection sites containing PHFτ plus aluminum by pretreatment with chelators suggests that aluminum remained closely associated with PHFτ in the injection site.

Previous studies showed that topographically separate PHFτ epitopes are eliminated from the PHFτ aggregates according to a distinct temporal sequence (Shin et al., 1993). For example, the carboxy-terminal τ epitope containing phosphorylated Ser^{396} (recognized by the PHF1 and T3P antibodies) persisted the longest (i.e., for many days to several weeks) in the rat brain, while amino-terminal τ epitopes (recognized by the ALZ50 MAb) were eliminated shortly (i.e., within a few hours) after an injection of PHFτ alone (Shin et al., 1993). In view of these observations, we characterized the temporal profile for the elimination of PHFτ epitopes in the injections sites containing PHFτ plus aluminum. These studies showed that aggregates of PHFτ co-injected with aluminum retained immunoreactivity for the epitopes recognized by the PHF1, T3P, and AT8 (which recognizes the epitope containing phosphorylated Ser^{202}) antibodies longer than any of the other epitopes studied here (i.e., >4 weeks postinjection; see Fig. 1*D*), while the amino-terminal epitope recognized by the ALZ50 MAb was eliminated far more rapidly (i.e., within <2 weeks postinjection; data not shown). However, all of the epitopes recognized by the antibodies to normal CNS τ and PHFτ were eliminated much more slowly from the injection sites containing PHFτ plus aluminum compared to PHFτ alone (data not shown). Thus, the temporal profile for the elimination of topographically separate epitopes in PHFτ is similar for injections of PHFτ with and without aluminum, but the rate at which these epitopes is eliminated appears to be far more attenuated when aluminum is co-injected with PHFτ.

Previous studies also showed that PHFτ induced co-deposits of Aβ, ubiquitin, and ACT at the injection sites (Shin et al., 1993). Further studies here confirmed these observations. In addition, we noted that injections of PHFτ alone also induced co-deposits of ApoE (data not shown). Similar co-deposits of Aβ (Fig. 1*G*), ubiquitin, ACT, and ApoE (Fig. 1*H*) were seen following injections of PHFτ with aluminum, and these co-deposits remained at the injection site for prolonged periods of time (i.e., for 2 weeks postinjection) relative to injections of the same PHFτ preparation without aluminum. Specifically, co-deposits of Aβ, ACT, ubiquitin, and ApoE were induced by injections of the same preparation of PHFτ without aluminum, but these co-deposits remained at the injection site for <1 d. Thus, injections of PHFτ plus aluminum lead to longer-lived co-deposits of Aβ, ubiquitin, ACT, and ApoE in the rodent brain than injections of the same preparation of PHFτ without aluminum.

Specificity of in vivo *co-deposition of Aβ with PHFτ*

In contrast to PHFτ, adult and fetal human CNS τ proteins injected into rat brains were eliminated far more rapidly (i.e., within 1 hr) from the injection site without forming aggregates (Shin et al., 1993). Further, injections of these τ proteins as well as injections of various synthetic peptides failed to induce co-deposits of Aβ, ubiquitin, ACT, and ApoE (data not shown). To determine if the proteolysis of adult and fetal human CNS τ would be influenced by co-injecting these forms of τ with aluminum, we performed additional injections of adult and fetal human CNS τ with and without aluminum as described above. Both adult (Fig. 2*A*) and fetal (Fig. 2*C*) CNS τ proteins co-injected with aluminum formed aggregates that were somewhat longer lived (e.g., as long as 2 d postinjection for adult τ and 1 week for fetal τ) relative to injections of the same proteins alone in the contralateral hemisphere of the same rats (data not shown).

The aggregates of adult human CNS τ plus aluminum retained only the T14 epitope (Fig. 2*A*) and not the epitopes recognized by the ALZ50 or T46 MAbs at 2 d postinjection. In contrast, the aggregates of human fetal τ plus aluminum retained the epitopes recognized by all these τ-specific antibodies in addition to the PHFτ and fetal τ-specific epitopes recognized by PHF1 (Fig. 2*C*), T3P, and AT8 at 2 d postinjection. Among the epitopes recognized by the aforementioned antibodies, those detected by ALZ50, T14, and T46 were removed from the injection sites containing fetal τ plus aluminum by 1 week postinjection, while the epitopes detected by PHF1, T3P, and AT8 were retained in these aggregates at this postinjection time point (data not shown). Thus, aluminum conveys a greater resistance to *in vivo* proteolysis for the epitopes recognized by PHF1, T3P, and AT8 in both PHFτ and in fetal human CNS τ. However, neither the aggregates of adult CNS τ plus aluminum (Fig. 2*B*) nor of fetal τ plus aluminum (Fig. 2*D*) exhibited the ability to induce co-deposits of Aβ. We also examined the effects of aluminum on several other proteins associated with plaques and tangles including ACT, HSPGs, and ApoE. All of the proteins injected without aluminum were rapidly cleared from the injection sites, while injections of ACT, HSPGs, or ApoE with aluminum delayed proteolysis and resulted in aggregates that were immunoreactive for either ACT (Fig. 2*E*), HSPGs (data not shown), or ApoE (Fig. 2*G*), respectively. However, none of these proteins injected with or without aluminum were able to induce co-deposits of Aβ (Fig. 2*F,H*). Further, no deposition of Aβ was seen in the rat brain following injections of aluminum alone (data not shown). Thus, only injections of PHFτ with and without aluminum were able to induce co-deposits of Aβ. These findings, together with data demonstrating that other proteins (e.g., NF-H) and τ peptides fail to induce co-deposits of Aβ (Table 3), suggest that the ability to induce co-deposits of Aβ in rat brain is a highly specific property that may be unique to PHFτ.

Several components of plaques and tangles induce co-deposits of ApoE

Although injections of $AlCl_3$ alone did not induce co-deposits of ApoE, injections of PHFτ with and without $AlCl_3$ and injections of adult or fetal CNS τ as well as ACT, HSPGs, and NF-H with $AlCl_3$ did induce co-deposits of ApoE. Further, injections

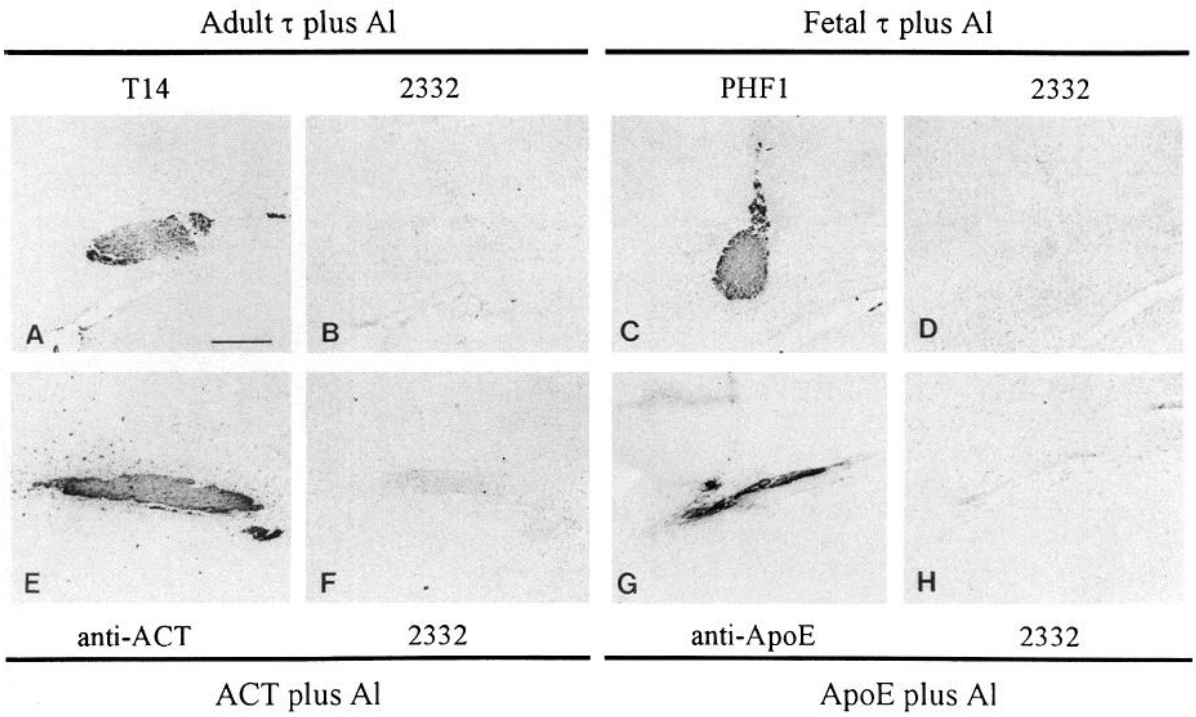

Figure 2. Photomicrographs of rat brains injected with purified human adult CNS τ (*A* and *B*), fetal CNS τ (*C* and *D*), recombinant ACT (*E* and *F*), and purified human ApoE (*G* and *H*) at a 2 d postinjection survival time. Each of the injections also included $AlCl_3$. All of the sections were pretreated with DFO at 37°C for 1 hr followed by immunostaining with antibodies to adult and fetal CNS τ as well as PHFτ (*T14* in *A*), PHFτ (*PHF1* in *C*), ACT (*E*), ApoE (*G*), and Aβ (*2332* in *B, D, F,* and *H*). The sections were counterstained with hematoxylin. All of the photomicrographs are at the same magnification. Scale bar (in *A*), 300 μm.

Table 3. Summary of injection data

Sample injected	Protein co-deposits				
	Aβ	Apoe	HSPG	ACT	UB
PHF τ	+	+	−	+	+
PHF τ + $AlCl_3$	+	+	−	+	+
Aβ	+	+	−	−	−
Aβ + $AlCl_3$	+	+	−	−	−
ACT	−	−	−	−	−
ACT + $AlCl_3$	−	+	−	+	−
APOE	−	−	−	−	−
APOE + $AlCl_3$	−	+	−	−	−
APOE3	−	−	−	−	−
APOE3 + $AlCl_3$	−	+	−	−	−
APOE4	−	−	−	−	−
APOE4 + $AlCl_3$	−	+	−	−	−
HSPG	−	−	−	−	−
HSPG + $AlCl_3$	−	+	−	−	−
A τ	−	−	−	−	−
A τ + $AlCl_3$	−	+	−	−	−
F τ	−	−	−	−	−
F τ + $AlCl_3$	−	+	−	−	−
Syn τ	−	−	−	−	−
NF-H	−	−	−	−	−
NF-H + $AlCl_3$	−	+	−	−	−
$AlCl_3$	−	−	−	−	−

This table summarizes the major results of the injection data described here. The first column lists the component that was injected while the subsequent columns indicate the rodent proteins that were (+) or were not (−) co-deposited with the injected material at the injection sites. The abbreviations are the same as those used in the text and in Table 1.

of Aβ, with and without $AlCl_3$, produced large Aβ aggregates. These injections also induced co-deposits of ApoE, but no co-deposits of the other plaque or tangle components studied here (data not shown). Thus, the induction of co-deposits of ApoE is not specific to PHFτ since several different plaque and tangle components were capable of inducing co-deposits of ApoE (see Table 2).

Acquisition of PHFτ-like immunoreactivity by rodent neurons adjacent to aggregates of PHFτ plus aluminum

Although injections of PHFτ with or without $AlCl_3$ did not induce overt neuropathological changes in the adjacent rat brain (i.e., changes over and above those seen after injections of vehicle alone or following injections of the other material described here), we did observe alterations in immunoreactivity in neurons lying within <1000 μm of the injection sites containing PHFτ plus aluminum. However, these changes were only observed in sections treated with chelators prior to immunohistochemical analysis. For example, preincubation of rat brain sections with DFO for 6 or more hours revealed that only those neurons near the injection sites containing PHFτ plus aluminum acquired *de novo* immunoreactivity for PHF1, AT8, and T3P (Fig. 3*A,B*). In addition, this same population of neurons also acquired Tau1 immunoreactivity only after the rat brain sections were pretreated with DFO and then subjected to enzymatic dephosphorylation with alkaline phosphatase (data not shown). However, using the same experimental procedures, these neurons were not immunostained with other phosphate-independent anti-τ antibodies such as ALZ50, T14 (Fig. 3*C*), and T46. Further, glial cells (which also contain low amounts of τ proteins; Shin et al., 1991) did not acquire PHFτ immunoreactivity. Thus,

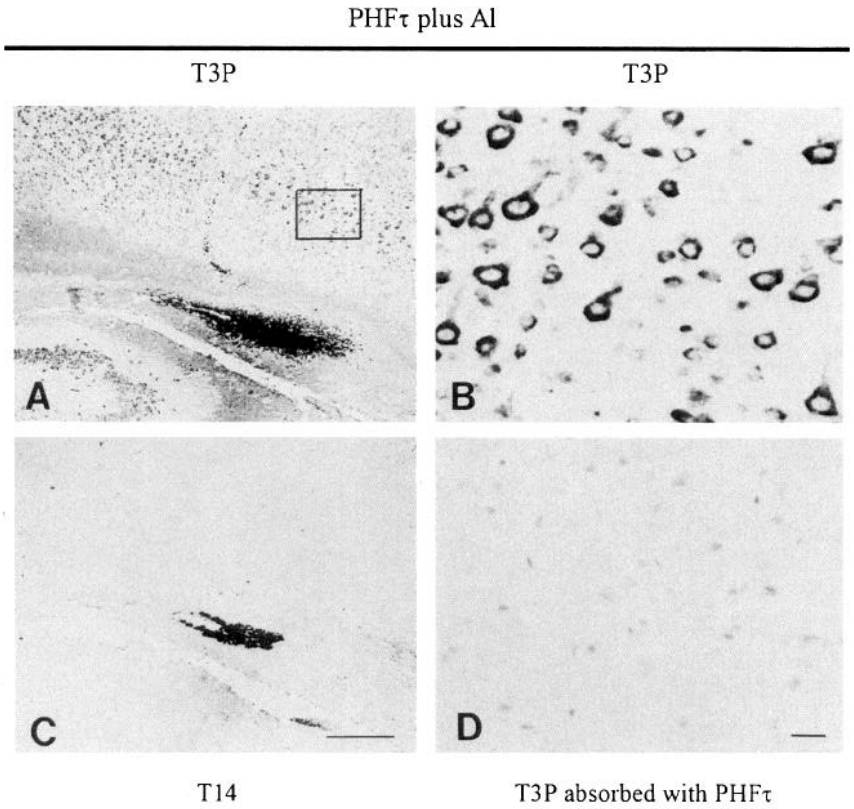

Figure 3. Photomicrographs of rat brains injected with PHFτ plus aluminum at 2 weeks postinjection. The sections were pretreated with DFO at 37°C overnight and probed with T3P (*A, B,* and *D*) or T14 (*C*). The T3P antiserum used in *D* was absorbed with PHFτ (20 μg/ml), which eliminated staining of neurofibrillary lesions in sections of AD brain. The sections were counterstained with hematoxylin. Note that many normal-appearing rodent neurons exhibit PHFτ (*T3P*) immunoreactivity near the injection site following pretreatment of the sections with DFO (*A* and *B*), and that this immunoreactivity is abolished after absorption of T3P with PHFτ (*D*). In contrast, T14 stains the aggregates formed at the injection site of PHFτ plus aluminum, but not any of the rodent neurons. The photomicrographs in *A* and *C* are at the same magnification, as are the photomicrographs in *B* and *D*. The *boxed area* in *A* is shown at higher power in *B*. Scale bars: *C*, 300 μm for *A* and *C*; *D*, 20 μm for *B* and *D*.

injections of PHFτ with $AlCl_3$ induced changes in the phosphorylation state of normal adult rodent CNS τ in neurons near the PHFτ/aluminum injection site such that several PHFτ-specific antibodies (i.e., PHF1, T3P, AT8) immunostained these neurons and this PHFτ staining was absorbed out by incubating antibodies with PHFτ (Fig. 3*D*). Notably, the detection of PHFτ immunoreactivity in the neurons described here required prolonged exposure to DFO. For example, similar results were not obtained following preincubations with DFO for brief periods of time (e.g., for 1 hr), or with the chelator EDTA. Finally, the rodent neurons that acquired *de novo* PHFτ immunoreactivity were confined to the region adjacent to the larger aggregates of PHFτ plus aluminum (i.e., within 1000 μm of the injection site.

Using the same DFO pretreatment, we examined sections from the rat brains (n = 62) that had received intracerebral injections of the other samples described above (e.g., CNS τ, ACT, ubiquitin, Aβ, ApoE, HSPGs, NF-H). In some of the brains (n = 3, 1 hr and 2 d postinjection survival times) from the series of rats described in an earlier study (Shin et al., 1993) that received injections of PHFτ alone, pretreatment with DFO revealed a small population of neurons that were immunostained by AT8, T3P, and PHF1. Further, these neurons also acquired Tau1 immunoreactivity following enzymatic dephosphorylation with alkaline phosphatase after initial treatment of the sections with DFO. These neurons were also confined to the injection site and were most abundant in the injection sites that contained large aggregates of PHFτ. In contrast, no PHFτ immunoreactivity was induced in rat neurons surrounding injection sites containing normal adult or fetal human CNS τ, ACT, HSPGs, ApoE, or NF-H with and without $AlCl_3$, and injections of AlCl;3 alone did not produce similar changes in protein immunoreactivity (data not shown).

Taken together, the observations from all of the rats studied here are most compatible with one of the following possibilities: (1) PHFτ injected with aluminum is capable of inducing the hyperphosphorylation of Ser^{202}, Ser^{396}, and Ser^{404} in rodent CNS τ proteins by activating kinases and/or blocking phosphatases; (2) PHFτ (or proteolytic fragments thereof) is internalized with the co-injected aluminum by rat neurons and that the PHFτ epitopes recognized by T3P, AT8, and PHF1 are protected from degradation within neurons by aluminum.

Immunoelectron microscopic studies of PHFτ injection sites

Previous studies showed that PHFτ proteins do not reassemble into PHFs or straight filaments *in vitro* following treatment with guanidine isothiocyanate, which is used to render these proteins water soluble for MT-binding studies (Bramblett et al., 1993) or for injection into the rat brain (Shin et al., 1993). Although rare T3P-positive straight filaments (~10–25 nm in diameter) were observed in one of two rats that survived 1 d following injections of PHFτ alone (Shin et al., 1993), examination of three additional rats injected with PHFτ without $AlCl_3$ did not reveal PHFτ-immunoreactive filaments (data not shown). Additional *in vitro* electron microscopic studies of the guanidine isothiocyanate–treated PHFτ preparations prior to injection into the rat brain also failed to reveal reassembled PHFs. Hence, the ability of PHFτ proteins to reassemble into filaments in the rat brain following guanidine isothiocyanate treatment appears to be very limited under the experimental conditions described here. Finally, recent *in vitro* studies indicate that aluminum does not induce τ to form PHFs (Scott et al., 1993).

Exogenous aluminum salts specifically interact with PHFτ in AD neurofibrillary lesions

To determine if aluminum salts interact with proteins in AD lesions *in situ*, we incubated 6-μm-thick paraffin sections of the

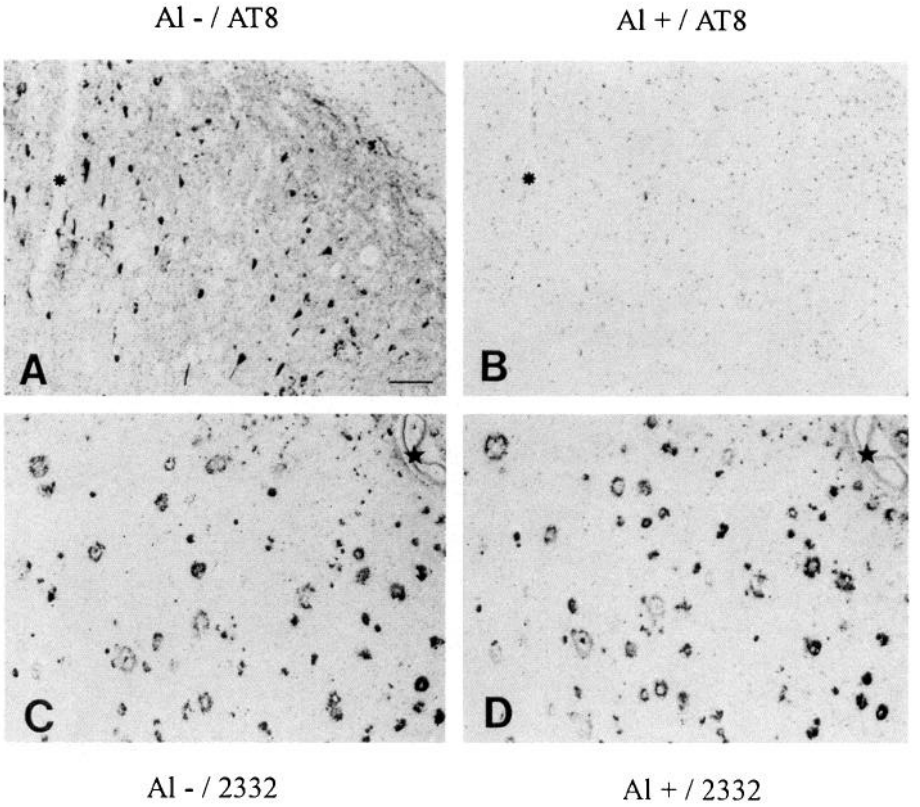

Figure 4. The selective elimination of PHFτ immunoreactivity from sections of the AD hippocampus by pretreating sections with aluminum. Closely adjacent sections of hippocampus from the AD brain were preincubated with control buffer (*A* and *C*) or with buffer containing 10 mM $AlCl_3$ (*B* and *D*) at 37°C overnight followed by immunostaining with antibodies to PHFτ (*AT8* in *A* and *B*) or to Aβ (*2332* in *C* and *D*). The sections were counterstained with hematoxylin. The *asterisk* in *A* and *B* and the *star* in *C* and *D* indicate the same blood vessel in each pair of photomicrographs, respectively. All of the photomicrographs are at the same magnification. Scale bar (in *A*), 100 μm.

AD hippocampus brain with $AlCl_3$ prior to probing these sections with antibodies to defined plaque and tangle components using immunohistochemistry. Remarkably, preincubation of AD hippocampal sections with $AlCl_3$ dramatically reduced or completely abolished the PHFτ immunoreactivity of NFTs, NTs, and dystrophic plaque neurites in studies conducted with the phosphate-dependent antibodies PHF1, T3P, and AT8 (compare Fig. 4*A,B*), whereas the immunostaining obtained with phosphate-independent anti-τ antibodies (i.e., ALZ50, T14, and T46) was unchanged or very mildly diminished (data not shown). Further, preincubation of sections with $AlCl_3$ did not affect the immunostaining of AD lesions using antibodies to Aβ, ubiquitin, ACT, ApoE, and HSPGs (see Fig. 4*C,D* for data on Aβ). Thus, the ability of exogenous aluminum salts to "mask" the immunoreactivity of proteins in amyloid and neurofibrillary lesions in sections of the AD hippocampus appears to be limited to PHFτ proteins, and especially those PHFτ epitopes that contain phosphorylated serine residues (i.e., Ser^{202}, Ser^{396}, and Ser^{404}). Indeed, neither the distribution nor the intensity of the immunoreactivity of several other proteins in amyloid or neurofibrillary lesions was affected by pretreating AD hippocampal sections with aluminum salts. Since aluminum binds avidly to phosphoproteins, and the conformation of a protein may be modified when aluminum binds to it (Webb et al., 1973; Siegel et al., 1983; Birchall and Chappell, 1988), the observations described here may be due to aluminum-induced conformational changes that result from the binding of aluminum to specific domains (i.e., the PHF1, T3P, and AT8 epitopes) in PHFτ that contain phosphorylated residues. Taken together with previous data demonstrating increased levels of aluminum in NFTs (Perl and Brody, 1980; Garruto et al., 1984; Picardo et al., 1988; Good et al., 1992; Perl and Good, 1992), the present findings imply that the aluminum in NFTs may bind selectively to PHFτ, and that these interactions between aluminum and PHFτ may alter the physical/chemical properties of these proteins.

Aluminum salts induce aggregation of PHFτ proteins in vitro *and alter their electrophoretic properties*

Immunoblot analysis (using PHF1, T3P, AT8, and Tau1) of the 350,000 × *g* supernatant derived from the AD brain extracts demonstrated the presence of small amounts of PHFτ proteins (i.e., immunobands with an M_r of 60–70 kDa that were reactive with PHF1, T3P, and AT8, but negative for Tau1) as well as some normal adult CNS τ proteins (i.e., the more rapidly migrating immunobands that were labeled by Tau1, but not by PHF1, T3P, or AT8) as seen in Figure 5 (compare lanes 3 in Fig. 5*A,B*). When negatively stained preparations of this fraction were examined by electron microscopy, some particulate material was noted, but there were no PHFs or straight filaments. These findings are consistent with the notion that a small amount of heat stable PHFτ may exist in a nonfilamentous and aqueous-soluble form in these high-speed supernatants. In contrast to the material from the AD brains, the 350,000 × *g* supernatant from the normal control brain extracts only contained normal adult CNS τ proteins that were indistinguishable from the normal CNS τ proteins in the AD brain (data not shown). Since small amounts of PHFτ remained in the final 350,000 × *g* supernatant with normal adult CNS τ, we deliberately selected these preparations for the *in vitro* analyses to assess the differential effects of $AlCl_3$ on PHFτ and normal adult CNS τ in the same AD brain extracts under identical conditions.

Following incubations of the AD and control brain 350,000 × *g* supernatants with varying concentrations of $AlCl_3$ (i.e., 0.1–1.0 mM) at 37°C for 1 hr, the solubility properties and electro-

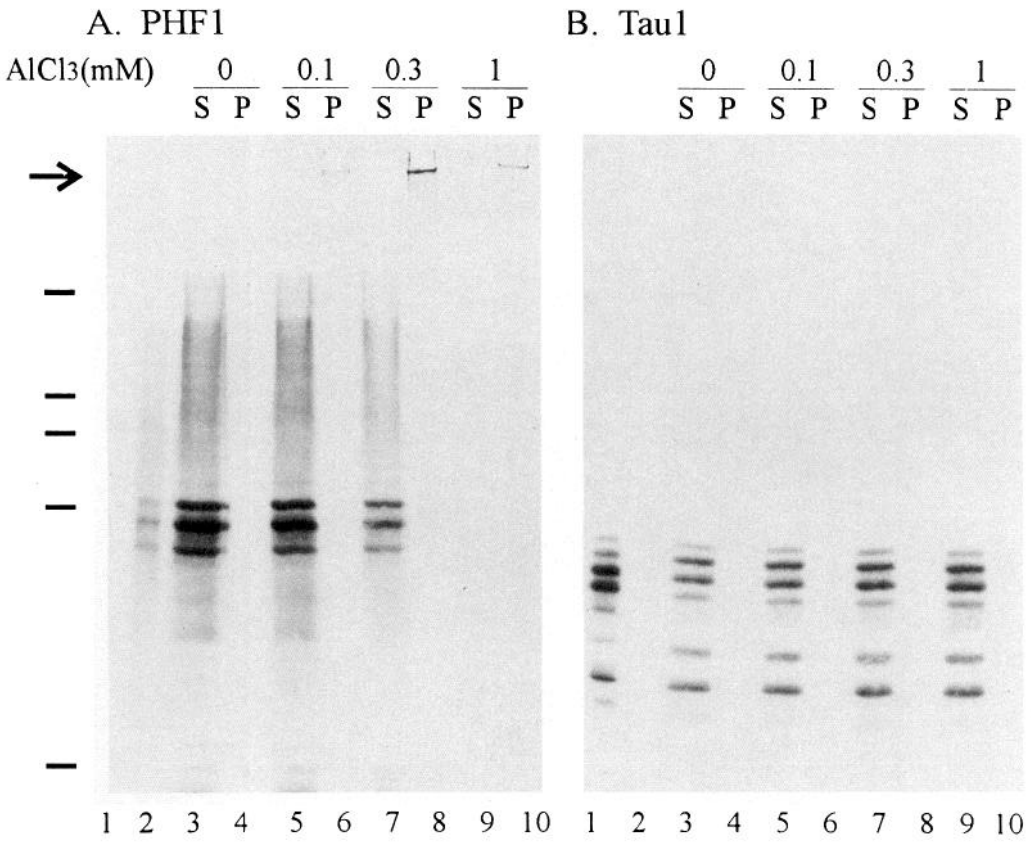

Figure 5. Immunoblot analysis of the effects of aluminum on PHFτ versus adult human CNS τ. Aliquots of the same 350,000 × *g* supernatant prepared from an AD brain were incubated with 0 mM (*lanes 3* and *4*), 0.1 mM (*lanes 5* and *6*), 0.3 mM (*lanes 7* and *8*), 1.0 mM (*lanes 9* and *10*) $AlCl_3$ at 37°C for 1 hr followed by centrifugation at 90,000 × *g* for 30 min. For each of these samples, the resulting supernatants (*lanes 3, 5, 7,* and *9*) and pellets (*lanes 4, 6, 8,* and *10*) were analyzed using 8.5% SDS-PAGE and immunoblots. After transfer to nitrocellulose paper, the gel replicas were probed with PHF1 (*A*) and Tau1 (*B*). As controls, *lanes 1* and *2* were loaded with a sample of normal adult CNS τ purified from a control brain (*lane 1* in *A* and *B*) as well as a sample of PHFτ purified from another AD brain (*lane 2* in *A* and *B*). The *arrow* at the top of the Western blot in *A* indicates the position of the top of the stacking gel. The position of M_r markers is indicated at the left of *A*. *From top to bottom,* these markers correspond to 205, 116, 97, 66, and 45 kDa, respectively.

phoretic mobility of the PHFτ proteins in the AD brain supernatants were dramatically altered (Fig. 5*A*), while no changes were noted in the normal adult CNS τ proteins in the AD (Fig. 5*B*) and control brain samples (data not shown). For example, increasing concentrations of $AlCl_3$ reduced the abundance of PHF1-positive PHFτ bands and induced the accumulation of PHF1-positive material at the top of the stacking gel. This SDS-insoluble material presumably represents aluminum-induced aggregates of PHFτ that did not enter the stacking gel (Fig. 5*A*), and similar effects of other aluminum salts on PHFτ were described recently (Scott et al., 1993). Indeed, at the higher concentrations of aluminum (i.e., at 1 mM $AlCl_3$), almost no immunoreactive PHFτ was present in the Western blots (see lanes 9 and 10 in Fig. 5*A*). In contrast, the normal adult CNS τ proteins that were present with PHFτ in the same AD brain supernatant fraction were unaffected by the same incubations with increasing concentrations of $AlCl_3$ (Fig. 5*B*). Similarly, no changes were observed in the normal adult CNS τ proteins that were present in the 350,000 × *g* supernatant obtained from the control brains (data not shown). Thus, although aluminum may interact with a number of different polypeptides including normal proteins under some conditions (Scott et al., 1993), we show here that aluminum preferentially interacts with and modifies specific physical and chemical properties of PHFτ relative to adult human CNS τ under the conditions described here.

Discussion

The data described here significantly extend previous findings on the induction of co-deposits of several different plaque and tangle components (i.e., Aβ, ubiquitin, ACT) in the rodent brain by intracerebral injections of PHFτ (Shin et al., 1993). The unique ability of PHFτ to induce co-deposits of Aβ, ubiquitin, and ACT in the rodent brain was reinforced here by showing that injections of several other plaque and tangle components (i.e., ACT, ApoE, and HSPGs) with and without aluminum failed to induce similar co-deposits. More importantly, we demonstrated that aluminum salts modulate the properties of injected PHFτ proteins in the rat brain by retarding the proteolysis of these proteins at the injection site, thereby extending the longevity of the co-deposits of Aβ, ACT, and ubiquitin. Furthermore, the complementary *in situ* and *in vitro* studies have partially characterized the more salient aspects of the interactions of $AlCl_3$ with PHFτ that might account for the *in vivo* observations reported here. Specifically, the *in situ* and *in vitro* data suggest that the effects of $AlCl_3$ on the physical and chemical properties of PHFτ injected into the rat brain may be due to

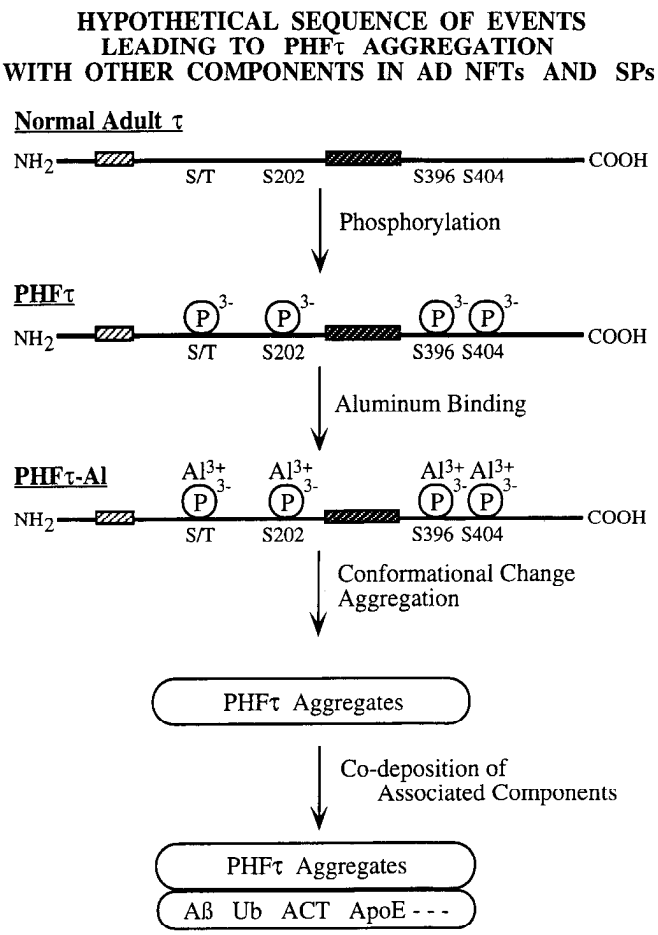

Figure 6. How interactions between aluminum and PHFτ could represent steps in a hypothetical sequence of events that lead to the aggregation of PHFs in NFTs and SPs. Normal CNS τ is shown in the *top* portion of the schematic oriented with the amino terminus (*NH_2*) on the left and the carboxy terminus (*COOH*) on the right. The amino-terminal (*light cross-hatching*) and MT-binding (*dark cross-hatching*) inserts are shown for this 441-aa-long form of adult CNS τ. Also shown are unidentified Ser/Thr (*S/T*) motifs and representative Ser residues (i.e., Ser^{202}, Ser^{396}, and Ser^{404}) that are known to be phosphorylated (*circled P with a 3− superscript*) in PHFτ, which is shown *below* normal CNS τ. The interaction of aluminum (*Al^{3+}*) with PHFτ (*PHFτ-Al*) also is depicted in the schematic. The figure shows the conversion of CNS τ into PHFτ by phosphorylation and the interaction of aluminum with PHFτ, which leads to the increased resistance of PHFτ to proteolysis, its aggregation, and association with other proteins found in SPs and NFTs such as *Aβ*, ubiquitin (*Ub*), *ACT*, and *ApoE* (*lower portion of the schematic*).

the ability of aluminum to bind selectively to domains in PHFτ that contain phosphorylated Ser or other residues, and this in turn might lead to the aggregation, diminished solubility, and delayed proteolysis of PHFτ *in vivo* (see schematic summary of these events in Fig. 6). The results described here are all the more remarkable, considering the fact that $AlCl_3$ may not remain soluble at the pH of the vehicle (pH 6.5) used here, or at the physiological pH in the brain (Martin, 1992).

Although the role of aluminum in AD is uncertain (McLachlan et al., 1992; Perl and Good, 1992; Marksbery and Ehmann, 1994), circumstantial evidence implicates aluminum in the pathogenesis of AD. This evidence includes (1) the detection of aluminum in some AD lesions as well as in tangle-bearing neurons (Perl and Brody, 1980; Garruto et al., 1984; Good et al., 1992); (2) epidemiological data linking aluminum levels in the water supply to the prevalence of AD (Martyn et al., 1989); (3) the modest therapeutic response to treatment of AD patients with DFO (Crapper-McLachlan et al., 1991); (4) the ability of aluminum to induce phosphorylation of normal τ (Yamamoto et al., 1990; Guy et al., 1991; Abdel-Ghany et al., 1993) and a small number of other CNS proteins (Johnson and Jope, 1988; Johnson et al., 1990); and (5) the ability of aluminum to promote aggregation of Aβ (Mantyh et al., 1993). Although aluminum does not induce normal CNS τ to form PHFτ-like structures *in vitro* (Scott et al., 1993), we have demonstrated here that aluminum interacts with PHFτ and alters its physical and chemical properties. We also show that aluminum enhances the longevity of PHFτ *in vivo* and extends the longevity of PHFτ-induced co-deposits of Aβ and plaque and tangle-associated proteins. Further, it also induces rodent neurons to acquire PHFτ-like immunoreactivity. The evidence here that aluminum enhances the resistance of PHFτ to *in vivo* proteolysis is compatible with the notion that aluminum may play a role in the pathogenesis of AD. If this indeed is the case, then our data imply that aluminum may serve as a cofactor in the formation of PHFτ-rich neurofibrillary lesions as well as in the convergence of PHFτ, Aβ, ubiquitin, ACT, and ApoE in amyloid-rich SPs.

One of the key events in the formation of PHFτ involves the hyperphosphorylation of normal adult CNS τ, which renders these abnormal proteins incapable of binding to microtubules (Bramblett et al., 1993). Recently, it was shown that a PHFτ-like species could be generated from normal adult recombinant human CNS τ *in vitro* using purified mitogen-activated protein kinases, glycogen synthase kinase-3, and proline-directed kinases (Drewes et al., 1992; Goedert et al., 1992; Hanger et al., 1992; Mandelkow et al., 1992; Vulliet et al., 1992) as well as by specific inhibitors of protein phosphatase $2A_1$ and protein phosphatase 2B (Goedert et al., 1992; Harris et al., 1993). Whether or not aluminum plays a direct role in the generation of PHFτ is unknown, but aluminum has been shown to modulate the phosphorylation and dephosphorylation of τ proteins (Guy et al., 1991; Abdel-Ghany et al., 1993). Further, an inhibitory effect of aluminum on the dephosphorylation of τ mediated by protein phosphatase 2A has been documented although there was no indication that aluminum was directly bound to this enzyme (Yamamoto et al. 1990). Additionally, aluminum is able to modify the conformation of proteins by inducing β-pleated sheet structure (Hollosi et al., 1992). Although neurons near the injection sites of PHFτ plus aluminum acquired PHFτ-like epitopes (as recognized by AT8, T3P, and PHF1 antibodies as well as by the Tau1 MAb following alkaline phosphatase treatment), the mechanism whereby this occurs is not clear, and previous studies of injections of aluminum alone into the brains of experimental animals produced accumulations of neurofilaments but no PHFτ inclusions (Selkoe et al., 1979; Munoz-Garcia et al., 1986; Troncoso et al., 1986; Wisniewski et al., 1986; Kowall et al., 1989). Further, patients with elevated aluminum levels due to dialysis develop an encephalopathy, but this is not due to the accumulation of AD-like pathology (Candy et al., 1992), and the accidental implantation of metallic aluminum in the human brain has not led to the induction of AD lesions (Foncin and El Hachimi, 1986). Nonetheless, the reproducible effect of injections of PHFτ plus aluminum on τ immunoreactivity in rodent neurons does suggest that aluminum in combination with PHFτ may modulate the phosphorylation state of CNS τ *in vivo*. However, further studies are needed to

determine if and how aluminum modulates the phosphorylation state of τ in the rodent and human CNS.

These and other questions concerning the pathogenesis of plaques, tangles, neuron loss, and other molecular lesions in the AD brain have yet to be fully answered even though there has been dramatic progress in the past decade toward elucidating the molecular composition of plaques and tangles (Goedert, 1993; Selkoe, 1993; Trojanowski et al., 1993b, 1994). In the absence of a complete animal model of the authentic human AD phenotype, we expect that experimental paradigms such as the one described here should prove effective in research efforts to arrive at a closer understanding of the mechanisms leading to the formation of complex AD lesions such as plaques and tangles.

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