Neurofibrillary Tangles May Interfere With Smad 2/3 Signaling in Neurons

Katy A. Chalmers, PhD, and Seth Love, PhD, FRCP, FRCPath

Abstract
Transforming growth factor (TGF)-β is a multifunctional cytokine with anti-inflammatory, reparative and neuroprotective functions. Increased levels of TGFβ in Alzheimer disease (AD) are associated with perivascular deposition of extracellular matrix, which may impair clearance of β-amyloid and contribute to the development of cerebral amyloid angiopathy. TGFβ signaling is transduced by Smad proteins: on TGFβ receptor activation, Smads 2 and 3 are released from sequestration by microtubules, phosphorylated (forming pSmad2/3), and, together with Smad 4, translocated to the nucleus, where they initiate the transcription of multiple genes. Neuronal microtubule assembly is disturbed in AD when tau, a microtubule-stabilizing protein, is hyperphosphorylated and forms neurofibrillary tangles. We have investigated the relationship between Ser202 phospho-tau and pSmads 2 and 3 in the temporal lobe in AD. Within neurons in control brains, pSmads 2 and 3 were almost exclusively intranuclear. In AD, pSmad 3 bound to phospho-tau (mostly insoluble tau) and accumulated in the cytoplasm of tangle-bearing neurons; this was accompanied by a marked decrease in nuclear pSmad3. pSmads 2 and 3 were also present in neuronal granulovacuolar inclusions. Our findings suggest that neurofibrillary tangles sequester pSmad3, preventing its translocation into the nucleus and the induction of gene transcription. Interference with the Smad signaling may adversely affect survival of tangle-bearing neurons in AD.

Key Words: Alzheimer disease, Neurofibrillary tangles, Smad signaling, Transforming growth factor β (TGFβ).

INTRODUCTION
The transforming growth factor (TGF)-β superfamily of cytokines comprises several homologous polypeptides that transduce a range of signals involved in cell growth and differentiation and the response to inflammation and tissue damage. Cellular responses to TGFβ family members (TGFβ, activin, nodal, and bone morphogenetic proteins) are transduced by intracellular mediators termed Smads (1, 2). Each ligand activates a specific group of Smads: TGFβ, activin, and nodal activate Smads 2 and 3, whereas bone morphogenetic proteins signal through Smads 1, 5, and 8.

On binding, each ligand induces the formation of a heteromeric complex of serine/threonine kinase receptors (types I and II). This initiates the mobilization of Smads to the receptor complex where they are phosphorylated at their carboxyl terminus by the type I receptor. The phosphorylation of Smads (to form pSmads) triggers the recruitment of the cofactor Smad4, and the entire Smad complex is translocated to the nucleus where it binds to DNA response elements and, together with transcription factors and coactivators such as activator protein-1 and cAMP response element-binding protein, promotes transcription of a range of target genes. Sustained signaling is limited by polyubiquitylation of pSmads and the subsequent proteasomal degradation of this complex (3) or by dephosphorylation by an as yet unidentified phosphatase that triggers its removal from the nucleus (4).

At least 2 members of the TGFβ superfamily have neuroprotective roles. Activin A can protect neurons from a number of insults including MPP+ (5), kainate (6), and ischemic injury (7) whereas TGFβ has also been observed to protect against ischemic (8–10) and excitotoxic (10) insults. These findings suggest that factors that affect TGFβ/activin or its downstream mediators, Smads 2 and 3, may be detrimental to neuronal survival.

The expression of TGFβs is abnormal in Alzheimer disease (AD). Immunohistochemical studies showed TGFβ1 and 2 to be present in plaques (11) and neurofibrillary tangles (NFTs) in AD (12, 13), and the concentration of TGFβ was elevated in cerebrospinal fluid and serum of patients with AD compared with controls (14). Furthermore, levels of TGFβ mRNA were 3-fold higher in the frontal cortex of AD brains than control brains (15).

Activation of the TGFβ signaling pathway is tightly regulated. TGFβ is itself produced in a latent form and is sequestered within the extracellular matrix (ECM), where its activation is dependent on the activity of specific proteases (16). In the cytoplasm, Smads 2, 3, and 4 are thought to be sequestered by microtubules (17) but dissociate after TGFβ receptor binding. Dong et al (17) showed that destabilization of microtubules (e.g. by treating cells with drugs such as nocodazole and colchicine) increased TGFβ-induced

phosphorylation of Smad 2 and increased target gene transcriptional activity. In AD, tau, a protein essential for the stabilization of microtubules, becomes hyperphosphorylated, leading to the formation of NFTs and neuropil threads. In this study we have investigated the relationship between tau and pSmads 2 and 3 in tangle- and nontangle-bearing neurons in postmortem brain tissue from AD patients and controls with varying degrees of tangle pathology. The findings were previously presented in abstract form (18).

MATERIALS AND METHODS

Brain Tissue

This study was approved by Frenchay Research Ethics Committee. Frozen and fixed brain tissue from 8 AD patients and 8 control subjects without dementia (Table) was obtained from the South West Brain Bank (Bristol, UK). These samples were primarily chosen neuropathologically to represent a spectrum of severity of tangle pathology. A diagnosis of AD was made in patients with a clinical presentation of dementia and a neuropathologic diagnosis of probable or definite AD according to criteria of the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) (19). The AD cases were selected to include examples of Braak tangle stages IV to V (20). Cases with concomitant CNS pathology such as Lewy bodies were excluded from the study. The control cases chosen included examples of Braak stages I to III and Braak “0” (i.e. with no tau pathology); none had shown clinical evidence of dementia during the weeks or months before death, and all had no or only sparse neuritic plaques.

Immunoperoxidase Staining

Paraffin-embedded blocks of left temporal lobe, encompassing the hippocampus, entorhinal cortex, and fusiform, inferior, middle, and superior temporal gyri, were cut at a thickness of 7 μm. Sections were immunolabeled with an antibody to pSmad2/3 (1:8000, raised against a pSmad3 peptide sequence, sc-11769R; Santa Cruz, distributed by Autogen Bioclear, Mile Elm, UK) or pSmad2 (1:8000, raised against a pSmad2 peptide sequence; Chemicon, Temecula, CA) after citrate buffer pH 6 microwave pretreatment. Antigen binding was visualized using a Super Sensitive Polymer-HRP detection system (BioGenex, Berkshire, UK) with diaminobenzidine as the chromogen.

Double Immunofluorescence

Neuronal localization of pSmad2/3 was assessed by double immunofluorescence with antibodies to pSmad2/3 (1:500) and microtubule-associated protein 2 (MAP2) (1:200, 13-1500; Zymed, San Francisco, CA) and visualized with species-specific Texas Red (1:100; Vector Laboratories, Peterborough, UK) and fluorescein isothiocyanate (1:100) fluorochromes, respectively.

The distribution of pSmad2/3 with respect to NFTs was assessed in sections double immunolabeled with antibodies to pSmad2/3 (1:500) and tau phosphorylated at serine 202 (Ser202 phospho-tau) (AT8, 1:800; Innogenetics, Ghent, Belgium). Visualization was as above.

The specificity of the pSmad2/3 antibody for NFTs was assessed by preadsorption with the pSmad 3 immunizing peptide (sc-11769P) used by Santa Cruz to raise the antibody. The pSmad2/3 antibody (1:500) was preadsorbed overnight at 4°C with a 20-fold molar excess (666 μmol/L) of the peptide. Sections were then incubated with the pSmad2/3 peptide solution overnight and visualized with species-specific fluorochromes. To further investigate the specificity of the pSmad2/3 antibody and its colocalization with NFTs, sections were double immunolabeled with antibodies to pSmad 2 (1:200) and Ser202 phospho-tau.

For all of the immunofluorescence, monochromatic images were acquired and merged using a Leica DM microscope.

| Table. Characteristics of AD and Control Cases |
|-----------------|--------|---------|---------------------------------|-----------------|
| Case | Age  | Gender | Postmortem Delay (hours) | Cause of Death | Braak Stage |
| 1    | 74   | F      | 59                  | Septicemia     | II          |
| 2    | 76   | F      | 46                  | Chronic renal failure | IV    |
| 3    | 82   | F      | 40                  | Congestive cardiac failure, ischemic heart disease | I |
| 4    | 94   | M      | 109                 | Ischemic heart disease | IV |
| 5    | 80   | F      | 19                  | Pulmonary embolism  | I      |
| 6    | 87   | M      | 36                  | Not available  | V |
| 7    | 73   | F      | 65                  | Carcinoma of breast | 0 |
| 8    | 93   | F      | 15                  | Congestive cardiac failure, ischemic heart disease | I |
| 9    | 93   | M      | 38                  | Carcinoma of colon | III |
| 10   | 63   | F      | 31                  | Acute subarachnoid hemorrhage (probable ruptured berry aneurysm) | II |
| 11   | 80   | M      | 49                  | Bronchopneumonia  | IV |
| 12   | 90   | M      | 5.5                 | Carcinoma of bladder | II |
| 13   | 89   | F      | 39                  | Dementia  | V |
| 14   | 74   | M      | 48                  | Bronchopneumonia, dementia | V |
| 15   | 73   | F      | 38                  | Bronchopneumonia, dementia | IV |
| 16   | 85   | F      | 85                  | Bronchopneumonia, dementia | V |

Counts of Tangle-Bearing and pSmad2/3-Positive Neurons

In the sections of temporal lobe that had been stained for pSmad2/3 by the immunoperoxidase method, 100 pyramidal neurons were assessed in the CA1 region of the hippocampus. The CA2/CA1 boundary was identified, and under 20× objective the distribution of pSmad2/3 immunoreactivity in the first 100 pyramidal neurons (progressing away from the CA2/CA1 boundary) was recorded. Each neuron was classified by its pSmad2/3 distribution as either neuronal pSmad2/3 only, cytoplasmic pSmad2/3 only, both neuronal and cytoplasmic pSmad2/3, or no pSmad2/3 immunoreactivity. Only neurons with a visible nucleus in the plane of section were included in the analysis.

ELISAs

To examine further the relationship between phospho-tau and pSmad2/3, we performed ELISAs using a Ser202 phospho-tau capture antibody and a pSmad2/3 detection antibody (the same antibodies as used for the double immunofluorescence). The tissue for ELISAs was obtained from the right superior temporal gyrus of 8 patients with CERAD-definite cases of AD (age range 61–98 years, mean = 78.9) and 4 age-matched controls (age range 73–90 years, mean = 83.3); we avoided the hippocampus as for these assays as we wished to maximize the difference between the 2 groups with respect to the presence or absence of phospho-tau. The tissue had been stored at −80°C before use. Samples (500 mg) of superior temporal gyrus were homogenized in lysis buffer (0.1 mmol/L NaCl, 10 mmol/L Tris (pH 6), 1 μmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1% sodium dodecyl sulfate in distilled water). The homogenate was centrifuged for 15 minutes at 16,000 × g at 4°C, and the supernatant was removed and stored at −80°C until required. Ninety-six-well plates (Nunc MaxiSorp, obtained from Fisher Scientific, Loughborough, UK) were coated with AT8 antibody (5 μg/mL in coating buffer) and left at 4°C overnight. Wells were washed thoroughly with wash buffer and incubated with blocking buffer (3% bovine serum albumin and 0.02% sodium azide in PBS) for 90 minutes at room temperature. Homogenates containing 5 to 40 μg of protein diluted in 50 μL of blocking buffer were added in triplicate and left at room temperature for 2 hours. The plates also included blank wells that contained blocking buffer without homogenate. After further washes, antibody to pSmad2/3 (5 μg/mL in coating buffer) was added, and plates were left for 60 minutes. The wells were washed and incubated with peroxidase anti-rabbit conjugate (Vector Laboratories) for 60 minutes. The wells were again washed, peroxidase substrate, ABTS (Vector Laboratories), was added, and plates were left for

FIGURE 1. Phosphorylated Smad (pSmad) 2/3 immunolabeling in the hippocampus. (A) In control brain, pSmad2/3 is largely restricted to nuclei, especially in the pyramidal neurons of the CA1 region. (B) In another control brain, this time from a patient with a small number of neurofibrillary tangles (NFTs) in the hippocampus, pSmad2/3 is present not only within neuronal nuclei but also within the NFTs (arrows). (C) In a section of hippocampus from a patient with Alzheimer disease, pSmad2/3 is present in numerous NFTs and also in granulovacuolar inclusions (arrow). Most of the neuronal nuclei are unlabeled. (D) Higher magnification view of pSmad2/3-labeled NFTs and granulovacuolar inclusions (arrows).
25 minutes in the dark. The plates were then read in a plate reader with a 405-nm filter. The mean readings from the blank wells were subtracted from the readings obtained from the wells containing homogenate.

For blocking peptide experiments, 5 μg of pSmad2/3 antibody was preadsorbed with 20x molar excess (666 μmol/L) of pSmad2/3 blocking peptide (Santa Cruz) in blocking buffer and left at 4°C overnight. After the plates had been coated with phospho-tau antibody and incubated with 10 Kgo protein homogenate, 50 μL of the antibody-blocking peptide complex was added to each well, and plates were incubated for 60 minutes. Specific binding was visualized as described previously.

**ELISAs on Soluble and Insoluble Tau Fractions**

In addition to the ELISAs using crude homogenates, the association between pSmad2/3 and NFTs was assessed in fractions of soluble and insoluble tau. Briefly, 500-mg samples of cortex from the superior temporal gyrus were homogenized in 1 ml of 4-morpholineethanesulfonic acid buffer with complete protease inhibitors (Roche Diagnostics, Burgess Hill, UK) and centrifuged at 300 × g for 5 minutes at 4°C. The supernatant was centrifuged at 20,000 × g for 30 minutes at 4°C and then at 100,000 × g for a further hour. The supernatant, which contained the soluble tau, was removed, and the pellet, which contained the insoluble tau, was resuspended in ELISA blocking buffer.

ELISAs using AT8 and pSmad2/3 antibodies were performed as described above, but on the soluble and insoluble tau fractions adjusted for protein concentrations, rather than on tissue homogenates.

**FIGURE 2.** Phosphorylated Smad (pSmad) 2/3 immunolabeling of temporal neocortex. (A) Section of control brain showing strong labeling of neuronal nuclei. (B) In Alzheimer disease, some pSmad2/3 staining is nuclear, but there is also labeling of neurofibrillary tangles (NFTs) and scattered neurites. (C) Many neurons containing pSmad2/3-positive NFTs lack nuclear staining. Other neurons lack pSmad2/3-positive NFTs but show nuclear labeling (arrow). (D) pSmad2/3 is present in plaque-associated neurites (arrows).

**FIGURE 3.** Double immunofluorescent labeling of phosphorylated Smad (pSmad) 2/3 (red) and microtubule-associated protein 2 (MAP2) (green). (A–F) Images of hippocampal neurons. There is cytoplasmic pSmad2/3 immunoreactivity in MAP2-positive neurons. There is cytoplasmic pSmad2/3 immunoreactivity in MAP2-positive neurons, in a distribution strongly suggesting labeling of neurofibrillary tangles. Granulovacuolar inclusions (arrow) are also immunopositive for pSmad2/3. In contrast, the neuronal nuclei in (B) and (C) are only weakly labeled and that in (E) and (F) is negative for pSmad2/3.
Statistical Analysis

Chi-square analysis with the help of SPSS version 12.0 was used to assess the relationship between nuclear and cytoplasmic pSmad2/3 staining. One-way analysis of variance with Bonferroni post-tests were used to assess the differences between pSmad and AT8 binding in AD and control brains as measured by ELISA. A Student t-test was used to compare pSmad binding to soluble and insoluble tau. A p < 0.05 was considered to be statistically significant.

FIGURE 4. Double immunofluorescent labeling of phosphorylated Smad (pSmad) 2/3 (red) and Ser202 phospho-tau (green) with 4',6-diamidino-2-phenylindole (DAPI) nuclear stain (blue); (A–L) are images of CA1 field of hippocampus and (M–P) are of temporal neocortex. (A–D) In a control brain that contains only sparse hippocampal neurofibrillary tangles (NFTs) and neuropil threads, many of the larger (neuronal) nuclei show colocalization of pSmad2/3 and DAPI (arrows). (E–H) In Alzheimer disease (AD), pSmad2/3 colocalizes with some of the Ser202 phospho-tau in NFTs (arrows). (I–L) Granulovacuolar inclusions (arrows) in a case of AD are strongly immunopositive for pSmad2/3 (arrows) and show only scanty colocalization with Ser202 phospho-tau. (M–P) An occasional neuron contains a NFT that is immunopositive for pSmad2/3 but negative for phospho-tau (arrow).
RESULTS

Intracellular Distribution of pSmad2/3

Initial immunoperoxidase assessment of the distribution of pSmad2/3 indicated that it was present within the neurons of the temporal lobe (Figs. 1 and 2). This was confirmed by double immunofluorescent labeling of sections for the neuronal marker MAP2 and pSmad2/3. pSmad2/3 was found in both the cytoplasm and nuclei of MAP2-positive neurons (Fig. 3).

In control brains pSmad2/3 was predominantly located within the nucleus of neurons. Nuclear labeling was particularly strong in the dentate gyrus and hippocampal pyramidal cell layer (Fig. 1A, B) but neuronal nuclei in the entorhinal cortex and neocortex were also immunopositive (Fig. 2A). Little or no pSmad2/3 was detected within the cytoplasm of most neurons. A few neurons in the CA1 region, subiculum, and entorhinal cortex in some of the controls contained NFTs; in these neurons the tangles were immunopositive for pSmad2/3, and the nuclei were only weakly labeled or unlabeled (Fig. 1B). Sparingly scattered neurites within these regions were also immunopositive for pSmad2/3.

In cases of AD, many neurons, especially in the CA1 region and subiculum but also elsewhere in the cortex, contained pSmad2/3-positive NFTs (Figs. 1C, D, and 2B, C). In these brains, pSmad2/3 was also present in moderate numbers of neurites. Some neurons, predominantly within the pyramidal cell layer of the hippocampus, showed punctate cytoplasmic staining of round structures measuring up to approximately 5 µm in diameter and having the appearance of granulovacuolar degeneration (Fig. 1C, D). Clusters of dystrophic neurites associated with neuritic plaques were also immunopositive for pSmad2/3 (Fig. 2D).

Colocalization of pSmad2/3 With Neurofibrillary Tangles

To confirm whether or not cytoplasmic pSmad2/3 immunoreactivity was associated with NFTs, we used double immunofluorescence for Ser202 phospho-tau and pSmad2/3. Immunofluorescence confirmed the nuclear localization of neuronal pSmad2/3 within control brains (Fig. 4A–D). In AD...
most of the immunolabeled pSmad2/3 colocalized with Ser202 phospho-tau-positive NFTs, although the extent of pSmad2/3 labeling of the NFTs was generally less than that of phospho-tau (Fig. 4E–H). In contrast, the granulovacuolar pSmad2/3 was largely unlabeled by the Ser202 phospho-tau antibody (Fig. 4I–L). A very occasional neuron contained a NFT that was immunopositive for pSmad2/3 but negative for phospho-tau (Fig. 4M–P).

The specificity of the pSmad2/3 antibody binding to NFTs was assessed by preadsorption with pSmad 3 blocking peptide and by use of antibody raised against the amino acid sequence of pSmad 2. Preincubation with the pSmad 3 blocking peptide abolished pSmad2/3 immunoreactivity (Fig. 5C). The pSmad 2 antibody showed nuclear localization (Figs. 6B and 7A–D) and some cytoplasmic staining in the form of granulovacuolar degeneration (Figs. 6A and 7E–H) but showed little or no labeling of NFTs.

**Relationship Between Tangle-Associated and Nuclear pSmad2/3**

Our assessment suggested strongly: 1) that the pSmad2/3 antibody predominantly labeled pSmad 3 rather than pSmad 2; 2) that nuclear pSmad 3 was largely restricted to nontangle-bearing neurons and, conversely; and 3) that many tangle-bearing neurons lack nuclear pSmad 3. To assess this further, we determined the following: the proportion of neurons in the CA1 field with nuclear pSmad2/3 immunopositivity; cytoplasmic pSmad2/3 immunopositivity only; both nuclear and cytoplasmic pSmad2/3 immunopositivity; or neither nuclear nor cytoplasmic pSmad2/3 immunopositivity. In each of the 8 AD cases and 8 controls, the CA1 field was examined in the same manner, progressing from the CA2 region toward the subiculum and counting all neurons with a discernible nucleus in the plane of section until 100 neurons that contained cytoplasmic and/or nuclear pSmad2/3 immunopositivity or had no pSmad2/3 immunopositivity had been counted. The results are shown in Figure 8. Chi-square analysis showed a highly significant relationship between the

**FIGURE 7.** Double immunofluorescent labeling of phosphorylated Smad (pSmad) 2 (red) and Ser202 phospho-tau (green) with 4',6-diamidino-2-phenylindole (DAPI) counterstain. (A–D) pSmad 2 is found within neuronal nuclei, including tangle-bearing neurons. (E–H) pSmad 2 is also present in granulovacuolar inclusions (shown here in a tangle-bearing neuron).

**FIGURE 8.** Horizontal bar chart showing the distribution of phosphorylated Smad (pSmad) 2/3 immunoreactivity in 100 CA1 neurons in each of 16 brains, representing a spectrum of Braak tangle stages. Neurons with cytoplasmic pSmad2/3 immunoreactivity were significantly less likely to contain nuclear pSmad2/3 (p < 0.0001). Red = cytoplasmic only; orange = nuclear and cytoplasmic; yellow = nuclear only; white = unlabeled.
presence of cytoplasmic pSmad2/3 and an absence of nuclear pSmad2/3 (p < 0.0001). Chi-square analysis also showed that the difference in distribution of pSmad2/3 between AD cases and controls was highly significant (p < 0.0001). There was no association between the distribution of pSmad2/3 and age, gender, or postmortem delay.

**ELISAs of Detergent-Resistant Interaction Between Phospho-Tau and pSmad2/3**

The use of a sandwich ELISA with a phospho-tau capture antibody and a pSmad2/3 detection antibody allowed us to investigate the interaction between phospho-tau and pSmad2/3 in detergent-solubilized homogenates of superior temporal cortex. In homogenates from normal brain, little signal was detected. Homogenates from AD brains showed an incremental relationship between the amounts of total protein (from 5 to 40 μg) added to the phospho-tau antibody–coated wells and the anti-pSmad2/3 signal (Fig. 9). The overall difference between AD and control cases was highly significant (p < 0.0001, 1-way analysis of variance) and Bonferroni post-tests showed that the difference in the ELISA signal between AD and control cases was significant at the p < 0.001 level for each amount of total protein. Preincubation of the pSmad2/3 antibody with the immunizing pSmad3 peptide completely abolished the ELISA signal.

**ELISA of Soluble and Insoluble Tau Fractions**

Examine of the soluble and insoluble tau fractions in homogenates of superior temporal cortex from 4 patients with AD showed most of the phospho-tau-bound pSmad 3 to be associated with the insoluble fraction (Fig. 10). The data were normalized by logarithmic transformation for statistical analysis by Student t-test, which showed the difference to be significant (p = 0.015).

**DISCUSSION**

We have demonstrated aberrant distribution of pSmad 3 in neurons that contain NFTs in hippocampus and in temporal neocortex from patients with AD. Whereas pSmad 3 within neurons in control brains was largely restricted to the nucleus, in AD pSmad 3 colocalized with Ser202 phospho-tau in NFTs, as demonstrated by double immunofluorescence. The binding of pSmad 3 to phospho-tau was confirmed by a modified ELISA. Furthermore, accumulation of pSmad 3 within the neuronal cytoplasm was associated with significantly reduced accumulation of pSmad 3 in the neuronal nucleus. In addition, both pSmad 2 and pSmad 3 colocalized with the inclusions of granulovacuolar degeneration, and pSmad 3 with a very occasional NFT that lacked Ser202 phospho-tau positivity. The sequestration of pSmads within the cytoplasm, particularly by NFTs, is likely to affect TGFβ and possibly activin and nodal signal transduction in AD, with potentially damaging consequences for affected neurons.

Evidence from previous immunohistochemical and biochemical studies indicates that TGFβ expression is upregulated in AD. Levels of TGFβ mRNA are increased in the frontal cortex of patients with AD, the extent of the increase correlating with the severity of cerebral amyloid angiopathy (15). Flanders et al (21) reported that TGFβ2 immunoreactivity was enhanced in tangle-bearing neurons. Neuronal TGFβ receptor I staining was also seen to be elevated (22). However, increases in TGFβ and TGFβ receptor do not necessarily lead to an increase in TGFβ-mediated activity. Transduction of the TGFβ signal is dependent on several factors, including the phosphorylation and nuclear translocation of Smad proteins.
Both in vitro (23–26) and in vivo (27–29) studies have shown that activation of Smads 2 and 3 by phosphorylation leads to the association of these proteins with Smad 4 and their subsequent translocation of to the nucleus, where binding of this complex to DNA response elements and transcription factors is crucial for the transduction of the TGFβ signal (1, 2). Our data indicate that pSmad2/3 is not translocated into the nucleus of tangle-bearing neurons and, further, that the NFTs themselves bind, and presumably sequester, pSmad2/3 within the cytoplasm. Through this action, NFTs are likely to hinder the transcription of TGFβ target genes, such as those involved in the synthesis of ECM proteins. In this context, it is of interest that we have demonstrated loss of the perineuronal net (specialized ECM that is particularly abundant around parvalbumin-positive GABAergic interneurons) from frontal cortex in AD (30). Brückner et al (31) found that cortical areas abundant in ECM chondroitin sulfate proteoglycans (CSPGs) contained fewer NFTs than did cortex lacking in CSPGs; in the latter regions of cortex AT8-positive NFTs were abundant. They concluded that the presence of a CSPG-rich perineuronal net made neurons less susceptible to the formation of NFTs. However, our data support a converse explanation for the findings, namely that NFT-rich regions are devoid of ECM because the NFTs interfere with TGFβ-induced neuronal synthesis of CSPGs.

Several studies have shown that Smads 2 and 3 interact with the cytoskeleton. Dong et al (17) demonstrated that in the absence of TGFβ stimulation, Smads 2 and 3 are bound to microtubules in epithelial, endothelial, and HeLa cells; exposure of the cells to TGFβ caused release of Smad 2/3 from microtubules, followed by Smad 2/3 phosphorylation, translocation into the nucleus, and initiation of target gene transcription. These authors observed that TGFβ-induced gene transcription could be augmented by exposure of cells to chemical agents such as nocodazole, which destabilized the microtubule network, releasing more Smad 2/3. Smad 2 became phosphorylated after the addition of nocodazole, even in the absence of TGFβ. The effects of nocodazole on TGFβ signaling were eliminated by pretreating the cells with the microtubule-stabilizing drug, Taxol, illustrating the importance of an intact microtubule network in the regulation of TGFβ signaling (17). Another cytoskeletal protein, filamin, also binds Smad proteins, including Smad 2, and was shown to be needed for TGFβ signaling in a melanoma cell line (32).

Within the neuron, microtubules are stabilized by the protein tau. Abnormal phosphorylation of tau, as occurs in AD, causes the tau to dissociate from the microtubules and to aggregate, forming NFTs. In the absence of tau, microtubules are unstable and disintegrate. Our data suggest that the aggregates of hyperphosphorylated tau bind Smad 3 even after TGFβ receptor–mediated phosphorylation of the Smads and, by interfering with their release, prevent their nuclear translocation. The presence of pSmads 2 and 3 in the inclusions of granulovascular degeneration may also represent an association between these Smads and tau, and possibly with other cytoskeletal elements as well; several studies have shown that granulovascular degeneration bodies contain cytoskeletal components including neurofilament proteins (33) and tau (34–36) epitopes, as well as kinases associated with tau phosphorylation (37, 38).

In conclusion, our findings raise the possibility that the sequestration of pSmads by NFTs may impair Smad signaling in neurons in AD. TGFβ has important roles in neuroprotection, ECM production, and reducing inflammation and the loss of these functions may be detrimental to the survival of tangle-bearing neurons.

NOTE ADDED AFTER SUBMISSION
Since our submission of this paper, Lee et al (39) have reported ectopic expression of pSmad 2 in the cytoplasm of neurons in AD. They used an antibody raised against pSmad 2 but described as showing possible cross-reactivity against pSmad 3; the specificity of the binding to pSmad 2 (or pSmad 3) was not assessed.

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