American Journal of Pathology, Vol. 137, No. 3, September 1990


# Molecular Analysis of Neurofibrillary Degeneration in Alzheimer's Disease

## An Immunohistochemical Study

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***Antibodies directed against three regions of tau, ubiquitin, and B-amyloid were used in a histologic study of neurofibrillary degeneration in Alzheimer's disease to distinguish two populations of neurofibrillary tangles. Intracellular tangles were immunolabeled exclusively by two antibodies raised against antigens in the fuzzy coat of the paired helical filament (PHF). Extracellular tangles were distinguished by selective immunolabeling with a monoclonal antibody raised against antigens in the PHF core. This was associated with removal of the fuzzy coat and exposure of PHF-core epitopes. In the transition from intracellular to extracellular compartments*** in vivo, ***tangles appeared to undergo changes similar to protease digestion*** in vitro. ***The transition was associated with the appearance of amyloid immunoreactivity. These findings suggest that tangle degradation occurs in a series of distinct stages, including ubiquitination of some unknown molecule, a change in tau immunoreactivity, and partial proteolysis of tangle-bound tau in extracellular tangles. (Am J Pathol 1990, 137:711–723)***

Alzheimer's disease (AD) is characterized histologically by the region-specific accumulation of senile plaques and neurofibrillary tangles (NFTs) in the brain. Immunohistochemical attempts to characterize plaques and tangles in molecular terms were based initially on antibodies raised against normal cellular constituents. Using this approach, many, although not all, of the elements making up the normal neuronal cytoskeleton were found in tangles, including neurofilament proteins,[1–5] microtubule-associated proteins tau[6–15] and MAP2,[6] vimentin,[16] and actin.[17] Tubulin has not been found. Noncytoskeletal antigens that have been found in tangles include ubiquitin[18–22] and B amyloid,[23,24] although the latter has been disputed.[25–27]

Paired helical filaments (PHFs) are known to contribute to the structure of both tangles and plaques. Structural studies of isolated PHFs have shown them to consist of a double helical stack with an axial periodicity of 3 nm.[28–30] Further biochemical and immunologic studies have demonstrated that the PHF has a protease-sensitive fuzzy outer coat, which accounts for about 20-kd per subunit, and a structurally more regular protease-resistant core. The latter accounts for a further 100 kd per subunit.[31]

Tau protein has been shown to contribute substantially to the fuzzy outer coat of the PHF.[32] It appears to be oriented so that the N-terminal 200 amino acids contribute to the pronase-sensitive fuzzy coat, whereas the microtubule binding domain, consisting of three[32,33] or four[34] tandem repeats of approximately 31 amino acids each, appears to be tightly bound within the pronase-resistant core. It also has been shown that an epitope recognized by a PHF-core–specific monoclonal antibody, 6.423, is partially occluded in most isolated PHFs by the fuzzy outer coat, but exposed after pronase digestion *in vitro*.[32]

We now report the results of a histologic study making use of antibodies directed against antigens associated with three portions of the tau molecule, the N-terminus, triple-repeat region, and the C-terminus, as well as findings with anti-ubiquitin and anti-amyloid antibodies. We show that it is possible to distinguish two mutually exclusive populations of tangles: one in which the fuzzy-coat antigens are present and the 6.423 epitope is occluded; another, in which fuzzy-coat antigens are absent but the 6.423 epitope is exposed. The former appears to be intracellular, the latter, extracellular. Extracellular NFTs have been described previously and identified by their micro-

Supported in part by the Della Martin Foundation.

Accepted for publication April 25, 1990.

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**Table 1.** *Sex, Age at Death, Duration of Dementia, and Histologic Diagnosis of Demented and Nondemented Subjects*

| Subject | Sex | Age at death | Duration (years) | Diagnosis |
|---|---|---|---|---|
| 36 | M | 70 | 6 | PD, AD |
| 37 | F | 87 | 1 | AD |
| 40 | M | 76 | 7 | AD |
| 41 | M | 74 | 11 | AD |
| 43 | M | 70 | 9 | AD |
| 49 | F | 71 | | AD |
| 51 | M | 87 | 7 | AD |
| 52 | M | 76 | 10 | AD |
| 529 | M | 40 | — | NP |
| 530 | M | 34 | — | NP |
| 531 | M | 23 | — | NP |
| 532 | M | 51 | — | NP |
| 533 | M | 86 | — | NP |

PD, Parkinson's disease; AD, Alzheimer's disease; NP, no significant pathology found.

scopic appearance,[35] certain staining properties,[36] their association with astrocytes,[37] and the absence of taulike epitopes.[38] Bancher et al[39] have suggested that taulike epitopes are hidden in extracellular tangles. We show that, although tangles with a typical extracellular appearance are not labeled by antibodies raised against tau N- and C-terminus antigens, they are labeled by antibodies directed against the triple-repeat region of tau. The converse is true for intracellular tangles. Therefore, in the transition from intracellular to extracellular stages, NFTs undergo changes that appear similar to those produced by protease digestion *in vitro*.

## Methods and Materials

Brain tissues were obtained after death from eight patients who died while hospitalized and satisfied DSM-III criteria for dementia[40] at some time before death. Abundant NFTs and senile plaques were found in the hippocampus and cerebral cortex (frontal and temporal lobes) in each patient, satisfying NIMH-ADRDA criteria for definite AD.[41] In addition to NFTs and senile plaques, Lewy bodies were found in neurons of the substantia nigra and the nucleus of the locus ceruleus (nLC) in one patient (Table 1) who had received a clinical diagnosis of Parkinson's disease 7 years before the diagnosis of dementia was made. The mean age at death of the AD patients was 76.4 years (SD, 6.5 years). The estimated duration of dementia varied from 1 to 11 years. Brains from five nondemented patients, demonstrating no pathology on routine neuropathologic examination, served as controls. The ages of the five controls were 23, 34, 40, 51, and 86 years. These data are summarized in Table 1.

Brains were removed 6 to 12 hours after death and divided in the midsaggital plane. From one half, specimens were taken of Brodmann areas 10 and 21, the pons, the basal forebrain caudal to the decussation of the anterior commissure, and the posterior hippocampus. These specimens were fixed overnight in 5% acetic acid in methanol, embedded in paraffin, and sectioned serially at 10 μm. The remainder of the sampled half-brain was fixed in 10% buffered formalin; the unsampled half was frozen.

### Immunocytochemical Methods

Serial sections for immunohistochemistry were deparaffinized in xylene, hydrated in the conventional manner, and treated with 1% aqueous hydrogen peroxide. They were treated with a solution of nonfat dry milk (2% in PBS) to block nonspecific labeling before being placed (1 hour) in a monoclonal or polyclonal (serum) antibody solution, the preparation of which is described below. The sections were subsequently incubated at room temperature with 1% peroxidase-conjugated rabbit immunoglobulin to mouse immunoglobulin or 1% peroxidase-conjugated swine immunoglobulin to rabbit immunoglobulin (DAKO-PATTS, Denmark), in 10% human serum in PBS. Perioxidase was visualized with diaminobenzidene (DAB) (1% in Sorensen's buffer at pH 6.0) and activated with hydrogen peroxide. Sections were counterstained with Ehrilch's hematoxylin (1.5% in distilled water). Dilutions of antibodies were empirically determined to produce maximal specificity with a minimum of background staining. Monoclonal antibodies 7.51, 6.423, and 4G8 were diluted 1:100, 9.42 1:10, and 5.25E 1:1000; polyclonal antibodies BR133 and BR134 were diluted 1:1000.

Monoclonal antibody 6.423, used in this study, has been previously characterized.[32] It and two other monoclonal antibodies (7.51 and 9.42) were prepared against subfractions of a highly enriched PHF fraction prepared from the brain of a deceased AD patient. Monoclonal antibodies 6.423[32] and 7.51 (unpublished data) have been shown to recognize a 9.5-Kd tau fragment extracted from the pronase-resistant core of the PHF by sonication in formic acid, which corresponds to the triple-repeat region of the molecule. Competative ELISA assays have shown that two epitopes recognized by 6.423 and 7.51 are located on the same peptide fragment (Harrington, unpublished data) demonstrating that 7.51, although not labeling isolated PHFs, is directed against a PHF core epitope. Monoclonal antibody 6.423, previously characterized,[32] has been shown to label isolated PHFs. BR133 and BR134 are antisera raised in rabbits immunized with synthetic peptides corresponding to residues 1 to 16 and 339 to 352, respectively, of human tau. Monoclonal antibody

4G8, raised in mice against a synthetic peptide corresponding to residues 1 to 24 of the B-amyloid sequence,[42] was the gift of Dr. D. L. Miller. Its histologic immunolabeling characteristics and those of 9.42 have been shown to be identical (Wischik et al, manuscript in preparation). Monoclonal antibody 5.25E, raised against PHF preparations and shown to react with ubiquitin,[43,44] was the gift of Senetek PLC (Aarhus, Denmark).

In each case, serial section series were prepared from the frontal and temporal cortices, hippocampus, basal forebrain, and pons. In each series, the first section was treated with 7.51, the second with 6.423, the third with BR133, the fourth with 9.42, and the fifth with 4G8. Additional series were prepared from the brainstem in each case and treated, sequentially, with the same antibodies in varying sequences. The latter brainstem series were used in a study of antibody distribution in the nucleus raphé dorsalis (nRD), which is found in the paramedian region of the pontine raphé in sections through the nLC. Additional sections of hippocampus were used with other antibodies (for example BR134) as required. In each series, two additional sections, one not treated with the primary antibody, the other (in the case of polyclonals) treated with antiserum plus the antigen used to generate it, served as controls. Specificity of antisera used was established by demonstrating the loss of immunoreactivity after preadsorption of each antiserum with its native antigen.

Photomicrographs were made on Ilford Pan F film with a green filter to emphasize the difference between immunolabeled (yellow-brown) objects and background (including nuclei), which were blue. The photographic density of the latter appeared artifactually enhanced.

## *Confocal Microscopy*

Serial sections of acetic methanol-fixed hippocampus were prepared from two demented subjects. They were deparaffinized, treated in nonfat dry milk, incubated with BR133 (as above), then incubated with fluorescence isothiocyanate (FITC)-conjugated goat anti-rabbit gammaglobulin for 1 hour. Sections were subsequently counterstained with Congo red according to the fluorescence method of Puchtler and Sweat.[45] Specimens, mounted in DPX on glass slides, were examined with an MRC-500 confocal imaging system (Bio-Rad, Cambridge, MA), using an argon ion laser.[46] Serial, confocal, optical 1 $\mu$m-thick sections through the 10-$\mu$m paraffin sections were combined to form projected images. Computer-generated stereo images also were examined. By means of two-channel filter blocks (A1 and A2) with 512-nm excitation, the FITC-labeled antibody (BR133) was readily visualized with reference to a Congo red-counterstained image. The two images were collected simultaneously and combined by means of software in such a way that the antibody appeared green and the counterstain red.

## *Electron Microscopy of Isolated PHFs*

Paired helical filaments were isolated as previously described,[31] with and without a pronase digestion step. The preparations were extensively dialized against distilled water. A small droplet of PHF suspension was placed on a carbon-coated grid (400 mesh) and allowed to evaporate partially. The grid was then placed on a drop of 0.1% gelatin in phosphate-buffered saline and blocked for 5 to 10 minutes. The grid was lifted, excess solution was blotted, and placed on a solution of 6.423 for 1 hour. The grid was then washed with a few drops of 0.1% solution of gelatin in phosphate-buffered saline, blotted, and placed on a solution of the appropriate second antibody conjugated to gold (Janssen Auroprobe EM goat anti-rabbit or goat anti-mouse IgG G5) for 1 hour. The grid was washed with a few drops of the gelatin solution, blotted, stained with a few drops of 1% lithium phosphotungstate, and allowed to air dry. Micrographs were recorded with a Philips electron microscope 301 (Eindhoven, The Netherlands), at an operating voltage of 80 kV, and at nominal magnifications between ×25,000 and ×45,000.

## *Results*

The descriptions that follow apply in a general way to all regions studied. They were readily apparent in the hippocampal formation and entorhinal cortex, which tend to be the most severely affected by Alzheimer-type histopathologic changes.

## *Tau-related Antigens*

Neurofibrillary tangles were studied in frontal and temporal cortices, hippocampus, nRD, nLC, and nucleus basalis of Meynert (nB) with polyclonal antibodies BR133 and BR134, and with monoclonal antibodies 7.51 and 6.423. These antibodies are directed against antigens associated with different portions of the tau molecule.

Two antigenically distinct and mutually exclusive classes of NFTs could be distinguished on the basis of reactivity with BR133/BR134 and 6.423 in tissues fixed in acetic methanol and embedded in paraffin wax. NFTs, decorated by BR133 and BR134, had a dense, flamelike appearance in hippocampal and cerebral cortical pyramidal cells (Figure 1) and had a more loosely structured,

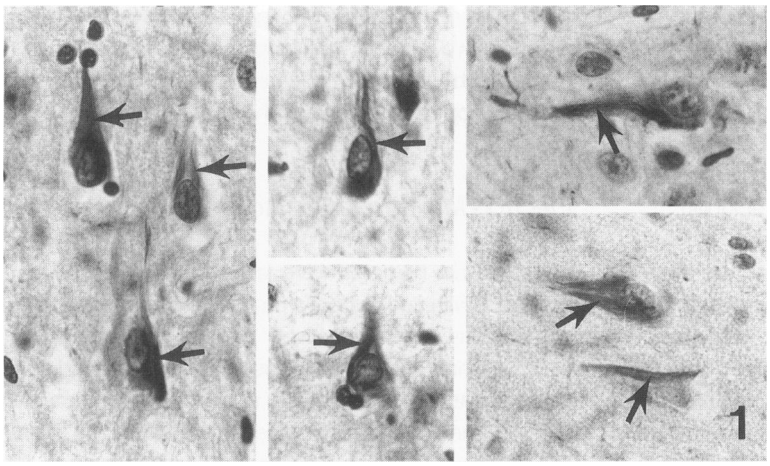


**Figure 1.** *Photomicrographs of BR133-labeled intracellular NFTs (arrows) in hippocampus (CA2-3) (acetic methanol fixation; original magnification ×400).*

globose appearance in neurons of nB, nLC, and nRD. Their appearance in these (and all other) neurons was similar to that seen typically after conventional silver staining. These BR133/BR134-positive NFTs were uniformly 6.423 negative. Other NFTs were 6.423 positive and BR133/BR134 negative. The dichotomy was particularly apparent in the hippocampal formation in which tangles decorated by BR133/BR134 tended to be relatively more abundant in the CA2-3 region, and those decorated by 6.423 were more abundant in CA1. Both classes of NFTs were 7.51 positive.

The appearance of a typical neuronal nucleus and a well-demarcated cell body suggested that NFTs decorated by BR133/BR134 were intracellular (Figure 1). Conversely the NFTs decorated by 6.423 appeared to be extracellular. They were not associated with a visible neuronal nucleus or plasma membrane when compact (Figure 2). These NFTs were either larger than those decorated by BR133/BR134 or they appeared as extracellular fragments. When less compact, they often showed considerable dispersion into the surrounding neuropil, which was especially noted in CA1 and entorhinal cortex (Figure 2).

With the confocal microscope, it was possible to establish more definitively that FITC-tagged, BR133-immunolabeled tangles were intracellular in location. This was apparent in serial optical sections (Figure 3) and three-dimensional

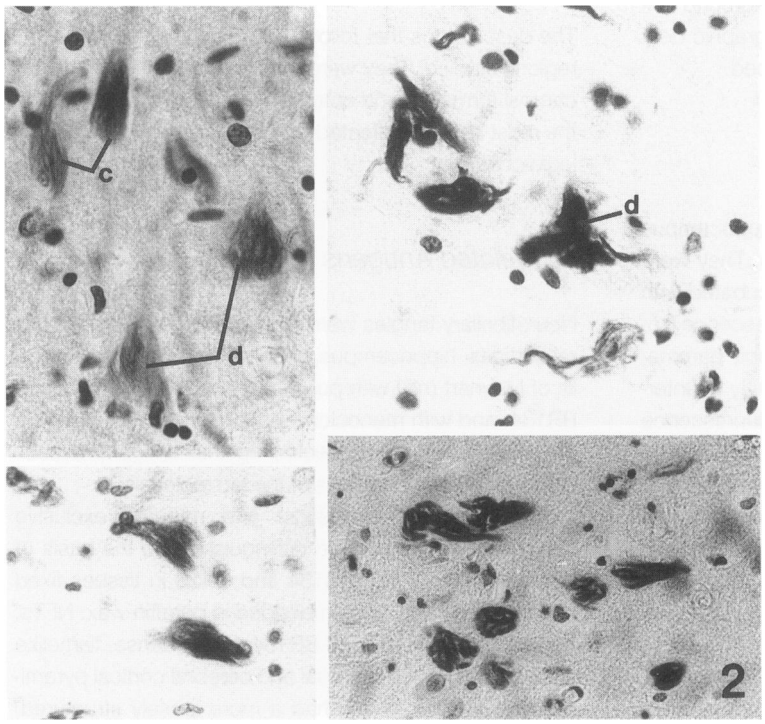


**Figure 2.** *Photomicrographs of 6.423-labeled extracellular NFTs in hippocampus (CA1). Examples of compact (c) and dispesed (d) types of extracellular NFTs are indicated (acetic methanol fixation; original magnification ×400).*

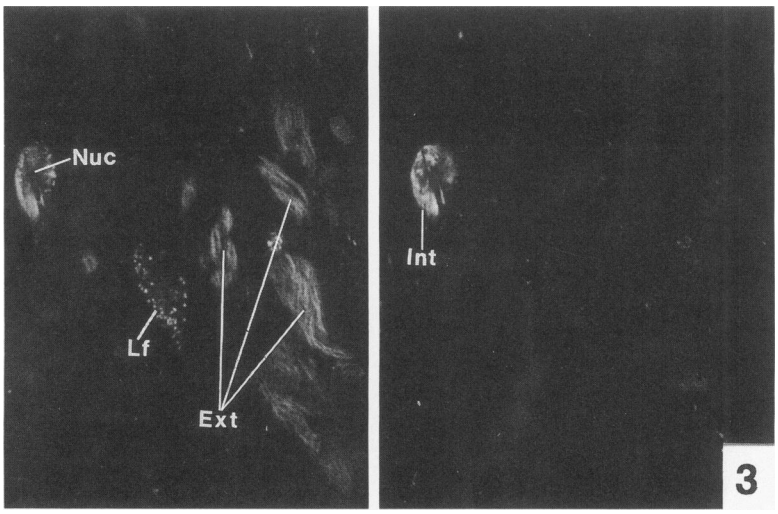


**Figure 3.** *Confocal microscopic fluorescence micrographs. A representative optical section is shown as it appeared in the red-transmitting channel (left, Congo red stained) and the yellow-green-transmitting channel (right, FITC labeled). The FITC-tagged BR133, visualized by itself in the right channel, selectively immunolabels an intracellular tangle (Int). This label was of such great intensity that it was also seen (less intensely) in the left channel. Intracellular NFTs, immunolabeled by BR133, are typically distributed around a central (unlabeled) nucleus (Nuc). Granular autofluorescent lipofuscin (Lf) is seen in the peripheral cytoplasm of a tangle-free neuron and in the NFT-laden neuron seen in the left channel. Several Congo red-labeled extracellular NFTs appear only in the left channel (Ext) (original magnification ×550).*

images of the pyramidal cell neuronal membrane and nucleus were clearly seen. Extracellular tangles, not associated with neuronal membranes or nuclei, were readily visualized in Congo red-counterstained preparations because of their bright red-orange fluorescence and distinctive, coarsely fibrillar, dispersed appearance (Figure 3).

## *Tau-related Immunolabeling of Neuropil and Plaque Elements*

In addition to tangles, neurites of various diameter and length were demonstrated by 7.51 and by BR133 and BR134. These appeared similar to those described by others.[47–49] While more neurites were sometimes labeled by 7.51 than by BR133/BR134, immunolabeling with 7.51 tended to be more erratic and required a greater concentration of antibody. Such elements were not found in nondemented controls. While some of these neurites appeared intact, most appeared to be dystrophic, that is, they were segmented and variably dispersed. They were found variously distributed in the neuropil, in the walls of blood vessels, and in the peripheries of plaques. In contrast to BR133/BR134 and 7.51, 6.423 did not stain long neuritic segments in the neuropil. Only some very short segments of irregular dimensions were stained, and no 6.423 immunolabeling could be demonstrated in blood vessel walls. Even these irregular elements were rare in plaque peripheries, and as a result plaques were usually inapparent in 6.423 preparations.

While plaque cores were never decorated with BR133/BR134 or 7.51, a faint, diffuse 'blush' of color, presumably associated with labeling of fine neurites in the periphery of plaques, was often seen. In addition, larger-diameter neurites, comparable to those demonstrated by silver stains, were sometimes immunolabeled in plaque peripheries by 7.51.

We note here, parenthetically, that immunolabeling with Alz[50,51] provided by Dr. P. Davies, was virtually indistinguishable from that observed with BR133/BR134 in two subjects who were tested.

## *Tau-related Immunolabeling of Isolated PHFs*

Whereas most PHFs isolated without a pronase digestion step were not labeled with 6.423 at dilutions greater than 1:200, a few were always found in such preparations that were strongly labeled below 1:1000 dilution (Figure 4). The latter labeling was typical of that seen usually after pronase digestion of isolated PHFs (Figure 4).

Labeling by BR133 or BR134 of PHFs isolated without pronase digestion was weak and variable. No labeling of isolated PHFs, whether pronase digested, could be demonstrated with 7.51.

## *Anti-ubiquitin and Anti-amyloidlike Immunoreactivity of NFTs*

Immunolabeling of tangles with an anti-ubiquitin monoclonal antibody (5.25E) was similar, but more intense, to that seen with 7.51 in all regions studied. Tangles ranged in appearance from well-formed dense fibrillar structures, clearly associated with neuronal nuclei within well-demarcated neuronal perikarya, to dispersed and enlarged extracellular tangle fragments. Love et al,[50] using an affinity-purified rabbit anti-ubiquitin, reported labeling only of intracellular NFTs.

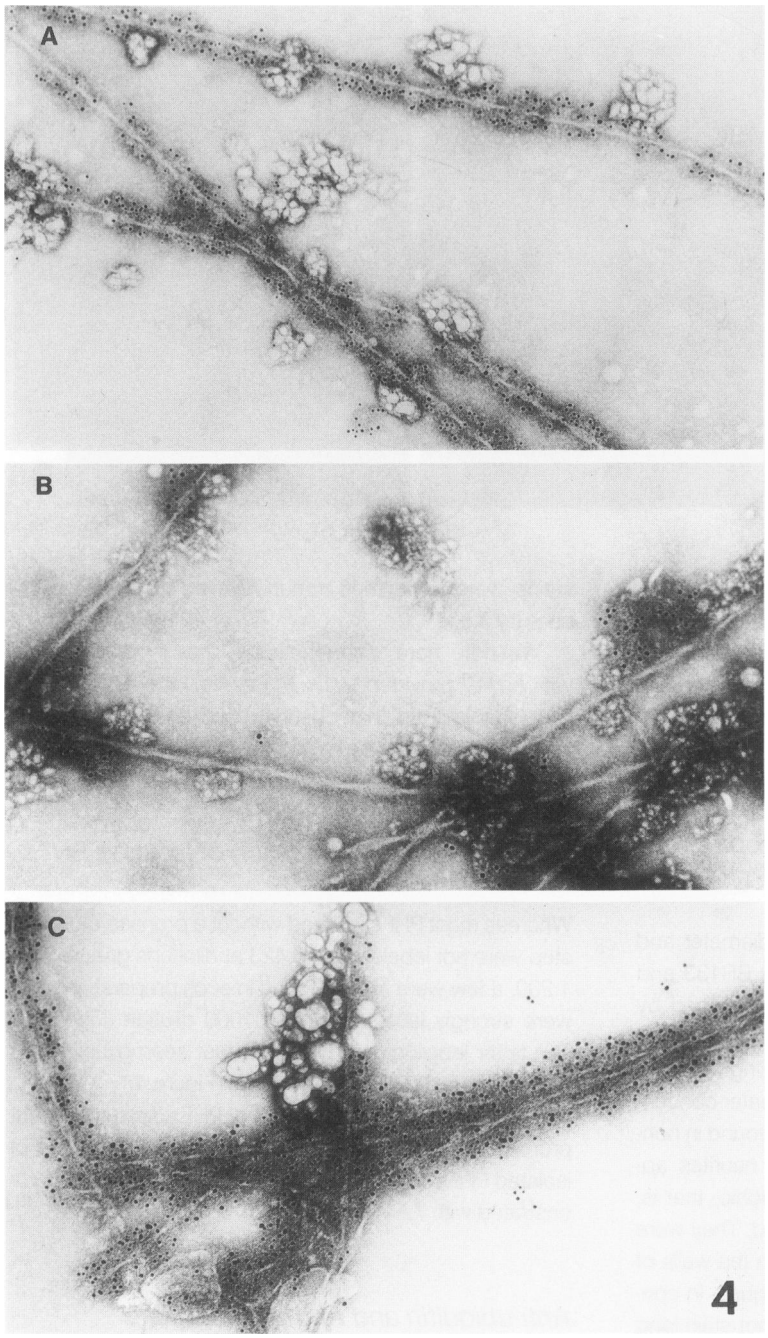


**Figure 4.** *Electron micrographs illustrating the effect of pronase digestion on gold-tagged-6.423 immunolabeling of isolated PHFs.* **A:** *MAb 6.423 (1:750) labels most PHFs after pronase digestion (original magnification ×90,000).* **B:** *Most PHFs isolated without pronase digestion are not immunolabeled by 6.423 (1:500) (original magnification ×90,000).* **C:** *A subset of PHFs is strongly labeled by 6.423 (1:1000) after isolation without pronase digestion (original magnification ×90,000).*

Dystrophic neurites in the neuropil appeared comparable in form and distribution, although more numerous than seen either with 7.51 or BR133/BR134. Larger neurites, as well as finer, diffusely distributed fibrillar elements, were immunolabeled in the periphery of senile plaques by 5.25E. This fine, fibrillar staining made plaques more clearly visible with 5.25E than with the tau-related antibodies. Plaque cores appeared weakly 'stained' with 5.25E. While they were never decorated with the typical, dense, yellow-brown DAB reaction product, staining (grey-brown) was distinct from that observed with anti-tau antibodies. Comparable staining of plaque cores was not seen in the nondemented controls. This reaction was, therefore, designated as '±' in Table 2.

## *Colocalization of Antibodies in the Hippocampus and nRD*

It was possible, in serial sections of hippocampus, to demonstrate regional colocalization of immunolabeling:

**Table 2.** *Relative Numbers of Histologic Structures Immunolabeled by Antibodies in Acetic Methanol-fixed, Wax-embedded, AD Brain Tissue*

| | Neuritic Plaques | | | Neurofibrillary tangles | | |
|---|---|---|---|---|---|---|
| | Periphery | | | | | |
| | Core | Fine fibrillar elements | Larger neurites | Intracellular | Extracellular | Neuropil, dystropic neurites |
| BR133 | — | + | +++ | ++++ | — | ++++ |
| BR134 | — | + | +++ | ++++ | — | ++++ |
| 6.423 | — | — | + | — | ++++ | + |
| 7.51 | — | ++ | +++ | +++ | +++ | +++ |
| 9.42 | ++++ | ++++ | — | — | ++[1] | — |
| 4G8 | ++++ | +++ | — | — | ++[1] | — |
| 5.25E | ± | +++ | +++ | +++ | ++++ | +++ |

++++, immunolabeling in virtually every case and unlabeled forms not seen; +++, immunolabeling in most cases and unlabeled forms sometimes found; ++, labeled forms and unlabeled forms frequently found; +, most cases not immunolabeled, although at least one case of definite labeling was found; ±, ambiguous (see text); [1], subset of extracellular tangles.

identifiable groups of intracellular NFTs, labeled by BR133/BR134, were not labeled by 6.423, which labeled extracellular NFTs in adjacent serial sections. Both groups were labeled by 7.51. A subset of extracellular 6.423-labeled NFTs was labeled by 4G8 (not illustrated) and 9.42 in CA1 (Figure 5). Immunolabeling in tissue sections by these two monoclonal antibodies is indistinguishable. In the hippocampus it was not possible to demonstrate colocalization in individual neurons. This was accomplished in serial sections of nRD, in which individual neurons were readily identified by their relationship to small blood vessels (Figures 6 and 7) and could be followed easily in serial sections. Neurofibrillary tangles labeled by 6.423 were never associated with nuclei and always appeared in some stage of degeneration in nRD; such tangles were never labeled by BR133. Both degenerating 6.423-labeled and intact BR133-labeled NFTs were labeled also by 7.51 (Figure 6). While not all 6.423-labeled tangles were labeled by 9.42, a subset was (Figure 7) and this subset was also labeled by 4G8. In both hippocampal region CA1 and nRD, the number of tangles labeled with 9.42 and 4G8 were fewer than those labeled with 6.423.

## Other Fixation Conditions

Although the mutually exclusive character of staining with BR133/BR134 and 6.423 was best demonstrated in acetic methanol-fixed, paraffin-embedded tissues, other fixations were tested in two subjects. In all the fixation conditions tested, BR133/BR134 immunolabeled only NFTs, judged to be intracellular by the criteria of their compact structure and close association with a neuronal nucleus. On the other hand, the distribution of 6.423 labeling was significantly extended in several fixation conditions to include intracellular tangles (Table 3).

## Controls

No immunolabeling was observed in any section of the four young controls. In the one older control subject, the few NFTs seen were labeled with with tau-reactive antibodies and 5.25E, and the few plaques seen were labeled with 9.42, 4G8, and 5.25E.

# Discussion

Tau, one of many proteins associated with NFTs, can be linked with certainty to PHFs, which are known to form the major structural constituent of the NFT. Predictably, anti-tau antibodies have been shown to label some NFTs in histologic preparations.[7-14,39] Unpredictably, tangles lacking tau immunoreactivity have been described also, particularly extracellular tangles.[38,39] In this report, we approached the problem of mapping tau antigens in relation to the neurofibrillary degeneration of AD with antibodies directed against three segments of the tau molecule. These segments were chosen to distinguish between the PHF-core-binding domain of tau (recognized by 6.423 and 7.51), and the N and C termini (recognized by BR133 and BR134) (Figure 8).

The tangle-labeling profiles of these antibodies in histologic preparations fell into three distinct categories: 6.423 labeled extracellular tangles predominantly; BR133 and BR134 labeled intracellular tangles exclusively; 7.51 labeled both types. The distribution of labeling in all brain regions studied was such that, in appropriate fixation conditions, 6.423-positive tangles and BR133/BR134-positive tangles formed complete and mutually exclusive subsets of tangles labeled with 7.51.

The distinction between intracellular and extracellular immunolabeling was supported by the following: 1) NFTs

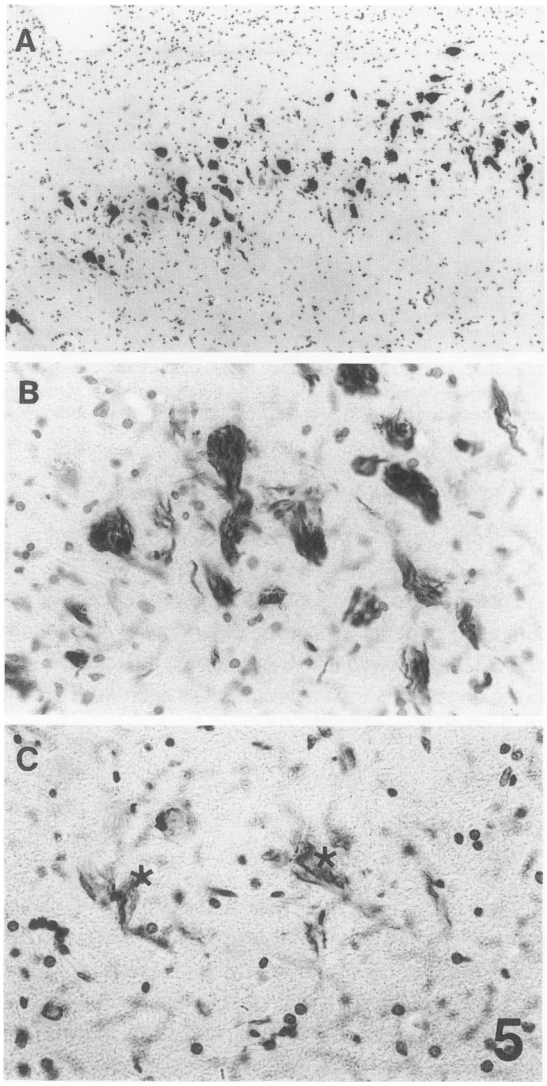


Figure 5. *Photomicrographs of hippocampus (CA1) showing immunolabeling in the same group of extracellular NFTs by 6.423* (A, B) *and 9.42* (C), *the latter indicated by* (acetic methanol fixation;* A: ×75; B, C: ×250).

labeled by BR133/BR134, typically, were associated with neuronal nuclei and soma, while those labeled by 6.423 were not; 2) while NFTs labeled by BR133/BR134 were always compact in appearance, those labeled by 6.423 were more coarsely fibrillar, more loosely arrayed to the point of often appearing partially disintegrated; 3) pyramidal neurons labeled in layers III and V of cerebral cortex by BR133/BR134 typically appeared intact, with a prominent apical dendrite directed toward the pial surface, whereas this characteristic morphology of pyramidal neurons was never demonstrated by 6.423; 4) images similar to dystrophic axons in the neuropil (as demonstrated by silver staining) were produced by immunolabeling with BR133/BR134, whereas only very few segments of neurites were labeled by 6.423; 5) a discrete population of globose NFTs in nRD, associated with a nucleus and clearly demarcated cell body, was immunolabeled by BR133/BR134, while those labeled by 6.423 were never associated with a nucleus, and typically appeared in some stage of disintegration; 6) tangles immunolabeled by BR133/BR134 in nRD neurons were never labeled by 6.423 and vice versa. That these two NFT populations in nRD neurons were regularly labeled by 7.51 emphasizes their separateness and confirms that 7.51 immunolabels both intracellular and extracellular tangles; 7) BR133-FITC–labeled NFTs were shown in three-dimensional images of pyramidal neurons generated by confocal microscopy to have an exclusively intracellular location.

The restriction of 6.423 labeling to extracellular tangles was shown to be fixation dependent, whereas the restriction of BR133/BR134 labeling to intracellular tangles was found to be independent of fixation procedure. The former appears to be explained by the partial occlusion of the 6.423 epitope by fuzzy-coat material, as shown previously by immunoelectron microscopy of isolated PHFs.[31] This explanation is supported here by the observation that BR133/BR134-positive tangles are 6.423 negative in certain fixation conditions.

The sensitivity of tau immunolabeling to fixation variables has been noted previously. For example, the demonstration by Kowall and Kosik[47] that tau epitopes are predominantly axonal in nondemented subjects, but somatodendritic in demented ones (although consistent with the sequestration of tau within PHFs in the somato-dendritic compartment of pyramidal cells[33]), appears to depend on the particular conditions of tissue preparation used.[47] Distinctive labeling of axonal tau was not demonstrated here in acetic methanol-fixed tissues (nor was it demonstrated after fixation in 10% buffered formalin, 5% glutaraldehyde, 4% paraformaldehyde, or Carnoy's fluid) with any of the antibodies that would be expected to recognize axonal tau.

While the observations reported here point to the existence of a clear antigenic difference between intracellular and extracellular tangles, it may be difficult with some tangles, particularly when the plane of section excludes or obscures the nucleus, to make an unambiguous assignment. On the one hand, some tangles, while intracellular in appearance, might be in the process of extracellular transition. On the other hand, some extracellular tangles might have escaped proteolysis. The observations reported here apply, therefore, to the numerically preponderant extremes of antigenic differences between intra- and extracellular tangles. The independent observation that some of the PHFs isolated without pronase digestion are labeled by 6.423 at antibody concentrations adequate for labeling of pronase-treated PHFs but not fuzzy-coated

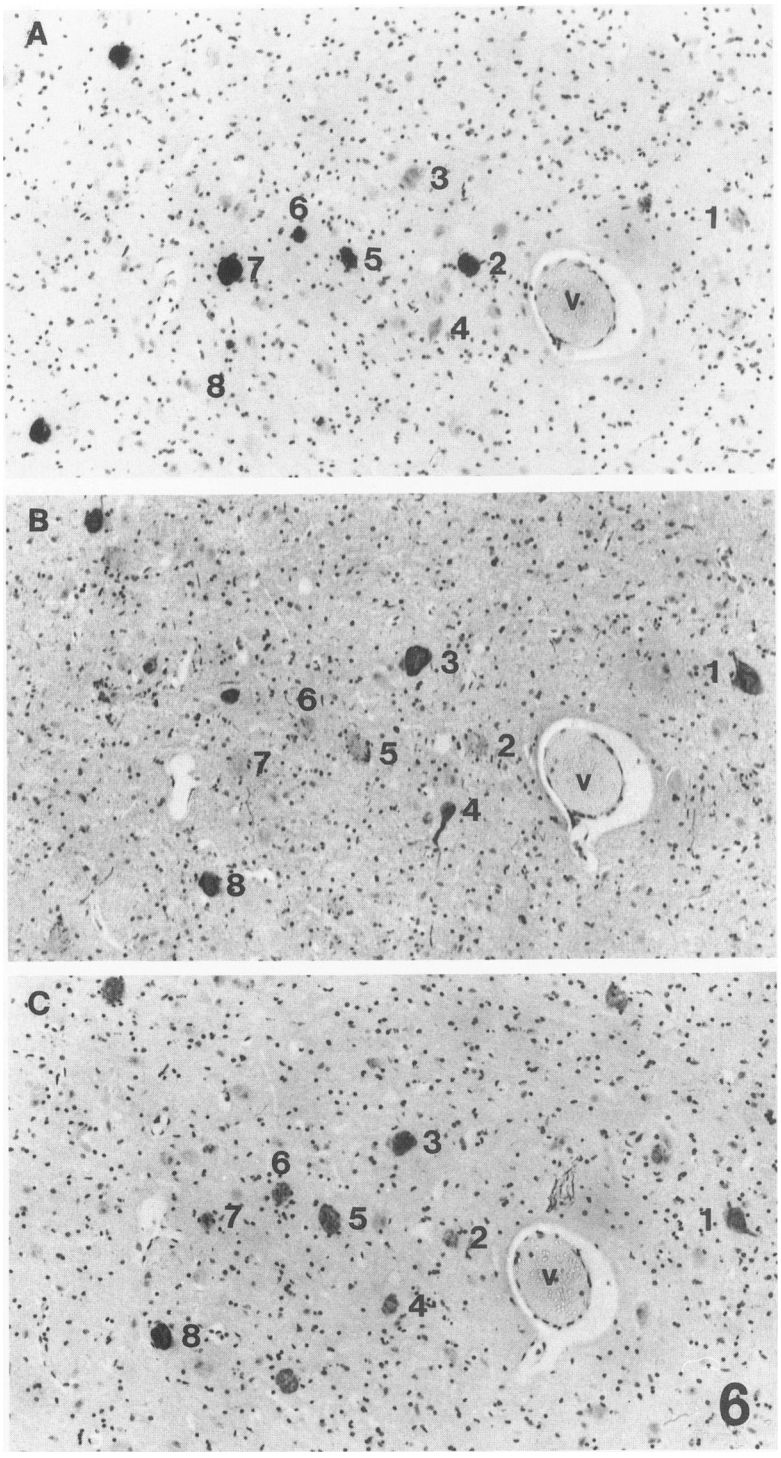


Figure 6. *Photomicrographs of serial sections of nRD showing immunolabeling of the same globose tangles (numbered) in the vicinity of a small blood vessel (v). NFTs labeled by BR133* (A) *are not labeled by 6.423* (B) *but are labeled by 7.51* (C) *(acetic methanol fixation; original magnification ×100).*

ones (Figure 4), suggests that such PHFs derive from extracellular tangles. It also suggests that such tangles have undergone some form of partial proteolytic degradation in the extracellular space *in vivo*. This is consistent with the absence of BR133/BR134 reactivity in extracellular tangles.

Earlier work[31] suggesting that the triple-repeat region of tau is protected from pronase digestion *in vitro* is supported by findings here that suggest that the triple-repeat region of tau is protected during the course of extracellular, proteolytic degradation of NFTs *in vivo*. It is supported by the fact that extracellular tangles are both 6.423 and

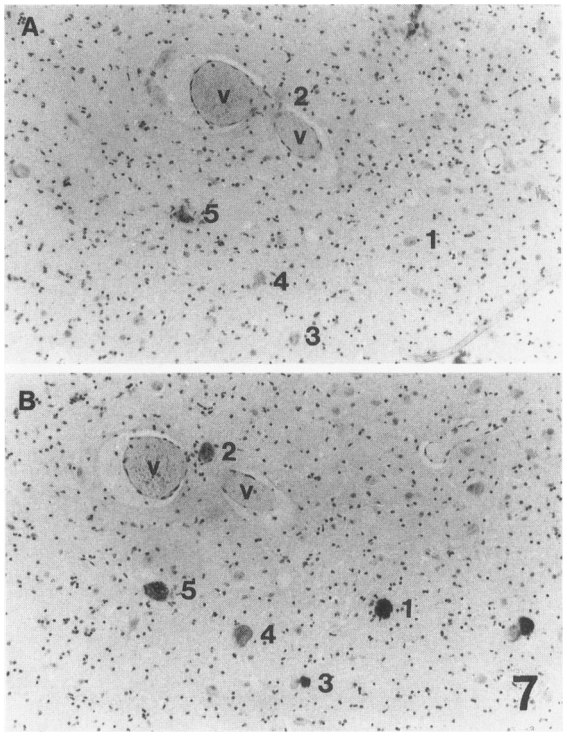


Figure 7. *Photomicrographs of serial sections of nRD showing immunolabeling of a subset (tangle 5) of 6.423-labeled NFTs* (B) *by 9.42* (A) *(acetic methanol fixation; original magnification × 100).*

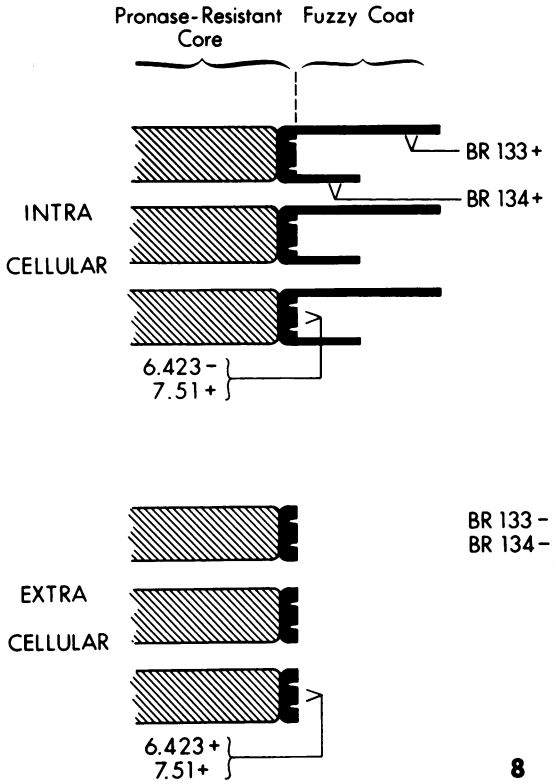


Figure 8. *Diagrammatic representation of the relationship between submicroscopic localization of epitopes and patterns of immunolabeling in intracellular and extracellular NFTs. One of the stack of subunits making a one-half PHF is indicated on the left by hatching. Tau protein is indicated by a solid dark line on the right, and consists of three segments containing epitopes recognized by tau antisera and monoclonal antibodies (redrawn from Wischik*[60]*).*

7.51 positive, but BR133/BR134 negative. The loss of the BR133/134 epitopes in extracellular tangles suggests that the N and C termi are not protected from protease digestion *in vivo* or *in vitro*. The association of ubiquitin with both intracellular and extracellular NFTs suggests that the process of neurofibrillary degeneration may require ubiquitination. Ubiquitin is believed to form conjugates with damaged or abnormal proteins marked for degeneration by an ATP-dependent nonlysosomal proteolytic system.[52,53] Its occurrence in normal-appearing neuronal processes, as well as in NFTs in varying states of degradation (but not in axons of deep white matter pathways) in AD, as reported here and by others,[20-22] supports the suggestion that ubiquitin is an integral component of PHFs.[19,44]

Early events in the course of tangle degradation may be closely associated with tau labeling in the somato-den-

Table 3. *Effect of Fixatives on Relative Numbers of Histologic Structures Immunolabeled by 6.423 and BR133 (parentheses) in Wax-embedded AD Brain Tissue*

| | Neuritic Plaques | | | Neurofibrillary tangles | | |
|---|---|---|---|---|---|---|
| | Periphery | | | | | |
| | Core | Fine fibrillar elements | Larger neurites | Intracellular | Extracellular | Neuropil, dystropic neurites |
| Acetic methanol | −(−) | −(+) | +(+++) | −(++++) | ++++(−) | +(++++) |
| Carnoy | −(−) | −(+) | +(+++) | −(++++) | ++++(−) | −(++++) |
| Formalin | −(−) | +(++) | +++(++++) | ++++(++++) | +(−) | −(++++) |
| Formalin acetic | −(−) | +(++) | +++(++++) | ++++(++++) | +(−) | +(++++) |
| Paraformaldehyde | −(−) | +(++) | +(++++) | ++++(++++) | +(−) | −(++++) |
| Glutaraldehyde | +(−) | +(++) | ++(++++) | ++(++++) | +++(−) | −(++++) |

++++, immunolabeling in virtually every case and unlabeled forms not seen; +++, immunolabeling in most cases and unlabeled forms sometimes found; ++, labeled and unlabeled forms frequently found; +, most cases not immunolabeled, although at least one case of definite labeling was found; ±, ambiguous (see text).

dritic compartment of affected neurons. The earliest change in tau reactivity that we detect is the appearance of BR133/BR134 immunolabeling. The subsequent loss of the fuzzy coat and the exposure of the 6.423 epitope appears to be associated closely with the breakdown of neuronal membranes and the introduction of partially degenerated NFTs into the extracellular compartment. Dispersion of NFTs, perhaps related to their invasion by astrocytic processes,[37] characterizes the extracellular phase of NFT degradation. At least in a subset of extracellular NFTs, this process is associated with the appearance of 4G8 and 9.42 labeling. Our findings do not elucidate the origin of amyloidlike material detected by these antibodies, but it seems likely that this 4G8- and 9.42-reactive material is neuronal in origin because neurons have been shown to synthesize the amyloid precursor protein in normal brain and in AD.[54,55] However vascular and/or glial contributions to the formation of extracellular amyloid cannot be discounted.[56–59]

Our histologic findings suggest that the process of NFT degradation is a complex series of closely associated molecular events (Figure 8). These include, at least, ubiquitination of some unknown intraneuronal protein; a change in the immunoreactivity of tau; a relocation of NFTs from intracellular to extracellular compartments that is associated with the loss of tau epitopes from the fuzzy coat of PHFs; a dispersion of NFT fragments in the extracellular compartment associated with the appearance of amyloidlike immunoreactivity.

While the temporal sequence of these events remains unknown, some of the changes that we have described can be understood at the molecular level in terms of partial proteolytic degradation. It can be anticipated that the further analysis of the events of NFT degradation *in situ* by immunohistochemical methods may shed light on the molecular events of NFT formation, as well as the processes underlying neuronal death in AD.

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