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The biochemical pathway of neurofibrillary degeneration in aging and Alzheimer's disease

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Article abstract—*Objective:* To determine the spatiotemporal mapping of neurofibrillary degeneration (NFD) in normal aging and the different stages of AD. *Background:* The pathophysiological significance of AD lesions, namely amyloid plaques and neurofibrillary tangles, is still unclear, especially their interrelationship and their link with cognitive impairment. *Methods:* The study included 130 patients of various ages and different cognitive statuses, from nondemented control subjects (n = 60, prospective study) to patients with severe definite AD. Paired helical filaments (PHF)-tau and A β were used as biochemical and histologic markers of NFD and amyloid plaques, respectively. *Results:* NFD with PHF-tau was systematically present in variable amounts in the hippocampal region of nondemented patients age >75 years. When NFD was found in other brain areas, it was always along a stereotyped, sequential, hierarchical pathway. The progression was categorized into 10 stages according to the brain regions affected: transentorhinal cortex (S1), entorhinal (S2), hippocampus (S3), anterior temporal cortex (S4), inferior temporal cortex (S5), medium temporal cortex (S6), polymodal association areas (prefrontal, parietal inferior, temporal superior) (S7), unimodal areas (S8), primary motor (S9a) or sensory (S9b, S9c) areas, and all neocortical areas (S10). Up to stage 6, the disease could be asymptomatic. In all cases studied here, stage 7 individuals with two polymodal association areas affected by tau pathologic states were cognitively impaired. *Conclusions:* The relationship between NFD and Alzheimer-type dementia, and the criteria for a biochemical diagnosis of AD, are documented, and an association between AD and the extent of NFD in defined brain areas is shown.

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AD is a neurodegenerative disorder leading to amnesia, cognitive impairment, and dementia. Two types of lesions characterize the disease: amyloid deposits resulting from the extracellular aggregation of A β

peptide, and neurofibrillary tangles composed of intraneuronal bundles of paired helical filaments (PHF). PHF result from the aggregation of pathologic tau proteins, named PHF-tau.¹ Both lesions ex-

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tend progressively to neocortical brain areas during the course of AD.^{2,3} The number and extent of lesions are used to define stages in the diagnosis of definite AD.⁴ The link between amyloid deposits and neurofibrillary tangles is not yet clear. Is amyloid the cause of the disease, as a neurotoxic agent,⁵ or is it a marker of a deadly neuronal A β precursor protein (β PP) dysfunction that causes neurofibrillary degeneration (NFD)?⁶ What is the threshold in the number and distribution of lesions that generate cognitive impairment, and what is the extent of brain lesions at the onset of clinical symptoms?

We approached these questions by analyzing the spatiotemporal distribution of the lesions in a large group of patients of different ages and different cognitive statuses ranging from normal aging to severe definite AD. Because AD is common, one can expect to find and study subclinical AD in nondemented patients. However, any study must be multidisciplinary and must include clinical, neuropathologic, and biochemical data. Nondemented patients must be recruited through a prospective study, and the identification of the lesions must be specific, using precise biochemical markers. The first biochemical marker is A β peptide, the basic component of amyloid deposition, which can now be quantified by biochemical means.^{7,8} The second marker is PHF-tau, the basic component of PHF, the histologic hallmark of NFD.¹

Using these criteria, we studied the biochemical and immunohistochemical distribution of amyloid deposits and NFD in the different cortical brain areas of 130 patients.

Materials and methods. We included 130 patients in this study. Clinical, neuropathologic, and biochemical data are given in tables 1 through 3, which can be obtained from the National Auxiliary Publications Service (NAPS; see Note at end of article).

Patients. Most of the nondemented patients (n = 60) and 35 demented patients were from the geriatric department of E. Roux Hospital at Limeil-Brevannes, France. They represented all patients who were hospitalized for various disorders and died at this hospital, excluding those whose families opposed autopsy or for whom the postmortem delay was >24 hours. The reasons for hospitalization were general health (26%), recurrent falls or gait disturbances (19%), cognitive impairment (10%), heart disease (9%), stroke (5%), and infections (5%). On admission, all patients received a full examination, including a standard neurologic examination. Cognitive status was evaluated using the Mini-Mental State Examination (MMSE) of Folstein et al.⁹ and the Clinical Dementing Rating (CDR) score.¹⁰ These tests were performed every 6 months by the same investigator. On occasion, the MMSE was either not applicable or not performed because of specific physical incapacities such as blindness, limb fracture, or paralysis. Detailed results of the MMSE scores are reported (table 1, available from NAPS) for nondemented patients and those with moderate cognitive impairment. The CDR value for each patient was determined by questioning their caregivers or relatives of the patient. CDR scores are as follows:

0 = no memory loss, 0.5 = questionable, 1 = mild, 2 = moderate, and 3 = severe dementia. When necessary, psychometric tests such as the Benton test were performed.¹¹ For demented patients, screening for treatable causes included CT, EEG, serologic tests for syphilis and HIV, vitamin B₁₂ and folate levels, serum calcium determinations, and thyroid function tests. Other possible conditions affecting cognition are mentioned in table 1 (available from NAPS). The levels of education, graded from low (1) to high (3), are given in table 1 (available from NAPS). The onset of illness was also recorded. The clinical criteria for dementia were those given in the Diagnostic and Statistical Manual of Mental Disorders, 3rd ed., revised (DSM-III-R)¹²; for AD, National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)¹³; for vascular dementia, National Institute of Neurological Disorders and Stroke-Association Internationale pour la Recherche et l'Enseignement en Neurosciences (NINDS-AIREN)^{14,15}; and for mixed dementia, a Hachinski score.¹⁶ Clinical diagnosis was summarized as AD (possible, probable), vascular dementia, mixed dementia (AD with a strong vascular involvement revealed by investigations), or dementia (for patients with an uncertain clinical diagnosis). The other patients, mostly with AD, came from the Department of Neurology and the Memory Clinic in Lille University Hospital and had undergone a comprehensive clinical and neuropathologic assessment.

Brain areas studied. One hemisphere was deep-frozen for biochemical experiments, and the other was formalin-fixed for neuropathologic examination. For most patients, 16 Brodmann areas (BA) were analyzed by immunoblotting. All dissections were done by the same investigator.

Immunologic probes. NFT and PHF-tau were immunostained with AD2, a monoclonal antibody against PHF that is directed against phosphorylated tau proteins.¹⁷ Amyloid plaques and A β peptides were detected using antibodies from Dako (Carpinteria, CA) (monoclonal A β 8 through 17) or from our laboratory⁷ (polyclonal antibodies against the N and C-terminal part of A β 1 through 40 and 1 through 42).

Neuropathologic examination. Amyloid deposition and NFD were classified in cortical and subcortical areas. Classification of amyloid deposits was determined in 10 different brain areas by counting the number of plaques per square millimeter, according to Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria: 0 = none, + = sparse, ++ = moderate, +++ = frequent¹⁸; amyloid plaques were detected using thioflavine S and antibodies against A β . For patients with no amyloid deposits detected by neuropathologic and biochemical approaches, we attempted to immunolabel the amyloid with polyclonal antibodies from our group, from other groups, or from commercial sources. We also tried to improve the immunostaining by pretreating with microwaves or by altering concentrations of formic acid.

Neurofibrillary tangles were semiquantified by counting their numbers per square millimeter, using the immunologic probe AD2 directed against PHF-tau,¹ and were also silver stained by the Bielchowski method. Other neuropathologic features were also studied, such as vascular involvement, atrophy, and glial reaction. Neuropathologic

examination of AD patients from Lille was expressed according to the stages of Braak and Braak.³

Biochemical studies. Immunoblots. Brain tissues were homogenized in Laemmli's sample buffer 1:10 (w/v) containing 5% sodium dodecyl sulfate and heat treated. Total brain homogenates (50-microgram proteins) were resolved by electrophoresis on gradients of 10% to 20% (w/v) polyacrylamide in the presence of sodium dodecyl sulfate and then transferred onto nitrocellulose sheets. PHF-tau proteins were immunodetected by Western blots as described by Sergeant et al.¹⁹ For PHF-tau immunostaining, monoclonal antibody AD2 was used because it is able to directly detect PHF-tau in brain homogenates, whereas commercially available antibodies were not sufficiently sensitive.¹⁹

Dot-blot analyses. For dot-blot quantification of A β , brain samples from frontal pole and parietal cortex were homogenized in Laemmli's sample buffer and centrifuged at high speed. The resulting pellet was used for A β extraction, using formic acid.⁷ Amyloid was detected and quantified by dot-blot, using the procedure developed by Permanne et al.⁷

Image analysis for immunoblot and dot-blot quantification. Immunoblots and dot-blots were quantified using the ImageMaster program developed by Pharmacia (Piscataway, NJ). Linearity of the signal was maintained, as previously described.¹⁹ A β peptide quantification by dot-blots was expressed in picomoles per milligram of brain tissue. PHF-tau was quantified by measuring the area of the peaks corresponding to tau 55, 64, and 69 detected in the different cortical homogenates, which were then scored by comparison with a temporal cortex homogenate from a patient with early onset considered as a positive internal standard, as already described.²⁰ The arbitrary value of 10 was given to this positive standard (an AD patient with severe NFD) and 0 for a young healthy cognitively unimpaired patient.

Results. Ten to 20 different brain areas from 130 patients were analyzed for their PHF-tau content using immunoblotting with the antibody AD2. A β peptide was also quantified in the frontal and parietal regions, using anti-A β antibodies. Clinical, neuropathologic, and biochemical results are summarized in NAPS tables 2 and 3. The 130 patients were ranked according to the extent of the degenerating process by using PHF-tau as a biochemical marker (figure 1). A stage was established if more than three patients had the same distribution of PHF-tau in the different Brodmann areas (figure 2).

NFD with PHF-tau was systematically present in variable amounts in the hippocampal region of nondemented patients >75 years old. When NFD was found in other brain areas, it was always along a stereotyped, sequential, hierarchical pathway. We have distinguished 10 stages, from S0 to S10, corresponding to the successive involvement of different brain areas.

Stage 0. Stage 0 included eight control subjects (MMSE = 30) with no PHF-tau proteins in any part of the brain, including amygdala and basal nucleus of Meynert. These nondemented patients were <73 years old, and no amyloid deposits or neurofibrillary tangles were detectable in any area of their brains. In younger nondemented patients (>20 patients, age 10 to 40 years), amyloid deposits and neurofibrillary tangles were also absent.

Stage S1. Stage S1 included three nondemented patients, 71, 78, and 83 years old, with PHF-tau proteins found in only one brain region, the transentorhinal cortex (Brodmann area 35). In the oldest nondemented patient, trace amounts of amyloid deposition were detected by immunohistochemistry. For these three patients, aggregated A β was not detected at the biochemical level.

Stage S2. Stage S2 individuals were four control subjects from 72 to 95 years old (mean 86 \pm 10) in whom two brain areas were affected by NFD, as revealed by tau pathologic state: the transentorhinal cortex and the entorhinal cortex (see figure 1). PHF-tau proteins and neurofibrillary tangles were not observed in other areas of the brain. The oldest patient was 95 years old and nondemented. A β peptide and amyloid plaques were not detectable in two patients, regardless of the antibody used. A β immunohistochemistry was found in low amounts in the other two patients.

Stage S3. Stage S3 included 16 individuals from 73 to 95 years old (mean 84 \pm 7) with PHF-tau proteins in three brain areas. The affected regions were the transentorhinal cortex, the entorhinal cortex, and the hippocampus. Amyloid deposits were absent to moderate (two patients with 10 pmol/mg and three with 20 pmol/mg). Two patients had a moderate MMSE score and a CDR of 0.5 with mild memory impairment. Six were demented, but the dementia was vascular in origin. Four older patients had significant amounts of PHF-tau in the hippocampal region but were not demented. One patient had a MMSE score of 30, determined 2 months before death. NFD was present in the hippocampal region, but no trace of amyloid deposits was found, regardless of anti-A β used.

Stage S4. Stage S4 included 10 patients age 69 to 98 years old (mean 88 \pm 9) in whom PHF-tau extended to Brodmann area 38 (see figure 1). This area corresponds to the anterior temporal cortex. Five patients were nondemented. Four had mild cognitive impairment. Amyloid deposits were absent to moderate, and the maximum A β concentration observed was 11 pmol/mg. The oldest patient was 98 years old, nondemented, with low amounts of amyloid deposits (A β at 2 pmol/mg).

Stage S5. Stage S5 included 12 patients age 76 to 98 years (mean 89 \pm 7) with PHF-tau in an additional brain area, Brodmann area 20, which corresponds to the inferior temporal cortex. Three patients were nondemented. Two patients with no amyloid deposits had CDR of 0.5 and 1, respectively. Three patients, two of whom had vascular dementia, had a CDR score of 1. Amyloid deposits ranged from absent (two patients) to moderate (nine patients; three with A β ~20 pmol/mg and one patient with 30 pmol/mg). The four last patients had significant quantities of PHF-Tau in the hippocampal region.

Stage S6. Stage S6 included 11 patients age 71 to 93 years (mean 86 \pm 7). The affected area was the medium temporal cortex (Brodmann area 21). One 88-year-old patient was nondemented, with normal MMSE score. He had low numbers of amyloid deposits. The amounts of amyloid deposits ranged from absent (one patient) to moderate, with one exception at 54 pmol/mg.

Stage S7. Stage S7 included 15 patients age 84 to 106 years (mean 96 \pm 6) with, in addition to and simultaneously, several brain areas affected by NFD. These addi-

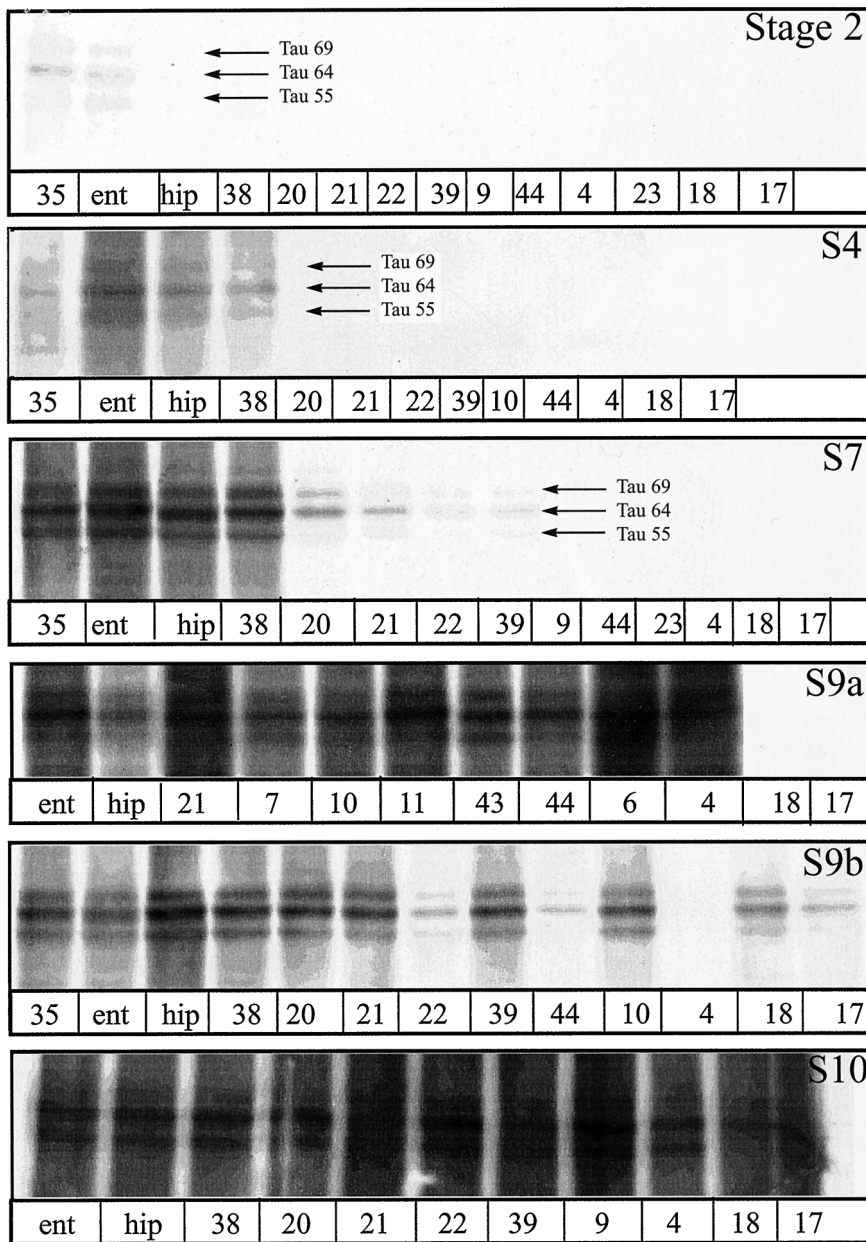


Figure 1. Western blot analysis of paired helical filaments (PHF)-tau in the different brain areas of patients at various stages of dementia. Brodmann areas studied are A35: transentorhinal cortex; temporal cortex: 38, 20, 21, 22; parietal cortex: 7, 39; frontal cortex: 11, 10, 9, 6, 43, 44, 4; anterior cingulum cortex: 23; occipital cortex: 18, 17. Ent = entorhinal cortex; Hip = hippocampus. PHF-tau were immunodetected with the monoclonal antibody AD2. The three main PHF-tau bands are marked by arrows: tau 69, 64, and 55. Smears of insoluble tau polymers are also observed in the lanes corresponding to brain samples strongly affected by neurofibrillary degeneration. Notice the sequential progression of the degenerating process in the different brain areas. At stage 6, progression of the disease is evident, from strongly affected regions with an important tau pathologic state to recently and mildly affected regions, with lower and decreasing amounts of PHF-tau. Notice also that S9 is heterogeneous. In S9a, the frontal motor cortex (A4) is affected, but not the occipital regions (18, 17), whereas it is the opposite in S9b and S9c.

tional affected brain areas were the cingulum cortex (BA23) and polymodal association cortical areas such as the superior temporal cortex (BA22), the inferior parietal cortex (BA39), and the anterior frontal cortex (BA10) (see figure 1). Most of the patients were mildly demented, except for one control subject, age 84, who had only trace amounts of PHF-tau proteins in the anterior frontal cortex and cingulum cortex. In this patient, diffuse amyloid deposits were concentrated in the same cortical areas mildly affected by NFD, as revealed by immunohistochemical and biochemical analysis. For the other patients, high amounts of amyloid deposits and PHF-tau were found in association areas. Patients with two association brain areas affected by NFD (PHF-tau >0.2) were demented. In contrast, four patients who were not impaired or mildly impaired had only traces of PHF-tau in one or several association areas.

Stage S8. Stage S8 included five patients age 77 to 91 years (mean 87 ± 6) with PHF-tau localized in addition in the inferior frontal gyrus, BA44 (Broca area). All patients

were demented. Amyloid deposits were low to significant (two patients with 88 pmol/mg).

Stage S9. Stage S9 included 19 patients age 65 to 100 years old (mean 81 ± 8) with sensory or motor cortex partially affected. This group was heterogeneous and was subdivided into three subgroups: S9a, S9b, and S9c. S9a included 6 patients age 74 to 100 years (mean 86 ± 9) and involved an additional change in the frontal motor cortex (BA4) (see figure 1, S9a). Occipital regions were spared. S9b included four patients age 65 to 89 years (mean 76 ± 11). These patients had an affected occipital secondary visual cortex (BA18) and primary visual cortex (BA17), with no PHF-tau in the motor region BA4 (see figure 1, S9b). S9c included nine patients age 69 to 86 years (mean 79 ± 5) in whom the only region spared was the occipital pole BA17 (primary visual cortex). All patients in stage 9 were demented. Amyloid deposits ranged from moderate to numerous, consistent with A β concentrations of 10 to 200 pmol/mg.

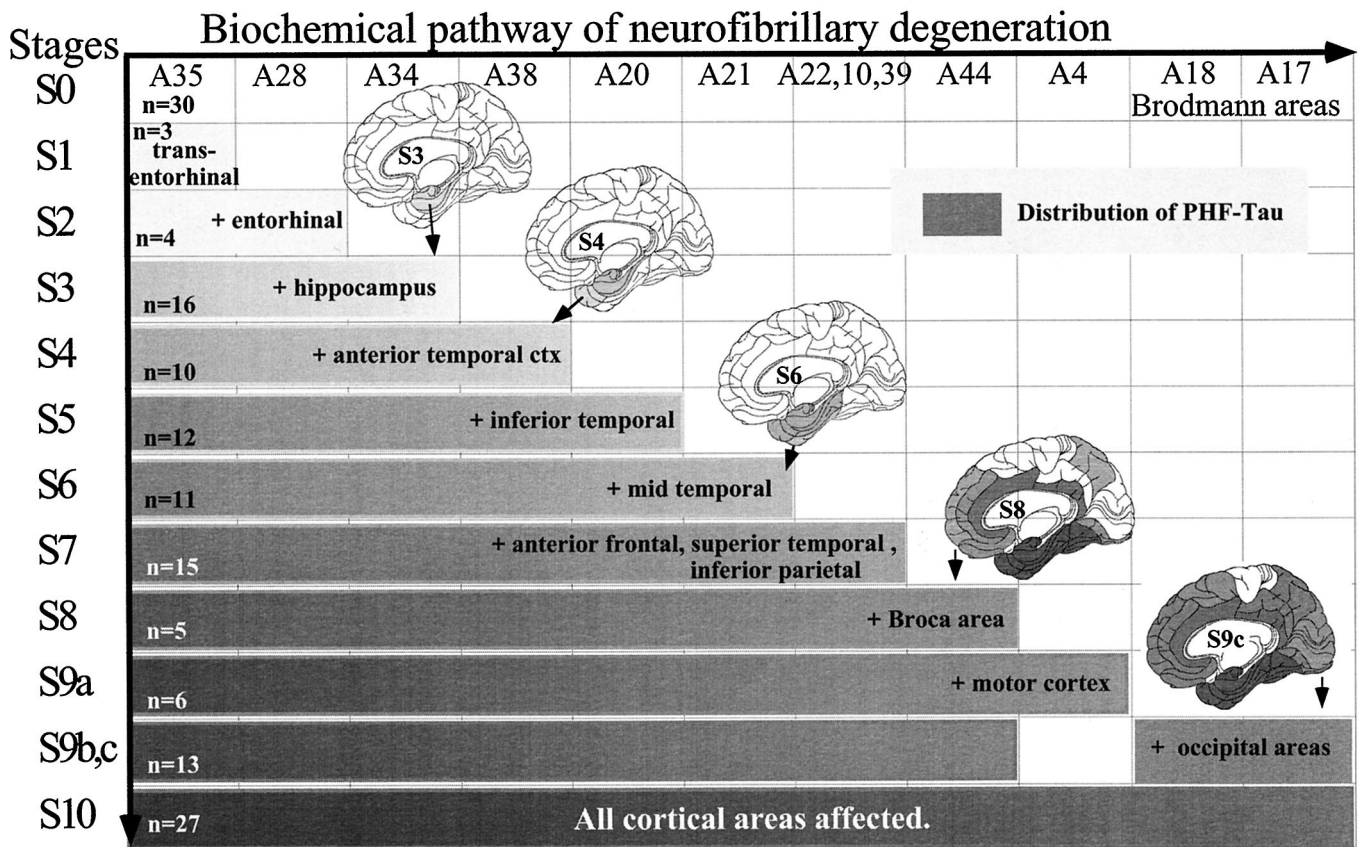


Figure 2. Pathway of neurofibrillary degeneration (NFD) in aging and AD. Paired helical filaments (PHF)-tau in the different brain areas, as a function of the stages, is shown in gray. Aged control subjects and non-AD patients were found at stages 0 to 3. Up to stage 6, NFD could be asymptomatic. All patients above stage 7 and with two association brain areas affected by tau pathology were patients with AD or mixed dementia. Note the heterogeneity of stage 9, with either the occipital areas or the frontal motor cortex affected.

Stage S10. Stage S10 included 27 patients age 37 to 90 years (mean 74 ± 13). All isocortical areas studied were affected by NFD (see figure 1). The amounts of immunodetected PHF-tau were generally very high but were sometimes heterogeneously distributed. The highest amounts were in the temporal cortex, with the heterogeneity mainly observed along the rostrocaudal axis. For example, one patient with very high amounts of PHF-tau in the occipital region had Balint syndrome. However, the occipital cortex (BA17) was generally the least affected region. In five patients, we compared the same brain areas from the left and right hemispheres and found that the amounts of immunodetected PHF-tau were always similar, with a difference of $<20\%$. The patients most affected were almost always the youngest ones, as described.²⁰

Discussion. In this study, we used immunoblot detection of PHF-tau to quantify NFD in different brain areas of 130 patients with various cognitive statuses. These results were analyzed in association with biochemical, neuropathologic, and clinical data, which allowed us to trace the pathway of NFD in normal aging and in AD.

The patients in this study came from two different centers because we worked simultaneously with two different populations, nondemented and AD patients, which cannot be found in large numbers in

one center. The 130 patients studied here do not reflect the normal population but do reflect different stages of cognitive impairment. Our goal was to study a large cohort of patients with various cognitive statuses, from normal aging to severe definite AD, and to study the different stages of the degeneration process. Because we also included patients with possible AD as well as mixed dementia or possible vascular dementia, it is not surprising that we found some patients with a vascular pathologic condition in addition to, or instead of, a pathologic state characteristic of AD.

Amyloid deposits were quantified by biochemical and immunohistochemical detection of A β . For some patients, we used a panel of antibodies and techniques to determine whether amyloid deposits were present in low amounts or not at all.

NFD was detected, typed, and quantified by immunoblot techniques. The Western blot tau profile is disease-specific^{21,22} and proportional to the number of tau neuronal inclusions.^{1,19}

The biochemical pathway of NFD. We focused our attention on PHF-tau and its relationship to cognitive status and amyloidosis. We were struck by the observation that the distribution of PHF-tau was never at random. In most cases, the distribution of

PHF-tau was stereotyped, sequential, and hierarchical. For example, we never found PHF-tau in the hippocampus and in the parietal cortex alone. If the parietal cortex was affected, many other "obligatory regions," such as all regions of the hippocampal and temporal cortex, were also involved. Similarly, the hippocampus was never affected by itself, but always in association with the transentorhinal and entorhinal cortex, showing that the degenerating process in the hippocampus follows that in transentorhinal and entorhinal cortices. The pathway of NFD in aging and AD corresponds to the 10 brain cortical regions that are successively affected. They define 10 major stages, into which the 130 patients studied here were easily and logically categorized (see figure 2). NFD progresses as follows: transentorhinal cortex (S1), entorhinal (S2), hippocampus (S3), anterior temporal cortex (S4), inferior temporal cortex (S5), medium temporal cortex (S6), polymodal association areas (prefrontal, parietal inferior, temporal superior) (S7), unimodal areas (S8), primary motor (S9a) or sensory areas (S9b, S9c), all cortical brain areas (S10).

Although this classification was performed independently of cognitive status, all nondemented patients were found at stages S0 to S5, whereas all AD patients were at stages S7 to S10. Also, we found eight demented people with very low amounts of PHF-tau distributed in only a few brain areas (S1 to S5). For these patients, vascular pathologic states explained their dementia, and their PHF-tau pattern was similar to that of control subjects, showing that vascular involvement did not affect the chemical nature and the distribution of NFD, at least at these stages of NFD progression.

Therefore, the biochemical pathway of PHF-tau presented here is similar to the NFD stages previously described in neuropathologic studies.^{2,3,23-25} However, our molecular approach gave complementary data and led us to additional conclusions, as follows.

NFD is systematically present in the hippocampal region of nondemented patients >75 years. In this study, the oldest patient totally devoid of PHF-tau was age 72. Conversely, all patients >72 had at least trace amounts of PHF-tau in the entorhinal region (transentorhinal and entorhinal cortex). Therefore, we conclude that during normal aging, NFD is present at least in the entorhinal region of people $>75 \pm 3$ years. These findings corroborate our previous studies.²⁶ Our results are significantly different from those of Braak and Braak²⁵ and Giannakopoulos et al.,²⁷ who found numerous neurofibrillary tangles in the hippocampus of young nondemented patients, even at the age of 45 years. We cannot explain these differences, because in our study, the neuropathologic and biochemical measurements gave similar results.

The hippocampal region is vulnerable and is electively affected by NFD. Our immunochemical results corroborate the observation that the hippocampal area is vulnerable and is specifically

affected by NFD during aging, especially after the age of 75 years.²⁸ All older nondemented patients were affected by NFD, but some of them were not affected by amyloid deposits, which is a prerequisite for AD.⁵ These results demonstrate that NFD in the hippocampal area of aged people does not necessarily mark the onset of AD but is more likely to be a consequence of the vulnerability of the hippocampal area.

Some patients were very mildly affected despite old age. Our data show that if NFD with PHF-tau starts invariably at the age of 75, this degeneration process is still weak at a very old age (95 years) in some patients.

A large part of the temporal cortex can be affected without obvious clinical symptoms. Some nondemented patients were at stages 4 to 6. Most of these patients had been monitored prospectively, confirming that some of them were nondemented based on MMSE and CDR scores and daily observations by caregivers. Our results demonstrate that the spread of NFD in the hippocampal region and the temporal cortex (anterior, inferior, and mid-region, except for temporal area BA22, which is a polymodal association area) can be asymptomatic, according to MMSE and CDR scores. Also, we found a nondemented patient with a trace of PHF-tau in one association brain area, the frontal cortex BA10 (one patient age 84, stage 7). This shows that neuronal plasticity, and in all likelihood other variables such as education, can overcome the degenerating process for a long time. However, these patients are likely to be at a preclinical stage of AD (next section).

Characterization of preclinical stages of AD. From our understanding of AD, preclinical AD patients should be considered asymptomatic, using MMSE and CDR scores, or with mild cognitive changes, and have very large amyloid deposits in the neocortex and PHF-tau in some brain areas.²⁻⁵ Such patients were mainly found at stages 4 to 6, with PHF-tau in the hippocampal region and the temporal cortex, and a generally moderate decline of cognitive functions. Our best example is an 83-year-old patient at stage 5, with a high level of education, an MMSE score of 30, and large deposits of amyloid. This is also true of other patients at stages 5 and 6, who had subtle alterations of memory, which can be considered pathologic in the context of their high levels of education and skills associated with their previous occupations. Also, one 85-year-old patient at stage 3, with important amyloid deposits, was probably in the earliest part of the preclinical stage of AD.

Involvement of polymodal association brain areas by NFD was always correlated with cognitive impairment. All patients with PHF-tau in two polymodal association areas were cognitively impaired and usually demented. All patients with a PHF-tau value >0.1 in the frontal cortex (BA10) and the parietal cortex (BA39) exhibited dementia of the Alzheimer's type. The relationship between the extent of NFD and cognitive impairment was nonlinear, because we observed a threshold for important clinical symp-

toms at stage 7. Together, these data are in excellent agreement with those reported at the neuropathologic level.^{23,29} They also fit well with the description of patients with important amounts of tangles in the inferior and medial temporal lobes (stage 6) and preclinical signs of AD.³⁰

The relationship between the histologic or biochemical presence of amyloid deposits and dementia is certainly more difficult to establish, mainly because amyloid deposition is diffuse, widespread, and heterogeneously distributed, whereas NFD is progressive, hierarchical, and along precise anatomic networks. However, there is a broad relationship, because nondemented patients have no or low amounts of amyloid (<10 picomoles of A β /mg of tissue), whereas AD patients invariably had moderate to high amounts of amyloid deposits (60 picomoles \pm 50). Therefore, using immunochemical parameters, it is possible to propose the following criteria to establish a (postmortem) biochemical diagnosis of AD (CEBDAD): A β peptide >30 pmol/mg of tissue and simultaneous PHF-tau in two polymodal association brain areas: Brodmann areas 9 and 39. With these criteria, there is no overlap between aging and AD. The diagnosis of infraclinical AD can also be proposed, taking into account the pathway of NFD. From our data, these criteria are PHF-tau in the hippocampal and temporal area (Brodmann areas 20 and 21) associated with amyloid deposits in cortical areas (at the histologic or biochemical levels). In the same way, the criteria for the status of non-AD control subjects are as follows: either absence of PHF-tau in the brain or presence of PHF-tau restricted to the hippocampal area in subjects >75 years old. Amyloid deposits should be absent or in low amounts. Thus, using such criteria, we can obtain an AD diagnosis close to 100% specificity and sensitivity. These criteria should be useful for the assessment of frozen brain samples from brain banks.

PHF-tau and the biological diagnosis of AD. Our results have theoretical consequences for the establishment of an early biological diagnosis of AD. On the one hand, they demonstrate that even when brain tissue is used, two brain areas and two biochemical markers are needed for a reliable biochemical diagnosis of AD. We therefore can predict that the biological diagnosis of AD will be difficult to establish. Conversely, we observed that patients with very early clinical signs of AD already had large amounts of PHF-tau in the temporal cortex. This means that at the first stage of AD, an extremely large number of neurons are degenerating and releasing PHF-tau antigens in the extraneuronal domain. If PHF-tau is not totally destroyed by microglia and astrocytes, it might be detectable in the CSF and thus provide the basis for a diagnostic test. Preliminary results showing increased tau amounts in the CSF of AD patients are encouraging in this respect.³¹

Heterogeneity of AD. The current study shows the spectrum of AD and its heterogeneity. For a

given stage, especially at stages 9 and 10, some patients had a degeneration process more focused in a specific brain region. The heterogeneity was found along the rostrocaudal axis, and correlated well with the heterogeneity of clinical manifestations, as already described.²⁰

PHF-tau spreading visualizes the sequential collapse of vulnerable networks of neurons. The progressive invasion of PHF-tau in the brain along a precise network of neurons also has implications for therapy. Our data show that there is a degeneration process in the hippocampal region that is expressed during aging, but not related to AD, because in some patients, amyloid deposits (a prerequisite to AD) were not present. Genetic studies demonstrate that beta-protein precursor (β PP) dysfunction followed by amyloid deposition is the etiologic factor of AD.⁵ In all studies,^{2,3,23-25} including the current one, amyloid deposits spread randomly within the entire cerebral cortex. From these observations, it is logical to conclude that the putative neurotoxic effect of amyloid peptide is not a direct one on neighboring neurons, because in that case a random process of NFD would be observed simultaneously in all brain areas where the amyloid deposits are found.^{2,3} Consequently, AD is a pathologic state of vulnerable networks of neurons, because we observed a hierarchical progression of NFD along a specific pathway of neuronal populations.

AD has been described as a disease resulting from a cascade of molecular dysfunctions.³² Our results support the observation that AD also depends on several dynamic processes. Variables that contribute to these dynamics are numerous. First, there is a selective vulnerability of neurons, because the hippocampal region is always affected in aging. A general brain dysfunction of β PP (loss of function or neurotoxicity of A β) should strike first on this vulnerable region and intensify the degeneration process initiated by aging. Second, the hierarchical collapse of subsets of neurons probably has its own dynamic. It is likely to result from the death of the most vulnerable neuronal populations, which are no longer able to supply the trophic factors to their connected neurons.³³ Once NFD has begun, it is likely to spread like a chain reaction, fueled by self-propagation and feedback amplification, under the constant burden of β PP dysfunction. Programmed cell death caused by the lack of neurotrophic factors could be part of this dynamic process.^{34,35} Third, there is a neuronal plasticity, which counterbalances the degenerating process for a time, because the disease can be asymptomatic up to stage 6. One way to slow down the disease would be to boost the production of adequate trophic factors^{36,37} (i.e. growth factors, cytokines, or neurosteroids) to stabilize vulnerable neuronal networks located along the pathway of NFD.

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Note. Readers can obtain 5 pages of supplementary material from the National Auxiliary Publications Service, 248 Hempstead Turnpike, West Hempstead, NY 11552. Request document no. 05468. This is not a multi-article document. Remit with your order, not under separate cover, in US funds only, \$15.00 for photocopies or \$5.00 for microfiche. Outside the United States and Canada, add postage of \$4.50 for the first 20 pages and \$1.00 for each 10 pages of material thereafter, or \$5.00 for the first microfiche and \$1.00 for each fiche thereafter. *There is a \$25.00 invoicing charge on all orders filled before payment.*

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