

# Cholinergic Nucleus Basalis Tauopathy Emerges Early in the Aging-MCI-AD Continuum

Marsel Mesulam, MD,<sup>1</sup> Pamela Shaw, BA,<sup>1</sup> Deborah Mash, PhD,<sup>2</sup> and Sandra Weintraub, PhD<sup>1</sup>

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The cholinergic denervation in Alzheimer's disease (AD) provides the rationale for treatments with anticholinesterases. The presence of this cholinergic lesion is solidly established in advanced AD. Whether it also exists in early disease remains unsettled. This question was addressed with thioflavin-S histofluorescence to identify neurofibrillary tangles (NFT) and two tau antibodies (AT8, Alz-50) to identify pre-tangle cytopathology in the nucleus basalis, the source of cortical cholinergic innervation. Methods for the concurrent visualization of tauopathy and choline acetyltransferase were used to determine if the cytopathology was selectively located within cholinergic neurons. Five elderly index cases who had died at the stage of mild cognitive impairment (MCI) or early AD were identified by longitudinal neuropsychological and behavioral assessments. They were compared to 7 age-matched cognitively normal subjects. NFT and AT8 (or Alz-50) immunostaining in cholinergic nucleus basalis neurons existed even in the cognitively normal subjects. The percentage of tauopathy-containing nucleus basalis neurons was greater in the cognitively impaired and showed a significant correlation with memory scores obtained 1-18 months prior to death. These results show that cytopathology in cortical cholinergic pathways is a very early event in the course of the continuum that leads from advanced age to MCI and AD.

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In 1976, two British teams reported that the presynaptic cholinergic marker choline acetyltransferase (ChAT) was depleted in the cerebral cortex of Alzheimer's disease (AD).<sup>1,2</sup> These reports rapidly transformed AD from an obscure neuropathological entity, descriptively characterized by the presence of plaques and tangles, into a disease with a potentially transmitter-based pathophysiology.<sup>3</sup> Numerous additional investigations confirmed that AD consistently leads to severe degenerative changes of cortical cholinergic axons and their cells of origin in the nucleus basalis.<sup>4</sup> However, these studies were conducted using patients at fairly advanced stages of AD and could not establish whether the cholinergic denervation was an early or late event. This question has been addressed more recently and has yielded the somewhat unexpected findings that early AD is not associated with a loss of either cortical ChAT or nucleus basalis neurons.<sup>5-7</sup>

The purpose of the study reported here was to reassess this relationship with a different marker of cholinergic pathway integrity. To this end, the distribution of

pathological cytoskeletal markers in the nucleus basalis was explored in a set of five neuropsychologically characterized elderly subjects at the stages of mild cognitive impairment (MCI) or early AD and seven cognitively normal controls. The results showed that such pathological changes in the nucleus basalis can be detected even during normal aging and that a further progression of this cholinergic cytopathology is associated with the early and putatively preclinical stages of AD. These results are consistent with the previously reported loss of nerve growth factor (NGF) receptors in the nucleus basalis of MCI, a situation that potentially could promote cytopathology by undermining the trophic effect of NGF upon these cholinergic neurons.<sup>8,9</sup>

## Subjects and Methods

### Subjects

Specimens came from a cohort of elderly volunteers who were healthy at the time of enrollment and who had agreed to annual testing and brain donation to the University of Miami Brain Endowment Bank. We scanned the database

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From the <sup>1</sup>Cognitive Neurology and Alzheimer's Disease Center, Northwestern University Feinberg School of Medicine, Chicago, IL; and <sup>2</sup>Department of Neurology, University of Miami School of Medicine, Miami, FL.

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Address correspondence to Dr Mesulam, Cognitive Neurology and Alzheimer's Disease Center, Northwestern University, Feinberg School of Medicine, 320 East Superior Street, Chicago, IL.  
E-mail: mmesulam@northwestern.edu

related to this cohort to identify subjects with cognitive impairments ranging from the very mild to the stage of early AD. We excluded subjects with other neurological or medical diseases that could account for these impairments and required multiple neuropsychological testings, at least one of which had to be done within 1.5 years of death. Only specimens that were free of major neuropathological markers other than those characteristic of aging and AD were included. The control group contained all specimens within the same age range for whom we had positive evidence of cognitive normalcy.

### Neuropsychology

Subjects were tested two to seven times before death; the last test occurred between 1 and 18 months before death (mbd). Tests included the Mini-Mental State Examination (MMSE)<sup>10</sup>; components of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) battery, such as word list learning and delayed recall, the 15-item Boston Naming Test (BNT), and graphomotor construction tasks<sup>11</sup>; the Logical Memory delayed recall subtest of the Wechsler Memory Scale-Revised (WMS-R)<sup>12</sup>; the Benton Visual Retention Test (BVRT)<sup>13</sup>; Trail Making Test Parts A and B; and the CFL word fluency test from the Multilingual Aphasia Examination.<sup>14</sup>

The range of cognitive performance deemed normal varies according to age, sex, and education. Some domains, such as language, remain relatively stable, whereas others, especially memory, show prominent age-related changes.<sup>15</sup> We compared our subjects' test scores against two standards: (1) normative scores for sex, education, and age<sup>16–19</sup> and (2) normative scores for 50 to 59-year-olds on the same tests (except for the CERAD BNT in which normative values were only available for the 50–69-year-old age range). Because norma-

tive scores are not typically available on many tests for those above 90, the oldest available group data (usually for 70–89 years) was used and an effort was made to consider education levels.

Each test score was ranked in one of three categories: (1) abnormal for age (more than 2 standard deviations below the mean score for age); (2) normal for age (within 2 standard deviations of the mean score for age); (3) normal for younger age (within two standard deviations for 50–59 or 50–69-year-olds). Subjects then were designated as (1) cognitively normal (CN) or (2) cognitively impaired (CI) based on all scores and daily living activities.

### Histology

Specimens had death-to-fixation intervals of 2.5 to 12 hours (Table 1). One hemisphere (usually left) was coronally sectioned into 1 to 2cm slabs and fixed as previously described.<sup>20</sup> The fixed slabs were frozen with dry ice and sectioned in whole-hemisphere sections at 40 $\mu$ m on a sliding microtome. Adjacent sections were processed with five procedures: (1) cresyl violet for the cytoarchitectonic identification of the nucleus basalis, (2) thioflavin-S histofluorescence for the identification of neurofibrillary tangles (NFTs) and compact/neuritic plaques, (3) immunohistochemistry for ChAT<sup>21</sup> to identify cholinergic nucleus basalis neurons, (4) immunohistochemistry with Alz-50 (Cases 7 and 12) and AT8 (all other cases), two antibodies that bind to abnormally phosphorylated (AT8) or abnormally folded (Alz-50) tau at various stages of neurofibrillary degeneration, including those that precede NFT formation,<sup>22–24</sup> and (5) the concurrent immunolabeling of AT8 (or Alz-50) with an alkaline phosphatase-based blue reaction product and of ChAT with a horseradish peroxidase-based brown reaction product. The Alz-50 antibody was a gift from Peter Davies and the AT-8

Table 1. Subject Characteristics

Subject No.	MMSE <sup>a</sup> (mbd)	Age at Death, Sex	Autopsy Interval (hr) <sup>b</sup>	Brain Weight (gm)	Braak Stage	CERAD Ent/STS	NB (Ch4) Tangles (%) <sup>c</sup>	NB (Ch4) AT8 or Alz-50 (%) <sup>c</sup>
1-CN	25 (12)	88, F	9	1,104	2	S/S	33/998 (3.3)	21/673 (3.1)
2-CN	29 (10)	89, F	9	1,180	1–2	S/S	2/614 (0.3)	16/597 (2.7)
3-CN	30 (13)	90, M	2.5	1,155	2	S/S	10/766 (1.3)	80/733 (11)
4-CN	28 (18)	91, M	12	1,309	2	S/S	7/1313 (0.5)	12/941 (1.3)
5-CN	28 (15)	92, M	7	1,290	3	S/S	1/593 (0.2)	16/600 (2.6)
6-CN	28 (4)	95, F	3.2	1,096	3	M/F	26/697 (3.7)	33/727 (4.5)
7-CN	NA	100, F	6.5	1,220	3	S/S	13/995 (1.3)	59/563 (10)
8-CI	24 (16)	89, F	4.5	1,280	2	M/S	41/1,009 (4.1)	229/864 (26)
9-CI	24 (1)	90, M	3	1,380	3	M/S	35/926 (3.8)	71/1139 (6.2)
10-CI	27 (9)	92, F	3.5	1,084	3–4	S/S	68/610 (11)	177/1046 (17)
11-CI	23 (11)	92, M	4.5	1,100	5	F/F	280/1,308 (21)	NA
12-CI	27 (1)	99, F	5	1,060	3–4	S/M	220/1,280 (17)	231/1591 (14)

<sup>a</sup>Scores at the last testing session.

<sup>b</sup>Elapsed time between death and autopsy.

The information for Braak & Braak and CERAD stages is derived from thioflavin-S-stained material examined with fluorescent microscopy. The Braak & Braak stages are based on tangle densities in the entorhinal, transentorhinal, fusiform, and inferotemporal cortices. The CERAD stages reflect compact plaque densities in Ent and in the cortex of the superior temporal sulcus (STS).

<sup>c</sup>In the anterior sector of the nucleus basalis (NB[Ch4]). The numerator shows the number of tangles (or immunoreactive neurons) and the denominator the number of nucleus basalis neurons. Cases 7 and 12 had immunostaining with Alz-50, all others with AT8.

MMSE = Mini-Mental State Examination; mbd = months before death; CERAD = Consortium to Establish a Registry for Alzheimer's Disease; Ent = entorhinal cortex; STS = superior temporal sulcus; S = sparse; M = moderate; F = frequent.

antibody was purchased from Pierce Endogen (Rockford, IL). Procedures for immunohistochemistry are described elsewhere.<sup>20,22,25,26</sup> Negative immunohistochemical controls were obtained by substituting the primary antibody with an irrelevant IgG (for AT8) or IgM (for Alz-50). For positive controls, we used sections from the brain of a 36-year-old with no known neurological disease.

### *Neuropathology*

Thioflavin-S was used to rate each specimen according to the Braak and Braak NFT stages<sup>27</sup> and the CERAD neuritic plaque stages.<sup>28</sup> Matching sections stained with thioflavin-S, AT8, and Alz-50 passing through the anterior part of the Ch4–nucleus basalis complex provided the major data. The Ch4–nucleus basalis complex is the source of all cortical cholinergic innervation, and the anterior sector chosen for this study contains its most extensive and conspicuous portion.<sup>20</sup>

For investigating the distribution of NFT, thioflavin-S-stained sections were placed on the computer-driven stage of a Nikon Eclipse E800 scope linked to Stereoinvestigator software (Microbrightfield, Inc., Colchester, VT). The image was digitized and displayed on a computer screen. The initial survey was done with a  $\times 4$  objective to draw the boundaries of the Ch4–nucleus basalis complex. The subsequent analysis was done with a  $\times 20$  objective while the stage was automatically advanced frame by frame to cover the entire area containing the Ch4–nucleus basalis complex under the guidance of software that prevented the same object from being counted twice. Fluorescent illumination at 528 to 553nm allowed magnocellular nucleus basalis neurons to be identified by their characteristically high content of lipofuscin that emits a reddish autofluorescence at that wavelength.<sup>20</sup> Illumination was switched to 380 to 420nm to identify thioflavin-S-positive NFTs within the nucleus basalis. Lipofuscin-rich magnocellular neurons and NFTs within the boundaries of the Ch4–nucleus basalis complex were marked on the digitized file by two different symbols. A similar approach was used to investigate the distribution of Alz-50 or AT8 labeling, but the illumination was switched to brightfield to identify immunolabeled neurons. In each of the 12 cases, the location of the nucleus basalis was verified in matching sections stained with cresyl violet and ChAT.

## **Results**

### *Neuropsychological Subject Characterizations*

All testing sessions were considered in the classification of the subjects as normal (CN) or impaired (CI). For the purpose of illustration, however, Figures 1 and 2 and Table 2 are based on scores from only the first and last sessions. Five CI index cases were identified, ranging in age at death from 89 to 99 years. The MMSE of these index cases (8-CI to 12-CI) ranged from 23 to 27 at the final testing session (see Figs 1 and 2, Table 1). The control group comprised all subjects within this age range for whom we had evidence of normalcy either by neuropsychological testing or, as in the case of one subject, by retrospective interviews of close acquaintances. Seven such cognitively normal controls ranging in age at death from 88 to 100 (1-CN to

7-CN) were identified with an MMSE range of 25 to 30. In the group of the cognitively impaired, one had the findings of early AD (11-CI), whereas four subjects (8-CI, 9-CI, 10-CI, and 12-CI) fit criteria for MCI, based on the presence of acquired cognitive deficits, especially in the area of memory, that are significant but not severe enough to curtail customary daily living activities.<sup>29,30</sup> All specimens in our collection that fulfilled criteria for index cases and their controls were included in this study.

1-CN. 1-CN, an 88-year-old woman with 12 years of education, was tested at age 81 years (78 mbd) and again at ages 82, 83, 84, 85, and 87 (12 mbd). She died at age 88 years. At the last testing, performance was better than what would have been expected for her age in Trail Making Part B, naming (BNT), and CERAD word list acquisition and constructions (see Fig 1, Table 2). There was even a slight improvement over time in the delayed recall score on the CERAD word list. Other memory tests were performed above the cutoff for age. Only the MMSE score decreased to the impaired range at the final testing. On the MMSE, she lost points for orientation (she thought it was February 15 when tested on February 18) and on word recall. Because the scores in the other individual memory tests were within acceptable limits for age and independent living activities were intact, including holding a volunteer job, she was deemed cognitively normal at the final testing session.

2-CN. 2-CN, a 89-year-old female with 16 years of education, was first tested at age 86 years (35 mbd) and at age 88 years (10 mbd). She had worked as a music teacher and librarian. In both testing sessions, scores were above the cutoffs for age, and in several instances normal for a younger age (see Fig 1, Table 2). The one exception was the CERAD constructions in the second session. Some scores improved over time, probably as a practice or familiarity effect. In both sessions, the CFL verbal fluency test, the BVRT non-verbal recall, word list acquisition and delayed recall, and the BNT were performed at a level that was normal for 50 to 69-year-olds. Daily living activities were intact. This subject was judged cognitively normal at both testing sessions.

3-CN. 3-CN, a 90-year-old man with 16 years of education who was self-employed as a tanner, was tested first at age 86 years (49 mbd), a second time at 88 years, and a final time at age 89 years (13 mbd). All test scores were normal either for age or for a younger age and remained generally stable across sessions (see Fig 1, Table 2). The subject remained independent and died of prostate cancer. He was judged cognitively normal at the last testing.

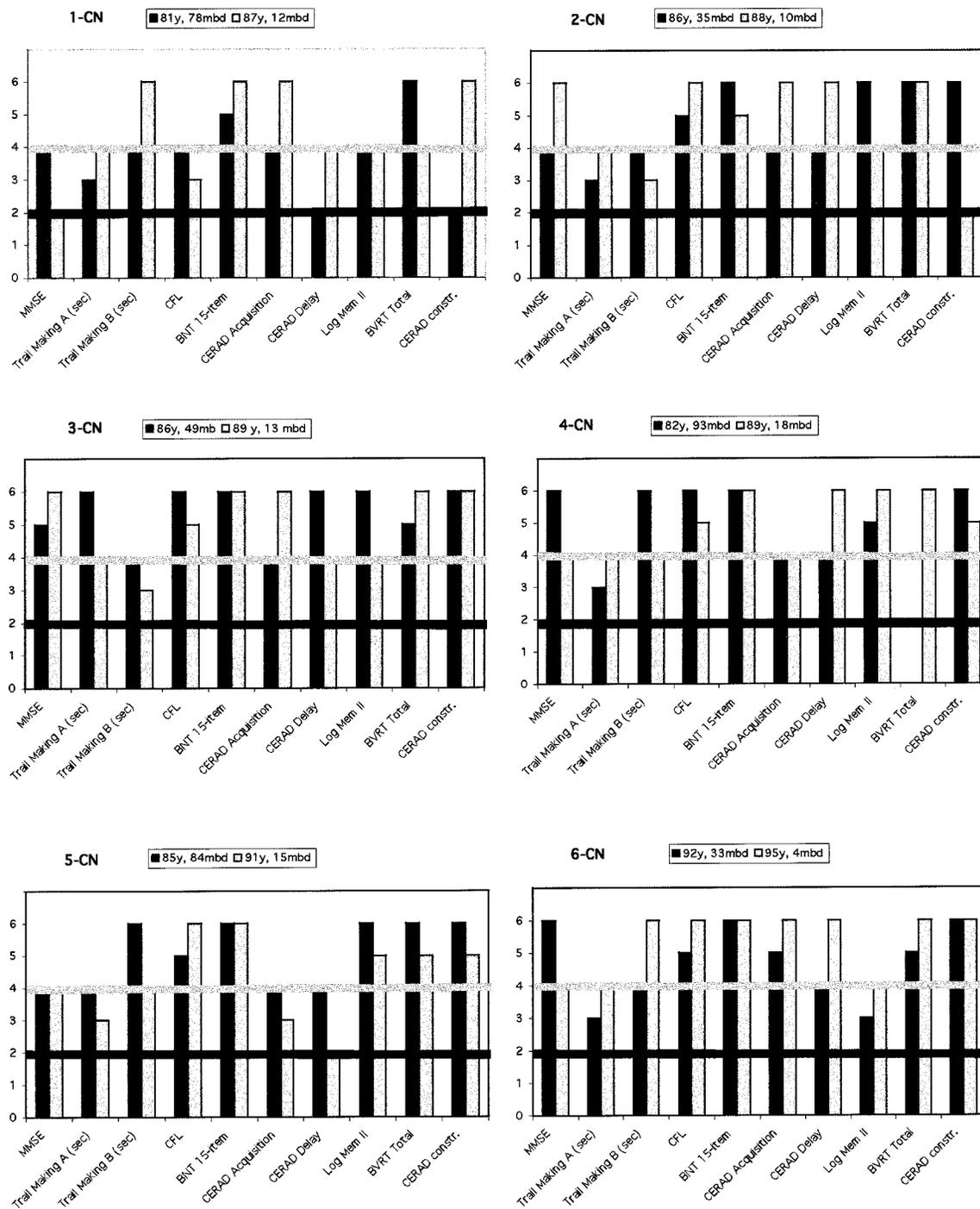


Fig 1. Neuropsychological profiles. Scores in neuropsychological tests at the first and last full testing sessions are shown in a semi-quantitative six-point nonlinear scale. The black vertical bars represent the first full testing, and the gray vertical bars represent the final testing. The subject's age at these sessions and the relationship to the date of death are indicated within the small boxes on top of each graph. The black horizontal bars show the cutoff for age 70 to 89 years, whereas the gray horizontal bars show the cutoff for the younger age range of 50 to 59 years (except for the CERAD BNT for which the cutoff is for the range of 50–69-year-olds). Grades 1 and 2 depict performance that is impaired for age, grades 3 and 4 depict performance that is normal for age, grades 5 and 6 depict performance that is normal even for the younger age range. mbd = months before death.

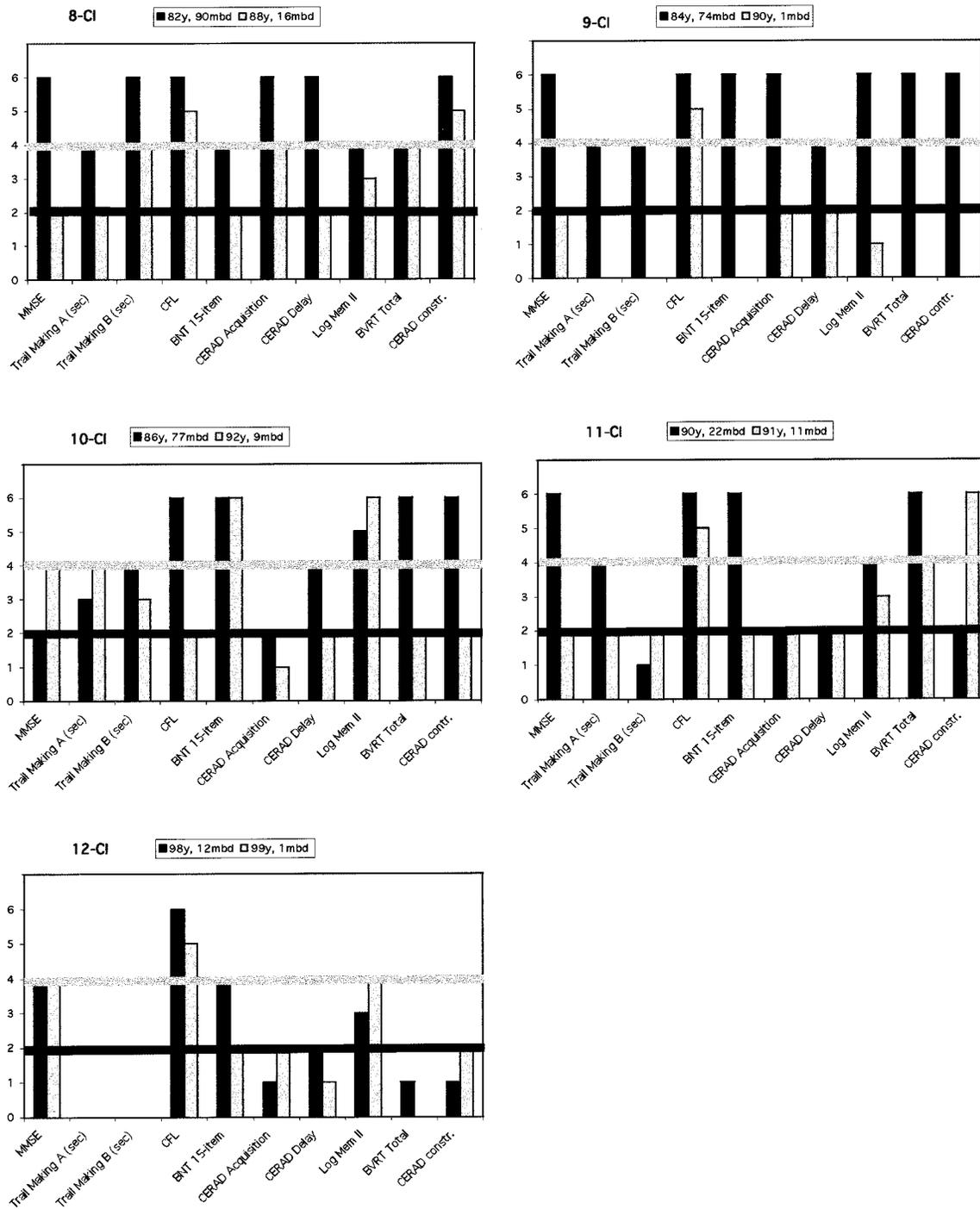


Fig 2. Neuropsychological profiles. Scores in neuropsychological tests at the first and last full testing sessions are shown in a semi-quantitative six-point nonlinear scale. The black vertical bars represent the first full testing, and the gray vertical bars represent the final testing. The subject's age at these sessions and the relationship to the date of death are indicated within the small boxes on top of each graph. The black horizontal bars show the cutoff for age 70 to 89 years, whereas the gray horizontal bars show the cutoff for the younger age range of 50 to 59 years (except for the CERAD BNT for which the cutoff is for the range of 50–69-year-olds). Grades 1 and 2 depict performance that is impaired for age, grades 3 and 4 depict performance that is normal for age, grades 5 and 6 depict performance that is normal even for the younger age range. mbd = months before death.

Table 2(A). Neuropsychological Scores

Normative Cutoffs (M/F)			Subject No./Gender, Education					
			1-CN		2-CN		3-CN	
			F, 12 yrs	F, 16 yrs	F, 16 yrs	M, 16 yrs		
50–59 yrs	70–89 yrs		78	12	35	10	49	13
		First and last testing in months before death						
		Age at test	81	87	86	88	86	89
≥29	≥27	MMSE (total = 30)	27	<b>25</b>	28	29	29	30
≤42	≤93	Trail Making Part A (sec)	62	43	74	63	42	49
≤128	≤249	Trail Making Part B (sec)	143	123	182	191	175	225
≥20/23	≥8/16	CFL Word Fluency (uncorrected total)	17	16	33	44	55	52
≥13	≥13	CERAD BNT (total = 15)	14	15	15	14	15	15
≥20	≥15	CERAD list acquisition (total = 30)	17	20	19	21	15	21
≥7	≥4/5	CERAD list delayed recall (10)	<b>4</b>	5	6	8	7	6
≥15	≥3	Logical Memory II (Delay) (Total = 50)	7	7	21	11	25	7
≥4	≥2	BVRT (total = 10)	5	3	5	5	5	6
≥9	≥9/8	CERAD constructions (total = 11)	<b>7</b>	11	10	7	11	11

The cutoffs for 70 to 89-year olds were derived from the literature. This is the closest to the age of our subjects. The cutoff scores for the 50 to 59-year range were based on data compiled at the Northwestern Alzheimer's Disease Center with the exception of the BVRT and CFL for which the information was obtained from the literature as indicated in Subjects and Methods. For the CERAD BNT, the cutoff scores are from published data for 50 to 69-year-olds (instead of 50–59). For some tests, there are gender differences in cutoffs. For the 11 subjects for whom neuropsychological information was available, test scores are shown for the first and last full testings. The timing of these sessions is indicated in terms of months before death. Boldface indicates that the scores are impaired for age.

4-CN. 4-CN, a 91-year-old man with 16 years of education, was tested at age 82 years (93 mbd) and at ages 83, 84, 85, 87, 88, and 89 (18 mbd). He had worked as a corporate vice president. Test scores at the initial visit were all either normal for age or better and remained so through the last test session (see Fig 1, Table 2). At the time of that session, he was leading a very active life style. He was considered to be cognitively normal.

5-CN. 5-CN, a 92-year-old man with 16 years of education, was first tested at age 85 years (84 mbd) and again at ages 86, 87, 88, 89, and 91 years (15 mbd). He had been a metallurgic engineer. Scores mainly fell in the reference range for age and in some instances for a younger age (see Fig 1, Table 2). At the last testing, 15 months before death, he reported engaging in regular exercise, travel, and hobbies such as making stained glass. Despite an abnormal score in one memory test (CERAD delay), other memory tests and the MMSE did not show a consistent memory problem. Based on test scores and activities of daily living, he was deemed cognitively normal at the last testing.

6-CN. 6-CN, a 95-year-old woman with 12 years of education, was tested first at age 92 years (33 mbd), again at age 93 years (22 mbd), and a third time at age 95 years (4 mbd). Test scores were normal for age or for younger ages and many improved over time (see Fig 1, Table 2). Daily living activities were maintained. This subject was judged cognitively normal.

7-CN. 7-CN, a woman who died at 100, before she could be tested. Her primary care physician who had been in contact with the subject until 3 days before her death was interviewed by phone. The information indicated that she had been cognitively intact up to her death. She was transferred to a nursing home 2 to 3 years before her death because of blindness and medical problems. A few weeks before her death, she appeared at a signing ceremony for a book she had just published about her missionary work. Those present at the ceremony commented on the preservation of her mental faculties, including memory for recent and distant events. Based on this information, she was judged to be cognitively normal within the week before her death.

8-CI. 8-CI, an 89-year-old woman with 18 years of education, was first tested at age 81 years (104 mbd) and again at ages 82 (90 mbd), 83 (74 mbd), 84 (62 mbd), 86 (44 mbd), 87 (32 mbd), and 88 years (16 mbd). The first test session was incomplete, and so the data shown in Figure 1 are for the second (age 82) and last (age 88) sessions. The subject taught Spanish literature. In the initial test session, 6 years before death, her scores in all domains were at least normal for age (see Fig 2, Table 2). By the last session, scores decreased to the impaired level on the MMSE, Trail Making Part A, naming (BNT), and CERAD delayed word list recall. At her last interview, she was disoriented to time and place in the MMSE. At that time, she was living independently with some help from fam-

Table 2(B). (Continued)

Subject No./Gender, Education															
4-CN		5-CN		6-CN		8-CI		9-CI		10-CI		11-CI		12-CI	
M, 16 years		M, 16 years		F, 12 years		F, 18 years		M, 14 years		F, 12 years		M, 16 years		F, 13 years	
93	18	84	15	33	4	90	16	74	1	77	9	22	11	12	1
82	89	85	91	92	95	82	88	84	90	86	92	90	91	98	99
30	28	28	28	29	28	29	<b>24</b>	30	<b>24</b>	<b>26</b>	27	29	<b>23</b>	27	27
50	57	46	68	48	44	64	<b>113</b>	67	NA	83	80	93	<b>101</b>	NA	NA
89	149	93	143	166	104	66	231	214	NA	154	213	<b>407</b>	<b>300</b>	NA	NA
46	44	31	32	33	41	33	27	44	20	31	<b>13</b>	31	27	36	33
15	15	15	15	15	15	13	<b>12</b>	14	NA	15	15	14	<b>12</b>	14	<b>12</b>
18	18	17	16	20	21	21	16	21	<b>13</b>	<b>14</b>	<b>10</b>	<b>13</b>	<b>13</b>	<b>13</b>	<b>14</b>
6	8	5	<b>3</b>	6	7	7	<b>2</b>	6	<b>2</b>	5	<b>2</b>	<b>3</b>	<b>3</b>	<b>3</b>	<b>1</b>
17	30	19	16	7	13	14	13	20	<b>0</b>	19	23	12	4	5	10
NA	5	8	4	5	6	3	3	4	NA	4	<b>1</b>	5	2	<b>0</b>	NA
11	9	11	9	11	11	11	9	10	NA	11	<b>2</b>	7	10	<b>4</b>	<b>5</b>

ily members. Based on the emergence of poor memory scores and disorientation, she was judged to have converted to a state of MCI at around the fifth testing, approximately 3 years before death, and that she had remained at that stage during subsequent sessions.

9-CI. 9-CI, a 90-year-old man with 14 years of education, was tested first at age 84 years (74 mbd) and again at 85 (62 mbd), 87 (40 mbd), 88 (28 mbd), 89 (16 mbd), and 90 years (<1 month before death). He had worked as an accountant and held several managerial positions. All test scores initially were normal either for age or even for a much younger age (see Fig 2, Table 2). Over time scores declined. At the final testing the MMSE and three of the memory scores had decreased to the impaired range, whereas scores in other domains remained above age cutoffs. Activities of daily living were preserved initially but, because his vision failed by the last examination, he engaged in fewer activities. A review of all testing sessions showed that he had entered the state of MCI by the sixth testing, 16 mbd.

10-CI. 10-CI, a 92-year-old woman with 12 years of education, was tested first at age 86 (77 mbd) and again at ages 89 (43 mbd), 91 (20 mbd), and 92 years (9 mbd). Despite significant arthritic pain, she was independent and managed her own finances. The initial testing was borderline because the MMSE and CERAD word list acquisition scores were just below the age-appropriate cutoffs (26 vs 27 and 14 vs 15).

Further impairments in memory scores, in the context of preserved independent daily living activities, were deemed most consistent with a conversion, first noted at the third testing 20 mbd, into a state of MCI (see Fig 2, Table 2).

11-CI. 11-CI, a 92-year-old man with 16 years of education, was tested first at age 90 years (22 mbd) and a second time at 91 years (11 mbd). He died at age 92. He was dependent on others for daily living activities by the second test. There was evidence for progressive decline of cognitive function and daily living activities (see Fig 2, Table 2). The MMSE score became abnormal during the year before death but was still 23 at the last testing. The diagnosis at the last session was consistent with early stages of AD, and it appeared that he had converted from MCI to mild AD between the first and second testing sessions.

12-CI. 12-CI, a 99-year-old woman with 13 years of education who had worked as an accountant, was tested first at age 98 years (12 mbd) and a second time at 99 years (<1 month before death). Performance in the first session was impaired on CERAD acquisition and delayed recall, the BVRT, and constructions. By the last session, performance was also abnormal on the BNT naming test (see Fig 2, Table 2). She had been working as a volunteer up to the time of death and in the prior 2 months and had been featured in a local newspaper article. She was deemed to be in a state of

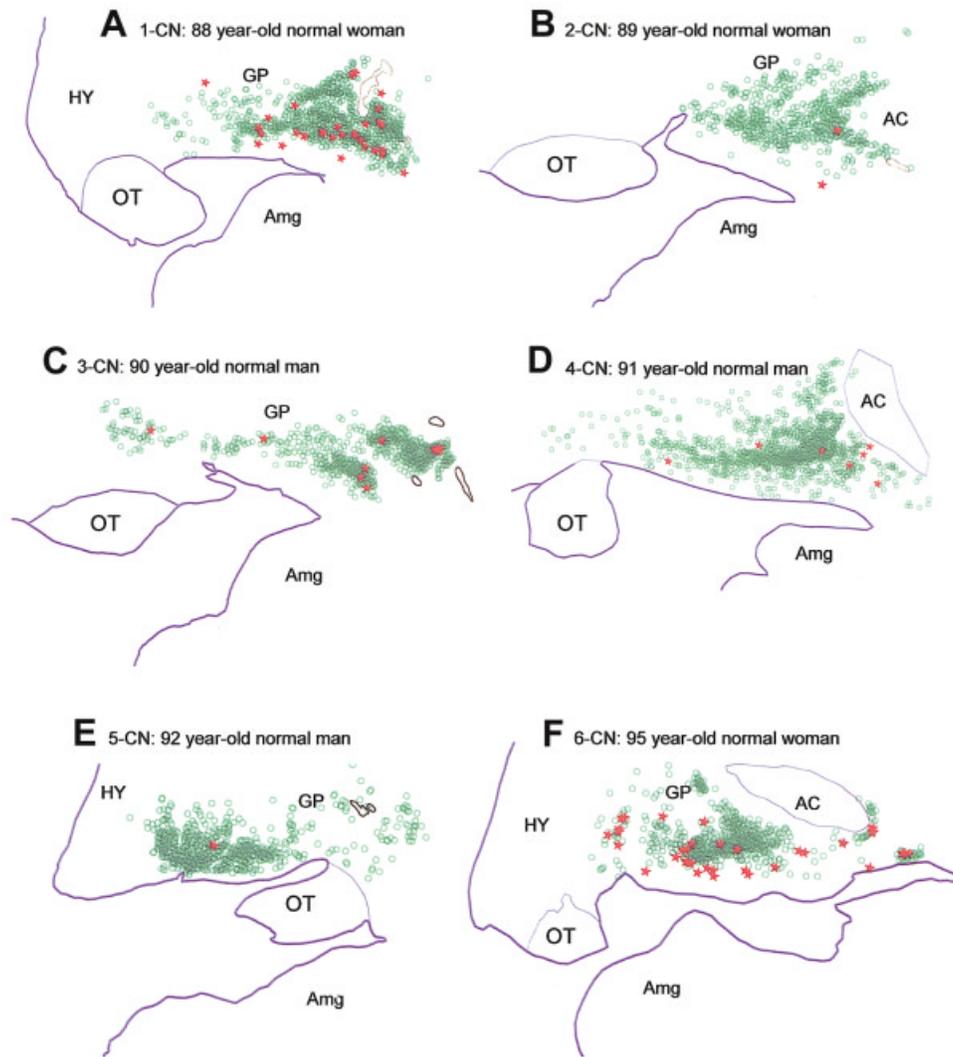


Fig 3. Neurofibrillary tangles in the nucleus basalis. The green circles show the magnocellular lipofuscin-rich nucleus basalis neurons. The red stars show the neurofibrillary tangles. AC = anterior commissure; Amg = amygdala; GP = globus pallidus; HY = hypothalamus; OT = optic tract.

MCI based on abnormal memory test scores and preservation of daily living activities.

#### General Neuropathology

Only Case 11-CI with the clinical picture of mild AD had a Braak stage 5 neurofibrillary degeneration and frequent neuritic plaques (see Table 1). In the other cases, the neurofibrillary degeneration ranged from stage 1 to stage 3 to 4 and the density of neuritic plaques ranged from sparse to moderate. In general, specimens with cognitive impairment tended to have a higher Braak NFT stage and more plaques than age-matched controls, but there was also considerable overlap.

#### Nucleus Basalis Tangles

All 12 specimens had matching thioflavin-S sections through the anterior part of the Ch4–nucleus basalis

complex (Figs 3 and 4). Two types of NFTs were identified in the Ch4–nucleus basalis complex, skein-like globose tangles and flame-shaped filamentous tangles. The highest NFT density (21%) was encountered in the early AD case (see 11-CI in Table 1, Fig 4E). The next two highest densities were noted in the two oldest MCI subjects, 11% in 10-CI and 17% in 12-CI (see Figs 4D, F). The five lowest tangle densities (<2%) were all seen in the cognitively normal subjects (2-CN to 5-CN and 7-CN). In general, CN subjects had a lower NFT density in the Ch4–nucleus basalis complex than age-matched MCI subjects (see Table 1). The only age-matched overlap between the two groups occurred in the case of Subject 1-CN who, at the age of 88 years, had an NFT density (3.3%) very close to the density seen in two of the MCI cases aged 89 and 90 years (4.1% in 8-CI and 3.8% in 9-CI). Note, however, that subject

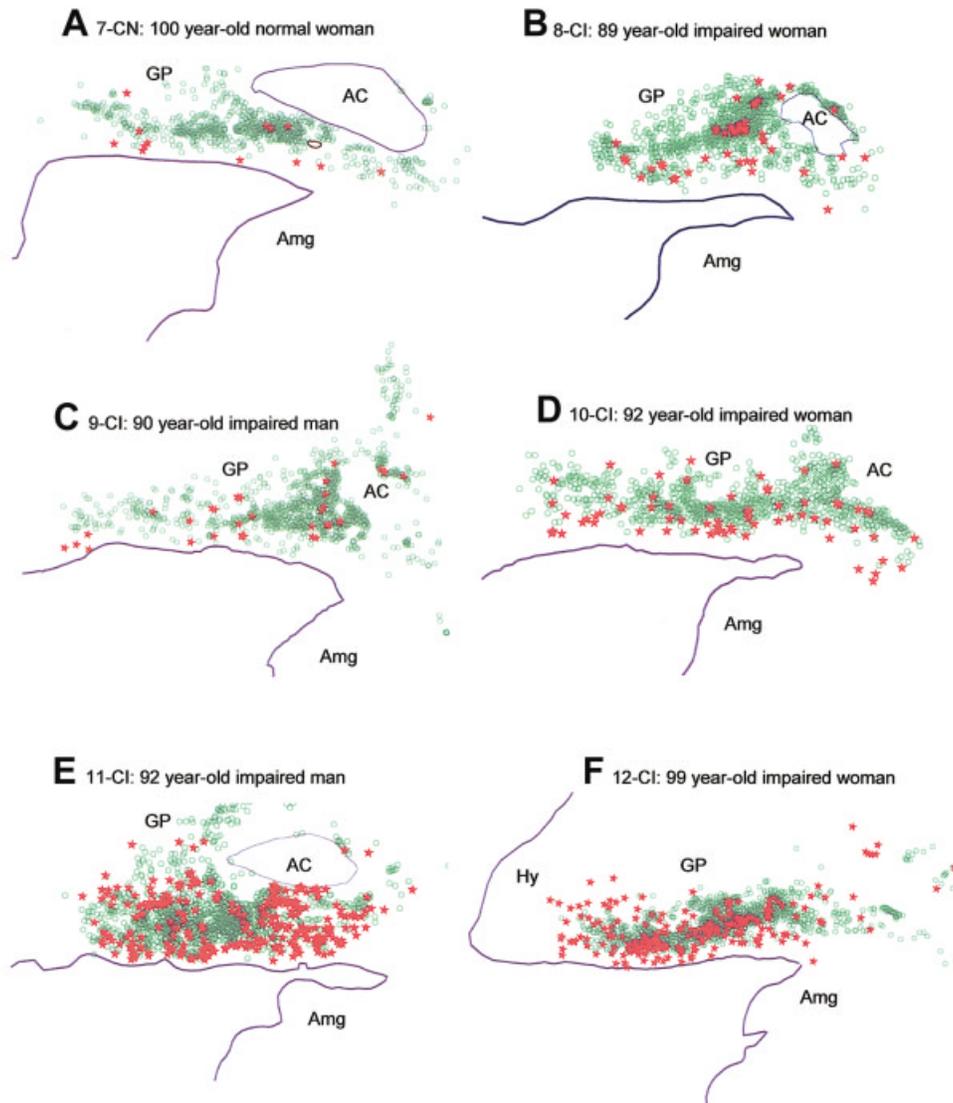


Fig 4. Neurofibrillary tangles in the nucleus basalis. The green circles show the magnocellular lipofuscin-rich nucleus basalis neurons. The red stars show the neurofibrillary tangles. AC = anterior commissure; Amg = amygdala; GP = globus pallidus; HY = hypothalamus; OT = optic tract.

1-CN also had the lowest MMSE score within the group of cognitively intact subjects (Table 1). In the five specimens at the Braak stages 1 and 2, the nucleus basalis was the only forebrain structure other than medial temporal areas consistently containing NFT.

#### Tau Immunoreactivity

The subject with mild AD (11-CI) did not have sections immunoreacted with Alz-50 or AT8. The other 11 cases had relevant sections immunoreacted with Alz-50 (Cases 7 and 12) or AT8 (all other cases). In two cases, matching sections stained with AT8 and Alz-50 showed that the two antibodies yielded virtually identical numbers of immunopositive perikarya in the nucleus basalis. Negative controls in which AT8 or

Alz-50 had been replaced by an irrelevant IgG or IgM did not show any staining in the nucleus basalis, establishing the specificity of the reaction product. The 36-year-old neurologically intact specimen displayed sparse, light, and punctate nucleus basalis immunostaining with both Alz-50 and AT8. However, this immunoreactivity was seen only in the small fusiform neurons, not in the magnocellular neurons that are damaged by AD.

The NFTs of AD contain hyperphosphorylated and abnormally folded tau that can be recognized by AT8 and Alz-50. Consequently, these antibodies showed perikaryal staining in the nucleus basalis of all immunoreacted cases (Figs 5 and 6). Although the thioflavin-S stain showed almost no neuropil threads,

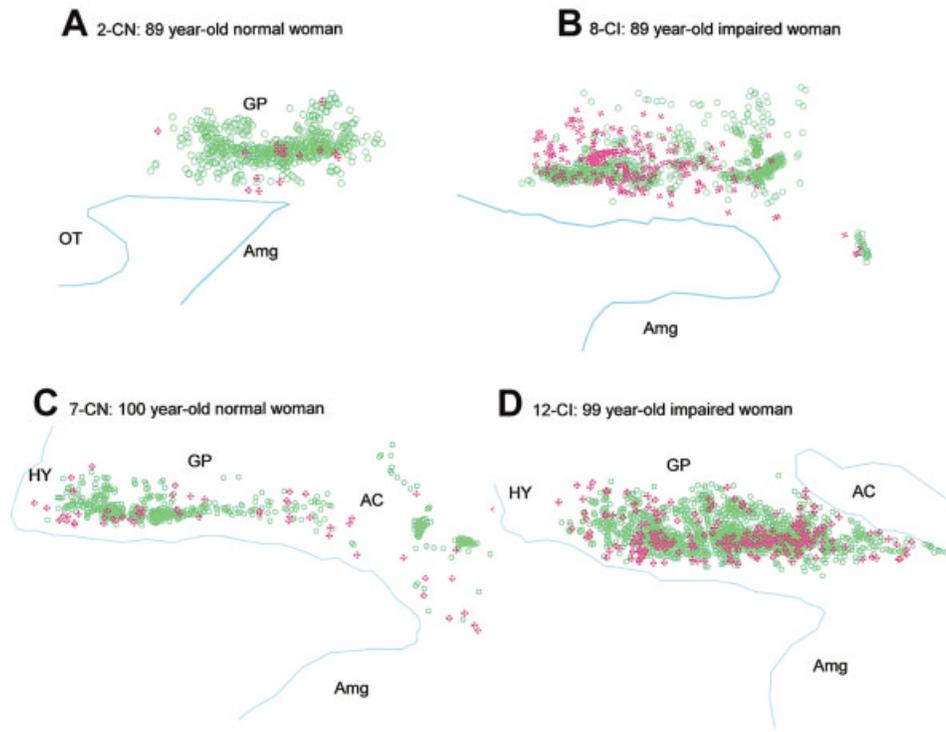


Fig 5. AT8 and ALZ-50 immunoreactivity in the nucleus basalis. The green circles show the magnocellular lipofuscin-rich nucleus basalis neurons. The magenta crosses show the immunoreactive neurons. Immunostaining was with Alz-50 in Cases 7 and 12, and with AT8 in all the others. AC = anterior commissure; Amg = amygdala; GP = globus pallidus; HY = hypothalamus; OT = optic tract.

many cases displayed immunoreactive neuropil staining that appeared partly dendritic and partly axonal (Fig 6). Immunoreactive dystrophic neurites were common. Some of the cytoplasmic perikaryal staining was punctate, some was diffuse and extended into proximal dendrites, and some had the morphological appearance of NFTs. Most of the reactive perikarya had normal-appearing morphology, whereas a few appeared to be at various stages of degeneration. The immunostaining was more extensive than the NFT distribution shown by thioflavin-S histofluorescence, suggesting that it included neurons at the pretangle stage of tau-related cytopathology (see Table 1). As shown in Figures 5 and 6, the MCI specimens displayed perikaryal and neuropil immunostaining that was distinctly more prominent than the age-matched cognitively normal control specimens. Sections processed for the concurrent demonstration of both ChAT and AT8 (or Alz-50) showed that nearly all the nucleus basalis neurons containing the tau-based cytopathology were also cholinergic (Fig 7). Rare exceptions occurred in the case of ghost tangles that were immunolabeled for phosphotau with AT8 but were not contained within ChAT-positive perikarya. The nucleus basalis was the only forebrain structure outside of the medial temporal lobe consistently displaying tau cytopathology in all specimens, including those at the Braak stages 1 and 2.

#### Quantitative Analyses

The nonparametric Wilcoxon rank-sum test showed that the cognitively normal group differed significantly from the impaired group for the percentage of NFT-containing ( $p = 0.004$ ) and AT8 or Alz-50-immunopositive neurons ( $p = 0.023$ ). The Wilcoxon matched-pairs signed rank test showed that the percentage of AT8 or Alz-50-immunoreactive neurons was higher than the percentage of NFT-containing neurons ( $p = 0.023$ ). We examined the relationship of cytopathology to the total score on the delayed recall trial of the CERAD word list. A Spearman rank correlation showed that the CERAD scores at the final assessment before death (Table 2) were negatively correlated with the percentage of NFT ( $r = -0.69$ ;  $p = 0.02$ ) and with the percentage of AT8 or Alz-50-immunoreactive neurons ( $r = -0.72$ ;  $p = 0.02$ ) in the nucleus basalis.

#### Discussion

The nucleus basalis lies under the globus pallidus and provides the source of nearly all cortical cholinergic axons. Most of its neurons are cholinergic and comprise the Ch4 cell group so that this nucleus is also known as the Ch4-nucleus basalis complex.<sup>20</sup> Cholinergic axons arising from the Ch4-nucleus basalis complex reach all parts of the cerebral cortex and therefore can

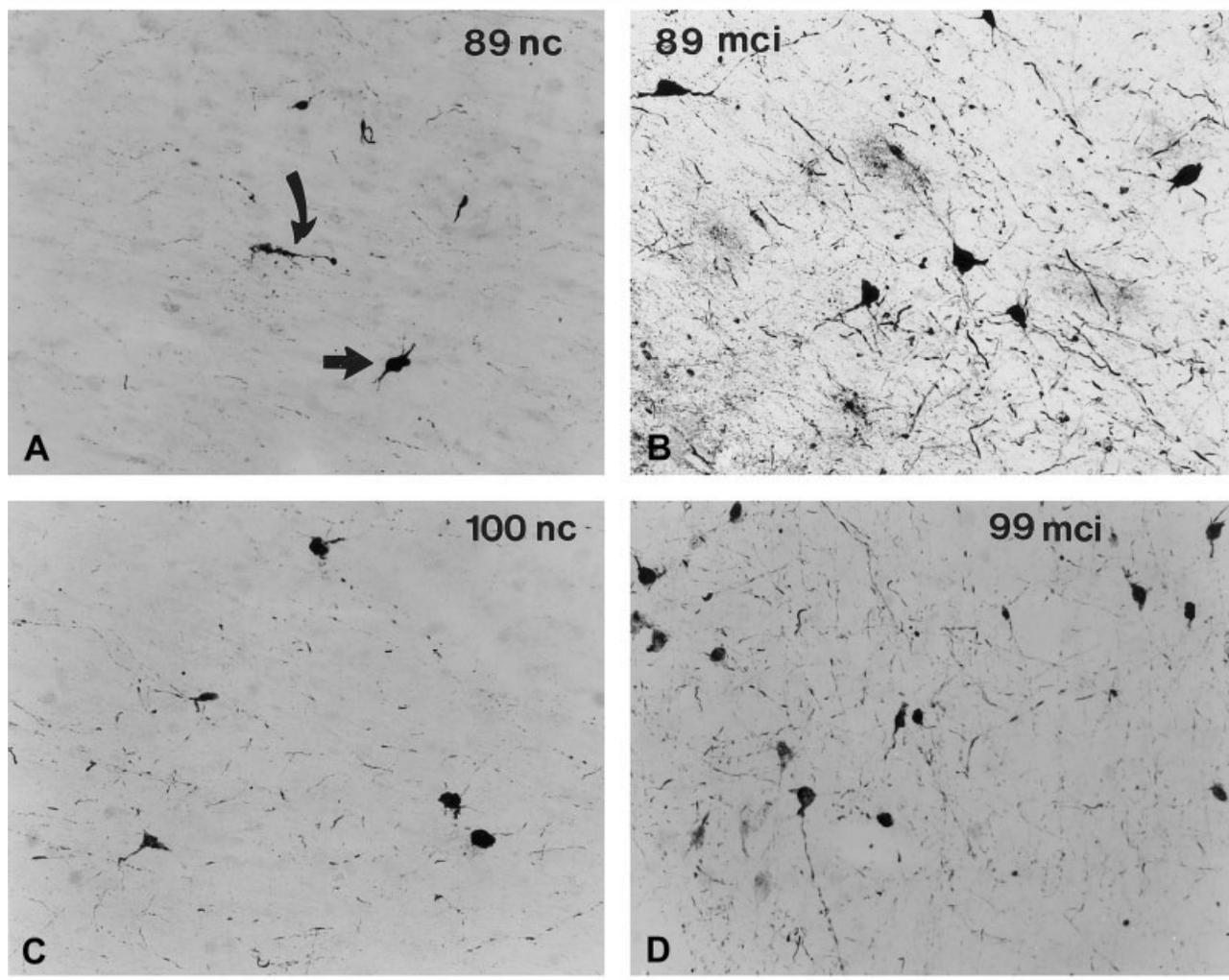


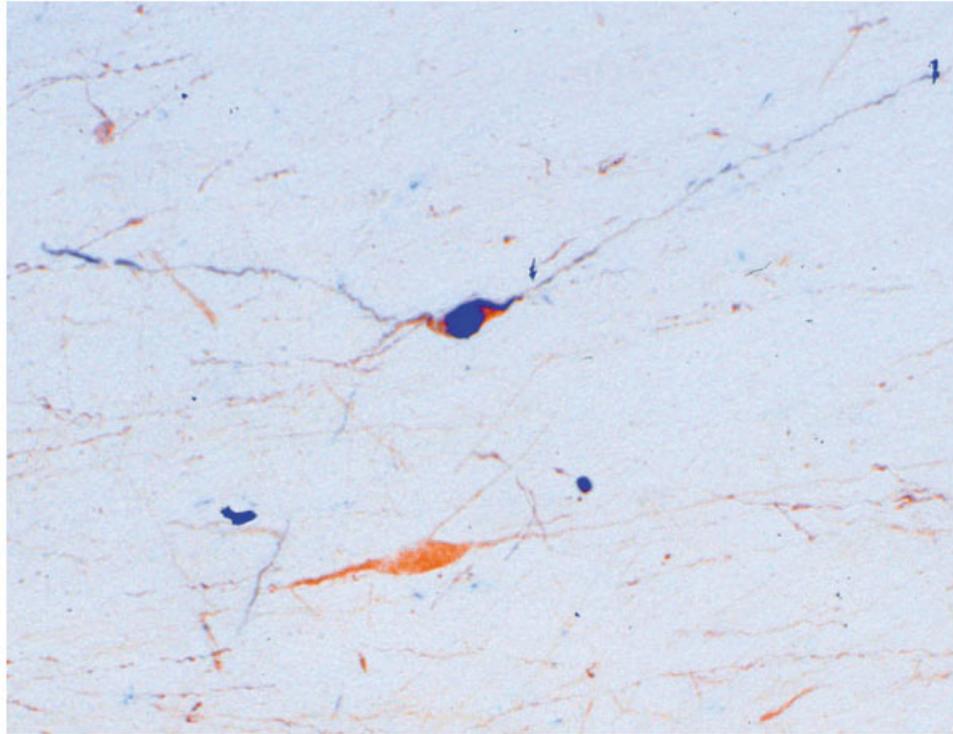
Fig 6. Tau cytopathology in the nucleus basalis in age-matched pairs of cognitively normal and of mild cognitive impairment (MCI) subjects. In all cases, the photomicrograph shows the area of maximal immunostaining in sections from the anterior part of the nucleus basalis. AT8 immunostaining in 2-CN, an 89-year-old normal subject (A) versus 8-CI, an 89-year-old MCI subject (B). The straight arrow in A points to perikaryal staining; the curved arrow points to abnormal neurite staining. Alz-50 immunostaining in 7-CN and a 100-year-old normal subject (C) and 12-CI a 99-year-old with MCI (D). Magnification  $\times 100$ . nc = normal control subject; mci = mild cognitive impairment subject.

influence all aspects of cognition, but especially attention and memory.<sup>3,31,32</sup> The association of advanced AD with severe neurofibrillary pathology in the nucleus basalis and an equally severe depletion of cortical cholinergic axons has been known for a long time. The principal outcome of this study was to show that this cytopathology starts during cognitively normal aging, that it becomes more severe at the MCI and early AD stages, and that its magnitude is correlated with memory function.

In all cases, the nucleus basalis contained fully formed NFT and Alz-50 and AT8-positive intracellular accumulations that appeared to reflect pretangle stages of tauopathy.<sup>22,23,33</sup> The fact that AT8 and Alz-50-immunoreactive neurons were more numerous than

NFT-containing neurons supports the contention that the NFT reflect a “tip of the iceberg” phenomenon preceded by multiple steps of tau-based cytopathology, some of which are recognized by these antibodies.<sup>25,34,35</sup> In the five specimens at the first two Braak stages of NFT, the nucleus basalis was the only fore-brain structure outside of medial temporal areas consistently displaying NFT and tauopathy, confirming its selective vulnerability to age-related and tau-based neurofibrillary pathology.<sup>36</sup>

Most of AT8 or Alz-50 accumulations were located within neurons that still had an active biosynthetic machinery as shown by the ChAT immunoreactivity of the cell body (see Fig 7). This observation is consistent with studies in which the number of cholinergic nu-



*Fig 7. Concurrent demonstration of at8 immunoreactivity (blue) and choline acetyltransferase (brown). The neuron on top has both reaction products, indicating that the AT8 immunostaining is located within a cholinergic neuron of the nucleus basalis. The neuron below is also cholinergic but lacks AT8.*

cleus basalis neurons in MCI and early AD were not found to be reduced when compared with age-matched controls.<sup>7</sup> Nevertheless, neurons at these putatively pretangle stages of cytopathology are unlikely to function normally because even early stages of NFT formation have been shown to interfere with protein synthesis and energy metabolism.<sup>37</sup> The intense immunoreactivity in the neuropil also indicated that the abnormal tau accumulations were not confined to the perikaryon but that they extended into neurites, most of which represent cholinergic axons directed to the cerebral cortex. Even in our cognitively impaired group, the percentage of immunoreactive neurons varied between 6 and 26%, a number that may not appear particularly impressive. However, note that the nucleus basalis has only 200,000 neurons, and that the number of cortical cholinergic axons exceeds this number by several orders of magnitude.<sup>38</sup> Cytopathology in a single nucleus basalis neuron thus may have widespread effects on cortical cholinergic innervation.

The extent to which the nucleus basalis cytopathology identified in this study influences the integrity of cortical cholinergic synapses remains to be determined. Circumstantial evidence favoring a destructive effect comes from post-mortem and in vivo studies in which nondemented elderly subjects (presumably having the sort of nucleus basalis cytopathology identified in this

report) displayed age-associated decrements of cortical cholinergic axons and synapses.<sup>39,40</sup> In our sample, the nucleus basalis cytopathology was significantly more pronounced in the cognitively impaired than cognitively intact subjects. Some studies suggest that this additional nucleus basalis cytopathology in MCI and early AD may not lead to further losses of cortical ChAT,<sup>5</sup> presumably because of reactive upregulations of enzyme activity in the unaffected cholinergic axons.<sup>6</sup> However, such an upregulation would not be expected to reverse structural and physiological damage in the synapses and axons emanating from the affected nucleus basalis neurons. Normal ChAT levels therefore do not assure the integrity of cholinergic innervation.<sup>3</sup>

This study relied on rigorous documentation of cognition. One impaired subject had the features of early AD, whereas the remaining impaired subjects fit the criteria for MCI, defined by the presence of acquired memory deficits that are significant but not severe enough to curtail customary daily activities.<sup>30</sup> When defined in this fashion, and when confined to elderly subjects with no other causes of cognitive dysfunction and no conspicuous neuropathological markers other than plaques and tangles, MCI is likely to represent a preclinical form of AD.<sup>29,41</sup> The classification of the five index subjects as cognitively impaired was based on cross-sectional normative cutoff scores. Multiple assess-

ments over many years also introduced a longitudinal dimension into the classification of subjects. We could document the presence of progressive impairment from a former baseline and, in some instances, approximate the time at which a "conversion" occurred from normal to MCI or from MCI to AD. Our definition of "impaired," based on two standard deviations from normative scores, is stringent so that those classified as MCI were very unlikely to represent outliers of the reference range.

The cytopathology in the nucleus basalis of the cognitively normal elderly initially may raise the possibility that it may have no functional relevance. Such an inference would be based on the assumption that "normal for age" reflects a stability of cognitive function. Table 2, however, indicates that a person in the age range of 70 to 100 may have experienced a considerable decline of performance from a former baseline and still remain within the reference range for age. Some of our subjects obtained scores normal even for 50 to 59-year-olds, but many did not and therefore may have undergone considerable cognitive decline while remaining within the age-appropriate range. The nucleus basalis cytopathology observed in our cognitively normal subjects could have contributed to the emergence of these age-related changes. Clearly, this would constitute one of numerous factors underlying age-associated cognitive changes, including the medial temporal NFT that were present in all cases.<sup>42</sup> The presence of a significant negative correlation between nucleus basalis cytopathology and delayed recall scores in the entire group of subjects, normal as well as impaired, also suggests that the influence of this cholinergic lesion on cognitive function extends to the stages of MCI and early AD as well, again in combination with numerous other factors.

In conclusion, our results show that cortical cholinergic pathways display clear-cut cytopathology early in the course of the aging-MCI-AD continuum. These abnormalities arise almost as early as those in medial temporal areas. The preclinical and initial stages of AD thus unfold on a background of age-related cholinergic cytopathology, whereas the transition from normal aging to MCI and AD is associated with a further exacerbation of this abnormality. Exactly how these pathological changes influence the functionality of cortical cholinergic synapses and their interactions with the other aspects of AD neuropathology will need to be clarified further.

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