Cholinergic Neuronal and Axonal Abnormalities Are Present Early in Aging and in Alzheimer Disease

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INTRODUCTION

Among the many cortical neurochemical changes in Alzheimer disease (AD), the most consistent and widespread is a marked loss of cortical cholinergic markers (see [1] for review). Virtually all cortical areas in the AD brain display depletion of their cholinergic axons (2), with a resulting reduction in the cholinergic synthetic enzyme choline acetyltransferase (ChAT) and the hydrolytic enzyme acetylcholinesterase (AChE) (3–5). In addition, the numbers of the basal forebrain cholinergic neurons (BFCNs), from which cortical cholinergic innervation emanates, are significantly decreased (6–8). The basal forebrain cholinergic system is involved in the cognitive processing of memory and attention (9–12), processes that are also deficient in AD. Moreover, the loss of cortical cholinergic innervation in AD shows a significant relationship with the severity of dementia (8, 13, 14). Therefore, it has been suggested that the cholinergic loss in AD contributes, at least in part, to the dementia observed in this disorder. These findings are also the rationale for cholinergic therapy in AD.

Beginning in the 1980s, a large body of evidence indicated that cortical cholinergic denervation occurs early in the course of AD. For example, in their study of neurochemical changes in temporal neocortex, Perry et al (15) divided AD cases into neuropathologically mild, moderate, and advanced groups on the basis of cortical plaque counts. In the mild group, only ChAT activity was significantly reduced, but other markers studied were normal. In the moderate group, in addition to ChAT, losses in dopamine β-hydroxylase and glutamic acid decarboxylase and an increase in substance P were found. In the severe group, an additional decrease was observed in cholecystokinin. Biopsy samples of the frontal lobe obtained within a year of the appearance of clinical symptoms displayed up to a 95% reduction of ChAT activity (16, 17). The brains of patients in whom biopsy samples at mild to moderately severe clinical stages of AD had shown a significant reduction in cortical ChAT activity displayed a further significant reduction of this marker at autopsy (13). In addition, nearly all comparisons have indicated that the loss of cortical cholinergic innervation in AD is more widespread and substantial when compared with the loss of a number of neurotransmitter systems (1). These observations have been interpreted as an indication of the selective vulnerability of the basal forebrain cholinergic system to early degeneration in AD.

More recent studies of mild cognitive impairment (MCI), the potential prodromal stage of AD, have cast doubt...
on the early vulnerability of cortical cholinergic innervation in this disorder. Two studies demonstrated that the levels of cortical ChAT activity showed no decrement in MCI and mild AD, and that there is a deficit only in moderate to severe AD cases (18, 19). One possible interpretation of these findings is that a deficit in the basal forebrain cholinergic system does not occur early in AD and is only a feature of more advanced disease. This interpretation has gained support by the finding that the number of BFCNs remains unchanged in MCI when compared with cognitively normal elderly (20).

Contrary to the above interpretation, Mesulam et al (21) have shown that BFCNs in cognitively normal elderly and in individuals with MCI display considerable accumulation of abnormal epitopes of tau in neurofibrillary tangles and pretangles. The present report extends this finding to earlier stages of life by analyzing the cortical cholinergic system in nondemented young, nondemented old, pathologically mild AD, and pathologically severe AD. The findings demonstrate that abnormalities in cortical cholinergic axons and tauopathy within the BFCNs occur very early in the course of life (i.e. as early as the third decade) and increase in frequency in old age and AD.

### MATERIALS AND METHODS

#### Case Information and Tissue Processing

Brains from 13 normal individuals without evidence of neurologic or psychiatric disorders ranging in age between 26 and 93 years and 10 brains from individuals with clinical and pathologic diagnosis of AD were studied. Acquisition of postmortem human tissue was carried out according to protocols approved by an institutional review board. Clinical records were available for every subject. In some of the young control cases in which the clinical record did not contain sufficient information to allow determination of cognitive function, this information was obtained from the next of kin. Brains of nondemented cases were obtained from autopsies at various hospitals. Brains of AD cases were obtained from a brain bank or from hospital autopsy services. Consecutive brains received at our laboratory from nondemented and demented individuals were used. All AD cases had been clinically diagnosed by a community neurologist; none of the nondemented or demented cases had undergone extensive neuropsychologic examination. Age, sex, postmortem interval, and cause of death for each subject are indicated in Table 1. The postmortem interval in the various groups of

#### Table 1. Characteristics of the Cases Studied

<table>
<thead>
<tr>
<th>Case</th>
<th>Age, Years</th>
<th>Sex</th>
<th>Postmortem Interval, Hours</th>
<th>Cause of Death</th>
<th>Braak Stage</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26</td>
<td>M</td>
<td>8</td>
<td>Lung failure after transplant</td>
<td>0</td>
<td>Normal young</td>
</tr>
<tr>
<td>2*</td>
<td>27</td>
<td>M</td>
<td>11</td>
<td>Gunshot wound to heart</td>
<td>0</td>
<td>Normal young</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>M</td>
<td>13</td>
<td>Non-Hodgkin lymphoma</td>
<td>I</td>
<td>Normal young</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>F</td>
<td>48</td>
<td>Myocardial infarction</td>
<td>I</td>
<td>Normal young</td>
</tr>
<tr>
<td>5</td>
<td>57</td>
<td>F</td>
<td>6</td>
<td>Pneumonia</td>
<td>I</td>
<td>Normal young</td>
</tr>
<tr>
<td>6</td>
<td>61</td>
<td>F</td>
<td>22</td>
<td>Metastatic carcinoma</td>
<td>I</td>
<td>Normal young</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>44.3 ± 6.1</td>
<td>18 ± 6.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>72</td>
<td>M</td>
<td>17</td>
<td>Pulmonary embolism</td>
<td>I</td>
<td>Normal old</td>
</tr>
<tr>
<td>8</td>
<td>73</td>
<td>M</td>
<td>16</td>
<td>Lung cancer</td>
<td>II</td>
<td>Normal old</td>
</tr>
<tr>
<td>9</td>
<td>77</td>
<td>F</td>
<td>15</td>
<td>Myocardial infarction</td>
<td>II</td>
<td>Normal old</td>
</tr>
<tr>
<td>10</td>
<td>82</td>
<td>M</td>
<td>24</td>
<td>Pneumonia</td>
<td>III</td>
<td>Normal old</td>
</tr>
<tr>
<td>11</td>
<td>83</td>
<td>F</td>
<td>23</td>
<td>Myocardial infarction</td>
<td>III</td>
<td>Normal old</td>
</tr>
<tr>
<td>12*</td>
<td>87</td>
<td>F</td>
<td>12</td>
<td>Cardiopulmonary failure</td>
<td>II</td>
<td>Normal old</td>
</tr>
<tr>
<td>13</td>
<td>93</td>
<td>M</td>
<td>22</td>
<td>Cerebellar hemorrhage</td>
<td>III</td>
<td>Normal old</td>
</tr>
<tr>
<td>Mean ± SE</td>
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<td>18.4 ± 1.7</td>
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<tr>
<td>14</td>
<td>77</td>
<td>F</td>
<td>12</td>
<td>Cardiopulmonary failure</td>
<td>IV</td>
<td>AD, pathology mild</td>
</tr>
<tr>
<td>15</td>
<td>80</td>
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<td>26</td>
<td>Pneumonia</td>
<td>IV</td>
<td>AD, pathology mild</td>
</tr>
<tr>
<td>16</td>
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<td>F</td>
<td>28</td>
<td>Myocardial infarction</td>
<td>V</td>
<td>AD, pathology mild</td>
</tr>
<tr>
<td>17</td>
<td>89</td>
<td>M</td>
<td>22</td>
<td>Myocardial infarction</td>
<td>IV</td>
<td>AD, pathology mild</td>
</tr>
<tr>
<td>18</td>
<td>89</td>
<td>F</td>
<td>21</td>
<td>Pneumonia</td>
<td>V</td>
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<tr>
<td>19</td>
<td>57</td>
<td>M</td>
<td>4</td>
<td>Cardiopulmonary failure</td>
<td>VI</td>
<td>AD, pathology severe</td>
</tr>
<tr>
<td>20</td>
<td>72</td>
<td>M</td>
<td>7</td>
<td>Cardiac arrest</td>
<td>V</td>
<td>AD, pathology severe</td>
</tr>
<tr>
<td>21</td>
<td>75</td>
<td>M</td>
<td>23</td>
<td>Pneumonia</td>
<td>VI</td>
<td>AD, pathology severe</td>
</tr>
<tr>
<td>22</td>
<td>87</td>
<td>M</td>
<td>23</td>
<td>Cardiac arrest</td>
<td>VI</td>
<td>AD, pathology severe</td>
</tr>
<tr>
<td>23</td>
<td>88</td>
<td>F</td>
<td>5</td>
<td>Pneumonia</td>
<td>VI</td>
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<tr>
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<td>75.8 ± 5.8</td>
<td>12.4 ± 4.3</td>
<td></td>
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</tr>
</tbody>
</table>

*Cases did not have complete material for some of the staining methods used and therefore were excluded from the quantitative analysis; they were used only for qualitative analysis.

AD, Alzheimer disease; F, female; M, male; SE, standard error.
subjects were not significantly different (p > 0.05). Additionally, there was no significant difference between age of AD cases compared with the nondemented elderly (p > 0.05).

One hemisphere of each brain was cut into 2- to 3-cm-thick blocks and placed in 4% paraformaldehyde (in 0.1 mol/L phosphate buffer, pH 7.4) for 36 to 40 hours at 4°C and then taken through sucrose gradients for cryoprotection (10%–40% in phosphate buffer). Blocks containing the regions of interest were sectioned at 40 μm and stored in 0.1 mol/L phosphate buffer at 4°C until used. Reports of neuropathologists from whom the brains were obtained indicated no abnormalities in any of the control brains used except age-appropriate changes (22) (e.g., small to moderate numbers of diffuse amyloid deposits in some elderly cases). The neuropathologic diagnosis of AD was made by the neuropathologists from whom brains were obtained according to the criteria suggested by the Consortium to Establish a Registry for Alzheimer’s Disease (23).

Brains from nondemented individuals younger than 65 years were designated as young, whereas those from individuals 65 years and older were designated as old. Based on the density of cortical plaques and tangles as well as the Braak staging of tangle progression (24), AD brains were divided into pathologically mild and pathologically severe groups (see Results section; Fig. 1; Table 1).

**AChE Histochemistry**

Acetylcholinesterase activity within cortical axons was visualized in a representative series of sections from each brain with the help of a highly sensitive histochemical method. The principles of this method (incubation in a dilute Karnovsky-Roots medium followed by metal ion-diaminobenzidine intensification) have been described by Hanke et al (25) and Tago et al (26). We have introduced several changes to this method that are described elsewhere (2, 27). We have demonstrated that AChE and ChAT, a specific cholinergic enzyme, visualize an identical population of cholinergic axons in normal and AD brains (2, 28). Thus, in the present study, histochemically visualized AChE activity in cortical fibers identifies cholinergic axons.

To ensure that the reaction product obtained was specific, the butyrylcholinesterase inhibitor ethopropazine (2 × 10⁻³ mol/L) was routinely added to the incubation medium.
Specificity of the AChE reaction was further ascertained by using 10^{-4} 	ext{ mol/L} of the specific AChE inhibitor BW284C51.

**Immunohistochemistry**

Series of sections from each brain were stained by immunohistochemistry using the avidin-biotin-peroxidase (ABC) method with the Vectastain Elite ABC kit (Vector Laboratories, Burlington, CA) as previously described (29). The antibodies used were a specific polyclonal antibody against ChAT (generous gift of Dr. Louis Hersh, University of Kentucky Medical School, 1/300–1/500), a monoclonal antibody against abnormally phosphorylated tau (paired helical filament 1 [PHF-1], recognizes tau phosphorylated at Ser 396/404; generous gift of Dr. Peter Davies) and a polyclonal antibody against amyloid-ß (Aß; generous gift of Dr. Dennis Selkoe). We have successfully used these antibodies in the past for visualizing each antigen (29–31). To control for nonspecific staining, sections stained using the previously discussed antibodies were compared with sections stained in the absence of primary antibodies or in the presence of nonspecific immunoglobulin G in place of primary antibodies. For visualization of ChAT within axons, the sections underwent silver intensification after immunohistochemical staining, as previously described (32).

**Quantitative Analysis of Matched Sections**

Matching sections stained for Aß, PHF-1, and AChE were available in all cases. Matching sections through the basal forebrain stained for ChAT were also available in all cases. Optimal ChAT staining for visualization of cortical cholinergic axons was obtained for 2 cases in each group. Therefore, only the AChE histochemistry results were used for qualitative analysis of cholinergic axons.

The density of cholinergic fiber abnormalities, Aß-immunoreactive plaques, and PHF-1-immunoreactive tangles and pretangles within the cerebral cortex was determined using a counting box with known dimensions (500 × 500 μm) at 200× magnification. The counting box was randomly placed at the cortical surface in 1 field in at least 3 sections, each within the cortical areas of interest. The box was systematically moved from the cortical surface to white matter, and the numbers of objects of interest in each box were counted. Counts were obtained from the entorhinal/perirhinal cortex (Brodmann area 28), middle temporal gyrus (area 21), inferior parietal lobule (areas 39–40), and anterior cingulate cortex (area 24). These areas were chosen because we and others (33–35) had shown them to be affected by pathologic alterations in AD. The counts from the 3 cortical columns in each case were expressed as numbers per square millimeter.

All PHF-1-positive magnocellular pretangles and tangles in 3 sections spanning the anterior, intermediate, and posterior sectors of the nucleus basalis of Meynert (nBM)-Ch4 (i.e. Ch4a, Ch4i, and Ch4p) were counted. We have previously shown that virtually all magnocellular nBM neurons are ChAT positive and are therefore cholinergic (36). Adjacent sections stained for ChAT were used to ascertain that counts are obtained from the cholinergic neurons only. Amyloid-ß-immunoreactive plaques were also counted within the nBM region. Counting was carried out at 100× magnification using a counting box placed in the eyepiece of a compound microscope. The data were compiled as the number of immunoreactive profiles per section.

The numbers of tangles and pretangles within the cerebral cortex and nBM were determined separately. Pretangles were defined as normal neurons with PHF-1 immunoreactivity but without the presence of characteristic morphologic features of tangles according to criteria described elsewhere (37).

In 3 nondemented cases, systematically sampled sections (1 in 25) through each structure were available. In all of these cases, the structures of interest were counted in all available sections and were expressed as number of immunoreactive profiles per section.

**Statistical Analysis**

Data for each portion of the study were subjected to a test for normality. All data were normally distributed, and therefore, parametric statistical analysis was used. Analysis of variance followed by appropriate post hoc tests were used to detect significant group differences. Relationships were determined using Pearson correlations. The probability for accepting a significant difference was set at p < 0.05.

**RESULTS**

The immunohistochemical and histochemical methods permitted visualization of AChE- and ChAT-positive cholinergic axons, PHF-1-immunoreactive pretangles and tangles, and Aß-positive plaques in the cerebral cortex and basal forebrain. Cholinergic neurons of the basal forebrain also displayed robust ChAT immunoreactivity.

**Cortical Plaques and Tangles**

Amyloid-ß-immunoreactive plaques were absent in the cerebral cortex of young brains. A subpopulation of old brains contained a low to moderate density of Aß deposits particularly prominent in the anterior cingulate cortex and middle temporal gyrus. The density of plaques displayed a substantial increase in AD brains.

Consistent with the susceptibility of the entorhinal/perirhinal cortex as a site of the formation of the first cortical tangles, a low density of PHF-1-positive pretangles was seen in this region in most young brains. The density of pretangles displayed an increase in the entorhinal/perirhinal cortex of old brains, and pretangles were also observed in neocortical areas in this group. An increased density of pretangles was observed in all cortical areas in AD. The distribution and density of tangles was similar to that of pretangles, except the density of tangles was higher, particularly in AD.

Quantitative analysis confirmed the distribution pattern of cortical plaques, pretangles, and tangles. Based on the counts of cortical plaques and tangles (Fig. 1) and the Braak staging of tangle formation, the AD cases were divided into mild (Braak Stages IV–V) and severe (Braak Stages V–VI) groups (Table 1).

With the exception of entorhinal/perirhinal cortex, the density of cortical Aß deposits displayed a dramatic increase...
in pathologically mild AD when compared with normal controls (p < 0.001; Fig. 1A). In severe AD cases, there was an additional slight but nonsignificant increase in the density of Aβ deposits compared with mild AD cases (p > 0.05) in all areas except the entorhinal cortex. In the entorhinal cortex, a significant increase in Aβ deposits was observed in severe when compared with mild AD (p < 0.01).

In the cingulate cortex and middle temporal gyrus, pretangles displayed a significant increase only in pathologically severe AD when compared with the normal control groups (p < 0.05; Fig. 1B). In the inferior parietal lobule, pretangles also displayed a significant increase in pathologically mild AD over controls (p < 0.05). In the entorhinal/perirhinal cortex, the density of pretangles showed a non-significant increase from young control to pathologically mild AD groups (p > 0.05). In all cortical areas examined, tangles displayed a dramatic increase in pathologically severe AD over the controls (p < 0.001; Fig. 1C, D) and over pathologically mild AD (p < 0.01).

**Cortical Cholinergic Axonal Abnormalities**

In the youngest cases (Cases 1 and 2; Table 1), AChE and ChAT staining revealed a dense plexus of cholinergic axons. Stained axons were thin and relatively homogeneous in diameter (Fig. 2A, B). In ChAT-immunostained sections, axons displayed small, fine varicosities (Fig. 2B). In all other areas examined, thickened axons and swollen and ballooned varicosities were seen in both AChE (C, D) and ChAT (F, H) stained sections. These included chandelier arrangements. Similar axonal abnormalities were seen in other nondemented old and AD cases. Scale bars = (A) 100 μm and also applies to (B); (C) 50 μm and also applies to (D–H).

![FIGURE 2.](http://jnen.oxfordjournals.org/) In the youngest case investigated (Case 1), acetylcholinesterase (AChE) activity (A) and choline acetyltransferase (ChAT) immunoreactivity (B) were present within homogeneously thin cholinergic axons. Immunohistochemistry for ChAT visualized small varicosities within axons (B; arrows). In the brain from an old nondemented case (Case 13; 93 years old), 1 or more of a number of abnormalities were seen in both AChE (C, E, G) and ChAT (D, F, H) stained sections. These included thickened axons (C, D) and swollen and ballooned varicosities at axonal endings (E–H), which often occurred in a chandelier arrangement. Similar axonal abnormalities were seen in other nondemented old and AD cases. Scale bars = (A) 100 μm and also applies to (B); (C) 50 μm and also applies to (D–H).
cases, the cholinergic axons stained with the above markers displayed several abnormalities. These included 1) considerably thicker axons with reduced or no branching when compared with adjacent normal axons (Fig. 2C, D), 2) swollen, ballooned terminals at axonal endings (Fig. 2E, F), and 3) collections of terminal swellings in a chandelier-like arrangement (Fig. 2G, H).

Excluding the youngest cases, nondemented young brains displayed a small number of the previously discussed abnormalities in cortical cholinergic fibers (Fig. 3). In every cortical area examined, the group of nondemented old individuals displayed significantly increased density of cholinergic fiber abnormalities when compared with the young (p < 0.05; Fig. 3). The numbers of cholinergic axonal abnormalities in the nondemented group of subjects showed a highly significant correlation with age (Table 2).

The numbers of cholinergic axonal abnormalities remained unchanged in pathologically mild AD when compared with the nondemented old group (p > 0.05; Fig. 3). In the entorhinal/perirhinal and inferior parietal cortices of pathologically severe AD, the numbers of cholinergic axonal abnormalities displayed a significant decrease when compared with pathologically mild AD.

| TABLE 2. Correlations Between Abnormalities and Age in Nondemented Cases |
|-----------------------------|-------------------|-------------|
| Type of Abnormality and Brain Region | Correlation | p          |
| Cholinergic axonal abnormalities |             |            |
| Entorhinal/perirhinal cortex | 0.8744 | p < 0.001 |
| Middle temporal gyrus | 0.8425 | p < 0.003 |
| Inferior parietal cortex | 0.8821 | p < 0.0008 |
| Anterior cingulate cortex | 0.8279 | p < 0.004 |
| Basal forebrain pretangles | 0.8032 | p < 0.003 |
| Basal forebrain tangles | 0.5656 | p = 0.07  |
(p < 0.05; Fig. 3), suggesting selective degeneration of cholinergic axons containing abnormalities. In the middle temporal gyrus, this decrease was not statistically significant (p > 0.05; Fig. 3), and in the anterior cingulate cortex of pathologically severe AD, the number of cholinergic abnormalities remained unchanged. Qualitative observations demonstrated loss of cortical cholinergic axons in all cortical regions of AD brains in a pattern consistent with our previous observations (2).

**Basal Forebrain Plaques and Tangles**

The area of basal forebrain in which the BFCNs are located was completely devoid of Aβ deposits in young cases. In nondemented old cases, this region contained sparse Aβ deposits. In pathologically mild AD, the number of basal forebrain Aβ-positive plaques showed a significant increase when compared with the normal old (p < 0.001; Fig. 4A). A further significant increase in the number of Aβ deposits was observed in the basal forebrain of pathologically severe AD cases when compared with pathologically mild AD (p < 0.001; Fig. 4A). Nearly all Aβ-positive plaques in the basal forebrain were of the diffuse type. Rarely were compact plaques seen in this region (see [38] for review).

As was the case in the entorhinal/perirhinal cortex, pretangles were detected in a small subpopulation of the nbM-Ch4 neurons in young cases (Figs. 4B, 5A, B). Slightly higher numbers of pretangles were seen in these neurons in old cases (Fig. 4B). In pathologically mild AD, the number of nbM-Ch4 pretangles displayed a significant increase when compared with nondemented old cases (p < 0.001; Fig. 4B), and these numbers remained unchanged in pathologically severe AD when compared with pathologically mild AD (p > 0.05). In the group of nondemented cases, the numbers of nbM-Ch4 pretangles displayed a significant correlation with age (Table 2).

Tangles were also present in the magnocellular nbM-Ch4 of a subpopulation of young brains and in higher numbers in old brains (Figs. 4B, 5A–D). Significantly larger numbers of tangles were seen within the nbM-Ch4 in

**Figure 5.** Paired helical filament 1-positive pretangles and tangles in the basal forebrain of young (A, B; Case 1), middle-aged (C; Case 6), old (D; Case 8), pathologically mild (E; Case 18), and pathologically severe AD (F; Case 20). Pretangles were present in the magnocellular basal forebrain neurons in the 26-year-old (A, B; Case 1) in whose brain no tangles or pretangles were found in the entorhinal/perirhinal cortex. In older cases, the numbers of tangles and pretangles within these neurons are increased. Scale bar = (A) 100 μm and also applies to (B–F).
pathologically mild AD when compared with the nondemented old group (p < 0.001; Figs. 4B, 5E), with a further significant increase in pathologically severe AD (p < 0.001; Figs. 4B, 5F). The correlation between the number of nBM-Ch4 tangles and age in nondemented individuals displayed a trend toward significance (p = 0.07; Table 2).

Of particular relevance to the vulnerability of the BFCNs, the brain of Case 1 (26 years old; Table 1), which contained no pretangles or tangles within the entorhinal/perirhinal or any other cortical region examined, contained pretangles in the BFCNs (Fig. 5A, B). Furthermore, of the 8 cases with the earliest stages of tangle formation (Braak Stages I and II; Table 1) with cortical pretangles and tangles only within the entorhinal/perirhinal cortex, all contained BFCN pretangles and tangles.

**DISCUSSION**

**Early Abnormalities in Cortical Cholinergic Axons**

The present results clearly demonstrate the presence of pathologic abnormalities in the basal forebrain cholinergic system early in the course of aging and AD. Abnormalities in cortical cholinergic axons were observed in nondemented young individuals, and they increased in frequency in the nondemented aged cases. Although the thickening of cortical cholinergic axons and ballooning of terminals may represent normal variations in axonal morphology rather than abnormalities, 2 observations argue against this possibility. First, in the youngest cases, all cholinergic axons and terminals were consistently thin and of the same diameter without any of the variations previously described. Second, the density of axonal changes displayed a significant decrease within most cortical areas in pathologically severe AD. Given that cortical cholinergic axons displayed a major depletion in AD (2), the previously described finding suggests that this loss occurs preferentially in axons that display the abnormalities we have observed. This line of reasoning is consistent with the qualitative observation that cortical cholinergic axons in rats with lesions in the BFCNs display similar abnormalities (39).

Some studies of cortical ChAT activity have shown upregulation of this enzyme in the hippocampus and frontal cortex in individuals with MCI and mild AD (18, 40). It is likely that the emergence of cholinergic fiber abnormalities in the form of thickened axons and ballooned terminals, which we found to contain ChAT immunoreactivity, is responsible, at least in part, for the increased ChAT activity in MCI and the reported preservation of ChAT activity in mild AD. Our present observations suggest that cortical ChAT activity alone does not provide an accurate indication of the health of the basal forebrain cholinergic system. Rather, direct visualization of cholinergic neurons and axons is necessary to identify the earliest alterations in this system in the course of normal aging and AD. The abnormalities in ChAT-containing axons and terminals identified are not likely to allow normal cholinergic transmission. Therefore, despite preserved cortical ChAT activity in MCI and mild AD, the cholinergic system seems to be functionally compromised.

**Early Pathology in BFCNs**

The magnocellular basal forebrain neurons contained pretangles and/or tangles very early in the course of normal aging, starting in the third decade of life. The numbers of these tangles and pretangles displayed a significant increase in pathologically mild AD and a further significant increase in pathologically severe AD. These observations are consistent with recent findings in individuals with MCI (21) and with qualitative observations of the basal forebrain during normal aging and AD (41).

Of great interest, basal forebrain pretangles and tangles were observed prior to such pathology in the entorhinal/perirhinal cortex, and the density of this pathology in the basal forebrain increased concurrent with the appearance of pathology in the above cortical area. Given the established observation that cortical tangles and pretangles first appear in the entorhinal/perirhinal cortex in the course of aging and then spread progressively to other cortical areas (24, 42), our findings suggest very early vulnerability of BFCNs to the process of tangle formation. Our observations further suggest that the BFCNs, rather than the entorhinal/perirhinal cortex, may be the site at which tau pathology appears first.

The general pattern of the appearance of Aβ-positive plaques in the basal forebrain followed that of the cerebral cortex. Furthermore, consistent with our earlier observations (38), compact Aβ deposits, which contain activated glia and dystrophic neurites and are considered the pathologic plaque variant (43), were absent from the basal forebrain. Therefore, it seems that the basal forebrain region within which the BFCNs are located does not display selective vulnerability to plaque formation. It remains to be determined whether intraneuronal accumulation of soluble Aβ oligomers, which have been shown to exert detrimental and toxic effects on neurons (44–46), occurs early within the BFCNs.

**Functional Implications of Early Cholinergic Neuron Alterations**

The early pathology observed in the cholinergic system of the basal forebrain and the involvement of this system in the cognitive processing of memory and attention supports current attempts of cholinergic therapy in AD. The morphologic abnormalities identified indicate that the cholinergic system likely suffers from functional deficits early in the course of AD despite preserved ChAT activity. Cholinesterase inhibitors are among a handful of therapeutic agents in AD and have been shown to result in modest improvements in cognition, behavior, and daily activities, primarily by inhibiting the cholinergic hydrolytic enzyme AChE, thereby increasing the available pool of the neurotransmitter acetylcholine (47, 48). Our observations indicate that current experimental therapeutic approaches, particularly those targeting tau and its abnormal phosphorylation, are likely to enhance the function of the basal forebrain cholinergic system early in the course of dementia and thereby result in preservation of cognition.

In addition to tauopathy and axonal abnormalities, 2 other early alterations have been described in BFCNs. First, these neurons have been shown to display a significant loss
of high-affinity (Trk-A) and low-affinity (p75NTR) neurotrophin receptors concomitant with the appearance of cognitive abnormalities in MCI (49, 50). Given the dependence of the BFCNs on nerve growth factor for protection against damage (51, 52), it is likely that the loss of neurotrophin receptors in MCI contributes to the loss of BFCNs in AD. In addition, we have observed a substantial and selective loss of the calcium-binding protein calbindin-D_{28K} from BFCNs in the course of aging in the human and nonhuman primates (53, 54). We have suggested that age-related loss of calbindin is likely to deprive the BFCNs of the capacity to buffer excess intracellular calcium, leaving them vulnerable to neurodegenerative insults with the potential to lead to excessive rises in intracellular calcium, with the potential to lead to excessive rises in intracellular calcium and degeneration due to calcium toxicity. The relationship between early BFCN tau pathology and alterations in nerve growth factor receptors and calbindin remains unexplored. It is likely that the previously described alterations will also be used for the development of novel therapeutic approaches aimed at improving the function of the cortical cholinergic system.

CONCLUSIONS

Along with the reported loss of neurotrophin receptors from the BFCNs in MCI and the age-related loss of calbindin from these neurons, the present findings provide clear evidence for the selective and early vulnerability of the basal forebrain cholinergic system to damage and loss in the course of aging and AD. They also justify continued therapeutic efforts aimed at ameliorating early cholinergic deficits in AD that are present despite preserved cortical ChAT activity. These observations, along with the vulnerability of this system in other neurodegenerative disorders, suggest that the basal forebrain cholinergic system can serve as a model for the investigation of the causes of selective neuronal vulnerability in neurodegenerative disorders.

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REFERENCES


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