Relationship Between Plaques, Tangles, and Loss of Cortical Cholinergic Fibers in Alzheimer Disease

CHANGIZ GEULA, M.-MARSEL MESULAM, DANIEL M. SAROFF, AND CHUANG-KUO WU

Abstract. Recent observations in our laboratory have indicated substantial and systematic regional variations in the loss of cortical cholinergic fibers in Alzheimer disease (AD). Previous attempts to study the relationship between cortical cholinergic loss and the density of cortical pathological lesions have resulted in conflicting findings. Furthermore, most reports have correlated density of plaques and tangles with the residual level of cholinergic innervation rather than its loss. The purpose of the present study was to determine the relationship between loss of cholinergic axons and density of tangles and β-amyloid (Aβ) deposits in various cortical areas of AD brains. Aβ deposits and tangles were observed throughout the cerebral cortex. Quantitative analysis revealed almost no correlation between loss of cholinergic fibers and the density of Aβ deposits. Qualitative observations revealed similar results when cored and neuritic plaques were considered separately. By contrast, cholinergic fiber loss displayed a significant correlation with the density of tangles (r = 0.52–0.79). However, in a few areas, such as the cingulate cortex, tangle density appeared to be unrelated to the loss of cholinergic fibers. These results indicate that cortical cholinergic denervation in AD is related to cytoskeletal pathology. However, the lack of a perfect relationship with cytoskeletal pathology implicates additional factors in the cholinergic pathology of AD.

Key Words: Amyloid plaques; Basal forebrain cholinergic neurons; Paired helical filaments.

INTRODUCTION

A marked loss of cortical cholinergic innervation is a consistent feature of Alzheimer disease (AD) (1–5). This loss occurs earlier, is more pronounced, and appears more widespread than the loss of other neurotransmitter-specific corticopetal systems (2, 6–9). Recent observations in our laboratory (5) have indicated that the depletion of cortical cholinergic axons in AD is not uniform, but displays systematic regional variations. For example, cortical areas within the temporal lobe, such as the entorhinal cortex, the visual association area (Brodmann areas 20 and 21), and the auditory association cortex (area 22) display greater than 80% loss of their cholinergic axons, while the cholinergic fibers in the anterior cingulate cortical zones (areas 24, 25 and 32) remain virtually intact.

The precise factors that contribute to the regional variation and extent of cortical cholinergic loss in AD remain controversial. The primary pathological features of AD, the amyloid plaque and the neurofibrillary tangle, have been considered as the main cause of the cortical cholinergic depletion. Some investigators have postulated the formation of tangles in the cholinergic neurons of the basal forebrain (Ch1-Ch4), which give rise to cortical cholinergic axons, as the main mechanism of cholinergic depletion in AD (10). Other evidence, however, indicates that cortical pathology is a likely contributor to the loss of cholinergic innervation. For example, the primary common feature of all subcortical nuclei that display neuronal loss and tangles in AD, including the basal forebrain Ch1-Ch4 neurons, is a widespread and diffuse cortical projection ([11], see [2] for review). Furthermore, among the subcortical nuclei that have both cortical and subcortical targets, such as the locus ceruleus, only the sectors that are known to project to the cerebral cortex display neuronal loss and tangles in AD (12).

Previous attempts to study the relationship between the cortical cholinergic loss in AD and the density of cortical pathological lesions have resulted in conflicting findings. The activity of the cholinergic enzyme choline acetyltransferase (ChAT) in many cortical areas of AD brains has been shown by some investigators to display a significant negative correlation with the density of plaques (7, 13–15). The size of this correlation, however, is quite variable, ranging from −0.34 to −0.82. Moreover, some studies show no significant relationship between the loss of cholinergic enzymes and plaque density (16–18). Similarly, some investigators have found a small but significant negative correlation (−0.38 to −0.58) between residual cortical ChAT levels and density of tangles (15, 16), while others have found no such correlation (14, 19).

There are two likely reasons for these inconsistent findings. First, most reports have correlated density of plaques and tangles with the residual level of cholinergic innervation rather than its actual loss. Our previous observations have indicated substantial variation in the density of cholinergic innervation among different cortical areas of the normal human brain (1, 20). Thus, the residual cholinergic innervation in a particular cortical area in
AD is not only a function of the extent of fiber loss, but also a function of the premorbid density of cholinergic innervation. Second, the biochemical determination of ChAT activity used in these experiments requires removal of a fresh sample of brain tissue and precludes pathological observations in the same sample. Thus, the majority of studies have determined the density of plaques and tangles at sites away from the exact area at which the ChAT activity was determined, making a correlation unreliable. This problem can be resolved through the use of histochemical methods in which cholinergic fiber loss and density of plaques and tangles can be determined from adjacent or closely matched sections.

In this report we present results of our histochemical experiments, demonstrating the lack of a relationship between cortical cholinergic denervation and the density of plaques, but a relatively strong relationship with the density of tangles.

**MATERIALS AND METHODS**

**Tissue Preparation and Pathological Observations**

Thirteen brains from normal aged individuals with no prior history of neurologic or psychiatric disorders and 15 brains from patients with a history of dementia of the Alzheimer type were used in these studies. Age (normal: 76.23 ± 9.8; AD: 76.6 ± 8.8; p > 0.05) and postmortem interval (normal: 12.8 hours; AD: 14.2 hours; p > 0.05) were not different in the 2 groups of subjects. Each brain was cut into 1–2 cm hemispheric coronal slabs and examined for the presence of atrophy, ventricular enlargement and other gross abnormalities. The slabs of tissue were placed in cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24–30 hours, then into graded concentrations of sucrose (10–40% in 0.1 M phosphate buffer, at 4°C) for cryoprotection. The slabs were sectioned at 40 μm on a freezing microtome into 0.1 M phosphate buffer and stored at 4°C until used. Representative sections from each tissue block were stained with hematoxylin-eosin, Bielschowsky silver and thioflavin-S for neuropathological observations, and with cresyl violet for delineation of cytoarchitectonic boundaries.

Only brains with no gross or microscopic abnormalities and no or very few cortical plaques and tangles, consistent with normal aging (21), were designated as normal. The brains from demented individuals contained numerous cortical plaques and tangles in a density and distribution consistent with the neuropathological diagnosis of AD (21). Brains which displayed other gross or microscopic neuropathological abnormalities were not used in this study.

**Acetylcholinesterase Histochemistry**

Previous work from our laboratory has demonstrated that in the human cerebral cortex, acetylcholinesterase (AChE) and ChAT, which is a specific cholinergic enzyme, visualize an identical population of cholinergic axons in normal and AD brains (5, 22). Thus, histochemically visualized AChE activity is a reliable marker of cholinergic axons. In the present study, AChE activity within cortical axons was visualized in a representative series of sections from each brain with the help of a new and 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 Harker et al (23) and Tago et al (24). We have introduced a number of changes in this method as described elsewhere (1, 5).

To inhibit butyrylcholinesterase (BuChE, non-specific cholinesterase), 2×10⁻⁴ M ethopropazine (MW 348.9) or 1×10⁻⁴ M ISO-OMPA (MW 342.4, Sigma Chemical Company, St. Louis, MO) were used in the incubation medium. The specific AChE inhibitor BW284C51 (MW 556.4 Sigma Chemical Company, St. Louis, MO) was added (10⁻⁴ M) to demonstrate the specificity of the AChE staining.

**Immunohistochemistry**

A series of sections adjacent or matched to the sections processed for AChE histochemistry were stained immunohistochemically for the visualization of β-amyloid (Aβ)-positive plaques using a sensitive antibody (1282 polyclonal antibody, 1/2000, kindly provided by Dr Dennis Selkoe, Harvard Medical School). Additional matching sections were processed with an antibody to paired helical filament fractions isolated from AD cerebral cortex, which also recognizes tau (PHF-tau, PAM polyclonal antibody, 1/1000, kindly provided by Dr Dennis Selkoe, Harvard Medical School), to visualize tangles and tau-positive neuropil threads and the neuritic component of plaques. The avidin-biotin-peroxidase (ABC) immunohistochemical procedure (25) was used employing the Vectastain Elite ABC kit (Vector Laboratories, Berlingame, CA). Control sections were incubated in irrelevant IgG in place of the primary antibody.

**Assessment of Density of Cholinergic Fibers, Plaques, and Tangles**

All sections processed for AChE histochemistry, Aβ and PHF-tau immunohistochemistry, and matching series of sections stained with thioflavin S were subjected to a thorough qualitative survey for the assessment of regional variations in the density of cholinergic fibers, plaques, and tangles. In addition to total Aβ-positive plaques, the distribution of cored plaques in thioflavin S-stained sections and of neuritic plaques in thioflavin S- and PHF-tau-stained sections were studied. Tangle distribution was investigated using the same thioflavin S- and PHF-tau-stained sections as above.

To substantiate the qualitative observations, quantitative measures were obtained from 6 normal and 6 AD brains. An intersect analysis was used to obtain an estimate of fiber density in 14 cytoarchitectonically and functionally distinct cortical areas (Table 1). The cortical areas were chosen so that regions that showed severe, modest, or minimal loss of cholinergic fibers in our previous studies (1, 5) were equally represented. Tissue sections processed for AChE histochemistry were viewed at 200X magnification through a square 10 × 10 grid (enclosing a 250 μm × 250 μm square of tissue) placed in the ocular of a Nikon compound microscope. The grid was adjusted such that one side of it was parallel to the cortical surface. The number of fibers intersecting the 10 lines parallel and perpendicular to the cortical surface were counted and recorded in lower lamina.
TABLE 1
Counts of Cholinergic Fibers in Various Cortical Areas of Normal and Alzheimer Brains

<table>
<thead>
<tr>
<th>Cortical area</th>
<th>Normal</th>
<th>Alzheimer disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temporal visual association (21)</td>
<td>524 ± 77</td>
<td>77 ± 43*</td>
</tr>
<tr>
<td>Temporal visual association (22)</td>
<td>617 ± 123</td>
<td>101 ± 59*</td>
</tr>
<tr>
<td>Entorhinal (28)</td>
<td>1,218 ± 170</td>
<td>239 ± 81*</td>
</tr>
<tr>
<td>Granular insula (Ig)</td>
<td>984 ± 167</td>
<td>262 ± 99*</td>
</tr>
<tr>
<td>Superior parietal association (7)</td>
<td>427 ± 45</td>
<td>135 ± 49*</td>
</tr>
<tr>
<td>Granular orbitofrontal (OFg)</td>
<td>734 ± 162</td>
<td>265 ± 122*</td>
</tr>
<tr>
<td>Inferior parietal lobule (39-40)</td>
<td>517 ± 54</td>
<td>200 ± 92*</td>
</tr>
<tr>
<td>Occipital visual association (18-19)</td>
<td>356 ± 103</td>
<td>148 ± 38*</td>
</tr>
<tr>
<td>Prefrontal association (9)</td>
<td>464 ± 48</td>
<td>198 ± 39*</td>
</tr>
<tr>
<td>Hippocampus (CA1 sector)</td>
<td>1,861 ± 505</td>
<td>856 ± 50*</td>
</tr>
<tr>
<td>Premotor association (6)</td>
<td>527 ± 105</td>
<td>304 ± 94*</td>
</tr>
<tr>
<td>Primary visual (17)</td>
<td>376 ± 96</td>
<td>231 ± 41**</td>
</tr>
<tr>
<td>Anterior cingulate (24)</td>
<td>1,193 ± 218</td>
<td>884 ± 123**</td>
</tr>
<tr>
<td>Primary motor (4)</td>
<td>619 ± 108</td>
<td>506 ± 35**</td>
</tr>
</tbody>
</table>

Cortical areas are listed in the order of decreasing magnitude of cholinergic fiber loss in Alzheimer disease. In most areas, Alzheimer brains displayed significantly lower densities of cholinergic fibers when compared with controls. * = p < 0.001; ** = p < 0.025; † = p < 0.05; n.s. = not significantly different from control.

III (lamina IIC) and upper lamina V of each cortical area examined. In areas with more primitive lamination, such as the hippocampus and the cingulate cortex, counting was performed in both a superficial and a deep layer. To ensure that interstices from all stained fibers within the full 40 μm thickness of each section were counted, the plane of focus on the microscope was systematically varied while counting. The best stained areas within each cytoarchitectonic region were chosen for this analysis. Aβ-positive deposits and thioflavin S-positive tangles within the boundaries of the same grid were counted in identical areas on adjacent or carefully matched sections. The counts obtained from the two grids (LIII and LV) were then combined to obtain an estimate of the density of cholinergic fibers, Aβ deposits, and tangles (Table 2).

The quantitative measures obtained were subjected to analysis of variance for repeated measures with Newman-Keuls post-hoc tests to determine significant differences between the various cortical areas. The counts of AChE-positive fibers in each area were averaged across all normal cases and used as the normative measure against which the loss of fibers in that area was calculated in each AD case. The relationships between the AChE-positive fibers remaining, and percent loss of fibers, and the density of Aβ-positive plaques and tangles were determined using Pearson correlations. This analysis was carried out in each AD case as well as in the combined AD group (Tables 3 and 4).

RESULTS

Cortical Cholinergic Fibers

Normal Pattern: A dense plexus of AChE-positive (cholinergic) fibers was present in all cortical areas of the normal brains (Figs. 2A, 3A). The staining within fibers was completely inhibited by the AChE inhibitor BW28C51, while the BChE inhibitor Iso-OMPA had no effect on staining. The density of these fibers displayed considerable regional variation (Table 1). The hippocampal formation contained the highest density of fibers (p < 0.001 in CA1). Paralimbic cortical areas such as the cingulate, entorhinal, orbitofrontal cortex, and insula displayed the next highest density of cholinergic fibers (p < 0.025). The primary auditory, somatosensory, and motor cortices

TABLE 2
Cholinergic Fiber Loss and Counts of Amyloid Deposits and Neurofibrillary Tangles Averaged Across Six Cases

<table>
<thead>
<tr>
<th>Cortical area</th>
<th>Fibers remaining</th>
<th>Fibers lost</th>
<th>% Fibers lost</th>
<th>Amyloid deposits</th>
<th>Tangles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temporal visual association (21)</td>
<td>77</td>
<td>447</td>
<td>85</td>
<td>49</td>
<td>61</td>
</tr>
<tr>
<td>Temporal visual association (22)</td>
<td>101</td>
<td>516</td>
<td>84</td>
<td>51</td>
<td>41</td>
</tr>
<tr>
<td>Entorhinal (28)</td>
<td>239</td>
<td>979</td>
<td>80</td>
<td>32</td>
<td>65</td>
</tr>
<tr>
<td>Granular insula (Ig)</td>
<td>262</td>
<td>722</td>
<td>73</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>Superior parietal association (7)</td>
<td>135</td>
<td>292</td>
<td>68</td>
<td>55</td>
<td>39</td>
</tr>
<tr>
<td>Granular orbitofrontal (OFg)</td>
<td>265</td>
<td>469</td>
<td>64</td>
<td>58</td>
<td>40</td>
</tr>
<tr>
<td>Inferior parietal lobule (39-40)</td>
<td>200</td>
<td>317</td>
<td>61</td>
<td>52</td>
<td>33</td>
</tr>
<tr>
<td>Occipital visual association (18-19)</td>
<td>148</td>
<td>208</td>
<td>58</td>
<td>53</td>
<td>34</td>
</tr>
<tr>
<td>Prefrontal association (9)</td>
<td>198</td>
<td>266</td>
<td>57</td>
<td>48</td>
<td>32</td>
</tr>
<tr>
<td>Hippocampus (CA1 sector)</td>
<td>856</td>
<td>1,005</td>
<td>54</td>
<td>17</td>
<td>54</td>
</tr>
<tr>
<td>Premotor association (6)</td>
<td>304</td>
<td>223</td>
<td>42</td>
<td>29</td>
<td>19</td>
</tr>
<tr>
<td>Primary visual (17)</td>
<td>231</td>
<td>145</td>
<td>39</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
<td>Anterior cingulate (24)</td>
<td>884</td>
<td>309</td>
<td>26</td>
<td>42</td>
<td>29</td>
</tr>
<tr>
<td>Primary motor (4)</td>
<td>506</td>
<td>113</td>
<td>18</td>
<td>18</td>
<td>8</td>
</tr>
</tbody>
</table>

Areas are listed in the order of the largest percentage of cholinergic fiber loss. Each point represents the average of counts obtained from 6 cases. The numbers in parentheses represent cortical areas according to the Brodmann classification. Counts were obtained from a 0.125 mm² area of cortex in layers III and V.
TABLE 3  
Correlation Between the Density of Amyloid Deposits and Loss of Cholinergic Fibers

<table>
<thead>
<tr>
<th>Case number</th>
<th>Fibers remaining</th>
<th>Fibers lost</th>
<th>% Fibers lost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>-0.51</td>
<td>-0.26</td>
<td>0.41</td>
</tr>
<tr>
<td>Case 2</td>
<td>-0.49</td>
<td>-0.07</td>
<td>0.52</td>
</tr>
<tr>
<td>Case 3</td>
<td>-0.41</td>
<td>0.58</td>
<td>0.67</td>
</tr>
<tr>
<td>Case 4</td>
<td>-0.56*</td>
<td>-0.09</td>
<td>0.56*</td>
</tr>
<tr>
<td>Case 5</td>
<td>-0.57*</td>
<td>-0.01</td>
<td>0.61*</td>
</tr>
<tr>
<td>Case 6</td>
<td>-0.13</td>
<td>-0.01</td>
<td>0.21</td>
</tr>
<tr>
<td>All cases combined</td>
<td>-0.57*</td>
<td>-0.21</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Cases are listed in the order of greatest overall loss of cholinergic fibers. * = p < 0.05. The density of cortical Aβ deposits shows no correlation with the actual number of lost fibers.

displayed an intermediate density of fibers and tended to contain slightly more fibers than most association cortical areas, while the primary visual and visual association areas displayed the lowest density of cholinergic fibers.

**Alzheimer Disease:** All cortical areas examined in AD brains displayed some degree of cholinergic fiber loss when compared with matching areas in normal brains. Comparison of individual cortical areas revealed marked regional variations in the extent of this loss (Table 1). In general, cortical areas within the ventral aspects of the hemisphere displayed a greater loss of fibers when compared with cortical regions within the dorsal portions of the hemisphere. Cholinergic fibers displayed a major depletion in the temporal neocortical areas, most of which were virtually empty of fibers. By contrast, the cholinergic fibers in the premotor cortex, the cingulate gyrus, the sensory/motor cortex, and in some of the frontal association areas seemed to be relatively well preserved.

Our quantitative analysis revealed that areas with the greatest loss (>80% reduction) of cholinergic innervation were all in the temporal lobe and included areas 21, 22, and 28 of Brodmann (Table 1, Fig. 3). The frontal, parietal, and occipital association areas and paralimbic areas such as the insula and orbitofrontal cortex showed an intermediate magnitude of loss (40–75%). The anterior cingulate gyrus, primary motor cortex, and primary visual cortex (Fig. 2) displayed less than 40% loss of cholinergic fibers (p > 0.05). The CA1 sector of the hippocampal formation displayed a moderate (54%) loss of its cholinergic fibers in AD brains.

**Relationship with Cortical Plaques**

**Aβ Deposits:** A dense accumulation of Aβ-positive plaques was observed in virtually all cortical areas of the AD brains (Fig. 1A). The majority of these plaques were of the diffuse variety. Particularly heavy deposits of amyloid were observed in the subpial-layer I region of most areas. In many cortical areas, supragranular layers contained much denser Aβ-positive plaques when compared with infragranular layers (in 9 of 14 quantified regions,

Fig. 1. Examples of amyloid-positive plaques and PHF-tau staining in an Alzheimer brain. (A) In the inferior temporal cortex (area 21), as well as in many other cortical areas, consistently more amyloid deposits were observed in supragranular layers when compared with infragranular layers. Small arrows delineate the border between cortical gray and white matter. (B) The PHF-tau antibody consistently stained tangles and neuritic processes in plaques (arrow). Magnification in (A), ×20 and in (B), ×312.
Fig. 2. (A) Cholinergic fibers in layer III of the primary visual cortex (area 17) from a normal elderly individual and (B) cholinergic fibers, (C) amyloid deposits, and (D) thioflavin S–positive tangle (arrow) in layer III of the visual cortex of an AD patient. This area displayed relatively well–preserved cholinergic fibers and contained a high density of amyloid deposits, but had very few tangles. Area 17 also contained a high density of plaques with a dense amyloid core (arrowheads in D). (Compare with Fig. 3). Magnification, 312×.
Fig. 3. (A) AChE-positive (cholinergic) fibers in the entorhinal cortex (area 28) from a normal elderly individual, and (B) cholinergic fibers, (C) amyloid deposits, and (D) thioflavin S–positive tangles (arrows) in the entorhinal cortex of an AD patient. The entorhinal cortex displayed one of the largest magnitudes of loss of cholinergic fibers in AD. It contained a similar density of amyloid deposits when compared with areas with a relatively small magnitude of fiber loss (see Fig. 3). The entorhinal cortex, however, displayed a high density of tangles. Magnification, ×312.
p < 0.05). In general, all association cortical regions contained a high density of Aβ deposits. The primary sensory and motor areas and most limbic and paralimbic areas, such as the hippocampus, entorhinal cortex, and insula, contained a relatively lower density of Aβ deposits.

Unlike the regionally variable loss of cholinergic fibers, the majority of cortical areas could not be differentiated from each other based on the density of Aβ deposits. Thus, areas which showed relatively severe loss of cholinergic fibers (e.g. area 21) displayed the same density of Aβ deposits (p > 0.05, Table 2) as areas with virtually preserved cholinergic fibers (e.g. area 24). The areas that showed significant variation from this pattern displayed the reverse density of Aβ deposits compared with that for cholinergic fiber loss. For example, the entorhinal cortex (Fig. 3) and the CA1 region of the hippocampus, which displayed relatively severe loss of their cholinergic fibers, contained lower densities of Aβ-positive plaques compared with all association regions and most other paralimbic regions (p < 0.01). Furthermore, these two regions contained the same density of Aβ-deposits as areas with the smallest loss of cholinergic fibers, such as the primary visual (Fig. 2) and primary motor cortices (p > 0.05). In addition, the laminar difference observed in the distribution of cortical Aβ deposits was not detected in the loss of cholinergic fibers.

Cored Plaques: Although some compact plaques with dense amyloid cores were identified in Aβ-immuno-stained sections, thioflavin S-stained sections proved most useful for the visualization of core plaques (Fig. 4). The overall density of cored plaques displayed considerable variation from brain to brain. In brains with significant numbers of cored plaques, the overwhelming majority were located within infragranular layers, a laminar pattern not detected in the loss of cholinergic axons. The majority of areas that displayed severe depletion of cholinergic fibers (e.g. areas 20, 21, 22, and 28) contained few cored plaques, and in some fields, none were detected (Fig. 4B). By contrast, many areas with negligible loss of cholinergic fibers, particularly the primary visual cortex (Fig. 4A), contained among the highest densities of cored plaques.

Neuritic Plaques: Both thioflavin S- and PHF-tau-stained sections visualized the neuritic component of plaques (Figs. 1B, 5). In most AD brains, a small to moderate proportion of the total population of Aβ-positive plaques appeared to be of the neuritic variety. In general, the distribution of neuritic plaques showed a better relationship with the extent of cholinergic fiber loss (particularly in association cortical areas) when compared with the density of Aβ deposits. However, discrepant relationships were observed in numerous cortical areas. For example, in the entorhinal cortex (Fig. 5A) and hippocampus, despite consistently high magnitude of cholinergic fiber loss, considerably lower densities of neuritic plaques were found as compared with most other cortical areas (Fig. 5B). In 4 of the AD brains, a high proportion of plaques were of the neuritic variety. In these brains, areas with relatively small magnitude of cholinergic fiber loss, such as area 24 and 17 (Fig. 5B), displayed much higher densities of neuritic plaques when compared with areas with a high magnitude of cholinergic fiber loss.

Relationship with Neurofibrillary Tangles

Thioflavin S and PHF-tau stained a large number of cortical tangles (Figs. 2-5). In addition to tangles, PHF-tau immunostaining visualized many neurons, particularly in the hippocampus, which appeared normal morphologically. In the majority of cortical areas, the highest density of tangles were observed in layers III and V. The density of tangles in these lamina was different only in the entorhinal cortex, in which layer V contained a significantly higher density (p < 0.02).

The overall distribution of tangles displayed a strong relationship with the regional loss of cholinergic fibers. Thus, areas with the highest magnitude of cholinergic loss (e.g. areas 21 and 28) displayed the highest density of tangles (p < 0.01, Table 2, Fig. 3), areas with an intermediate magnitude of fiber loss (e.g. areas 9, 18, 19, 39, and 40) displayed intermediate densities of tangles, and areas with a relatively small magnitude of cholinergic loss (e.g. area 4 and 17) displayed the lowest density of tangles (p < 0.01, Fig. 2). There were a few exceptions to this rule. The most prominent of these occurred in area 24, which displayed among the lowest magnitudes of cholinergic fiber loss, but contained a density of tangles not significantly different from most other cortical areas (p > 0.05; except areas 21 and 28).

Correlation Analysis

Aβ Deposits: The density of amyloid deposits showed no correlation with the absolute number of lost cholinergic fibers in any of the cases used for quantitative analysis (Table 3). It showed a weak but significant correlation with the number of fibers remaining and with the percentage of fibers lost in 2 of 6 cases, and with the number of fibers remaining when all cases were combined.

Tangles: The density of thioflavin S-positive tangles displayed a significant correlation with the absolute number of lost cholinergic fibers and the percentage of lost fibers in 5 of 6 cases and in all cases combined (Table 4). By contrast, tangle density showed no correlation with the number of remaining cholinergic fibers.

DISCUSSION

Consistent with our earlier observations (1, 5), the results of the present study revealed substantial and systematic regional variations in the loss of cortical cholinergic
Fig. 4. (A) The primary visual cortex (area 17), which displayed only a relatively small magnitude of loss of its cholinergic fibers, contained a high density of plaques with dense amyloid cores (arrows) in thioflavin S–stained sections. (B) The temporal visual association cortex (area 21), which displayed among the greatest loss of cholinergic fibers in AD brains (85% loss), contained a very low density of cored plaques (large arrows). This area also contained many tangles (small arrows). Magnification, ×150.
Fig. 5. Many fields in AD brains, such as the portion of entorhinal cortex (area 28) shown here (A) that displayed a marked loss of its cholinergic fibers (80% loss), contained few neuritic plaques (arrow). Others, such as the portion of area 17 depicted above (B), which displayed a relatively small loss of its cholinergic fibers, contained many neuritic plaques (arrows). The entorhinal cortex and the auditory association cortex (area 22) both displayed a severe loss of their cholinergic fibers. Staining for PHF-tau showed many tangles (arrowheads) and very few neuritic plaques in the entorhinal cortex (C), and tangles and many neuritic plaques (short arrows) in the auditory association cortex (D). Both areas contained many PHF-tau–positive neuropil threads (long arrows). Magnification, ×312.
TABLE 4
Correlation Between the Density of Neurofibrillary Tangles and the Loss of Cholinergic Fibers

<table>
<thead>
<tr>
<th>Case number</th>
<th>Fibers remaining</th>
<th>Fibers lost</th>
<th>% Fibers lost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>-0.18</td>
<td>0.77*</td>
<td>0.76*</td>
</tr>
<tr>
<td>Case 2</td>
<td>-0.28</td>
<td>0.44</td>
<td>0.74*</td>
</tr>
<tr>
<td>Case 3</td>
<td>0.05</td>
<td>0.72*</td>
<td>0.48</td>
</tr>
<tr>
<td>Case 4</td>
<td>-0.03</td>
<td>0.88*</td>
<td>0.70*</td>
</tr>
<tr>
<td>Case 5</td>
<td>-0.06</td>
<td>0.83*</td>
<td>0.66*</td>
</tr>
<tr>
<td>Case 6</td>
<td>0.04</td>
<td>0.85*</td>
<td>0.63*</td>
</tr>
<tr>
<td>All cases combined</td>
<td>-0.07</td>
<td>0.81*</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Cases are listed in the order of greatest overall loss of cholinergic fibers. * = p < 0.05; † = p < 0.005; ‡ = p < 0.0005.

Density of cortical tangles shows a strong correlation with the actual number and percentage of lost cholinergic fibers.

contain components from other neurotransmitter and peptide systems (37–39), make it quite unlikely that there is a direct relationship between plaques and degenerating cholinergic fibers in AD. Furthermore, histochemical studies in our laboratory have demonstrated that the AChE associated with plaques differs enzymatically from the AChE associated with normal fibers and neurons with respect to optimum histochemical pH and inhibitor sensitivity (40–42). In addition, many plaques in the AD brain display butyrylcholinesterase (BChE) activity, while BChE activity is a minor component of normal fibers and neurons. It is therefore difficult to support the suggestion that plaques are formed by degenerating cholinergic axons or that plaque formation is the major cause of cholinergic depletion in AD.

In contrast to plaque density, tangle density displayed a strong relationship with cholinergic loss in the sample of AD cases used in the present study. Furthermore, tangles showed a significant correlation with the actual loss of cholinergic fibers; tangle density was not related to the residual density of fibers. Thus, it appears that cortical pathology is related to cholinergic fiber loss, but that the density of tangles and not plaques is the predictor of the magnitude of this loss. However, the lack of a relationship between cholinergic fiber loss and density of tangles in some cortical areas (e.g. area 24) indicates that other factors are likely to participate in the pathology of cortical cholinergic innervation in AD. One such factor is pathology in the area of origin of these fibers in the basal forebrain.

Both plaques and tangles are found in the Ch1-Ch4 cholinergic cell groups within the basal forebrain (10, 43). While Aβ deposits are intermingled with these cholinergic neurons, we have found that the density of these deposits is far below that found in the cerebral cortex and that virtually all of these plaques are of the diffuse, nonneuritic variety (unpublished observations). Many of the remaining Ch4 neurons in AD brains contain neurofibrillary tangles (10, 44, 45). In fact, the cholinergic neurons of the basal forebrain are among the first neuronal groups vulnerable to tangle formation in the normal elderly and in mildly demented persons (30, 46, 47). It appears, therefore, that tangles formed within the Ch1-Ch4 neurons and within their target cortical areas are likely contributors to damage and loss of the Ch1-Ch4 neurons and their axons.

A significant relationship has been reported between cortical plaques and Ch4 neuronal loss and shrinkage by some investigators (48, 49), but not by others (50). Two studies that have attempted to correlate neuronal loss in various sectors of Ch4 with plaque counts in anatomically related cortical areas found a larger correlation between Ch4 neuronal loss and plaques in anatomically related cortical sites as compared with unrelated cortical sites (51, 52). A simple interpretation of these findings would
appear to run counter to the results of the present study. It should be pointed out, however, that some presumably related cortical areas in these studies showed little correlation with Ch4 neuronal loss (52). Furthermore, inconsistencies have been detected between Ch4 sectors and the assumed cortical targets used in one of these studies (53).

A further contribution to the loss of Ch1–Ch4 cholinergic neurons might come from age-related neurochemical changes within these neurons. For example, work in our laboratory and that of others has demonstrated an age-related loss of the calcium binding protein calbindin-D_{28K} from the human Ch1-Ch4 neurons in normal individuals (54–56). The loss of the calcium buffering capacity of calbindin in normal aged individuals is likely to leave these cholinergic neurons vulnerable to an excessive rise in intracellular calcium levels, such as those induced by certain pathological processes, and in turn, to the resultant neuronal damage and degeneration.

One interpretation of the findings of the present study would be that plaques, and particularly Aβ deposits, do not make a significant contribution to the loss of cholinergic fibers in AD. However, in light of recent findings, such an interpretation may be premature. A number of in vivo and in vitro studies (32), including our recent observations in the nonhuman primate (57), have indicated that the Aβ found in plaques can be directly toxic to neurons. More importantly, Aβ has been shown to be able to cause phosphorylation of tau similar to that observed in tangles (57, 58). Thus, it may be argued that the sequence of the pathological cascade in AD begins with the deposition of Aβ in diffuse plaques, followed by maturation of plaques and abnormal phosphorylation of tau, formation of tangles within cortical and Ch1-Ch4 neurons, and resultant damage to cholinergic axons in the cortex and cholinergic neurons in the basal forebrain. However, regardless of the role that plaques and amyloid may play in the cholinergic loss, our findings strongly suggest that the process which results in the formation of tangles is a more likely indicator of cholinergic fiber loss in AD.

A significant negative correlation has been reported between cortical ChAT activity and the degree of dementia in AD as determined by neuropsychological tests (7, 13, 16, 18). The extent of Ch4 neuronal loss has also been shown to be correlated with the degree of dementia (59). A large number of recent investigations have indicated that the distribution and total density of cortical plaques display little relationship with the presence, and particularly the severity, of dementia (60–62), while the density of tangles displays a strong relationship with both the presence and severity of dementia (60, 61, 63). Together with the results of the present study, these findings indicate that tangle formation is a major pathological force in AD that is strongly related to the loss of cortical cholinergic innervation, and that the formation of tangles and cholinergic loss both contribute to the dementia characteristic of AD.

REFERENCES

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PLAQUES, TANGLES AND CHOLINERGIC LOSS IN ALZHEIMER DISEASE


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