CORTICAL CHOLINERGIC FIBERS IN AGING AND ALZHEIMER'S DISEASE: A MORPHOMETRIC STUDY

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Abstract—A histochemical method for acetylcholinesterase was used to assess the regional density of acetylcholinesterase-rich (putatively cholinergic) axons in the cerebral cortex. A dense plexus of these fibers was observed in all cortical areas. The entorhinal, cingulate and inferotemporal regions were used for quantitative analysis. The paralimbic cortical areas (entorhinal and cingulate) contained a higher density of acetylcholinesterase-rich fibers than the association cortex of inferotemporal area TE. The more superficial layers in all three regions contained a denser net of these fibers than did the deep layers. Aging was accompanied by a modest loss in the density of acetylcholinesterase-positive fibers in the entorhinal and inferotemporal cortex but not in the cingulate area. In brains from patients with Alzheimer's disease, a dramatic loss of these fibers was observed in all three areas examined but the loss was relatively less pronounced in the cingulate area.

The results demonstrate that the cholinergic innervation of the cerebral cortex in the human brain displays considerable regional and laminar variations. Regional variations were also observed in the alterations that the cortical cholinergic fibers displayed as a result of normal aging and Alzheimer's disease.

Cholinergic innervation is widely distributed in the cerebral cortex of the mammalian brain and displays marked regional variation. In general, the level of cholinergic markers is highest in limbic and paralimbic areas, lowest in fronto-temporo-parietal association areas and of intermediate intensity in the primary sensorimotor regions. This cholinergic innervation is almost exclusively extrinsic, originating in the cholinergic cells of the basal forebrain.

The effect of age upon the cholinergic innervation of the cerebral cortex has attracted considerable attention. In the rodent, where these changes have been investigated most extensively, acetylcholine (ACh) synthesis and release, choline uptake, choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activity, conduction velocity in corticopetal cholinergic axons and the response of neurons to ACh application have each been shown to display some degree of age-related reduction. Morphological studies have revealed what appears to be a pathological swelling of ChAT-positive axons in the cerebral cortex of aged rats. In the mouse, the cholinergic cells of the basal forebrain show an age-related shrinkage in volume but do not appear to be reduced in number.

The effects of aging on the cortical cholinergic innervation of the human brain have not been investigated as thoroughly. A slight reduction in ChAT and AChE activity has been reported in various regions of the human brain as a consequence of aging. A significant drop in the number of magnocellular nucleus basalis of Meynert (nBM) neurons in the aged human brain has been reported by some investigators, while no change has been observed by others.

Not all reports are in agreement regarding the depletion of cholinergic markers in the course of normal aging. Many investigators have failed to find age-related deficits in a number of species, including humans. One explanation for this apparent inconsistency could be the existence of regional differences which would be difficult to identify when biochemical analysis of brain tissue are carried out without cytoarchitectonic guidance. In the present study, we used a histochemical technique which allows the visualization of AChE-rich (putatively cholinergic) fibers in architectonically distinct subregions of the human cerebral cortex. We used this material to study age-related changes in the regional density of cholinergic fibers. We also compared age-related changes to those observed in Alzheimer's disease (AD), a disease which is known to be accompanied by a marked depletion of cortical cholinergic innervation.
EXPERIMENTAL PROCEDURES

Preparation of human brain tissue

Six brains from normal individuals 22 to 91 years of age with no prior history of dementia and six brains from individuals with a clinical history of dementia of the Alzheimertype were used in this study. The characteristics of the subjects are summarized in Table 1. The interval between death and autopsy varied from 2 to 24 h. The tissue was cut into 1-2-cm 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 7.3) for 24-34 h, then into graded concentrations of sucrose (10-40% in 0.1 M phosphate buffer) and stored at 4°C. The coronal blocks of interest were then sectioned at 40 μm on a freezing microtome into 0.1 M phosphate buffer.

Histology

Representative sections from several cortical and limbic structures were stained with hematoxylin–eosin, Bielschowski's silver method, Thioflavin-S and Cresyl Violet (or Thionin) for neurofibrillary tangles and other pathological changes were observed in the brains of the 22-, 35-, 43- and 76-year-old normal individuals. The brains of 68- and 91-year-old non-demented individuals contained neuritic plaques (in cortical areas) and neurofibrillary tangles (only in limbic areas) with a density and distribution consistent with normal aging. In the brain of the 91-year-old, in addition to the terminal cerebellar hemorrhage, rare cortical microinfarcts were also noted. The brains of the six demented individuals contained numerous plaques and neurofibrillary tangles in all cortical areas. The density and distribution of these lesions were consistent with a definitive diagnosis of AD. Acetylcholinesterase histochemistry

The observations reported in this study are based on the visualization of cholinesterase activity with the help of a new and highly sensitive method. The principles of this method (incubation in a dilute Karnovsky–Roots medium followed by metal diaminobenzidine intensification) have been described by Hakker et al. and Tago et al. We have introduced a number of changes in this method as outlined below. The cholinesterase solution was prepared by adding to 1000 ml distilled water, 17 186 mg maleic acid (disodium salt), 147 mg sodium citrate, 75 mg copper sulfate and 16.4 mg potassium ferricyanide. The pH was adjusted to the value of 8.0 by titrating with drops of glacial acetic acid or 10 N sodium hydroxide. To this solution, 106 mg acetylthiocholine iodide (mol. wt 289.2) or the equimolar amount of 116 mg butyrylthiocholine iodide (mol. wt 317.2) were added in order to demonstrate AChE or butyrylcholinesterase (BChE) activities, respectively. To inhibit BChE, 7.2 mg of ethopropazine (mol. wt 348.9) or 34 mg of iso-OMPA (mol. wt 342.4, Sigma Chemical Company, St Louis, MO) were added when acetylthiocholine was the substrate. The specific AChE inhibitor BW284C51 (mol. wt. 556.4, Wellcome Research Laboratories, Beckenham, U.K.) was added (0.06–0.6 mg/l) to demonstrate the specificity of the AChE staining.

Brain sections were mounted on slides, air dried overnight and rinsed in 0.1 M maleate buffer (at the same pH as the cholinesterase incubation) for 5–10 min. The sections were then incubated in the cholinesterase solution for 2–5 h. At the end of the incubation, the sections were rinsed in a 0.1 M Tris buffer at a pH of 7.6 for 4–10 min. This was followed by an intensification step for 10 min in a solution of 0.5% (w/v) cobalt chloride in the same Tris buffer. The sections were then rinsed for 5–10 min in the Tris buffer and incubated for 3–5 min in a solution containing 50 mg of diaminobenzidine tetrahydrochloride and 3.3 ml of 0.3% (v/v) hydrogen peroxide per 100 ml of the Tris buffer. This was followed by a final rinse in the Tris buffer and the sections were then air-dried, dehydrated in graded alcohols, cleared in xylene and coverslipped under permount.

Evaluation of cholinergic fiber density

For a quantitative analysis of AChE fiber density, 8 × 30–40 in. panels containing full depth cortical photomontages at ×270 magnification (Fig. 1) were prepared of the densest AChE staining within three regions: (1) entorhinal cortex at the level of amygdalohippocampal junction; (2) agranular cingulate cortex, and (3) infratemporal area TE (sensory association cortex). The density of AChE-rich fibers was assessed with a grid drawn directly on the photomontages. The grid consisted of lines drawn at one inch intervals parallel (for determination of vertical fibers) and perpendicular (for determination of horizontal fibers) to the cortical surface. The number of fibers intersecting each line was counted and recorded for different lamina of each cortical area examined. These counts were subjected to analysis of variance with appropriate post-hoc tests to determine significant differences in regional and laminar fiber density. In an earlier report, we described qualitative changes in AChE fiber density in AD as compared with aged individuals using a few of the same cases used in this study (43-, 69- and 91-year-old normal and two 88-year-old AD cases).

RESULTS

Our analysis is based on the regions that appeared to contain the heaviest concentration of AChE-rich fibers and thus were used in the analysis of variance.

Table 1. Cases used for histochemistry

<table>
<thead>
<tr>
<th>Case number</th>
<th>Post-mortem interval (h)</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Hemisphere</th>
<th>Alzheimer's disease</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>F</td>
<td>22</td>
<td>L</td>
<td>-</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>F</td>
<td>35</td>
<td>R</td>
<td>-</td>
<td>Rheumatic heart disease</td>
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<tr>
<td>3</td>
<td>17</td>
<td>F</td>
<td>43</td>
<td>L</td>
<td>-</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>F</td>
<td>69</td>
<td>R</td>
<td>-</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>M</td>
<td>76</td>
<td>L</td>
<td>-</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>M</td>
<td>91</td>
<td>R</td>
<td>+</td>
<td>Cerebellar hemorrhage</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>F</td>
<td>86</td>
<td>R</td>
<td>+</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>M</td>
<td>87</td>
<td>L</td>
<td>+</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>F</td>
<td>88</td>
<td>R</td>
<td>+</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>F</td>
<td>88</td>
<td>L</td>
<td>+</td>
<td>Pneumonia</td>
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<tr>
<td>12</td>
<td>12</td>
<td>M</td>
<td>89</td>
<td>R</td>
<td>+</td>
<td>Cardiorespiratory arrest</td>
</tr>
</tbody>
</table>
fibers within each of the three architectonic regions under investigation. The results, therefore, most specifically address the "peak density" of cholinergic innervation in these regions. AChE fiber staining in layer 1 was inconsistent and difficult to interpret and therefore not included in the analysis. In the entorhinal cortex, layer 2 was identified by the presence of stellate cell islands in matching sections stained for Nissl substance, layer 3 by its position between layer 2 and the lamina dissecans, and the deep layers (5 and 6) by their location between the lamina dissecans and the white matter. In the cingulate region, we analysed the AChE-rich fiber density in a "superficial" region marked by the presence of small pyramidal neurons and a "deep" region marked by the presence of larger pyramidal neurons. The fiber density in area TE was analysed in layer 2 (the external granular lamina), layer 3 (the pyramidal layer lying between layer 2 and the internal granular layer 4) and layers 5–6 (the infragranular layers).

In all cortical areas, the histochemical staining revealed a dense plexus of AChE-rich axons as well as a variable density of AChE-rich cells (Figs 3–5). The specificity of the AChE reaction was demonstrated by the absence of reaction-product in these structures when butyrylthiocholine iodide was substituted for acetylthiocholine iodide in the incubation solution. The AChE reaction was consistently and reliably inhibited by $10^{-4}$–$10^{-5}$ M of the specific AChE inhibitor BW 284C51 while the specific BChE inhibitor iso-OMPA had no effect on such staining even at a concentration as high as $10^{-4}$ M.

### Normal distribution of cortical acetylcholinesterase-rich fibers

In the group of non-demented individuals, within-groups analysis of variance with adjustment for unequal sample sizes, showed significant differences in the density of vertical and horizontal fibers (Table 2) for variables of area, layer and subject ($P < 0.0001$). The probability values referred to hereafter are for the Newman–Keuls post-hoc test.

The entorhinal cortex contained the highest overall density of AChE-rich fibers (Fig. 3) followed by cingulate cortex (Fig. 4) and inferotemporal cortex (Figs 2, 5 and Table 2). This difference was statistically significant ($P < 0.001$). In layer 1 of all three areas (when staining was observed), fibers travelled horizontal to the cortical surface. In other laminae, slightly higher numbers of vertical fibers were encountered as compared with horizontal fibers. The difference between the density of horizontal and vertical fibers was more pronounced in the entorhinal and cingulate cortex than in area TE (Table 2). Excluding layer 1, which was not included in the quantitative analysis, fiber density was higher in the superficial layers than in the deeper layers. For example, in the entorhinal cortex, layer 2 (stellate cell layer) contained the densest net of AChE-rich fibers followed by layer 3 and the deeper layers ($P < 0.001$).

### Table 2: Interictal data for vertical and horizontal cortical acetylcholinesterase-rich fibers

<table>
<thead>
<tr>
<th>Orientation</th>
<th>Layer 1</th>
<th>Layer 2</th>
<th>Layer 3</th>
<th>Layer 4</th>
<th>Layer 5</th>
<th>Layer 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vertical</td>
<td>10.1 ± 3.5</td>
<td>8.6 ± 3.3</td>
<td>7.8 ± 3.2</td>
<td>7.0 ± 2.4</td>
<td>6.3 ± 1.8</td>
<td>6.1 ± 1.4</td>
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<tr>
<td>Horizontal</td>
<td>9.6 ± 4.1</td>
<td>8.5 ± 3.1</td>
<td>7.8 ± 2.6</td>
<td>7.7 ± 2.4</td>
<td>7.4 ± 1.9</td>
<td>7.2 ± 1.7</td>
</tr>
</tbody>
</table>

Each point represents the mean ± standard deviation of the number of fibers in a 200-mm-long horizontal slice drawn vertical or horizontal to the cortical surface. Each point represents data from at least six different rats, N.A., not available.
Fig. 1. Full cortical depth photomontage of AChE-rich fibers in the inferotemporal cortex (area TE) of the 22- (A), 43- (B) and 91-year-old (C) normal subjects. Approximate laminar boundaries are shown in the figure. Note the higher density of AChE-rich fibers in the more superficial layers as compared with the deep layers. This area displayed the largest age-related loss of AChE-rich fibers observed in this study. In addition to AChE-rich fibers, many AChE-rich cells can also be observed, particularly in layers 3 and 5. Arrow in C points to an AChE-positive plaque in the brain of the 91-year-old individual. Scale bar = 200 μm. Magnification × 137.

Fig. 2. Tracings of AChE-rich fibers in the entorhinal cortex (A), cingulate cortex (B) and inferotemporal cortex (area TE) (C) of the 22-year-old normal subject. Approximate laminar boundaries are shown in the figure. The tracings were made from full depth photomontages. The entorhinal cortex (A) displayed the highest density of AChE-rich fibers, followed by the cingulate cortex (B) and the inferotemporal cortex (C), respectively. Note the higher density of these fibers in superficial layers as compared with the deep layers. Scale bar = 200 μm. Magnification × 107.
Fig. 3. Photomicrographs of AChE-rich fibers in layer 3 of entorhinal cortex of the 22- (A), 43- (B) and 91-year-old (C) normal subjects and of an 89-year-old patient with Alzheimer's disease (D). This area displayed the densest AChE-rich fiber staining observed in this study. Note the modest age-related decrease in the density of these fibers in the normative sample (A-C) and the drastic decline in the AD brain (D). Magnification × 370. Cortical surface is on the top and white matter towards the bottom.
Fig. 4. Photomicrographs of AChE-rich fibers in the superficial layer of the cingulate cortex of the 35- (A), 69- (B) and 91-year-old (C) normal subjects and of an 89-year-old patient with Alzheimer's disease (D). Note that the density of the AChE-rich fibers in this area is well preserved, even in the 91-year-old (C). Although the cingulate cortex in the AD brain (D) displays a drastic reduction in the density of these fibers when compared to normal (C), the extent of the reduction is less than in entorhinal and inferotemporal cortex. Magnification × 370. Cortical surface is on the top and white matter towards the bottom.
Fig. 5. Photomicrographs of AChE-rich fibers in layer 3 of inferotemporal cortex (area TE) of the 43- (A), 76- (B) and 91-year-old (C) normal subjects and of an 89-year-old patient with Alzheimer's disease (D). This area displayed the lowest density of AChE-rich fibers among the three areas studied. The age-related (compare A and C) and AD-related (compare C and D) loss was more pronounced in this area than in the entorhinal and cingulate cortex. Magnification × 370. Cortical surface is on the top and white matter towards the bottom.
In the cingulate cortex, the superficial layer contained a denser net of AChE-rich fibers than the deep layer (P < 0.0025). The inferotemporal cortex contained the densest AChE-rich fiber pattern in layer 2 with decreasing density in layers 3 and 5–6 (P < 0.001). In addition, no laminar differences in the density of AChE-rich fibers were observed. Differences could also be observed in the thickness of these fibers. In general, fibers in the deep layers appeared thicker and showed higher staining intensity (Fig. 1).

The histochemical method used in this study also stained many cortical AChE-rich cells. The majority of these cells were pyramidal in shape and were most frequently noted in layers 3 and 5. Regional and laminar distributions of these AChE-rich cortical cells have been described elsewhere. 

**Age-related changes in the distribution of cortical acetylcholinesterase-rich fibers in non-demented subjects**

With advancing age, a modest but progressive decline in the density of AChE-rich fibers was observed in the three cortical areas examined (Table 2 and Figs 1, 3–5). When the 22-year-old brain was compared with that of the 91-year-old, the magnitude of fiber loss was 31% in the inferotemporal cortex, 23% in the entorhinal cortex and 13% in the cingulate cortex for vertical fibers and 34% in the cingulate cortex for horizontal fibers.

The inferotemporal cortex displayed the greatest age-related fiber loss (Fig. 1, Table 3), followed by the entorhinal cortex and cingulate cortex, respectively. In addition, in all regions, horizontal AChE-rich fibers displayed a greater age-related loss than did vertical fibers (Table 2). Analysis of variance applied to the combined vertical and horizontal fibers for all layers in the young group (three youngest subjects) and the old group (three oldest subjects) revealed an age by region interaction (P < 0.0001). The group of older non-demented subjects had 20% fewer AChE-rich fibers in area TE and 17% fewer in entorhinal cortex when compared to the group of young subjects. These differences were significant (Newman-Keuls, P < 0.001). In the cingulate cortex, the age-related difference in AChE-rich fibers (11%) did not reach significance (Table 3).

The entorhinal cortex was the only area in which the density of AChE-rich fibers decreased consistently in all subjects with advancing age (Table 2). Although age-related loss of AChE-rich fibers was greater in inferotemporal cortex than in the other areas (Fig. 1), there was also a great deal of individual variability. Thus, the brain of the 76-year-old (Fig. 3B) displayed higher density of AChE-rich fibers than the brains of the 68- and the 43-year-old (Fig. 3A) subjects. Likewise, denser AChE-rich fibers were observed in this area of the brain of the 35-year-old when compared with the 22-year-old (Table 2).

In addition to regional differences in age-related AChE-rich fiber loss, laminar differences were also observed. In the entorhinal cortex and the inferotemporal cortex, age-related loss of AChE-rich fibers was greater (P < 0.01) in the superficial layers (L2 and L3, Fig. 1) than in the deep layers (L5 and L6). In the cingulate cortex, however, no significant differences were found between the age-related fiber loss in the deep and superficial layers.

**Loss of cortical acetylcholinesterase-rich fibers in Alzheimer's disease**

In the AD brains, the quantitative analysis was confined to layer 3 of entorhinal and inferotemporal cortex and to the superficial region of the cingulate area. A dramatic loss of AChE-rich fibers was observed in all six AD specimens (Figs 3–5). This loss was greatest in the inferotemporal cortex, followed by entorhinal cortex and cingulate cortex, respectively. The AD-related AChE-rich fiber loss was in the order of 90% in the inferotemporal cortex, 78% in the entorhinal cortex and 59% in the cingulate cortex when compared to the group of normal old subjects (P < 0.0001).

**DISCUSSION**

Our intersect analysis of AChE-rich (putatively cholinergic) axonal density in the human brain revealed three major findings: (1) the existence of regional and laminar variations in the density of cortical cholinergic fibers; (2) a modest age-related decline in this innervation; and (3) a dramatic and regionally selective decrement of cholinergic innervation in AD.

The density of the AChE-rich axons in the normative brains was higher in the entorhinal cortex and cingulate cortex than in the inferotemporal cortex. In all three areas, the superficial layers displayed a denser net of these fibers than the deep layers. Similar...
patterns in the laminar distribution of biochemically
determined ChAT and AChE activity have been
reported in the frontal (area 9) and midtemporal
cortex of the human brain. Our observations of
greater density of cholinergic (AChE-rich) fibers in
paralimbic areas (i.e. entorhinal and cingulate cortex)
as compared with sensory association cortex (i.e.
area TE) is in keeping with previous findings in the
cerebral cortex of the monkey brain. Similar
observations have been made in the cat and the rat.

The age-related loss of AChE-rich fibers that we
have observed is consistent with the results of several
experiments showing an age-dependent decrease in
cholinergic markers in many cortical areas. For
example, biochemical studies by DeKosky et al.
demonstrated an age-dependent decrease in ChAT
activity in all laminae of the frontal cortex (area 9)
in man. These investigators, however, were unable
to detect significant age-related decrements in AChE
activity in this same area. The reason for the latter
finding is most likely the presence of numerous
AChE-positive, presumably cholinceptive cells in
the cortex. A biochemical method would not dis-
tinguish between the AChE which belongs to cortical
cholinergic fibers and that belonging to the cortical
AChE-positive cells.

It is important to emphasize that the age-related
decline of AChE-rich fibers was quite modest. In the
three regions that we studied, the change was signifi-
cant in entorhinal and inferotemporal association
cortex but not in the cingulate region. Even in the
entorhinal and inferotemporal areas, the putative
age-related difference (from the second to the ninth
decade) was in the order of 20%. This contrast with
the dramatic decline (up to 90%) we noted in the
aging and AD brains from the AD patients. The decline in the
density of AChE-rich fibers in aging and AD was of
greatest magnitude in the inferotemporal cortex and
least pronounced in the cingulate cortex.

Our findings of greater AChE-rich fiber loss in
temporal lobe structures (i.e. inferotemporal and
entorhinal cortex) in AD is consistent with prior
neurochemical reports showing a greater loss of
cholinergic markers in the temporal lobe than else-
where in the AD brain. Conceivably, a similar
phenomenon of selective vulnerability may exist in
the course of normal aging.

The age-related decline of AChE-rich fibers was
consistent and appeared linear in entorhinal cortex
but not in area TE. In the latter region, the 35-year-
old subject displayed a higher density of AChE-rich
fibers than did the 22-year-old, and the 76-year-old
subject had a higher density of these fibers than did the
43- and the 68-year-old. This contrasts with
observations of greater density of AChE-rich fibers
in aging and AD was of
time in keeping with previous findings in the
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phenomenon of selective vulnerability may exist in
the course of normal aging.

These considerations support our contentention that
the variations of AChE-rich fibers that we have
observed most likely reflect variations in the cortical
cholinergic innervation arising from the nucleus bas-
alis and associated cell groups (the Ch4 complex).
Whether this loss of cortical cholinergic innervation
in the elderly is accompanied by a parallel loss in the
density and size of Ch4 neurons remains to be deter-
mined. In comparison, the dramatic loss of cortical
cholinergic innervation in AD is consistently associ-
ated with a depletion of cell number, volume and
AChE activity in the nucleus basalis.

Modest changes in learning and memory capacity
have been reported with advanced age. There is
now a large body of evidence demonstrating the influ-
ence of the cholinergic system on learning and mem-
ory in man and animals. It is therefore possible that the modest loss of cortical cholinergic
innervation in the elderly observed in this study is,
least in part, responsible for the cognitive deficits
observed in these individuals.

The most characteristic feature of AD is a severe
and incapacitating amnesia. The dramatic patho-
logical changes in limbic structures (e.g. cell loss,
neuritic plaques, neurofibrillary tangles) constitute
some of the major anatomical correlates for this
amnesia. The loss of cortical cholinergic innervation
may provide an additional substrate for this memory
loss and perhaps also for the other behavioral
changes seen in AD. Although it is customary to
emphasize the apparent continuity between aging and
AD, we want to emphasize how modest the changes
associated with advanced senescence are when com-
pared to the near-total depletion that occurs in
comparable regions of the brain in patients with AD.
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