Neuron Number in the Entorhinal Cortex and CA1 in Preclinical Alzheimer Disease

Joseph L. Price, DPhil; Andy I. Ko, BA; Marcus J. Wade, BA; Sarah K. Tsou; Daniel W. McKeel, MD; John C. Morris, MD

Objectives: To determine whether nondemented subjects with pathological evidence of preclinical Alzheimer disease (AD) demonstrate neuronal loss in the entorhinal cortex and hippocampus, and whether the onset of cognitive deficits in AD coincides with the onset of neuronal degeneration.

Methods: Preclinical AD cases have been defined by the absence of cognitive decline but with neuropathological evidence of AD. The hippocampus and entorhinal cortex were examined in 13 nondemented cases (Clinical Dementia Rating [CDR] 0) with healthy brains, 4 cases with preclinical AD, 8 cases with very mild symptomatic AD (CDR 0.5), and 4 cases with severe AD (CDR 3, hippocampus only). The volume and number of neurons were determined stereologically in 2 areas that are vulnerable to AD—the entorhinal cortex (as a whole and layer II alone) and hippocampal field CA1.

Results: There was no significant decrease in neuron number or volume with age in the healthy nondemented group and little or none between the healthy and preclinical AD groups. Substantial decreases were found in the very mild AD group in neuron number (35% in the entorhinal cortex, 50% in layer II, and 46% in CA1) and volume (28% in the entorhinal cortex, 21% in layer II, and 29% in CA1). Greater decrements were observed in CA1 in the severe AD group.

Conclusions: There is little or no neuronal loss in aging or preclinical AD but substantial loss in very mild AD. The findings indicate that AD results in clinical deficits only when it produces significant neuronal loss.

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Several clinicopathologic studies of older adults with slight cognitive decline before death, just at the threshold for clinical detection, demonstrated large numbers of neurofibrillary tangles and amyloid plaques, sufficient for the diagnosis of Alzheimer disease (AD). Reasoning that these pathological lesions develop over time, the pathobiological processes that underlie AD must begin in a preclinical stage that precedes clinically detectable cognitive change, probably by years. This preclinical AD stage would be a critical target for therapeutic intervention.

As used herein, "preclinical AD" indicates a stage in which there is no impairment in memory or other cognitive functions. Although this term has been used in other studies for slight cognitive decline before dementia, results of careful clinicopathologic studies indicate that such decline, in the absence of known explanation, consistently indicates a neuropathological disorder, usually AD. Indeed, subtle cognitive decline before dementia diagnosis often includes deficits in multiple cognitive domains that are sufficient to cause functional interference.

A previous study by 2 of us (J.L.P. and J.C.M.) reported cases of preclinical AD that did not have any clinical indication of cognitive decline (Clinical Dementia Rating [CDR] 0) but had substantial pathological changes indicative of AD. These cases were defined specifically by the presence of neuritic plaques, but they also had high numbers of total senile plaques and neurofibrillary tangles comparable to the density of these markers in very mild AD cases. Preclinical cases represent the earliest definable stage of AD, between healthy aging and clinically detectable AD. Subsequent analyses of longitudinal psychometric assessments from preclinical AD cases confirmed that they were free from subtle cognitive decline.

Several studies of neuronal number in the hippocampus and entorhinal cortex have shown that there is essen-
MATERIALS AND METHODS

The brains used in this study were taken from a previous clinico-pathological series8 and included all cases that had sufficient sections through the structures to be counted. The cognitive status of most subjects (including all of the preclinical AD cases) was assessed within a year of death using the CDR as part of a longitudinal study.21,22 These cases were supplemented with others that were assessed by a validated postmortem interview with an informant who knew the subject well.23 Twenty-two brains were available with sections through the entorhinal cortex that were rated CDR 0 (non-demented) or 0.5 (very mild AD) (Table 1). Cases assessed as CDR 0/0.5 (reflecting a less certain stage of CDR 0.5) have been shown in previous studies to have no pathological distinction from the CDR 0.5 group8 and were included in the very mild AD group in this study. The CDR 0 cases had no indication of any cognitive impairment or decline. An overlapping group of 29 brains rated CDR 0, 0.5, or 3 (severe AD) was available with sections through hippocampal field CA1 (Table 1). Frozen sections were cut at 50 mm from 1-cm-thick coronal blocks. Series of 1 in 22 sections were stained using the Bielschowsky silver method, immunohistochemical stains for β-amyloid and paired helical filaments, and the Nissl method. The Nissl-stained sections were used in this study for volume measurements and cell counts. The Bielschowsky and immunohistochemical stains were used in the previous study6 to analyze neurofibrillary tangles and amyloid plaques.

Cases were divided into healthy nondemented brains (CDR 0), preclinical AD brains (CDR 0), and very mild AD brains (CDR 0/0.5 or 0.5) (Table 1). Because the extreme cell loss in severe AD was documented previously in the entorhinal cortex16 but not in CA1, a fourth group of severe AD (CDR 3) cases was added for CA1 (Table 1). The criteria for the groups were the same as in the previous study.6 Healthy nondemented brains were rated CDR 0 and had either no amyloid plaques or cortical patches of diffuse plaques only. These brains also had a variable number of tangles, especially in the entorhinal and perirhinal cortex.6 Preclinical AD brains were defined as CDR 0 cases that had neuritic and diffuse plaques widely distributed throughout the neocortex; when so defined, the cases also had substantial numbers of tangles.6 In the entorhinal cortex analysis, there was no significant difference in age between the healthy nondemented and preclinical AD groups, but the very mild AD cases were significantly older. There was no significant difference in age between the groups analyzed for CA1.

All cases were assessed using 4 pathological standards for AD: (1) a modified version of the Khachaturian criteria,24,25 which has been used in other studies from the Alzheimer Disease Research Center (ADRC) of Washington University, St Louis, Mo4,12,22; (2) the Braak stages for neurofibrillary and amyloid change26; (3) the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) neuropathological criteria27; and (4) the National Institute on Aging/Reagan Institute criteria for likeliness of AD28 (Table 1). Preclinical AD and very mild AD cases were rated similarly, although more of the very mild AD cases were rated as “probable AD” by the CERAD criteria because of their positive clinical history of dementia. Other aspects of some of the cases studied here have been analyzed in previous studies.1,2,4-8,15,16,22

Two separate analyses of the entorhinal cortex, as a whole and layer II alone, were done by 2 different examiners (A.I.K. and M.J.W., respectively). The boundaries of the entorhinal cortex were delineated by the sharply defined layer II, often grouped into cell islands, and the lamina

RESULTS

ENTORHINAL CORTEX

Correlational analysis of the relation between age and neuron number in the healthy nondemented group indicated that there was a nonsignificant 0.7% per year decrease in total numbers of neurons with age (slope = −85,000 neurons per year, r = −.04, P = .12) (Figure 1A). A similar but significant 1% per year decrease in the volume of the entorhinal cortex was also found with age (slope = −12.7 mm^3 per year, r = −.69, P = .01).

Because layer II of the entorhinal cortex previously has been shown to be among the most severely affected structures in AD,8,16,26 it was analyzed separately. The correlation with age again indicated nonsignificant decreases with age in layer II neuron number (slope = −16,000 neurons or 1.2% per year, r = −.45, P = .15) (Figure 1B) and tissue volume (slope = −0.4 mm^3 or 0.4% per year, r = −.27, P = .4).

For all measures, preclinical AD cases were intermixed with healthy nondemented cases (Figure 1). The mean volume and number of neurons in the entorhinal cortex also showed essentially no difference between the preclinical AD and healthy nondemented groups in either the whole cortex or layer II (P = .71–.97 for all comparisons) (Table 2 and Figure 2). In contrast, most very mild AD cases had fewer neurons and smaller volume than most healthy nondemented or preclinical AD cases (Figure 1). The mean volume and number of neurons were substantially decreased in the very mild AD group (by 21% to 50%) (Table 2 and Figure 2). All the decreases were significant (P = .004 for neuron number and P = .01 for volume in the whole entorhinal cortex, and P < .02 for neuron number in layer II), except that in volume of layer II, which showed a strong trend toward significance (P = .055). When age was inserted in the ANOVA as a covariant, the difference in neuronal num-

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dissected deep to layer III, using the criteria of Amaral and Insausti. Layer II was distinguished by its greater cellular density compared with layer III and included the cell islands and intervening cells.

A third investigator (S.K.T.) measured hippocampal field CA1. Because of tissue demands for diagnostic and other purposes, sections through the entire extent of CA1 were not available. A counting region was defined in the middle portion of CA1, bounded rostrally where field CA2/3 was first distinguished by its narrower pyramidal cell layer and caudally by the posterior edge of the entorhinal cortex. The values therefore do not represent the total volume or neuronal number in CA1. The boundaries of CA1 with CA2/3 and the subiculum were demarcated as defined by Amaral and Insausti.

All counts were done on coded slides, with investigators masked to the age, cognitive status, and neuropathological diagnosis of the case. Measurements were made using a stereological system (C.A.S.T.-Grid; Olympus, Albertslund, Denmark), which includes a computer-linked microscope with a motorized stage for automated selection of counting fields and a z-axis sensor to determine depth within the section. A video camera on the microscope and interfaced to the computer allowed accurate marking of cells within the counting volume. A regularly spaced series of 10 to 12 sections through the entire rostrocaudal extent of the target structure was selected for random, systematic sampling, beginning randomly with the first section available. The entorhinal cortex, layer II, or CA1 was outlined on each section, and the Cavalieri principle was used to determine the overall area. The total reference volume for each structure was determined as the sum of areas of the counted sections divided by the number of sections counted and then multiplied by the total number of sections through the entorhinal cortex and the distance between sections (1100 μm). Because tissue necessarily was lost between blocks, 1 to 3 sections were added for each transition between blocks. These extra sections slightly increased the absolute number of cells but had little effect on relative differences between groups.

The computer set counting fields that were regularly spaced but randomly positioned within the outlined area. Approximately 100 to 150 fields were counted for each brain. Cell numbers were estimated using the optical dissector method. The actual thickness of the sections on the slide was measured and an optical dissector depth was set to that thickness minus 1-mm guard volumes on top and bottom. Because the 50-mm frozen sections collapsed during dehydration, the measured section thickness was typically 10 to 12 mm and the dissector depth was 8 to 9 mm. Neurons were distinguished from glia by their stained cytoplasm and nucleolus and their generally larger size and nonspherical shape. At least 100 neurons were counted for each brain. The total number of neurons counted was divided by the volume sampled (number of counting fields × shrinkage factor × dissector depth × counting field area) to calculate volumetric cell density. Shrinkage factor is the thickness at which the sections were cut (50 mm) divided by the measured section thickness. Cell density was then multiplied by the calculated reference volume of the entire structure to give the total number of cells.

Data were analyzed across the 3 groups using 1-way analysis of variance (ANOVA). Subsequently, post hoc analysis of differences between groups was done using the Tukey “honestly significant difference” test for groups with unequal numbers (Spjotvoll/Stonine test), which corrects for multiple comparisons. Because of possible age effects, the ANOVAs were repeated with age as a covariant. The Spearman rank order correlation (r) was also calculated for the relation between age and volume or neuron number in the healthy nondemented brain group.

There was considerable variance in the neuronal count in hippocampal field CA1 within each group, probably owing to the difficulty in identifying the boundaries of CA1. There was one outlying case in each of the healthy nondemented, very mild AD, and severe AD groups that deviated from the mean by 2 SDs or more (Figure 3A). To reduce the variance and allow comparisons between groups, these outliers were eliminated from further analyses and from Table 1.

Correlational analysis indicated no change in the number of neurons in CA1 with age within the healthy nondemented group (slope=0) (Figure 3B). Individual preclinical AD cases were distributed among healthy nondemented cases, whereas the very mild and severe AD cases had fewer neurons than most nondemented cases.

The AD-related changes in CA1 were similar to those in the entorhinal cortex. The mean number of neurons in preclinical AD cases decreased by only 11% compared with the healthy nondemented group (Table 3 and Figure 3C) (P=.95). On the other hand, very mild and severe AD cases decreased by 46% and 65%, respectively. These decreases were significant (P<.02 for both), even when age was covaried out (P=.02 for both). The small number of cases limited the power for statistical comparison, but power analysis indicated that a decrease in the preclinical AD group would have been detected if it approached that in very mild AD (Table 3).

Although the total volume of hippocampal field CA1 was not determined (see the “Materials and Methods” section), the analyzed portion of CA1 showed volume changes similar to those in the entorhinal cortex. There was virtually no change (+6%) in the average volume of the preclinical AD group compared with the healthy nondemented group (P=.95). In contrast, vol-

HIPPOCAMPAL FIELD CA1

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ume decreased by 29% and 38% in the very mild and severe AD groups, respectively. ANOVA indicated that there was a significant variation among the groups \((P = .047)\), but results of post hoc analysis with the stringent Tukey honestly significant difference test for unequal groups did not indicate significant differences between specific groups \((P = .3)\). If age was used as a covariant, there was only a trend toward significance among groups \((P = .06)\).

**COMMENT**

The main finding of this study is that preclinical AD cases resemble healthy nondemented cases in the volume and number of neurons in the entorhinal cortex and hippocampal field CA1. Preclinical AD cases do not show the decreases that are seen in clinically detectable, very mild AD. Although preclinical AD cases have substantial numbers of plaques and tangles, indicating the beginning of the pathobiological process underlying AD, the disease has not yet progressed to the point of producing identifiable neuronal degeneration.

The observations also confirm the results of previous studies\(^6,^{19,31}\) that there is little or no loss of neurons in the entorhinal cortex or CA1 during healthy aging. A slight, nonsignificant decrease in neurons with age was found in the entorhinal cortex but not in CA1. Absent a neurodegenerative condition such as AD, aging is associated with little if any neuronal loss.

The 35% loss of neurons in the entorhinal cortex and 50% loss in layer II in very mild AD cases agrees closely with results of previous studies. Gomez-Isla et al\(^8\) reported that the number of neurons decreased in very mild AD by 32% in the whole entorhinal cortex and 57% in layer II. More recently, Kordower et al\(^10\) reported similar losses in layer II of 64% and 58% in MCI and “mild to moderate” AD, respectively. They also reported 26.5% loss in volume of layer II in MCI.

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**Table 1. Summary of Cases***

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*CDR indicates Clinical Dementia Rating; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; AD, Alzheimer disease; NIA, National Institute on Aging; and EC, entorhinal cortex.
†The NIA/Reagan Institute likelihood was indicated as 2 levels (eg, low/intermediate) when there was disagreement between the neurofibrillary and plaque criteria.
‡Plaque and tangle densities in this case were slightly below the modified Khachaturian criteria for AD.
which corresponds well to the 21% decrease reported herein. Cases of MCI are generally rated CDR 0.5,32 and the Mini-Mental State Examination scores reported by Kordower et al20 for MCI are comparable to those recently reported by Morris et al 14 for CDR 0.5 (MCI) cases. That same study 14 showed that MCI generally represents very mild AD.

In contrast to the relative decreases, the absolute number of cells counted in the healthy entorhinal cortex in this study was higher than in the other studies.16,20 In a third study, West and Slomianka initially reported31 numbers close to the present results but later reported33 a technical correction that reduced their values by 0.6, resulting in values close to those of the other studies. Because the studies all used similar stereological counting methods, it is difficult to explain the differences. It is possible that an unrecognized technical correction should be made in numbers obtained in this study, as in the study by West and Slomianka.31 Such a correction, however, would not change relative values between groups or the primary conclusion that there is substantial neuronal loss in very mild AD but little or none in aging or preclinical AD.

The definition and pathological analysis of preclinical AD cases were previously described in detail,8 but the major points are reviewed herein. Very mild AD cases consistently have extensive diffuse and neuritic amyloid plaques throughout the neocortex and neurofibrillary tangles in and around the hippocampus and meet pathological criteria for AD.1-8 Because these cases are at the threshold for detection of cognitive decline, 4,34 and because the pathological lesions develop over some time, the disease process must begin before it can be detected clinically. Preclinical AD cases resemble very mild AD cases pathologically but do not have cognitive impairment or decline.

Virtually all healthy nondemented cases have neurofibrillary tangles,8,35 and these increase exponentially with age, overlapping with very mild AD cases.3,8 Although tangles clearly are pathological, the pattern of tangle development resembles an age-related process. By themselves tangles do not provide a criterion that could be used to distinguish individual preclinical AD cases from healthy aging cases8 (see also Schmitt et al36). In con-

Table 2. Neuron Number and Volume in the Entorhinal Cortex*

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<tr>
<th>Group</th>
<th>Cases, No.</th>
<th>Neuron No., Millions (% Change)</th>
<th>Volume, mm³ (% Change)</th>
<th>Neuron No., Millions (% Change)</th>
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<td>Healthy nondemented</td>
<td>12</td>
<td>11.85 ± 0.61 (−9)</td>
<td>1211 ± 56</td>
<td>1.29 ± 0.14 (−9)</td>
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<td>1.41 ± 0.16 (−9)</td>
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<td>6</td>
<td>7.65 ± 0.79 (−35)</td>
<td>864 ± 721 (−29)</td>
<td>0.65 ± 0.06 (−50)</td>
<td>78 ± 6 (−21)</td>
</tr>
</tbody>
</table>

*Data are given as mean ± SE. AD indicates Alzheimer disease. For the preclinical AD group, power analysis indicated that the statistically detectable change from the healthy nondemented group would be 26% in neuron number and 30% in volume for the whole cortex and 39% in neuron number and 17% in volume for layer II at power = 0.8 and α = .05.
†P < .05 (.004-.017) vs the healthy nondemented group.
‡P = .055 vs the healthy nondemented group.

Figure 1. Relation between age and neuron number in the whole entorhinal cortex (A) and layer II only (B) in individual cases. The line of regression lines relate age to neuron number in healthy nondemented cases. Although the lines have a slight downward slope, neither correlation is significant. AD indicates Alzheimer disease.

Figure 2. The average number of neurons (A) and tissue volume (B) in the entorhinal cortex as a whole and in layer II alone in the healthy nondemented, preclinical Alzheimer disease (AD), and very mild AD groups. There was no significant difference between the first 2 groups, but the very mild AD group had significantly fewer neurons than all the other groups. Asterisk indicates P < .05 vs the healthy nondemented group; dagger, P = .055 vs the healthy nondemented group. Error bars represent SE.
trast, plaques are not found in all healthy nondemented cases. Many cases have no plaques, and others have only diffuse plaques, but a few cases with no cognitive impairment have neuritic and many diffuse plaques widely distributed throughout the neocortex. When identified by the presence of neuritic plaques, these cases approach the very mild AD cases in tangle and plaque density and generally meet pathological criteria for AD. These cases therefore satisfy strict criteria for preclinical (presymptomatic) AD.

When effective disease-modifying therapies for AD become available, the preclinical stage as defined herein would be the ideal period for treatment initiation because it precedes the stage of significant neuronal death. At present, however, preclinical AD cannot be detected during life. By definition, it cannot be detected even with sensitive clinical assessment. Structural magnetic resonance imaging also might be insufficient because significant volume loss was not found in the entorhinal cortex or hippocampus in preclinical AD cases. Magnetic resonance imaging studies reporting volume reductions very early in AD either found that the volume changes coincided with memory decline or included cases with MCI in their “preclinical” group, indicating that the subjects were symptomatic and not strictly preclinical. Functional imaging might be better able to recognize AD before structural and cognitive decline, although a method to detect β-amyloid in vivo might best diagnose preclinical AD.

Cross-sectional psychometric analyses do not identify reliably the earliest stages of AD in individuals because the range of normal performance is greater than the decline in very mild AD. Longitudinal analysis can detect decline in individuals but is not more sensitive than careful clinical assessment. A recent study reported longitudinal psychometric data from 24 cases analyzed in the present study. None of the individual preclinical AD cases declined in psychometric performance with time;
their mean performance was close to that of the healthy non-demented group. The very mild AD group, on the other hand, consistently showed a decline in psycho-metric performance, and their mean performance was significantly below that of the healthy non-demented and preclinical AD groups.

Our data indicate that the onset of cognitive decline correlates closely with the onset of neuronal loss in the hippocampus and entorhinal cortex, 2 areas that are particularly critical for memory processing. Of course, this correlation does not mean that neuronal death is more important than other causes of neuronal dysfunction, such as synaptic loss. It would be expected that synaptic dysfunction would precede neuronal death, but, within the resolution of available data, neuron loss in the hippocampus and entorhinal cortex and identifiable cognitive decline occur at the same stage of AD.

These and previous results support a model in which AD has a subtle transition from healthy aging, beginning before identifiable cognitive loss (Table 4). In this model, neurofibrillary changes occur in everyone during aging but affect relatively few neurons at younger than 85 to 90 years. Although tangles and other neurofibrillary changes undoubtedly indicate a degenerative process, age-related neurofibrillary change is insufficient to cause significant cellular loss or cognitive impairment. If substantial amyloid plaques develop, however, the rate and amount of neurofibrillary change increases during the preclinical stage of AD. There is insufficient neuronal degeneration to produce detectable cognitive impairment, but autopsied cases in this stage generally meet pathological criteria for AD. Further amyloid deposition and accelerated neurofibrillary change result in significant neuronal dysfunction and death and associated MCI, which characterizes the earliest clinically detectable stage of AD.

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