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# Hippocampal neurons in pre-clinical Alzheimer's disease

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**Abstract**

In a previous study of hippocampal neurons in aging and AD [Lancet 344 (1994) 769], we demonstrated that the loss of neurons in the CA1 region was disease-specific and not related to aging. In the present study, we examined for loss of hippocampal neurons in preclinical AD, a period during which there are abundant amyloid deposits in the brain but no evidence of cognitive decline. We examined the postmortem brains of 33 subjects from the Baltimore Longitudinal Study of Aging and the Johns Hopkins Alzheimer's Disease Research Center. Using unbiased stereology, we estimated the total number of neurons in the granule cell layer, hilus, CA3-2, CA1, and subiculum of AD ($n = 14$), preclinical AD ($n = 8$), and age-matched control subjects ($n = 11$). The results from the present study confirm our previous finding of significant neuronal losses in the CA1 (48%), hilus (14%), and subiculum (24%) in AD [Lancet 344 (1994) 769]. However, we did not observe a significant loss of neurons in CA1 or any of the other subdivisions of the hippocampus in preclinical AD.


*Keywords:* Hippocampal neurons; Subiculum; Alzheimer's disease (AD)

## 1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized clinically by dementia and neurobehavioral deterioration [9,24]. In demented subjects with AD, the pathological hallmarks are Aβ amyloid deposits, senile plaques (SP), neurofibrillary tangles (NFT), degeneration of synapses [34], and loss of neurons [10,26,39]. However, not all of these pathological changes have been characterized before the onset of cognitive decline, i.e., in the preclinical stages of AD. The development of NFT and SP has been described in preclinical AD [25], but there is only a single stereological study of the number of neurons in the hippocampus in preclinical AD [26]. The validity of the concept of a preclinical stage in AD is supported by longitudinal clinical studies of mild memory changes followed by an accelerated decline [6,15,19,33] and by clinical-pathological studies that report the presence of the characteristic lesions of AD in cognitively normal individuals [1,23,35,36]. Some of these studies suggest that the Aβ deposits in the brain may precede the onset of cognitive decline by a decade or more [27,36], indicating the presence of a wide temporal window for neuronal protective therapeutic intervention. An important caveat with the concept of preclinical AD is that we are making the assumption that those individuals, had they live long enough, would have developed clinical AD. This is not certain and we cannot rule out the possibility that some individuals can tolerate substantial AD neuropathological lesions, but never become demented.

In a previous study we described the pattern of loss of hippocampal neurons in normal aging and AD [39] and concluded that the most distinctive lesion of AD was the loss of neurons in the CA1 region. Moreover, we found that the degeneration in CA1 was disease-specific and not related to aging, per se. These findings have prompted us to examine whether or not a significant loss of hippocampal neurons, in particular in CA1, occurs in preclinical AD, that is, during a

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period in which there are abundant amyloid deposits present in the brain but no evidence of cognitive decline. Thus, in the present study, we extend our stereological evaluation of the number of hippocampal neurons to the preclinical stage of AD.

## 2. Materials and methods

We used unbiased stereological techniques to examine the postmortem brains of subjects followed by the Baltimore Longitudinal Study of Aging (BLSA) and the Johns Hopkins University Alzheimer's Disease Research Center (ADRC). Estimates of the total number of neurons in each of five subdivisions of the hippocampus were made in the left hippocampus of 33 subjects.

### 2.1. Subjects

The subjects were placed into one of three diagnostic categories: AD, preclinical AD, and age-matched controls. The AD ($n = 14$) subjects had clinical histories of dementia and their brains contained a sufficient number of neuritic Aβ plaques (CERAD plaques scores B or C) to meet the CERAD criteria for definite AD [21]. The preclinical AD subjects ($n = 8$) were cognitively intact and their MMSE scores were $\geq$26, but their brains contained substantial numbers of neuritic Aβ plaques (CERAD plaque scores B or C) [21]. The age-matched controls ($n = 11$) were cognitively intact and their MMSE were $\geq$26, and their brains had no or minimal pathologic changes that ranged from no Aβ deposits, to a few diffuse Aβ plaques, to sparse neuritic Aβ plaques (CERAD neuritic plaque score 0 or A). Many controls exhibited NFTs in hippocampus and entorhinal cortex.

The subjects for this study were recruited from the BLSA ($n = 25$) and JHU-ADRC ($n = 8$). The BLSA is a longitudinal study of normal aging conducted by the National Institute on Aging (NIA). At enrollment and at subsequent 2 year intervals, all BLSA subjects were examined according to BLSA protocols [29], complemented by a comprehensive medical history, a standardized neurological examination, and a battery of neuropsychological tests [17,35]. This battery included the Blessed BIMC [3], the Mini-Mental State Examination (MMSE) [8], Free and Cued Selective Reminding Test-Delayed recall [11], Category Fluency (animals) [22], Letter Fluency ("S") [2], and Trails A and B [5]. In the year between clinical evaluations, participants were administered the telephone version of the BIMC (T-BIMC) [16]. Subjects who showed a decline of two or more points in the T-BMIC were invited to an in-person evaluation. In addition, we completed a postmortem Dementia Questionnaire (DQ) [7,16,30,31] for each participant, interviewing a reliable informant within 1 year of death. ADRC subjects received neurological and neurocognitive evaluations when they entered the program and at subsequent 1 year intervals. The neurocognitive evaluation was similar to that of the BLSA subjects. Table 1 shows the demographic information for all subjects. Neuropsychological test scores (means and ranges) are summarized in Table 2. Subjects with preclinical AD had scores that were similar or even superior to the scores of the control subjects.

### 2.2. Clinical diagnoses

The diagnostic status of each participant was routinely evaluated during a multidisciplinary diagnostic conference that took into consideration the outcome of evaluations described above and medical records from external sources. Criteria from the Diagnostic and Statistical Manual of Mental Disorders IIIR (DSM IIIR) were used to make the diagnosis of dementia and NINCDS-ADRDA criteria [20] were used to make the diagnosis of AD. Dementia was diagnosed on the basis of the patient's history and examination. The clinical diagnosis was made without knowledge of the pathologic diagnosis.

### 2.3. Neuropathology

The autopsies of all of the subjects were performed by the JHU-ADRC and for most of them the interval between the last clinical evaluation and death was less than 18 months. At autopsy, the left half of the brain was immersion-fixed in 10% buffered formaldehyde for 2 weeks prior to being sectioned coronally into 1 cm thick slabs. For diagnostic purposes, tissue blocks were dissected from frontal, temporal, parietal, occipital, and cingulate cortices, basal ganglia, amygdala, entorhinal cortex; hippocampus; thalamus; brain stem; and cerebellum. The tissue blocks were embedded in paraffin, cut at 10 μm, and stained with hematoxylin–eosin and the Hirano silver method [40]. Selected sections were immunostained with antibodies for Aβ (directed against residues 1–28, a gift from Elan Corporation) and tau (Tau-2 from Sigma) using a standard protocol [18]. The neuropathological diagnosis of AD followed CERAD [21] guidelines. Silver stains were used to determine Braak neurofibrillary scores [4].

### 2.4. Stereology

For estimating the total number of neurons in the various hippocampal subdivisions, 5–6 large blocks of tissue (1 cm thick) that contained the entire hippocampus region, from the pez to the tenia tecta, were dissected from the 1 cm thick coronal slabs on the left side. These blocks were then embedded in paraffin and exhaustively sectioned at a microtome thickness setting of 50 μm. For each individual, a systematic random sample of sections, cut in the coronal plane and selected at 30 section intervals, was collected from the entire series of sections selected from all of the blocks. This resulted in 8–14 sections that spanned the

Table 1
Demographic and neuropathological information on cases and controls

| ID # | Age | Sex | MMSE | CERAD plaque score | Braak NFT stage | Pathological diagnoses |
|---|---|---|---|---|---|---|
| Controls | | | | | | |
| 01 | 84 | M | 30 | 0 | II | Control/parietal infarct |
| 02 | 72 | M | 29 | 0 | II | Control/lacune |
| 03 | 73 | F | 30 | 0 | II | Control/infarct remote |
| 04 | 81 | M | 30 | 0 | II | Control |
| 05 | 90 | M | 30 | 0 | II | Control |
| 06 | 69 | M | 29 | 0 | II | Control |
| 07 | 90 | M | 29 | 0 | II | Control |
| 08 | 80 | M | 29 | A | III | Control |
| 09 | 96 | M | 29 | A | IV | Control |
| 10 | 83 | M | 28 | 0 | I | Control |
| 11 | 77 | M | 26 | A | II | Control |
| Preclinical AD | | | | | | |
| 12 | 75 | M | 29 | C | III | AD possible/lacune |
| 13 | 91 | M | 30 | C | IV | AD possible |
| 14 | 72 | M | 30 | C | II | AD possible |
| 15 | 93 | M | 30 | C | IV | AD possible |
| 16 | 82 | F | 29 | C | II | AD possible |
| 17 | 87 | M | 28 | C | III | AD possible |
| 18 | 79 | M | 28 | B | II | AD possible |
| 19 | 94 | M | 26 | B | IV | AD possible |
| AD cases | | | | | | |
| 20 | 74 | M | 23 | B | IV | AD |
| 21 | 83 | M | 24 | C | III | AD |
| 22 | 81 | M | 19 | C | V | AD |
| 23 | 73 | F | 18 | C | V | AD |
| 24 | 92 | F | 16 | C | IV | AD |
| 25 | 59 | M | 6 | C | VI | AD |
| 26 | 86 | M | 5 | C | IV | AD |
| 27 | 85 | F | 4 | C | VI | AD |
| 28 | 96 | M | 4 | B | V | AD |
| 29 | 83 | F | 3 | C | VI | AD |
| 30 | 80 | F | 3 | C | VI | AD |
| 31 | 89 | F | 0 | C | VI | AD |
| 32 | 92 | M | 0 | B | V | AD |
| 33 | 87 | F | 0 | C | VI | AD |

Pathological diagnoses according to CERAD [21].

Table 2
Means and ranges of neuropsychological test scores in controls, preclinical AD subjects and AD cases

| | MMSE | Delayed recall | Category fluency (animal) | Letter fluency ("S") | Trail A seconds to completion | Trail B seconds to completion |
|---|---|---|---|---|---|---|
| Controls | 29 (26–30) | 11.4 (6–15) | 14.4 (9–22) | 14 (9–23) | 42.3 (28–61) | 96.4 (54–152) |
| Preclinical AD | 28.7 (26–30) | 12.8 (11–15) | 18.1 (14–23) | 18 (12–22) | 43 (33–73) | 126.6 (68–193) |
| AD cases | 8.9 (0–24) | 2.8 (0–10) | 4.1 (0–10) | 5.5 (0–12) | 247 (126–300) | 280 (180–300) |

In each box, the mean is the top number and the range is in parentheses.

entire length of the hippocampus of each individual. These sections were stained by the Nissl method and the boundaries of principal cell layers of five different hippocampal "subdivisions" were defined [37]. The subdivisions were: (1) The dentate granule cell layer (GRAN), (2) the dentate hilus (HIL), (3) CA3-2, (4) CA1, and (5) subiculum (S).

### *2.5. Estimating the total number of neurons in a hippocampal subdivision*

The optical fractionator method [38] was used to estimate the total number of neurons in each hippocampal subdivision of each individual used in the study. This stereological

method combines fractionator sampling [12] with optical disector counting [13,37]. The number of neurons counted with optical disectors, $\sum Q^-$, in a known fraction of the section thickness, tsf; under a known of fraction of the area of the sectional profiles of a subdivision, asf; on a known fraction of the sections that pass through the region of interest, ssf, were used to make an unbiased estimate of the total number of neurons, *N*, in an individual subdivision. (See Table A.1 and Appendix A for more details.)

### 2.6. Data and statistical analyses

Summary statistics (mean and median) were derived from the individual estimates of total neuron number made in each of the five hippocampal subdivisions of controls, preclinical AD cases, and AD cases. Statistical differences in the region specific estimates of total neuron number, among the three groups studied, were evaluated using the Wilcoxon Sign Test. This is a rank order non-parametric test suitable for small sample sizes or when values are not normally distributed. For each comparison between groups of size $n_1$ and $n_2$, each value is assigned a rank order between 1 and $n_1+n_2$. Significance testing is based on a comparison of the sum of the rank scores derived for each group and expressed as a *Z*-score and associated *P*-value. The three groups were compared to each other using the fractional cell count rank for each of the five subdivisions of the hippocampus.

## 3. Results

Table 1 summarizes the neuropathological observations. Fig. 1 and Table 3 summarize the cell count data for each hippocampal subdivision of each of the three groups. The box plots in Fig. 1 indicate the mean (i.e., horizontal line within the box) and the interquartile range (i.e., the upper and lower limits of the box).

Compared to age-matched controls, the brains of AD cases had significantly fewer neurons in CA1 (48%; $P < 0.001$), subiculum (24%; $P < 0.05$), and hilus (14%; $P < 0.05$). However, no differences in neuron number were present between these two groups in either the granule or the CA2-3 subdivision.

The significant loss of neurons in CA1, detected between controls and AD, persisted (37%; $P < 0.05$) when the analysis was limited to the brains of males (Table 4). However, the difference in the number of neurons in either hilus or subiculum, in males, was not statistically significant.

Compared to the age-matched control group, no differences were observed in any subdivision of the preclinical AD group. In contrast, the comparison between the preclinical AD group and the AD group revealed significantly lower numbers of neurons in the CA1 (44%; $P < 0.05$), but not in other subdivisions of the AD group (Table 3). No significant differences were observed between preclinical AD and control groups or between preclinical AD and AD groups when the analysis was restricted to males (Table 4).

## 4. Discussion

The results from the present study confirm our previous finding of substantial neuronal loss in the CA1, hilus, and subiculum in AD [39]. This confirmation is notable in that two totally different cohorts and two different unbiased stereological methods were used in these studies. Furthermore,

Table 3
Total number of neurons in hippocampal subdivisions of all cases and controls

| Subregion | Controls, $N = 11$ | | Preclinical AD (P-AD), $N = 8$ | | AD, $N = 14$ | | Comparison | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | Median | Mean | Median | Mean | Median | Control vs. P-AD | Control vs. AD | P-AD vs. AD |
| Granule | 12.27 | 12.16 | 13.9 | 14.382 | 11.03 | 10.02 | NS | NS | NS |
| Hilus | 0.74 | 0.70 | 0.74 | 0.74 | 0.64 | 0.59 | NS | $P < 0.05$ | NS |
| CA3-2 | 1.70 | 1.59 | 1.74 | 1.90 | 1.52 | 1.48 | NS | NS | NS |
| CA1 | 6.17 | 6.40 | 5.70 | 5.69 | 3.21 | 2.82 | NS | $P < 0.001$ | $P < 0.05$ |
| Subiculum | 2.46 | 2.54 | 2.16 | 1.87 | 1.88 | 1.77 | NS | $P < 0.05$ | NS |

Mean and median are expressed in millions of neurons. NS: not significant.

Table 4
Total number of neurons in hippocampal subdivisions of male cases and controls

| Subregion | Controls, $N = 11$ | | Preclinical AD (P-AD), $N = 7$ | | AD, $N = 7$ | | Comparison | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | Median | Mean | Median | Mean | Median | Control vs. P-AD | Control vs. AD | P-AD vs. AD |
| Granule | 11.9 | 11.7 | 13.43 | 13.05 | 11.32 | 10.10 | NS | NS | NS |
| Hilus | 0.74 | 0.62 | 0.69 | 0.72 | 0.74 | 0.72 | NS | NS | NS |
| CA3-2 | 1.62 | 1.58 | 1.62 | 1.62 | 1.77 | 1.65 | NS | NS | NS |
| CA1 | 6.14 | 6.46 | 5.55 | 5.23 | 3.89 | 3.44 | NS | $P < 0.05$ | NS |
| Subiculum | 2.41 | 2.42 | 1.98 | 1.86 | 2.49 | 2.73 | NS | NS | NS |

Mean and median are expressed in millions of neurons. NS: not significant.

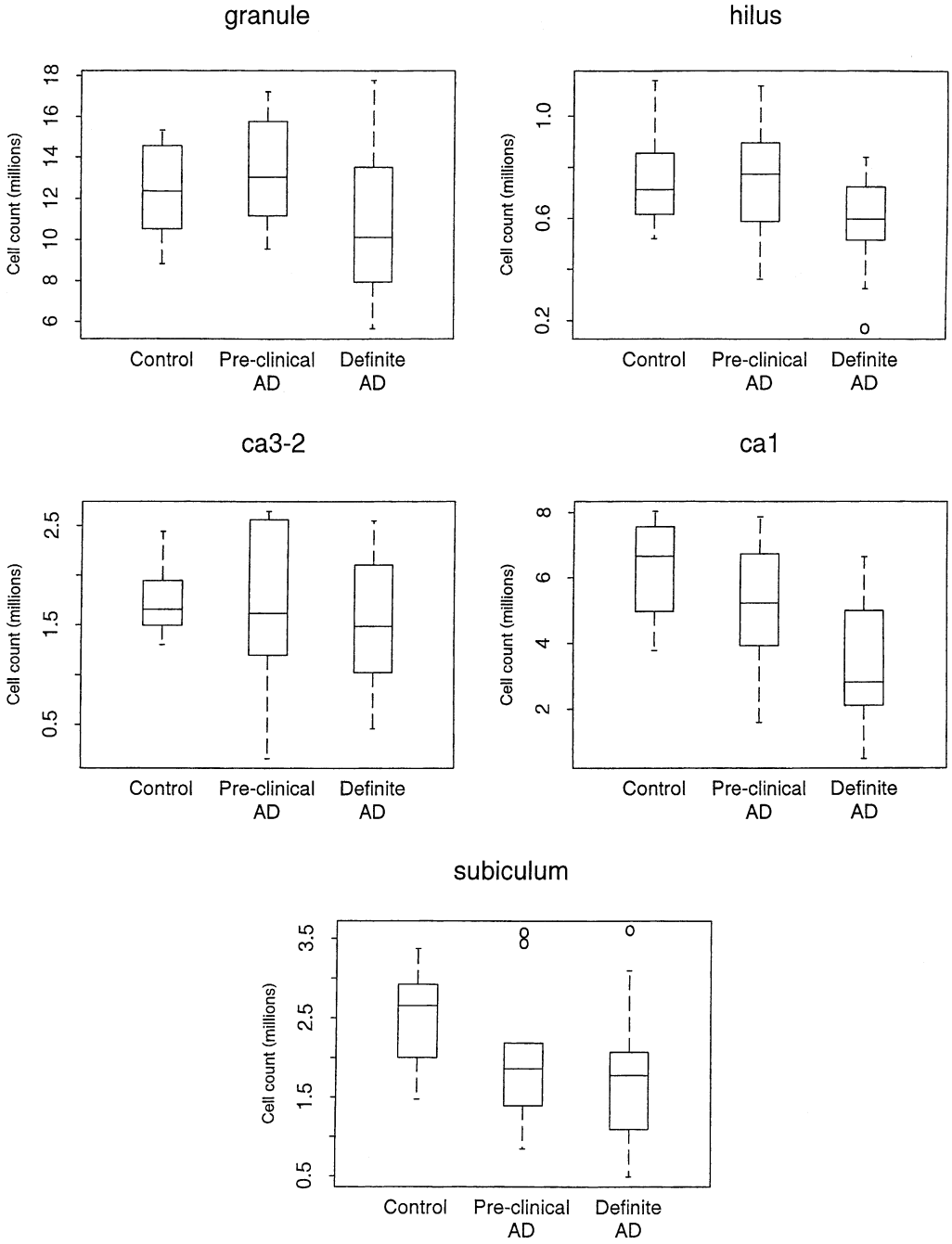


Fig. 1. Total number of neurons in subdivisions of the hippocampus. The box plots indicate the mean (i.e., horizontal line within the box) and the interquartile range (i.e., the upper and lower limits of the box) of the total number of neurons in each of the five subdivisions of the hippocampus.

the present study indicates that preclinical Alzheimer's disease is not characterized by a significant neuronal loss in CA1 or any of the other subdivisions of the hippocampus.

The first major finding of our study is the confirmation of loss of neurons in CA1, subiculum, and hilus in the AD group. With regard to gender composition and severity of disease, the AD group examined in the present study differs from the sample used in our previous study. Whereas in our original study, all the AD cases were males and in the later stages of the disease, in the present study half of the cases were females and 5 out of 14 cases were of mild to moderate severity, i.e., with MMSE $> 16$. To examine a possible gender effect on the results, we conducted an additional analysis that excluded females from all diagnostic categories. This analysis demonstrated a significant difference between males in the AD and control groups for CA1 neurons ($P = 0.02$), but not for subiculum or hilus. It should be noted that three of the seven males in the AD group had MMSE scores

that were ≥19. The inclusion of this relatively large number of cases of mild severity may have resulted in a smaller difference between the two groups than was observed in our first study [39]. The relatively mild clinical severity of the disease in the male AD cases, together with the large variability in the preclinical group and the reduction in the size of the groups when making comparisons among the males, may also be responsible for our inability to demonstrate significant differences in the number of hippocampal neurons between preclinical AD and AD groups (Table 4).

Our observations are also consistent with a recent report which described substantial losses of neurons in CA1 both in mild and severe AD (46 and 65%, respectively) [26]. However, another study [32] failed to show AD-related changes in CA1. This result may be explained by contamination of the age-matched control group with mild AD cases. This is a distinct possibility since the clinical data in that report was retrospective and it is not clear if the clinical evaluations were designed to detect changes in mental status or cognitive decline.

The present study of the number of neurons in the hippocampus of preclinical AD cases demonstrates that the total number of neurons in CA1, or any other hippocampal subdivision, is not significantly different from those of controls. However, we found a significant loss ($P < 0.05$) in CA1 cell count in cases of AD compared to preclinical cases. These results are in close agreement with those in a previous study [26], which was based on the examination of a segment of the hippocampus and also provided information on the entorhinal cortex. Our results suggest that a majority of the decline in the CA1 cell count is likely to occur between the preclinical phase of the disease and the overt clinical phase. We had only limited power (i.e., 12.6%) to detect a difference between controls and preclinical AD cases. As such, we cannot state whether the process of cell death begins around the preclinical phase. Follow-up studies are required to address this question. Calculations indicate that a sample of 164 preclinical and 164 controls would be required to have 80% power to detect the difference we observed in this study. Although our study had limited power to detect a difference in the number of hippocampal neurons between controls and preclinical AD cases, it should be underscored that the agreement of our observations with those of a previous study [26], also with limited statistical power, strengthens the findings in both.

The absence of differences in hippocampal neuronal counts in the preclinical stage of the disease, compared to controls, implies that the deposition of Aβ and the development of neuritic plaques in the neocortex precedes significant neuronal death in the hippocampus. In view of the early appearance of NFT in the hippocampus [4], the preservation of hippocampal neurons in preclinical AD is an unexpected observation. An important caveat to our observations, however, is that the preservation of hippocampal neurons does not necessarily mean that these neurons are not affected by the disease. It is possible that pathological changes at the cellular level are well underway and affect the neuronal processes and synapses of these neurons without overt perikaryal changes. In view of the recent study [26] describing the sparing of neurons in entorhinal cortex in preclinical AD, this phenomenon does not appear to be unique to the hippocampus. To determine the temporal relationship of neuronal loss in entorhinal cortex and hippocampus in AD, it would be valuable to examine these two regions in the same brains.

In the present study, the optical fractionator stereological method was used. Accordingly, neurons are directly counted in a known fraction of the region of interest using optical disector probes. This number times the reciprocal of the fraction of the region sampled is an estimate of the total number of neurons in the region (see Appendix A). In our earlier study [39], we used a two step method to estimate total neuron number, $N$. This involved determining the numerical density of neurons $N_V$ with optical disector probes and then multiplying this density by the volume of the region of interest obtained by point counting, $V_{\mathrm{REF}}$. Accordingly, $N = N_V V_{\mathrm{REF}}$. The optical fractionator was chosen for the present study in order to reduce potential biases related to defining the anatomical borders of regions of interest. It is also less sensitive than the $N_V V_{\mathrm{REF}}$ method to tissue deformation caused by changes in the thickness of the sections during staining and mounting. The estimates of total neuron number reported in the present study are slightly lower than those of the earlier study. This most likely reflects the lack of inclusion of partial sections at the leading and trailing edges of the 1 cm thick blocks that contained hippocampal tissue. In theory all sections, whether complete or not, should enter into the sampling scheme.

The existence of significant amyloid deposits and neuritic plaques in the neocortex, prior to the loss of hippocampal neurons, suggests that there is a hierarchical order of involvement of various brain regions during the development of AD. According to this scenario, which is based on the idea that amyloid deposition is a primary event in the development of the disease [28], the disease first affects the neocortex (as evidenced by the presence of abundant amyloid) and then spreads to the hippocampus. The lack of change in hippocampal neuron number, at this stage, may then contribute to the preservation of normal cognitive function in preclinical AD.

Another possible explanation of the severe amyloid and neuritic pathologic changes in the neocortex without significant loss of neurons in hippocampus is that the two are not causally related. It may well be that the loss of hippocampal neurons is not related to the amyloid pathology but to tau abnormalities or other types of insults that may lead or contribute to neuronal death, such as aging, hypoxia, or stress. Studies aimed at evaluating the relationship between these other factors and hippocampal neuronal death may provide a more complete understanding of the mechanisms involved in the loss of neurons in this region, which appears to have

Table A.1
Sampling scheme for estimating the total number of neurons (*N*) in each hippocampal subdivision

| | GRAN | HIL | CA2-3 | CA1 | SUB |
|---|---|---|---|---|---|
| Section sampling fraction (ssf) | 1/30 | 1/30 | 1/30 | 1/30 | 1/30 |
| Area sampling fraction (asf) | 1/545 | 1/66 | 1/79 | 1/534 | 1/133 |
| Thickness sampling fraction (tsf) | 1/2.3 | 1/2.3 | 1/2.3 | 1/2.3 | 1/2.3 |
| Total fraction | 1/37674 | 1/4554 | 1/5451 | 1/34500 | 1/9177 |
| *a*(frame), optical disector area ($\mu m^2$) | 414 | 10208 | 3710 | 3685 | 10208 |
| *x* and *y* step ($\mu m$) | 475 | 823 | 542 | 1408 | 1164 |
| *a*(step) ($\times 10^3\ \mu m^2$) | 226 | 677 | 294 | 1982 | 1355 |
| *h*, optical disector height ($\mu m$) | 20 | 20 | 20 | 20 | 20 |
| *t*, mean section thickness ($\mu m$) | 46 | 46 | 46 | 46 | 46 |

*N*: $\sum Q^-(1/\text{ssf} \times 1/\text{asf} \times 1/\text{tsf})$, $\sum Q^-$: number of neurons counted in all disectors in a specific subdivision of a specific individual, ssf: 30 (sections taken at 30 section intervals, after a random start within the first 30 sections), asf: *a*(frame)/*a*(step) where *a*(step) = *x* step × *y* step, tsf: *h*/*t*.

a direct relationship with the initial phases of cognitive decline in AD patients.

In conclusion, the results from the present study indicate that the number of neurons of the various hippocampal subdivisions remains stable in preclinical AD, during which there are significant amounts of amyloid deposition in the neocortex but no cognitive changes. This suggests that in spite of severe amyloid pathology there may still be a large temporal window during which cognitive changes, perhaps resulting from hippocampal neuronal loss, can be prevented.

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## Appendix A

An example of sampling scheme used to make the estimates in each of the subdivisions of the hippocampus is shown in Table A.1. The CE (coefficient of error = S.E.M./mean) was calculated according to published methods [14]. The mean CEs, for the individual estimates made in each subdivision, were less than 0.10, indicating that the precision of the estimates derived with this sampling scheme were adequate for the stereological analysis described here [38].

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