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# Volume and Number of Neurons of the Human Hippocampal Formation in Normal Aging and Alzheimer's Disease

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ABSTRACT

In order to observe changes owing to aging and Alzheimer's disease (AD) in the volumes of subdivisions of the hippocampus and the number of neurons of the hippocampal formation, 18 normal brains from subjects who died of nonneurological causes and had no history of long-term illness or dementia (ten of these brains comprised the aged control group) and 13 AD brains were analyzed. An optimized design for sampling, measuring volume by using the Cavalieri principle, and counting the number of neurons by using the optical disector was implemented on 50 μm-thick cresyl-violet sections.

The mean total volume of the principal subdivisions of the hippocampal formation (fascia dentata, hilus, CA3-2, CA1, and subiculum) showed a negative correlation with age in normal subjects ($r = -0.56$, $2P < 0.05$), and a 32% mean reduction in the AD group compared with controls ($P < 0.001$). This finding supports the measurement of the coronal cross-sectional area and the volume of the hippocampal formation in the clinical diagnosis of AD.

There was an inverse relationship between the age of normal subjects and the number of neurons in CA1 ($r = -0.84$, $2P < 0.0001$) and subiculum ($r = -0.49$, $2P < 0.05$) but not in other subdivisions. Pronounced AD-related reductions in neuron number were found only in the subiculum and the fascia dentata. Compared with controls, both losses represented 23% of neurons ($P < 0.05$). These results 1) confirm that AD is a qualitatively different process from normal aging and 2) reveal the regional selectivity of neuron loss within the hippocampal formation in aging and AD, which may be relevant to understanding the mechanisms involved in the neuron loss associated with the two processes. J. Comp. Neurol. 379:482–494, 1997. 

**Indexing terms: cortex; memory; stereology; neurobiology**

There are many good reasons why it is important to know the number of neurons. The number of neurons determines the functional capacity of the brain or any particular neural structure (Bok, 1959; Jerison, 1973). An increase in the number of neurons of the central nervous system is the main phylogenetic trend in the evolution of mammals, especially primates and humans (Brodmann, 1909; Pearson and Pearson, 1976). Because all neurons in the human cerebral cortex are postmitotic at the moment of birth and, therefore, cannot be regenerated, a loss can lead to significant and irreversible behavioral changes (Rakic, 1985).

Many studies have proposed that the loss of neurons in the hippocampus is a morphological correlate of the memory impairment seen in aging and disease-related dementia (Ball, 1977; Burke and Light, 1981; Mani et al., 1986; Morris and Kopelman, 1986). Alzheimer's disease (AD) is accompanied by very early cell degeneration in the hippocampus, which is associated with the deterioration of memory processes (Hyman et al., 1984). In advanced AD

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cases, the hippocampus is one of the most profoundly affected regions of the brain (Braak and Braak, 1991), and the consistency of hippocampal histopathology has led to a description of AD as a "hippocampal dementia" (Ball et al., 1985).

However, the relation of AD to the aging process of the normal human brain has remained unclear. Because the brains of most nondemented elderly individuals contain neurofibrillary tangles (NFTs), senile (neuritic) plaques (SPs), and neuropil threads (NTs) at autopsy, it has been proposed that the difference in neuron loss between aging and AD could be only quantitative (Selkoe, 1982; Terry and Katzman, 1983; Berg, 1985; Khachaturian, 1985). Because it is not possible to determine whether NFTs, SPs, and NTs represent nonspecific, age-related changes or are signs of early preclinical AD (Ulrich, 1985), it has been suggested that there is an overlapping continuum (or "clinicopathologic spectrum") in the pathologic process between elderly nondemented individuals, those with early stages of AD, and those with AD (Mann et al., 1984). The finding that the distribution of AD-type changes in nondemented elderly individuals roughly matches the AD pattern supported this view of a commonality in the molecular pathology that leads to NFTs and SPs in both aging and AD (Arriagada et al., 1992).

On the other hand, in the last several years, many authors have rejected the concept of AD as an exaggerated (or accelerated) normal aging process, because many genetic, biological, and clinical factors suggest that AD involves separate degenerative processes (Friedland et al., 1988; Mann, 1994; Selkoe, 1994; Hardy, 1996; Sandbrink et al., 1996). Although the AD process may take years to manifest itself, the atrophic phase develops relatively rapidly (Jarvik, 1978; Jobst et al., 1994). It seems that, with this phase, beyond a certain "threshold" (Roth, 1986), some specific AD-related process is associated with neuronal loss (West et al., 1994).

In an attempt to resolve the relationship of AD to normal human brain aging, we were guided by the pioneering work of West and collaborators (West and Gundersen, 1990; West, 1993; West et al., 1994). By studying the five major subdivisions of the hippocampal formation (granule cell layer, hilus, CA3 and 2, CA1, and subiculum) in three groups of subjects (subjects who died of AD, normal subjects above 65 years of age as controls, and normal subjects under 65 years of age), the aims of our study were 1) to determine possible losses in the volume and number of neurons related to normal aging and 2) to compare the regional pattern of volume and neuron reduction between normal aging and AD. We started from the assumption that, if a difference in the regional pattern of volume and neuronal loss in the normal aging and AD groups could be proven, then this would mean that AD involves a specific disease process that is not necessarily linked with the aging process of the brain.

Most studies dealing with the number of neurons are based on profile, but not particle, estimation (Agduhr, 1941; Floderus, 1944; Abercrombie, 1946) and rarely fulfill requirements for lack of bias (Gundersen, 1986). In such studies, the data represent indirect estimates of neuron density obtained from improperly sampled sections, and, usually, nothing is known about the volume of the investigated structure. Consequently, the results are unreliable and incompatible (Coleman and Flood, 1987; Swaab and Uylings, 1987; Coggeshall, 1992; Coggeshall and Lekan, 1996).

TABLE 1. Information About the Patients From Whom Brains Were Obtained

| Case no. | Sex | Age (years) | Source[1] | Cause of death |
|---|---|---|---|---|
| Normal aging | | | | |
| 1 | F | 16 | ZC | Traffic accident |
| 2 | M | 17 | ZC | Hyperthermia |
| 3 | M | 20 | ZC | Murder |
| 4 | M | 25 | ZC | Traffic accident |
| 5 | M | 28 | ZC | Traffic accident |
| 6 | M | 33 | ZC | Perforation of duodenal ulcer |
| 7 | M | 47 | ZC | Traffic accident |
| 8 | F | 52 | ZC | Traffic accident |
| Age-matched controls | | | | |
| 9 | M | 71 | HBB | Cardiopulmonary insufficiency |
| 10 | M | 75 | HBB | Myocardial infarction |
| 11 | F | 76 | HBB | Pneumonia |
| 12 | F | 77 | HBB | Bronchopneumonia |
| 13 | M | 78 | HBB | Myocardial infarction |
| 14 | F | 78 | HBB | Traffic accident |
| 15 | M | 80 | HBB | Myocardial infarction |
| 16 | M | 80 | HBB | Rupture of aorta |
| 17 | F | 88 | HBB | Lung cancer |
| 18 | F | 99 | HBB | Traffic accident |
| Alzheimer's disease | | | | |
| 19 | F | 65 | HBB | Pulmonary embolism |
| 20 | M | 77 | HBB | Massive hemorrhage from esophagus |
| 21 | M | 80 | HBB | Rupture of aorta |
| 22 | M | 83 | HBB | Pulmonary embolism |
| 23 | F | 83 | HBB | Bronchopneumonia |
| 24 | F | 86 | HBB | Bronchopneumonia |
| 25 | F | 86 | HBB | Cardiopulmonary insufficiency |
| 26 | M | 87 | HBB | Cardiopulmonary insufficiency |
| 27 | F | 88 | HBB | Cardiopulmonary insufficiency |
| 28 | F | 88 | HBB | Liver cancer |
| 29 | F | 90 | HBB | Bronchopneumonia |
| 30 | F | 92 | HBB | Massive intestinal hemorrhage |
| 31 | M | 92 | HBB | Pulmonary embolism |

[1]ZC, Zagreb Collection of Human Brains; HBB, Huddinge Brain Bank.

## MATERIALS AND METHODS

### Materials

The study was based on 18 normal and 13 AD postmortem human brains from the Zagreb Collection of Human Brains and the Huddinge Brain Bank. All brains were obtained at routine autopsies in accordance with the laws of both countries and with the permission of the ethical committee. The brains were divided into three groups. The first group included brains from 18 subjects who died of nonneurological causes and had no history of long-term illness or dementia (mean age 58 years, range 16–99). The second group of brains was a subgroup of the first and consisted of ten subjects (mean age 80 years, range 71–99). The mean age of this control group approximately matched the mean age of the AD group. The third group included the brains from 13 patients with clinically and pathologically confirmed AD (mean age 84 years, range 65–92). Clinical diagnosis was based on combined DSM-III-R (American Psychiatric Association, 1987) and NINCDS-ADRDA (McKhann, 1984) criteria. The neuropathological diagnosis of definite AD was determined by using CERAD criteria (Mirra, 1993; Bogdanović and Morris, 1995). Brains from AD patients with other major neuropathological findings, e.g., multiinfarct dementia, were excluded from the analysis.

All of the brains were fixed within 24 hours of death. Basic data about the patients from whom brains were obtained, and the causes of death are shown in Table 1.

## Methods

***Sectioning and histology.*** After fixation in 4% formaldehyde buffered with 0.1 M phosphate buffer for 8–32 months, the left hippocampus was removed from each brain and cut in the rostrocaudal direction in 3-mm-thick slabs, with a random position for the first cut within the first rostral 3 mm interval. To determine shrinkage of the tissue in the period from cutting the slabs to final mounting of sections, the areas of three randomly chosen slabs were determined for every hippocampus before the embedding procedure by point counting under a dissecting microscope by using a quadratic grid with an interpoint distance of 1 mm. The slabs were dehydrated through a graded series of ethanol solutions (70%, 70%, 96%, 96%, 100%, and 100%; 12 hours each) and ether-absolute alcohol solution (with ether and alcohol in equal parts) for 180 minutes twice. The slabs were then embedded in 2% celloidin (Cedukol, Merck, cat. no. 4363) for 24 hours, 4% celloidin for the next 24 hours, and finally in 8% celloidin until adequately hardened.

One 50-µm-thick section was cut from the rostral part of each slab. The sections were collected in 70% ethanol, put in 50% ethanol, then put in 5% ethanol for 2 minutes, then put in distilled water for 5 minutes, and finally in staining solution. Staining solution was comprised of 1 part of 0.5% cresyl-violet in distilled water mixed with 4 parts of distilled water. The mounted sections were placed in the staining solution until adequate staining was achieved. After that, the sections were placed in distilled water twice for 5 minutes and then through a graded series of alcohol solutions [50% ethanol, 70% ethanol with a few (two to four) drops of 25% acetic acid in distilled water, 70% ethanol, and 95% ethanol] for 10 minutes each. Finally, the sections were placed in ether-alcohol solution (2 parts of ether and 1 part of absolute alcohol) for 5 minutes, rinsed with xylene for 5 minutes, and mounted with a cover glass using Permount mounting medium. For determination of the extent of tissue shrinkage, the areas of the three previously selected slabs were then reestimated (Uylings et al., 1986).

***Estimates of the reference volume and numerical density of the neuron-containing subdivisions of the hippocampal formation.*** All measurements were carried out on the left temporal lobe with an Olympus Video Stereological Analysis System (manufactured by BICO, Copenhagen). The system was based on a Zeiss Axioskop microscope mounted with a Hamamatsu C3077 CCD video camera with a 50 mm Canon lens. The camera was interfaced via a Sony camera adapter CMA-D2 and a Commodore Amiga 2000 computer to a Sony Trinitron color monitor.

For generation of the point counting grids of required interpoint distance and the counting frames of required areas (both were superimposed on the screen picture) and for the control of the x and y movement of the microscopic stage through two Multicontrol 2000 programmable stepping motors, the GRID v2.0 software (Interactivision ApS, Silkeborg) was used. Measurement of the vertical movement of the stage (i.e., the height of the disector) was implemented by using a Heidenhain MT-12 digital microcator, which measures movements with a precision of 0.5 µm. Analysis of the brains was made without the investigator's knowledge of the group identity or the age of the subjects.

The first step in quantification was delineation of the subfields of the hippocampal formation (HF). This was made by using a low-power magnification of the video image imported from the section and was printed on a Sony color video printer Mavigraph UP-5000P. Definitions of the neuron-containing layers of the HF were made according to well-known architectonic and topographic data (Lorente de Nó, 1934; Braak 1972, 1974, 1980; Stephan, 1975; Schwerdtfeger, 1984; Rosene and Van Hoesen, 1987; Duvernoy, 1988). For description of demarcation lines, see West and Gundersen (1990).

In the second step, estimates of the reference volume of the delineated subdivisions of the HF were made according to the classic mathematical principle of Cavalieri (1966): The volume of an object is the sum of the areas of individual profiles of the object on a set of systematically positioned parallel sections through the object (with random placement of the first section) multiplied by a known constant distance (in our case, 3 mm) between profiles. The areas of the delineated profiles of the subfields on the sections were estimated by counting the points that hit the profiles in a quadratic lattice superimposed on the printed video image. The coefficient of error (CE) for the estimates of volumes of the subfields less than 0.10 was achieved by using the nomogram developed by Gundersen and Jensen (1987). This nomogram determines what the interpoint distance of the point lattices should be, so that 50–100 points would hit a given set of 8–15 sections per hippocampus. According to the nomogram and suggestions given in West and Gundersen (1990), we used point-counting grids with different interpoint distances for different subdivisions: 2 mm for subiculum and CA1, 1 mm for CA3-2 and hilus, and 0.2 mm for the granule cell layer.

The third step was measuring the numerical density of neurons in the HF subdivisions by using the disector method (Sterio, 1984; Gundersen et al., 1988a,b). An estimate of numerical density within an individual with a predetermined CE of less than 0.10 was achieved with about 100 observations per one subdivision in one hippocampus performed in a systematic random manner. Assuming that all cells to have one, and only one, nucleus, estimates were based on counting nuclei. The cells were classified either as neurons or as glial cells. Classification of a cell as a neuron was made on the basis of a combination of the following observations: 1) neuronal nuclei were larger and less stained than those of glial cells, and 2) neurons mostly contained a distinct nucleolus and clearly defined cytoplasm containing Nissl bodies. Cells that could not be classified as neurons were categorized as glial cells; therefore, they were not counted. The areas of the counting frames were dimensioned, so that one to two neuronal nuclei would typically be sampled in a disector of a 20 µm height. This height was chosen, because it was a comfortable distance over which to make observations. The dimensions of the counting frames used to obtain the sampling intensity mentioned had different sizes in the different subdivisions of HF: 8,112 $\mu m^2$ for subiculum and hilus, 3,588 $\mu m^2$ for CA1 and CA3-2, and 391 $\mu m^2$ for the fascia dentata, as shown in Figure 1. The systematic random disector samples were obtained by stepping using the meander path function of the GRID v2.0 program. The particular hippocampal subdivision was first outlined on the screen at a low magnification by using the cursor. Then, a $\times$100 oil-immersion objective with a numerical aperture of 1.40 was moved into place, and the appropriate counting frame was superimposed on the screen. The

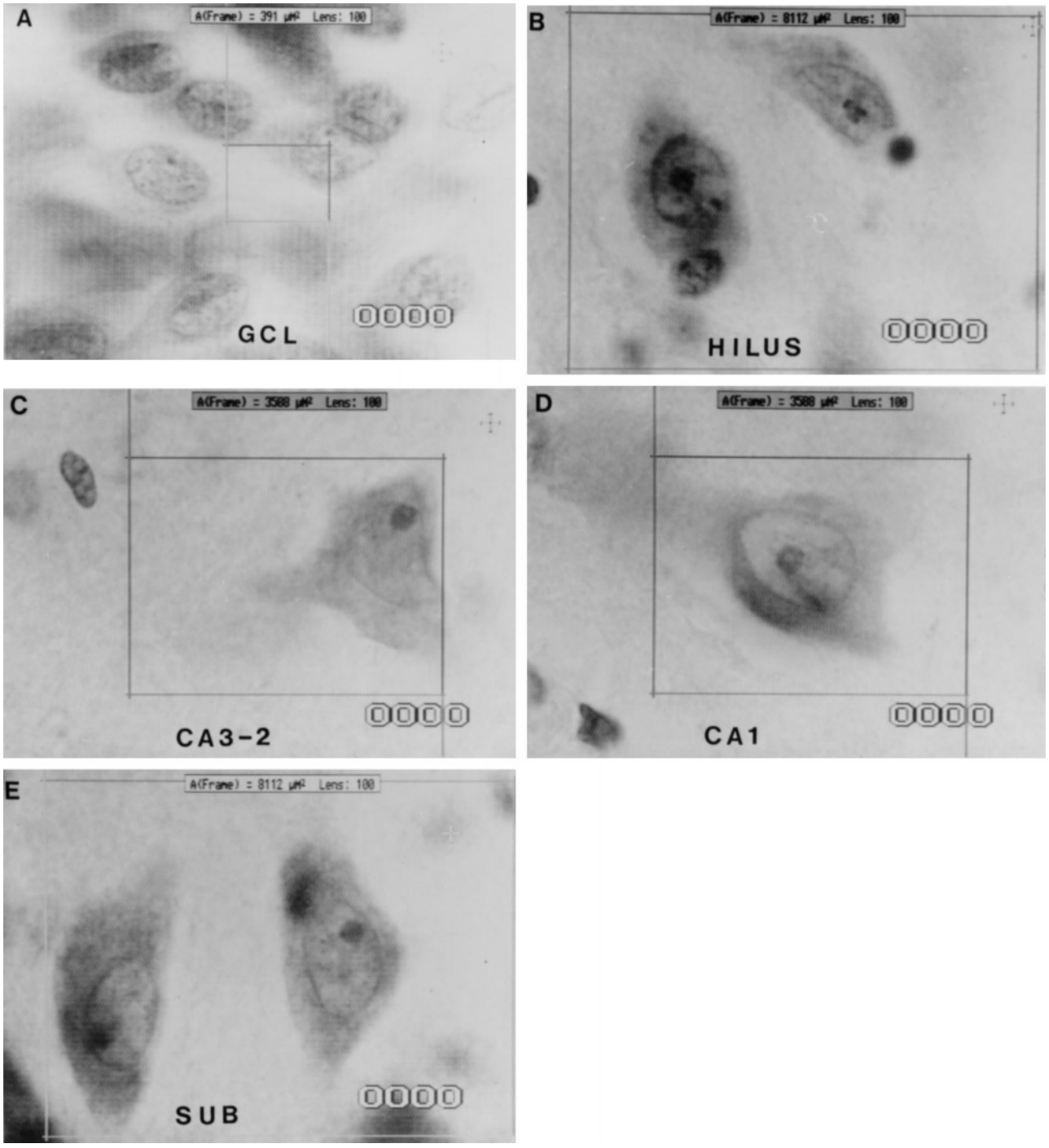


Fig. 1. Counting frames used in the different subdivisions of the hippocampal formation. The dimensions of the frames are indicated at the top of each micrograph. **A:** Granule cell layer (GCL). **B:** Hilus. **C:** CA3-2. **D:** CA1. **E:** subiculum.

desired horizontal and vertical step lengths, for which highly precise servocontrolled motors move the sections in a raster pattern, giving systematic disector samples, were dimensioned in such a way that approximately 100 disector samples per hippocampus were obtained. Frames that did not fall entirely within the outlined area were counted as fractional frames (depending on the number of corners of the frame hitting the outlined area). Neurons for which the clearest nuclear profiles fell within the disector volume and did not touch either of the two (left and bottom) forbidden lines or the "look-up" plane were counted. The total number of neurons was obtained by multiplying the numerical density of the particular hippocampal subdivision with its reference volume.

TABLE 2. Data About the Number of Digitations, Shrinkage, and Reference Volumes of the Hippocampal Formation Subdivisions Studied

| Case no. | Age (years) | Digitations | Shrinkage (%) | FD ($mm^3$)[1] | HIL ($mm^3$)[2] | CA2-3 ($mm^3$) | CA1 ($mm^3$) | SUB ($mm^3$)[3] | $V_{tot}$ ($mm^3$)[4] |
|---|---|---|---|---|---|---|---|---|---|
| Normal | | | | | | | | | |
| 1 | 16 | 4 | 42.3 | 52 | 252 | 129 | 633 | 498 | 1,564 |
| 2 | 17 | 4 | 45.7 | 71 | 225 | 142 | 634 | 451 | 1,523 |
| 3 | 20 | 4 | 38.7 | 73 | 220 | 126 | 605 | 438 | 1,462 |
| 4 | 25 | 2 | 44.1 | 80 | 272 | 175 | 755 | 482 | 1,764 |
| 5 | 28 | 7 | 40.1 | 63 | 266 | 148 | 669 | 486 | 1,632 |
| 6 | 33 | 4 | 42.1 | 77 | 298 | 142 | 570 | 438 | 1,525 |
| 7 | 47 | 2 | 37.7 | 61 | 196 | 111 | 651 | 422 | 1,441 |
| 8 | 52 | 2 | 39.6 | 58 | 199 | 156 | 611 | 403 | 1,427 |
| Mean | 29.8 | | 41.3 | 66.9 | 241.0 | 141.1 | 641.0 | 452.3 | 1,542.3 |
| S.D. | 13.5 | | 2.7 | 9.8 | 36.7 | 19.6 | 55.1 | 33.5 | 112.3 |
| Age-matched controls | | | | | | | | | |
| 9 | 71 | 4 | 39.9 | 46 | 187 | 138 | 637 | 464 | 1,472 |
| 10 | 75 | 1 | 45.6 | 55 | 233 | 146 | 609 | 473 | 1,516 |
| 11 | 76 | 4 | 39.9 | 59 | 220 | 131 | 665 | 512 | 1,587 |
| 12 | 77 | 2 | 41.2 | 81 | 259 | 151 | 675 | 500 | 1,666 |
| 13 | 78 | 4 | 37.5 | 54 | 176 | 135 | 486 | 377 | 1,228 |
| 14 | 78 | 2 | 41.0 | 67 | 149 | 124 | 511 | 381 | 1,232 |
| 15 | 80 | 3 | 39.2 | 48 | 145 | 120 | 544 | 333 | 1,190 |
| 16 | 80 | 1 | 40.2 | 51 | 250 | 173 | 678 | 322 | 1,474 |
| 17 | 88 | 3 | 38.1 | 89 | 172 | 126 | 570 | 320 | 1,277 |
| 18 | 99 | 3 | 36.8 | 37 | 181 | 144 | 540 | 358 | 1,260 |
| Mean | 80.2 | | 39.9 | 58.7 | 197.2 | 138.8 | 591.5 | 404.0 | 1,390.2 |
| S.D. | 7.9 | | 2.5 | 16.1 | 40.7 | 15.7 | 70.9 | 75.6 | 171.8 |
| Alzheimer's disease | | | | | | | | | |
| 19 | 65 | 3 | 42.3 | 58 | 225 | 117 | 623 | 376 | 1,399 |
| 20 | 77 | 3 | 35.1 | 38 | 159 | 82 | 289 | 264 | 832 |
| 21 | 80 | 3 | 38.4 | 60 | 197 | 91 | 379 | 337 | 1,064 |
| 22 | 83 | 1 | 43.0 | 28 | 117 | 56 | 257 | 207 | 665 |
| 23 | 83 | 3 | 42.7 | 34 | 108 | 80 | 220 | 159 | 601 |
| 24 | 86 | 1 | 36.5 | 37 | 114 | 76 | 286 | 208 | 721 |
| 25 | 86 | 4 | 41.6 | 67 | 211 | 105 | 519 | 282 | 1,184 |
| 26 | 87 | 3 | 42.4 | 72 | 215 | 100 | 555 | 438 | 1,380 |
| 27 | 88 | 2 | 40.6 | 45 | 215 | 96 | 344 | 237 | 937 |
| 28 | 88 | 2 | 37.9 | 50 | 169 | 93 | 371 | 217 | 900 |
| 29 | 90 | 2 | 39.5 | 62 | 200 | 104 | 377 | 282 | 1,025 |
| 30 | 92 | 3 | 36.1 | 33 | 145 | 86 | 486 | 226 | 976 |
| 31 | 92 | 2 | 37.2 | 27 | 102 | 73 | 201 | 142 | 687 |
| Mean | 84.4 | | 39.5 | 47.0 | 167.5 | 89.2 | 377.5 | 259.6 | 951.6 |
| S.D. | 7.3 | | 2.8 | 15.4 | 46.1 | 16.1 | 132.7 | 84.3 | 258.5 |

[1]FD, fascia dentata.
[2]HIL, hilus of the dentate gyrus.
[3]SUB, subiculum (includes subiculum and prosubiculum).
[4]$V_{tot}$, total volume of the subdivisions (FD + HIL + CA1 + CA3-2 + SUB); S.D., standard deviation.

Statistical analysis was carried out by means of the Statistica v4.0 program (Statsoft, Inc.). The age-related reduction in volume and loss of neurons were described with Pearson's coefficient of correlation (r) and were evaluated for each group of hippocampi (normal, controls, AD) by testing the linear regression. Regressions with 2*P* values of Pearson's coefficient of correlation less than 5% were defined as statistically significant. AD-related volume and neuronal loss were evaluated with unpaired (one-sided) Student's t tests: data from subjects in the AD group were compared with the age-matched control group. The value of *P* less than 0.05 was chosen as the criteria for the level of confidence.

## RESULTS

### Shrinkage measurements

There was no correlation in either direction between the shrinkage and the age of the subjects, as can be seen in Table 2. Although the extent of the shrinkage was large, the difference in shrinkage between brains was rather small. Therefore, an identical areal shrinkage correction factor (40%) was used for all brains. For determination of the shrinkage in the third dimension (assuming that shrinkage is similar in all three dimensions), we used the value of the square root of the areal shrinkage as a correction factor on slab thickness used in the Cavalieri formula.

### Qualitative observations

Localized regions of neuronal loss in the CA1 field were an almost constant finding in normal older brains (Fig. 2). Similar changes, usually of a greater extent, were also observed in AD hippocampi (Fig. 3). On the other hand, unlike normal brains, in most AD cases, an apparent diffuse loss of neurons from almost all subfields of HF was also observed (Fig. 4). A predominant mechanism of neuronal death in all subdivisions was necrotic death characterized by cell swelling and gliosis (Saper, 1985). Neurons in the early stage of this change (except granule cells of the fascia dentata) had ballooned cell bodies, and most of them contained an amorphous poorly stained zone that corresponded to the developing NFTs (Fig. 5A). At a later stage, the neuron dies, leaving behind an insoluble tangle ("ghost tangle") and accompanying gliosis. In AD brains as well as in older aging brains, astrocyte-like cells surrounding dying neurons were predominant, particularly in the subiculum. A small proportion of AD neurons (and, very rarely, of normal aging neurons) in all subfields (particularly CA1) of HF, except the fascia dentata, underwent granulovacuolar degeneration (Fig. 5B). Most of them contained NFTs, and those with diminished nuclei were not counted. In addition, localized regions of neuronal loss in the CA3-2 field (a so-called "resistant sector" of HF) were occasionally observed in normal older brains as well as in AD brains (Fig. 6), and a normal hippocampus with seven digitations was found (Fig. 7).

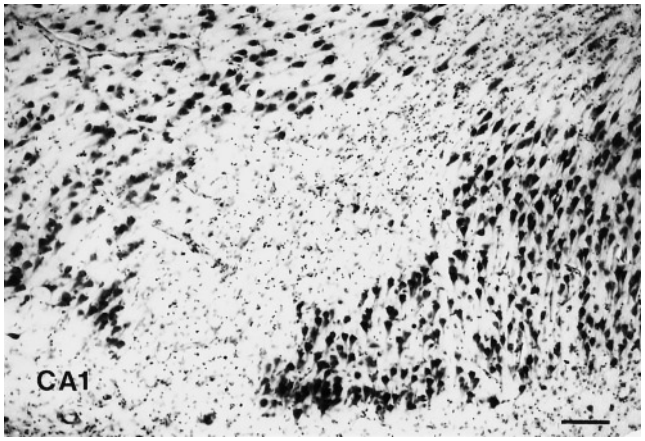


Fig. 2. An example of localized region of neuron loss in the CA1 field of a normal elderly person (88-year-old subject no. 17) on a frontal section through the rostral part of hippocampal formation. At the place of neuron loss, gliosis and revascularization are seen. Scale bar = 0.1 mm.

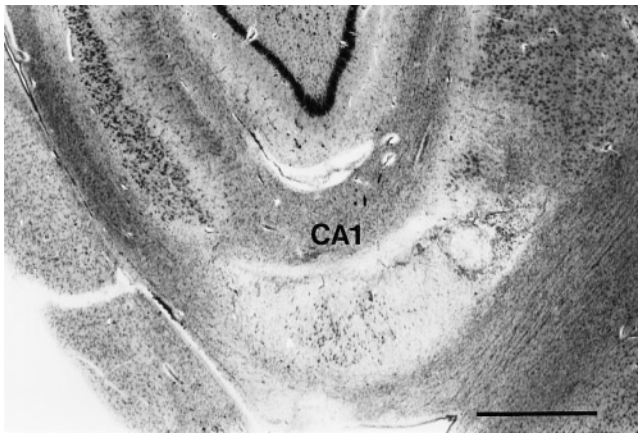


Fig. 3. Big necrotic region in the CA1 field of an Alzheimer's disease (AD) patient (87-year-old subject no. 26). Scale bar = 1 mm.

## Estimates of the volume and number of neurons of the neuron-containing subdivisions of the HF

The data concerning the volumes (corrected for tissue shrinkage in all three dimensions) for each subdivision of the HF are presented in Table 2. The total volume, i.e., the sum of the volumes of all five principal subdivisions of the HF (fascia dentata, hilus, CA3-2, CA1, and subiculum), for each hippocampus is plotted as a function of age in Figure 8. A significant negative correlation between the total volume of the HF subdivisions with age was found ($r = -0.56$, $2P = 0.02$). Compared with controls, a significant reduction of 32% in the mean total volume of the HF subdivisions of AD brains was found ($t = 4.54$, d.f. = 21, $P = 0.0001$).

Data concerning the number of neurons in each subdivision of the HF as well as the total number of neurons in each hippocampus are presented in Table 3. Figure 9 shows the total number of neurons in each of the hippocampal subdivisions for every individual. Significant negative

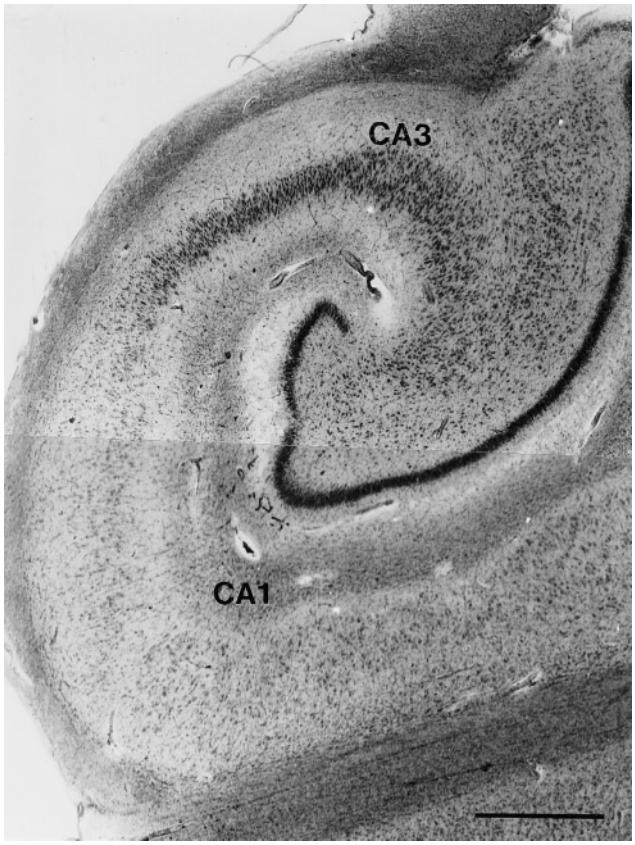


Fig. 4. Diffuse loss of neurons from almost all parts of hippocampal formation of an AD patient (88-year-old subject no. 28). Frontal section through the hippocampal body. Scale bar = 1 mm.

regressions were seen between age and neuron numbers in CA1 ($r = -0.84$, $2P = 0.00001$) and subiculum ($r = -0.49$, $2P = 0.04$) as well as between age and the total number of neurons ($r = -0.73$, $2P = 0.001$). The loss of neurons predicted on the basis of regression lines was from 25.994,800 neurons at the age of 16 to 8.465,200 at the age of 99 (overall loss of 67% of neurons) for CA1 and from 6.190,200 neurons at the age of 16 to 4.198,200 at the age of 99 (overall loss of 32% of neurons) for the subiculum. There were no statistically significant correlations between age and the numbers of neurons in the fascia dentata, the hilus, and the pyramidal cell layer of CA3-2.

Compared with age-matched controls, statistically significant AD-related reductions in the number of neurons were found in the subiculum ($t = 1.81$, d.f. = 21, $P = 0.04$) and the granule cell layer ($t = 2.71$, d.f. = 21, $P = 0.007$) but not in other subdivisions. Compared with controls, both of these losses represented 23% of neurons. A significant positive correlation between the total number of neurons in the HF subdivisions (particularly of CA1) and the number of hippocampal digitations was found ($r = 0.50$, $2P = 0.02$; for CA1 $r = 0.45$, $2P = 0.03$; Fig. 10A,B).

## DISCUSSION

We find celloidin blocks in combination with cresyl-violet dye very suitable for counting neurons, because the neurons are reliably stained and the sections have a very good

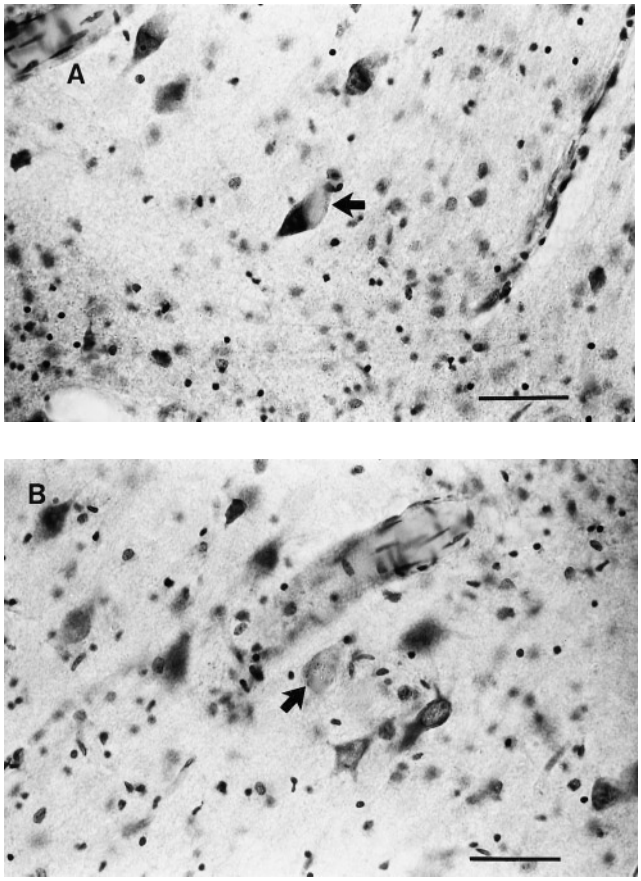


Fig. 5. **A:** Neuron in the CA1 of an AD patient (90-year-old subject no. 29) in the early stage of necrotic cell death with an amorphous, poorly stained zone that corresponds to the developing neurofibrillary tangles (NFT; arrow). **B:** Another example of a dying neuron (arrow) in the CA1 in the same patient. The neuron is full of degenerating vacuoles. Most of such neurons contained NFT, and those with diminished nuclei were not counted. Scale bar = 500 μm.

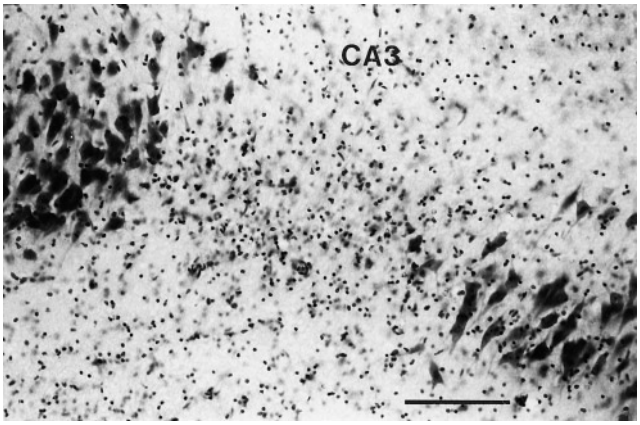


Fig. 6. An example of sharply demarcated region of neuron loss in the CA3 field in an AD patient (92-year-old subject no. 30). Like in Figure 2, at the place of neuron loss, gliosis and revascularization are seen. Scale bar = 0.1 mm.

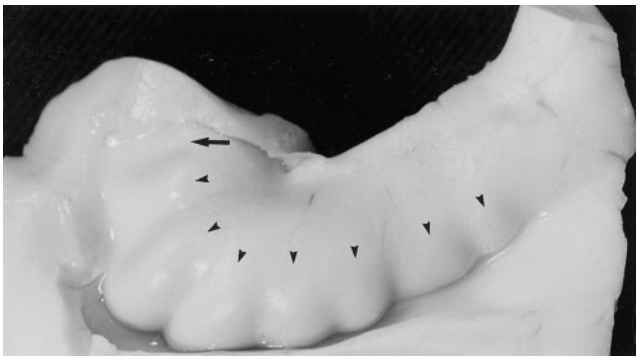

Fig. 7. Normal hippocampus with seven digitations (arrowheads; 28-year-old subject no. 5). Arrow shows vertical digitation (which is not counted). This is a rare finding, because only one to five digitations are usually found.

quality. Hippocampal atrophy is a common feature of advanced AD (Tomlinson et al., 1970) and a relatively good radiographic marker of the disease (Killiany et al., 1993; Lehericy et al., 1994). For example, hippocampal atrophy was found in 87% of AD cases in one large study by using computerized tomography (De Leon et al., 1989). In this study, we have found a mean reduction of 32% in the total volume of the subdivisions of the HF in AD patients compared with controls. These results are in accordance with the previously reported mean reductions of 10% (De la Monte, 1989) and 31% (Huesgen et al., 1993) in the area of the hippocampus on the coronal cross section in AD patients, and they are also compatible with the results of MRI studies that reported reductions in total hippocampal volume of 36–60% (Bobinski et al., 1995), 40% (Seab et al., 1988), 41% (Erkinjuntti et al., 1993), and 48.8% (Kesslak et al., 1991) in AD patients vs controls. In conclusion, our results support the measurement of the area of the hippocampus in the frontal plane on CT and MRI scans (and, if possible, volume) in shaping the antemortal diagnosis of AD.

The normal hippocampus with seven digitations found in this study is a rare finding, because only one to five digitations are usually found (with the incidence of five

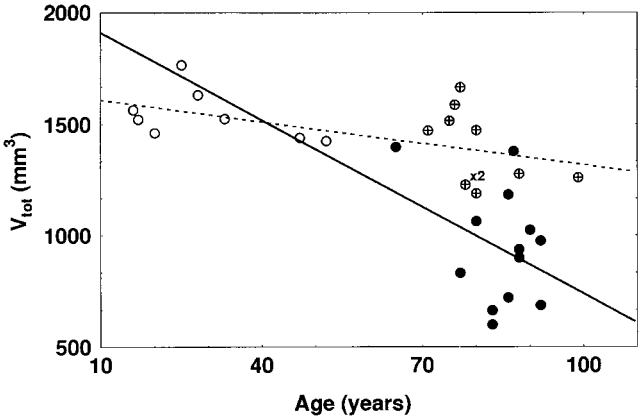


Fig. 8. Total volume of the neuron-containing subdivisions of the hippocampal formation. The open circles represent brains from normal individuals, the crossed open circles represent controls, and the solid circles represent AD brains. The dashed line represents a significant regression for the normal brains, and the full line represents regression for the AD group. Among normal subjects, there is a significant negative correlation with age ($2P < 0.05$). The values of the AD group are significantly smaller than in the controls ($P < 0.001$).

TABLE 3. Data About the Number of Neurons in Each Subdivision of the Hippocampal Formation and the Total Number of Neurons in Each Hippocampus

| Case no. | Age (years) | FD ($10^6$)[1] | HIL ($10^6$)[2] | CA2-3 ($10^6$) | CA1 ($10^6$) | SUB ($10^6$)[3] | $N_{tot}$ ($10^6$)[4] | CE ($N_{tot}$)[5] |
|---|---|---|---|---|---|---|---|---|
| Normal | | | | | | | | |
| 1 | 16 | 17.7 | 2.2 | 3.1 | 26.3 | 5.0 | 54.3 | 0.115 |
| 2 | 17 | 19.8 | 1.4 | 2.8 | 30.2 | 5.3 | 59.5 | 0.353 |
| 3 | 20 | 20.7 | 1.6 | 2.4 | 26.7 | 4.5 | 55.9 | 0.277 |
| 4 | 25 | 14.9 | 1.8 | 2.5 | 17.1 | 6.7 | 43.0 | 0.256 |
| 5 | 28 | 18.0 | 1.3 | 2.3 | 17.6 | 7.5 | 46.7 | 0.169 |
| 6 | 33 | 17.0 | 1.9 | 2.7 | 22.5 | 4.7 | 48.8 | 0.098 |
| 7 | 47 | 20.9 | 1.5 | 3.0 | 14.8 | 6.5 | 46.7 | 0.201 |
| 8 | 52 | 11.6 | 1.5 | 3.0 | 11.6 | 5.2 | 32.9 | 0.117 |
| Mean N | 29.8 | 17.6 | 1.7 | 2.7 | 20.9 | 5.7 | 48.5 | |
| S.D. | 13.5 | 3.1 | 0.3 | 0.3 | 6.6 | 1.1 | 8.4 | |
| CV[6] | 0.45 | 0.18 | 0.18 | 0.11 | 0.32 | 0.19 | 0.17 | |
| Mean CE[7] | | 0.080 | 0.098 | 0.066 | 0.069 | 0.060 | | 0.216 |
| BV (N)[8] | | 0.161 | 0.139 | 0.075 | 0.302 | 0.191 | | |
| $BV^2/OCV^2$ (%)[9] | | 80 | 67 | 56 | 95 | 91 | | |
| Age-matched controls | | | | | | | | |
| 9 | 71 | 15.7 | 1.6 | 2.3 | 10.5 | 6.2 | 36.3 | 0.142 |
| 10 | 75 | 14.2 | 1.0 | 2.3 | 10.8 | 3.2 | 31.5 | 0.091 |
| 11 | 76 | 15.9 | 1.2 | 2.6 | 12.4 | 2.7 | 34.8 | 0.245 |
| 12 | 77 | 20.3 | 1.3 | 2.4 | 10.9 | 5.0 | 39.9 | 0.271 |
| 13 | 78 | 21.8 | 2.1 | 3.1 | 16.2 | 5.3 | 48.5 | 0.334 |
| 14 | 78 | 15.3 | 1.2 | 2.3 | 9.8 | 3.5 | 32.1 | 0.319 |
| 15 | 80 | 12.3 | 1.1 | 2.2 | 9.6 | 3.4 | 28.6 | 0.243 |
| 16 | 80 | 11.8 | 1.2 | 2.5 | 12.2 | 3.4 | 31.1 | 0.109 |
| 17 | 88 | 24.3 | 1.5 | 2.9 | 11.2 | 3.9 | 43.8 | 0.281 |
| 18 | 99 | 13.9 | 1.3 | 2.4 | 14.3 | 5.1 | 37.0 | 0.171 |
| Mean N | 80.2 | 16.6 | 1.4 | 2.5 | 11.8 | 4.2 | 36.4 | |
| S.D. | 7.9 | 4.2 | 0.3 | 0.3 | 2.1 | 1.1 | 6.2 | |
| CV | 0.10 | 0.25 | 0.21 | 0.12 | 0.18 | 0.26 | 0.17 | |
| Mean CE | | 0.072 | 0.105 | 0.076 | 0.071 | 0.067 | | 0.230 |
| BV (N) | | 0.239 | 0.170 | 0.080 | 0.154 | 0.262 | | |
| $BV^2/OCV^2$ (%) | | 91 | 72 | 53 | 82 | 94 | | |
| Alzheimer's disease | | | | | | | | |
| 19 | 65 | 13.2 | 1.8 | 3.0 | 13.1 | 4.1 | 35.2 | 0.155 |
| 20 | 77 | 10.9 | 1.0 | 1.8 | 11.3 | 2.9 | 27.9 | 0.202 |
| 21 | 80 | 15.7 | 1.6 | 2.2 | 9.2 | 3.8 | 32.5 | 0.323 |
| 22 | 83 | 12.9 | 1.3 | 2.1 | 8.4 | 2.8 | 27.5 | 0.329 |
| 23 | 83 | 11.4 | 1.5 | 2.2 | 10.1 | 3.8 | 29.0 | 0.149 |
| 24 | 86 | 10.3 | 1.5 | 2.4 | 13.6 | 5.8 | 33.6 | 0.337 |
| 25 | 86 | 15.1 | 1.1 | 2.3 | 11.4 | 3.6 | 33.5 | 0.229 |
| 26 | 87 | 18.5 | 1.2 | 2.9 | 17.9 | 4.6 | 45.1 | 0.300 |
| 27 | 88 | 14.2 | 1.4 | 2.8 | 7.5 | 2.6 | 28.5 | 0.197 |
| 28 | 88 | 10.2 | 1.4 | 2.0 | 5.4 | 1.3 | 20.3 | 0.285 |
| 29 | 90 | 12.4 | 1.5 | 2.9 | 12.4 | 3.1 | 32.3 | 0.222 |
| 30 | 92 | 11.6 | 1.2 | 2.4 | 9.8 | 2.3 | 27.3 | 0.199 |
| 31 | 92 | 8.8 | 1.2 | 2.1 | 4.9 | 1.0 | 18.0 | 0.159 |
| Mean N | 84.4 | 12.7 | 1.4 | 2.4 | 10.4 | 3.2 | 30.1 | |
| S.D. | 7.3 | 2.7 | 0.2 | 0.4 | 3.5 | 1.3 | 6.8 | |
| CV | 0.09 | 0.21 | 0.14 | 0.17 | 0.34 | 0.41 | 0.23 | |
| Mean CE | | 0.098 | 0.088 | 0.099 | 0.085 | 0.093 | | 0.246 |
| BV (N) | | 0.186 | 0.095 | 0.113 | 0.329 | 0.399 | | |
| $BV^2/OCV^2$ (%) | | 78 | 53 | 57 | 94 | 95 | | |

[1]FD, fascia dentata.
[2]HIL, hilus of the dentate gyrus.
[3]SUB, subiculum (includes subiculum and prosubiculum).
[4]$N_{tot}$, total number of neurons in neuron-containing layers (FD + HIL + CA + CA3-2 + SUB); S.D., standard deviation.
[5]CE($N_{tot}$), coefficient of error of the total neuron number for one subject. The formula for CE($N_{tot}$) determination is given in Appendix A.
[6]CV, coefficient of variation (CV = S.D./mean).
[7]Mean CE, the mean coefficient of error of all individual estimates of the total neuron number, i.e., computable variation (OCE) of the stereological estimate of N [CE($\Sigma$N) is calculated in the same manner as CE($\Sigma$P), as described in Appendix A, where $N_i = Q_i \times P_i/F_i$]; mean CE($N_{tot}$), coefficient of error of the total neuron number for the group. The formula for CE($N_{tot}$) determination is given in Appendix A.
[8]BV (N), biological variation, i.e., estimate of the true biological variance of N; $BV(N) = \sqrt{OCV^2(N) - OCE^2(N)}$,
where $OCV^2(N)$ is the total observed variance, and $OCE^2(N)$ is the estimated stereological sampling variance. For details, see Kroustrup and Gundersen (1983).
[9]$BV^2/OCV^2$, proportion of the biological variance contributing to the observed variance of the total neuron number among the hippocampal subdivisions.

digitations less than 1%) (Gertz et al., 1972). Because a significant positive correlation between the total number of neurons of the HF subdivisions (particularly of CA1) and the number of hippocampal digitations was found ($r = 0.50$, $2P = 0.02$; for CA1 $r = 0.45$, $2P = 0.03$), we suppose that hippocampal digitation does not reflect cortical atrophy (Zuckerkandl, 1887) or some "obstruction" during development (Giacomini, 1884) but is a result of the "gyration" of the hippocampus. This is in agreement with the fact that CA1 is the main component of the digitations and is the field that contributed most to the evolutionary progression of the retrocommissural hippocampus (Stephan and Manolescu, 1980; Stephan, 1983).

Considering neuron numbers, the only study comparable to ours is the one by West and collaborators (West et al., 1994), which is a continuation of the two previously published studies (West and Gundersen, 1990; West, 1993). In that study, significant negative regressions were found between age and neuron number in the hilus and subiculum, representing 37% and 43% reductions, respectively, over the ages studied. The mean numbers of neurons per hippocampus in the hilus, CA1, and the subiculum of the AD group were significantly smaller than those of the age matched control group ($1.16 \times 10^6$ vs. $1.54 \times 10^6$ for hilus, $4.40 \times 10^6$ vs. $13.75 \times 10^6$ for CA1, and $2.93 \times 10^6$ vs. $5.51 \times 10^6$ for subiculum). The most pronounced AD-

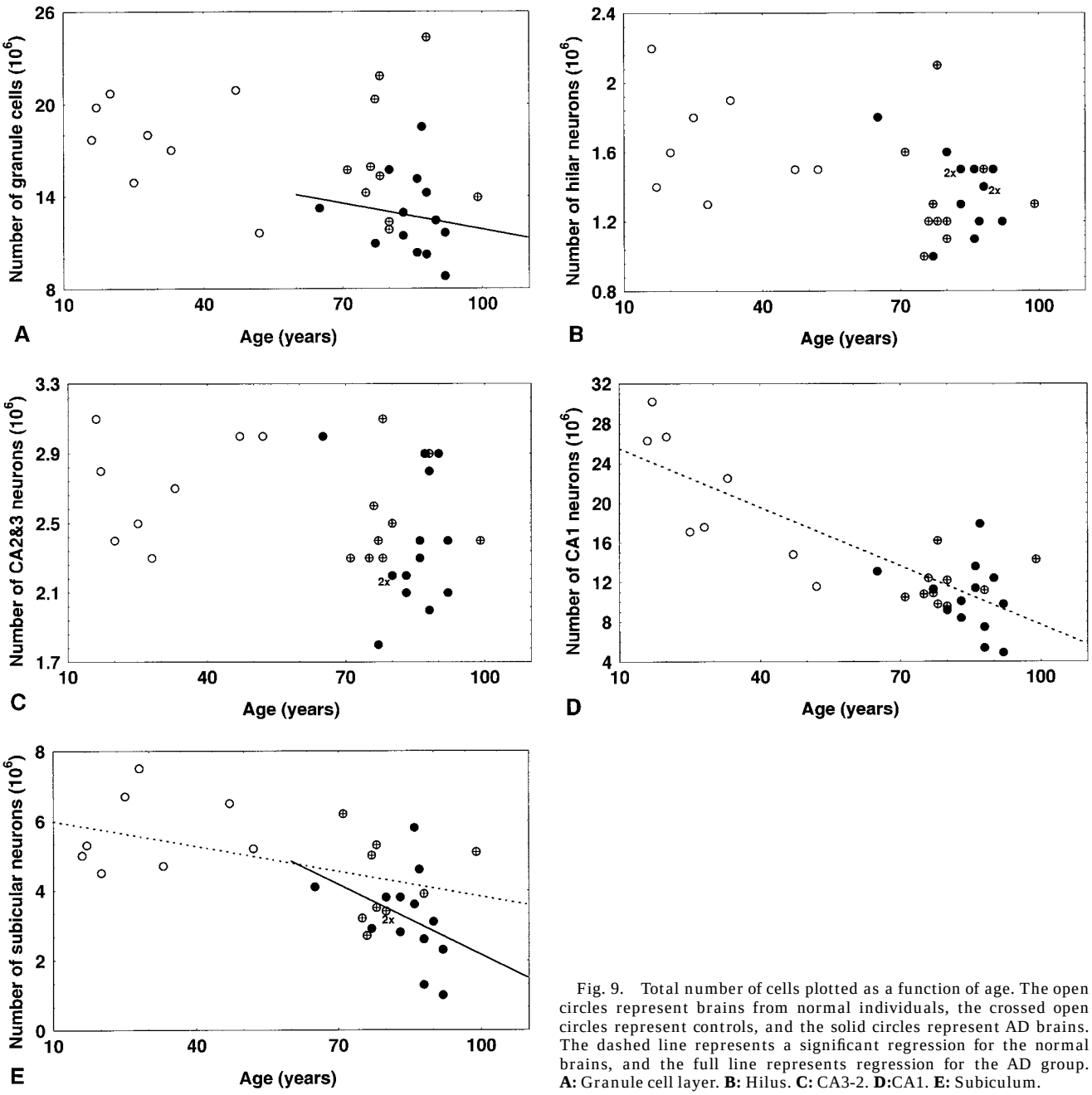


Fig. 9. Total number of cells plotted as a function of age. The open circles represent brains from normal individuals, the crossed open circles represent controls, and the solid circles represent AD brains. The dashed line represents a significant regression for the normal brains, and the full line represents regression for the AD group. **A:** Granule cell layer. **B:** Hilus. **C:** CA3-2. **D:**CA1. **E:** Subiculum.

related neuronal reduction was observed in CA1, where an average of 68% of neurons were lost.

The magnitude of number of neurons that we estimate in the separate HF subdivisions is similar to that estimated by West et al. (1994). However, the main differences in our findings are: 1) in a normal aging brain, we found a significant loss of neurons in CA1, but not in the hilus, and 2) in the AD group, we found a significant loss of neurons in the fascia dentata, but not in CA1 (although the mean of the number of neurons in the CA1 for the AD group was less than that in the age-matched control group; this difference was not statistically significant). The fact that we found no difference in the number of CA1 neurons between AD brains and controls is a consequence of the fact that we found a larger overlap of age-matched controls with the AD values in CA1. If one assumes that the properties of the disector method enable reliable neuron counts and comparison of results between laboratories, then why does the age-related loss in CA1 in these two studies differ?

Because it is well known that specific, extensive neuron loss can occur in CA1 with ischemia of only 15 minutes duration (Zola-Morgan et al., 1986, 1992), the first possible explanation is that some of the aged individuals in our normal aging group may have experienced some sort of agonal ischemia not experienced by the aging group presented in the study of West and collaborators. However, we think that this is not the case, because 1) all of our patients

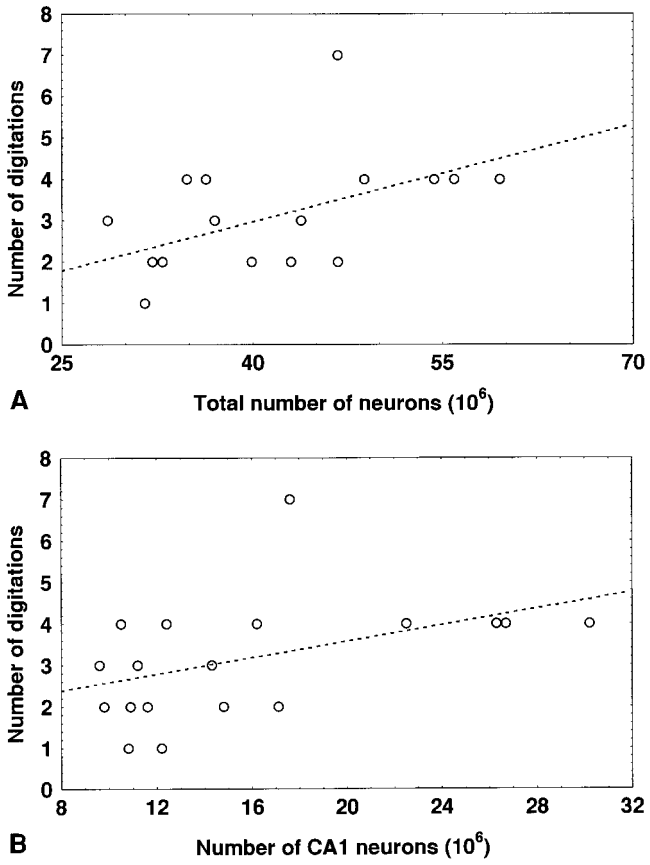


Fig. 10. **A:** A significant positive correlation between the total number of neurons and the number of digitations ($r = 0.50$, $2P < 0.05$). **B:** A significant positive correlation between the CA1 number of neurons and the number of digitations ($r = 0.45$, $2P < 0.05$).

in this group except two died in hospital in circumstances of maximum health care with no clinical evidence for premortal agonal state, 2) we were not able to demonstrate shrunken "red neurons" in control haematoxylin-eosin preparations of the opposite (right) hippocampi as a sign of ischemia, and 3) all of these patients had a very low content of initial NFTs and, under the clinical and pathological criteria described in the Materials and Methods section, were not considered as "initial AD" (therefore, we chose them as controls).

From our point of view, a second and more plausible explanation is that interindividual differences are very large, so the sample size of both studies seems to be too small to reduce the standard error of the mean values of the two overlapping groups (AD group and controls) to a point where the effects of aging and AD can be detected reliably. Our data differ especially in the AD group. In the AD group, we have six cases with a larger number of CA1 neurons than were reported by West et al. (1994). This leads to an increased overlap between the AD and age-matched control groups. However, we find our results to be in very good agreement with recent findings of DNA damage patterns in the AD hippocampus (Paul Lucassen, unpublished data, personal communication).

One interesting aspect of our findings is that there is loss of neurons in CA1 during normal aging but no apparent atrophy in CA1. In AD brains compared with age-matched controls, there is a loss of volume but not much change in number. These data suggest that there is neuron loss in CA1 during normal aging and that it is particularly atrophy in CA1, and not neuron loss, that distinguishes AD brains from the controls. We assume that atrophic changes in the neuropil of CA1 in AD brains may be the consequence of a lowered compensatory neuronal plasticity and degeneration of dendrites that receive subicular and perforant pathway projecting axons (Mizutani and Kasakava, 1995).

Nevertheless, beside the differences mentioned, it can be concluded that both studies confirm the differences in the pattern of neuron number reduction associated with normal aging and that associated with AD and can be considered compatible in a wider context. This is particularly true for the subiculum. A significant reduction of subicular neurons may represent a most profound and detrimental deficit in AD patients compared with age-matched controls, because these fibers constitute the "back projections" necessary for establishing declarative "long-term memories" (Lopes da Silva et al., 1990; Rolls, 1991; Traub and Miles, 1991) and serve as a major efferent component of HF information processing to entorhinal cortex and to other cortical and subcortical regions (Rosene and Van Hoesen, 1977; Van Hoesen, 1982). Cell loss documented in other subfields of HF may further compromise the flow of information through the intrinsic hippocampal circuitry and produce severe anterograde amnesia (Zola-Morgan et al., 1986). Another contribution to the isolation of HF from the association cortex in AD ("disconnection syndrome") is the inevitable severe loss of entorhinal layers II and III stellate and small pyramidal neurons, which isolates the hippocampal input via the perforant pathway (Braak and Braak, 1991; Hyman et al., 1984, 1986), as well as the loss in layer IV large multipolar neurons, which prevents receipt of the hippocampal output (Hodges et al., 1992).

To avoid misinterpretation of the results, it should be emphasized that the relationship between the quantity and location of NFTs, and SPs, cell loss, and the degree of dementia is not straightforward (Alafuzoff et al., 1987; Tomlinson, 1989; Braak and Braak, 1991; Terry et al., 1991). In our preliminary data (not published), we were also unable to find any correlation between NFTs and the number of neurons in the HF subdivisions, especially in the initial, preclinical stage of AD. Moreover, a certain extent of neuron loss is tolerated without concomitant functional loss because of redundant neuronal elements and compensatory structural, physiological, and biochemical processes (Hyman et al., 1987; Gertz et al., 1991). There are certainly limits to the degree to which a reduction in the number of neurons can be tolerated, but the question of how and when neuron loss translates to functional impairment is still not resolved.

On the basis of qualitative findings and the data obtained, we suppose that localized losses of CA1 and subicular neurons during normal aging may be morphological correlates of the so-called "benign senile forgetfulness" (Winocur, 1988; Braak and Braak, 1991; Bouras et al., 1993; Hof and Morrison, 1994). For example, one such clinically and pathologically well-documented case was reported by Yamazaki et al. (1993). At the same time, a diffuse degeneration and neuron loss of vulnerable neuronal populations, such as the projecting neurons of subiculum and fascia dentata, together with simultaneous

changes in other cortical areas and the septohippocampal system, may be the cause of a more severe disruption of hippocampal circuitry and the memory decline seen in AD (Van Hoesen et al., 1991; DeLacoste and White, 1993).

In conclusion, it is likely that the regional pattern of neuronal loss differs qualitatively and quantitatively between the normal aging group and the AD group, particularly in the subiculum and fascia dentata. The loss of neurons in these regions is a result of the pathological process involved in AD but not in normal aging. Our data, thus, argue further against the hypothesis that AD is purely an acceleration of aging.

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## APPENDIX A. FORMULAE FOR THE CE CALCULATION

The CE ($N_{tot}$) for one subject is calculated by using the formula

$$CE(N_{tot}) = \sqrt{\Sigma(CE(\Sigma P))^2 + \Sigma(CE(\Sigma Q/\Sigma F))^2}$$

where

$$CE\left(\sum_{i=1}^{n} P_i\right) = \frac{\sqrt{(3A + C - 4B)/12}}{\sum_{i=1}^{n} P_i},$$

and

$$A = \sum_{i=1}^{n} P_i \cdot P_i,\ B = \sum_{i=1}^{n-1} P_i \cdot P_{i+1},\ C = \sum_{i=1}^{n} P_i \cdot P_{i+2},$$

and

$$CE(\Sigma Q/\Sigma F) = \sqrt{CE^2(\Sigma Q) + CE^2(\Sigma F) - \frac{2. \cdot Cov(\Sigma Q,\Sigma F)}{\Sigma Q \cdot \Sigma F}},$$

where CE(ΣQ) and CE(ΣF) are calculated in the same manner as CE(ΣP) described above, and the covariance is calculated by using the formula

$$Cov(\Sigma Q,\Sigma F) = \frac{3D + G - 4E}{12},$$

where

$$D = \sum_{i=1}^{n} Q_i \cdot F_i,$$

$$E = \sum_{i=1}^{n} \frac{F_i \cdot Q_{i+1} + F_{i+1} \cdot Q_i}{2}$$

and

$$G = \sum_{i=1}^{n} \frac{F_i \cdot Q_{i+2} + F_{i+2} \cdot Q_i}{2}$$

(P, number of grid points counted on each section; Q, number of the nuclei counted; F, number of the disector frames; n, total number of sections; i, individual sections). The CE ($N_{tot}$) for the group of m subjects is calculated by using the formula

$$\overline{CE}(N) = \sqrt{\frac{1}{m}\sum_{j=1}^{m} CE^2(N)}.$$

For details, see Gundersen and Jensen (1987) and West and Gundersen (1990).