

Correlates of Hippocampal Neuron Number in Alzheimer's Disease and Ischemic Vascular Dementia

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The cornu ammonis 1 region of the hippocampus (CA1) sector of hippocampus is vulnerable to both Alzheimer's disease (AD)-type neurofibrillary degeneration and anoxia-ischemia. The objective of this article is to compare number and size of neurons in CA1 in AD versus ischemic vascular dementia. Unbiased stereological methods were used to estimate the number and volume of neurons in 28 autopsy-derived brain samples. For each case, the entire hippocampus from one cerebral hemisphere was sliced into 5mm slabs (5–7 slabs/case), cut into 50 μ m sections, and stained with galloxyanine. Using the optical dissector, we systematically sampled the number and size of neurons throughout the extent of CA1 and CA2. The total number of neurons was significantly less in AD compared with ischemic vascular dementia ($p < 0.02$), but there was no significant difference in neuron size. The greatest loss of neurons was observed in two cases with combined AD and hippocampal sclerosis. Regardless of causative diagnosis, the number of CA1 neurons correlates with magnetic resonance imaging-derived hippocampal volume ($r = 0.72$; $p < 0.001$) and memory score ($r = 0.62$; $p < 0.01$). We conclude that although CA1 neuron loss is more consistently observed in AD than ischemic vascular dementia, severity of loss shows the expected correlation with structure and function across causative subtype. Reductions in magnetic resonance imaging-derived hippocampal volume reflect loss, rather than shrinkage, of CA1 neurons.

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Although Alzheimer's disease (AD) is characterized in part by loss of cognitive function, cortical neuron loss, and the development of neurofibrillary tangles and senile plaques, ischemic vascular dementia (IVD) is not a single entity from a neuropathological viewpoint. Rather, it encompasses dementias characterized by intellectual deterioration caused by ischemic brain injury, including multiinfarct dementia, small-vessel disease, and leukoariosis.¹

The cornu ammonis 1 region of the hippocampus (CA1) sector of hippocampus is vulnerable to both AD-type neurofibrillary degeneration and anoxia-ischemia. Although a small number of studies have compared the numbers of hippocampal neurons in AD versus normal aging,^{2,3} to date, no study has estimated the number of hippocampal neurons in brains from patients with vascular dementia. Preliminary studies using magnetic resonance imaging (MRI) have demonstrated a similar significant loss of hippocampal volume in both AD and

IVD cases compared with control subjects, but a much greater decrease in the neuronal marker *N*-acetyl aspartate in AD than IVD for the same cases.⁴ The question arises: What is the substrate for these findings if the hippocampus of IVD cases does not show the formation of neurofibrillary tangles? Is the loss of hippocampal volume caused by loss of cells or, if the number of cells is unchanged, by cell shrinkage?

In this study, an optimized design was implemented for sampling, measuring volume, measuring the size, and estimating the number of neurons in CA1 and CA2 subfields of the hippocampus using the optical dissector for cases with the pathological diagnosis of AD or IVD and normal control subjects.

Subjects and Methods

Case Selection

Cases were obtained from two prospective longitudinal studies: the Ischemic Vascular Dementia (IVD) Program Project

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and the Honolulu Asia Aging Study (HAAS). All subjects or their guardians gave written informed consent before participation in the study, which was approved by the institutional review board at each institution.

Neuropsychological Testing

Three psychometrically matched measures were used to assess global cognitive ability (Global), memory (Memory), and executive (Executive) function. These tests were derived from a standardized battery of neuropsychological tests in common clinical use. Details of scale derivation and validation have been previously reported.⁵ Memory was derived from delayed and cued recall and selected immediate recall trials of the word list learning task of the Memory Assessment Scales.⁶ Donor scales for Global included: (1) total recall on trials 1 and 2 of the Memory Assessment Scales word list learning task; (2) digit span forward and backward from the Wechsler Memory Scale-Revised⁷; (3) letter fluency (the letter *A* from the FAS test⁸; and (4) Animal category fluency.⁸ The Executive scale used letter fluency (*F*, *A*, and *S*), digit span backward, visual span backward, and the Initiation-Perseveration subscale of the Mattis Dementia Rating Scale as donor scales.⁹ These tests broadly assess cognitive domains relevant to AD and IVD, have a broad range of measurement without appreciable floor or ceiling effects, and have linear measurement properties across this broad ability range.

Magnetic Resonance Imaging Acquisition and Hippocampal Volume Determination

Serial MRI studies were performed for 20 cases in this study. The interval between last MRI and death averaged 2.6 (± 1.8) years. Hippocampal volumes were determined using commercially available software (Medtronic Surgical Navigation Technologies, Louisville, CO).¹⁰ Although MRI volumes were determined for each hippocampus, only data from the same cerebral hemisphere in which counts were performed were used in this analysis.

Tissue Acquisition

At the time of autopsy, the entire brain was extracted from the cranium, weighed, photographed, and immersed in 10% neutral-buffered formalin. After 10-day fixation, the brain was again weighed, photographed, embedded in agar, and sliced coronally at 5mm intervals using a motor-driven rotary slicer.¹¹ Beginning at the level of the pes, the entire hippocampus from one hemisphere, comprising five to seven slices, was blocked for stereology. An entire hippocampus was obtained from 28 cases (13 female and 15 male cases; Table 1).

Histology

From each 5mm-thick slab, 50 μ m-thick sections were cut on a freezing microtome, mounted on glass slides, and stained overnight in galloxyanine. The remainder of each slab was embedded in paraffin and used for routine assessment of AD and vascular pathology. Stains included hematoxylin and eosin, cresyl violet, Congo Red, Bielschowsky silver, thioflavine S, Klüver-Barrera, and glial fibrillary acidic protein, ubiquitin, and α -synuclein immunostains.

Routine Pathology

Cases were evaluated for neurofibrillary tangle load (Braak score),¹² neuritic plaque burden (Consortium to Establish a Registry for Alzheimer's Disease [CERAD] rating),¹³ vascular lesions including infarcts (cerebrovascular disease [CVD] score),¹⁴ Lewy bodies (McKeith Lewy body score),¹⁵ and Vonsattel rating of cerebral amyloid angiopathy¹⁶ (CAA), expanded to grade IV where there is evidence of CAA-associated microangiopathy. Hippocampal sclerosis (HS) was given a score of 0 when there was no HS, 1 when the segmental neuron loss was limited to a single CA segment, 2 when the HS extended from CA1 to the prosubiculum, and 3 when the HS involved the entire pyramidal layer or affected two or more sectors of the hippocampus.

The severity of cerebrovascular ischemic brain injury was rated using a new CVD-pathology scoring system developed within this project.¹⁴ The CVD-pathology score (0–60 points) reflects the number and location of cystic infarcts, lacunar infarcts, and microinfarcts in the hippocampus and several gray and white matter regions. The HS score was included in the CVD-pathology score but acute infarcts were not.

Diagnostic Classification of Alzheimer's Disease and Ischemic Vascular Dementia

We used Braak and Braak staging to confirm the diagnosis of AD, AD being defined by a Braak stage higher than IV. There are no consensus criteria for the pathological diagnosis of IVD. We arbitrarily used CVD-pathology score of 4 or greater for a working diagnosis of IVD. This corresponds to our neuropathologists' (H.V.V., W.G.E.) belief that there was sufficient cerebrovascular pathology to account for dementia in a given patient.

Systematic Sampling

One galloxyanine-stained section was systematically chosen from each slab, and the CA1 and CA2 regions were identified and outlined on the coverslip using waterproof markers.¹⁷ The slide was placed on the microscope stage in a random orientation and overlaid with a uniform grid.

Counts were performed using an SPLAN 100X oil-immersion objective (N.A. = 1.25) on a Nikon Microphot FX microscope fitted with a Mertzhauser XYZ-stage and driven by StereoInvestigator (MicroBrightfield, Williston, VT) image analysis system. Using the motor-driven stage, we determined the outline of each region of interest. The stage was programmed to move in a buxtrophenal pattern with stage steps ranging from 250 to 1,000 μ m in each direction. A stage micrometer was used to calibrate the stage step and the counting frame. The stage step was tailored so that approximately 80 to 120 neurons were counted in each region for each section. The counting frame for the dissector was a 30 \times 30 μ m square. The left and bottom edges of the counting frame were designated as forbidden boundaries. The starting plane was 1 to 5 μ m below the surface of the section to avoid cutting artifacts and surface irregularities. The height of the fractionator volume was 12 μ m. Counts within each position were performed by focusing through the section. Neurons were counted when a clearly visible nucleolus located within the counting frame came into focus. Cells

Table 1. Characteristics of the Autopsy Brains Sampled

Age (yr)	Sex	Pathological Diagnosis	Braak	CERAD	CVD	CAA	HS	DRSTot	GLOB	MEM	EXEC
69	F	AD	VI	Moderate	0	0	0				
82	F	AD	VI	Frequent	3	1	0				
83	F	AD	VI	Frequent	2	2	0	114	56.90	59.66	59.64
87	F	AD	VI	Frequent	0	0	0				
91	F	AD	VI	Frequent	0	2	0				
81	M	AD	VI	Frequent	0	0	0	103	31.79	32.07	48.64
83	M	AD	V	Frequent	0	0	0	112	74.76	57.41	91.36
86	M	AD	V	Frequent	2	2	1	84	78.69	46.62	84.01
89	M	AD	V	Moderate	2	3	1	81	11.38	36.13	39.29
77	F	IVD	II	None	16	1	0	123	54.40	75.87	58.20
77	F	IVD	I	None	7	0	1				
78	F	IVD	0	None	9	0	0	139	116.58	94.58	114.86
68	M	IVD	0	None	11	0	1	80	40.84	43.38	37.25
87	M	IVD	0	None	10		1				
92	M	IVD	III	Sparse	10	0	0	135	106.54	97.42	101.89
86	F	MIXED	V	Moderate	5	4	3	115	56.90	52.41	59.64
97	F	MIXED	VI	Frequent	12	4	3	120	54.40	49.62	63.75
93	F	NMC-AD	III	Sparse	1	1	1	131	97.56	77.57	94.68
82	M	NMC-AD	II	None	4	0	0	144	92.80	107.33	90.29
88	M	NMC-AD	III	Frequent	1	3	1				
94	M	NMC-AD	IV	Frequent			0	129	86.04	85.07	80.91
75	F	NC	II	None	0	1	0	142	100.62	107.33	116.51
85	F	NC	II	None	0	0	0	138	76.75	91.95	72.29
80	M	NC	I	Sparse	0	0	0	142	112.30	103.77	110.18
80	M	NC	0	None	0	0	0	144	112.30	115.30	110.18
81	M	NC	IV	Sparse	0	0	0	120	74.76	49.62	73.42
85	M	NC	III	None	1	3	0	141	82.45	89.50	75.62
86	M	NC	III	Sparse	0	3	0				

AD = Alzheimer's disease; CAA = cerebral amyloid angiopathy; CERAD = Consortium to Establish a Registry for Alzheimer's Disease; CVD = cerebrovascular disease; DRSTot = Dementia Rating Scale total score; EXEC = executive; GLOB = global; HS = hippocampal sclerosis; IVD = ischemic vascular dementia; MEM = memory; nc = normal controls; NMC-AD = cognitively impaired not meeting criteria for AD.

without nucleoli or with nucleoli that intersected the lower or left edge of the counting frame were excluded.

Neuron size was measured with the nucleolator component of StereoInvestigator using a minimum of five rays. Each neuron not excluded by the above criteria was measured by the operator. The estimated volume (in cubic micrometers) was determined by StereoInvestigator.

The sampling scheme and counting protocol described above results in a counting accuracy with a coefficient of error for the estimate of the number of neurons (N), calculated as follows:

$$CE(\Sigma Q^-) = \frac{\sqrt{(\text{VAR}(N))}}{\Sigma Q^-}$$

where CE = coefficient of error; Q^- = counts; Var (N) = total variance.

Statistical Analyses

Analyses were two tailed with significance set at $p < 0.05$ and were performed with the interactive software SigmaStat 3.1 (SPSS, Chicago, IL).

Results

Neuropathological Findings

For 19 of 28 cases, the brain was weighed both in the fresh state and after fixation. The average fresh brain weight was 1,210gm. On average, there was a 1.7% increase in weight after fixation. Postmortem delay ranged from 3.0 to 70.5 (11.9 ± 13.6) hours. The average age of all cases is 83.6 (± 6.9) years.

Case Classification

Braak and Braak, CERAD, and McKeith Lewy body scores were determined for all cases. Eleven cases had Braak and Braak scores of V or VI. Nine cases rated Definite AD on the CERAD scale; three cases scored Possible AD because of a lower prevalence of cortical neuritic plaques. The McKeith score for all cases was 0 because no Lewy bodies were seen in the cortex or substantia nigra using hematoxylin and eosin, ubiquitin, or α -synuclein immunostains.

Fourteen cases had multiple large and small subcortical and white matter infarcts or cortical microinfarcts,

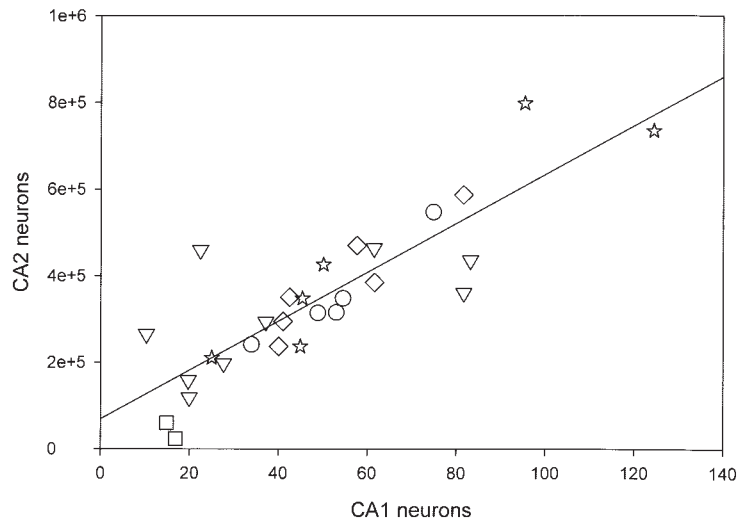


Fig 1. Estimated number of neurons in cornu ammonis 1 region of the hippocampus (CA1) is strongly correlated with the estimated number of neurons in CA2. Stars indicate healthy control subjects; circles indicate cognitively impaired not meeting criteria for Alzheimer's disease (AD); triangles indicate AD; squares indicate AD with hippocampal sclerosis; and diamonds indicate ischemic vascular dementia.

or both. Infarct size ranged from 8mm³ to 30cm³. Many of these had been noted by the radiologist on the premortem MRI, although others occurred in the interval between MRI and death. Arteriosclerosis was moderate to severe in 10 cases and mild in 3 cases. Moderate to severe white matter pallor was observed in eight cases. Nine cases had Vonsattel grade I or II CAA, whereas six cases had Vonsattel grade III or IV CAA. Nine cases had HS scores of 1 or greater. AD changes, vascular lesions, and atherosclerosis were absent in six cases.

Accordingly, nine cases were assigned the pathological diagnosis of AD, six cases had IVD, two cases were mixed AD/IVD/HS/CAA, five cases were cognitively impaired not meeting criteria for AD, and six cases were healthy control subjects (see Table 1).

Number of Neurons and Volumes of CA1 and CA2

The number of neurons in CA1 was correlated with the number of neurons in CA2 ($r = 0.48$; $p = 0.009$) (Fig 1). The volume of CA1 was correlated with the volume of CA2 ($r = 0.59$; $p = 0.009$) (Fig 1). The coefficient of error for these estimates averaged 0.10 ± 0.02 for number of neurons in CA1 and 0.12 ± 0.04 for number of neurons in CA2.

Premortem Magnetic Resonance Imaging Hippocampal Volume Correlates with Neuron Number

Hippocampal volumes were determined from premortem MRIs (Fig 2). MRI hippocampal volumes were highly correlated with the number of neurons in CA1 ($r = 0.72$; $p < 0.0004$) and volume of CA1 ($r = 0.54$;

$p < 0.02$) but not CA2. In addition, MRI hippocampal volumes were highly correlated with brain weight ($r = 0.58$; $p = 0.01$).

Relations between Pathology Scores and Neuron Number

The number of neurons in CA1 was negatively correlated to Braak score ($r = -0.73$; $p = 0.0002$), CERAD score ($r = -0.75$; $p = 0.0001$), and HS score ($r = -0.63$; $p = 0.003$) (Fig 3). The number of neurons in CA2 was negatively correlated to HS score ($r = -0.55$; $p = 0.01$). AD cases (Braak score = V or VI) have 48% fewer CA1 neurons compared with non-AD cases ($p < 0.00002$) and have a 39% reduction in CA1 volume ($p = 0.002$). For CA2, there are 35% fewer neurons in AD cases ($p < 0.02$) and 35% less volume ($p < 0.03$). Cases with Braak stage III to IV had a statistically nonsignificant 8% loss of CA1 neurons. IVD cases could not be distinguished from control subjects either by the Braak score or number of neurons (Fig 3). No significant correlations were found among number of neurons or volume of region and age, brain weight, or presence, absence, or number of vascular lesions.

Neuropsychological Testing and Number of Neurons

Neuropsychological examinations were administered to 20 subjects. The interval between testing and death was an average of 2.0 years (range, 4 months to 7 years). The composite Global and Memory scores, as well as Demential Rating Scale score, correlated with the number of neurons in CA1 (Fig 4), despite the sometimes long interval between testing and death (Ta-

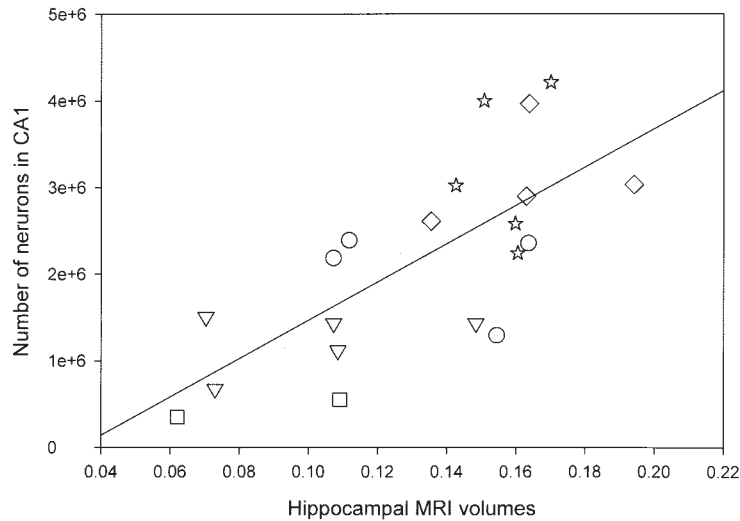


Fig 2. Estimated number of neurons in cornu ammonis 1 region of the hippocampus (CA1) is strongly correlated with the volume of the hippocampus determined from magnetic resonance imaging. Stars indicate healthy control subjects; circles indicate cognitively impaired not meeting criteria for Alzheimer's disease (AD); triangles indicate AD; squares indicate AD with hippocampal sclerosis; and diamonds indicate ischemic vascular dementia. MRI = magnetic resonance imaging.

ble 2). Hippocampal volume from the MRI was significantly related to the Memory and the Demential Rating Scale scores but not the Executive score. None of the cognitive test scores was significantly related to number of neurons in CA2.

CA2 neurons are larger than CA1 neurons but equally affected across disease states. Neuron volume was estimated for an average of 403 CA1 and 295 CA2 neurons per case. CA2 neurons are consistently and significantly larger than CA1 neurons. On average, CA2 neurons are $6.62 \times 10^6 \mu\text{m}^3$ ($\pm 9.73 \times 10^7 \mu\text{m}^3$), whereas CA1 neurons are $5.58 \times 10^6 \mu\text{m}^3$ ($\pm 9.82 \times$

$10^7 \mu\text{m}^3$), a difference of 15.6% ($p < 0.0001$). Although, in each region, neuron volume for non-AD cases was slightly larger (6%) than those for AD cases, the difference was not statistically significant.

The vulnerability of both CA1 and CA2 hippocampal neurons to disease, as measured by duration of illness, is similar for both regions. Duration of illness, defined as the onset of cognitive difficulties (either as reported by a caregiver or as noted on neuropsychological examination) to the time of death, was negatively correlated with number of neurons in CA1 ($r = -0.55$; $p = 0.004$) and CA2 ($r = -0.49$; $p = 0.01$).

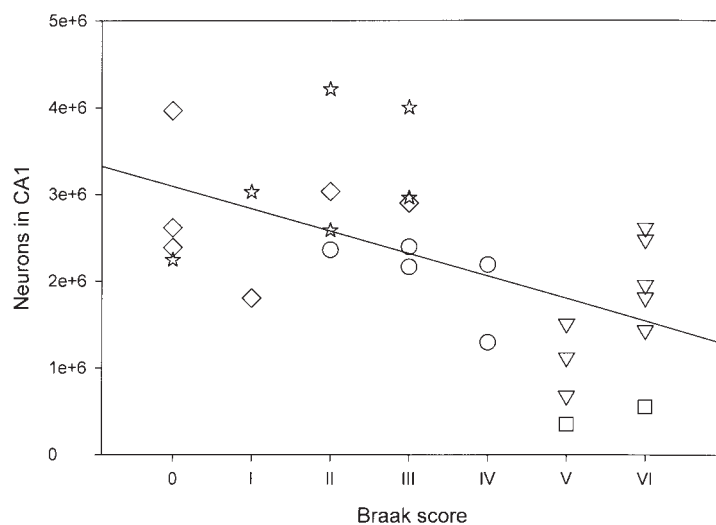


Fig 3. Number of neurons in cornu ammonis 1 region of the hippocampus (CA1) versus Braak score. Stars indicate healthy control subjects; circles indicate cognitively impaired not meeting criteria for Alzheimer's disease (AD); triangles indicate AD; squares indicate AD with hippocampal sclerosis; and diamonds indicate ischemic vascular dementia.

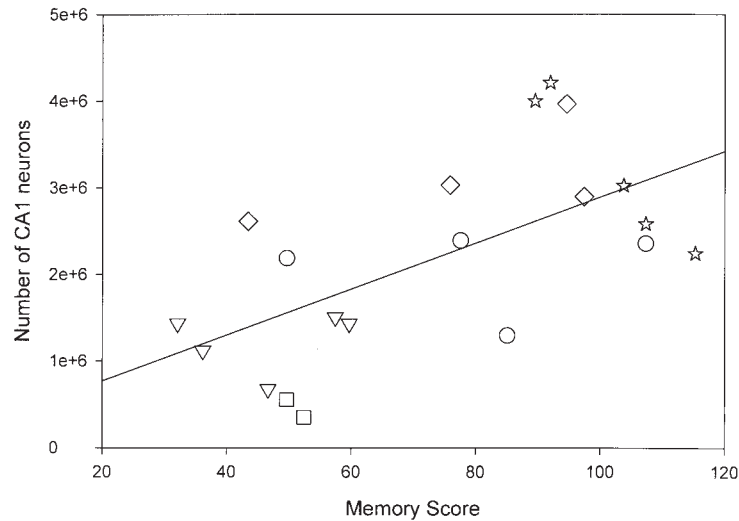


Fig 4. Number of neurons versus memory scores. Stars indicate healthy control subjects; circles indicate cognitively impaired not meeting criteria for Alzheimer's disease (AD); triangles indicate AD; squares indicate AD with hippocampal sclerosis; and diamonds indicate ischemic vascular dementia.

Discussion

Relatively few studies have quantified the severity of hippocampal neuron loss in AD and IVD compared with healthy control subjects. We found that although the estimated number of neurons in CA1 and CA2 was decreased in AD, the estimated number of neurons in IVD was comparable with control subjects. The greatest reduction of neurons was observed in cases with combined AD and HS that also met our operational criteria for mixed AD/IVD. Importantly, however, when diagnostic categories were set aside, numbers of CA1 neurons were significantly correlated with MRI volume and memory performance.

Memory impairment is widely recognized as an early sign of AD. Reductions in MRI hippocampal volume range from 15 to 22% for mild impairment to 40% for more severe impairment in patients with AD.¹⁸ Using nonbiased stereology methods, West and colleagues¹⁹ observed a 69% reduction in CA1, but not CA2, neurons in seven autopsied AD cases. Our nine cases with isocortical stage AD showed a 48% reduction, and our four cases with limbic stage AD showed an 8% reduction in CA1 neurons. Thus, our findings are in agreement with the literature.

In our six cases with IVD, the mean number of CA1 neurons did not differ significantly from control subjects. Our findings are similar to those of Korbo and colleagues,²⁰ who studied six cognitively normal and six non-AD cases and found no difference in the estimated number of neurons for the dentate gyrus, CA4, CA3-2, or CA1. Three of the six non-AD cases would have met our criteria for IVD. In contrast, Kril and colleagues²¹ noted significant loss of CA1 neurons in four cases with IVD, which was similar in magnitude to their five cases with AD (50% and 69%, respectively). Collectively, these data suggest that there may be a wide range and variable degree of CA1 neuron loss in IVD.

The most profound loss of CA1 neurons (83%) was noted in two cases with both isocortical stage AD and HS. We define HS as selective neuronal loss with gliosis in the absence of cystic cavitation. It usually occurs in the CA1 sector of the hippocampus, extending into the prosubiculum and subiculum. It has been reported to be a common neuropathological finding in patients with dementia who are very old (>80 years),²² among 12% of elderly people with dementia²³ or cardiac disease.²⁴ The pathogenesis of HS is still disputed, but

Table 2. Neuron Numbers and MRI Volumes vs Cognitive Test Scores (r values)

	DRSTot	Global	Memory	Executive
CA1 neurons	0.57 ^a	0.48 ^b	0.62 ^a	0.37, ns
CA2 neurons	-0.01, ns	0.002, ns	0.06, ns	-0.011, ns
Hippocampus MRI volume	0.58 ^a	0.37, ns	0.70 ^c	0.27, ns

^a $p < 0.01$; ^b $p < 0.05$; ^c $p < 0.001$.

CA1 = cornu ammonis I region of the hippocampus; MRI = magnetic resonance imaging; ns = not significant.

systemic hypoxia–ischemia²⁵ or ischemia caused by intrinsic CVD are commonly hypothesized to be of causative importance. Although HS is generally considered to affect only CA1, we found strong correlations between HS and the number of neurons in both CA1 and CA2.

In a case series composed of 12 healthy control subjects, Harding and colleagues²⁶ found a strong, statistically significant correlation between cerebral volume and the number of neurons in CA1. Using brain weight as a proxy for volume, we were unable to corroborate this finding. However, we found a strong correlation between brain weight and hippocampal volume, regardless of disease state.

This study is unique in providing quantitative neuropathological validation of MRI-derived hippocampal volume and neuropsychological testing. Lye and colleagues²⁷ found that the left hippocampal volume was an important predictor of verbal memory scores in nondemented community-dwelling elderly people. Peterson and colleagues²⁸ reported a similar finding in AD where the volume of the left hippocampus correlated with performance on verbal tasks using delayed recall measures. Because of our small sample size (limited by the number of cases where hippocampal neurons were counted in both hemispheres), we were unable to separate our cases into left and right whereas maintaining an even match on diagnostic category in each group. Nevertheless, we demonstrated that in our sample, CA1 neuron counts correlate with MRI-derived hippocampal volume and Global and Verbal Memory but not Executive scores. These findings confirm the expected brain–behavior relations regardless of the type of underlying pathology.

We estimated the size of neurons in both CA1 and CA2 to determine if the change in MRI-determined hippocampal volume could be caused by neuron shrinkage, as well as neuron loss. Contrary to our expectations, we found no difference in neuron size in high Braak versus low Braak cases for either CA1 or CA2 neurons. This may be a consequence of the counting criteria, which established namely that cells must have a readily identifiable nucleolus to be counted. Cells that did not have a readily identifiable nucleolus were excluded. By these criteria, degenerating cells, either swollen or pyknotic, were not measured. Caveat aside, the data suggest that reduction in MRI hippocampal volume reflects loss, not shrinkage, of neurons.

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