Hippocampal size and neuron number are reduced in a number of conditions, including temporal lobe epilepsy and Alzheimer’s disease. Furthermore, a decrease with advancing age has also been suggested. The present study examined the entire hippocampal formation of 12 subjects aged from 46 to 85 years and free from neurological disease. The volume of seven subregions (CA1, CA2–3, CA4, dentate gyrus, subiculum, presubiculum and white matter) was determined and the number of neurons estimated in each of these grey matter subregions using the optical dissector technique. There was a significant relationship between CA1 neuron number and cerebrum volume. Multivariate analysis showed the greater contribution to the variance in CA1 neuron number was made by cerebrum volume (69%) rather than age (2%) or sex (1%). The findings of this study show that, in neurologically normal individuals, brain size is a major determinant of the number of CA1 neurons.

**Introduction**

A small decrease in brain size is often reported with increasing age (Dekaban, 1978). However, most brain size measurements have been made at a single time point (i.e. autopsy) for each individual and cross-sectional population data used to derive information concerning changes during ageing. It is therefore not surprising that controversy exists as to whether these data represent a true loss of brain tissue with ageing or are the result of cohort differences in the populations studied (Miller and Corsellis, 1977; Kretschmann et al., 1979). There is increasing evidence that gross cerebral atrophy is not a normal consequence of ageing but may be a reflection of degenerative processes. In a recent cross-sectional post-mortem study brain tissue loss was minimal (~2 ml/year) in patients without neuropathological abnormalities (Double et al., 1996). In addition, this reduction in tissue volume was restricted to the white matter, suggesting age-related changes in myelination rather than a large loss of neurons with age. A longitudinal tomographic study of subjects >60 years of age found only a slight annual reduction in brain size (0.8%/year) of which atrophy of the cerebral cortex was a small proportion (0.1%/year) (Akiyama et al., 1997). These recent studies suggest that the effects of age on the cerebral cortex may be less than previously assumed.

The loss of brain tissue with age has always been assumed to be a result of neuron loss. However, controversy exists over the measurement of this variable, particularly as the neuron content of individuals cannot be sampled longitudinally. Older studies reported decreased neuronal density with increasing age (Brody, 1955; Samorajski, 1976), and these values were erroneously taken to reflect a large loss of neurons (see Haug and Eggers, 1991; Wickelgren, 1996 and commentaries Neurobiology of Aging 1994:15). A recent study using unbiased estimates of the total number of cortical neurons in a cross-sectional sample of patients spanning 70 years in age (range 20–90 years old) found a 9.5% decrease in neuron number (Pakkenberg and Gundersen, 1997). Excluding any cohort effects, this work suggests a small loss of neurons with age (~0.1% reduction/year), supporting the idea that substantial neurodegeneration is not a prominent feature of the ageing brain.

A brain region suspected of having marked age-related changes is the hippocampus. Age-related hippocampal atrophy has been repeatedly reported using both neuroimaging (Golomb et al., 1994; Convit et al., 1995) and pathological (Simic et al., 1997) techniques with magnetic resonance imaging (MRI) measurements of the hippocampus currently used in the diagnosis of a variety of neurological diseases (Jobst et al., 1994; Gilmore et al., 1995). A number of studies have reported an age-associated decrease in the neuron content of particular hippocampal subdivisions using both density measures (Ball, 1977; Miller et al., 1984) and unbiased stereological techniques (West and Gundersen, 1990; West, 1993; West et al., 1994). West and colleagues initially described a decrease in the number of CA1 neurons (West and Gundersen, 1990), but on expansion of their sample found only a significant reduction in the number of CA4 and subiculum neurons with age (West, 1993; West et al., 1994). Overall, these findings have led to the belief that there is a significant decrease in hippocampal size with age and that this decrease is as a result of neuron loss. However, these studies have not analysed their results in association with brain size, a variable which may be associated with a particular cohort, and which results in a similar age-related change. The study described here analyses age and volume variables to determine the contribution of these variables to the number of hippocampal neurons. In this way we hope to clarify the relative effect of age on hippocampal neuronal populations.

**Materials and Methods**

**Case Selection and Preparation**

The brains from eight males (aged 46–85, mean 68 ± 12 years) and four females (63–84, mean 72 ± 9 years) were selected for study. These cases were collected from a large cohort of ~600 patients referred for neuropathological assessment (all adult patients necropsied between 1989 and 1992 at the Royal Prince Alfred Hospital). In particular, no case had any neuropathological abnormality. All cases had assessment of silver-stained sections of association cortices and the medial temporal lobe for evidence of Alzheimer’s disease or any other neurodegenerative condition. Furthermore, all cases chosen for analysis had no history of neurological disease as ascertained by retrospective examination of the medical records and a questionnaire sent to the next of kin. In particular, no case was included which had a history or neuropathological evidence of head trauma, cerebrovascular disease, epilepsy, neurological disease, alcoholism or substance abuse. All cases consumed <20 g of ethanol per day and had a Clinical Dementia Rating Scale of 0 (Rubin et al., 1998). This study was approved by the ethics review committees of Central Sydney Area Health Service and South Eastern Sydney Area Health Service.
The weight and volume of each brain was determined at the time of autopsy and following fixation for 2 weeks in 15% buffered formalin (Harper et al., 1984). The cerebellum and brainstem were separated from the cerebrum and the volume of the cerebrum determined prior to embedding in 3% agar and sectioning at 3 mm intervals in the coronal plane using a rotary slicer. Average slice thickness was determined in each case by measuring the anteroposterior distance of the cerebral hemisphere and dividing by the number of slices obtained (average thickness 3.34 ± 0.19 mm). Microscopical examination was performed on representative areas including the cerebral cortex, hippocampus, diencephalon, cerebellum, midbrain, pons and medulla. These examinations were used to exclude the neuropathological abnormalities as outlined above.

**Quantitation of the Hippocampus**

The hippocampal formation was dissected from the right hemisphere of each of the 3 mm slices (on average 11 blocks/brain), cryoprotected in 30% sucrose for 48 h and three 48 µm thick sections cut from the caudal face of each block using a Leica freezing microtome. Frozen section thickness was verified in a single case by measuring the stage + block prior to sectioning and then remeasuring after cutting a 48 µm thick section. Sections were mounted onto gelatinized slides and the first section stained with haematoxylin and eosin, the second with cresyl violet (the Nissl substance was overstained to a very light blue in order to see the section borders, Fig. 1) and the third with the nickel peroxidase stain to identify plaque and tangle pathology (Cullen, 1994). As described in our previous study of the hippocampal formation in alcoholics (Harding et al., 1997), the boundaries of the hippocampus and its subregions were identified and drawn using the Nissl-stained sections (Figs 1 and 2) and a microfiche reader (magnification ×19). These regions were delineated according to the cellular criteria of Duvernoy (1988), which are similar but not identical to those of Amaral and Insausti (1990) and West and Gundersen (1990). Using these criteria, nine subregions (CA1, CA2, CA3, CA4, dentate gyrus, subiculum, pre- and parasubiculum, and white matter) could be defined (Figs 2 and 3), although the differentiation between the CA2 and CA3 regions and the pre- and parasubicular regions were unreliable between investigators, and therefore these regions were analysed together as the CA2–3 and presubiculum respectively. If, at low magnification, the cellular configuration of the hippocampus could not be determined with confidence, the border region in doubt was analysed under higher power to determine the cellular configuration for adequate subregion delineation (Fig. 2). Each hippocampal subregion was present in 6–12 sections (average of 9 sections/region was sampled for each case). Point counting in conjunction with Cavalieri’s principle was used to estimate volume (point size = 0.0123 mm², average slice thickness = 3.34 mm) as previously described (Double et al., 1996; Harding et al., 1997). There was no significant difference between the volume of any subregion determined using drawings by different investigators from the same case (<5% difference in points counted for every subdivision).

The total number of neurons in each subregion was estimated using the unbiased optical dissector technique described by West and Gundersen (1990) as used in our previous study (Harding et al., 1997). As significant shrinkage of frozen section occurs in the z plane during staining (Harding et al., 1994), the dissector height equivalent to the known tissue height prior to staining (48 µm) was used in all calculations. No upper or lower exclusion borders were used in the z plane as our previous analyses demonstrated that no significant variation in neuronal density occurred whether such borders were used or not (Harding et al., 1994). There is no tissue shrinkage in the x-y plane when using frozen sections as the sections are stuck to the glass slide. The dissector frames for the current protocol had dimensions of 60 × 60 µm for the CA1, CA2–3 and presubiculum; 120 × 120 µm for the subiculum and CA4; and 20 × 20 µm for the dentate gyrus. These dissector frames were generated in a regular array by a computer program and optically superimposed through a camera lucida onto the section at 60× magnification for the subiculum and CA4 and 100× magnification for the CA1, CA2–3, presubiculum and dentate gyrus. The first frame was randomly placed within the subregion and then subsequent frames sampled at regular intervals of 2.4 mm for the CA1, subiculum and presubiculum, 1.2 mm apart for the CA2–3 and CA4, and 0.4 mm apart for the dentate gyrus. As the area of each subregion in each section differed, the number of dissector frames sampled per region also differed, and an average of 85 dissector frames were sampled for the CA1, 56 in the CA2–3, 36 in the CA4, 26 in the subiculum, 31 in the presubiculum, and 48 in the dentate gyrus.

Neurons were identified by the presence of a prominent nucleolus within the nucleus and were counted throughout the section thickness. No neuron was observed to contain more than one nucleolus, such that only nucleoli contained within each dissector frame, or touching the inclusion borders of the frame (lower and left borders) were counted. In our sections we did not observe sectioned nucleoli (nuclear diameter in all dimensions was measured at 3 µm on average with the smallest measuring 2.5µm). This suggests that cut fragments of nucleoli do not remain within the frozen sections, as previously described for paraffin sections (Cammermeyer, 1967). On average four neurons were sampled per dissector frame. The average number of neurons counted in each region was 183 (20 neurons/region/section) and an average of 1096 neurons were sampled/hippocampus (119 neurons/section); however, significant variation occurred depending on the size of the region. The lowest average number of neurons counted for any subregion was 160 for the CA4 subregion, and the largest average number of neurons counted was 244 neurons for the CA1 region. The number of neurons counted

Figure 3. (A) Low-power photomontage of a cresyl violet stained section through the middle of the body of the hippocampus. (B) Reduced line drawing of the entire cresyl violet stained section indicating the subregions of the hippocampus and the area displayed in the micrograph above (box). This section is also illustrated as number 6 in Figure 3.
Figure 2. High-power photomicrographs of a cresyl violet stained section of the hippocampus at the level of the lateral geniculate nucleus demonstrating the cytoarchitecture of the various cellular regions. Cortical surface is to the top of the page. Scale is equivalent for all micrographs. (A) The CA1 pyramidal layer has pale staining neurons with a regular orientation. (B) The CA2–3 pyramidal neurons are larger, more basophilic and more densely populated than the CA1 neurons. (C) Pyramidal neurons in the CA4 are large and irregularly oriented. (D) The dentate granule cells are small and round in a tightly packed narrow band. (E) Pyramidal neurons in the superficial layer of the subiculum are smaller and arranged in clusters compared with the deeper layer of neurons (see G). (F) Small neurons are characteristic of the ‘clouds’ in layer II of the presubiculum. These are the smallest neurons found in the hippocampus. (G) Pyramidal neurons in the deep layer of the subiculum. (H) Pyramidal neurons in the deep layer of the presubiculum. These pyramidal neurons are significantly smaller than the neighbouring pyramidal neurons in the deep layers of the subiculum and entorhinal cortex.
Figure 3. Delineations of the hippocampal subdivisions analysed in spaced serial coronal sections through the hippocampal formation as indicated at right. Section intervals were \(\sim 3\) mm apart and the average hippocampal length was \(35 \pm 3\) mm. SUB, subiculum; PS, presubiculum; WM, white matter. The CA1 occupied \(34.5 \pm 5.0\)% of the grey matter volume, the SUB \(28.7 \pm 6.0\)%; the PS \(17.5 \pm 0.9\)%; the CA4 \(9.1 \pm 1.5\)%; the CA2–3 \(7.5 \pm 1.3\)% and the dentate gyrus \(2.7 \pm 0.5\)%.
within the sample volume determined the neuronal density, which when multiplied by the regional volume determined by point counting gave an unbiased estimate of neuron number. There were no significant differences in regional neuronal densities counted by different investigators from the same cases. Coefficient of variance (SD/mean) and coefficient of error (SEM/mean) for each subdivision can be calculated from the data given in Table 3. The coefficient of variance for the regional volume measurements ranged from 0.138 in the presubiculum to 0.260 for the dentate gyrus. The error ranged from 0.040 to 0.075 for subiculum, and for estimated neuron number, 0.231 for the subiculum to 0.453 for the dentate gyrus. No relationship was found to be significant with white matter volume, but a decreasing CA1 neuron number with age was found \( r = 0.309, P = 0.06 \) \( P < 0.01 \). Table 2 lists the cases studied in this study, their age and brain size, details for each of the cases included in this study and the volume for each of the subregions of the hippocampus.

### Table 1

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<th>Case no.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Cerebral volume (ml)</th>
<th>Hippocampal subregional volumes (mm³)</th>
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### Table 2

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### Volume Measures

The volume of the hippocampal formation and each of the subregions for each case is listed in Table 1. No significant correlations with age were found (Table 2). A trend was found towards decreased cerebrum volume with age \( r^2 = 0.26; P = 0.09 \) (Fig. 4A), as previously shown (Samorajski, 1976; Haug and Eggers, 1991). There was a similar trend towards a correlation between the volume of the hippocampus and the volume of the cerebrum \( r^2 = 0.31; P = 0.07 \) (Table 2). Further analysis revealed no significant relationship with white matter volume, but a significant correlation was found between the volume of the cerebrum and hippocampal grey matter volume \( r^2 = 0.48; P = 0.013 \).

### Neuronal Measures

Total estimated neuron number for each subregion of each case is listed in Table 3. No significant correlation was found between age and the number of neurons in any of the subdivisions of the hippocampal formation (Table 2). However, a trend towards decreasing CA1 neuron number with age was found \( r^2 = 0.31; P = 0.06 \) (Fig. 4B). This represents an average decrease of \( \sim 1\% \) of CA1 neurons per year, although neuronal loss was highly variable in older cases. This relationship was remarkably similar to that observed between cerebrum volume and age (see above). No other significant relationships between neuron number and age were identified (Table 2). The estimated number of CA1 neurons was the only hippocampal subregion to be correlated with

### Results

#### Cases Studied

Table 1 lists the cases analysed in this study, their age and brain volume. Retrospective examination of the medical records for all cases revealed no evidence of neurological disease. Pathological examination demonstrated that no case fulfilled the criteria for a diagnosis of Alzheimer's disease (Tierney et al., 1988; Mirra et al., 1991). Furthermore, all cases had a Braak and Braak (1991) grade of 0, indicating the absence of neurofibrillary degeneration.
Relationship between CA1 neuron number and cerebrum volume. Sex or age did not significantly correlate with CA1 neuron number but accounted for 15 and 25% of the variance in this model respectively.

\[ r^2 \text{ change for } x_1 = 0.15, F(1,10) = 1.73, P = 0.22 \]
\[ r^2 \text{ change for } x_2 = 0.25, F(1,10) = 3.66, P = 0.09 \]

In contrast, cerebrum volume significantly correlated with CA1 neuron number accounting for 33% of the variance using this model.

\[ r^2 \text{ change for } x_3 = 0.33, F(1,9) = 9.58, P = 0.015 \]

In the second model, cerebrum volume was the first regressor forced into the analysis followed by age and sex. Cerebrum volume significantly correlated with CA1 neuron number accounting for 69% of the variance.

\[ r^2 \text{ change for } x_3 = 0.69, F(1,10) = 22.04, P = 0.001 \]

Only a further 2% of the variance was accounted for by age using this model. Sex contributed a further 1% to the variance. These were not significant contributions.

\[ r^2 \text{ change for } x_2 = 0.02, F(1,9) = 0.75, P = 0.41 \]
\[ r^2 \text{ change for } x_1 = 0.01, F(1,9) = 0.36, P = 0.57 \]

When all variables were entered together, the statistical package chose the same order as model 2. The \( \beta \) coefficient for cerebrum volume was 0.70 (\( P = 0.015 \)), for age \(-0.12 (P = 0.57) \) and for sex \(-0.12 (P = 0.57) \). This indicates that cerebrum volume influences CA1 neuron number to a much greater extent than age (by a factor of \(-4\)).

**Discussion**

The findings of the present study demonstrate a large, highly significant and previously unrecognized relationship between the estimated number of neurons in the CA1 region of the hippocampal formation and the volume of the cerebral hemispheres. The relative size of the CA1 region is known to increase in humans (Seres, 1988), suggesting a possible relationship with cortical size; however, proof of this relationship has not previously been reported.
The effect of cerebral volume has not been considered in past analyses of age-related changes in CA1 neuron number (West and Gundersen, 1990; Simic et al., 1997). Moreover, our results suggest that this variable is likely to be a significant contributing factor to the number of CA1 neurons at any age. Age-associated neurodegeneration in the hippocampus has been reported using both classical (Miller et al., 1984) and unbiased (West, 1993; West et al., 1994; Simic et al., 1997) quantitative techniques. In their most recent studies West and colleagues showed a small but significant reduction in CA4 and subiculum neurons with age (West, 1993; West et al., 1994), while Simic and colleagues (1997) found a small but significant age-associated decline in CA1 and subiculum neuron numbers. These apparently contradictory findings may be reconcilable if brain size was also taken into account. It should be noted that the variations in age and cerebrum volume in our study population do not fully account for all the variance in the number of CA1 neurons. Other factors such as genetic variation between individuals and, possibly, environment may also contribute. There have been no studies analysing the effect of these variables on hippocampal size or neuron number in humans.

We have shown that the previously demonstrated relationship between brain volume and age (Miller and Corsellis, 1977; Dekaban, 1978) is mirrored for CA1 neuron number and age. Whether an ageing process significantly contributes to these brain tissue changes is difficult to answer with certainty, but our study suggests that the magnitude of any ageing effect on the number of CA1 neurons is considerably less than previously thought, particularly when brain size is accounted for. The number of individuals who can be examined in the intense fashion necessary for the measurement of these variables is relatively few, and therefore all such studies are subject to biases from case selection. In addition, all studies are by necessity cross-sectional in design and are therefore subject to cohort differences in the populations studied. This is of paramount importance in studies of brain size and neuron number as brain size is known to have changed during the past century (Miller and Corsellis, 1977). In addition, other differences between the populations sampled potentially exist. For example, major changes in nutrition, vaccination and other public health issues between those born earlier this century and those since World War II may contribute to cohort differences and therefore influence studies of ‘ageing’.

Few previous studies have considered cerebrum volume as a variable when evaluating the number of neurons in any brain region as it has been assumed that larger brains had a similar number of bigger neurons. Cullen and colleagues demonstrated a correlation between cerebral volume and the number of neurons in the nucleus basalis (Ch1), a relationship which the authors suggest may be as a result of the innervation of parenchymal blood vessels by Ch1 neurons (Cullen et al., 1997a, b). These findings are of particular interest as the quantitation of neurons was performed using the fractionator method which is independent of the volume of the structure, in contrast to the optical dissector technique. This indicates that the relationship between cerebrum volume and neuron number is unlikely to be simply an artefact of the quantitative technique used. The particularly striking relationship between cerebrum volume and neuron number in only one of the seven hippocampal regions analysed indicates a selectivity in the interaction between these variables; however, the underlying functional and anatomical correlates of this relationship are purely speculative at this time. Seres (1988) suggested that the increased size of the CA1 region in humans is attributable to increased intracortical connections that might be responsible for more sophisticated functions. A recent unbiased stereological study showed that larger brains have more neocortical neurons (Pakkenberg and Gundersen, 1997). Our results may indicate that CA1 neuron number reflects cortical neuron number. The significance of any such relationship requires further investigations.

Methodological Considerations

In any quantitative study it is necessary to understand any biases and sources of error to be confident of the interpretation of the data. The neuronal estimates were calculated using the unbiased optical dissector technique except that no inclusion/exclusion borders were used in the z plane. Several previous studies have emphasized that this would introduce significant bias into the estimates (Williams and Rakic, 1988; Oorschot, 1994). In our preparations the major tissue-processing artefact occurs during the staining and coverslipping procedures. There is no x–y shrinkage (sections are stuck to gelatinized slides) but there is substantial shrinkage in the z plane. This makes the measurement of smaller fractional dissectors in the z plane cumbersome as the dissector height needs to be corrected to the real section thickness for each individual slide. Counting nucleoli throughout the entire section thickness obviates the need for such corrections and thus improves efficiency. Depending on the artefacts introduced by tissue processing, the lack of z plane exclusion borders on the dissector frames would mean either fewer (lost nucleoli) or more (split nucleoli) estimated neurons (Williams and Rakic, 1988; Oorschot, 1994). We could not find any cut nucleoli in our preparations (see Materials and Methods), indicating that the bias in our frozen section preparations is due to lost caps. However, based on the number of nucleoli within the dissectors and the nucleoli-to-dissector volume ratio, the error in density measure due to missing nucleoli would be <0.005% (sample nucleoli volume/sample volume). This negligible error could have been predicted based on the size of the object relative to the section thickness (Coggeshall and Lekan, 1996). In addition, we have previously empirically tested whether the use of the full section thickness versus a fractionated thickness introduces substantial bias in the estimates and shown no significant difference between density counts using either method (Harding et al., 1994). Thus we believe that our optical dissector methods do not introduce significant bias into the estimates of neuronal number. Furthermore, should any errors have been introduced, these would have been uniform across the cases and therefore would not have influenced the relationship between cerebrum volume and CA1 neuron number demonstrated in this study.

Implications for the Study of Diseases Involving the Hippocampus

Our results have particular implications for the design of diagnostic techniques using in vivo imaging to distinguish age-related neurodegenerative conditions. With the development of more sophisticated and sensitive imaging techniques, brain atrophy, and in particular hippocampal atrophy, is being used to identify early Alzheimer’s disease (Jobst et al., 1994; Bobinski et al., 1997). Neurodegeneration in Alzheimer’s disease is known to affect particularly the CA1 region (Ball, 1977; Double et al., 1996; Bobinski et al., 1997). Our results suggest that the measurement of cerebrum volume may aid in standardizing in
measurements of hippocampal volume and allow small neurodegenerative changes in the CA1 region to be identified with greater certainty. In fact, changes in these volume ratios may be a more sensitive marker for the degenerative changes characterizing Alzheimer’s disease (not yet tested clinically) than current in vivo volume measurements using patients and age-matched controls. Of course, for clinical studies the possibility exists that patients with early neurodegenerative disease may be erroneously included in any aged control group, although recent longitudinal data suggests that very few cognitively normal individuals have significant neuropathology at autopsy (Rubin et al., 1998). The inclusion of patients with early neurodegenerative disease in correlative studies of structural changes with age would significantly bias the results towards finding reduced hippocampal volume with age. In vivo longitudinal imaging studies of patients taken to post-mortem would allow the analysis of degenerative changes and neuron numbers in association with volume changes over time. This may provide a more definitive evaluation of the contribution of disease versus age to any tissue pathology observed.

Notes
We wish to thank Amanda Wong, Bronwyn Lakay and Michelle Svoboda for their matchless preparation of the hippocampus sections, the application of quantitative techniques and for assistance with the figurework. This work was supported by the National Health and Medical Research Council of Australia (no. 960755) and the Medical Foundation of The University of Sydney. J.J.K. is a Medical Foundation of The University of Sydney. J.J.K. is a Medical Foundation Fellow.

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