Neuronal Volumes in Hippocampal Subfields in Delayed Post-stroke and Ageing-Related Dementias

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Running title: Hippocampal neuron volume in post-stroke dementia

Word count: Title: 93 characters; Running title: 49 characters; Number of text pages: 23; Number of figures: 3; number of tables: 3 + supplementary table; Abstract: 200 words; Total: 4879 words.

Grant sponsor: Research Councils UK; **Grant numbers**: G0700718; G0500247

Abstract

Hippocampal atrophy in relation to Alzheimer's disease (AD) is widely known. Whether neurons within hippocampal subfields are similarly affected in other ageing related dementias, particularly after stroke remains an open question. We investigated hippocampal CA3 and CA4 pyramidal neuronal volumes and densities using 3-dimensional stereological techniques in post mortem samples from a total of 67 subjects; post-stoke demented (PSD), non-demented stroke survivors (PSND) and PSD patients from the CogFAST cohort, elderly controls, and subjects diagnosed with vascular dementia, AD, and mixed AD and VaD. We found CA3 and CA4 neuronal volumes were reduced in PSD compared to PSND. The CA3 and CA4 neuronal volumes were positively correlated with post-stroke global cognitive function, but were not associated with the burden of AD pathology. There were no changes in total neuronal densities from either subfield in any of the groups studied. Our results indicated that the selective reduction in CA4 and to a lesser extent CA3 neuronal volumes was related to post-stroke cognitive impairment and ageing-related dementias. This suggests that CA4 neurons were more vulnerable to disease processes, and supports our previous finding that a reduction in hippocampal neuronal volume predominantly reflects vascular mechanisms as being causative of dementia after stroke.

Key words: Alzheimer's disease; hippocampus; neuron; post-stroke dementia; stroke; vascular dementia

Introduction

Stroke is a major risk factor for dementia (1), and up to 50% of initially non-demented stroke survivors will ultimately go on to develop delayed post-stroke dementia (PSD) (2). However, the underlying mechanisms which increase the vulnerability of stroke survivors to delayed PSD months to years after a stroke are unclear. Medial temporal lobe atrophy and hippocampal neurodegeneration are key factors in dementia, particularly Alzheimer's disease (AD) but little is known how these degenerating structures associated with learning and memory change in dementias caused by vascular disease.

We previously found the volumes of CA1 and CA2 hippocampal neurons were related to post-stroke cognitive function, and that delayed PSD subjects had 10-20% smaller neuronal soma volumes than non-demented stroke survivors (PSND) and age-matched controls. CA1 and CA2 neuronal volumes were similarly reduced in patients with vascular dementia (VaD), AD and mixed AD with VaD (3). We reasoned this reduction in neuronal volume reflected disease processes causing changes in neuronal morphometry, resulting in disrupted hippocampal circuitry and cognitive impairment. The finding that neuronal volumes were equally reduced in CA2 as was surprising as neurons in the CA1 subfield are selectively vulnerable to damage after hypoxia and in AD, whereas the CA2 is considered to be more resistant to damage (4-6). This therefore suggests that neurons in the other hippocampal subfields may also be similarly affected.

Pyramidal neurons in the CA3 and CA4 form extensive contacts with CA1 and CA2 as part of the hippocampal circuit, and CA3 neurons are particularly closely physiologically linked to CA1 through the Schaffer collaterals synapsing on CA1

dendrites as part of the classical trisynaptic hippocampal circuit (7). CA3 and CA4 neurons are also exposed to similar pathological insults as the CA1 and CA2 due to their close proximity within the hippocampal formation. Therefore, we investigated neuronal volume and density in CA3 and CA4 to determine whether neuronal changes within these subfields were also implicated in the pathogenesis of post-stroke and ageing-related dementias.

Methods

Subject Selection, Clinical Diagnosis and Tissue Acquisition

Neuronal volumes and densities were measured in the CA3 and CA4 subfields of the same hippocampal sections studied previously. Subject demographics and pathological findings are presented in Table 1. Analysis was performed on post-mortem hippocampal tissue from 24 subjects from the prospective Cognitive Function After Stroke (CogFAST) study (8). Non-demented stroke survivors >75 years old were recruited 3 months post-stroke, and received annual clinical assessments and neuropsychological testing from baseline 3 months post-stroke, including the Cambridge Assessment of Mental Disorders in the Elderly CAMCOG test, which generated subscores for cognitive domains including memory and executive function (9, 10). To investigate the effects of different disease processes, analysis was also carried out in 12 cognitively normal elderly controls, 11 VaD, 10 mixed AD and VaD, and 10 AD subjects. Final diagnoses of dementia was assigned based on Diagnostic and Statistical Manual of Mental Disorders Third Edition Revised (DSM III-R) criteria for dementia, and established neuropathological diagnostic criteria. Haematoxylin and eosin staining was used for assessment of structural integrity and infarcts, cresyl fast violet and luxol fast blue for cellular and

myelin loss, Bielschowsky silver impregnation for Consortium to Establish A Registry for Alzheimer's Disease (CERAD) rating of neuritic plaques (11), and tau immunohistochemistry for Braak staging of neurofibrillary tangles (12). A diagnosis of VaD was made based on the presence of multiple or cystic infarcts, lacunae, microinfarcts and small vessel disease with Braak stage < III (13). A diagnosis of AD was made when there was significant Alzheimer-type pathology (Braak stage V–VI and moderate to severe CERAD score) in the absence of severe vascular pathology. A diagnosis of 'mixed dementia' was made when there was evidence of VaD with AD. In patients from the CogFAST study, the burden of global vascular pathology was also calculated from the sum of ratings of vascular lesions (including arteriolosclerosis, amyloid angiopathy, perivascular space dilation, myelin loss and infarcts) in the hippocampus, frontal lobe, temporal lobe and basal ganglia to generate a score /20 (VD., RK.), as described in detail in (14). Control subjects were selected if they demonstrated no evidence of cognitive impairment or any neurological or psychiatric disease. Neuropathological examination of the control samples were confirmed to have no significant pathology.

Tissue Acquisition

Brain tissues were acquired from the Newcastle Brain Tissue Resource (Newcastle, UK), except four control cases which were obtained from the Medical Research Council London Brain Bank for Neurodegenerative Diseases (Institute of Psychiatry, London, UK). Ethical approval and permission for post-mortem research using brain tissue was granted for this project. Three 30μm thick sections were cut from pre-defined paraffin-embedded blocks of the hippocampus according to the Newcastle Brain Map (15), at the level of the pre-geniculate nucleus and the pulvinar at which

the emergence of the ventricle is visible. Sections were stained using cresyl fast violet to visualize neuronal cell bodies and nucleoli, and checked for quality and staining consistency. All cases were collected, treated and analysed in a standardized manner to minimize differential effects from processing and staining.

Stereological Analysis

Stereological analysis of neuronal soma volumes and densities was carried out using identical equipment and techniques described previously (3). Slides were coded so analysis was carried out blind to disease group. Sections were viewed using a X2.5 objective and the reference areas were delineated using stereological analysis software (Visiopharm Integration System, Hørsholm, Denmark). CA3 and CA4 subfields were defined according to *The Human Hippocampus* (16), where the CA4 was completely enclosed by the dentate gyrus, and CA3 began at the opening of the dentate gyrus where neurons became densely packed in a curve leading to the thinner band of CA2 neurons (Figure 1). 3D stereological analysis of neuronal volume and density was carried out at X100 magnification. Pyramidal neuronal density was estimated using the optical disector method (17). Each disector frame had an x-y area of 2548.66μm^2 and a depth of 18μm, excluding a guard volume ≥4μm from the top and bottom of each section, measured using a Heidenhain z-axis microcator accurate to 0.5μm (Heidenhain GB Ltd, London, UK). Pyramidal neurons were identified using established criteria, i.e. characteristic triangular soma, with darkly stained single nucleolus (18). Neuronal soma volume was measured using an independent uniform random orientated nucleator probe when the nucleolus came into focus as the probe was traversed through the z axis (19). An average of 116 neurons (±2SE = 5) in CA3 and 106 (±2SE = 8) neurons in CA4 were analysed per subfield per case. Coefficient of error values were within the acceptable range

demonstrating a high level of precision (neuronal volume in CA3 p=0.052 and CA4 p=0.073, neuronal density in CA3 p=0.051 and CA4 p=0.07)(20). Further details of equipment used are described in (3).

Statistical analyses

Statistical analyses were conducted using SPSS Version 19.0. Data were checked for normal distribution and homogeneity of variance using the Shapiro-Wilk test and Levene's tests. Group means were analysed using one-way ANOVA with post-hoc Tukey's HSD. Correlations were performed using Pearson's rank correlation. Results were considered significant when $p<0.05$.

Results

Subject demographics

Subject demographics are presented in Table 1. and the clinical features of post-stroke subjects are presented in Table 2. Fixation length and post-mortem delay (PMD) were different across all groups [$F(5,83) = 2.9$, $p = 0.019$] and [$F(5, 56) = 2.7$, $p = 0.028$] respectively. Post-hoc comparisons using the Tukey HSD test indicated that the control group mean PMD was shorter than PSD ($p = 0.054$), and the MD group mean fixation time was significantly longer than PSD ($p = 0.015$). However, neither was correlated with CA3 or CA4 neuron measurements. There were no differences in age between groups. There were no differences between PSD and PSND in CERAD, Braak or vascular pathology scores. Majority of the PSD cases met pathological criteria for VaD whereas four samples had some AD pathology and were classed as mixed VaD with AD (21).

Neuronal densities and volumes

Neuronal densities were greater in CA3 than CA4 in all groups ($p < 0.001$). There were no differences in CA3 or CA4 neuronal densities between groups. Neuronal volumes were greater in CA4 than CA3 in all groups ($p < 0.01$) except PSND ($p = 0.059$).

CA3 neuronal volumes were different between the groups [$F (5, 60) = 6.3$, $p < 0.001$]. Compared to controls, CA3 neuronal volume was reduced in PSD ($p = 0.065$) and mixed dementia ($p < 0.001$). Compared to the PSND group, CA3 neuronal volumes were reduced in PSD ($p = 0.043$), MD ($p < 0.001$). Mixed dementia CA3 neuronal volumes were lower than VaD ($p = 0.04$)(Figure 1).

CA4 neuronal volumes also differed between groups [$F = (5, 61) = 9.4$, $p < 0.001$]. Compared to controls, CA4 neuronal volumes were reduced in PSD ($p < 0.001$), mixed dementia ($p < 0.001$), AD ($p = 0.001$), and there was a trend to significance with the VaD group ($p = 0.089$). Compared to the PSND group, CA4 neuronal volumes were reduced in PSD ($p = 0.001$), mixed dementia ($p < 0.001$), and a trend to significance in AD ($p = 0.052$). Neuronal volumes in CA4 in mixed dementia were lower than in VaD ($p = 0.025$).

Neuronal volume group means are presented as a percentage of control means in Supplementary Table S1. There were no differences in neuronal volumes or densities between male and female subjects.

Clinicopathological correlations in stroke survivors

The CA3 and CA4 neuronal volumes were positively correlated with CAMCOG scores ($r = 0.526$, $p = 0.012$ and $r = 0.572$, $p = 0.004$ respectively). There were no correlations between neuronal volume and memory or executive function subscores. Neuronal density was not correlated with CAMCOG scores. Neither CA3 nor CA4 neuronal volumes were correlated with AD pathology (Braak staging or CERAD scores), global vascular pathology, or age. Correlations between neuronal volumes and CAMCOG scores remained significant when corrected for age.

Correlations between hippocampal subfields

Neuronal volumes in CA3 and CA4 were positively correlated across all subjects ($r = 0.718$, $p < 0.001$), and also correlated with previous neuronal volume measurements in CA1, CA2 and ECV (Table 3). In the post-stroke subjects only, neuronal volumes were positively correlated between CA3 and CA4 ($r = 0.718$, $p < 0.001$), CA3 and CA1 ($r = 0.612$, $p = 0.002$), CA3 and CA2 ($r = 0.418$, $p = 0.024$), CA4 and CA1 ($r = 0.750$, $p = 0.005$) and CA4 and CA2 ($r = 0.619$, $p = 0.002$).

Neuronal densities in CA3 were not correlated with densities in CA4. However, CA3 neuronal densities were positively correlated with ECV neuronal densities ($r = 0.481$, $p < 0.001$), and CA4 neuronal densities were also correlated with CA1 neuronal densities ($r=0.317$, $p=0.003$). In the post-stroke subjects only, CA3 neuronal densities were correlated with ECV neuronal density ($r=0.503$, p0.02). There were trends towards negative correlations between neuronal volume and density in CA3 ($r = -0.373$, $p = 0.08$) and CA4 ($r = -0.403$, $p = 0.051$).

Discussion

We found novel evidence of reduced neuronal volumes in hippocampal subfields CA3 and CA4 in post-stroke and ageing-related dementias. The CA3 and CA4 neuronal volumes were reduced by ~20% in PSD patients compared to non-demented stroke survivors and elderly controls, and neuronal volumes were related to post-stroke cognitive function. These results support those we previously found in CA1 and CA2, where neuronal volumes were also reduced by 10-20% in the dementia groups. Taken together, these findings suggest that neurons within all hippocampal CA subfields are similarly affected in PSD, and reflect pathological mechanisms contributing to cognitive decline.

The other dementia groups also had reduced neuronal volumes compared to controls and PSND. CA3 and CA4 neuronal volumes were reduced in mixed dementia, and CA4 neuronal volumes were reduced in AD and there was a trend in VaD. We did not find any relationships between neuronal volumes and AD pathology including amyloid or neurofibrillary tangle burden (Braak stage or CERAD score), which suggests a role for non-AD-specific processes in neuronal volume loss. However, the mixed dementia group had the most severely reduced neuronal volumes in all CA subfields, indicating that both vascular and neurodegenerative disease processes may have exacerbated mechanisms causing neuronal soma shrinkage.

CA3 and CA4 neuronal volumes were related to stroke survivors' global cognitive function but not memory scores, unlike our previous study which reported CA1 and CA2 neuronal volumes were associated with memory function. This may reflect differing roles of the CA3/CA4 neurons compared to CA1/CA2 neurons in

hippocampal information processing, as CA1 forms major outputs from the hippocampus and has been shown to be able to function independently of CA3 inputs (7, 22).

Our findings suggest that reduced neuronal volumes contribute to hippocampal atrophy widely observed in post-stroke, vascular and neurodegenerative dementias (23-28), particularly in early stages of cognitive impairment prior to significant neuron loss. However, the finding that CA3 neuronal volumes were not reduced in AD and VaD subjects may suggest that CA3 neurons were more resistant to disease specific insults inflicted by either vascular or neurodegenerative disease. In the MD group, the coexistence of both AD and CVD processes resulted in the most severely reduced neuronal volumes in all CA subfields including CA3. This may simply reflect damage to CA3 neurons caused by collective insults from both disease processes, or alternatively it may reflect increased damage to remote susceptible neurons which communicate with CA3 neurons, resulting in increased loss of targets and deafferentation. This may have caused the retraction of processes and loss of axo-dendritic arbour in CA3 neurons, which has previously been implicated in the cause of neuronal volume loss. (29, 30).

Neuron volumes in all CA subfields were significantly correlated with one another. The strongest correlations were generally found between adjacent subfields (CA4 - CA3, CA4 - CA2, CA3 - CA2 and CA2 - CA1), which make up the major connections within the hippocampal circuit (7). These relationships may be due to similar levels of exposure to disease processes, or may reflect secondary morphological changes to neurons, caused by loss of connections from or to the neurons they contact. Loss of

axo-dendritic arbour has been suggested to cause reductions in neuronal soma volume in dementia (30), and studies have found synapse loss to be an important correlate of cognitive impairment in dementia (31). However, further work is needed to determine whether neuronal soma volume changes reflect loss of axo-dendritic arbour and/or synapses in PSD.

We did not find any differences in CA3 and CA4 neuron density in PSD, VaD, mixed dementia or VaD compared to controls. Interpretation of this finding is limited by the use of neuron density rather than total neuron numbers as an indicator of neuron loss, as discussed in detail previously (3). However, studies of other brain disorders including depression and HIV-AIDS with cognitive dysfunction have also reported reductions in neuronal volumes without neuron loss (32, 33). Our results build on these findings, suggesting that neuronal volume reductions can occur in response to a variety of disease processes, resulting in changes to neuronal morphology and cognitive dysfunction even without significant neurodegeneration.

Although this study was of a relatively substantial size for a study of human brain tissue, it would require greater numbers to investigate the relationship between the observed neuronal changes and factors such as age, risk factors and number and size of infarcts. There were no associations between neuronal shrinkage and age, however as this study only investigated neuronal volumes in 75+ year olds, further work in younger controls without age-associated neuropathology would be required to determine whether neuronal volume loss also occurs in normal ageing. We did not find any associations between the number of vascular risk factors and neuronal changes in PSND and PSD subjects, which may have been limited by the sample

size, as a previous study of the whole CogFAST cohort (n ~400) found that the presence of two or more vascular risk factors was a predictor of dementia (21). We also found that it was not possible to accurately establish whether further strokes had occurred at follow-up, therefore in this subgroup of subjects it was not possible to investigate relationships between lesion number and hippocampal neuronal changes. A further limitation of this study was that tissue from controls, VaD, MD and AD subjects was collected from parallel prospective studies rather than part of the CogFAST study. However, the robust results demonstrating differences between the PSND and PSD subjects within the same cohort and almost equal burden of vascular pathology at baseline, were not attributable to differences in tissue processing or other unforeseen factors. Furthermore, all tissue was collected, treated and analyzed in a standardised manner to minimize differential tissue effects from processing and staining all cases, allowing accurate and valid comparisons to be made.

These findings provide further evidence that hippocampal neuronal soma volumes are decreased in delayed PSD and ageing related dementias, and that reduced neuronal volumes are associated with impaired cognitive function. CA4 neuronal volumes were similarly decreased in AD and VaD, indicating that neuronal volume loss occurred as a response to pathological mechanisms in distinct disease aetiologies. We did not find any significant differences in CA3 or CA4 neuron density between controls, PSND and dementia groups. Taken together, our findings suggest that the selectively reduced neuronal volumes reflect mechanisms contributing to dementia and post-stroke cognitive impairment even in the absence

of significant neuron loss or AD pathology. Further work is needed to establish the underlying vascular mechanism driving neuron volume loss.

Acknowledgements

We are very grateful to the patients, families and clinical staff for their cooperation in this study. We are indebted to Dr Tuomo M Polvikoski for his assistance with the pathological diagnosis of the PS cohort. We thank Michelle Widdrington, Carein Todd, Jean Scott, Deborah Lett and Anne Nicholson for assistance in managing and screening the cohort. Our research work is supported by grants from the Newcastle Centre for Brain Ageing and Vitality (BBSRC, EPSRC, ESRC and MRC, LLHW), UK Medical Research Council (MRC, G0500247), and the Alzheimer's Research UK (UK). Tissue for this study was collected by the Newcastle Brain Tissue Resource, which is funded in part by a grant from the UK MRC (G0400074), by the Newcastle NIHR Biomedical Research Centre in Ageing and Age Related Diseases award to the Newcastle upon Tyne Hospitals NHS Foundation Trust, and by a grant from the Alzheimer's Society and Alzheimer's Research UK (ARUK) as part of the Brains for Dementia Research Project. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, or the Department of Health.

Contributions

EG contributed to the conception and design of the study, collected, analysed and interpreted the data, and wrote the first draft of the article. RK contributed to the conception and design of the study, interpretation of data and revisions of the article

for important intellectual content. ET, AK, RH, AEO contributed to the collection and interpretation of the data and revision of the article for important intellectual content. LA provided the clinical data and computed the diagnostic scores and JO led the dementia diagnosis consensus. RK and VD performed, quantified and interpreted the neuropathological data. All authors gave final approval of the version to be published.

Conflict of Interest

The authors have no competing interests to declare.

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Tables

Table 1. Demographic details of the subjects.

	Controls	PSND	PSD	VaD	Mixed	AD
N	12	13	11	11	10	10
Age, years Mean (range)	81.9 (72-92)	84.5 (80-94)	88.7 (80-98)	86.4 (71-97)	84.6 (76-93)	82.4 (70-91)
PMD, hours Mean (range)	22.9 (8-48)	44.8 (24-96)	40.4 (10-96)	51.2 (24-84)	34.6 (11-63)	40.9 (6-72)
Section thickness, µm Mean ±2SE	25.1 (1.4)	26.3 (0.4)	27.1 (0.2)	27.3 (1.6)	25.9 (2.4)	25.8 (1.6)
Braak Stage * Mean (range)	0-1	2.8 (1-5)	2.3 (0-4)	2.1 (1-4)	4.4 (1-6)	5 (4-6)
CERAD score * Mean (range)	0-1	1.6 (0-2)	1 (0-3)	1.2 (0-2)	2.4 (1-3)	3 (3)
Vascular pathology Mean (range)	N/A	12.5 (10-16)	11.5 (8-16)	N/A	N/A	N/A

* indicates significant ($p<0.05$) differences found between group means .

Abbreviations: PSND = post-stroke non-demented; PSD = post-stroke dementia; VaD = vascular dementia; mixed = mixed VaD and Alzheimer's disease; AD = Alzheimer's disease; PMD = post-mortem delay, CERAD = Consortium to Establish a Registry for Alzheimer's disease score; n, number, N/A = no data available.

Table 2. Clinical findings in post-stroke subjects.

	PSND	**PSD**
Time from baseline-death (mo), Mean ±2SE	68.5 (32.6)	54.2 (14.4)
Total CAMCOG score (/100), Mean (range)	88.5 (76-98)	63 (24-80)
Memory subscore (/27), Mean ±2SE	22 (1.18)	17.3 (3.6)
Executive function subscore (/28), Mean ±2SE	16.9	9.6 (3)
Hemisphere with visible lesion on CT, (right, left, both none)	(3, 1, 3, 4)	(2, 4, 2, 2)

Abbreviations: CAMCOG = Cambridge Assessment of Mental Disorders in the Elderly; mo = months; PSND = Post-stroke non-demented; PSD = post-stroke dementia.

Table 3. Neuronal volume correlations between all regions

	CA3	CA2	CA1	ECV
CA4	r = 0.718, p < 0.001	r = 0.627, p < 0.001	r = 0.462, p < 0.001	r = 0.373, p = 0.001
CA3	-	r = 0.555, p < 0.001	r = 0.386, p < 0.001	r = 0.325, p = 0.005
CA2	-	-	r = 0.406, p < 0.001	r = 0.311, p = 0.012
CA1	-	-	-	r = 0.231, p = 0.05

r represents Pearson's correlation coefficient.

Figure legends

Figure 1 CA3 and CA4 subfields in the human hippocampus, stained using cresyl fast violet.

Figure 2 Neuronal volumes in CA1-4 and ECV. PSND = post-stroke non-demented, PSD = delayed post-stroke dementia, VaD = vascular dementia, MD = mixed vascular and Alzheimer's dementia, AD = Alzheimer's disease; *indicate difference to controls, + indicate difference compared to PSND; black = $p<0.05$, grey = $p<0.1$.

Figure 3. CA4 neuronal volume vs total CAMCOG score. O = Post-stroke non-demented, x = Post-stroke dementia.