Pyramidal neurones of the prefrontal cortex in post-stroke, vascular and other ageing-related dementias

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Running title: Selective neuronal changes in prefrontal cortex of post-stroke dementia

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Summary

Dementia associated with cerebrovascular disease is common. We previously found that almost 30% of elderly stroke survivors develop delayed dementia as post-stroke dementia (PSD), most of which was diagnosed as vascular dementia (VaD). The pathological substrates associated with PSD or VaD are poorly understood, particularly those associated with executive dysfunction. Three separate yet interconnecting circuits control executive function within the frontal lobe involving the dorsolateral prefrontal cortex (dlPFC), anterior cingulate cortex (ACC) and the orbitofrontal cortex (OFC). We used unbiased stereology along with immunohistological and related morphometric methods to examine densities and volumes of pyramidal neurones of the dlPFC, ACC and OFC in the frontal lobe from a total of 90 elderly subjects (age range 71-98 years). Post-mortem brain tissues from PSD and post-stroke survivors with no dementia~~non-demented stroke survivors~~ (PSND) were derived from our prospective Cognitive Function After Stroke study. ~~but w~~We also examined in parallel samples from ageing controls and similar age subjects pathologically diagnosed with Alzheimer's disease (AD), and mixed AD and VaD dementia and VaD. We found pyramidal cell volumes in layers III and V in the dlPFC of PSD and VaD and, of mixed and AD subjects to be reduced by ~~as much as~~ 40% compared to ~~those in~~ PSND and controls. There were no significant changes in neuronal volumes in either the ACC or OFC. Remarkably, pyramidal neurones within the OFC were also found to be smaller in size when compared to those in the other two neocortical regions. To relate the cell changes to cognitive function, we noted significant correlations between neuronal volumes and total CAMCOG, orientation and memory scores and clinical dementia ratings. Total estimated neuronal densities were not significantly changed between PSD and PSND groups or ageing controls in any of the three frontal regions. In further morphometric analysis of the dlPFC, we showed that neither diffuse cerebral atrophy nor neocortical thickness explained the selective neuronal volume effects. We also noted that neurofilament protein SMI31 immunoreactivity was increased in PSD and VaD compared to PSND subjects and correlated with decreased neuronal volumes in the PSD and VaD subjects. Our findings indicate selective regional pyramidal cell atrophy in the dlPFC rather than neuronal density changes *per se* ~~to be~~ are associated with dementia and executive dysfunction in PSD and VaD. The

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changes in dlPFC pyramidal cells were not associated with neurofibrillary pathology suggesting there is a vascular basis for the observed highly selective neuronal atrophy.

Key words: Ageing, Alzheimer's disease, executive function, prefrontal cortex, post-stroke dementia, stroke, vascular dementia

INTRODUCTION

It is estimated that 20% of older people suffer a stroke, and 30% of these individuals develop vascular dementia (VaD) or vascular cognitive impairment (Savva and Stephan, 2010). There is an approximately 9-fold increased risk of incident dementia immediately after the stroke and rising to a cumulative incidence of more than 23% within 10 years (Kokmen et al., 1996). We previously reported that during the follow up of a mean time of 3.8 years, more than 24% of elderly subjects had developed dementia following the first-ever cerebral ischaemic event. The underlying pathological processes determining which stroke survivors develop dementia and which remain cognitively stable are largely unknown. Dementia occurring after stroke regardless of the underlying pathology ~~included in AD or mixed dementia~~ is described as post-stroke dementia (PSD) (Leys et al., 2005). We have previously shown that the most common form of PSD fits the criteria for VaD, accounting for over 75% of all cases (Allan et al., 2011).

PSD patients exhibit a decreased ability to perform certain executive functions (Pohjasvaara et al., 1998; Pohjasvaara et al., 2002), such as working memory, planning, orientation and problem solving. This is thought to reflect changes in one or more of the three separate yet interconnecting pre-frontal circuits which control specific aspects of executive function~~;~~: the dorsolateral prefrontal cortex (dlPFC), the orbitofrontal cortex (OFC), and the anterior cingulate cortex (ACC) (Tekin and Cummings, 2002). Previous studies have linked lesions in these fronto-subcortical circuits with the executive dysfunction commonly associated with VaD (Swartz et al., 2008), where pathological changes such as vascular damage and degeneration are thought to lead to white matter degeneration commonly found in the frontal lobes (Craggs et al., 2013; Ihara et al., 2010). The white matter changes have been linked to neuronal dysfunction and degeneration, proposing a potential mechanism for decline in executive function. Pathological changes in the white matter of the frontal lobes or within the centrum semiovale in cerebrovascular disease suggests that damaged connections between these circuits may reflect loss of the large pyramidal cells (Ihara et al., 2010; Ishii et al., 1986; Pasquier et al., 2000). It is therefore plausible that similar factors affect those who develop PSD or vascular cognitive impairment (Burton et al., 2003) involving the pre-frontal circuits which may disrupt executive function including working memory.

In previousior studies, we reported that hippocampal pyramidal neurones in PSD exhibited reduced soma volumes compared to non-demented stroke survivors and ageing controls, and that this reduction was related to global cognitive dysfunction and memory impairment (Gemmell et al., 2012). We hypothesised that similar changes in the glutamatergic pyramidal neurones (Kirvell et al., 2010) in layers III (which largely project within the neocortex) and V which make up the frontal circuits and connect to subcortical pathways to the basal ganglia and thalamus (Khundakar et al., 2009; Tekin and Cummings, 2002) may relate to executive dysfunction in dementia caused by cerebrovascular disease (Allan et al., 2011). Prior investigators (Cotter et al., 2005; Rajkowska et al., 2005) have reported layer specific reductions in pyramidal neurones of older depressed subjects, a syndrome postulated to have its basis in frontal vascular pathology (Alexopoulos et al., 1997; Ongur et al., 1998). Thus the question arises whether there is a similar global loss in PSD, in which depression is also manifested (Allan et al., 2013). We therefore investigated the status of pyramidal neurones in the dlPFC, OFC and the ACC as indicators of disease mechanisms driving executive dysfunction and related cognitive status in elderly stroke survivors. By also analysing pyramidal neurones in these three circuits in subjects with VaD, Alzheimer's disease (AD) and mixed AD and VaD dementia, we aimed to elucidate the specific roles of different circuit neurones within key regions controlling frontal lobe function(s).

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Khundakar A, Morris CM, Oakley A, McMeekin W **Thomas AJ** (2009) Morphometric Analysis of Neuronal and Glial Cell Pathology in the Dorsolateral Prefrontal Cortex in Late-life Depression. *British Journal of Psychiatry* **195**(2): 163-169.

Khundakar AA, **Thomas AJ** (2009) Morphometric changes in early- and late-life major depressive disorder: evidence from postmortem studies. *International Psychogeriatrics* **21**(5): 844-54.

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Kohler S, **Thomas AJ**, Barnett NA, O'Brien JT (2010) The pattern and course of cognitive impairment in late-life depression. *Psychological Medicine* **40**(4): 591-602.

Thomas AJ, Kalaria RN, O'Brien JT (2004) Depression and vascular disease: what is the relationship? *Journal of Affective Disorders* **79**: 81-95.

MATERIALS AND METHODS

Study Design and subject demographics and clinicopathological assessment

We analysed brains from a total of 90 subjects. The demographic details of all the subjects are shown in Table 1. Brains from post-stroke survivors were acquired at autopsy from the stroke subjects recruited as part of the Cognitive Function After Stroke (CogFAST) study (Allan et al., 2011; Gemmell et al., 2012). Briefly, first time stroke patients >75 years old who remained cognitively intact 3 months post-stroke received baseline and annual comprehensive clinical and neuropsychological assessments as described previously (Desmond et al., 2000). The neuropsychometric assessments included the revised Cambridge Cognition Examination (CAMCOG) battery (Huppert et al., 1995), from which we

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generated sub-scores for cognitive domains including memory, orientation and other domains of executive function. Stroke survivors were diagnosed as having post stroke dementia (PSD) if they had met the Diagnostic and statistical manual of mental disorders (DSM) IV criteria for dementia before death. Stroke survivors who did not meet DSM IV criteria for dementia and had MMSE scores >25 and CAMCOG scores > 85 were designated as post-stroke no dementia (PSND) (Table 2). Subjects were excluded from entry to the study if they i) were younger than 75 years old, ii) had significant neurological deficits or physical illness, iii) had MMSE ≤24 points and iv) were diagnosed with dementia (DSM IV).

Brains from the AD, VaD and mixed dementia subjects were obtained from our prospective memory clinic studies as described previously (Ballard et al., 2000). Ageing control subjects aged >70 years were either part of previous prospective studies or referrals to the Newcastle Brain Tissue Resource (NBTR). They were only selected to include in this study if they had not been diagnosed with any neurological or psychiatric illness and did not have cognitive impairment. Ethical approvals for the CogFAST and prospective dementia studies were granted by local research ethics committees of the Newcastle upon Tyne Foundation Hospitals Trust. Permission for use of brains for post-mortem research was also granted by consent from next of kin or family. All the brain tissues were retained in and obtained from the NBTR.

Neuropathological examination

~~In general, n~~Neuropathological assessment was carried out as described previously (Allan et al., 2011; Gemmell et al., 2012). Briefly, haematoxylin-eosin staining was used for assessment of structural integrity and infarcts, Nissl and luxol fast blue staining for cellular patterns and myelin loss, Bielschowsky's silver impregnation and amyloid β for CERAD rating of neuritic plaques, Gallays for neuritic pathology, and tau immunohistochemistry for Braak staging of neurofibrillary tangles. A clinical diagnosis of VaD was made when there were multiple or cystic infarcts, lacunae, microinfarcts and small vessel disease, and Braak stage <III (Kalaria et al., 2004). A clinical diagnosis of AD was confirmed on evidence of significant Alzheimer's- type pathology, namely a Braak stage V-VI score, a moderate-severe CERAD score and an absence of significant vascular pathology. Mixed dementia was classified

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when there was sufficient degree of pathology to reach Braak V-VI and significant vascular pathology (Ballard et al., 2000). Vascular pathology scores were derived from the presence of vascular lesions in brain areas, including the frontal lobe at the level of the olfactory bulbs, temporal lobe at level of the anterior hippocampus, and basal ganglia at level of mamillary body. Lesions including arteriolosclerosis, cerebral amyloid angiopathy, perivascular haemosiderin leakage, perivascular space dilatation in the deep and juxtacortical white matter (WM), myelin loss, and cortical micro (<0.5 cm) and large (>0.5 cm) infarcts were recorded with increasing severity resulting in greater scores (Deramecourt et al., 2012). Tissues from control subjects bearing insufficient pathology to reach threshold for any diagnosis for dementia was classified as 'no pathological diagnosis' (Table 1). Except for the neuropathological examination (by TMP and RK), all of the morphological analyses were always undertaken under operator blinded conditions. Samples were appropriately identified with coded sequential numbers. In addition, at least 2 each positive and negative controls were included in the sample pool.

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Unbiased Stereological analyses

Paraffin-embedded coronal blocks were selected to include Brodmann areas 9, 11, and 24 containing the dlPFC, ACC and OFC respectively (Perry and Oakley, 1993). Thirty µm-thick sections were cut using a microtome and stained with cresyl fast violet using an established protocol (Khundakar et al., 2009) and then viewed using a Zeiss Axioplan Photomicroscope. Cortical layers III and V were distinguished from other layers by the presence of larger pyramidal neurones (Figure 1) according to (Khundakar et al., 2009). The reference area was mapped out at x2.5 objective using Visiopharm Integrator System (VIS) software. Approximately 40 frames were measured using a uniform random sampling technique within the reference area. At least three sections were analysed from each case, resulting in > 100 neurones analysed per case which brought the sampling error to an acceptable level of CE of P=<0.15.

Morphometric analysis of neuronal volumes and densities was carried out as described previously (Gemmell et al., 2012). Estimation of pyramidal neurone density was achieved using the optical disector method at 100x magnification. Although ideally we

would have estimated neuronal number, it is not possible to demarcate these prefrontal brain areas in order to estimate their volumes and so we used neurone density instead as previously REFS KHUNDAKAR. Neuronal volumes were estimated using an independent uniform random orientated nucleator probe (Gundersen, 1988) (Figure 2). Neuronal density was calculated from the number of cells counted within a disector box using the following equation (Sterio, 1984): $Nv = \frac{\sum^{p^-} Q^-}{P \cdot V}$ where: Nv = Numerical density, p^- = Disector samples, Q^- = Number of objects counted, P = Total number of dissectors and V = Disector box volume.

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For Brain we may get a stereologist reviewer and so I think best to try to counter this

Conventional two-dimensional analyses

Using a selection of the same Nissl stained sections as for the 3D stereological analysis images of randomly selected pyramidal neurones in cortical layers III and V of the dlPFC and OFC were taken using a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) and image capture software (Infinity Capture v4.6.0, Lumenera Corporation, Ontario, Canada) at 40x magnification. The length and width of each neurone was recorded at their longest and widest points. Using these values it was possible to work out the length to width ratio of each neurone using the following formula: Ratio=Length/width. Images were also analysed using Image Pro analysis (Craggs et al., 2013; Yamamoto et al., 2009).

To confirm volume difference observed between the OFC and the dlPFC, we also performed analyses using 2D techniques. Images of individual pyramidal neurones in the dlPFC and OFC from control cases were delineated using the wand tool. The number of pixels within the area of the delineated cell was assessed and defined using Image Pro Plus. Neurones containing more pixels were deemed to have a larger volume than those containing fewer pixels.

We determined the degree of atrophy in the dlPFC of the brains from the PSND compared to those from PSD subjects. Using the methods proposed by White and colleagues, we calculated a z score for atrophy using three markers for atrophy including the ratio of brain weight to intracranial volume, the ratio of cortical thickness to head diameter,

and neuronal loss (Gelber et al., 2012) (and personal correspondence). Brain weights were recorded at post-mortem examination of the CogFAST cases. Intracranial volume was measured from the MRI scans taken during the CogFAST study (Burton et al., 2003).

Cortical thickness was assessed from the sulcus of Brodmann area 9 of the dlPFC at 2.5x magnification. Three measurements were taken from each side of the sulcus and averaged. This was done to avoid any artefact which may result from sections that might have been cut obliquely with cortical depth appearing wider than actual size.

Head diameter was derived from a population mean as established previously (Ching, 2007). Neuronal loss was scored on a 1-8 scale (1 no loss, 8 severe) in the region of the dlPFC. All raw data were converted into a Z score allowing for each individual marker to be compared to one another. The equation $Z= (x-u)/\sigma$, where X= raw score, U= mean and σ=standard deviation was used. Each marker was assigned a percentage weight indicating how much it's Z score would influence the final result (Gelber et al., 2012): Brain weight vs. intracranial volume accounting for 50%, cortical thickness vs. head diameter for 40% and neurone density as 10%.

Additionally, we determined cortical thickness in another 60 Nissl stained cortical sections from the dlPFC (10 x 6 groups). This was assessed by on screen measurements of the prefrontal cortex at 2.5X magnification using a Zeiss Axioplan Photomicroscope. The sulcus of the relevant area (Brodmann area 9) was measured at 4-6 separate points. To remove bias produced by potential variations in the angle of cut, cortical thickness measurements were taken from opposing sides of the sulcus. The value of each side was used to calculate an overall average for the cortical region. Measurements were taken from the edge of the pial surface directly to the edge of the WM following the general direction of the neurones.

Neurofilament protein immunohistochemical analyses

Paraffin wax embedded tissue blocks containing the PFC and ACC were serially cut into 10µm or 30µm sections. Tissue sections first underwent antigen retrieval by heating in the microwave with citrate buffer for 12 minutes before being quenched with TBS and 3%

hydrogen peroxide. Sections were then blocked with serum of the secondary antibody before being immunostained with the primary antibody. For the neurofilament protein markers, tissue sections were incubated in either monoclonal antibody AT8 to phosphorylated tau (dilution 1:2,000, Innogenetics, Autogen Bioclear, UK), SMI31 (1:50,000, Alpha Center, Maryland, US) or SMI32 (dilution 1:1,000, Convance, California, US) overnight. Sections were then washed before being stained with the secondary antibody for 30 minutes. After the final wash phase the immunocomplexes were detected with diaminobenzidine (DAB). Each section stained with AT8 antibody was then qualitatively analysed and assigned a score out of 6 or quantified using 2-dimensional in vitro image analyses (Burke et al., 2013). SMI31 counts were performed on at least 10 images, taken at x10 magnification, of each case to quantify the level of damaged neurones within layers III and V of the dlPFC. The 6 x 4 grids were superimposed onto the image to aid counting and any pyramidal neurone cell body positive for SMI31 immunoreactivty independent of intensity was counted.

For the microvascular markers, 30µm thick serial sections were immunostained with antibodies to the Glucose Transporter 1 (GLUT1) (1:200, ThermoScientific UK), a marker for endothelial cells in microvessels. GLUT1 immunostained microvessel profiles were then quantified by using 3 dimensional stereological analysis s described previously (Burke et al., 2013).

Quantification of white matter changes

Ten µm coronal sections at the level which contains the dlPFC and OFC from the disease and control groups were stained with Luxol Fast Blue (LFB) and analysed using image pro as essentially described previously (Ihara et al., 2010). The median grey level of each quartile was then calculated, for example; 14.4, 43.1, 71.9, 100.6 as an estimate of staining intensity. The value was then multiplied by the % area in each quartile to calculate the myelin loss index.

For SMI32 WM analysis, Image Pro software (Mediacybernetics, USA) analysis software was used to calculate the quantity of staining, by measuring the total area of

immunoreactivity and expressing it as a percentage of area in 10 images (at 10 x magnification) of the white matter of all cases as a marker for axonal damage. To correct for the apparent white matter changes, SMI32 immunoreactivity scores were normalised to myelin index scores.

Quantification of microvascular changes

To assess the degrees of arteriolosclerosis, sclerotic index (SI) and perivascular spaces were quantified in the grey and white matter vessels of disease cases and controls. The Vascal programme (Yamamoto et al., 2009) was used to measure the external diameter (Dext) of the vessel and the diameter (Dint) of the lumen. These values were then used to calculate the SI and PVS for each vessel using the equation: $SI = 1 - (Dint/Dext)$

Statistical Analysis

Statistical analysis was carried out using SPSS Version 19.0 with the level of significance set at $p<0.05$. Normal distribution of values was first tested using the Shapiro-Wilk test. In prior analysis, data found to be not normally distributed were analysed using non-parametric methods. Group means such as PSND, PSD, VaD and AD were compared using analysis of variance (ANOVA) with post hoc Tukey tests for normal data or Kruskall Wallis and the Mann-Whitney U tests for non-normally distributed values. Spearman's rank (r^2) correlation was used to assess correlations between clinical and neuropsychometric variables or specific protein immunoreactivity measures and neuronal changes. The correlation coefficients were expressed as r.

RESULTS

Clinicopathological features of the sample

The mean ages at death were not different between the groups (Table 1). To compare PSND subjects against those who developed delayed dementia (PSD), we had divided the

post-stroke cases into two groups based on cognitive status (Table 2), which was determined at the mean time of 7.6 months prior to death. There was no significant difference between the groups in average survival time post ischaemic injury event. Thus, the presence of dementia and executive dysfunction were the only features that separated the two groups. There were no apparent differences in the burden of neurofibrillary pathology (Braak staging), amyloid β plaques (CERAD) or vascular pathology scores or the time from stroke to death between PSND and PSD groups (Table 1). PSD and VaD subjects exhibited minimal neurofibrillary pathology compared AD and mixed dementia subjects (Figure 2).

To account for the presence of any intracellular pathology in the cortical sections that could influence neuronal changes in the various dementias, we also quantified the density of hyperphosphorylated tau pathology evident by AT-8 immunoreactivity. There was negligible AT8 immunostaining which revealed no differences in hyperphosphorylated tau burden between controls, PSND, PSD, and VaD (p=1.00). However, as expected both the mixed dementia and AD groups had 4-5 fold greater tau burden compared to controls, PSND, PSD or VaD subjects (0.001) (Table 1). AT-8 immunostaining in the frontal cortex signifying local NFT pathology was correlated with the Braak scores for total brain tau burden (r=0.769, p=0.001). AT-8 staining also correlated with CERAD scores (r=0.706, p=0.001), MMSE (r=-0.543, p=0.005), and CAMCOG scores (r=-0.471, p=0.019) (Table 1).

More than 70% of the PSD cases met pathological criteria for a final diagnosis of VaD, the remainder exhibited mixed AD and VaD. The mean scores of vascular pathology in terms of small infarcts, microinfarcts, arteriolosclerosis, perivascular spacing and cerebral amyloid angiopathy (Deramecourt et al., 2012) was similar in PSND, PSD and VaD cases (Table 1). None of the cases had visible large infarcts in grey or white matter that could confound the neuronal assessments (below) but exhibited variable demyelination and axonal changes. There were no clear associations between lesion location and delayed PSD (p = 0.743).

Frontal lobe neuronal densities

We analysed neuronal densities and volumes of pyramidal cells in neocortical layers III and V in the dlPFC, ACC and OFC regions. In ageing controls, mean neuronal densities in layer III were estimated to be (per mm^3) 35,110 in the dlPFC, 49,372 in the ACC and 33,321 in the OFC in the three neocortical regions. Whereas densities in layer V were found to be (per mm^3) 39,436 in the dlPFC, 41,057 in the ACC and 30,960 in the OFC. There were no striking differences in densities between cortical layers III and V in any of the groups although pyramidal neurone densities in the ACC and OFC tended to lower in numbers in layer V. We found no significant changes related to dementia status in pyramidal neurone densities in the dlPFC or ACC. In fact, there was a consistent lack of difference in neuronal densities between PSD and PSND and ageing controls in both cortical layers and in all of the neocortical regions including the OFC (Table 3). However, neuronal densities in both layers III and V of the OFC tended to be decreased in VaD, mixed dementia and AD groups compared to ageing controls but only found to be significant when compared to the mixed dementia group (p=0.001 for both cortical layers). Neuronal densities in layers III and V were also significantly lower in mixed dementia compared to PSD (p=0.049 and p=0.028) (Table 3). The primary analysis also gave no evidence to indicate that the time period of post-mortem delay (PMD) or length of fixation (up to 40 weeks) influenced neuronal densities or volumes between the various dementia groups or controls (p>0.05).

Frontal lobe neuronal volumes

Unlike neuronal densities, there were distinct differences in neuronal size between the three cortical regions. In ageing controls, mean neuronal volumes in layer III were found to be (in μl^3) 1129 in the dlPFC, 1087 in the ACC and 732 in the OFC III in the three neocortical regions. Whereas those in layer V were found to be (in μl^3) 993 in the dlPFC, 959 in ACC and 729 in OFC. Neuronal volumes in the ACC were greater than both the dlPFC and the OFC ($p \geq 0.05$) (Figure 3) with the following order of size: ACC >dlPFC >OFC. The mean size of neurones in both layers III and V of the OFC throughout the different groups was found to be on average 50% smaller compared to those in the dlPFC (p=0.001) (cf. Figure 3). This was true for pyramidal neurones within both layers III (p=0.010) and layer V (p=0.027) of the OFC. This apparent unique observation was proposed by Constantine von Economo (von

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Economo, 2009) but precise size measurements were not reported. Our additional two-dimensional analyses showed that neurones in the OFC were significantly longer in relation to their width in both layers III (p=0.012) and V (p=0.002) compared to those found in the dlPFC. This analysis also confirmed the neuronal volume differences between the OFC and the dlPFC regions. Pyramidal neurones within the dlPFC were again found to be significantly larger than those in the OFC in layers III (p=0.010) and layer V (p=0.027).

In contrast to the neuronal densities, pyramidal neuronal volumes were found to be markedly affected by dementia. They were reduced in the dementia groups in the dlPFC compared to controls and PSND cases (p=<0.05) in both layers III and V (Figure 3). The PSD (p=0.027), VaD (p=0.012), mixed dementia (p=0.03), and AD (p=0.035) groups had reduced neuronal volumes in the dlPFC compared to controls and PSND in layer III. There were no differences in neuronal volumes in the PSND compared to controls (p=0.843). Compared to PSND, pyramidal neuronal volumes were reduced in the PSD (p=0.01), VaD (p=0.001), mixed dementia (p=0.004), and AD (p=0.005) subjects (Figure 3). Similarly, there were differences between groups within layer V of the dlPFC (p=0.05). Pyramidal neuronal volumes were reduced in PSD (p=0.007), VaD (p=0.002), mixed dementia (p=0.008), and AD (p=0.015) subjects compared to controls. Neuronal volumes in the dlPFC were also reduced in VaD subjects compared to PSND (p=0.034) subjects, who exhibited similar burden of vascular pathology (Figure 3). Different from the dlPFC, there were no measureable effects of dementia or disease on neuronal volumes in either the ACC or OFC.

Neuronal volumes and cognitive function

To relate neuronal changes to cognitive function, we examined relationships between neuronal volumes in the dlPFC and neuropsychometric measures. We found that neuronal volumes in layer III were correlated with the total CAMCOG scores (r=0.495, p=0.027), MMSE (r=0.367p=0.021) and sub-scores for orientation (r=0.509, p=0.018), as one of components of executive function. Neuronal volumes in layer V were correlated with clinical dementia ratings (r=-0.-756, p=0.003) and memory scores (r=0.486, p=0.026).

Neocortical atrophy and interlaminar neuronal volumes comparisons

We considered whether global cerebral atrophy or diffuse neocortical ribbon thinning was a factor that could explain the reduced neuronal volumes in PSD compared to PSND cases. First, concentrating solely on alterations in cortical thickness between the disease groups, or between disease groups and age-matched controls, we found there were no significant differences in cortical thickness variation between the groups in the dlPFC (Table 4). We also found no relationships between cortical thickness and length of tissue fixation (P>0.05) which could cause tissue shrinkage.

In further analysis, we found no significant differences between any marker of atrophy in any groups (p=0.193, ANOVA). However, brain weight to volume ratio was significantly lower in PSD compared to PSND subjects (p=0.022, independent t-test). Similarly, when all three factors were combined into the atrophy formula, ANOVA revealed no significances between the groups (p=0.193). The total Z score for the PSD group was calculated to be -0.160 whereas that for PSND was 0.216. There was no evidence that general atrophy differed between the two groups (p>0.05).

In an attempt to disclose differential degenerative processes within cortical cell layers across diseases, we also made interlaminar comparisons between cell volumes in layers III and V (Table 4). We found that while there was an overall correlation of neuronal volumes in the dlPFC, the interlaminar correlations for neuronal volumes were not significant in cases with and type of vascular pathology e.g. PSD, VaD, mixed dementia and PSND groups although significant relationships were noted in AD (p=0.026) and ageing controls (p=0.012). Layer III and V neurones in PSD tended to be smaller in actual volumes compared to those in VaD, and mixed dementia and PSND groups suggesting individual neuronal atrophy in different disease sates may not occur similarly across cortical layers. There were no other striking regional differences in neuronal volumes across disease types between layers III and IV (data not shown).

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Comparison of neurofilament markers in PSND, PSD and VaD in dlPFC

To further differentiate PSD and PSND subjects and compare with VaD pathology, we assessed the widely recognised neurofilament protein markers SMI31 and SMI32 in the grey and white matter, respectively. Compared to PSND subjects , SMI31 immunoreactivity in layer III was increased by 2.6-fold in PSD and by 2.3-fold in VaD cases compared to PSND subjects (Figure 4). The PSD ($p=0.004$) and VaD ($p=0.031$) subjects showed increased SMI31 neuronal immunoreactivites in layer III compatible with the decreased neuronal volumes (cf. Figure 3). However, similar degrees of increases in SMI31 were not apparent in layer V neurones either in PSD and VaD compared to PSND ($p>0.05$). Increased immunoreactivity of SMI32 was also correlated with decreased neuronal volumes in PSD and VaD cases ($r = 0.619$, $p= 0.008$) (Supplement Figure S1).

Consistent with the widespread changes in the axonal architecture, we found that SMI32 immunoreactive profiles of axons in the white matter were not significantly altered in PSD or VaD compared to PSND subjects ($p>0.05$). In parallel analyses, we found that only cases with mixed pathology had increased SMI32 immunoreactivity (data not shown). This indicated that although neuronal abnormalities (SMI31) were apparent in the grey matter, there were even more widespread and variable axonal anomalies apparent in the PSND, PSD and VaD cases in the white matter.

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Microvascular pathology in the frontal lobe in PSD and VaD

Based on our previous methods (Ihara et al., 2010), we analysed various markers in the underlying white matter to identify substrates which explain the neocortical differences in the dlPFC in PSD and PSND subjects. We found that frontal WM had nearly similar frontal myelin loss and there were no significant differences in the myelin index between PSD and PSND cases ($p=0.514$), or the combined score from all post-stroke subjects against ageing controls ($p=0.103$). However, the analysis showed VaD subjects to have significantly higher myelin loss when compared controls ($p=0.034$). Overall, the demented subjects showed comparable levels of myelin loss with no clear differences found between PSD, VaD, mixed, or AD groups ($p>0.05$).

We also determined degrees of arteriolosclerosis within the WM. This analysis did not reveal any differences between any of the groups although the sclerotic index values were greater in the white matter (0.44) compared to the cortical grey matter (0.40) in the dlPFC ($p < 0.05$). Similarly, we found no differences across the dementias or controls in either the grey or white matter (data not shown). Exploring the hypothesis that the microvasculature of the dlPFC would increase in density with increased neuronal atrophy in demented subjects, we also assessed the length density (L_v) of microvessels labelled with GLUT1 in the PSD, VaD, mixed dementia and VaD subjects compared to PSND and ageing controls. We found no significant differences between any of the groups in the dlPFC (Kruskal-Wallis $p=0.627$).

DISCUSSION

We provide novel evidence for reduced pyramidal neurone volumes in layers III and V in the dlPFC of subjects with PSD compared to PSND subjects and ageing controls. This was a regionally selective change in that the ACC and OFC were not affected. The PSD and PSND subjects had comparable burdens of vascular pathology but in the general absence of Alzheimer type neurofibrillary pathology. We further found that VaD subjects exhibited similar ~25% reduction in pyramidal neuronal volumes in the dlPFC. We also noted that AT8 immunostaining within the frontal cortex revealed negligible or no tau burden in the PSD (or VaD) and PSND subjects agreeing with the lack of differences in Braak scores between the vascular disease groups. These observations were also corroborated by the finding of increased SMI31 immunoreactivities indicating selective neuronal abnormalities in dlPFC layer III of PSD and VaD subjects. While we noted similar degree of SMI31 in layer V neurones in PSD and VaD compared to PSND ($p>0.05$) the differences in the findings between layers III and V suggests different neurodegenerative processes occur within cortical cell layers as a result of the vascular changes.

Our observations support a vascular basis for the highly specific pyramidal neurone atrophy in those subjects who develop cognitive impairment or dementia after stroke or acquire VaD. This also indicates that Alzheimer type of pathology does not play a role in the neuronal atrophy in PSD and VaD. However, neuronal volumes in layers III and V in the

same brain region of the frontal lobe were also reduced in subjects with mixed dementia and AD. While neurofibrillary pathology (AT8 and amyloid- β immunoreactivites) could have influenced neuronal size (Giannakopoulos et al., 1997). It is plausible that the observed atrophic changes in the dlPFC result from different pathogenetic mechanisms not withstanding changes in intracellular regulatory proteins within different organelles or nuclei (Love et al., 1999; Salehi et al., 1996). It has been suggested that the brain has a limited repertoire to insults, with pathologies from unrelated aetiologies display similar end stage changes (Wardlaw et al., 2003). However, this does not negate the notion that vascular disease per se or small vessel disease pathology could also play a substantial role in influencing the frontal lobe in subjects who develop AD and mixed dementia (Kalaria, 2000; Kalaria and Ihara, 2013).

The lack of a relationship between cortical thickness and disease suggests diffuse atrophy or shrinkage of the cortical ribbon, within the dlPFC is not a pathological substrate for the development of dementia or that our results are produced by artefacts of post-mortem tissue shrinkage. Furthermore, using the atrophy formula we calculated total Z scores using three different indices for the PSD and PSND cases. Overall, these findings were consistent with our observations on the selective pyramidal cell atrophy and lack of neuronal number loss in dlPFC of PSD (and VaD) subjects compared to PSND and ageing controls. However, when brain weight to volume ratios were considered as a predictor of atrophy separately, PSD ratios were shown to be significantly lower compared to PSND subjects. PSD subjects therefore appear to lose more brain mass than the PSND subjects. This suggests a more widespread pathology in which the other regions of the brain succumb to atrophy and possibly accounts for the temporal lobe (Firbank et al., 2007) and white matter (Burton et al., 2004).

The overall correlations between volumes and densities of layer III and V neurones across the dementias and controls provided internal consistency of our assessment methods. However, we found evidence to suggest that in the PSD and VaD cases particularly there were differential effects in cell volume changes between layers III and V. This is consistent with the observation of a selective atrophy and anatomical properties of the pyramidal neurones whereby those in layer III largely innervate neocortical domains

whereas those in layer V project to subcortical structures including the basal ganglia and thalamus (Molnar and Cheung, 2006).

The changes in neuronal volumes were also related to post-stroke cognitive function. We found positive correlations between neuronal volumes in layer III of the dlPFC with total CAMCOG scores and orientation, and between neuronal volumes in layer V with total memory and CDR scores. A reduced neuronal volume in the dlPFC may reflect smaller dendritic or axonal arbours with fewer connections between pyramidal neurones and aberrant neuronal networks within the fronto-subcortical circuits (Burton et al., 2003) resulting in a possible disconnection between the three major circuits and the observed cognitive function deficits (Freeman et al., 2008). This is consistent with our previous findings linking hippocampal neuronal volumes and memory function (Gemmell et al., 2012). We did not find any changes in neurone densities differentiating the PSD and PSND subjects as determined using 3-D stereology in any of the three frontal lobe regions. These observations are in agreement with previous studies (Khundakar et al., 2009; Rajkowska et al., 1999) suggesting that neuronal loss is not necessarily a prerequisite for executive dysfunction.

In comparing neuronal volumes in ageing controls and PSND subjects first, we noted that pyramidal neurones within both layers III and V in the OFC were substantially smaller than those in the dlPFC and the ACC. This was also true across the dementias. These results were confirmed using both 2-D and 3-D analyses. Upon measuring the length and width of individual neurones, it was evident that pyramidal neurones of the OFC were slender than those found in layers III and V of the dlPFC. This finding suspected previously by von Economo (von Economo, 2009) may relate to the specialised functions of the OFC neurones (Viskontas et al., 2007).

While our analysis included a substantial number of cases, it would require greater numbers to examine relationships between the observed neuronal changes and factors such as age, risk factors and more pathological markers. Another limitation of the study was 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 dlPFC neuronal changes. A further limitation of this study was

that tissue from controls, VaD, mixed 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 disease at baseline, were not attributable to genral cerebral atrophy, 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.

Conclusions

We found a highly selective effect in the frontal lobe of elderly post-stroke subjects who develop delayed dementia and in VaD subjects that is explained in the absence of any discernable neurofibrillary pathology or proteinopathy. We noted pyramidal neurone atrophy rather than loss of neuronal numbers within the dlPFC but not in the ACC and OFC suggests localised pathological changes are associated with distinct cognitive processes. We also found reduced pyramidal neuronal volumes in the OFC compared to the ACC and the dlPFC that is likely an anatomical trait of the OFC rather than related to any pathological process. Our study showed that neuronal volume reduction or atrophy rather than neuronal number loss is apparent in PSD cases suggesting high potential for therapeutic strategies (Kirvell et al., 2010) to maintain or recover neuronal function in these disease states. Further substantial work is needed to explore the differential status of dendritic arborisation and synaptic density in the three frontal lobe regions.

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Author contribution

Vincent Foster performed all experiments, developed the methodology and analysis and wrote several drafts of the manuscript.

Arthur E Oakley advised on and interpreted the stereological analysis and correct drafts of the manuscript.

Janet Y Slade performed the initial immunocytochemical analysis and advised on the analysis.

Ros Hall offered their technical skills in cutting a selection of sections for the 3D analysis.

Tuomo M Polvikoski advised on the study and performed the neuropathological analysis.

Matthew Burke performed the microvascular analysis and wrote the relevant sections of the manuscript.

Alan Thomas provided clinical input to the CogFAST study, participated in the diagnostic consensus conferences and gave intellectual support.

Ahmad Khundakar advised on and the interpretation of the stereological analysis.

Louise Allan provided clinical input to the CogFAST study, participated in diagnostic consensus conferences and gave intellectual support.

Raj N Kalaria conceived the study, performed some of the neuropathological analysis, corrected several drafts and obtained the funding.

Conflict of interest

The co-authors have no disclosures with regard to this report. The study was not industry-sponsored. There are no conflicts of interest.

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Table 1: Details of all the subject demographics and pathological features

Variable	Control	PSND	PSD	VaD	Mixed dementia	AD
Number of subjects (n=90)	Total 25	11	13	15	13	13
Age, years (range)	81.5 (71-98)	83.2 (78-88)	87.2 (80-98)	83.8 (71-97)	83.9 (72-94)	85.6 (76-96)
PMD, hr (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)
Braak stage (range)	1.5 (1-4)	2.6 (1-4)	2.6 (1-4)	1.9 (1-4)	4.9 (4-6)*	5.3 (4-6)*
CERAD (range)	NPD	1.7 (1-2)	1.3 (1-3)	0.9 (1-2)	2.7 (1-3)*	3 (3)*
Vascular pathology score† (range)	NPD	13.5 (13-14)	13.3 (9-17)	14.3 (14-15)	11.0 (6-14)	5.0* (4-5)
AT-8 Score 0-6 (range)	1.3 (1-3)	1 (1)	1.3 (1-3)	1.3 (1-3)	4.8 (2-6)	4.4 (2-6)

Numbers represent mean values with the range of values in parentheses. The causes of death included bronchopneumonia, cardiac arrest and carcinoma with no particular distribution in any group. The time period (weeks) of tissue fixation was in range 8-40 weeks for all the cases.† Vascular pathology scores were derived as described previously (Deramecourt et al., 2012). Significance: *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; NPD, no pathological diagnosis.

Table 2: Clinical features in post-stroke and VaD subjects

Variable		PSND	PSD	VaD
Time from baseline- death (months)	Mean (±2SE)	63.5 (22)	64.4 (14)	Dementia
Total CAMCOG score (/100)	Mean (range)	88.0 (83-98)	61.5 (24-80)	58 (36-80)
Memory sub-score (/27)	Mean (2SEM)	21.4 (2.8)	15 (4.3)	<15
Executive function sub-score (/28)	Mean (SEM)	16.6 (1.2)	11.1 (1.9)	<11
Clinical Dementia Rating (CDR)	Mean (2SEM)	0.1 ±0.4	1.28 (0.25)	3.0 ±0
Hemisphere with visible change or not on CT	None, right, left, both	4, 3, 1, 3	2, 2, 6, 3	na

Abbreviations: PSND = non-demented post-stroke subjects, PSD = delayed post-stroke dementia; VaD = vascular dementia, na, not available

Table 3: Neuronal densities in layers III and V of dlPFC, ACC and OFC in PSD and PSND subjects

Control and disease group	Layer III (dlPFC)	Layer V (dlPFC)	Layer III (ACC)	Layer V (ACC)	Layer III (OFC)	Layer V (OFC)
Ageing Controls	35110 ±202	39436 ±220	49372 ±397	41057 ±375	33321 ±214	30960 ±214
PSND	31584 ±216	36821 ±260	44727 ±322	42639 ±489	39605 ±408	40346 ±242
PSD	39166 ±340	41620 ±420	40692 ±287	38097 ±378	36243 ±491	41402 ±679
VaD	38148 ±291	37902 ±307	43177 ±304	38840 ±344	27027 ±217	26760 ±286*
Mixed	37205 ±433	44370 ±479	43720 ±435	44582 ±433	22737 ±139*	23645 ±325*
AD	35480 ±202	38154 ±171	39292 ±297	33252 ±397	26669 ±317	27050 ±294*
Total	36132 ±118	39739 ±132	43497 ±141	39745 ±161	31151 ±140	31627 ±160

Values represent mean ± standard error of the mean (SEM) of counts of neurones in layers III and V of the three frontal lobe regions. Total numbers are given to show consistency of numbers within layers. Significance: *indicates significant ($p<0.05$) differences found against ageing controls. There were no differences in the means between controls and PSND groups (>0.05). Abbreviations: PSND = post-stroke non-demented; PSD = post-stroke dementia; VaD = vascular dementia; mixed = mixed VaD and Alzheimer's disease; AD = Alzheimer's disease.

Table 4: Neocortical thickness and Interlaminar neuronal volumes correlations in the dlPFC

Thickness in mm	**Ageing Controls**	**PSND**	**PSD**	**VaD**	**Mixed**	**AD**
Mean (±SEM)	2.96 ±0.13	2.75 ±0.11	2.89 ±0.17	2.78 ±0.10	2.98 ±0.12	2.86 ±0.13
Volumes						
Layer III vs V R (p value)	0.696 (0.012)	**0.468 NS**	**0.181 NS**	**0.610 NS**	**0.378 NS**	0.695 (0.026)

For cortical thickness, numbers show mean ±SEM for n= 10-11 cases. Cortical depth was determined within the sulci as described in the Methods. There were no significant differences (P>0.05) in cortical thickness variation between any of the groups. For interlaminar comparisons, numbers show r values, correlation coefficients from Pearson's analysis and p values in parentheses. Interlaminar neuronal volumes were not correlated in PSND, PSD, VaD and Mixed cases (in BOLD) suggesting differential cellular changes between layers III and V. Interlaminar correlations in the ACC and OFC were consistently significant ($p<0.05$, data not shown). Abbreviations: NS, not significant ($p>0.05$), PSND = post-stroke non-demented; PSD = post-stroke dementia; VaD = vascular dementia; mixed = mixed VaD and Alzheimer's disease; AD = Alzheimer's disease..

Figure legends

Figure 1. Frontal lobe regions and Image of the nucleator principle for assessing neuronal volumes. A, shows the regions of the dlPFC, ACC and OFC where neurones were sampled per coronal atlas of the human brain (Perry and Oakley, 1993). B, an example of a pyramidal neurone in which six randomly oriented rays originating from the nucleolus were marked where they crossed the border of the neuronal soma and then the soma volume was calculated using the nucleator. P = pyramidal cell, N = non-pyramidal cell, G = glial cell. Red and green lines delineate the disector frame. Bar 20 µm

Figure 2. Neocortical neurofibrillary pathology in the dlPFC in post-stroke, vascular and other dementias. The first column shows a H&E stained section from a control subject. Cortical columns show AT8 immunoreactivity as hyperphosphorylated tau in tangles in controls, PSND, PSD, VaD, Mixed and AD subjects. Bar = 320 µm

Figure 3. Neuronal volumes in the prefrontal cortex of PSD and PSND subjects. Histograms show pyramidal cell volumes (in μl^3) in layer III (A) and layer V (B) in the dlPFC (filled columns), ACC (hatched) and OFC (stippled) in ageing controls, PSND, PSD, VaD, Mixed and AD subjects. Asterisks indicate significantly different to ageing controls ($p<0.01$) or PSND ($p<0.05$). There were no differences in the means between controls and PSND (>0.05). Abbreviations: Ctrl = Ageing controls, PSND = non-demented post-stroke subjects, PSD = delayed post-stroke dementia, VaD = vascular dementia, 'mixed' mixed Alzheimer's and vascular dementia, AD= Alzheimer's disease.

Figure 4. Intralaminar cortical and white matter neurofilament protein localization in dlPFC of PSD and PSND subjects. Panels A-D show SMI31 immunoreactivity in layer III in PSND, PSD and VaD subjects. Panels E-H show SMI32 immunoreactivity in the white matter in PSND, PSD and VaD subjects. Histograms (D and H) show mean $\pm$SEM immunoreactivities (counts) of SMI31 and SMI32 in the three groups. Significance: *indicates significant ($p<0.05$) differences found against PSND. Bar = A-C =50 µm (SMI31), E-G, 200 µm (SMI32).

Supplement Figure S1. Relationship between dlPFC layer III SMI31 reactivity in neurones and pyramidal cell volumes in PSD (red symbols) and VaD (black) subjects. There was a strong correlation between SMI31 counts and neuronal volumes ($r = 0.619$, $p= 0.008$).