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

Vincent Foster, Arthur E. Oakley, Janet Y. Slade, Roslyn Hall, Tuomo M. Polvikoski, Matthew Burke, Alan J. Thomas, Ahmad Khundakar, Louise M. Allan and Raj N. Kalaria

Institute for Ageing and Health, Newcastle University, Campus for Ageing and Vitality, Newcastle upon Tyne, NE4 5PL, UK

Correspondence to: Prof R.N. Kalaria, Institute for Ageing and Health, Newcastle University, Campus for Ageing and Vitality, Newcastle upon Tyne NE4 5PL, UK
E-mail: r.n.kalaria@ncl.ac.uk

Correspondence may also be addressed to: Mr V Foster, Institute for Ageing and Health, Newcastle University, Campus for Ageing and Vitality, Newcastle upon Tyne NE4 5PL, UK. E-mail: v.foster@ncl.ac.uk

Dementia associated with cerebrovascular disease is common. It has been reported that ~30% of elderly patients who survive stroke develop delayed dementia (post-stroke dementia), with most cases being diagnosed as vascular dementia. The pathological substrates associated with post-stroke or vascular dementia 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, anterior cingulate cortex and the orbitofrontal cortex. We used stereological methods, along with immunohistochemical and related cell morphometric analysis, to examine densities and volumes of pyramidal neurons of the dorsolateral prefrontal cortex, anterior cingulate cortex and orbitofrontal cortex in the frontal lobe from a total of 90 elderly subjects (age range 71–98 years). Post-mortem brain tissues from post-stroke dementia and post-stroke patients with no dementia were derived from our prospective Cognitive Function After Stroke study. We also examined, in parallel, samples from ageing controls and similar age subjects pathologically diagnosed with Alzheimer’s disease, mixed Alzheimer’s disease and vascular dementia, and vascular dementia. We found pyramidal cell volumes in layers III and V in the dorsolateral prefrontal cortex, anterior cingulate cortex and orbitofrontal cortex in the frontal lobe from a total of 90 elderly subjects (age range 71–98 years). Post-mortem brain tissues from post-stroke dementia and post-stroke patients with no dementia were derived from our prospective Cognitive Function After Stroke study. We also examined, in parallel, samples from ageing controls and similar age subjects pathologically diagnosed with Alzheimer’s disease, mixed Alzheimer’s disease and vascular dementia, and vascular dementia. We found pyramidal cell volumes in layers III and V in the dorsolateral prefrontal cortex, anterior cingulate cortex and orbitofrontal cortex in the frontal lobe from a total of 90 elderly subjects (age range 71–98 years). Post-mortem brain tissues from post-stroke dementia and post-stroke patients with no dementia were derived from our prospective Cognitive Function After Stroke study. We also examined, in parallel, samples from ageing controls and similar age subjects pathologically diagnosed with Alzheimer’s disease, mixed Alzheimer’s disease and vascular dementia, and vascular dementia. We found pyramidal cell volumes in layers III and V in the dorsolateral prefrontal cortex, anterior cingulate cortex and orbitofrontal cortex in the frontal lobe from a total of 90 elderly subjects (age range 71–98 years). Post-mortem brain tissues from post-stroke dementia and post-stroke patients with no dementia were derived from our prospective Cognitive Function After Stroke study. We also examined, in parallel, samples from ageing controls and similar age subjects pathologically diagnosed with Alzheimer’s disease, mixed Alzheimer’s disease and vascular dementia, and vascular dementia. We found pyramidal cell volumes in layers III and V in the dorsolateral prefrontal cortex, anterior cingulate cortex and orbitofrontal cortex in the frontal lobe from a total of 90 elderly subjects (age range 71–98 years). Post-mortem brain tissues from post-stroke dementia and post-stroke patients with no dementia were derived from our prospective Cognitive Function After Stroke study. We also examined, in parallel, samples from ageing controls and similar age subjects pathologically diagnosed with Alzheimer’s disease, mixed Alzheimer’s disease and vascular dementia, and vascular dementia. We found pyramidal cell volumes in layers III and V in the dorsolateral prefrontal cortex, anterior cingulate cortex and orbitofrontal cortex in the frontal lobe from a total of 90 elderly subjects (age range 71–98 years).
prefrontal cortex—rather than neuronal density changes per se—are associated with dementia and executive dysfunction in post-stroke dementia and vascular dementia. The changes in dorsolateral prefrontal cortex pyramidal cells were not associated with neurofibrillary pathology suggesting there is a vascular basis for the observed highly selective neuronal atrophy.

**Keywords**: ageing; Alzheimer’s disease; executive function; prefrontal cortex; post-stroke dementia; stroke; vascular dementia

**Abbreviations**: ACC = anterior cingulate cortex; CAMCOG = Cambridge Cognition Examination; OCSP = Oxford Community Stroke Project; OFC = orbitofrontal cortex; PFC = prefrontal cortex

**Introduction**

It is estimated that 20% of older people suffer a stroke, and 30% of these individuals seem to develop chronic vascular dementia or vascular cognitive impairment rather than an Alzheimer’s disease type of dementia (Altieri et al., 2004; Savva and Stephan, 2010). There is a ~9-fold increased risk of incident dementia immediately after the stroke and rising to a cumulative incidence of >23% within 10 years (Kokmen et al., 1996). We previously reported that during the follow-up of a mean time of 3.8 years, >24% of elderly subjects had developed dementia after 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, is described as post-stroke dementia (Leys et al., 2005). We have previously shown that the most common form of post-stroke dementia fits the criteria for vascular dementia, accounting for >75% of all autopsied cases (Allan et al., 2011). Depressive illness is similarly high (32%) among elderly stroke survivors (Allan et al., 2013) and even more frequent (41%) in patients with minor stroke (Altieri et al., 2012).

Patients with post-stroke dementia exhibit a decreased ability to perform certain executive functions (Pohjasvaara et al., 1998, 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 prefrontal circuits that control specific aspects of executive function: the dorsolateral prefrontal cortex (PFC), 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 vascular dementia (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 (Ihara et al., 2010; Criggs et al., 2013). 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 suggest that damaged connections between these circuits may reflect loss of the large pyramidal cells (Ishii et al., 1986; Pasquier et al., 2000; Ihara et al., 2010). It is therefore plausible that similar factors affect those who develop post-stroke dementia or vascular cognitive impairment (Burton et al., 2003) involving the prefrontal circuits, which may disrupt executive function including working memory.

In previous studies, we reported that hippocampal pyramidal neurons in post-stroke dementia 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 hypothesized that similar changes in the glutamatergic pyramidal neurons (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) (Tekin and Cummings, 2002; Khundakar et al., 2009) may relate to executive dysfunction in dementia caused by cerebrovascular disease (Allan et al., 2011). We and others (Cotter et al., 2005; Rajkowska et al., 2005; Khundakar and Thomas, 2009; Khundakar et al., 2009) have reported layer-specific reductions in pyramidal neurons of older depressed subjects, a syndrome postulated to have its basis in frontal vascular pathology (Alexopoulos et al., 1997; Ongur et al., 1998; Thomas et al., 2004; Kohler et al., 2010). Thus the question arises whether there is a similar global loss in post-stroke dementia, in which depression is also manifested (Altieri et al., 2012; Allan et al., 2013). We therefore investigated the status of pyramidal neurons in the dorsolateral PFC, OFC and the ACC as indicators of disease mechanisms driving executive dysfunction and related cognitive status in elderly stroke survivors. By also analysing pyramidal neurons in these three circuits in subjects with vascular dementia, Alzheimer’s disease and mixed Alzheimer’s disease and vascular dementia, we aimed to elucidate the specific roles of different circuit neurons within key regions controlling frontal lobe function(s).

**Materials and methods**

**Study design and subject demographics and clinicopathological assessment**

We analysed brains from 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 ischaemic stroke patients >75 years old received baseline and annual comprehensive clinical and neuropsychological assessments based on the paradigm established previously (Desmond et al., 2000). They were enrolled into the study 3 months post-stroke to enable resolution of acute post-stroke delirium with a standardized battery comprised of medical history, Mini-Mental State Examination (MMSE) score, assessment of neurological deficits, a blood screen, and review of CT brain scan...
underwent the time of the stroke. Stroke was defined according to the WHO definition and classified according to the Oxford Community Stroke project (OCSP) classification (Allan et al., 2011). Medical histories taken from the participants were supported by review of hospital charts for diagnoses of previous stroke (including whether there was any residual disability from previous stroke), hypertension (a documented history of blood pressure >140/90 mm Hg or treatment of hypertension), atrial fibrillation, ischaemic heart disease, peripheral vascular disease, hypercholesterolaemia, diabetes (documented or treated) and history of smoking prior to stroke (Ballard et al., 2003a, b).

Table 2 provides the location of the hemispheric lesions on CT and the OCSP classification of cases analysed in this study. The neuropyschometric assessments included the revised Cambridge Cognition Examination (CAMCOG) battery (Huppert et al., 1995), from which we generated subscores for cognitive domains including memory, orientation and other domains of executive function. Stroke survivors were diagnosed as having post-stroke dementia 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 with no dementia (Table 2). Subjects were excluded from entry to the study if they (i) were <75 years old; (ii) had significant neurological deficits or physical illness; (iii) had MMSE ≤24 points; and (iv) were diagnosed with dementia (DSM-V). A clinical diagnosis of whether the dementia syndrome was present was made independently of neuropathological data before monthly clinicopathological consensus meetings where clinicians met with the pathologists to designate a final diagnosis for autopsied subjects.

Brains from the Alzheimer’s disease, vascular dementia and mixed dementia cases came from subjects from our prospective memory clinic studies (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 be included 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 Newcastle Brain Tissue Resource.

Neuropathological examination

Neuropathological assessment was carried out as described previously (Allan et al., 2011; Gemmell et al., 2012). Briefly, haematoxylin and 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 Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) rating of neuritic plaques, Gallays stain for neuritic pathology, and tau...
immunohistochemistry for Braak staging of neurofibrillary tangles. A clinical diagnosis of vascular dementia was made when there were multiple or cystic infarcts, lacunae, border-zone infarcts, microinfarcts and small vessel disease, and Braak stage ≤ IV (Kalaiarasan et al., 2004). A clinical diagnosis of Alzheimer’s disease 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 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 the anterior hippocampus, and basal ganglia at level of mamillary body. Lesions including arteriosclerosis, cerebral amyloid angiopathy, perivascular haemosiderin leakage, perivascular space dilatation in the deep and juxtacortical white matter, 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 had occasional ageing-related pathology and were classified as ‘no pathological diagnosis’ (Table 1). Except for the neuropathological examination (by T.M.P. and R.K.), all of the morphological analyses were always undertaken under operator blinded conditions. Samples were appropriately identified with coded sequential numbers. In addition, at least two of both positive and negative controls were included to monitor the quality of staining.

**Cellular morphometry and stereological analyses**

Paraffin-embedded coronal blocks containing no visible infarcts or other lesions were selected to include Brodmann areas (BA) 9, 24 and 11 containing the dorsolateral PFC, ACC and OFC, respectively, at the same level from each brain (Perry and Oakley, 1993). Thirty-micrometre thick sections (average size 6 × 5 cm) 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. Previously, a minimum thickness for paraffin-embedded section of 20 μm has been recommended to minimize discrepancies due to shrinkage (Harding et al., 1994). We analysed thicker sections (30 μm) to allow a 4 μm guard volume to be removed from the top and bottom of the section to eliminate artefact caused by potential mechanical damage and maintain this recommended minimum thickness. Cortical layers III and V were distinguished from other layers by the presence of larger pyramidal neurons (Fig. 1) according to Khundakar et al. (2009). The reference area was mapped out at ×2.5 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 neurons analysed per case, which brought the sampling error to an acceptable level of coefficient of error (CE) of P < 0.15. This was consistent with the previous recommendations by Harding et al. (1994).

Morphometric analysis of neuronal volumes and densities was carried out as described previously (Gemmell et al., 2012). Estimation of pyramidal neuron density was achieved using the optical dissector method at ×100 magnification. Although ideally we would have estimated total neuronal numbers, it was not possible to demarcate these prefrontal brain areas in order to estimate their whole volumes and so we used neuron density instead as previously described (Khundakar et al., 2011). The protocol we routinely used to dissect all brains with suspected cerebrovascular disease brains involves sampling of coronal blocks from alternative slices (Hachinski et al., 2006). Neuronal volumes were estimated using an independent uniform random orientated nucleator probe (Gundersen, 1988) (Fig. 2). Neuronal density was calculated from the number of cells counted within a dissector box using the following equation (Sterio, 1984): \[ \text{Nv} = \frac{\sum_{i=1}^{N} Q_i - \sum_{i=1}^{N} \sum_{j=1}^{P_i} \frac{Q_i}{p_{ij}}}{P} \]

where: Nv = numerical density, p = dissector samples, Q = number of objects counted, P = total number of dissectors and V = dissector box volume.

**Neocortical neuron size measurements**

Using a selection of the same Nissl stained sections as for the stereological analysis images of randomly selected pyramidal neurons in cortical layers III and V of the dorsolateral PFC and OFC were used to determine their longest and widest points. The length to width ratio
of each neuron was calculated using the following formula: ratio = length/width.

To confirm cell differences, we took images of individual pyramidal neurons in the dorsolateral PFC and OFC from control cases using the wand tool and determined the number of pixels within the area of the delineated cell. Neurons containing more pixels were deemed to have a larger volume than those containing fewer pixels. All images were also analysed using Image Pro analysis (Yamamoto et al., 2009; Craggs et al., 2013).

Cerebral atrophy and cortical thickness assessments

We determined the degree of atrophy in the dorsolateral PFC of the brains from the post-stroke patients with no dementia compared to those from subjects with post-stroke dementia. Using the methods proposed by White and colleagues (Gelber et al., 2012; and personal correspondence), 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. 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 BA 9 of the dorsolateral PFC at ×2.5 magnification. Three measurements were taken from each side of the sulcus and averaged. This was done to avoid any artefact that 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 scale of 1–8 (1 = no loss, 8 = severe) in the region of the dorsolateral PFC. 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 $\sigma$ = 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 versus intracranial volume accounting for 50%, cortical thickness versus head diameter for 40% and neuron density as 10%.

Additionally, we determined cortical thickness in another 60 Nissl stained cortical sections from the dorsolateral PFC (10 × 6 groups). This was assessed by on screen measurements of the prefrontal cortex at ×2.5 magnification using a Zeiss Axioplan Photomicroscope. The sulcus of the relevant area (BA 9) was measured at four to six 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.

Figure 2 (A–F) Fluorescent stained images from the dorsolateral PFC layer III of a control subject (A), post-stroke patients with no dementia (B), patients with post-stroke dementia (C), vascular dementia (D), mixed Alzheimer’s disease/vascular dementia (E) and Alzheimer’s disease (F). Background autofluorescence from lipofuscin and red blood cells is evident in all images. E and F show abundant amyloid plaques (open arrow) and neurofibrillary tangles (solid arrows). (G) Neocortical neurofibrillary pathology in the dorsolateral PFC in post-stroke, vascular and other dementias. The first column shows a haematoxylin and eosin stained section from a control subject. Cortical columns show AT8 immunoreactivity as hyperphosphorylated tau in tangles in controls, post-stroke patients with no dementia (PSND), subjects with post-stroke dementia (PSD), vascular dementia (VaD), and mixed Alzheimer’s disease (AD). Scale bars: A–F = 50 μm; G = 320 μm; WM = white matter.
Measurements were taken from the edge of the pial surface directly to the edge of the white matter following the general direction of the neurons.

**Immunohistochemical and immunofluorescence analyses**

Paraffin wax-embedded tissue blocks containing the PFC and ACC were serially cut into 10-µm or 30-µm sections. Tissue sections first underwent antigen retrieval by heating in the microwave with citrate buffer for 12 min before being quenched with Tris-buffered saline 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 1:2000, Innogenetics, Autogen Bioclear), SMI31 (1:50000, Alpha Centre) or SMI32 (dilution 1:1000, Convance) overnight. Sections were then washed before being stained with the secondary antibody for 30 min. 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 2D in vitro image analyses (Burke et al., 2013). SMI31 counts were performed on at least 10 images, taken at ×10 magnification, of each case to quantify the level of damaged neurons within layers III and V of the dorsolateral PFC. The 6 × 4 grids were superimposed onto the image to aid counting, and any pyramidal neuron cell body positive for SMI31 immunoreactivity, independent of intensity, was counted.

To reveal the extent of both tau and amyloid-β containing structures in the dementia groups, fluorescence immunolocalization was performed in adjacent sections. The large sections were de-waxed in xylene before being rehydrated. Subsequent to antigen retrieval in boiling 10% citrate buffer (10 min) and formic acid bath (4 h), sections were blocked with both horse and goat serum (5% in PBS) for 45 min before incubation with primary antibodies, AT8 (anti-rabbit, Invitrogen) and 4G8 (anti-mouse, Covance) (1:200) overnight. Secondary antibodies were applied by first washing sections in PBS and incubating with DyLight red 550 and green 488 (1:200) for 1 h. Finally, sections were mounted in Vectashield medium (Vector Laboratories) and stored in the dark at 4 °C before examination.

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

**Quantification of white matter changes**

Ten-micrometre coronal sections at the level which contains the dorsolateral PFC and OFC from the disease and control groups were stained with Luxol Fast blue and analysed using Image-Pro software (Media Cybernetics) 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 per cent area in each quartile to calculate the myelin loss index.

For SMI32 white matter analysis, Image Pro software 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 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 normalized to myelin index scores.

**Quantification of microvascular changes**

To assess the degrees of arteriolosclerosis, sclerotic index 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 sclerotic index (SI) and perivascular spaces 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 previous analyses, data found to be not normally distributed were analysed using non-parametric methods. Group means such as post-stroke patients with no dementia, post-stroke dementia, vascular dementia and Alzheimer’s disease were compared using 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 ρ (rho) correlation was used to assess correlations between clinical and neuropsychometric variables or specific protein immunoreactivity measures and neuronal changes.

**Results**

**Clinicopathological features of the sample**

The mean ages at death were not different between the groups (Table 1). To compare post-stroke patients with no dementia against those who developed delayed dementia (post-stroke dementia), we 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 before 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. We also noted lack of differences in the distribution of stroke territories according to the OCSP classification between the patients with post-stroke dementia and post-stroke patients with no dementia (Table 2). The majority of the post-stroke cases (43%) were classified as lacunar strokes. There were also 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 post-stroke with no dementia and post-stroke dementia groups (Table 1). Subjects with post-stroke dementia and those with vascular dementia exhibited minimal neurofibrillary or amyloid pathology compared to Alzheimer’s disease and mixed dementia subjects (Fig. 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 AT8 immunoreactivity. There was negligible AT8 immunostaining that revealed no differences in hyperphosphorylated tau burden between controls, post-stroke with no dementia, post-stroke dementia, and vascular dementia groups (P = 1.00). However, as expected, both the mixed dementia and Alzheimer’s disease groups had 4- to 5-fold greater tau burden compared with control subjects, post-stroke subjects with no dementia, subjects with post-stroke dementia or subjects with vascular dementia (P < 0.001) (Table 1). AT8 immunostaining in the frontal cortex signifying local neurofibrillary tangle pathology was correlated with the Braak scores for total brain tau burden (ρ = 0.776, P = 0.001). AT-8 staining also correlated with CERAD scores (ρ = 0.714, P = 0.001), MMSE (ρ = −0.530, P = 0.002), and CAMCOG scores (ρ = −0.431, P = 0.035) (Table 1).

More than 70% of the cases with post-stroke dementia met pathological criteria for a final diagnosis of vascular dementia, the remainder exhibited mixed Alzheimer’s disease and vascular dementia. The mean scores of vascular pathology in terms of small infarcts, microinfarcts, arteriolar sclerosis, perivascular spacing and cerebral amyloid angiopathy (Deramecourt et al., 2012) was similar in post-stroke cases with no dementia, subjects with post-stroke dementia and vascular dementia (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 post-stroke dementia (P = 0.743).

**Frontal lobe neuronal densities**

We analysed neuronal densities and volumes of pyramidal cells in neocortical layers III and V in the dorsolateral PFC, ACC and OFC regions. In ageing controls, mean neuronal densities in layer III were estimated to be (per mm³) 35 110 in the dorsolateral PFC, 49 372 in the ACC and 33 321 in the OFC, whereas densities in layer V were found to be (per mm³) 39 436 in the dorsolateral PFC, 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 neuron densities in the ACC and OFC tended to be lower in number in layer V. We found no significant changes related to dementia status in pyramidal neuron densities in the dorsolateral PFC or ACC. In fact, there was a consistent lack of difference in neuronal densities between patients with post-stroke dementia, post-stroke patients with no dementia 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 the vascular dementia, mixed dementia and Alzheimer’s disease groups compared to ageing controls, but were 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 the mixed dementia compared to post-stroke dementia groups (P = 0.049 and P = 0.028) (Table 3). The primary and secondary analyses also gave no evidence to indicate that the time period of post-mortem delay or length of fixation (up to 40 weeks) influenced neuronal densities or volumes between the various dementia groups or controls (P > 0.05).

### Table 3 Neuronal densities in layers III and V of dorsolateral PFC, ACC and OFC in subjects with post-stroke dementia and post-stroke patients with no dementia

<table>
<thead>
<tr>
<th>Control and disease group</th>
<th>Layer III (dIPFC)</th>
<th>Layer V (dIPFC)</th>
<th>Layer III (ACC)</th>
<th>Layer V (ACC)</th>
<th>Layer III (OFC)</th>
<th>Layer V (OFC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ageing controls</td>
<td>35 110 ± 202</td>
<td>39 436 ± 220</td>
<td>49 372 ± 397</td>
<td>41 057 ± 375</td>
<td>33 321 ± 214</td>
<td>30 960 ± 214</td>
</tr>
<tr>
<td>Post-stroke with no dementia</td>
<td>31 584 ± 216</td>
<td>36 821 ± 260</td>
<td>44 727 ± 322</td>
<td>42 639 ± 489</td>
<td>39 605 ± 408</td>
<td>40 346 ± 242</td>
</tr>
<tr>
<td>Post-stroke dementia</td>
<td>39 166 ± 340</td>
<td>41 620 ± 420</td>
<td>40 692 ± 287</td>
<td>38 097 ± 378</td>
<td>36 243 ± 491</td>
<td>41 402 ± 679</td>
</tr>
<tr>
<td>Vascular dementia</td>
<td>38 148 ± 291</td>
<td>37 902 ± 307</td>
<td>43 177 ± 304</td>
<td>38 840 ± 344</td>
<td>27 027 ± 217</td>
<td>26 760 ± 286*</td>
</tr>
<tr>
<td>Mixed</td>
<td>37 205 ± 433</td>
<td>44 370 ± 479</td>
<td>43 720 ± 435</td>
<td>44 582 ± 433</td>
<td>22 737 ± 139*</td>
<td>23 645 ± 325*</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>35 480 ± 202</td>
<td>38 154 ± 171</td>
<td>39 292 ± 297</td>
<td>33 252 ± 397</td>
<td>26 669 ± 317</td>
<td>27 050 ± 294*</td>
</tr>
<tr>
<td>Total</td>
<td>36 132 ± 118</td>
<td>39 739 ± 132</td>
<td>39 497 ± 141</td>
<td>39 745 ± 161</td>
<td>31 151 ± 140</td>
<td>31 627 ± 160</td>
</tr>
</tbody>
</table>

Values represent mean ± standard error of the mean (SEM) of counts of neurons 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 post-stroke patients with no dementia groups (> 0.05).

dIPFC = dorsolateral PFC.
Neuronal volumes in the prefrontal cortex of subjects with post-stroke dementia and post-stroke patients with no dementia. Histograms show pyramidal cell volumes (in μm$^3$) in layer III (A) and layer V (B) in the dorsolateral PFC (filled columns), ACC (hatched) and OFC (stippled) in ageing controls, post-stroke patients with no dementia, subjects with post-stroke dementia, vascular dementia, mixed and Alzheimer’s disease. Asterisks indicate significantly different to ageing controls ($P<0.01$) or post-stroke patients with no dementia ($P<0.05$). There were no differences in the means between controls and post-stroke patients with no dementia ($P>0.05$). 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.

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 patients with post-stroke dementia compared with post-stroke patients with no dementia. 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 dorsolateral PFC (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 subjects with post-stroke dementia compared to post-stroke patients with no dementia ($P=0.022$, 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 post-stroke dementia group was calculated to be $-0.160$ whereas that for post-stroke patients with no dementia was $0.216$. There was no evidence that general atrophy differed between the two groups ($P>0.05$).

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 dorsolateral PFC, the interlaminar correlations for neuronal volumes were not significant in cases with any type of vascular pathology e.g. post-stroke dementia, vascular dementia, mixed dementia and post-stroke with no dementia groups although significant relationships were noted in Alzheimer’s disease (P = 0.026) and ageing controls (P = 0.012). Layer III and V neurons in post-stroke dementia tended to be smaller in actual volume compared to those in vascular dementia, and mixed dementia and post-stroke with no dementia groups suggesting individual neuronal atrophy in different disease states may not occur similarly across cortical layers. There were no other striking regional differences in neuronal volumes across disease types between layers III and V (data not shown).

| Table 4 Neocortical thickness and interlaminar neuronal volumes correlations in the dlPFC |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Thickness (mm)  | Ageing controls | Post-stroke with no dementia | Post-stroke dementia | Vascular dementia | Mixed Alzheimer’s disease |
| Mean (±SEM)     | 2.96 ± 0.13     | 2.75 ± 0.11     | 2.89 ± 0.17     | 2.78 ± 0.10     | 2.98 ± 0.12     |
| Volumes         | 0.696 (0.012)   | 0.468 n.s.      | 0.181 n.s.      | 0.610 n.s.      | 0.378 n.s.      |

For cortical thickness, numbers show mean ± SEM for n = 10–11 cases. Cortical depth was determined within the sulci as described in the ‘Materials and methods’ section. 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 post-stroke with no dementia, post-stroke dementia, vascular dementia and mixed cases (in bold) suggesting differential cellular changes between layers III and V. Interlaminar correlations in the ACC and OFC were typically significant (P < 0.05, data not shown). n.s. = not significant (P > 0.05).

**Comparison of neurofilament markers in post-stroke with no dementia, post-stroke dementia and vascular dementia groups in the dorsolateral prefrontal cortex**

To further differentiate subjects with post-stroke dementia and post-stroke with no dementia and compare with vascular dementia pathology, we assessed the widely recognized neurofilament protein markers SMI31 and SMI32 in the grey and white matter, respectively. Compared to post-stroke patients with no dementia, SMI31 immunoreactivity in layer III was increased by 2.6-fold in patients with post-stroke dementia and by 2.3-fold in cases with vascular dementia compared with post-stroke patients with no dementia.
dementia (Fig. 4). The subjects with post-stroke dementia ($P = 0.004$) and vascular dementia ($P = 0.031$) showed increased SM131 neuronal immunoreactivities in layer III compatible with the decreased neuronal volumes (cf. Fig. 3). However, similar degrees of increases in SM131 were not apparent in layer V neurons either in patients with post-stroke dementia and vascular dementia compared with post-stroke patients with no dementia ($P > 0.05$). Increased immunoreactivity of SM131 was also correlated with decreased neuronal volumes in cases with post-stroke dementia and vascular dementia ($p = 0.573$, $P = 0.016$) (Supplementary Fig. 1).

Consistent with the widespread changes in the axonal architecture, we found that SM132 immunoreactive profiles of axons in the white matter were not significantly altered in subjects with post-stroke dementia or vascular dementia compared with post-stroke patients with no dementia ($P > 0.05$). In parallel analyses, we found that only cases with mixed pathology had increased SM132 immunoreactivity (data not shown). This indicated that although neuronal abnormalities (SM131) were apparent in the grey matter, there were even more widespread and variable axonal anomalies (SM132) apparent in the white matter of cases with post-stroke with no dementia, post-stroke dementia and vascular dementia.

### Microvascular pathology in the frontal lobe in post-stroke dementia and vascular dementia

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 dorsolateral PFC in subjects with post-stroke dementia and post-stroke patients with no dementia. We found that frontal white matter had nearly similar frontal myelin staining loss and there were no significant differences in the myelin index between cases with post-stroke dementia and post-stroke with no dementia ($P = 0.514$), or the combined score from all post-stroke subjects against ageing controls ($P = 0.103$). However, the analysis showed subjects with vascular dementia to have significantly higher myelin staining loss when compared with controls ($P = 0.034$). Overall, the demented subjects showed comparable levels of myelin loss with no clear differences found between post-stroke dementia, vascular dementia, mixed, or Alzheimer’s disease groups ($P > 0.05$).

We also determined degrees of arteriolosclerosis within the white matter, which upon scanning CT or MRI had shown variable degrees of leukoaraiosis (Burton et al., 2004). 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 dorsolateral PFC ($P < 0.05$). 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 dorsolateral PFC 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 subjects with post-stroke dementia, vascular dementia, mixed dementia and vascular dementia compared with post-stroke patients with no dementia and ageing controls. We found no significant differences between any of the groups in the dorsolateral PFC ($P = 0.627$, Kruskal-Wallis test).

### Discussion

We provide novel evidence for reduced pyramidal neuron volumes in layers III and V in the dorsolateral PFC of subjects with post-stroke dementia compared to post-stroke patients with no dementia and ageing controls. This was a regionally selective change in that neurons in the ACC and OFC regions did not show similar changes. The subjects with post-stroke dementia and those post-stroke subjects with no dementia had comparable burdens of vascular pathology, but in the general absence of Alzheimer’s disease-type neurofibrillary pathology. We further found that subjects with vascular dementia exhibited a similar ~25% reduction in pyramidal neuronal volumes in the dorsolateral PFC. We also noted that AT8 immunostaining within the frontal cortex revealed negligible or no tau burden in the subjects with post-stroke dementia (or vascular dementia) and post-stroke patients with no dementia agreeing with the lack of differences in Braak scores between the vascular disease groups. These observations were also corroborated by the finding of increased SM131 immunoreactivities indicating selective neuronal abnormalities in dorsolateral PFC layer III of subjects with post-stroke dementia and vascular dementia. Although we noted similar degree of SM131 in layer V neurons in patients with post-stroke dementia and vascular dementia compared with post-stroke patients with no dementia ($P > 0.05$), 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 suggest a vascular basis for the highly specific pyramidal neuron atrophy in those subjects who develop cognitive impairment or dementia after stroke or acquire vascular dementia. This indicates that the Alzheimer’s disease type of pathology does not play a role in the neuronal atrophy in post-stroke dementia and vascular dementia. 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 Alzheimer’s disease. These observations are also consistent with the differential degrees of atrophy as revealed by assessing cortical volumes even in the frontal lobe in Alzheimer’s disease (Halliday et al., 2003). However, while neurofibrillary pathology (AT8 and amyloid-β immunoreactivities) could have influenced neuronal size (Giannakopoulos et al., 1997), it is plausible that the observed atrophic changes in the dorsolateral PFC result from different pathogenetic mechanisms not withstanding changes in intracellular regulatory proteins within different organelles or nuclei (Salehi et al., 1996; Love et al., 1999). It has been suggested that the brain has a limited repertoire to insults, with pathologies from unrelated aetiologies displaying 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 Alzheimer’s disease 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 dorsolateral PFC, seems not to be a pathological substrate for the development of dementia or that our results are necessarily produced by artefacts of post-mortem tissue shrinkage (Harding et al., 1994). Furthermore, using the atrophy formula we calculated total Z-scores using three different indices for the post-stroke dementia and post-stroke with no dementia cases. Overall, these findings were consistent with our observations on the selective pyramidal cell atrophy and lack of neuronal number loss in dorsolateral PFC of post-stroke dementia (and vascular dementia) subjects compared with post-stroke patients with no dementia and ageing controls. When brain weight to volume ratios were considered as a predictor of atrophy separately, post-stroke dementia ratios were shown to be significantly lower compared to post-stroke with no dementia. Subjects with post-stroke dementia therefore seem to lose more brain mass than the post-stroke patients with no dementia. 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 neurons across the dementias and controls provided internal consistency of our assessment methods. It is plausible that due to a high interindividual variations in atrophy rates or neuronal densities, we were unable to detect any significant changes in neuronal densities in any of the frontal lobe cortical regions as would be predicted by atrophy volumes in different disorders (Kril et al., 1997). However, we found evidence to suggest that in the cases with post-stroke dementia and vascular dementia 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 neurons 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 dorsolateral PFC with total CAMCOG scores and orientation, and between neuronal volumes in layer V with total memory and Clinical Dementia Rating scores. A reduced neuronal volume in the dorsolateral PFC may reflect smaller dendritic or axonal arbours with fewer connections between pyramidal neurons 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). Although it is possible that the resultant neuronal changes are explained by diaschisis, our observations here are consistent with previous findings linking hippocampal neuronal volumes and memory function (Gemmell et al., 2012). We did not find any changes in neuron densities differentiating the subjects with post-stroke dementia and post-stroke with no dementia as determined using 3D stereology in any of the three frontal lobe regions. These observations are in agreement with previous studies (Rajkowska et al., 1999; Khundaker et al., 2009) suggesting that neuronal loss is not necessarily a prerequisite for executive dysfunction.

When comparing neuronal volumes in ageing controls and post-stroke patients with no dementia first, we noted that pyramidal neurons within both layers III and V in the OFC were substantially smaller than those in the dorsolateral PFC and the ACC. This was also true across the dementias. These results were confirmed using both 2D and 3D analyses. On measuring the length and width of individual neurons, it was evident that pyramidal neurons of the OFC were slender than those found in layers III and V of the dorsolateral PFC. This finding, suspected previously by von Economo (2009), may relate to the specialized functions of the OFC neurons (Viskontas et al., 2007).

Although 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. It was also not possible to establish accurately 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 dorsolateral PFC neuronal changes. A further limitation was that tissue from controls, subjects with vascular dementia, mixed and Alzheimer’s disease was collected from parallel prospective studies rather than part of the CogFAST study. However, the robust results demonstrating differences between the post-stroke patients with no dementia and those with post-stroke dementia within the same cohort and almost equal burden of vascular disease at baseline, were not attributable to general cerebral atrophy, differences in tissue processing or other unforeseen factors. A technical limitation was lack of availability of the whole reference volume (the dorsolateral PFC, OFC and ACC). The tissue was obtained from predefined coronal slices and only one to two blocks per case were available for sampling. As a result of these inherent issues, unlike others (Thune et al., 2001; Halliday et al., 2003; Courchesne et al., 2011) we were unable to estimate total neuron numbers per cortical area, especially of the dorsolateral PFC. The total numbers of neurons in the adult PFC are estimated to be 1.16–1.55 billion (age range 2–85 years) (Thune et al., 2001; Courchesne et al., 2011).

However, if one assumes that the density of total neurons within a structure such as the PFC acts as a proxy for their total number, one must also make the assumption that the reference volume of the structure itself remains unchanged across the groups measured. Though we cannot entirely rule out the effects of some shrinkage, all sections were treated identically and assessment of section thickness revealed no significant differences between groups indicating actual relative densities were measured between groups. Furthermore, all tissue was collected, processed and analysed in a standardized manner to minimize differential tissue effects and staining in all cases, allowing accurate and valid comparisons to be made.
Conclusion

We found a selective effect in the frontal lobe of elderly post-stroke subjects who develop delayed dementia in and vascular dementia subjects that is explained in the absence of any discernable neurofibrillary pathology or proteinopathy. We noted pyramidal neuron atrophy rather than loss of neuronal numbers within the dorsolateral PFC, but not in the ACC and OFC, suggests localized pathological changes are associated with distinct cognitive processes. We also found reduced pyramidal neuronal volumes in the OFC compared with the ACC and the dorsolateral PFC 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 cases with post-stroke dementia 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 arborization and synaptic density in the three frontal lobe regions.

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Supplementary material

Supplementary material is available at Brain online.

References


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