Mechanisms of Regeneration in Post Stroke Dementia

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Dissertation submitted for the degree of Doctor of Philosophy in Newcastle University
October, 2013

Abstract

Stroke is a leading cause of morbidity, disability and cognitive impairment. Approximately, 25% of post stroke survivors develop dementia in the subsequent five years after initial cerebrovascular injury. However, the majority of individuals maintain their cognitive function. The cognitive function after stroke (CogFAST) study was established to assess the long term consequences of ischemic injury in the elderly, determine the types of dementia inherent in stroke survivors and to elucidate the pathological substrates of cognitive impairment. The aim of this specific study was to determine whether regenerative mechanisms, specifically angiogenesis and neurogenesis, could explain the variance in cognitive status of post stroke survivors; those who succumbed to dementia (PSD) compared to those who did not (PSND). We also compared subjects with Alzheimer's disease (AD) and vascular dementia (VaD) and aged match controls. Quantitative histopathological methods were used to assess microvascular density and neurogenic markers in post-mortem human tissue.

Vascular density was assessed using a morphometric technique based on stereology and found significant increases in both PSD and AD compared to all other groups. A significant increase in vessel diameter was also observed in PSND compared to other groups. Changes in expression of pro-angiogenic molecules hypoxic inducing factor 1α, integrin and vascular endothelial growth factor were assessed, with an increase reported in PSD and AD. Differences in expression of neurogenic markers in the dentate gyrus were noted, generally an increase was observed in a number of markers in VaD subjects compared to other groups. Significant decreases in expression in PSND and AD were also found compared to control groups. A reduction in expression was reported, in all dementia groups, for Hu C/D. Further analysis was performed using the neuronal marker Hu C/D in the hippocampus, which has been suggested to be involved in neuronal maintenance and plasticity. An increased in expression in the Cornu Ammonis 1 (CA1) in PSND subjects compared to all dementia groups. Significant reductions were also reported in VaD cases in a number of regions of the hippocampus compared to controls and PSND subjects.

Although, significant differences were found between PSND and PSD cases, the data did not produce conclusive evidence that either angiogenesis or neurogenesis could explain the

maintenance of cognitive function in PSND subjects. A number of similarities were also found between PSD and AD subjects, which may suggest an underlying similar mechanism.

Acknowledgements

First and foremost, I would like to thank my supervisor, Professor Raj Kalaria, who has given me this opportunity and supported me throughout my PhD with his encouragement, enthusiasm and outstanding knowledge of dementia neuropathology, especially in regard to vascular impairment. His personal support and expert supervision have been invaluable, contributing greatly to the completion of this work.

My eternal gratitude goes to Mr. Arthur Oakley; words cannot express the thanks I owe to him. He has always listened and answered my questions, helped me generate lots of good ideas, assisted me whenever I have required help and provided encouragement, advice, good teaching and even better company.

I would like to give special thanks to Dr. Ahmad Khundakar, who introduced me to stereology, which constituted one of the vital chapters in this thesis.

I have been fortunate to be involved with a truly excellent group of people in my daily work. I am grateful to Janet Slade, Ros Hall and Mary Johnson for sharing their great knowledge and extensive techniques in immunohistochemistry. I also wish to extend my appreciation to Dr. Lucy Craggs, Elizabeth Gemmell, Vincent Foster, Dr. Rufus Akinyemi, Paul Keane and especially Dr. Yumi Yamamoto, who developed the VasCalc software. I would like to thank for their assistance Lucy Nelson, Emma Yates, Zeynep Elcioglu, Ross Fisher and all the staff at the Newcastle Brain Tissue Resource Centre for their support and expertise. The research documented within this thesis could not have been performed without the sacrifice and generosity of all the patients that have been involved in the cognitive function after stroke study and my heartfelt thanks goes to them and their families.

My PhD was funded by Alzheimer Research UK. I thank them for their support and I look forward to continuing a successful relationship with them, throughout my career. Finally, I would like to thank Eleanor Glendenning and my family for supporting me throughout my studies

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List of figures and tables

Abbreviations

AChE	Acetylcholinesterase
AD	Alzheimer's disease
Ang	Angiopoietin
ANOVA	Analysis of variance
AOI	Area of interest
APES	Aminopropyltriethoxysilane
APP	Amyloid precursor protein
Aβ	Amyloid plaques
BA36	Brodmann area 36
BM	Basement membrane
BRC	Brain reserve capacity
BrdU	Bromodeoxyuridine
CA	Cornu Ammonis
CAA	Cerebral amyloid angiopathy
CBF	Cerebral blood flow
CE	Coefficient of Error
CogFAST	Cognitive function after stroke
COL4	Collagen type 4
DAB	3,3′-Diaminobenzidine
DCX	Doublecortin
DG	Dentate gyrus
DLB	Dementia with Lewy bodies
Dll4	Delta-like 4
DLPFC	Dorso-lateral prefrontal cortex
dpi	Dots per inch
EC	Entorhinal cortex
ECM	Extracellular matrix
ECs	Endothelial cells
EGFL	Epidermal growth factor like domain
ELAV	Embryonic lethal abnormal vision
EPO	Erythropoietin

FGF	Fibroblast growth factor
GCL	Granular cell layer
GLUT1	Glucose transporter 1
Hif-1α	Hypoxia inducible factor 1α
IHC	Immunohistochemistry
IOD	Integrated optical density
L_v	Length density
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MEOX	Mesenchyme homeobox 2
MRI	Magnetic resonance imaging
MSI1	Musashi 1
NBTR	Newcastle brain tissue resource
NFT	Neurofibrillary tangle
NOS	Nitric oxide synthases
PCA	Posterior cerebral artery
PDGF	Platelet derived growth factor
PS	Post stroke
PSAnCAM	Poly-sialylated neural cell adhesion
PSD	Post stroke dementia
PSND	Post stroke non dementia
RBCs	Red blood cells
RBP	Rostral migratory stream
ROBO	Roundabout receptor
SGL	Subgranular layer
SMA	smooth muscle α-actin
SNP	Single nucleotide polymorphism
SVD	Small vessel disease
SVZ	Subventricular zone of the lateral ventricles
Tg	Transgenic
TGF	Transforming growth factor
VaD	Vascular dementia
VEGF	Vascular endothelial growth factor
WM	White matter

WMH	White matter hyperintensities

List of Original Publications

This thesis is based on the following original publications and some unpublished data

I. **Matthew Burke,** Janet Y Slade, Lucy Nelson, Arthur E Oakley, Ahmad A Khundakar, Raj N Kalaria. Morphometry of the hippocampal microvasculature in post-stroke and age-related dementias. *Accepted Neurobiology and Applied Neuropathology 05 Sep 2013, electronic publication ahead of print*

II. **Matthew Burke,** Mary Johnson, Arthur E Oakley, Johannes Attems, Elaine Perry, Raj N Kalaria. Hippocampal Neuronal Progenitor Cell Markers in Post-Stroke Dementia. *In preparation*

III. **Matthew Burke,** Janet Y Slade, Mary Johnson, Ross Fisher, Arthur Oakley, Raj N Kalaria. Changes in expression of Hu C/D across the hippocampus in cases of dementia. *In preparation*

Chapter 1 Introduction

1.1 Introduction

Cerebrovascular disease is one of the major causes of disability and death in many developed countries. Improving stroke mortality rates (Centers for Disease and Prevention, 1999, Kleindorfer and Khatri, 2013), means patients can often be left with disabilities and cognitive impairment such as dementia. It is essential that pathogenetic mechanisms are understood, which should help to reduce the outcomes associated with the risk factors.

Stroke is the result of a reduction in blood flow to a region of the brain or globally causing a loss of glucose and oxygen, refered to as ischemia, both of which are required for normal cell function. Strokes or ischemic incidents are graded differently dependent on the region affected; globally (entire brain tissue) or focally (specific region surrounding affected vessel). Global ischemia is caused by temporary interpution or severe reduction of blood flow possibly as a result of cardiac arrest. Focal ischemia is the result of interruption of blood flow to a single vessel. Stroke can also vary in type, thereare two variations ischemic or haemorraghic. Ischemia describes when a vessel is blocked as a result of an embolism, thrombosis or the result of arteriosclerosis. Rupture of blood vessel and the expulsion of blood and subsequent damage to the surrounding tissue are classified as a haemorrahage. Clinical syndromes of a stroke will vary depending on the location of the lesion but symptoms may include contraleteral hemiparesis, homonymous hemianopsia, aphasia, apulia and ipsalateral gaze preference and there on set will often be sudden within seconds or minutes.

Dementia is a clinical syndrome manifesting itself from a progressive degenerative disorder, which mainly affects the elderly population. It is clinically characterised by loss of memory, aggression, language difficulties and executive function. Stroke increases the risk of dementia whereas cognitive impairment itself could increase the risk of a stroke (Pendlebury and Rothwell, 2009). Due to the high risk of stroke in the elderly population there is a growing need to understand the clinical and pathological progression leading to post stroke dementia (PSD).

Alzheimer's disease (AD), vascular dementia (VaD) and dementia with Lewy bodies (DLB) are the most common forms of dementia. These dementias are considered as distinct separate diseases of old age unconnected in terms of pathogenesis, but it is now widely recognised that there can be a high degree of overlap and common risk factors are associated with these dementias (Premkumar et al., 1996). In 1997, Snowdon et al reported that those who have experienced lacunar infarcts in the basal ganglia, thalamus, or deep white matter (WM) required less of the classical pathological hallmarks of AD to develop dementia. Patients with lacunar infarcts in these regions had a higher prevalence of dementia, at the time of meeting the AD criteria. However, if they did not meet criteria there was only a weak association between infarcts and dementia (Snowdon et al., 1997). Subsequent studies have also supported this finding (Heyman et al., 1998, Kalaria and Ballard, 1999).

Although, a stroke will increase a person's risk of developing dementia, a number of cases maintain their cognitive function and there are reports that people may improve their score on a clinical test over time (Desmond et al., 1996, Ballard et al., 2003a). It has been speculated that some people may posses a "reserve" (brain or cognitive), so that they are able to maintain cognitive functions (Stern, 2009). This study will examine the possibility that regenerative mechanisms, particularly angiogenesis and neurogenesis, are able to explain

why subjects can maintain cognitive function, post stroke (PSND). The mechanisms and history, including arguments for and against involvement in post stroke (PS) and dementia, will be discussed below.

1.1.1 Post stroke dementia

Stroke is associated with a fivefold increased risk of dementia and people who suffer with dementia are at an increased risk of a stroke (Pendlebury, 2009). Post stroke dementia (PSD) is defined as any dementia occurring after a stroke. Although, VaD is often a consequence of stroke and is associated with ischemic incidents, not all who have a stroke necessarily develop dementia that would be defined as VaD but could develop AD. Therefore there is a need to differentiate cases and categorising cases as PSD allows for this (Leys et al., 2005).

Varying results from epidemiological studies have suggested that the prevalence of PSD after one year may range from 4.8-53.1%. The highest percentages are associated with hospital based studies, with recurrent stroke and pre-stroke dementia cases often being included, whilst the lower rates are observed in population based studies and when pre-stroke is excluded (Pendlebury and Rothwell, 2009). A long term study reported 7% of subjects had PSD after the first year and 48% had developed PSD after 25 years (Kokmen et al., 1996). Separate studies have reported cases where a number of stroke survivors maintain their cognitive function and may even improve over time (Desmond et al., 1996, Tham et al., 2002).

A recent systematic review and meta-analysis of the prevalence, incidence and risk factors of PSD found that approximately 10% of first time stroke patients and 30% of recurrent stroke patients developed dementia shortly after their stroke. However the rate of development of dementia in the years afterwards was 1.7-3.0% per year. The importance of the stroke was

highlighted by a review of risk factors, which showed that previous symptomatic, prior asymptomatic, haemorrhagic and recurrent stroke in addition to number, size and location of lesion were significantly associated with PSD rather than background vascular risk factors. A number of non-stroke risk factors were also associated with PSD including age, low education attainment, race, medial and temporal lobe atrophy (Pendlebury and Rothwell, 2009).

Most of the risk factors identified for PSD are also associated with AD and VaD. Furthermore, pre stroke cognitive decline is associated with higher levels of PSD which propose a possible neurodegenerative mechanism, which could suggest underlying AD involvement (Leys et al., 2005, Pendlebury, 2009). Studies have shown that patients with both vascular lesions and Alzheimer's pathology have a more severe form of dementia compared to subjects with similar levels of only one type of lesion (Heyman et al., 1998, Kalaria and Ballard, 1999). Some studies suggest that infarcts in multiple areas instead of a single infarct or numerous lesions within the same region are likely to increase the risk of dementia, which suggests that interruption of numerous circuits are particularly important in dementia or that infarcts are associated with cortical disconnection as a result of extensive WM damage (Saczynski et al., 2009). However, recent experimental studies in rats showed that occluding one penetrating vessel caused enough damage to produce a cognitive deficit, showing that strategic infarcts could be equally important (Shih et al., 2013). Due to the decline in stroke mortality (Rothwell et al., 2004, Kleindorfer and Khatri, 2013) and increasing size and extent of the ageing population, it is likely that the burden from PSD will increase in the future (Mackowiak-Cordoliani et al., 2005).

1.1.2 Cognitive function after stroke study

To try to address the long term consequences of ischemic injury in the elderly, the cognitive function after stroke (CogFAST) study was established in Newcastle in 1999. It was a major prospective study that would follow PS survivors throughout life with annual clinical assessments and magnetic resonance imaging (MRI) scans as well as investigating the brain structure and pathology at autopsy. The main aims of the study was to try and determine mechanisms and risk factors associated with PSD and to establish differences in those that remained cognitively stable (PSND).

The study recruited elderly subjects over the age of 75 years from the North East of England who had suffered a stroke. The inclusion criteria were that patients suffered from no physical disabilities as a result of the stroke and were not clinically diagnosed with dementia at baseline (at least three months PS). For these reasons it was established that many patients had suffered similar ischemic events, likely a result of small vessel disease (SVD) and small cortical infarcts but few large vessel strokes with a high degree of disability. In total over 300 patients were recruited, so far more than 50 participants have donated their brain at post mortem.

To date the study, has made a number of key findings including:

- Stroke survivors suffer with impairment to working memory, executive functions and reaction time without a diagnosis of dementia compared to controls (Ballard et al., 2003b).
- Improvement in cognitive performance was observed in the majority of patients between 3 and 15 month time points PS (Ballard et al., 2003a).

- Significant increases in white matter hyperintensities (WMH) on MRI scans in key areas for PSD cases. Correlations were found with cognitive processing speed and performances on measures of attention with frontal WMH volume (Burton et al., 2004).
- Regression analysis indicated memory impairment was related to medial temporal atrophy and not WMH volume. Suggesting a greater role for Alzheimer type than vascular pathology (Firbank et al., 2007).
- Identification of a single nucleotide polymorphism (SNP) at codon 298 in nitric oxide synthases (NOS) increased the risk of dementia in cohort , possibly as a result of reduced NOS and cerebral perfusion (Morris et al., 2011).
- The median survival rate from the incidence of stroke was found to be 6.72 years. In this time, 23.9% of subjects were known to have developed dementia whilst 76.1% remained alive without dementia or had died without dementia. The greatest risk factors for dementia were low baseline cognitive scores including executive function and working memory, depression and three or more cardiovascular risk factors. At autopsy 75% cases displayed neuropathological criteria to meet a diagnosis of VaD (Allan et al., 2011).
- Global cerebral blood flow (CBF) ratios of cortex to WM were the best predictor of dementia in PS survivors. Hippocampus volume was also significantly decreased in both PSD and AD subjects. Results suggest both the direct impact of the stroke and subsequent development of AD-type changes play a role in etiology of PSD (Firbank et al., 2011).
- Putamen lacunar infarcts correlated with cognitive impairment, whilst WMH and hippocampal volume both correlate with global dysfunction. PSND cases showed a

greater number of lacunar infarcts in the caudate nucleus. Microinfarcts could not be accurately measured as analysis was performed by magnetic resonance imaging (MRI, Lopes et al., 2011).

- Neuronal volume was decreased in the PSD cases compared to PSND, although neuronal density was similar. Neuronal volume positively correlated with global cognitive and memory function. CA1 but not CA2 neuronal density was affected in PSD, AD and VaD compared to controls (Gemmell et al., 2012).

Overall the CogFAST study has suggested that approximately 25% of those who suffer a stroke in later life will go on to develop dementia during their life. A number of risk factors and morphological changes have been shown to be related to developing dementia including low baseline cognitive scores, depression, medial temporal lobe atrophy, reduced CBF and infarcts in the putamen. Furthermore approximately 75% of cases at autopsy met criteria to meet VaD diagnosis, suggesting that the stroke did not unmasked Alzheimer type pathology. Although, a number of MRI studies suggested similar morphological changes in PSD similar to AD.

It has previously been difficult to study groups of individuals in order to establish the initial commencement of dementia and also to compare that data to people who have suffered a similar incident but have different cognitive outcomes; PSND and PSD. This study gives us a unique opportunity, with its prospective nature, to allow comparisons between clinical information and pathological findings.

1.1.3 Vascular dementia

Vascular cognitive impairment (VCI) is considered to result from all causes of vascular injury or disease (Hachinski et al., 2006). VaD is the result of vascular lesions that lead to

impairment of cognitive function (Jellinger, 2013) and usually shows evidence of a clinical stroke or another vascular brain injury (Thal et al., 2012). Common vessel disorders include atherosclerosis, arteriolosclerosis and cerebral amyloid angiopathy [CAA, (Vinters, 2001, Kalaria and Kalimo, 2002, Kalaria and Erkinjuntti, 2006)]. These disorders increase in frequency with age (Jellinger and Attems, 2010). As mentioned before, VaD as a result of its nature often develops suddenly and progresses stepwise, with sudden large reductions in cognitive function followed by periods of stability (Roman, 2003). Impaired frontal and subcortical functions such as memory retrieval, attention and executive functions are characteristic whilst hippocampal functions such as episodic and recognition memory are spared (Desmond et al., 1999, Looi and Sachdev, 1999, Traykov et al., 2002).

Although VaD is believed to be the second most common form of dementia, it has been often difficult to diagnose, due to its similarities with other disorders. Detailed criteria for the diagnosis of VaD were proposed by the National Institute of Neurological Disorders and Stroke-Association Internationale pour la Recherché et l'Enseignement en Neurosciences [NINDS-AIREN] consortium (Roman et al., 1993). Clinico-pathological studies reported moderated sensitivity and variable specificity with current used diagnosis criteria (Gold et al., 2002, Bacchetta et al., 2007, Jellinger, 2007). However the demonstration of cerebrovascular lesions by neuroimaging techniques or at autopsy does not guarantee the cause of the dementia (Markesbery, 1998).

Pathological hallmarks in VaD include large and small ischemic lesions with other cerebrovascular lesions and can involve multiple or individual locations. Multifocal lesions are defined by multiple lacunar or microinfarcts in the cortical and subcortical areas and are often as the result of large vessel diseases including atherosclerosis of the circle of Willis or embolic events, CAA and SVD or haemorrhages. Others may include subcortical

arteriosclerotic encephalopathy (Binswanger's disease), which describes a widespread demyelination and axonal loss caused by SVD (Farkas et al., 2006, Thal et al., 2012). Strategic infarcts affect a particular area that is functionally significant such as the hippocampus or thalamus and leads to cognitive impairment. They are caused by large vessel disease and SVD, cardiac embolic events, hemodynamic events and cerebral ischemia of various etiologies (Jellinger, 2013). As mentioned in the post stroke dementia section there is conflicting information regarding the severity and effect between multinfarct and strategic infarct in the importance of cognitive impairment (Saczynski et al., 2009, Shih et al., 2013). Whilst previous studies suggested that the mean number of infarcts, irrespective of their volume, in VaD was 5.8–6.7 compared to 3.2 in non-demented subjects (del Ser et al., 1990).

The size of the lesion is also a factor in cognitive outcome. Earlier studies by Tomlinson et al in 1970 suggested that there was no significant difference in mean volumes of lesion size between controls and demented cases. But demented patients have significantly more lesions > 50ml in volume compared to controls (Tomlinson et al., 1970). This study suggested that relatively small infarcts may not cause dementia and subsequently others have since suggested that volume of infarction is not consistently related to dementia and that cases reported a total of less than 100/ ml^3 of infarct volume (del Ser et al., 1990). Location of the lesion therefore must also play a critical role in the outcome of the patient. Bilateral thalamic infarction and lacunar lesions in basal ganglia, head of the caudate nucleus and inferior genu of the anterior capsule and additionally hippocampal infarcts and sclerosis either alone or in combination with other vascular lesions have been related to dementia (Van der Werf et al., 2003, Attems and Jellinger, 2006, Hachinski et al., 2006, Jellinger, 2013).

Unlike other dementias which are associated with deposition of proteins, VaD is characterised in regard to its pathology associated with macro and microscopic infarcts,

increase in perivascular spaces and demyelination. Although vascular lesions are often reported in pathological studies the heterogeneity of cerebrovascular lesions and their causative factors makes it difficult to perform accurate assessment (Jellinger, 2008). Therefore, no widely accepted morphological criteria for quantifying vascular lesions and no validated pathological criteria for VaD has been produced. (Roman, 2008). To try to address this situation, a study involving this group was established to quantify vascular lesions in sampled 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 mammillary body. Scores were given dependent on the presence of arteriolosclerosis, CAA, perivascular hemosiderin leakage, perivascular spaces dilatation in the deep and juxtacortical WM, myelin loss, and cortical micro (<0.5 cm) and large infarcts (>0.5 cm) with increasing severity resulting in greater scores. A maximum score of 20 was available for each case, a single point was given on the presence of any lesion in a region, no prefence was given for one type over another. The study found that vascular lesions were common among all types of dementia with the most frequent lesions being arteriolosclerosis and increased perivascular spaces (Deramecourt et al., 2012). However, this study was not able to identify what affect these lesions had on cognitive impairment and requires further validation.

1.1.4 Alzheimer's disease

AD is characterised by the accumulation of two proteins amyloid-β (Aβ) and tau. Aβ fibrils are formed by the cleavage of the amyloid precursor protein (APP), a transmembrane glycoprotein ubiquitously expressed in the body (Sisodia and Price, 1995). Aβ was isolated and identified (Glenner and Wong, 1984), which was shown to be toxic (Yankner et al., 1990). Aβ aggregates form oligomers and then accumulate into insoluble plaques within the parenchyma. Senile or neuritic plaques consist of a dense core of Aβ and often contain

hyperphosphorylated tau aggregates surrounded by a rim of paired helical filament dystrophic neurites, often in close association with reactive microglia and astrocytes (Dickson, 1997a). The majority of plaques are located in the cortex and follow a distinctive pattern of deposition, which have been defined into a number of stages. Initially, Aβ is exclusively deposited in the neocortex, followed by allocortical regions including the hippocampus followed by the striatum, then the brain stem and finally the cerebellum (Thal et al., 2002). Along with subsequent genetic analysis, mapping the APP gene on chromosome 21 and linking it with a rare form of early onset AD (St George-Hyslop et al., 1987), lead to the amyloid cascade hypothesis (Hardy and Allsop., 1991). Subsequently, the amyloid hypothesis was formulated and proposes that Aβ plaques or fibrils were the main source of toxicity in the AD brain and has led to many years of basic research and development of therapeutic targets to try to inhibit the deposition or removal of Aβ plaques, although to date no treatment has been successful at stoping or limiting memory impairment (Hawkes and McLaurin, 2007).

The other neuropathology hallmark of AD is the presence of intracellular neurofibrillary tangles (NFTs). Tau is a microtubule protein involved with regulating the trafficking of vesicles from the cell body to the synapse. In AD, this protein becomes hyperphosphorylated and is unable to perform its standard conformational change to regulate vesicles. As a result of hyperphosphorylation the microtubules become unstabilised and degenerate to form NFTs. As a result the neuron degenerates and is eventually destroyed. Similar to Aβ, NFTs appear in a predicatable progression. The first deposits are in a very restricted region of the brain, the transentorinhal region, before advancing into the hippocampus and then increasing deposition in the neocortex spreading out from the temporal lobe into other regions including

the pre-motor and occipital cortex. At the same time increasing deposition is found in the earlier regions as the stages progress (Braak et al., 2006).

Whilst these two pathologies have come to define AD, there have been a number of controversies regarding the importance and affect they have on cognitive impairment. These have developed from research observations including persons without dementia but advanced levels of pathology (Katzman et al., 1988, Ince, 2001, Price et al., 2009). Studies have suggested a poor correlation with Alzheimer pathology and cognitive impairment, possibly as widespread diffuse plaques have been described as advanced neuropathological criteria for AD regardless of the number and distribution of NFTs. It has now been suggested that plaques in the absence of other neurodegenerative pathology or lesions are not a sufficient substrate for severe dementia (Nelson et al., 2012). This has been recognised in the latest update of pathological criteria for AD (Montine et al., 2012). It is suggested that only neuritic plaques appear to correlate with cognitive impairment (Wisniewski et al., 1982, Dickson, 1997b, Tiraboschi et al., 2004). Additional changes may occur including age related brain atrophy, synaptic pathology, WM dysfunction, neuronal loss and neuroinflamation but they are not considered specific features for AD (Montine et al., 2012). Assertions from clinical trial data, especially with regard to immunotherapy of Aβ have generally been negative (Nicoll et al., 2006, Roher et al., 2011).

There is a growing body of evidence that vascular risk factors also play a critical role in the development of AD. In 1997, Snowdon reported using a unique cohort of 678 Roman Catholic Nuns, refered to as the NUN study, which eliminated most of the factors that confound other epidemiologic studies i.e. diet, social activities, smoking and drinking excessively by studying a homogenous group due to their strictly controlled lifestyle and living arrangements. This study exposed an important difference at post mortem in patients

who were clinically diagnosed with AD. Patients who had lacunar infarcts gained lower scores on cognitive tests whilst displaying fewer NFTs (Snowdon et al., 1997).

The Rotterdam Study was a large population based prospective study involving nearly 8000 patients over the age of 55. The aim was to look for the frequency and risk factors of dementia. Risk factors identified had a vascular basis including hypertension (in't Veld et al., 2001), atrial fibrillation (Ott et al., 1997), atherosclerosis and apolipoprotein E (APOE, (Hofman et al., 1997), diabetes mellitus (Ott et al., 1999) and smoking (Ott et al., 1998). These findings suggested that risk factors capable of causing or contributing to cerebral hypoperfusion were important in AD and that there is a close association between AD and VaD (de la Torre, 2000).

Aβ has been shown to be removed from the brain along the basement membrane (BM) of the capillaries and arteries to lymph nodes in the neck (Carare et al., 2008). The accumulation of Aβ in the ageing brain is conceivably caused by the clearance mechanism being damaged or insufficient. With increasing age arteries stiffen such that the perivascular drainage system is unable to remove Aβ. As a result, soluble and insoluble Aβ accumulates in vessel walls (CAA) and the parenchyma (Weller et al., 2008). Severe CAA correlates with severe dementia (Ince, 2001) and is also recognised in VaD (Vinters, 2001). Furthermore, both Aβ plaque and NFT deposits have both been shown to increase following hypoxia and ischemic injury (Wen et al., 2004, Guglielmotto et al., 2009).

1.1.5 Brain and Cognitive Reserve hypotheses

As suggested above, pathological changes do not always correlate with disease progression and cognitive impairment (Katzman et al., 1988, Ince, 2001, Nelson et al., 2009, Price et al.,

2009). This has led to the development of theories that people are able to cope with different amounts of pathology due to a “reserve”.

The initial theory described was brain reserve, which describes the physiological element. For instance a larger brain is able to withstand more insults due to a greater number of neurons, which support brain function. This led to the threshold theory initially being described (Satz, 1993). Satz’s described how two brains could be differently affected by a similar insult dependent on the individual level of brain reserve capacity (BRC). If BRC was reduced beyond a particular threshold then cognitive impairment was inevitable. Additionally, repetitive insults could lead to further depletion of BRC or in neurodegenerative disorders, such as AD, with increasing time BRC would be reduced as a result of the disease progressing with increasing pathology beyond the threshold leading to clinical diagnosis of dementia (Figure 1.1). However, there are some key limitations to this theory. Firstly, it assumes that functional impairment will happen for everybody beyond a certain threshold. Secondly, it presumes that a lesion will have the same effect in different individuals and that repetitive lesions will have a summation effect.

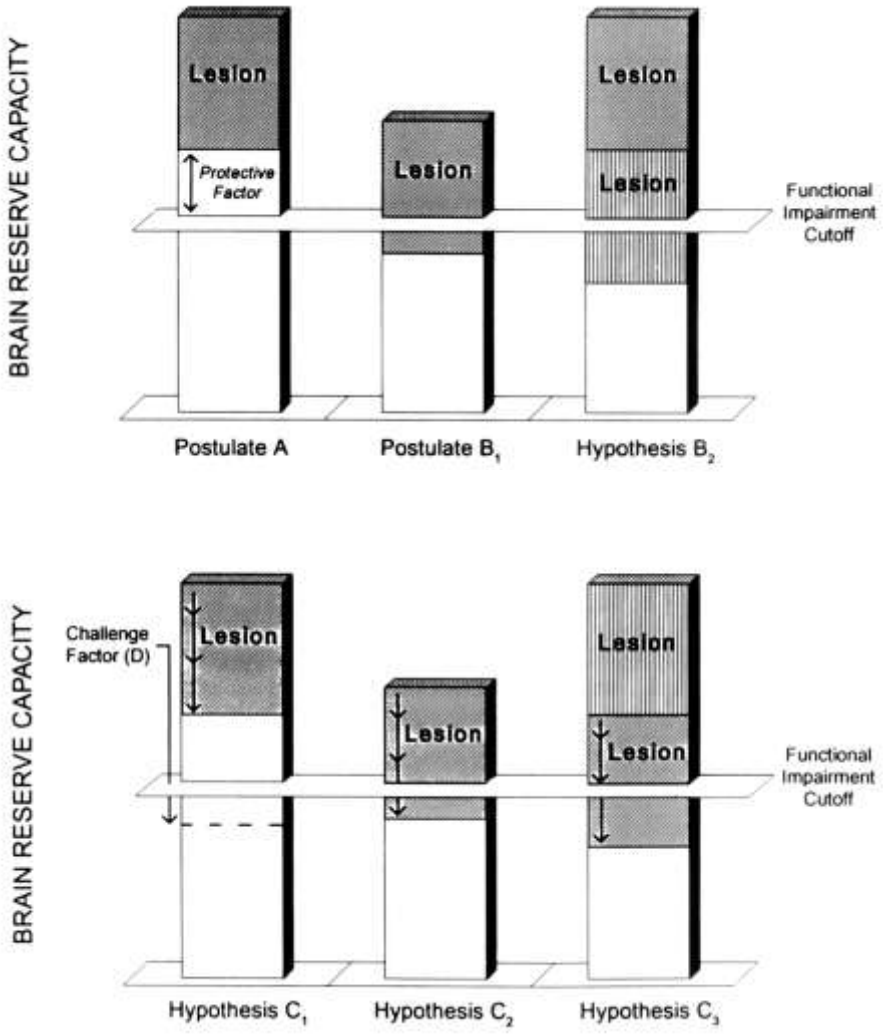


Figure 1.1 Graphical representation of the theory of threshold brain reserve. Postulate A shows that after a lesion the subject does not suffer with impairment as there is a greater brain reserve. B_1 shows that from a similar lesion as A the subject suffers impairment as they pass the threshold. B_2 repetitive lesions are required to bring about impairment and by pass the threshold level. Hypothesis $C_{1\text{-}3}$ is a nearer description to neurodegeneration similar to AD, as the lesion is progressive over time. C_1 although having similar level of degenerative lesion does not suffer with impairment due to brain reserve, unlike C_2. C_3 shows two different lesions combined to induce functional impairment, whilst the individual lesions would not surpass the threshold level. D states that a lesion that remains subthreshold but is undetected due to protective factor of BRC until an appropriate assessment challenge is presented. Image from (Satz, 1993).

In 2002, another theory of cognitive reserve was suggested, which built on the brain reserve concept by suggesting the brain was able to cope with lesions and damage by using pre-existing cognitive processes or by enlisting compensatory processes (Stern, 2002). Instead of there being a finite amount of BRC that was different from person to person, this theory suggests that cognitive reserve was able to tolerate greater damage before impairment arose

(Stern, 2009). Both theories must be linked, for cognitive reserve to occur there must be a physiological basis. The difference between cognitive and brain reserve is in terms of the level of analysis; cognitive reserve is coordinated by synaptic organization or utilisation of specific brain regions, while brain reserve implies differences in the quantity of available neural substrate (Stern, 2009).

In epidemiological studies, it has been shown that the risk of dementia is increased in subjects with low education and low lifetime job attainment (Stern et al., 1994). As life experiences can have a negative effect it was shown that there can be positive aspects in subjects who were active in intellectual, social and physical activities by having a reduced risk of cognitive impairment (Christensen et al., 1996, Scarmeas et al., 2001). Education, occupation and premorbid IQ are continually linked with lower risks of incident dementia (Valenzuela and Sachdev, 2006) and lower rates of cognitive decline in normal ageing (Butler et al., 1996, Farmer et al., 1995, Snowdon et al., 1989). However, it is difficult to know with epidemiological studies if single factors can have such far reaching effects. Higher levels of education could be linked to a better quality of life and reduced risk factors for dementia. Does participating in stimulating activities promote improved cognitive performance or are people with good cognitive functions more likely to participate? It has been shown that individuals with higher levels of intellectual ability and function lead highly intellectually active lives and have the least decline in cognitive function over long periods of time, up to 20 years (Gold et al., 1995, Hultsch et al., 1999, Schooler and Mulatu, 2001). However other studies have suggested that the interest in intellectual activities is lost with the development of cognitive decline (Hultsch et al., 1999).

Prospective studies have suggested that once AD emerges, those with greater cognitive reserve decline the fastest (Stern et al., 1999, Scarmeas et al., 2006). This may be at odds with what had been proposed in the theory for cognitive reserve. However, subjects with high levels of cognitive reserve are able to tolerate more pathology and therefore the "point of inflection", where memory declines, will occur after a longer time interval (Figure 1.2).

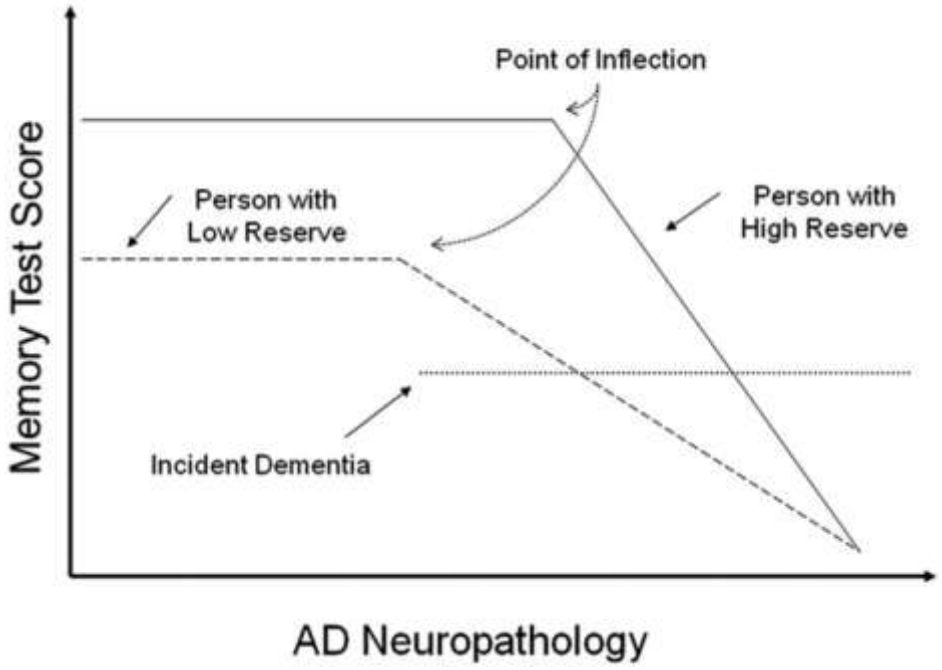


Figure 1.2 Theoretical illustration of the effect of cognitive reserve on the incident of dementia. It is assume that AD pathology increases over time, and this is graphed on the x-axis and cognitive function is shown on the y-axis Assuming that AD pathology increases over time to affect cognitive function, the following predictions can be made about the individual with high cognitive reserve 1) the point of inflection, where memory begins to be affected, will be later; 2) clinical diagnostic criteria for AD will be reached later, when pathology is more severe; 3) after the point of inflection, clinical progression will be more rapid. Image from (Stern, 2009)

This idea was confirmed when a prospective study measured cognitive function over time in healthy volunteers until they became demented. They retrospectively modelled the time of the point of inflection. It was found that for every additional year in education lead to an additional 0.21 years prior to the point of inflection but the rate of memory decline was increased (Hall et al., 2007).

Recent studies have shown evidence for the reserve hypotheses. Wilson et al (Wilson et al., 2013) measured neuronal density in the brain stem from 150 older volunteers who had been involved in a longitudinal study to measure cognitive change. The study found that higher noradrenergic neuronal density in the locus ceruleus was associated with reduced cognitive decline when adjusted for neuropathological lesions within the rest of the brain and Lewy bodies in the brain stem. Another study measured years in education, as a marker of cognitive reserve. It was shown that there is a positive correlation associated with glucose metabolism analysed via a fludeoxyglucose positon emission tomography (FDG-PET) scan in cognitively stable age matched controls in temporal regions compared to a decrease in those with preclinical AD, defined by low cerebral spinal fluid Aβ (Ewers et al., 2013).

Currently no marker has yet been found which identifies cognitive reserve. However, animal studies have shown a significant increase in neurogenesis in animals exposed to exercise and enriched environments (Brown et al., 2003, van Praag et al., 2005). Combined with the knowledge that exercise and an intellectual stimulation may have an effect on cognitive reserve, it may be suggested that neurogenesis may have an involvement with cognitive or brain reserve. Additionally, it has been well established that neurogenesis and angiogenesis are closely linked (Palmer et al., 2000). As the majority of risk factors for dementia are cardiovascular and the cerebrovascular system is continually linked with the importance of offsetting dementia (de la Torre, 2000, Iadecola et al., 2009), then it is possible that there is a vascular form of brain reserve in addition to neuronal.

1.2 Hippocampal anatomy and function

The hippocampus has come under particular scrutiny in dementia research and is the most studied brain region for this condition. Investigating the hippocampus is a natural step due to

the structures association with memory formation and there is deterioration of the structure, including hippocampal atrophy (Rowe et al., 2007) and deposition of pathology (Braak et al., 2006, Thal et al., 2006), which links with the primary symptom of dementia being short term memory impairment.

1.2.1 Neuronal anatomy and connectivity

The hippocampus is a unique brain region located in the medial temporal lobe, being structurally different from the vast majority of the other regions. Predominantly, the brain consists of cortical regions, which are well defined layers of neurons surrounding a central area of WM. The hippocampus is a bilaminar structure involving the cornu ammonis (CA) and the dentate gyrus (DG). The DG is a narrow body of archicortex forming a V or U shape encompassing the CA4 region; consisting of three layers, an outer molecular layer, a middle granular layer and a deep polymorphic layer. The characteristic granule cells are packed tightly together whilst their dendritic trees diverge out into the molecular layer where they synapse with the pyramidal cells of the CA3 region. The proportion of the granular layer located between the CA3 and CA1 region is referred to as the suprapyramidal blade, whilst the opposite proportion is known as the infrapyramidal blade. The CA region is also a three layered strip of archicortex, made up of polymorphic, pyramidal cell and molecular layers. The large pyramidal neuronal cells which are characteristic to the CA regions are divided up into four separate fields. The CA1 occupies the majority of the region between the DG and the subiculum, a transition region from archicortex to the six layered isocortex. The CA2 is a small and thin transitional region between CA1 to the CA3. CA3 occupies the majority of the remaining region often referred to as the inferior region. The CA4 is a transitional zone between the CA and the DG ((Burt, 1993), Figure 1.3)

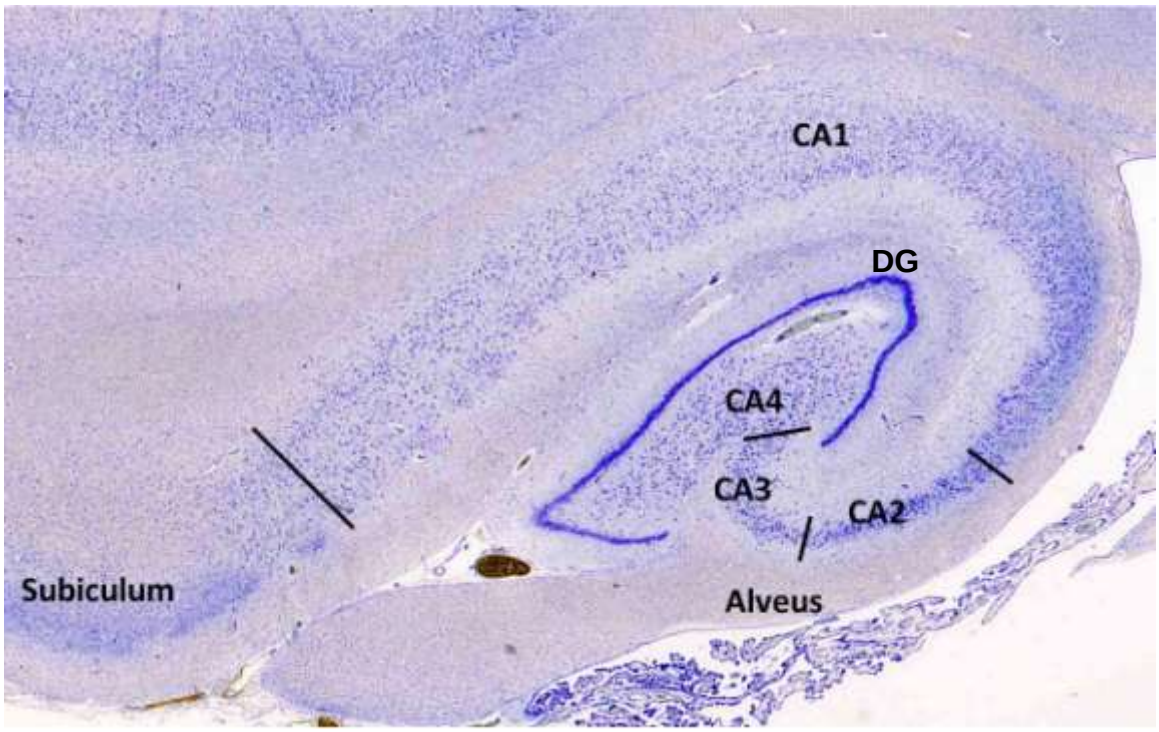


Figure 1.3 Scan of a section of human hippocampus with a Nissl stain to highlight cellular composition and structure of the region. The image shows the hippocampus divided into separate sub regions including the CA fields, subiculum and DG. Image scanned at 9,600 dots per inch (dpi)

A common feature of neurons in the neocortex is that they are largely reciprocal i.e. region A projects to region B then region B often sends a reverse projection back to region A. However, in the hippocampus projections follow a very specific and unidirectional route with signals propagating in the entorhinal cortex (EC) and then passing to either the CA1 or DG. Cells in the superficial layers of the EC project to the DG via the perforant pathway. From here cells project to CA3 and then via CA2 to CA1. This is the major input pathway to the CA1 via the Scaffer collaterals, however some axons do project directly to the CA1 from layers III and V of the EC. From the CA1, axons are projected back to the deep layers of the EC directly or via the subiculum (Amaral, 2006). This circuitry is often referred to as the tri-synaptic circuit. This representation covers the basic and prominent circuitry of the hippocampus, however there are also a number of projections acting directly from other brain

regions including the amygdaloid complex (Pitkanen et al., 2000), the perirhinal and postrhinal cortices (Agster and Burwell, 2009) and posterior hypothalamus ((Magloczky et al., 1994), Figure 1.4).

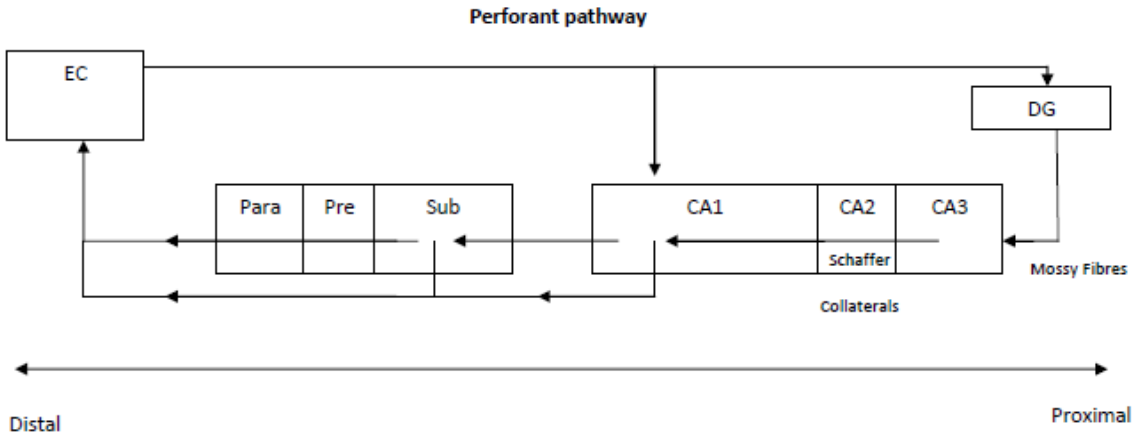


Figure 1.4 Representation of the tri-synaptic circuitry of the hippocampus. Signals enter the hippocampus from the EC; axons send messages to the CA1 either directly or via the DG and CA3. The signal then is sent back to the EC directly or via the subiculum (Sub) and pre and para subiculum

The DG is classically separated into three layers, the focal region being the middle granular layer, principally consisting of granular cells, which have an elliptical cell body. The total number of granular cells has been estimated to be $1.2x10^6$ in the rat (West et al., 1991). The molecular layer is a relatively cell free region containing the dendrites of the granular cells, a small number of interneurons and a number of fibres from the perforant pathway. Whilst the polymorphic layer consists of a number of varying cell types, with the most prominent being the mossy cells, which have distinctive proximal dendrites covered by large and complex spines called thorny excrescences (Lavenex, 2012).

Likewise the CA fields are classically defined as being archicortex and its defining cell type is the pyramidal neuron. Pyramidal neurons in the CA1 are on average smaller than those found in the CA2 and CA3 regions. Regardless of where the neuron is located within the

CA1 field it has approximately the same total dendritic length and configuration unlike the pyramidal cells of the CA3 which are quite variable in size and organisation. Dependent on the location of cells in the CA3 region, there can be a variable effect on dendrite projections. Those that are in the limbs of the DG have few dendrites in the striatum lacunosum-moleculare and therefore these cells receive little or no direct connections from the EC and are instead mainly influenced by the granular cells (Amaral, 2006). Less is known in regards to CA2 pyramidal cells, and although the soma appears similar to the CA3 neurons, differences have been observed in the dendrites with no input coming directly from the DG (Lauer and Senitz, 2006).

1.2.2 Hippocampal vascular structure and blood supply

As with all cells in the brain, successful functionality is dependent on a healthy supply of both oxygen and glucose. The hippocampus is the beneficiary of a generous arterial supply, originating predominately from the posterior cerebral artery and its branches and to a lesser extent from the anterior choroidal artery (Erdem et al., 1993). Three arteries vascularise the hippocampus: the anterior, middle and posterior hippocampal arteries. The middle and posterior arteries supply the body and tail, whilst the anterior supplies the hippocampal head (Duvernoy, 2005). Venous return from the hippocampus is supplied by branches of the basal vein. The superficial veins of the hippocampus form two longitudinal superficial venous arches covering the fimbriodentate and the superficial hippocampal sulci (Duvernoy, 2005).

Despite this, vascular supply might be interrupted and the hippocampus is particularly vulnerable to hypoxia in comparison to the neocortex (Scharrer, 1940). Further experiments have shown specifically the CA1 is most susceptible (Schmidt-Kastner and Freund, 1991, Wu et al., 2008). Although the sensitivity of hippocampal cells to glutamate toxicity may be

a reason for this effect (Ekstrom, 2010). However with increasing age there is a well defined decline in cerebrovascular parameters such as CBF, metabolic rates of glucose and oxygen and a compromised structural integrity of the cerebral vasculature, increasing the risk of ischemia and neurodegeneration (Farkas and Luiten, 2001).

Cerebral vessels are made of a number of separate cells which work together to form a functional unit called a neurovascular unit. The neurovascular unit is composed of a monolayer of endothelial cells (ECs), the basement membrane (BM), vascular smooth muscle cells (VSMC), pericytes and astrocytes. ECs are the crucial component of the neurovascular unit, they allow for the movement of oxygen, glucose and blood borne cells between the blood and surrounding tissue. The BM is a multilayer membrane consisting of a number of extracellular matrix (ECM) proteins including collagen and laminin. The BM anchors down the ECs and is essential for the structure of the vessel. Pericytes are small undifferentiated cells, which are mainly found within capillaries. They sheath most of the BM but have long processes which penetrate through and are in contact with the ECs layer. Astrocytes are positioned between neurons, pericytes and ECs and communicate with these cells via their numerous end foot processes (Zlokovic, 2005). For larger vessels such as arterioles and arteries, there is an additional layer of smooth muscle cells which allows the vessel to contract and maintain flow of blood under changing pressure in the cardiac cycle (Figure 1.5).

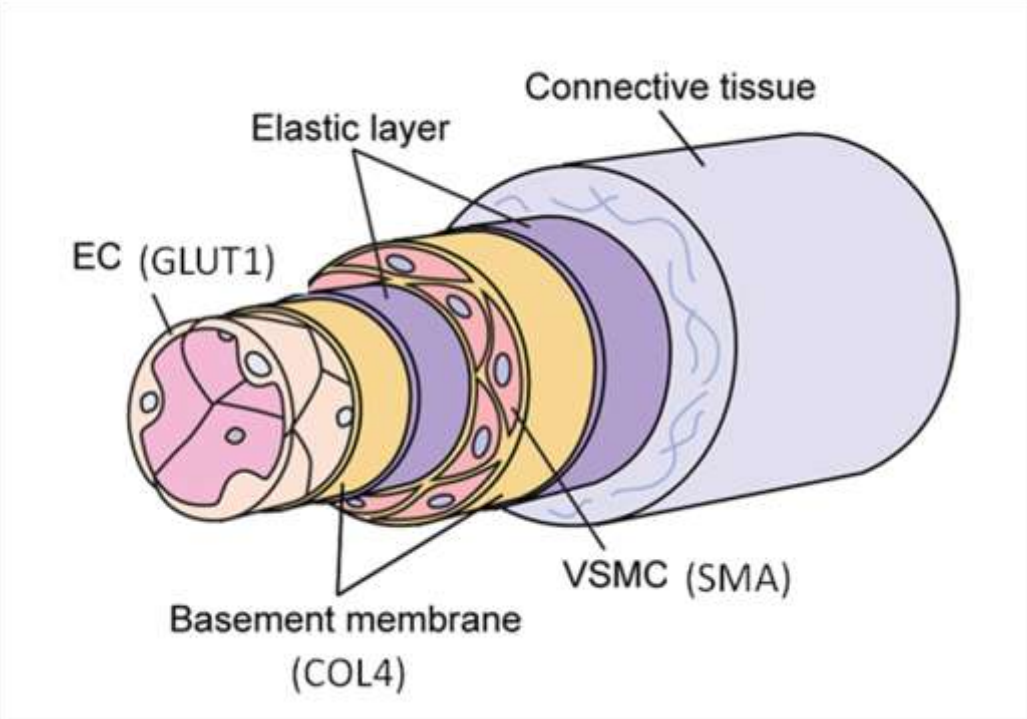


Figure 1.5 Schematic representation of a blood vessel detailing the separate layers along the vessel. Image also shows the antibodies used to stain different aspects of the vessel used in this thesis. Image provided by Y. Yamamoto

Cerebral vessels have an important and significant difference compared to peripheral vasculature, the presence of the blood brain barrier (BBB). The monolayer of ECs is the primary constituent of the BBB. ECs are normally connected at junctional complexes by tight junctions and adherence junctions. Tight junction proteins with their adaptor molecules link the tight junction with the cellular cytoskeleton and can be affected by acute and chronic disease of the brain (Zlokovic, 2008). The tight junctions are responsible for the severe restriction of the diffusion pathway between ECs and physically block the movement of penetration from the blood to the brain by macromolecules (Abbott et al., 2010).

1.2.3 Hippocampal function in learning and memory

The hippocampus has long been associated with memory and learning. However, it was studies of the epileptic patient HM, who had segments of his hippocampus surgically

removed in an attempt to cure seizures which has been credited with initiating the link between the hippocampus and memory. After the surgery HM displayed severe anterograde amnesia and some moderate retrograde amnesia although the seizures were diminished (Scoville and Milner, 1957). Later analysis of HM by MRI, suggested that the resection may have extended further than previously considered and included the medial temporal polar cortex, most of the amgydaloid complex, most of the entire EC and approximately half of the rostrocaudial intraventricular portion of the hippocampal formation. No other major abnormalities were found that could have explained the amnesia (Corkin et al., 1997).

After the initial findings by Scoville and Milner, there was an increased interest in the link between memory and the hippocampus leading to a number of animal studies. However, in both primate and rodent tests there was limited proof of deficits in memory but an increase in anxiety and hyperactive response (Orbach et al., 1960, Kimble, 1963). This led to the development of new theories suggesting that there may be more than one type of memory and that only one was involved with hippocampal function (Gaffan, 1974, Hirsh, 1974). The two theories that emerged proposed that memory had two forms, those set in a spatiotemporal context and those for semantic items, such as facts (Tulving, 1972, Winograd, 1975). We now recognise that the cortex is the major storage site for memories and that the hippocampus is involved with the process of preparing information for storage.

To date only two brain regions have been identified as being locations of endogenous neurogenesis in humans; the subgranular layer (SGL) of the DG and the subventricular zone of the lateral ventricles (SVZ). Neurogenesis has been implemented as possibly being important in both learning and memory and linked as an example of a patient's reserve capability and will be discussed in more detail later in this thesis.

1.2.4 Hippocampal involvement in dementia

As previously described AD is characterised by the accumulation of pathological hallmarks plaques and NFT's. Both of these lesion types have been characterised into specific stages of progression and they have been shown to follow a predictable sequence pathway with both appearing in the hippocampus. Deposition is seen especially early of NFTs, whilst plaques begin in the neocortex before appearing in the hippocampus and later the brainstem and cerebellum (Braak et al., 2006, Thal et al., 2002). Hippocampal infarcts and sclerosis, either alone or in combination with other vascular lesions, including demyelination, have been related to dementia in AD and VaD (Van der Werf et al., 2003, Attems and Jellinger, 2006). As previously suggested the CA1 region is particularly vulnerable to hypoxia (Schmidt-Kastner and Freund, 1991). Another striking change of the brain structure associated with dementia is tissue shrinkage, causing ventricular enlargement and sulcal widening. The hippocampus is also affected by atrophy. Memory impairment appears to correlate with hippocampal volume and not with the volume of other structures including the amygdala and the complete temporal lobe (Walker, 2006). Hippocampal atrophy is not exclusively associated with dementia and is often associated with epilepsy (Jack, 1994) and depression (Schweitzer et al., 2001). It has also been associated with many forms of dementia including VaD (van de Pol et al., 2011), PSD (Firbank et al., 2007) and DLB (Barber et al., 1999).

1.3 Regenerative Mechanisms

1.3.1 Angiogenesis

Angiogenesis is the mechanism by which new capillaries sprout from pre-existing vessels throughout life. Capillaries are essential for the diffusion exchange of oxygen, nutrients and metabolites to the surrounding tissue. Angiogenesis is an essential mechanism and often a

reactive response required during a number of biological functions not least the formation of the vascular system during development. Other conditions when angiogenesis is active include wound healing, inflammation and post ischemic incidences (Krupinski et al., 1994, Flamme et al., 1997, Lingen, 2001). The body also generates new vessels as a result of disease such as diabetic retinopathy and solid tumours (Ferris et al., 1984, Leon et al., 1996). Tumours require the recruitment of new blood vessels in order to grow to a large mass (Folkman et al., 1971). Otherwise the tumour is limited in size as the dividing cells at the tumour edge are cancelled out by the death of cells at the centre of the tumour (Carmeliet, 2003). This has led to great interest in angiogenesis research as a possible therapeutic target.

1.3.1.1 Angiogenic mechanism

During development blood vessels are formed by the process of vasculogenesis. Both ECs and blood cells differentiate from a common source, the mesoderm stem cell. Mesoderm stem cells via haemangioblast cells give rise to either haematopoietic stem cells or angioblasts, which in turn create haematopoietic cells and ECs (Adair and Montani, 2010). Vasculogenesis occurs with the early development of a primitive network of vessels to produce the basic circulatory system. By the time the future nervous system is developing, vasculogenesis has been completed and angiogenesis leads to the development of the cerebral vasculature.

For successful angiogenesis to occur in any situation a complex mix of growth factors and signalling molecules are required. Ligands can have multiple effects dependent on the receptor they bind with. A number of ligands and receptors interact to regulate these events and a selection of them are mentioned here (Table 1.1).

Effectors	Target	Effect	Reference
Vascular endothelial growth factor (VEGF)	VEGFR-1	Negative regulation of VEGF	(Terman et al., 1994)
	VEGFR-2	Angiogenesis, vasculogenesis	(Waltenberger et al., 1994)
Hypoxia inducing factor (HIF)-1α	Hypoxia response elements (HRE) on target genes	Increased release of growth factors	(Carrero et al., 2000, Semenza, 2001)
Angiopoieten (Ang) 1	Tie-2	Sprouting angiogenesis, vascular remodelling and ECs survival	(Dumont et al., 1994)
Ang-2	Tie-2	Antagonist of receptor	(Maisonpierre et al., 1997)
Unknown	Tie-1	Protecting cells from apoptosis	(Kontos et al., 1998)
Erythropoietin (EPO)	EPOR	Regulation of Ang1 and development of a vascular network	(Kertesz et al., 2004)
Fibroblast growth factor (FGF)	FGFR	Stimulator of endothelial growth	(Javerzat et al., 2002)
Transforming growth factor (TGF) β	TGFβR	Increases VEGF expression	(Pertovaara et al., 1994)
Epidermal growth factor like domain (EGFL) 7	Notch receptors	Stimulates vascular regeneration by suppressing inflammatory response in ECs and elongation of stalk.	(Badiwala et al., 2010, Schmidt et al., 2007)
Platelet derived growth factor (PDGF)	PDGFR-β	Pericyte proliferation	(Bergers and Song, 2005)
SLIT	Roundabout receptor (ROBO)	Stabilises vascular network, inhibiting pathogenic angiogenesis	(Jones et al., 2008)
Fibrinogen, vitronectin	Integrins (α5β3 and α5β5)	Adhesion, migration and proliferation	(Brooks, 1996)
Notch	Delta Like 4 (Dll4)	Arterial/venous cell fate decision, proliferation, filopodia protrusion	(Duarte et al., 2004, Liu et al., 2003, Hellstrom et al., 2007)

Table 1.1 A list of a number of ligand and receptors involved in angiogenesis. The table lists ligand and receptors and some of their possible effects as a result of binding. References are given for each result. There are many more other molecules involved in angiogenesis besides those listed.

It is now accepted that there are two distinct forms of angiogenesis; sprouting and intussusceptive. Sprouting angiogenesis has been identified for over two centuries and is

better understood, whilst intussusceptive (splitting) was recently discovered by Burri et al in postnatal rat lung parenchyma (Burri and Tarek, 1990). Sprouting describes the classic description of new vessel formation from an already established blood vessel caused by a stimulus such as a nearby hypoxic region, whilst intussusceptive involves splitting of a vessel to increase vascular density (Risau, 1997).

Sprouting angiogenesis follows a specific process, initially the BM is degraded by protyolytic enzymes including matrix metalloproteases (MMPs), recent studies have also shown that MMPs play additional roles including releasing sequestered pro-angiogenic factors from the ECM and detaching supportive pericyte cells from the vessel (Rundhaug, 2005). This is followed by ECs proliferation and the directed migration of ECs usually along a chemotaxin pro-angiogenic gradient e.g. VEGF and Ang-1, which is secreted by cells in response to a hypoxic environment. For successful migration of the sprouting vessel ECs must play their role but not all ECs can respond to this stimulus in the same way. An endothelial tip cell guides the new vessel through the ECM (Adair and Montani, 2010). Experiments in *Drosophila* identified that tip cells contain long filopodia, whilst the following ECs (known as stalk cells) are devoid of these morphological features. These filopodia secrete proteolytic enzymes to degrade the ECM and are attracted to the secreted pro-angiogenic factor by high concentrations of VEGF receptors at their surface (Marin-Padilla, 1985, Gerhardt et al., 2003). When a number of filopodia have anchored on to the source of the pro-angiogenic factor, contraction of actin fibres pulls the ECs behind the tip cell toward the chemoattractant source (Huber et al., 2003). At the same time stalk cells can push the tip cell forward. In the absence of EGFL7, a vascular specific ECM protein secreted by stalk cells, leads to new ECs accumulate at the base instead of sprouting (Schmidt et al., 2007). To ensure that ECs behind the lead tip cell do not branch out to form competing tip

cells the ECs become quiescent and have limited response to VEGF, by expressing a high number of VEGFR-1s, which act as a dummy receptor to VEGF. These cells were recently described as phalanx cells (Mazzone et al., 2009).

ECs behind the tip cell, known as stalk cells are important in order to elongate the stalk and follow the tip cell; therefore they have a high proliferation rate. Stalk cells must also form the lumen and connect to allow blood to circulate (Gerhardt et al., 2003). The battle to become a tip cell over a stalk cell is regulated by Delta-like 4 (Dll4) and Notch. Notch signalling reduces response to VEGF and inhibits the production of filopoda, whilst inhibiting notch promotes tip cell like characteristics (Hellstrom et al., 2007). Once the stalk has been formed and elongated, a lumen is developed to allow for continued circulation through the new vessel. Understanding of lumen formation has been limited for some time; initial hypothesis suggested that lumen formation was caused by cell hollowing (Folkman and Haudenschild, 1980, Davis and Camarillo, 1996). This theory was based around the idea that ECs formed large intracellular vacuoles, thus giving rise to a large seamless lumen. More recent studies have suggested that cord hollowing is a more likely mechanism (Jin et al., 2005, Blum et al., 2008, Herbert et al., 2009). In this mechanism, VEGF causes an alteration in the cytoskeleton, the opposing apical ECs separate, whilst at the same time elongating and flattening (Lammert and Axnick, 2012). When tip cells of two or more capillary sprouts converge on the source of the pro-angiogenic signal, tip cells fuse together to create a continual vessel. Maturation and stabilisation of the new capillary requires the recruitment of pericytes and deposition of a BM ((Adair and Montani, 2010), Figure 1.6).

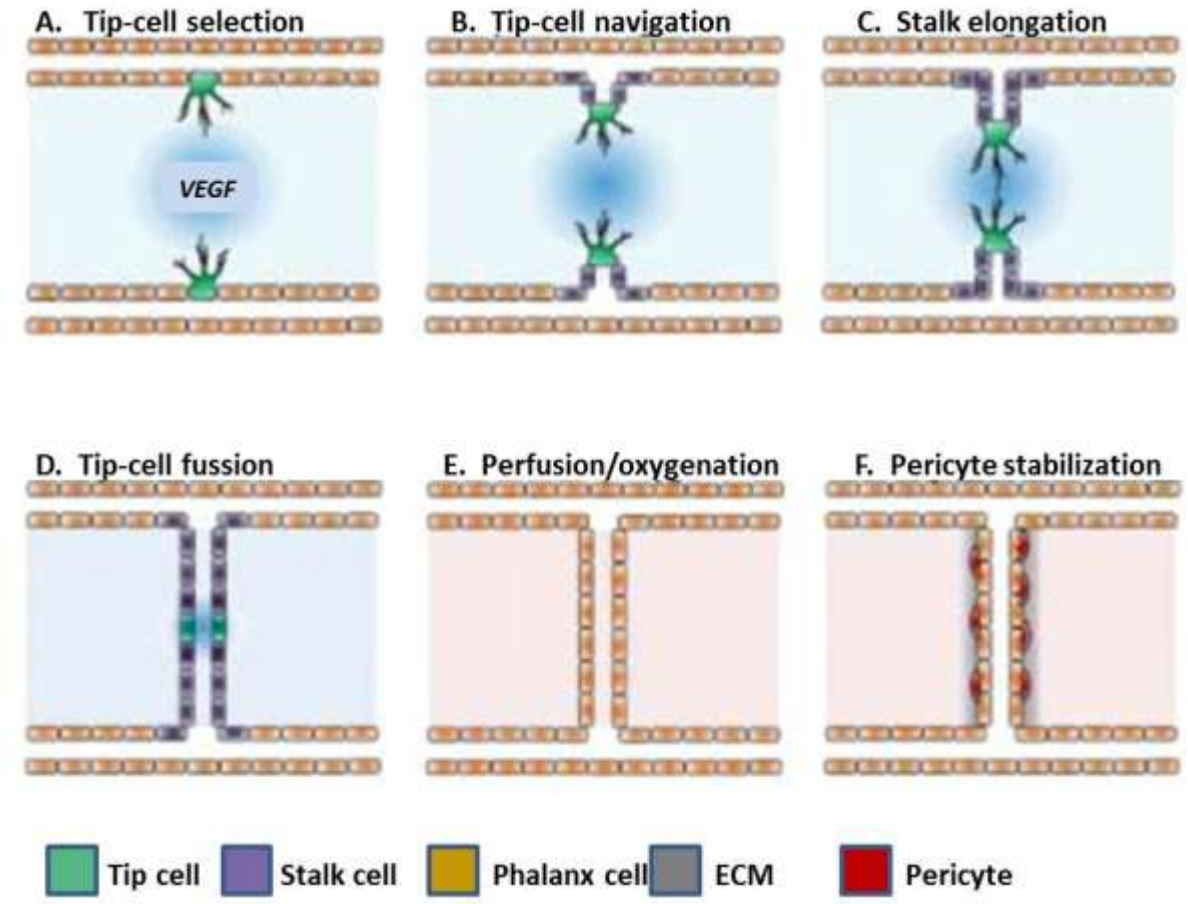


Figure 1.6 Schematic representation of the sprouting angiogenesis mechanism. A shows tip cell selection and the cell responding to an angiogenic chemotaxin stimulus. B shows tip cells with long filopodia navigating the tip cell. C displays stalk cells behind the tip cell moving the tip cell toward the stimulus. D shows the two sprouting vessels fusing together to complete a new vessel. Images E and F show how the vessel is stabilised and reaches maturity. Image from (Adair and Montani, 2010)

Additionally to sprouting angiogenesis, intussusceptive angiogenesis has also been identified, although considerably less is known about the mechanism works. Due to the lack of cell proliferation it is believed it would be a much faster and metabolically economical process than sprouting angiogenesis (Burri et al., 2004). The process was identified in both rat and human lung parenchyma (Burri and Tarek, 1990) and has subsequently been identified in a number of other regions including the kidney and heart (van Groningen et al., 1991, Makanya et al., 2005). This form of angiogenesis was identified looking at corrosion casts using scanning electron microscopes; the authors initially questioned why they could not see

evidence of angiogenesis. Then they noticed that the structure was pitted with thousands of tiny holes. The authors suggested these holes were evidence of new transcapilly pillars, which increased vascular density and complexity. The theory of this process might work is highlighted in the following figure (Figure 1.7). Although the mechanism is still poorly understood, flow alterations such as acceleration and pressure may produce a biochemical cascade in response leading to activation of intussusceptive angiogenesis (Djonov et al., 2002, Kurz et al., 2003).

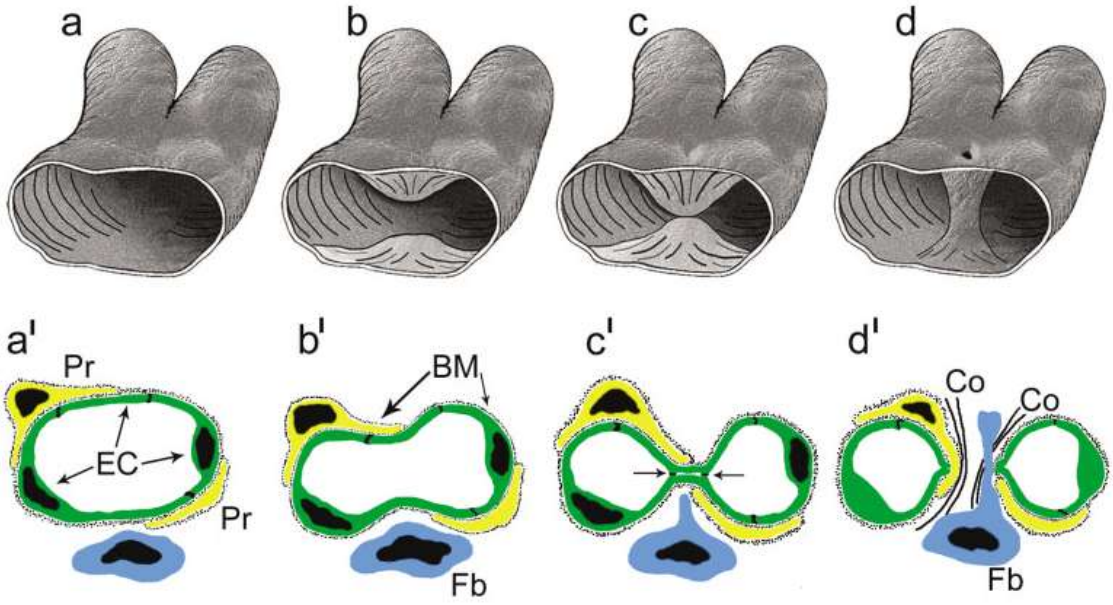


Figure 1.7 Schematic imaging showing the intussusceptive angiogenesis process. The process begins with the protrusion of opposing capillary walls into the vessel lumen (a'). After establishing an interendothelial contact (c'), the ECs and the BM are perforated centrally and the newly formed pillar increases in girth after being invaded by fibroblasts (Fb) and pericytes (Pr), which produce collagen fibrils (Co, d'). Image from (Kurz et al., 2003)

1.3.1.2 Evidence of angiogenesis post stroke and in dementia

1.3.1.2.1 Experimental Stroke Studies

Studies of rodents with either transient or permanent occlusion of the middle cerebral artery (MCA) have demonstrated the up regulation of angiogenic markers including VEGF and

Ang-1 around 6-24 hours following occlusion and an increase in vessel density (Marti et al., 2000, Beck et al., 2000, Hayashi et al., 2003). Studies in humans have shown that active angiogenesis takes place approximately 3-4 days following a stroke in the penumbra (Krupinski et al., 1994). Furthermore, it was shown that there is a correlation between rates of survival in PS patients who have shown active angiogenesis in the ischemic boarder region (Krupinski et al., 1994). However, it is difficult to know how long active angiogenesis can occur for and how successful the mechanism is at completing in varying conditions. It has been suggested that after ischemia, angiogenesis in mice might last for up to 21 days (Hayashi et al., 2003).

Genes linked with angiogenesis PS have been identified via complementary DNA array methods of ischemic mouse brains and have demonstrated up to 42 different genes are expressed compared to controls (Hayashi et al., 2003). A number of these gene and protein expression changes have also been identified in humans. Although most studies have been conducted in animal studies, genes and protein expression changes include VEGF (Issa et al., 1999, Slevin et al., 2000)), VEGF receptor (VEGFR)-1 (Kovacs et al., 1996), VEGFR-2 (Hayashi et al., 1997), plasminogen growth factor (Carmeliet et al., 2001), Ang-2 (Beck et al., 2000, Lin et al., 2000, Zhang et al., 2002b), nitric oxide (Chen et al., 2005) and fibroblast growth factor (Hara et al., 1994).

It had previously been thought that neovascularisation occurs exclusively through angiogenesis from fully formed pre-existing blood vessels. But recent evidence shows that circulating endothelial progenitor cells (EPCs) can additionally contribute to vascular repair and homeostasis, by a process more akin to vasculogenesis (Asahara et al., 1997). Circulating EPCs accumulate at the site of repair and differentiate into ECs (Asahara and Kawamoto, 2004). Studies in mice have shown that bone derived EPCs participate in neovascularisation

after ischemia (Zhang et al., 2002a, Hess et al., 2002, Beck et al., 2003). Human studies, subsequent to these initial animal studies showed that circulating EPCs were higher in patients PS compared to high stroke risk control subjects but not with normal control subjects (Yip et al., 2008). Furthermore, EPCs were associated with improved neurological outcomes PS (Willing et al., 2003, Yip et al., 2008). Another study suggested increased circulating EPCs after ischemic stroke is associated with reduced infarct area and might participate in neurorepair leading to improved neurological outcome (Sobrino et al., 2007). This alternative angiogenic mechanism further supports the role of angiogenesis PS and improvement or maintaining cognitive outcome after the ischemic incident. Although, a separate study has suggested that there are low levels of EPCs in stroke patients compared to age matched controls (Ghani et al., 2005).

1.3.1.2.2 Angiogenesis in old-age and Dementia

The subject of angiogenesis in dementia, specifically in AD, where most of the studies have been conducted, is a controversial issue. With a number of studies in both animal and human claiming evidence of both increased angiogenesis and vascular dysfunction in dementia. If angiogenesis is occurring it is unclear what role it is playing either positive, as an attempt to restore homeostasis, or negative as a result of another factor such as inflammation or uncontrolled pathological angiogenesis. As shown earlier in this chapter the successful regulation and completion of angiogenesis involves several stages and key regulators to play a number of roles at specific times. In addition, proteins such as VEGF play various roles in other mechanisms apart from angiogenesis (Storkebaum et al., 2004, Schanzer et al., 2004), therefore individual findings maybe more complicated then reported. VaD is characterised by vascular diseases including arteriosclerosis, SVD and CAA showing vascular dysfunction. As previously mentioned there is a large cross over in vascular risk factors for both AD and

VaD and patients often present with mixed pathologies thereby suggesting that vascular dysfunction and degeneration is common in all dementias.

A number of studies have previously mentioned that angiogenesis does not occur in AD even in the presence of pro-angiogenic factors, suggesting the mechanism is either delayed or impaired (Buee et al., 1994). Studies have shown that Aβ is a dose-dependent anti-angiogenic, in vitro and ex vivo (Paris et al., 2004b, Patel et al., 2008). However a similar study looking at both $A\beta_{1-40}$ and $A\beta_{1-42}$ suggested they had a pro-angiogenic effect (Boscolo et al., 2007). Furthermore, reports have suggested there is a decrease in vascular density in AD cases (Buee et al., 1997, Kitaguchi et al., 2007) and an increase in string vessels in both AD and VaD (Brown and Thore, 2011).

However, other studies in AD have indicated that there is an upregulation or modulation of pro-angiogenic proteins and vascular growth factors and pro-angiogenic markers (Kalaria et al., 1998, Thirumangalakudi et al., 2006, Fernando et al., 2006, Desai et al., 2009, Grammas et al., 2011). Increased levels of pro-angiogenic factors over a sustained period of time can be toxic to cerebral vasculature. Further controversy surrounds possible angiogenic signals, mesenchyme homeobox 2 (MEOX2) gene, described as being at low levels in AD, has been reported as both pro (Wu et al., 2005) and anti-angiogenic (Gorski and Leal, 2003). A number of studies have suggested increased microvessel density in AD subjects (Bell and Ball, 1981, Richard et al., 2010, Schwartz et al., 2010), models of AD in mouse (Meyer et al., 2008, Biron et al., 2011) and zebrafish (Cameron et al., 2012). With another proposal going further, suggesting that AD is an angiogenesis dependent disorder (Vagnucci and Li, 2003).

1.3.2 Neurogenesis

1.3.2.1 Historical review of neurogenesis

The concept of the adult brain generating new neurons is relatively recent. The first reported observation of neurogenesis in the adult hippocampus came from Altman in the 1960s who labelled cells with thymidine-H (3), to study cell division and DNA synthesis, injected into rats at post natal day 30 and examining the brains four months later (Altman, 1962, Altman and Das, 1965a, Altman and Das, 1965b). Kaplan and Hinds study identified neurogenesis in the olfactory bulb of three month old rats and is often seen as the first evidence of adult neurogenesis. This study also used thymidine-H (3) and electron microscopy to reveal positive cells in both the olfactory bulb and granular cell layer [GCL, (Kaplan and Hinds, 1977)].

In the early 1990s, Gould and colleagues were studying the effects of stress on the brain. They had noted that the DG did not appear to be sensitive to stress induced cell death like other regions of the hippocampus (Kempermann, 2011). They showed that neurogenesis was linked to the turnover of neurons and therefore removing the effect of corticosterone (Cameron et al., 1993). Follow up studies demonstrated that increasing levels of stress and corticosterone levels lead to negative regulation of adult hippocampal neurogenesis, whilst adrenalectomy (removal of all stress hormones) increased adult neurogenesis (Cameron and Gould, 1994).

These studies and others led to the development of the work by Fred Gage and colleagues at the Salk institute. They used immunohistochemistry (IHC) to detect bromodeoxyuridine (BrdU) as a replacement for the thymidine-H (3) autoradiography. This allowed for double and triple labelling of cells and greater understanding of neuronal circuitry involved in

neurogenesis. In 1998, BrdU was administered to cancer patients to assess the proliferactivity of the tumour cells. At autopsy, the hippocampus and subventricular zone of the caudate nucleus were examined and showed positive labelling for BrdU with the oldest patient being 72 years of age [(Eriksson et al., 1998) Image 1.8].

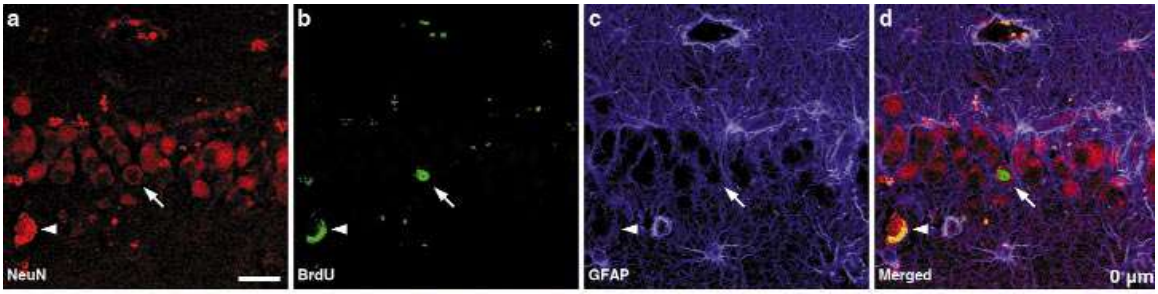


Figure 1.8 Image showing BrdU positive cells in the human DG showing adult human neurogenesis. Simultaneous detection of immunofluorescent labels for NeuN (a; scale bar represents 25 mm), BrdU (b) and GFAP (c) for detection of astrocytes and a merge of these signals in x-, y- and z registration (d) Image from (Eriksson et al., 1998)

1.3.2.2 Mechanism of neurogenesis in the adult hippocampus

Neurogenesis in the adult hippocampus is exclusively found in the DG. The SGL is where new neurons are formed and as they mature they become excitatory granular cells in the GCL. The granular cells are fundamental in the hippocampal tri-synaptic circuitry system and they receive the majority of input projections from the EC before relaying the input to the CA3 pyramidal neurons (see Figure 1.5).

Unlike new neurons produced in the subventricular zone (SVZ) which migrate to the olfactory bulb in rodents by the rostral migratory stream (RMS), the new granular cells are much more locally confined, with only a few cells migrating towards the molecular layer of the DG (Kempermann et al., 2003). Additionally, neurogenesis in the hippocampus produces only a small number of new neurons; in the order of hundreds in the mouse compared to thousands in the olfactory bulb. Therefore, hippocampal neurogenesis is described as being cumulative rather than replacement (Ninkovic et al., 2007, Imayoshi et al., 2008).

The SGZ is ideal for the development of new neurons as it offers an almost unique environment which provides a good possibility that the new neurons will reach full development. Areas like this are often referred to as neurogenic niches. This environment includes astrocytes, which support the developing new neurons with diffusible and membrane bound supportive factors that promote neurogenesis (Seri et al., 2001, Song et al., 2002). Furthermore, the SGZ is a highly vascularised region, with clusters of progenitor cells often found near to blood vessels leading to the theory of vascular niches. Also, 37% of all dividing cells are immunoreactive for endothelial markers (Palmer et al., 2000). This theory was further supported by the finding that VEGF is involved directly with neurogenesis (Palmer et al., 2000, Cao et al., 2004). However it seemed in vivo that VEGF had a survival promoting effect, with reduced apoptosis and no change in proliferation unlike in vitro where VEGF showed direct involvement in proliferation (Schanzer et al., 2004). There is a growing body of evidence that suggests that the link between angiogenesis and neurogenesis is not just serendipity; one study has now shown that the process of neurogenesis is directly related to blood-borne factors such as chemokines CCL_2, CCL_{11}, CCL_{12} and CLL_{19} and that this may explain the decline in neurogenesis with age (Villeda et al., 2011). It has been shown that blood vessels direct the migration of neurogenic cells (Andreu-Agullo et al., 2009).

The development of mature granular cells from progenitor cells follows a basic linear model. The mechanism is initiated by stem cells which develop into progenitor cells followed then by blast cells and concluding with differentiated cells. Specifically within the hippocampus the cells in this mechanism are divided into a numbering system. Primarily, the self-renewing multipotent stem cells are referred to as type-1 cells. When these cells develop they form transiently amplifying progenitor cells; initially apical (type 2a cell) followed by basal (type

2b). These then become the type 3 migratory neuron-determined blast cells, which via a terminal division produce a post-mitotic progeny (Kempermann, 2011).

Type 1 cells have a characteristic morphology resembling radial glia and display astrocytic properties (Filippov et al., 2003). Type 1 cells express glial fibrillary acidic protein (GFAP) but do not express the other astrocytic marker S100β (Seri et al., 2004). Due to their complexity including long apical branches and interactions with vasculature, it would be difficult to imagine that type 1 cells would successfully be able to complete division. However, it has been shown they will complete asymmetrical division. The orientation and greater morphology of the cell will be maintained during division and the daughter cell would bud at the base (Kempermann, 2011).

Type 2 cells are type 1 daughter cells (Filippov et al., 2003, Fukuda et al., 2003). Cell division occurs in clusters and is associated with the vasculature, as previously described; the cells then begin to migrate into the GCL (Kuhn et al., 1996). The first signs of neuronal determination are seen in type 2 cells, leading to the separation of this group into a and b cells. Type 2B cells express precursor identity and markers for neuronal lineage such as the marker doublecortin (DCX), the transcription factor NeuroD (Steiner et al., 2006) and an alteration in electrophysiological properties (Fukuda et al., 2003).

Type 3 cells are exclusively neuronal and do not express astrocytic markers or show a combined electrophysiological signature like earlier type 1 and 2a cells. The cells display dendrites although dendritic spines are not clear (Plumpe et al., 2006). This stage compromises the transition from proliferative state to post mitotic immature neuron (Figure 1.9).

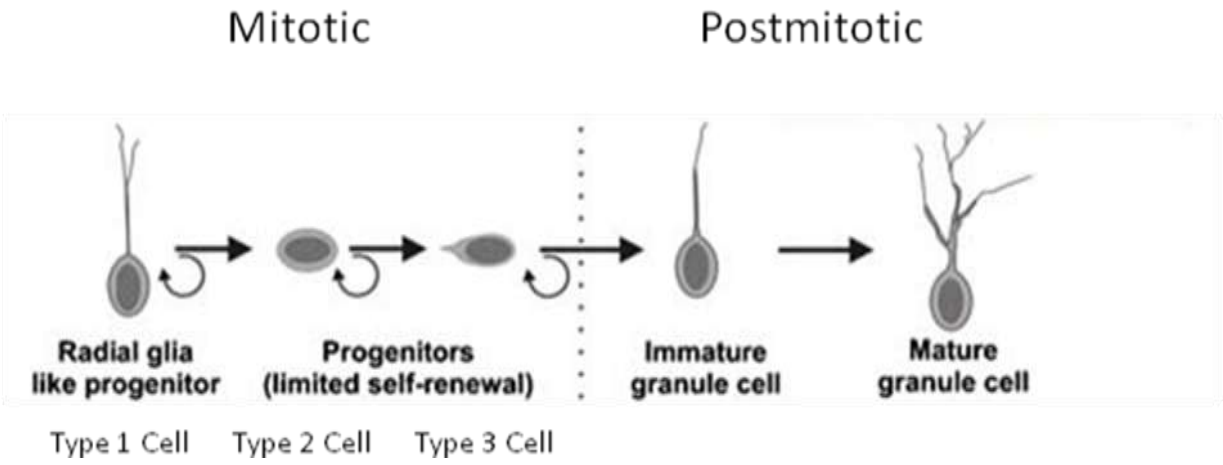


Figure 1.9 The linear process of neurogenesis. Image shows the mitotic and post mitotic stages and includes the different cell types. Image adapted from (von Bohlen Und Halbach, 2007).

1.3.2.3 Function

The important question is whether the development of new neurons in the hippocampus leads to an increase in function and if that is beneficial, possibly by increasing cognition or limiting damage? As with most research in neurogenesis the majority of this work has been studied in rodent models, little is known how this translates to human. Individual granular cells can fire action potentials, which can lead to alterations at the network level, which can be analysed by measuring differences in the hippocampal circuitry. At the system level, these changes can make alterations in the ability of learning and memory.

Most of the evidence that these newly developed cells are functional is taken from electrophysiological measurements of isolated precursor cells. In 2002, it was shown that when co-cultured with primary neurons and astrocytes, neural stem cells developed into active neurons and integrated into networks with functional synaptic transmission (Song et al., 2002). However, in vivo studies using patch clamp techniques targeting GFP+ cells promoted by the nestin gene have also been described (Filippov et al., 2003, Fukuda et al., 2003).

For integration to occur the new neurons have to demonstrate that they have fully extendable axons and dendrites which can synapse with target cells. Granular cells were shown to have such dendrites along the mossy fibre tract (Stanfield and Trice, 1988). This was followed by work that showed rapid extensions of axonal projections from the new granular cells to the CA3 region and that the axons are surrounded by synaptic vesicles (Hastings and Gould, 1999, Markakis and Gage, 1999). Synaptic integration was confirmed using a pseudorabies virus expressing GFP. The virus is actively transported along axons and dendrites to synapses, where it passes on to neurons upstream within a circuit (Card and Enquist, 2001). After injection of the virus labelled with GFP into CA1 neurons, the marker was later found in BrdU labelled neurons in the GCL suggesting that the neurons were integrated into the circuit (Carlen et al., 2002).

The hippocampus contains a specific unidirectional circuit, which is often referred to as the tri-synaptic circuit. The development of new neurons in the DG alters the circuit primarily at the point between the EC and CA3. Firing patterns in the DG are sparse and the number of granular cells far outweighs the number of cells in the EC, which has led to the suggestion that each granular cell might only carry a small and distinct fraction of the input (Leutgeb et al., 2004). The DG is connected with the CA3, the second station of the tri-synaptic circuit and it is thought data compression by the DG would stop a process called catastrophic interface, by which information would be constantly overwritten as the information does not have time to be modulated and stored. Formation of long term memory requires the transfer of memory from the hippocampus into storage in the cortex. This is believed to occur predominately in the CA1 region. As the DG is two steps in the circuitry away from the CA1, it is believed that neurogenesis functionally contributes to data processing and independent of storage (Kempermann, 2011).

A number of studies have suggested that neurogenesis is involved in up to five overlapping functions in learning and memory. See Table 1.2 for details:

Function	References
Improving temporal resolution, pattern separation and avoiding catastrophic interference	Impaired spatial pattern separation, without navigation and locomotion, post ablation of adult neurogenesis (Clelland et al., 2009) Impairment in reversal task of the Morris water maze (Garthe et al., 2009) Additive network to avoid interference, computer modelling (Appleby and Wiskott, 2009)
Increased ability to add temporal emotional and other contextual information	Requirements of new neurons for anxiety like behaviour (Santarelli et al., 2003) Relevance of new neurons in contextual fear conditioning (Saxe et al., 2006, Winocur et al., 2006)
Improving learning and memory	Improved probe trial performance in water maze (Imayoshi et al., 2008, Deng et al., 2010)
Apparent improved processing speed	Correlation between neurogenesis and acquisition speed in water maze (Kempermann and Gage, 2002)
Improving formation of allocentric representations and increasing flexibility to integrate new relative details	New neurons only improve hippocampus dependent aspects of water maze performance (Dupret et al., 2008) Neurogenesis improves reversal learning and flexible use of advanced spatial strategies (Garthe et al., 2009)

Table 1.2 Alterations in function of learning and memory and involvement in neurogenesis. Table adapted from (Kempermann, 2011).

1.3.2.4 Evidence of neurogenesis in relation to stroke and cognitive impairment

The hypothesis that neurogenesis may be able to reduce the effect of cognitive impairment and cortical injury by replacing lost or damaged neurons is an attractive option due to it being a naturally occurring mechanism. Studies have shown an improvement in cognitive outcome with enhanced neurogenesis after traumatic brain injury in mice (Han et al., 2011). With regards to stroke, both global and local hypoxia can induce neurogenesis in animal models

including cerebral artery occlusion models (Liu et al., 1998, Kee et al., 2001, Jin et al., 2001). However, it has been shown in animal models neurogenesis declines with age and with one study reporting no evidence of hippocampal neurogenesis but evidence of neurogenesis in the olfactory bulb in older mice [aged up to 24 months (Jin et al., 2004a)]. A later study showed similar decreases with age but also noted that there was an up regulation of neurogenesis in 15 month old rats that underwent middle cerebral artery occlusion (MCAO) compared to sham animals. In addition to showing evidence of hippocampal neurogenesis and suggesting that this ischemic incident might initiate the mechanism again in later life. It was also shown that neurogenesis in the SVZ was less affected than that in the SGZ (Darsalia et al., 2005). There is evidence that neurons formed in the SVZ separate from their usual migratory stream to the olfactory bulb and instead migrate to the site of injury post ischemia (Arvidsson et al., 2002, Yamashita et al., 2006).

A limited number of studies suggest neurogenesis occurs post stoke in humans (Jin et al., 2006, Marti-Fabregas et al., 2010). These studies used markers established for cell proliferation including Ki-67 and polysialylated neural cell adhesion molecule (PSAnCAM) respectively, suggested cell proliferation in and around the ischemic penumbra and in the ipsilateral SVZ.

A number of additional studies have looked at the relationship between neurogenesis and AD, based on the theory that inhibition of neurogenesis may be linked with cognitive impairment. However, studies in transgenic (Tg) mouse models reported that AD pathology, specifically Aβ, has an up regulatory effect on cell division (Lopez-Toledano and Shelanski, 2007, Rohe et al., 2008). Studies have shown there is a significant decrease (Verret et al., 2007, Hamilton et al., 2010). Another study showed a decrease only in the hippocampus with the olfactory bulb spared (Zhang et al., 2007). There is additional controversy regarding the

toxicity of Aβ to precursor cells (Haughey et al., 2002, Lopez-Toledano and Shelanski, 2007). It has also been suggested that APP may itself be instrumental in playing a role in neuronal cell differentiation (Sugaya et al., 2007).

Studies in brain post mortem tissue suggested that neurogenesis might be increased in AD, due to an increase in the marker DCX (Jin et al., 2004b). Other studies have indicated that neurogenesis expression is altered in relation to Braak staging, with varying results dependent on the different markers used with increasing Braak stage (Perry et al., 2012). Whilst, growth factor promoters such as bone morphogenic protein (BMP6) have been shown to be up regulated in AD human hippocampal tissue and Tg mice, compared to controls, although this study found a decrease in neurogenic expression (Crews et al., 2010). Whilst glia and vascular changes are associated with an increase in Ki-67 and DCX in the CA fields, there were no changes associated with the DG in cases of presenile AD (Boekhoorn et al., 2006). A study of the SVZ of VaD post-mortem tissue suggested an increase in proliferating markers (Ekonomou et al., 2011).

1.4 Aims and outlines of thesis

The work described in this thesis had two major aims. To ascertain if there is evidence of an increase in both vascular and neuronal regenerative mechanisms (angiogenesis and neurogenesis) in PSND cases compared to PSD. Although there are other possible mechanisms including gene interaction, these regenerative mechanisms may provide a cellular explanation for the "brain/cognitive reserve" hypothesis. This hypothesis attempts to explain why some cases can have different cognitive outcomes over time even with a similar level of neuropathology or that they may have suffered from a similar specific neurological event, such as a stroke. The PS cases were compared with control subjects, VaD and AD

cases to discover if there are particular similarities between the PS groups and other disease progressions.

To investigate these mechanisms, post mortem human hippocampal tissue was studied from elderly PS survivors and compared with that obtained from elderly subjects with different dementias. The hippocampus was chosen for this study because of its importance to memory and learning and also its dysfunction in dementia. Previous, CogFAST studies have also reported that medial temporal lobe atrophy was a key substrate of cognitive impairment in post-stroke decliners (Firbank et al., 2007). Furthermore, the hippocampus has specific vulnerability to ischemia and is one of the few sites of endogenous neurogenesis in humans. The study aim was to investigate changes in vascular density and previously established markers of neurogenesis in post mortem human tissue. Furthermore, changes in expression of growth factors were also to be investigated in order to assess their influence in either or both of the aforementioned mechanisms.

Chapter 2 Materials and Methods

2.1 Introduction

This chapter provides the diagnostic criteria for the CogFAST, VaD, AD cases and discusses the definition of controls. It also outlines the brain sampling methods and immunohistochemical protocols and details of techniques used for image analysis.

2.1.1 Study Subjects

2.1.1.1 Cognitive tests

Cognitive function of CogFAST patients were evaluated by clinical psychologists using the following cognitive tests.

2.1.1.2 Mini-mental state examination

Mini-Mental State examination (MMSE) is a short questionnaire test for cognitive impairment screening (Folstein et al., 1975). It is the most commonly used cognitive test, although age, sex, depressive symptomology and educational level can give rise to false positive cases (Gagnon et al., 1990, Crum et al., 1993). The MMSE is comprised of five sections: attention and calculation, language and praxis, orientation, recall and registration. A cut off score of 24 is most frequently used and a score of 23 or less suggests the presence of cognitive impairment (Mungas, 1991). For classification purpose, scores of 27-30 are considered normal, a cut off of 26 gives optimal sensitivity and specificity to detect mild/moderate dementia (Mungas, 1991, van Gorp et al., 1999).

2.1.1.3 Cambridge Cognitive Examination

Cambridge Cognitive Examination (CAMCOG), which is a cognitive test section of CAMDEX assessment (Cambridge Mental Disorders of the Elderly Examination) and was designed to assess various cognitive functions for the diagnosis and grading of dementia (Roth et al., 1986). The commonly-used cut off point of CAMCOG is 79/80 (Roth et al., 1986). It covers a broader range of cognitive functions than MMSE including orientation, language, memory, praxis, attention, abstract thinking, perception and calculation and is modified by age and educational level. Therefore it performs well compared to the MMSE test and has demonstrated excellent sensitivity and specificity for dementia (Huppert et al., 1995).

2.1.2 Diagnosis and definition of groups

2.1.2.1 COGFAST cases

CogFAST study is a prospective cohort study with cognitive function assessment of post-stroke patients. The aims of the study were to address the long term consequences of ischemic injury in the elderly and to elucidate the mechanism of delayed cognitive impairment after stroke and determine factors influencing the fate of post-stroke survivors (Ballard et al., 2003b, Allan et al., 2011). Stroke patients' ≥75 years old were selected, provided they were not demented three months post-stroke and did not exhibit disabilities that would prevent them from completing cognitive testing. They received annual clinical assessments and a neuropsychological test battery from baseline including MMSE and CAMCOG tests which generated sub-scores for various cognitive domains including memory and executive function (Ballard et al., 2003a, Allan et al., 2011). Autopsy was performed 24-92 hours after death and the brain was then fixed in 10 % buffered formalin for

5-32 weeks. Cases range in age from 78 – 96 years. Braak stage and CERAD (consortium to establish a registry for Alzheimer's disease) score were determined by a neuropathologist. A vascular severity scale was initiated and performed on these cases as previously described, with a maximum score of 20 with a single point given for the presence of any vascular pathology in a region analysed (Deramecourt et al., 2012, see chapter 1 for more details). Thal staging (Thal et al., 2002) for Aβ plaque deposition was also performed. The hippocampal formation and medial temporal lobe were stained with the 4G8 antibody and each case was graded, dependent on staging criteria orgionally reported and mentioned in Chapter 1. Thal staging is divided into six stages, which involves the entire brain and brain stem. Thal has also specifically reported the deposition of Aβ plaques in the medial temporal lobe. In stage 1, deposits begin in the temporal neocortex and then in stage 2 depositis expand to subiculum and the CA1 region. By stage 3, Aβ deposits are observed in the WM and the molecular layer of the DG. Finally in stage 4, Aβ deposits are seen in CA4 and with each increasing stage further deposition of Aβ is seen in the regions where deposits have started. However, as the only regions investigated were in the temporal lobe, a maximum score of 4 could be assigned to cases. Higher scores could only be given with additional analysis of separate regions including the cortex, brain stem and cerebellum. Analysis showed a significant increase in Thal staging of PSND compared to PSD. Significant decreases in both MMSE and CAMCOG were observed in the PSD group. There were no significant difference in APOE genotype, stroke details including anatomical location, previous history of stroke and time from stroke to death. Details regarding the CogFAST cases are available in Table 2.1

	PSND	PSD
Total number of controls or cases analysed	23	14
Age, y mean (Range)	84.1 (79-91)	87.8 (80-98)
PMD, h mean (± 2SEM)	44.7 (11.2)	47.15 (14.6)
Gender (% Male)	52.2	42.9
MMSE score (Range)	27.1 (24– 30)	17.5 (12-24)*
CAMCOG score (Range)	89.1 (82-99)	62.9 (24-80)*
Braak stage median (Range)	2.0 (0-5)	3.0 (0-4)
CERAD median (Range)	2.0 (0-2)	1.0 (0-3)
Thal stage median (Range)	3.0 (2-4)	1.0 (0-3)*
Vascular pathology median (Range)	13.0 (7-16)	12.0 (7-18)
Vascular pathology hippocampus (range)	2.0 (1-3)	2.0 (1-3)
APOE genotype (% of cases)	3/3 (43.8), 3/4 (37.5), Other (18.8)	3/3 (58.3), 3/4 (16.7), Other (25.0%)
Time from stroke, months (± 2SEM)	60.7 (21.7)	57.5 (12.2)
Previous Stroke (% of cases)	Yes (52.6), No (42.1), Unknown (5.3%)	Yes (30.8), No (61.5), Unknown (7.7)
Lesion Location (% of cases)	Parietotemporal (17.4), Deep WM (13.0) and Cerebellum (8.7), Unknown (60.9)	Parietotemporal (35.7), Deep WM (21.4), Cerebellum (7.1), Unknown (35.7)
Brain side of Lesion (% of cases)	Left (8.7), Right (21.7), Both (26.1), None (26.1), Unknown (17.4)	Left (35.7), Right (21.4), Both (14.3), None (21.4), Unknown (7.1)
Vascular territory involved (% of cases)	MCA (30.4), PCA (8.7), Unknown (60.9),	MCA (57.1), Ventrobasilar (7.1), Unknown (35.7)

Table 2.1 Detailed information of the CogFAST cases analysed in this study. Fixation The time period (weeks) of tissue fixation was in range 4-32 weeks for all the cases. Significant differences ($p \leq 0.05$) are highlighted by * MCA – middle cerebral artery, PCA – posterior cerebral artery

2.1.2.2 Control, VaD and AD groups

Subjects were classified as demented if they met DSM-IIIR criteria for dementia. Controls >70 years old were only selected if they had not been diagnosed clinically with cognitive impairment. Final classification of demented subjects was assigned based on established neuropathological diagnostic criteria (Kalaria et al., 2004). Briefly, Haematoxylin-eosin staining was used for assessment of structural integrity and infarcts, Nissl and Luxol fast blue staining for cellular pattern and myelin loss, Bielschowsky's silver impregnation for 'CERAD' rating of neuritic plaques and tau IHC for 'Braak' staging of NFT's. A diagnosis of VaD was made when there were multiple or cystic infarcts, lacunae, microinfarcts, SVD, and Braak stage <III (Kalaria et al., 2004). The diagnosis of AD was assigned when there was evidence of significant Alzheimer's type pathology namely Braak stage V-VI, moderate-severe CERAD score and absence of significant vascular pathology. Both Thal staging (Thal et al., 2002) and Vascular pathology scores (Deramecourt et al., 2012) were also performed where possible, as previously described. Control subject tissue was determined not to have sufficient pathology to reach threshold to ascertain a diagnosis for dementia (details for all groups can be seen in table 2.2).

	Controls	VaD	AD
Total number of controls or cases analysed	18	15	14
Age, y Mean (range)	81.9 (72-98)	85.1 (71-97)	83.5 (70-90)
PMD, h Mean (± 2SEM)	35.1 (11.1)	34.86 (13.4)	58.67 (15.82)
Braak stage Median (range)	2.0 (1-4)	2.0 (1-4)	5.5 (4-6)*
CERAD Median (Range)	NPD	1.0 (0-2)	3.0 (3-3)*
Thal Stage Median (Range)	NPD	2.0 (0-3)	4.0 (3-4)*
Vascular Pathology (Range)	NPD	14.0 (12-18)	NPD

Table 2.2 – Demographic information for all subjects used throughout studies in control, VaD and AD groups. Fixation - The time period (weeks) of tissue fixation was in range 4-40 weeks for all cases. Significant differences ($p \leq 0.05$) are highlighted by * NPD = No pathological data available.

2.2 Brain Tissue

Formalin fixed, paraffin embedded blocks of human brain tissue were obtained from the Newcastle Brain Tissue Resource Centre (NBTR), Institute for Ageing and Health, Newcastle University. Additional control subjects were provided by London Neurodegenerative Disease Brain Bank, Institute of Psychiatry, King's College, London. Ethical approval was granted by local research ethics committees for this study (Newcastle upon Tyne Hospitals Trust, UK) and permission for post-mortem research using brain tissue was granted for this project. The primary neuropathological diagnosis and observations were transcribed from post mortem reports. Brains with fixation length of less than 40 weeks were selected for the studies in order to reduce the variability in IHC staining intensity due to excessive fixation time. Additional selection criteria were made so that control cases with a history of myocardial infarction and other cardiovascular causes of death were excluded from the study. The majority of work in this study has been carried out in the hippocampus region.

Additional analysis has also been performed in the EC, Brodmann area 36 (BA36) and Dorso-lateral prefrontal cortex (DLPFC).

2.2.1 Hippocampus and Cortical Regions

Hippocampus and adjacent EC are important areas for autobiographical, declarative and episodic memory (Eichenbaum, 2004). Information from other areas of the brain is sent to CA fields via layers of the EC, which acts as the interface between the hippocampus and other brain regions (Amaral, 2006). The flow of information is predominately unidirectional in that signals propagate in the EC, pass through the hippocampus and are then sent back to the EC (Amaral, 2006). The temporal lobe tissue block was selected from coronal level 18-20 of the Newcastle Brain Map [(Perry, 1993) figure 2.1]. Hippocampal areas and EC were defined as described by the others (Insausti, 1990, Insausti et al., 1998). The DG's principle cell is the granular neurone, which is tightly packed into a layer that encompasses the CA4 region in coronally cut hippocampus. The CA2 region forms a tight band of large pyramidal cells near the hippocampal fissure; the band broadens to form the CA1 region containing a substantially heterogeneous group of pyramidal neurones. Neurons in the EC are organised into six distinct layers and form many connections with both the hippocampus and a number of separate brain regions including the DLPFC (Goldman-Rakic et al., 1984).

The DLPFC, located in the frontal lobe, is an area synonymous with planning tasks and executive functions. The frontal lobe is known to be affected in AD, with large deposits of Aβ (Rowe et al., 2007). VaD is often characterised by impairments of executive function such as planning and judgement, which is associated with the frontal lobe (Roman and Royall, 1999). The DLPFC is particularly vulnerable to cerebrovascular disease (Xi et al., 2011). The DLPFC, which is roughly equivalent to Brodmann areas 9 and 46, is involved in

the integration of processes such as working memory, problem solving and planning. Those with damaged DLPFC displayed impaired reasoning and lack the ability to maintain attention. Sections were selected from levels 6 of the Newcastle brain map [(Perry, 1993) Figure 2.1].

2.3 Immunohistochemistry

2.3.1 Standard Protocol

Paraffin-embedded brain tissue blocks were cut into either 10 or 30 μm sections, dependent on requirements for experiments, stereological experiments need thicker tissue to take into account the z axis. After incubation at 60 ˚C for 30 minutes, sections were transferred into xylene to dewax, followed by rehydration in decreasing concentrations of alcohol. The antigen retrieval was performed by various forms of pre-treatment such as heat treatment in the form of microwaving sections for 10 minutes or pressure cooking them for 2 minutes in 0.01M boiling citrate buffer (pH 6.0). Prior to COL4 staining, enzyme antigen retrieval was used; sections were incubated with 0.6% Subtilisin A, type VIII bacterial protease (Sigma, UK) solution for 10 minutes at room temperature. Sections were quenched by 3% H_2O_2 for 15 minutes, unless stated otherwise, when they were quenched with a 10% H_2O_2 methanol solution for 30 minutes. Blocking of non specific binding was performed with normal horse serum (anti-mouse antibody), normal goat serum (anti-rabbit antibody) or normal rabbit serum (anti-goat antibody) for 30 minutes, dependent on primary antibody. Primary antibodies were diluted to a specific concentration with buffer and were applied to the sections. Sections were then incubated for the specified length of time at either room temperature or in a fridge (2 hours at room temperature or overnight at 4˚C). Biotinilated secondary antibody was applied to the sections with the blocking serum for 30 minutes, followed by incubation in tertiary antisera stripped avidin biotinalted complex (SABC) for 30 minutes for increased specificity. Sections were treated with chromogens including 3,3‘-Diaminobenzadine (DAB), Nickel-DAB or Vector grey for a predetermined time to visualize the positive antibody reaction. Sections were then rinsed in water and counterstained with haematoxylin, strength determined on a stain by stain basis, using standard procedure.

Sections were dehydrated in ethanol and taken back through xylene in preparation for mounting in DPX mounting medium (Sigma, UK). After each step, except blocking, sections were rinsed in buffer (PBS/TBS) for 5 minutes x 3 times.

Dual labelling followed a similar protocol as above, a single antibody was labelled initially and then post staining protocol would be repeated specifically for the second antibody.

2.3.2 Fluorescent Labelling

Six micron sections were cut on a microtome and were then used for fluorescent staining. As previously described sections were dewaxed and a heat antigen retrieval step was used. No quenching step was required but an extended blocking step was used with normal horse serum applied to all cases. Selected antibodies were then incubated with sections at 4x concentration compared to those used for brightfield analysis; overnight at 4˚C. For double labelling both antibodies were incubated simultaneously with section. Sections were then incubated with appropriate secondary antibody at 1:200 concentration, anti-rabbit IgG Dylight 549 (red) or anti-mouse IgG Dylight 488 (green) in the dark lighting conditions. Excess secondary was removed and sections were washed in PBS. The sections were then stained with 0.5% Sudan Black to quench autofluorescence. Sections were mounted with Vectashield (Vector Labs, UK). All staining was performed with a PBS buffer adjusted to pH7.5.

2.4 Image Analysis

2.4.1 Brightfield Image Capture

Images were captured using either a Zeiss Axioplan 2 or AX10 research grade microscope coupled to a Luminera Infinity 2 camera, magnification was selected dependent on staining. When required the goniometer stage of the Axioplan 2 was used to enable all images to be

taken in the same orientation. The number of images taken per stain was calculated, dependent on the magnification, in order to allow as complete a coverage of the region of interest as possible, without taking execisive number of images or imaging the same area multiple times.

2.4.2 Digital image analysis

Images were analysed using Image Pro software (Mediacybernetics, USA). The software allowed for calculation of the quantity of staining, by measuring the total area of immunoreactivity and expressing it as a percentage of the total area of interest (AOI). It also allowed for the quantification of staining intensity by measuring the integrated optical density (IOD), which calculates the intensity of staining per pixel selected (Figure 2.2).

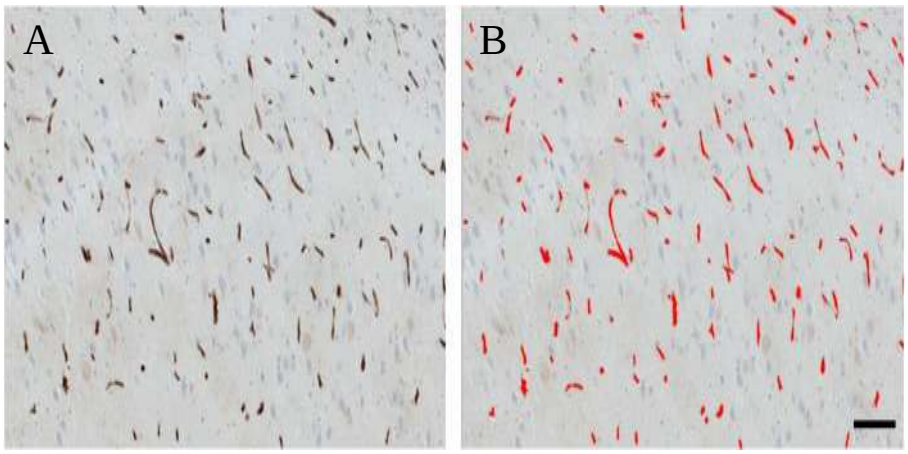


Figure 2.2 Image analysis performed by Image Pro software. A screen shot image to show (A) the image analysed and (B) analysis with Image Pro, displaying how the software is able to identify the subject of interest (DAB stained microvasculature, brown) from background and haematoxylin (blue) counterstained neurones. 10 μm thick section, bar = 100 μm

2.4.3 Vessel diameter analysis

Vessel diameter was measured using software developed within the group [Y. Yamamoto - VasCalc, (Craggs et al., 2013)]. The software is used to calculate the length of an object by

using digitally generated lines drawn by the operator across the subject matter of interest. The lines are calibrated at the first stage by measuring a known length from a graticule at the magnification selected for analysis.

2.4.4 Stereology

Stereology allows for the estimation of structural characteristics relating to parameters such as volume, surface area, length, number and thickness using unbiased sampling (Mayhew, 1991, Nyengaard and Gundersen, 2006, Dockery and Fraher, 2007). It reduces the issues in traditional approaches to quantify 3D biological structures in 2D reference space by efficient sampling through the x, y and z axis. This allows for efficient and unbiased sampling of entire regions of interest removing the complexity of measuring these parameters in human tissue and therefore allows more precise analysis of parameters in fewer cases than traditional methods.

In this thesis, two parameters are described and measured using stereology, length density (L_v, Chapter 4) and cell density (Chapter 7). Both of these parameters were measured on the same system, which included Stereologer 2000 software (Stereologer, WV, USA) installed on a computer connected to a Zeiss Axioplan microscope, with a motorised stage and JVC live image camera. Fifteen serial 30 μm-thick sections were cut from predefined blocks containing the region of interest and for each study three sections were selected per case with a periodicity of five. Each AOI is mapped out at low magnification, with a number of equally spaced grids produced by the software to allow for accurate and complete sampling.

There has been a long history of attempting to calculate the length of an object. In the 18th century, Count Buffon first demonstrated with the needle problem (Buffon, 1777). He showed that needles of an unknown but equal length will intersect parallel lines with a

predictable probability. The number of intersections will be directly proportional to the length of the needle and inversely proportional to the distance between the lines. This rule can be stated by the mathematical formula - $P = (2/\pi)x(l/d)$, P is the probability of the needle and line intersecting, l the length of the needle and d the distance between the lines. The factor $2/\pi$ refers to the isotropic (uniformity in all directions) intersections between the lines on the floor and the needle; $2/\pi$ includes all integral angles between the needle and lines on the floor (Calhoun and Mouton, 2000). Buffon performed a few measured observations, where he would throw the needles into the air and count how many intersected with the lines on the floor. After a number of repeated measurements he was able to determine a constant probability for an intersection between a needle and the lines on the floor. From this information he was able to accurately estimate the length of the unknown needle.

As with the above example, stereological estimations of length depend on random intersections between the probe and the object of interest i.e. microvasculature. Smith and Guttman (1953), proposed a three dimensional application of Buffon's Needle principle, in which the total L_v (length per unit volume) is proportional to the number of random intersections between linear objects and a two dimensional sampling probe, giving the formula - $L_v = 2.\sum Q/\sum A$. $\sum Q$ is equal to the number of random intersections and $\sum A$ the area of sampling probe. L_v was shown to be directly related to the total surface area and total length with no further assumptions required (Mouton et al., 2002).

Unlike number, length is an orientation dependent parameter and to ensure accurate estimates of length, either the tissue or the probe must have an isotropic orientation. Gokhale (Gokhale, 1990) demonstrated that a sphere was the ideal probe for estimating the length of anisotropic structures as it was perfectly isotropic in three dimensions. There are a number of

techniques utilizing a spherical probe to estimate length in tissue, including vasculature (McMillan, 1994, Lokkegaard et al., 2001).

As the tissue analysed in this study was from a brain bank environment and therefore had previously been cut into vertical uniform random (VUR) sections, the option to randomly orientate the tissue is not possible. Therefore the probe must be isotropic to give unbiased estimation of length. A digital spherical probe was developed by (Mouton et al., 2002). It was this, spherical probe, principle that was selected in these studies (see chapter 4).

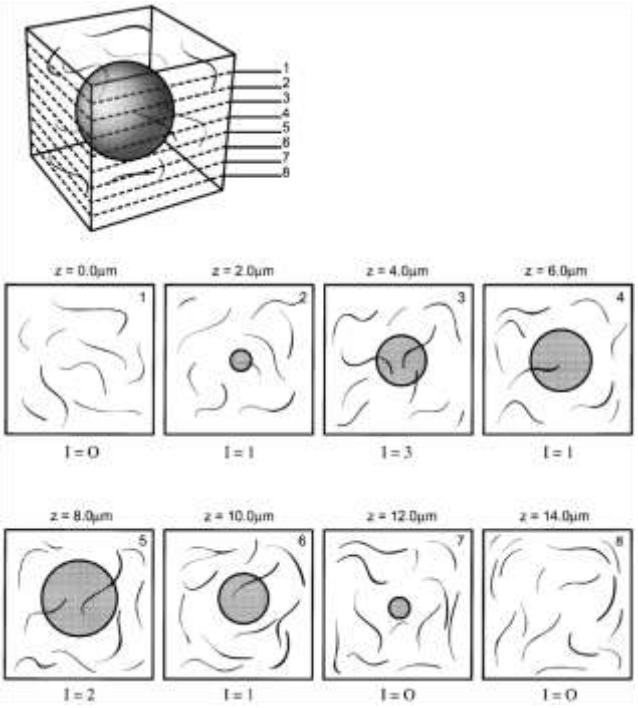


Figure 2.3 A Schematic impression of the methodology for using the spherical probe to measure length. The 3D spherical probe is viewed at eight 2D focal planes, 1 µm apart in the z axis. The total number of intersections (I) is equal to eight and is proportional to the length of the linear fibres within the tissue. The first and last focal planes are above and below the probe respectfully and therefore show no interactions. Image from (Mouton et al., 2002)

Cell density was analysed using the optical disector probe technique (Sterio, 1984, West et al., 1991). Similarly to the spherical probe method, three sections were analysed per case and the reference space was mapped out at low magnification. At high magnification the motorised stage positions the section relative to each grid point and at this time the software displays a dissector frame superimposed upon the microscopic image. Within this frame the operator counts all the objects of interest provided the object is found entirely within the

counting frame or if the object touches one of the inclusion lines. Objects are excluded if any part of them touches the exclusion line (Image 2.4).

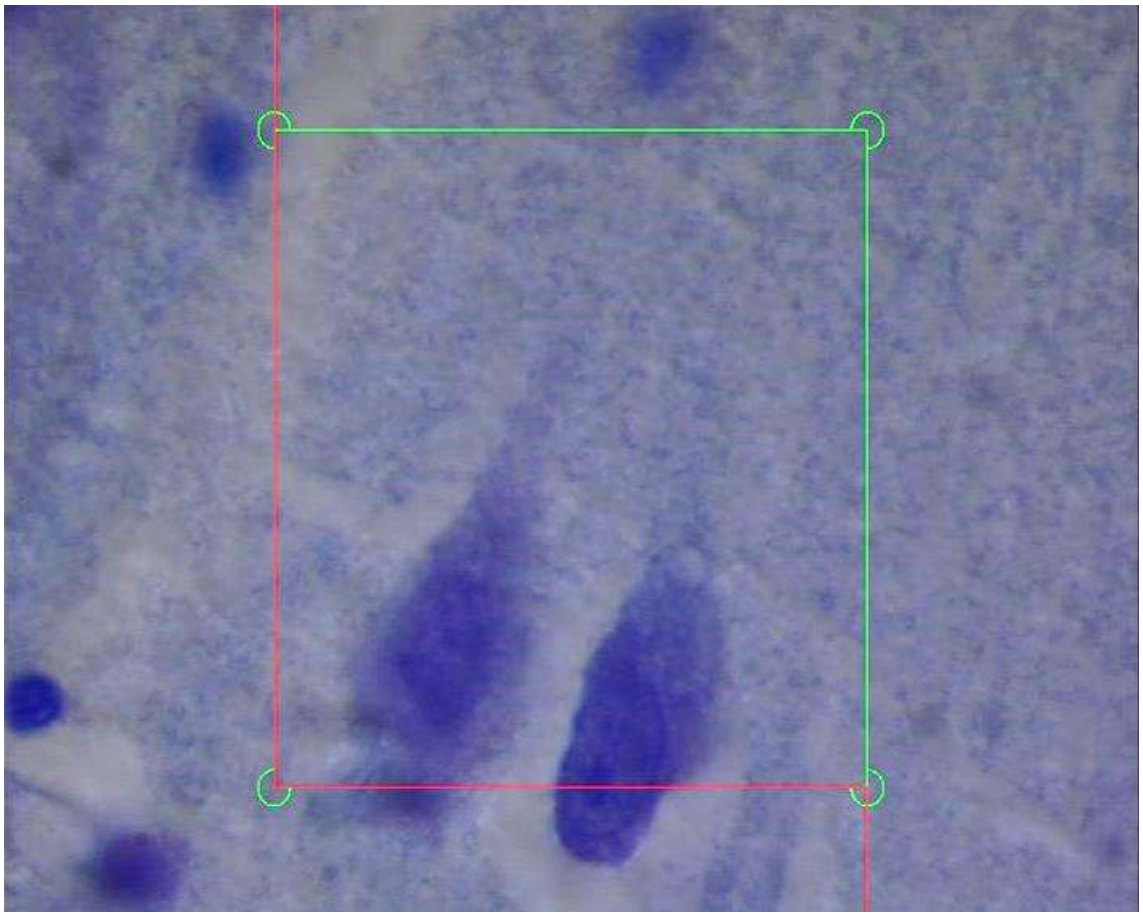


Image 2.4 Disector Probe for analysis of cell density. Nissl stain section using disector probe for cell density count. Probe shown as a square, cells inside the cell are counted. If any part of the cells touch the exclusion lines (red lines), then they are not counted however if a cell is not inside the dissector box but is touching the inclusion line cells (green lines) then will be counted. The lines guarantee that the same cell will not be counted twice even if two probes are adjacent.

2.4.5 Confocal fluorescent image capture

All imaging was performed at the Newcastle University Bio Imaging Unit. Assistance for image capture was provided by Dr. T Booth and all images were taken using a Leica TCS SP2 AOBS (UV) microscope (Leica, Germany). The confocal microscope allows images to be acquired at all magnifications, up to and inclusive of 100x lens magnification, whilst displaying the maximum resolution attainable with fluorescent imaging. This is achieved by

utilizing a laser source traversing through the "Z" plane, whilst acquiring images at known focal depths. The individual "Z" plane images are then reconstructed into either a 2D or 3D image. The advantage of confocal microscopy compared to standard fluorescent microscopy is to be found in the clarity of the fluorescent signal with respect to background noise, as lasers exhibit a small depth of focus compared to standard fluorescent lamps. Also, confocal microscopy allows the use of wavelength channels to filter noise when images are being acquired, which is not normally integrated into standard fluorescent systems.

2.5 Statistical Analysis

Statistical analysis was performed using SPSS version 19 (IBM, USA). Shapiro-Wilk analysis was used to test normality of data. When normal distribution was expected, One-Way ANOVA with Tukey's or Dunnett's T3 post hoc test was used as required. In cases of non-normally distributed data Kruskal-Wallis test was performed to assess significant differences between groups, followed by Mann–Whitney U test to obtain p-values for each pair. Spearman (rho) correlation was used to test the relationships between ages, length of fixation, post mortem delay and quantified variables. For analysis of cumulative frequency data in regard to vessel diameter analysis, Minitab 16 (Minitab Inc, USA) was used to analyse distribution of the cumulative frequency data by χ^2 analysis. Significance was recorded at $p \leq 0.05$ and is shown throughout the thesis as * on graphs. Significance of $p \leq 0.01$ is recorded as ** and a trend toward a significance difference was recorded at $p \leq 0.075$ and on graphs differences are shown as +.

Chapter 3 Microvascular Analysis of Hippocampal Subfields

3.1 Introduction

The human brain is a highly vascularised organ. It receives approximately 15% of the total blood flow and is responsible for around 25% of the oxygen consumption rate whilst only counting for 2% of the total body weight (Clark, 1999). Therefore any change to vasculature or blood flow can have severe consequences on the health of the brain. Previous epidemiological studies have shown that vascular disease risk factors play a key role in the progression of dementia (Hofman et al., 1997, Snowdon et al., 1997). These include increased blood pressure (Skoog et al., 1996), atrial fibrillation (Elias et al., 2006), diabetes mellitus (Pasquier et al., 2006) and other vascular disease-related factors. Pathological studies have shown an increase in cerebrovascular changes in AD, including CAA, compared to non neurological demented controls, correlating with the severity of AD neuropathology (Jellinger and Attems, 2005). This emphasises the importance of vascular pathology in AD (Premkumar et al., 1996). It has previously been well documented that in VaD cases, the numerous vascular dysfunctions are associated with the condition leading to cognitive impairment, inclusive of: atherosclerosis, hypertension and increased presence of WMH, caused by myelin loss and axonal damage, which is also associated with increased cerebrovascular events (Debette and Markus, 2010).

However, the specific role of the cerebral microvasculature in aging-related dementias still remains unclear. A number of human pathological studies using various methods suggest brain microvascular density, especially in AD, is decreased (Buee et al., 1997, Paris et al.,

2004b, Kitaguchi et al., 2007), whereas others claim that vascular density is increased (Bell and Ball, 1981, Richard et al., 2010, Schwartz et al., 2010). Differences in the methods of analysis and brain regions investigated may explain the variability in results.

Studies involving Tg mice models, which over-express APP to simulate AD pathology, have reported decreased angiogenesis in the presence of Aβ peptides (Paris et al., 2004a, Miao et al., 2005). A study using the Tg2576 mouse model showed increased microvascular density and extensive disruption to tight junctions leading to a new hypothesis of amyloidogenesis (Biron et al., 2011). A recent study using zebrafish also suggests Aβ plays a role in angiogenesis (Cameron et al., 2012). Vascular corrosion casts in a Tg AD mouse, formed by intravascular infusion of resin followed by removal of intervening tissue, showed increased vessel density, although the vessels had degenerated, shown by resin pouches around the vessel. Vessels terminated abruptly at Aβ plaques and surrounding vessels were narrowed with an appearance of angiogenesis (Meyer et al., 2008).

The status of microvascular density is generally lacking in VaD in the hippocampus. Brown et al 2007 analysed cases with leukoaraiosis, detected as WMH on MRI scans, for demyelination and reactive gliosis using brightfield microscopic examination. Leukoariosis is associated with VaD (van Gijn, 1998). In 2007, Brown reported significantly reduced microvascular density in leukoaraiosis lesioned deep WM compared to corresponding WM areas in controls. However, there was no significant difference in cortical vascular density. Furthermore, it should be noted that no previous study has been reported elucidating microvascular density analysis of long term survivors of stroke in human.

Additionally, human post mortem studies have so far suggested that there is a decrease in vascular diameter in cases of AD compared to controls (Bell and Ball, 1981, Bouras et al.,

2006). Lower mean capillary diameter was independently associated with greater antemortem cognitive disability, as measured by the Clinical Dementia Rating scale, even after controlling for the burden of senile plaques and NFTs (Bouras et al., 2006). Whilst, BM thickening was observed in cortical capillaries compared to controls (Mancardi et al., 1980). Similar findings have been observed in a Tg mouse model of AD (Wyss-Coray et al., 2000). However, there are no reported studies of vessel diameter analysis in VaD cases. These changes lead to an alteration in physiological CBF and the ability to control fluctuations in blood flow and pressure and direct increased hypoperfusion, which is commonly associated with dementia (Osawa et al., 2004).

These observations have led to proposed mechanisms, regarding reduced cerebral hypoperfusion in dementia caused by reduced CBF in cases of AD (Schuff et al., 2009) and similar findings have also been observed in our PSD cohort (Firbank et al., 2011). This reduced flow increases oxidative stress, which damages vessels and the surrounding tissue (Zhu et al., 2005). The decrease in CBF was believed to have been a result of neurodegeneration in dementia, where the cerebral microvasculature is restricted or modulated due to lower demand for glucose and oxygen. However, an alternative view has also been proposed, suggesting that the decrease in CBF may be the cause or instigator of neurodegeneration (de la Torre, 2000). Therefore it is believed subjects who maintain cognitive function will show unperturbed vasculature structure compared to those clinically diagnosed with dementia

The use of post mortem tissue from the CogFAST study gave a rare opportunity to assess the extent of hippocampal microvasculature density and diameter in a well characterised range of dementias and cognitively stable subjects, especially comparing differences between the PSND and PSD group. The aims of this study were to perform standard two dimensional

density analysis of microvasculature using markers of both ECs with glucose transporter 1 (GLUT1), and the BM layer with collagen 4 (COL4). in the CA1, CA2 and GCL within the hippocampus. Further analysis of the vessel diameter in the CA1 region would be performed on COL4 sections.

It was reasoned that this would provide some information regarding the structural status of the microvasculature in the disease groups. Areas were selected, as described previously, related to 1) the varying degree of damage associated with both ischemia and Alzheimer pathology for CA1 and CA2 and 2) to progress that part of the study relevant to the investigation of regional specific neurogenesis. Additionally, using both markers on serial sections allowed a simple ratio of vessel density to be applied, which gave an indication of either angiogenesis (GLUT1:COL4 > 1) or vessel degeneration (GLUT1:COL4 < 1). It was expected that dementia cases would show significant reductions in both vessel diameter and vasculature density.

3.2 *Materials and Methods*

3.2.1 Subjects

Demographic details of the subjects for this study from which the hippocampal brain samples were obtained at autopsy are given in Table 3.1

	Controls	PSND	PSD	VaD	AD
Total number of controls or cases analysed	13	23	13	15	14
Age, y Mean (range)	80.4 (72-94)	84.3 (78-94)	86.3 (80-96)	85.1 (71-97)	83.5 (70-91)
PMD, h Mean (± 2SEM)	28 (6.1)	45 (11.2)	47.2 (14.6)	35 (13.4)	59 (15.8)

Table 3.1 Demographics of groups including sample number, age and post mortem delay. The time period (weeks) of tissue fixation was in range 4-40 weeks for all the cases.

For vessel diameter analysis, 6 cases from each group were randomly selected. There was no significant difference in any of the demographics for this edited cohort (more detailed information on this cohort can be found in chapter 4). Cases were selected based on the criteria described in chapter 2. As expected significant increases were observed in Braak, CERAD and Thal pathological scores in AD cases compared to all other groups. Although vascular pathology, not recorded for AD or controls, showed no significant difference between PSND, PSD and VaD (chapter 2 for specific values).

3.2.2 Immunohistochemistry

Formalin fixed, paraffin embedded posterior hippocampal brain tissue blocks were serially cut into 10-µm-thick sections. Sections were mounted onto slides coated in 2% aminopropyltriethoxysilane (APES) solution and standard single label IHC protocol was followed as described in Chapter 2. Heat induced epitope retrieval was performed for GLUT1 (ThermoScientific, UK, 1:200 for 2 hours room temperature), and a protolytic enzymatic induced epitope retrieval was performed for sections stained with COL4 (Sigma Aldrich, UK, 1:500 for 2 hours room temperature). Both groups of slides were treated with

DAB for at least 4 minutes. Double label staining was performed using the same methods above; GLUT1 was stained with DAB and COL4 with Vector Grey (Vector Labs, UK)

3.2.3 Image Capture and Analysis

Sections were viewed under a Zeiss Axioplan 2 brightfield microscope at 10x magnification and numerical aperture 0.30. The CA1, CA2 and GCL region were specifically identified and imaged individually. Regions were detectable by the morphometric differences of pyramidal neurons in CA fields and the unique GCL structure in the hippocampus, which were visible due to haematoxylin counterstain. Where possible, six fields were captured from the CA1, three fields of the CA2 and five fields of the GCL for both GLUT1 and COL4 labelled sections.

The images were analysed using Image Pro Plus 4 software (Mediacybernetics, USA), an established program for image analysis as described in Chapter 2, which calculated the percentage of the image occupied by vasculature.

Images to establish vessel diameter were captured using the Zeiss microscope, at 40x magnification and the vessels imaged were all from the CA1 region. Approximately thirty images were taken of each CA1 region, this number of images allowed for an almost complete coverage of the region. Images were taken in planed and ordered columns across the stratum pyramidale from the striatum oriens to the striatum radiatum, with a small gap being left between each column to reduce the possibility of imaging the same area twice.

3.2.4 Hippocampal Area

The complete hippocampal area of the sections were analysed using a Zeiss Axioplan Photomicroscope with a Pixelink PL-B623CF colour digital camera and a motorised stage

attached. The microscope was connected to stereological software, Visopharm (Integration System, Denmark). The boarder of the hippocampus was mapped out as the mask of the AOI using a low magnification (2.5x). The criteria established for mapping each hippocampus was primarily to follow the structure around the hippocampal fissure, then around the external edge of the CA1, along the CA1 with the boarder with the WM until finally arriving at the start of the sulcus. The software then calculated the area for the regional mask, which was calculated in μm^2.

3.2.5 Vascular diameter analysis

All visible vessels were measured from the images captured; in total 4,082 vessels were sampled. Each vessel profile was only measured once to reduce over sampling bias. Due to orientation or size of the vessel, especially arterioles, it was occasionally difficult to determine if the diameter measured was the full external diameter, these vessels were ignored unless the vessel was cut trans-sectional and the complete diameter could be visualised.

Vessel diameter was measured using software developed within the group (VasCalc, Yamamoto Y, (Craggs et al., 2013)). The software is calibrated by measuring a known length, using a graticule, at the magnification selected for analysis. Length is calculated by using digitally generated lines drawn by the operator across the subject matter of interest. When the first line is drawn, the centre is marked by a cross. Two lines then intersect this line through the cross at approximately 30-45˚ angles from the original line; this reduces the possibility of bias caused by the angle of the original line drawn across the vessel. The average diameter of the vessel is then calculated from these three lines. For a vessel cut longitudinally, the first line is perpendicular to the length of the vessel and the two lines intersect this line. For a trans-sectional cut vessel the operator orientates the first line so that

it passes through the centre of the vessel at its longest diameter, which is again intersected by two lines at 30-45˚. Each vessel was measured three times; the average from these measurements was recorded as the vessel diameter (Figure 3.1).

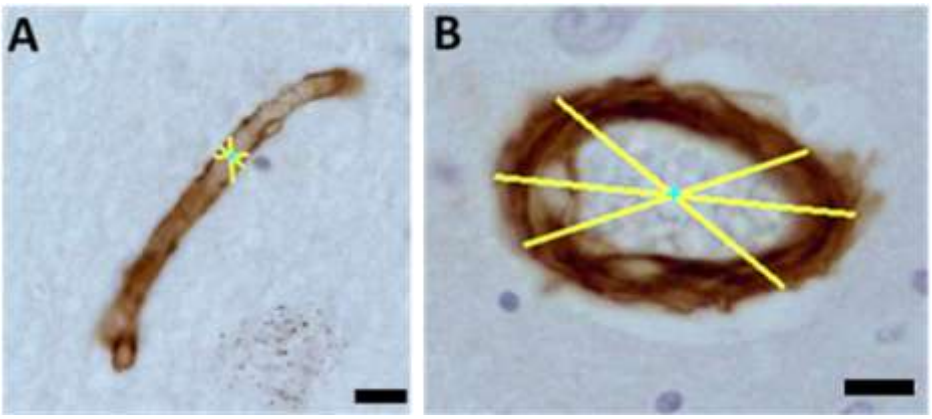


Figure 3.1 VasCalc measurement of vessel diameter. Longitudinal vessel (A) and trans-sectional vessel (B). Vessel stained with COL4. Mag bar = 10 μm

3.2.6 Statistical analysis

Regarding staining density, ANOVA or Kruskal-Wallis test was used to test for differences between groups dependent on normality of data. In variables were there was significant differences the Tukey and Dunnett T3 post hoc (parametric data) or the Mann-Whitney U test (non parametric data) was utilised to obtain p values for individual group pairs. Pearson's Correlation was used to test possible correlations.

For vessel diameter, the Komolgrov-Smirnof test, instead of the Shapiro-Wilk test, was used to verify normal distribution, as collective data was analysed from each group due to the individual measurements of each vessel. Non parametric Kruskal Wallace test was used to analyse data and the Mann-Whitney U test to establish significance between groups. Cumulative frequency analysis was performed of vessel diameter to determine if subpopulations of vessels were responsible for any changes in group mean. Comparisons

between group cumulative frequencies were analysed using Multitab 16 Statistical Software (Multitab Inc, USA).

3.3 Results

Examination of the sections revealed consistent staining of vasculature across all regions (Figure 3.2 A and B). GLUT1 antibody was used as a marker of the glucose transporter 1 protein found in mammalian cells for the transport of glucose across the plasma membrane; it is particularly abundant in ECs of vasculature and facilitates transport across the blood brain barrier.

Staining with GLUT1 often appears to give a discontinuous effect, suggesting the protein is not continuous across the entire vasculature; as a result the vessel structure appeared to change across its length, with a noticeable decrease in vessel size (Figure 3.2 C). Additionally immunoreactivity included occasional staining of red blood cells (RBCs) within and around the vessel, as tissue was not perfused it was often difficult to visualise the vascular lumen, however it was occasionally possible (Figure 3.2E).

COL4 antibodies stained only the BM of the vasculature. Staining was continuous along the vasculature and the vascular lumen can be clearly visualised in trans-sectional vessels. An increase in intensity appeared where the vessel had been cut, giving a clear boarder between the vessel and the parenchyma (Figure 3.2 D and F). Both stains appeared to be relatively specific to components of the neurovascular unit, with no obvious staining of astrocytic end feet or pericytes.

There was no obvious difference in total density between the two stains in all regions, although GLUT1 appears more discontinuous, therefore staining may not appear as intense as in COL4. Some COL4 vessels appeared as if the BM was disentangling or was damaged, as weakly stained unstructured staining appeared to be separating from more defined vasculature, although there was no obvious increase of this feature in a particular group and may be due to tissue processing. Some COL4 vessels appeared to have uneven edges and the border between the vessel and the parenchyma, as previously described, which could suggest twisting. Whilst, a number of possible string vessels were visible in sections stained with COL4, their small size and weak staining intensity often made them difficult to visualise. In both stains, there is some light intercellular background staining, which was especially noticeable in the CA2 and CA4 regions (Figures 3.3 A-F).

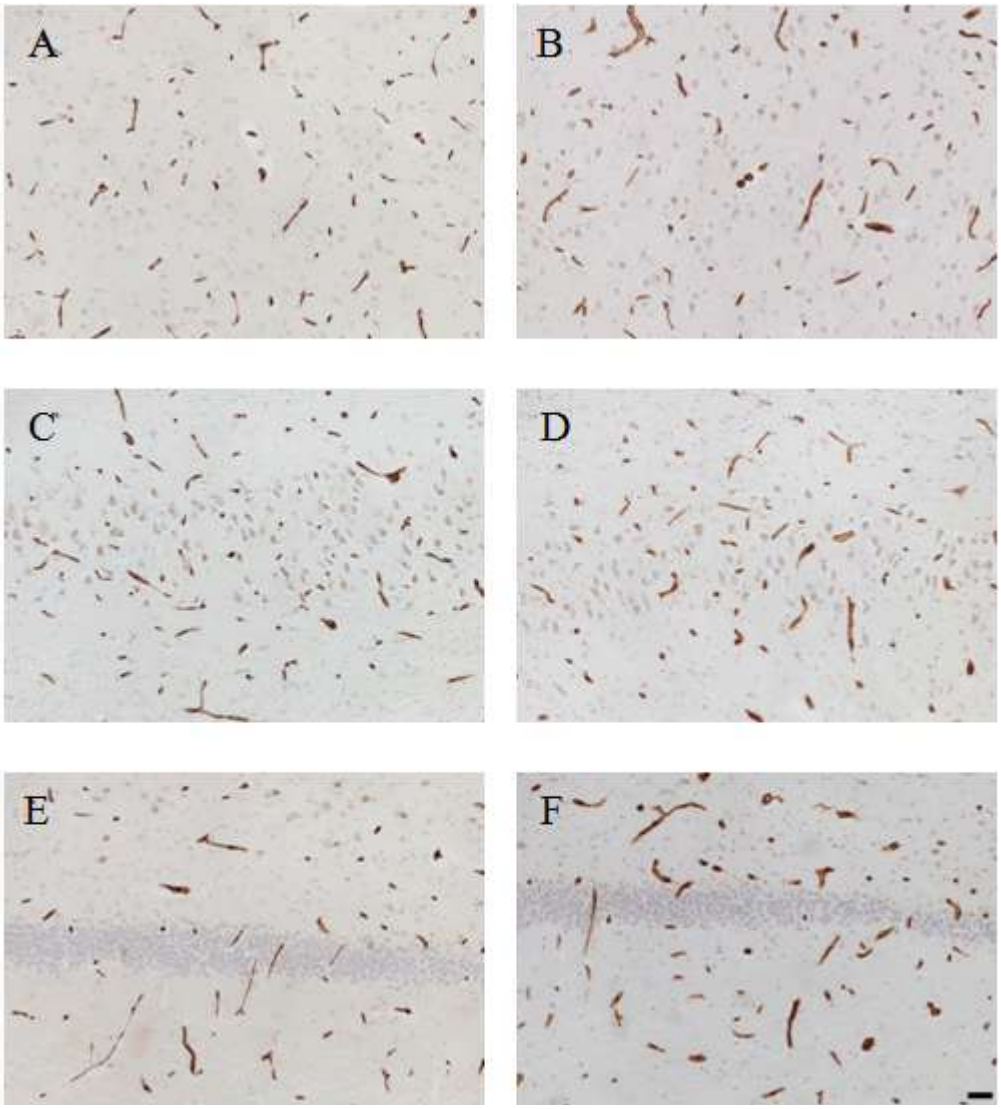


Figure 3.3 Staining with vascular markers in CA1, CA2 and DG. GLUT1 A,C and E and COL4 B, D and F. Counterstain with haematoxylin. Mag bar = 50 μm

Initial double labelling was performed to observe, if it was possible to visualise either, new vessel generation or vessel degeneration. In the majority of incidences the two stains were co-localised and the COL4 (grey) stain appeared to ensheath GLUT1 staining (brown, Figure 3.4 A). We observed some possible string vessels (Figure 3.4 B). At a number of points there appeared to be just one stain present and this may suggest vascular regeneration or degeneration (Figure 3.4 C). It was probable that this staining would be impossible to

quantify as structures were adjacent and hence software dissociation of one type from another would be unreliable.

Figure 3.4 shows double labelling of vasculature with GLUT1 (DAB) and COL4 (Vector Grey). Image A shows co-localisation of stains with GLUT1 endothelial layer visible within the COL4 stained BM layer. B shows probable string vessel formation, with the loss of GLUT1 and the collapse of vessel structure, with COL4 still present. C shows single staining of either GLUT1 (closed arrow heads) or COL4 (open arrow heads) on a double labelled section, suggestive of either vessel regeneration or degeneration. Mag bar = 10 μm for A-C

3.3.1 Microvascular density analysis

Although there were no significant differences resulting from the Kruskal-Wallis test for CA1, CA2 or GCL for the percentage per area results (p=0.058, p=0.265 and p=0.817 appropriately) differences were observed in GLUT1 antibody staining in both the CA1 and CA2 regions of individual group pairs (Mann-Whitney U test). Significant increases of AD cases compared to PSND and PSD groups (p=0.011 and 0.037 respectively) in the CA1 region and a trend toward significance was observed for a decrease in PSND compared to controls (p=0.073). Significant increase of AD cases contrasted to VaD cases (p=0.040) in the CA2 region. No significant differences were apparent in the GCL (Figure 3.5).

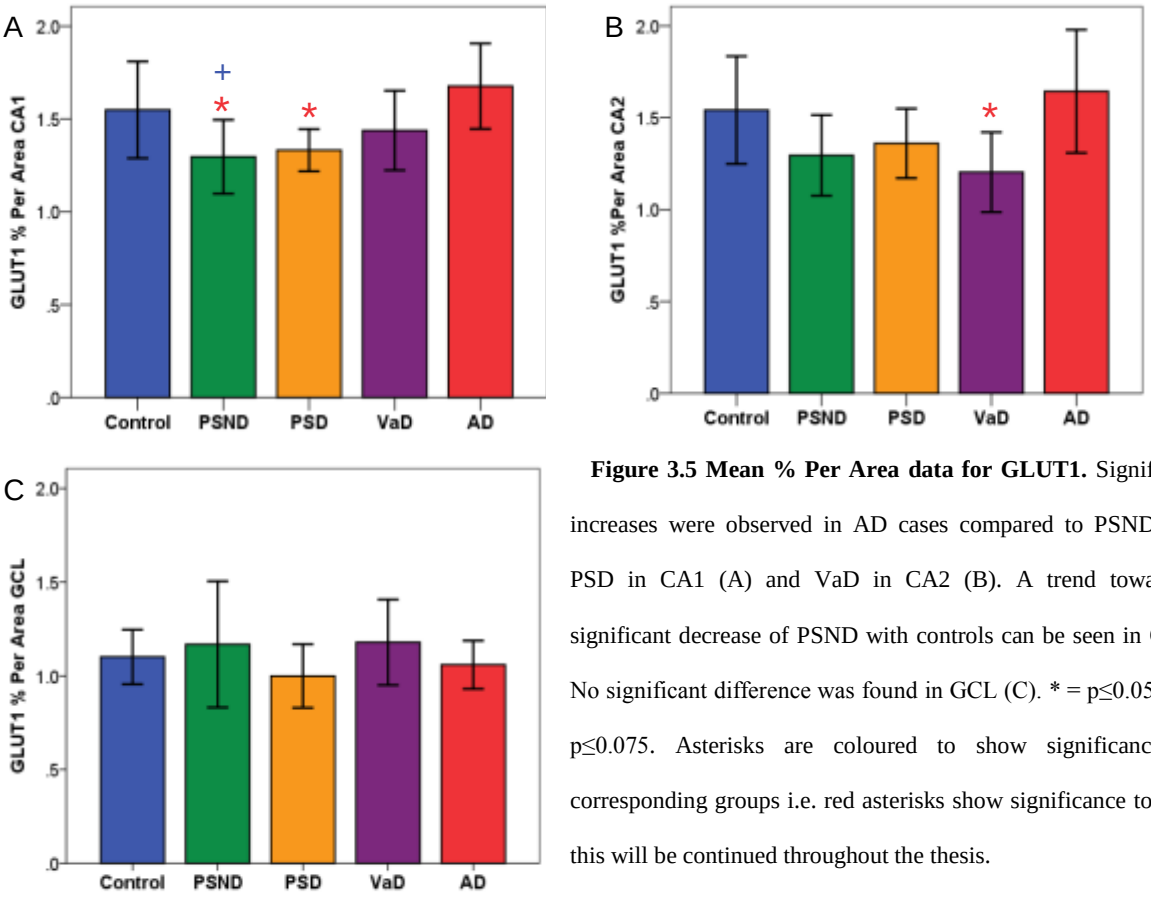


Figure 3.5 Mean % Per Area data for GLUT1. Significant increases were observed in AD cases compared to PSND and PSD in CA1 (A) and VaD in CA2 (B). A trend toward a significant decrease of PSND with controls can be seen in CA1. No significant difference was found in GCL (C). * = p≤0.05, + = p≤0.075. Asterisks are coloured to show significance to corresponding groups i.e. red asterisks show significance to AD, this will be continued throughout the thesis.

Similar to GLUT1, COL4 antibody staining density was increased in AD cases. However, significant increases noted in the CA1 region were compared to control and VaD cases (p=0.024 and p=0.050). In addition, a trend toward a significant increase in PSND cases was found compared to controls in CA1 (p=0.069). Furthermore, PSND was significantly increased compared to controls in the GCL (p=0.011) and there was a trend toward a significant increase with PSD against controls (p=0.075, Figure 3.6).

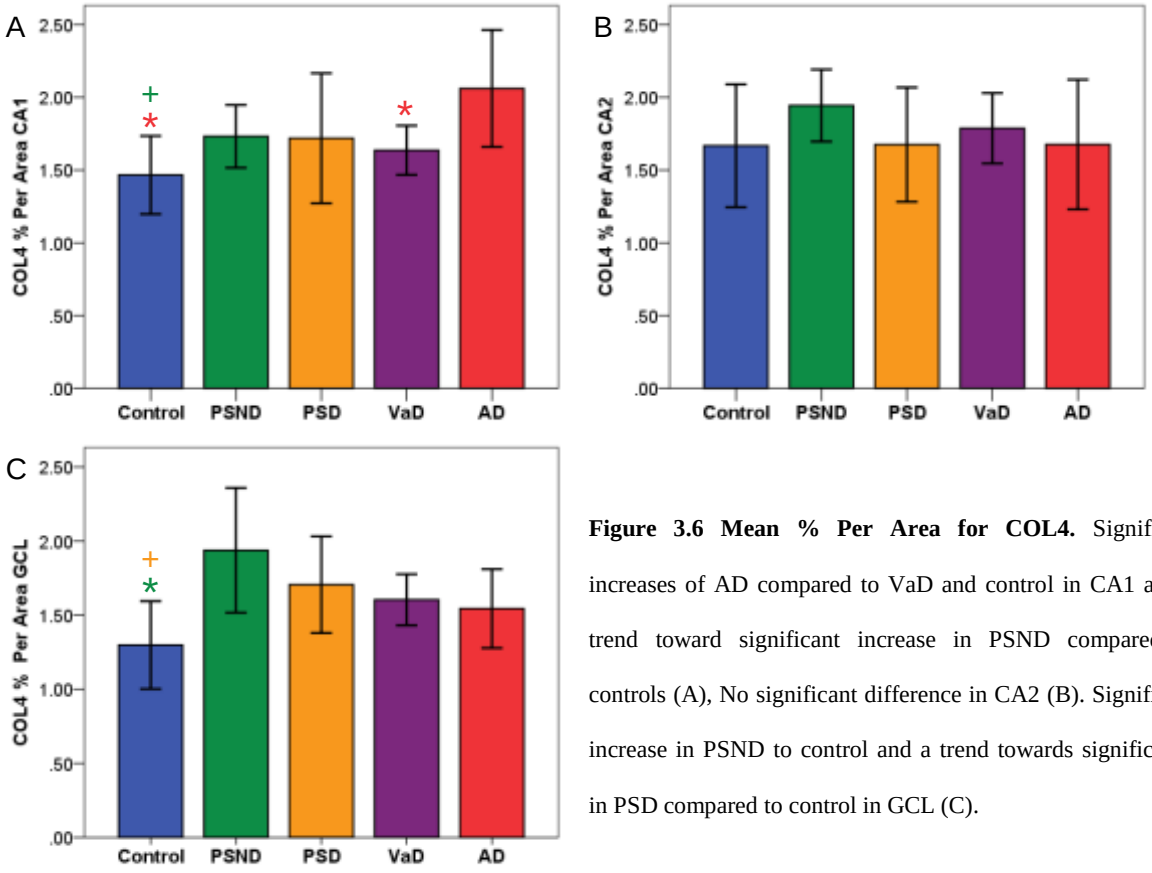


Figure 3.6 Mean % Per Area for COL4. Significant increases of AD compared to VaD and control in CA1 and a trend toward significant increase in PSND compared to controls (A), No significant difference in CA2 (B). Significant increase in PSND to control and a trend towards significance in PSD compared to control in GCL (C).

As previously described, an additional aim of this investigation was to ascertain if it was possible to detect whether the vasculature was undergoing an angiogenic or degenerative process by examining the ratio of the % per area measurement for the ECs marker, GLUT1, compared to the BM marker COL4. As a result a ratio was produced of the GLUT1 and COL4 results. Each ratio value was converted, so that a 1:1 ratio gave a value of 0 instead of

1, therefore if angiogenesis was occurring a positive result would be observed and vessel degeneration would be expressed as a negative value.

In all regions the data were determined to be non parametric, Kruskal-Wallis tests revealed no significant differences in any region. Paired group analysis with Mann-Whitney U test, showed a significant decrease compared to control subjects in cases of PSND in the CA1 (p=0.006) and GCL (p=0.019). AD ratio was significantly decreased in the CA1 (p=0.042), although the group had expressed highest per area results with both markers. The resulting ratio this was caused by a large increase in quantity of COL4 compared to GLUT1. Whilst, PSD also displayed a trend towards a significant decrease compared to controls in the GCL (p=0.059, Figure 3.7).

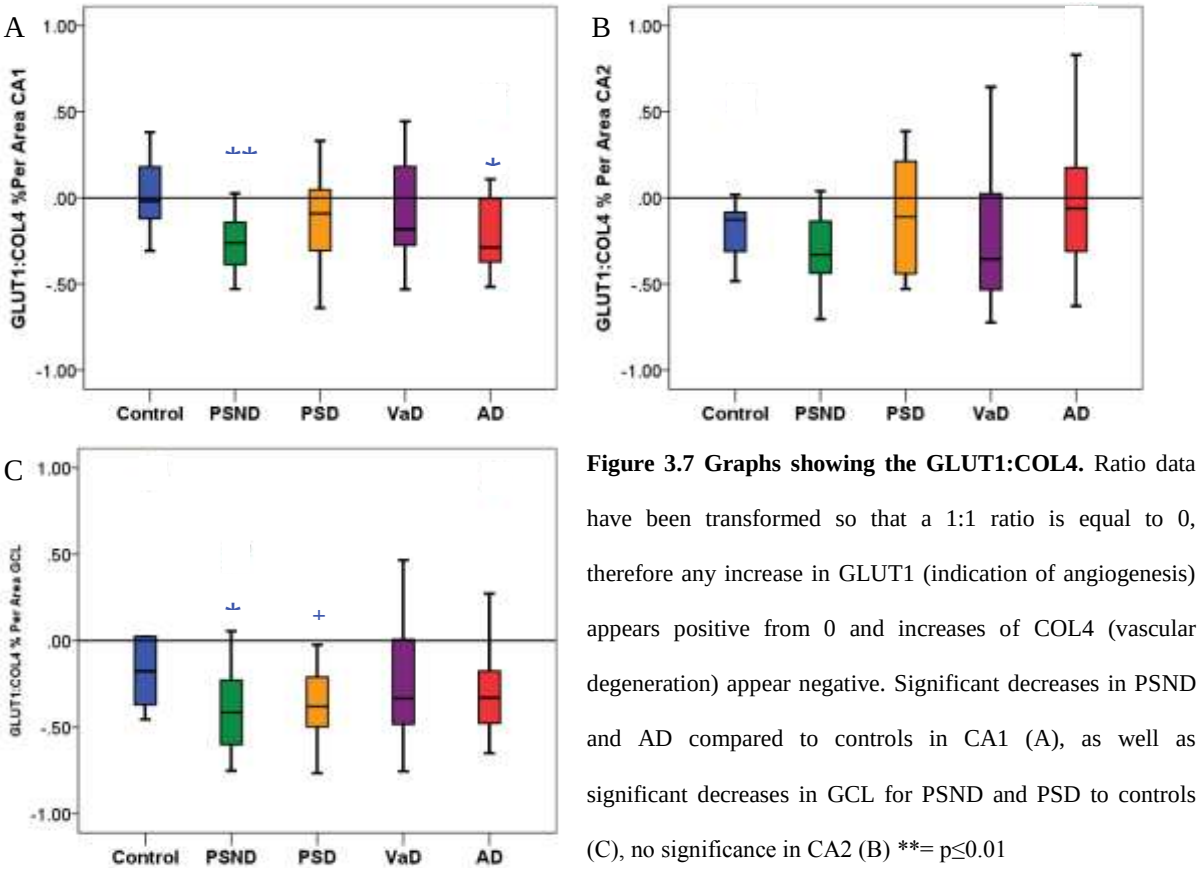


Figure 3.7 Graphs showing the GLUT1:COL4. Ratio data have been transformed so that a 1:1 ratio is equal to 0, therefore any increase in GLUT1 (indication of angiogenesis) appears positive from 0 and increases of COL4 (vascular degeneration) appear negative. Significant decreases in PSND and AD compared to controls in CA1 (A), as well as significant decreases in GCL for PSND and PSD to controls (C), no significance in CA2 (B) **= p≤0.01

These vascular density results consistently showed significant increases in both GLUT1 and COL4 for AD cases against other diseases. Further investigation was initiated to determine if this effect was consistent in other regions of the temporal lobe, specifically the EC and BA 36. Both of these areas are known to develop higher burdens and at an earlier time point in the deposition of Alzheimer pathology than the hippocampus (Thal 2002 and Braak 2006).

An initial study examined 10 cases from control, PSND, PSD and AD groups in cases where these regions were discernible and undamaged by processing on slides stained for GLUT1. This ensured staining consistency from the original study. ANOVA analysis of both regions with post hoc Tukey test demonstrated that there was no significant difference between the groups (EC p=0.193 and BA36 p=0.808, Figure 3.8).

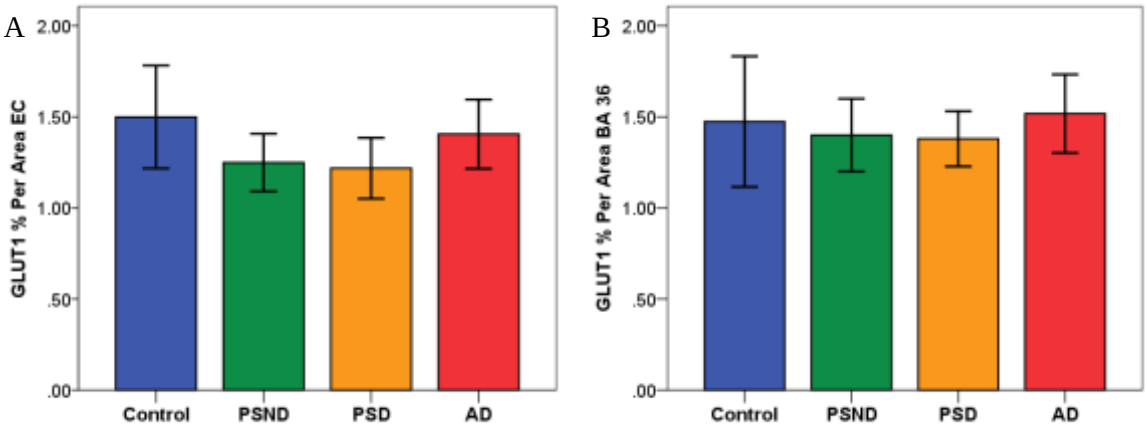


Figure 3.8 % Per Area of EC and BA 36. No significant difference of microvascular density in either EC (A) or BA 36 (B).

3.3.2 Hippocampal area

Due to these results, the possible reasons for the increased density in AD cases, was explored. Atrophy is a well known phenomenon especially within the hippocampus of AD cases and has previously been well defined using MRI scans over the progression of the disease (Jack et al., 1992, Whitwell et al., 2007) and has also been observed at post mortem

with increased hippocampal sulcus and reduced gyrus sizes. The amount of atrophy correlates with stage and duration of AD (Bobinski et al., 1995). This phenomenon is not only unique to AD but has been described in other dementias including DLB (O'Brien et al., 2001), frontotemporal dementia (Chan et al., 2001) and VaD (van de Pol et al., 2011).

While, MRI atrophy data were unavailable for all of these cases, making it impossible to make measurements of changes in hippocampal size over time and to compare with the microvascular density results. However, we believed it was important to identify if there were significant changes in size of the post mortem hippocampus measured as a marker of possible atrophy. The theory being that the possible increase in vessel density seen in AD may have been due to increased atrophy, which would reduce the volume of parenchyma and therefore produce false positive results of increased density as the vessels are brought closer together (Kalaria, 1996).

Hippocampal area was measured as described in materials and methods section of this chapter. Significant difference were found for data with Kruskal-Wallis test (p=0.020). Significant increases of hippocampal area was shown for PSND and PSD cases compared to controls (p=0.015 and p=0.011 respectively). In addition a trend toward a significance decrease of AD was seen compared to PSND and PSD (p=0.059 and p=0.062) and no significant difference was observed between AD cases and controls (Figure 3.9).

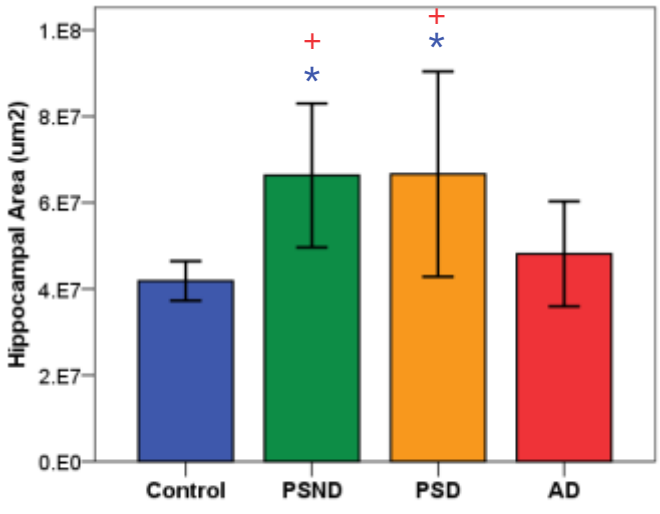


Figure 3.9 Hippocampal Area (µm^2). Significant increase in PSND and PSD compared to controls, whilst trend toward significant increase in PSND compared to AD.

3.3.3 Vessel diameter

A total of 4,082 microvessels were assessed from all groups collectively in the CA1. As shown by staining density, there were differences between groups; therefore, to guarantee all the groups had an equal number of data points the first 100 vessels measured in each case were analysed. This number was selected due to the observation that the quantity of vessels analysed per case had ranged from 111 to 199. The 600 data points for the groups, derived from the 100 data points per case in each group, were combined to calculate the mean vessel diameter for each group. AD and VaD groups exhibited the narrowest mean diameter, 7.01 and 7.04 µm, respectively. The widest mean diameter was found in the PSND measuring 8.03 µm. However, controls and PSD had similar mean diameters of 7.47 and 7.44 µm, respectively. After demonstrating that the data were non parametric (Kolmogorov-Smirnov test), significant increase was observed between the PSND and all other groups (Mann-Whitney, $p \leq 0.01$ for all groups). A significant decrease was also shown between controls and PSD, compared to AD and VaD ($p \leq 0.01$ in all cases, Figure 3.10).

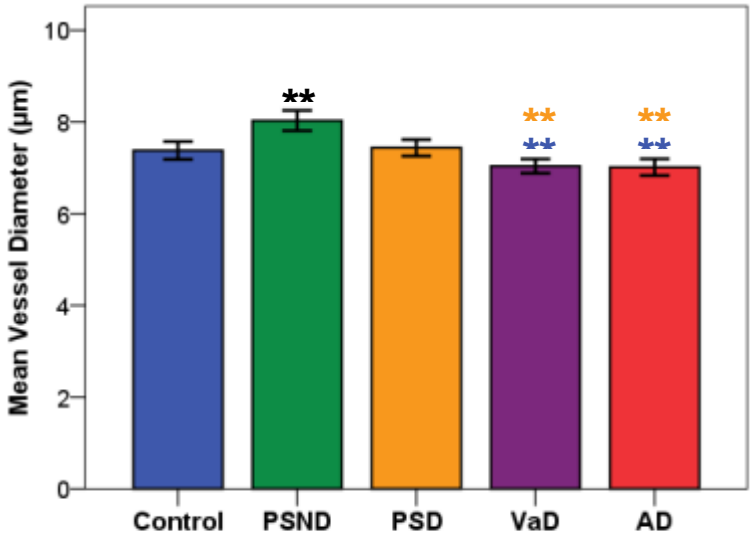


Figure 3.10 Mean vessel diameter in the CA1. Black asterisks show significance to all groups, coloured asterisks show significance with the group of the same colour.

Data were re-analysed to

compare the changes in vessel population, by calculating cumulative frequency of increasing vessel diameter per group, i.e. each diameter measured per group is given an equal value as a percentage of the total number of measurements and is arranged in assending order (Figure 3.11). Results show that as expected the vast majority of vasculature in the CA1 is capillary, with a very small number of vessels ≥ 15-μm in diameter. Significant differences were found between distributions using χ^2 distribution analysis (Table 3.2). Significant differences were found between all groups with PSND except VaD. Other significant differences were observed in VaD cases compared with controls, PSD and AD (all $p \leq 0.001$) and PSD data compared to AD ($p=0.013$ Table 3.2 and Figure 3.11 A-E). The figure shows that the increase in the number of vessels in PSND between 7 and 15 μm significantly changes the distribution of increasing vessel diameter.

	Controls	**PSND**	**PSD**	**VaD**	**AD**
Controls					
PSND	49.11 ($p \leq 0.001$)				
PSD	3.51 (N/S)	23.74 ($p \leq 0.001$)			
VaD	63.56 ($p \leq 0.001$)	1.49 (N/S)	34.90 ($p \leq 0.001$)		
AD	0.535 (N/S)	55.24 ($p \leq 0.001$)	6.12 ($p=0.013$)	69.89 ($p \leq 0.001$)	

Table 3.2 – χ^2 distribution analysis for changes in vessel diameter between groups. Significant differences between all groups with PSND except VaD.

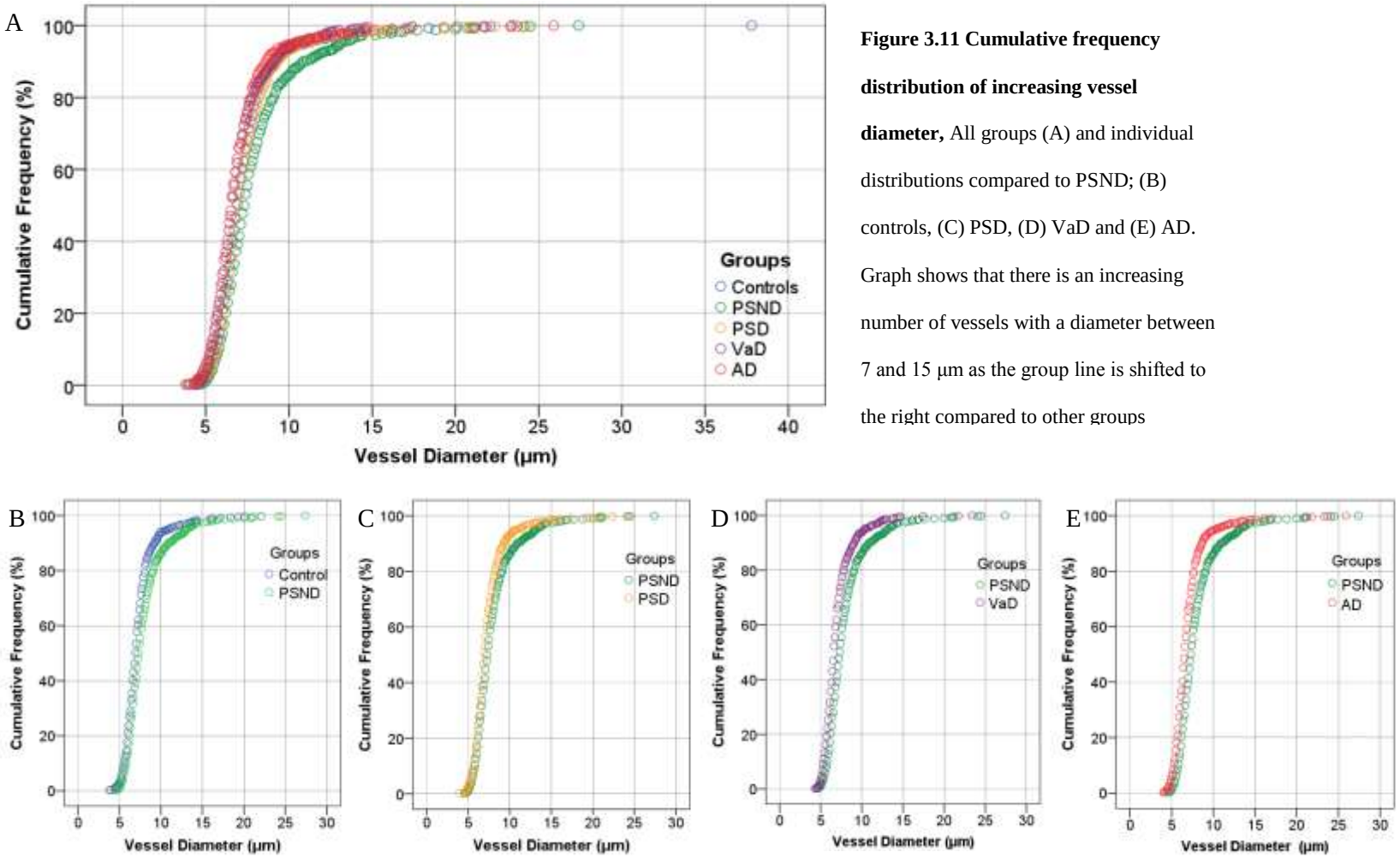


Figure 3.11 Cumulative frequency distribution of increasing vessel diameter, All groups (A) and individual distributions compared to PSND; (B) controls, (C) PSD, (D) VaD and (E) AD. Graph shows that there is an increasing number of vessels with a diameter between 7 and 15 µm as the group line is shifted to the right compared to other groups

3.4 Discussion

Recent advances have shown that cerebral microvascular pathology is related to age-related cognitive decline (van Dijk et al., 2008, Brown and Thore, 2011,, Hunter et al., 2012). The present study quantitatively examined the vascular density and diameter of the hippocampus using a standard two dimensional analysis approach. In contrast to our hypothesis, no significant increase of microvascular density was observed in the groups which had maintained their cognitive function compared to the groups which had be clinically defined as suffering from dementia in life. However, an increase was consistently observed in cases of AD compared to other groups, significant increases were found in GLUT1 staining in the CA1 to PSND and PSD, and compared to the VaD group in the CA2. There was no significant difference between groups in other regions analysed including EC and BA36. Meanwhile, AD cases were also significantly increased compared to controls and VaD when stained with COL4 in the CA1. Other groups only showed significant changes in vascular density with COL4; the PSND group displayed increased density compared to controls in the GCL and a trend toward significant increase in the CA1, PSD showed a trend towards increase compared to controls in the GCL.

Consistent with previous studies, significant decreases in mean vessel diameter were identified in AD (Bouras et al., 2006, Bell and Ball, 1981). Similar, decreases were observed in VaD cases, which may be associated with reduced CBF previously described in dementia and GLUT1 protein density (Kalaria, 1996). Microvessel diameter was significantly increased in PSND, compared to controls and PSD. This may either suggest that vessels in PSND are more vasoreactive and responsive to factors such as hypoperfusion or that they are inherently plastic and undergo re-modelling PS. String vessels, (Kalaria and Hedera, 1995, Brown and Thore, 2011) were often observed but not quantified due to difficulty with

guaranteeing accuracy. These reductions in vessel diameter may promote occlusion and progressive stenosis resulting in acute or chronic ischemia to the surrounding region (Lammie, 2000). Additionally, atherosclerotic changes to vessels may cause loss of elasticity to dilate and constrict in response to changes in systemic blood pressure (Kalaria and Erkinjuntti, 2006).

The initial hypothesis was that cases that maintained cognitive function would show the largest vascular network, thus the highest microvascular density and widest vessel diameter. Loss of vasculature, especially in the hippocampus, we thought might explain findings in a number of studies as previously described using arterial spin labelling showing decreased CBF (Schuff et al., 2009, Firbank et al., 2011) and may indicate a vascular response to the reason some individual maintain cognitive function.

These increases in microvascular density, may infer that angiogenesis is or has occurred in AD. Similar findings and discussions regarding angiogenesis in AD have been previously proposed (Vagnucci and Li, 2003,Meyer et al., 2008). This trend was not found in other regions beyond the hippocampal structure; the EC and BA36, as part of the temporal lobe were also analysed. The EC and BA36 had been hypothesised to show greater vascular density due to increased pathology in these regions, if there was a direct association between vascular density and pathology (Thal et al., 2006, Braak et al., 2006, Boscolo et al., 2007). Previous unpublished work showed an association between plaques and capillaries.

It is proposed that there is a hypoxic cerebral environment in AD cases (Grammas et al., 2011) leading to an upregulation of pro-angiogenic proteins and vascular growth factors (Kalaria et al., 1998). However, the results suggest that this attribute is mainly specific to CA1. It has been well documented that the CA1 is specifically susceptible to damage from

Alzheimer-type pathology, ischemia and hypoperfusion, which may alter vascular density expression (Leifer and Kowall, 1993, Wu et al., 2008).

Due to these results, it was reasoned that atrophy, the shrinkage of tissue caused by neurodegeneration and loss of parenchyma, may explain the increase of microvascular density in CA1 in AD cases and that it was not caused by angiogenesis. Some studies have reported that Aβ is a potent anti-angiogenic molecule (Patel et al., 2008, Paris et al., 2004b) and that it may lead to vessel degeneration (Kalaria, 1997). As MRI volumetric data, a standard methodology for measuring atrophy (Thompson et al., 2004, Jack et al.2004, Ridha et al., 2008), were not available on all these cases. Analysis was performed to measure the hippocampal area of sections from 10 selected cases. Whilst significant differences were found between groups, an increase in PSND and PSD groups compared to controls and a trend toward an increase in PSND and PSD compared to AD. No difference was found between PSND and PSD groups or controls and AD. No significant loss of hippocampal area in AD was reported compared to any other group. This result was unexpected as an MRI study of seventy nine PS survivors showed that medial temporal lobe atrophy, 2 years PS, predicted cognitive decline (Firbank et al., 2007). It has also been well established that there is significant hippocampal atrophy in cases of AD (Jack et al., 1992). Hippocampal area results therefore may not explain the true observation of atrophy in these cases; although all analysis was performed in similar posterior hippocampus it is inconceivable that sections would be taken from the exact same section plane and therefore may skew the results. Furthermore, without having the original size reference point for each hippocampus to measure, the possibility that any changes found are caused by natural differences such as brain size or differing effect of post mortem processing on tissue. Finally, an affiliated study (Gemmell, 2012, Stroke) using the same cases, cut from the same block found significant

neuronal atrophy, defined as decreased individual neuronal volume, in all the demented groups compared to controls and PSND groups. As a result we would have expected to see a decrease in area for all demented groups.

Although the results suggest angiogenesis is occuring in AD cases, as indicated by the increase in vascular density. One of the additional aims of this experiment was to investigate if comparing the ratio of GLUT1 to COL4 staining, could confirm if there was an increase in either ECs or BM of vasculature as an indication of either angiogenesis or vessel degeneration. The results show there is a significant decrease in GLUT1:COL4 in the CA1 compared to controls for both AD and PSND. Furthermore, there is a significant decrease in PSND and a trend toward decrease in PSD compared to controls in GCL. This result may be altered by the fact that as we described GLUT1 staining is discontinuous, therefore these results may suggest a greater loss of GLUT1 in these groups. However, the result could be interpreted as possible vascular degeneration in these groups; as a result an increase in string vessels, remnants of capillaries, would be expected in these cases, which is similar to previous findings in AD (Perlmutter and Chui, 1990, Kalaria and Kroon, 1992, Buee et al., 1994). Whilst, it is plausible that vasculature would be degenerating in PSND, it was expected that the VaD group would show signs of vessel degeneration although no significanct difference was found. Furthermore, this affect would appear to be regionally specific as there is no difference in the CA2 between the groups.

Regarding vessel diameter, a number of studies have previously suggested that Aβ may play a direct role in increasing vasoconstriction (Niwa et al., 2001) or that nitric oxide, a natural vasodilator, is altered in dementia (Price et al., 2001) and may have therefore affected average vessel diameter in these groups. Another study has suggested that the vasoconstrictor endothelin-1 (ET-1) may play a role in both AD and VaD. However changes in mRNA of the

endothelin converting enzyme (ECE-1) were small and increases found in ET-1 are most likely caused by Aβ mediated up regulation of the converting enzymes (Palmer et al., 2010, Palmer et al., 2012). Furthermore, inhibiting angiotensin II, a potent vasoconstrictor, with pharmacological treatments has been suggested to improve cognitive function in AD and VaD by ameliorating hypotension. However, the angiotensin II mechanism is involved with inhibiting the release of acetylcholine and upregulation of inflammatory response (Kehoe and Passmore, 2012, Wharton et al., 2012). Cases of AD are less likely to have been prescribed angiotensin II receptor blockers, which may affect vessel diameter (Davies et al., 2011).

Cumulative frequency analysis shows that there were significant differences between the groups, suggesting variations in subpopulations of hippocampal microvasculature. In the PSND group mean vessel diameter was significantly wider than all the other groups. Visualizing the data, via cumulative frequency, suggested that there were greater proportions of vessels with a diameter between approximately 7 and 12 μm compared to the other groups and the mean diameter increase was not caused by analysing a larger number of large vessels including arterioles (Figure 3.11). As suggested before, this could propose that the PSND group had microvasculature which is more adaptable within their environment and responsive to hyoperfusion. Distribution analysis showed that there were significant difference between the PSND group and all groups but VaD, suggesting significant variation in the composition of microvasculature while vasculature are thinner in VaD they follow a similar overall distribution.

Whilst, the data regarding vessel diameter are interesting and gives important information regarding microvasculature and the distribution of vessels across the hippocampus, it does not give a clear understanding on the condition of the vessel and presumptions such as vasoconstriction and or remodelling are made without definitive evidence. The VasCalc

software used was initially developed to calculate sclerotic index [Y Yamamoto, (Craggs et al., 2013)]. Sclerotic index (SI) is the measurement of vessel wall thickness as a factor of the size of the vessel and was first mention by Lammie et al (Lammie et al., 1997). The internal and external diameter of a trans-sectional cut vessel is measured using the same method as described in this chapter. SI is calculated by using the equation SI=1-(internal diameter/external diameter), where a healthy vessel has an SI of 0.2-0.3 and increases to 0.3-0.5 in cases of SVD (Craggs et al., 2013). However, for accurate measurements this form of analysis must be carried out on arteries and large arterioles and due to the high oxygen/glucose demand and small area of the hippocampus, there are very few large vessels to measure. Although the surrounding cortical and WM regions could be investigated to identify changes in the temporal lobe, and similar results may apply to the hippocampus. Additionally to SI analysis, lumen and perivascular space area can also be calculated, which may give an indication of degeneration and vessel wall thickening. However, this is outside the remit of this thesis but future analysis should be performed.

Although these data are encouraging and worth further investigation there are possibilities that the standard 2D analysis employed regarding density measurements may introduce some bias as a result of vessel orientation. Whilst this two dimensional form of analysis has benefits in pathological assessment and is widely used, it has limitations for analysing density of string-like structures such as vasculature and axons, which can have an infinite number of orientations. For these reasons, further analysis is required to investigate changes in the quantity of vasculature where the orientation of the vessel is not a concern and determine if this believed issue has an effect on these results. This can be performed using recently developed stereological techniques, using a spherical probe, which has been previously used to estimate changes in length of axons (Mouton et al., 2002).

In conclusion, these results suggest that there is increased microvascular density in AD. The argument for and against increased vascular density is controversial. Also, issues regarding the methodology used, which may add bias to the data due to differing orientation and size of the vessels means that the current data may not provide the true picture. Therefore the opportunity to reanalyse the cases using recently developed stereological approaches is being taken up to verify if similar results are found. Finally, there was a significant increase in mean vascular diameter in PSND cases compared to all groups. Further analysis of data suggests there is an increase in the proportion of microvasculature with a diameter between 7 and 12 μm in PSND and there is not a significant increase in the proportion of larger vasculature i.e. arterioles. The decrease in diameter in other groups may reduce CBF and increase hypoperfusion, a known effect in dementia. There is no conclusive evidence of an increase in the proportion of narrower vessels, which may have been an indication of increased angiogenesis.

Chapter 4 Stereological Assessment of Microvascular Density

4.1 Introduction

Results from chapter 3 suggested an increase in microvasculature in AD compared to a number of other groups. Although concerns were raised regarding potential bias of results caused by orientation and vessel size. It was clear that a more accurate analysis technique was required to discern differences between groups. This reasoning led to the investigation of microvascular density using a stereological principle.

Stereology is based around the principles of stochastic geometry, which predicts the properties of geometric shapes within three dimensional spaces. This allows the estimation of structural characteristics relating to parameters such as volume, surface area, length, number and thickness using unbiased sampling (Mayhew, 1991, Nyengaard and Gundersen, 2006, Dockery and Fraher, 2007). Stereology has been widely used in a number of fields including geology and engineering. However, it was not until the 1960s, that biologists realised its potential in the measurement of the structural components of the human body, such as cells, blood vessels and nerve fibres. The advent of high resolution microscopy led to a drive for more reliable and accurate forms of analysis to quantify microstructures associated with health and disease.

Due to the scale and complexity of the brain, measuring all components of the brain precisely would be impossible. However, stereology offers a solution to this issue, by making precise estimates of total amounts using thorough and efficient sampling protocol. Sampling is performed through three dimensions, the x and y axis is accounted for via the microscope and

use of stereological software. The z axis is sampled by sectioning through the known volume of the region of interest.

To measure different geometric properties, a number of probes have been developed, which must be assessed in an unbiased way. This is accomplished by using random encounters between test probes and sectional images. The probe is superimposed on to the section, using specialist software, and is randomly positioned and orientated to reduce sampling and analysis bias. Today, there are a variety of probes available to measure a number of dimensions including the point probe, which can be used to measure both area and volume of a structure (West, 2012) or the optical disector, which can be used to count numbers of cells [(Van Otterloo et al., 2009, Khundakar et al., 2011) see chapter 7].

For the purpose of measuring length, the sphere has been shown to be the ideal shape for a probe, as it has an isotropic orientation and therefore removes the anisotropic orientation issue caused by measuring string-like structure such as blood vessels and axons. More detail of the history and development of this technique can be found in Chapter 2. A digital spherical probe was developed by Mouton et al., 2002, giving isotropic orientation of a probe in predefined tissue. The probe was superimposed through the depth of the tissue and allowed for estimates of L_v (cumulative vessel length per unit volume of tissue) of acetylcholinesterase (AChE) stained nerve fibres (Figure 2.3). The probe would appear at systematic random locations throughout a pre-determined reference space. At each location the spherical probe was focused through the z axis and at each focal plane concentric circles of increasing, then decreasing, diameter appeared and the number of intersections between the probe and the stained nerve fibres were counted using computerised software. As the sphere is of a known radius, and therefore the volume of each sphere is known, it is possible to calculate the L_v (see figure 2.3).

In this chapter, the spherical probe technique was selected for the measurement of microvasculature L_v labelled with GLUT1 and COL4 antibodies to reanalyse microvascular density in the hippocampus as described in chapter 3. Arterioles are also stained with smooth muscle actin (SMA) and analysed. Larger vessels could not be analysed previously using the 2D analysis as their size would significantly affect the % per area results. As stated previously, this technique provides the most precise estimate of analysis for measuring vascular density, as an indicator of total length. There have been very few previous studies, using this spherical probe methodology to investigate microvascular density in human post mortem brain tissue. Two studies were performed on AD cases (Schwartz et al., 2010, Richard et al., 2010), and both showed a significant increase in L_v compared to controls. Although an AD animal model showed a significant reduction in capillary length compared to wild type littermates (Gama Sosa et al., 2010). Studies using the same technique performed on schizophrenic brain tissue and found no difference compared to controls in a number of regions (Kreczmanski et al., 2005, Kreczmanski et al., 2009). To our knowledge no analysis has been performed to date on VaD or PS cases. Initial analysis was performed in the CA1 and CA2 regions and additional analysis was then performed in EC and DLPFC. It is expected that, from the initial hypothesis, cases which maintained cognitive function, PSND and controls, will show unperturbed microvascular changes and therefore it is contemplated that significant increase will be seen compared to PSD and VaD cases. A significant loss of vessels is expected in cases of VaD due to vascular degeneration. From previous studies and including our own work in chapter 3, we hypothesise that there will be an increase in L_v in AD this may be a result of atrophy.

4.2 Materials and Methods

4.2.1 Subjects

Six subjects per group were selected from the previous cohort used in chapter 3. Tissue was selected based on proximity to a specific plane within the posterior hippocampus, adjacent to anterior pole of the lateral geniculate body in the coronal plane. The same sections were appropriate for analysis of the EC and the same cases were selected for analysis of DLPFC, except two replacement control cases. The demographics between DLPFC and hippocampal cases showed no significant difference.

	Controls	**PSND**	**PSD**	**VaD**	**AD**
Age, y Mean (range)	78.5 (72-91)	84 (80-87)	85.5 (80-93)	85.3 (76-90)	82 (70-88)
PMD, h Mean (± 2SEM)	34.5 (21.6)	49.3 (30.1)	49.4 (23.1)	36 (13.9)	60 (24)
Braak stage Median (range)	NPD	2.5 (2-4)	2.83 (1-4)	2.33 (1-4)	5.0 (4-6)
CERAD Median (Range)	NPD	1 (0-2)	0.83 (0-2)	1 (0-2)	3 (3-3)
Thal Stage Median (Range)	NPD	1.4 (0-3)	0.75 (0-2)	2 (0-3)	3.66 (3-4)
Vascular Pathology* (Range)	NPD	13.5 (10-16)	13.7 (8-17)	13 (12-14)	NPD

Table 4.1 Demographics for Stereology Cohort. No significant difference in age or post mortem delay between any groups. Fixation ranged from 4-32 weeks. Significant increase in Braak and CERAD stages for AD compared to all groups, significant difference in Thal Stage for AD compared to both PS groups. No significant difference for vascular pathology. NPD = Non pathological data

4.2.2 Tissue Sectioning

For complete stereological analysis, the experimental design requires the entire structure of interest to be equally sectioned into blocks and sampled. Only one block per case was available for this analysis due to confines of all tissue being sourced from a brain bank (NBTR). Microtome sections of paraffin wax embedded blocks were cut into fifteen serial sections. Each section was cut at 30-μm-thickness, and mounted onto Superfrost + slides (Fisher scientific, UK); the sections were air dried in a 37˚C oven for a minimum of 48 hours. Every fifth section was then chosen so that three sections per case were selected for uniform random sampling, in order to analyse the tissue through a total thickness of 450 μm. This section selection was performed prior to staining with each of the markers for IHC: GLUT1, COL4 and SMA.

4.2.3 Immunohistochemistry

Standard IHC technique was used as previously described in Chapter 2. Antigen heat retrieval was utilized by placing the sections in boiling 0.1M citrate buffer for 10 minutes, the double labelled COL4/SMA sections underwent a second heat retrieval step of 5 minutes on the second day of the assay. The COL4 antibody was selected to be the second antibody to partner SMA, as testing had previously shown that dual immunostaining with GLUT1 did not always label the full length of arterioles, probably due to a loss of ECs.

It has been previously shown that the optimal antigen retrieval for collagen in paraffin embedded tissue is an enzymatic retrieval (Wakamatsu et al., 1997). However, initial testing showed that sections which had been subjected to heat antigen retrieval previously were increasingly prone to section damage when treated with enzymes. It was found that with extra heat antigen retrieval there was no reduction in the quantity of COL4 staining; however

staining intensity was noticeably reduced. Nonetheless, stereological analysis is not dependent on staining intensity and at 100x magnification the vessels are clearly discernible. The same primary antibodies and concentrations were used as those in chapter 3, whilst additional SMA staining (Sigma Aldrich, UK, 1:1000, 2 hours room temperature) was included. Antigens were visualized using either 3,3'-Diaminobenzidine (DAB, GLUT1 and SMA) or Vector SG (COL4) and counterstained with haematoxylin.

4.2.4 Stereological Analysis

The analysis was carried out using Stereologer 2000 software (Stereologer, WV, USA) installed on a computer coupled to a Zeiss Axiolab microscope, motorised stage (Prior Scientific, UK) and JVC camera. The spherical probe option for analysis was used to measure L_v of the microvasculature. The spherical probe was generated with a diameter of 18 μm; this was selected to allow for section shrinkage and an appropriate guard volume. An outline was drawn denoting the entire AOI corresponding to the relevant hippocampal subfield at low magnification (5x); neuronal subfields could be distinguished by the haematoxylin counter staining. Due to the unique structure and amount of vasculature bisecting the GCL of DG an entirely different experimental protocol would be needed to accurately estimate L_v of microvasculature and was therefore removed this region from analysis. A digitally generated, equally spaced grid was overlaid and used to ensure random sampling within the x and y axis of the mask region of the reference area. Dependent on reference area, the grid dimensions were assigned to produce approximately 100 frames in CA1 and 40 frames in CA2. Number of frames was determined when the coefficient of error (CE) value (a measure of the sampling error) reached a satisfactory level from pilot studies.

L_v was calculated by counting the number of random intersections between the probe and the parameter, in this instance microvasculature (ΣQ), and the area of sampling probe (ΣA) ($L_v = 2(\Sigma Q/ \Sigma A)$) at 100x magnification (Mouton et al., 2002). The number of intersections was used to estimate the L_v for each case within the known sampled section (Figure 4.1).

4.3 Results

Initial examination of the sections showed no discernible differences in the pattern or density of the vasculature between groups or tissues stained with GLUT1 and COL4/SMA antibodies. As before in 10 μm-thick sections, GLUT1 revealed more discontinous profiles in comparison to COL4. Single labelling of vasculature with SMA, consistently stained the end regions of vessels. As a result arterioles appear as two concentric circular shapes. Therefore a double label was selected to visualize the entire length of arteriole within the section. Figure 4.2 shows the staining of an arteriole with SMA and COL4 through a depth of tissue.

4.3.1 Hippocampal Stereological Microvascular L_v Analysis

L_v was normally distributed for all markers in both the CA1 and CA2 regions (P>0.05, Shapiro-Wilk normality test). GLUT1 immunoreactivity in the CA1 region displayed significant changes in L_v between groups (ANOVA p = 0.000). Tukey post hoc test showed significant increases in mean L_v in AD compared to all groups except PSD (Figure 4.3 A). Coefficient of error (CE) values, which gives an indication of the accuracy of the value estimated by stereology, were controls = 0.13, PSND = 0.13, PSD = 0.11, VaD = 0.14 and AD = 0.10. There was no significant difference in L_v in the CA2 region (ANOVA p = 0.459, Figure 4.3 B). The CE values were controls = 0.20, PSND = 0.18, PSD = 0.18, VaD = 0.18 and AD = 0.18.

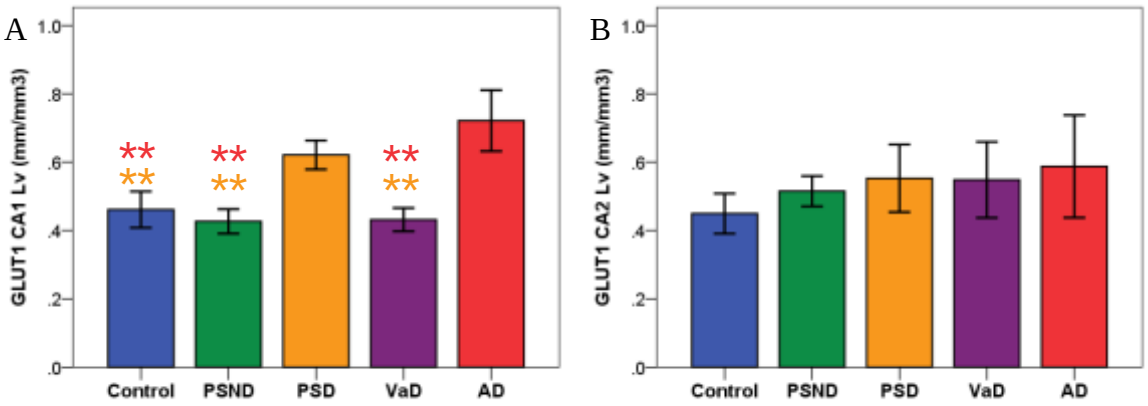


Figure 4.3 L_v Results for GLUT1 in both CA1 (A) and CA2 (B) region. ** = p≤0.01

COL4 L_v in the CA1 was also different between groups (ANOVA p=0.000). Significant increases were again observed for L_v in AD compared to the controls, PSND and VaD. L_v in PSD cases were increased compared to PSND and VaD. The mean L_v of PSD and controls were not significantly different as it was in GLUT1 L_v (Figure 4.4 A). However, only five cases of both PSD and controls could be analysed due to tissue processing damage in sections. It would be expected that with the extra cases, this difference would reach significance, similar to GLUT1 results due to the strong correlation between GLUT1 and

COL4 L_v (Figure 4.6). CE values observed were from controls 0.13, PSND = 0.12, PSD = 0.11, VaD = 0.14 and AD = 0.10. Similarly, as with GLUT1 there were no significant differences in COL4 L_v in CA2 (ANOVA p = 0.443) (Figure 4.4 B). CE values were controls = 0.17, PSND = 0.20, PSD = 0.19, VaD = 0.18 and AD = 0.19.

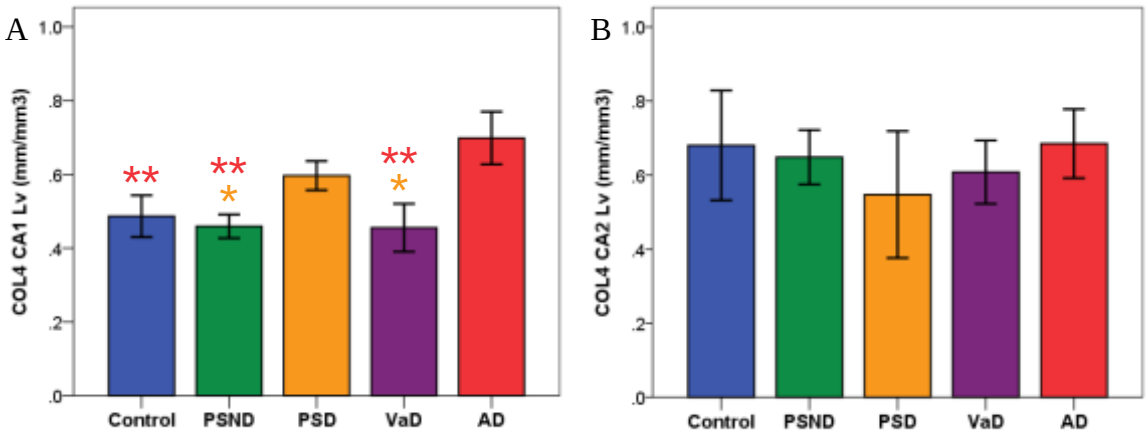


Figure 4.4 COL4 L_v data for CA1 (A) and CA2 (B) region. . * = p≤0.05 and ** = p≤0.01

As expected, SMA L_v was lower in all groups compared to other vascular markers, due to its specificity for larger vessels, such as arterioles; unlike GLUT1 and COL4 which label all vasculature. However significant differences were apparent in the CA1 between groups (ANOVA p= 0.004). Significant increases were observed in AD compared to controls and VaD. There were no significant differences observed in CA2 (ANOVA p= 0.959). SMA staining had a much greater CE values in all CA fields compared to other L_v, due to the relative low vessel density of arterioles, suggesting these results may be inconsistent. CA1 CE values were as followed controls = 0.37, PSND = 0.30, PSD = 0.29, VaD = 0.42 and AD = 0.26 (Figure 4.5).

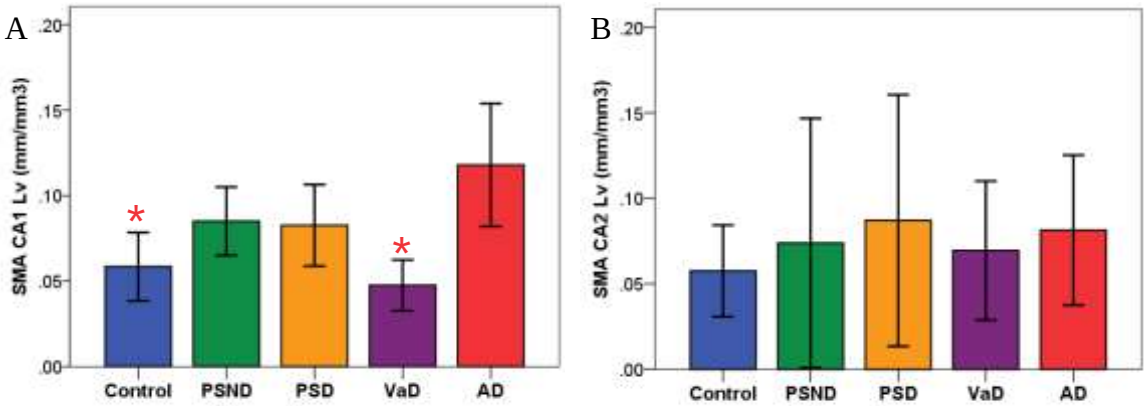


Figure 4.5 SMA Lv CA1 (A) and CA2 (B). Much reduced L_v and greater error bars compared to GLUT1 and COL4. Significance still reached in CA1 with increase of AD compared to controls and VaD. * = p≤0.05

The robustness of findings were strengthened by the strong correlation between GLUT1 and COL4 in the CA1 (r^2 = 0.687, p=0.000). This indicated internal consistency of the results obtained for individual microvascular L_v (Figure 4.6). Similar conclusions can be made for the COL4 results as for those found in GLUT1. Thus, changes in L_v in the CA1 and lack of those in CA2 were reflected in both microvascular elements and profiles across all groups. The correlation also shows that the group separates into three clusters, AD cases, PSND cases and at baseline level VaD, PSND and control groups.

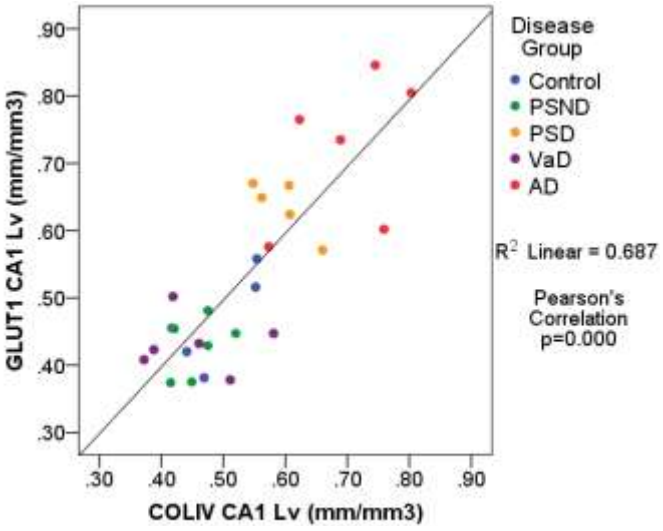


Figure 4.6 Correlation between GLUT1 and COL4 CA1 L_v data

As had previously been shown with the two dimensional % per area analysis, a ratio of the GLUT1:COL4 values were used to establish whether the microvasculature was proliferating or degenerating. The data were shown to be parametric and ANOVA analysis of the groups, indicated there were no significant differences between the groups (p=0.480, Figure 4.7).

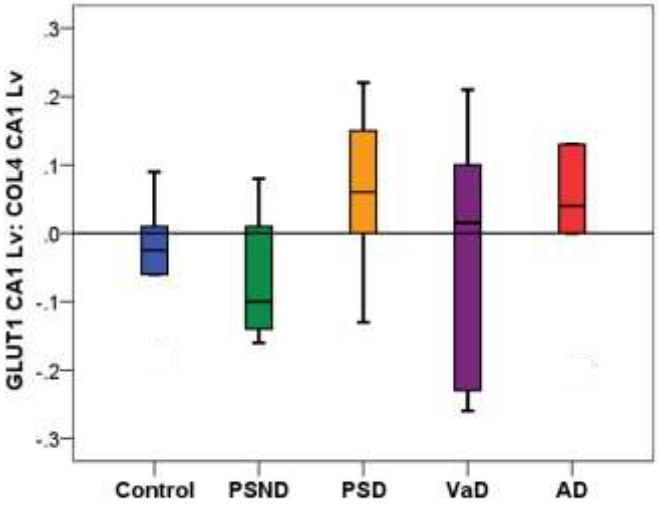


Figure 4.7 Ratio of GLUT1:COL4 of CA1 L_v data

The 10-µm-thick sections stained with GLUT1 and COL4 from the thirty cases for the stereology cohort were reanalysed with two dimensional analysis., To determine if an increase in AD and PSD would be observed by the previous method using these specific cases and if we had potentially biased the results by selecting these cases. Both markers showed no significant difference between groups (GLUT1 ANOVA p=0.149 and COL4 Kruskal-Wallis p=0.988, Figure 4.8).

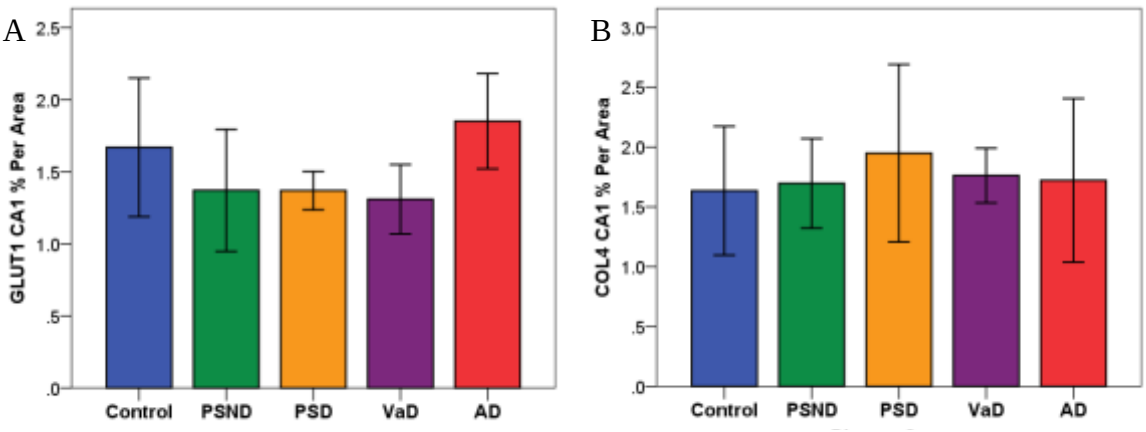


Figure 4.8 % Per Area CA1 results for stereology cohort for GLUT1 (A) and COL4 (B). No significant difference observed.

Although the stereological analysis was limited to six cases per group, power analysis of the data showed a strong level of observed power in the CA1 for the L_v of all microvascular IHC markers, power results and maximum L_v differences between all cases are reported in Table 4.2.

	GLUT1	COL4	SMA
Statistical power	1.00	1.00	0.93
Maximum difference in L_v (mm/mm^{-3})	0.30	0.24	0.07

Table 4.2 Statistical power and maximum difference for each vascular marker for L_v data.

No significant correlation was found between L_v and age of subject, post mortem delay or fixation time.

4.3.2 Cortical Stereological Microvascular L_v Analysis

Following the hippocampal results, analysis of cortical regions was proposed, as this investigation would add to the overall understanding of microvascular density changes

throughout the brain in differing conditions. The cortical region is more vascular in nature than the hippocampus and plays a significant role in the pathophysiology of dementia.

The EC was selected for its proximity and close association to the posterior hippocampus including a shared branch of the major artery the supplies the hippocampus, the posterior cerebral artery, before dividing into specific regional arteries and arterioles. As mentioned previously, significant Alzheimer pathology is deposited in the EC prior to the hippocampus, suggesting it maybe one of the first regions affected by AD (Thal et al., 2002, Braak et al., 2006).

Additionally, the DLPFC (Brodmann Area 9) in the frontal lobe was analysed.. VaD is often characterised by impairments of executive function such as planning and judgement, which is associated with the frontal cortex (Roman and Royall, 1999). High deposits of Aβ are also found within the frontal lobe of cases of AD (Rowe et al., 2007). The DLPFC is particularly vulnerable to cerebrovascular disease (Xi et al., 2011). A previous study has also analysed this region for changes in microvasculature in schizophrenic cases and found no significant difference compared to controls (Kreczmanski et al., 2005). Furthermore, unpublished work from the group had shown similar findings in this region to that of Gemmell 2012 study, with significantly reduced neuronal volume in layers three and five of the cortical region in all demented groups compared to control and PSND, indicating possible neuronal atrophy. The hypothesis was that the vasculature of the EC would show similar results to the hippocampal data and DLPFC would show increases in L_v in cases with increased neuronal atrophy.

The same methodology and cases were used in previous analysis within the hippocampus, with the entire cortical layer being analysed. Due to tissue availability and damage, two

control cases had to be replaced in the DLPFC cohort. Only GLUT1 was analysed for L_v, as the hippocampal data had shown strong internal consistency between GLUT1 and COL4.

Both pieces of data were non parametric, Kruskal-Wallis analysis showed significant difference between groups in EC (p=0.038). Significant increases were identified in AD compared to PSND and PSD (p=0.004 and 0.016 respectively). Additionally, PSND was significantly decreased compared to controls (p=0.015, Figure 4.9 B). No significant differences were found between any of the groups in the DLPFC (Kruskal-Wallis p=0.627, Figure 4.9 A).

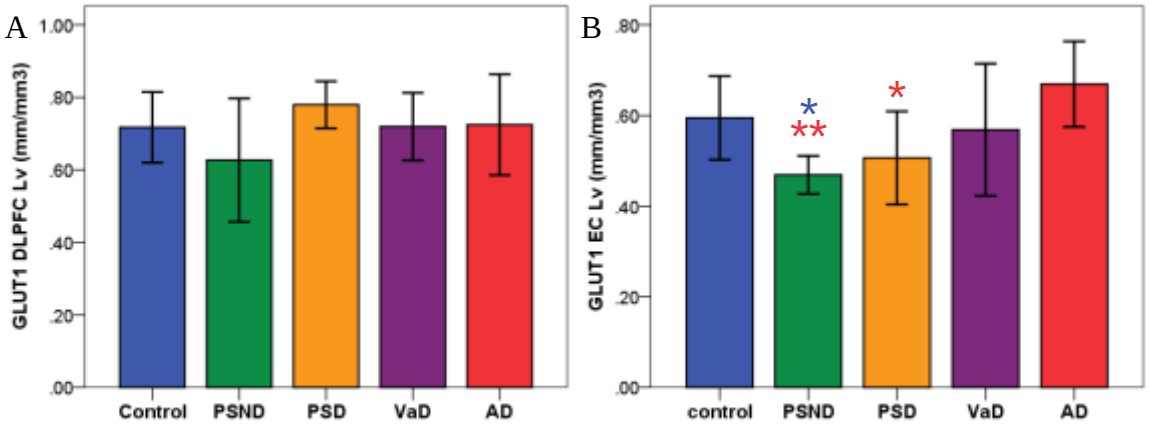


Figure 4.9 GLUT1 L_v results for DLPFC (A) and EC (B). Significant decrease in L_v PSND compared to controls and significant increase in AD compared to PSND and PSD in the EC.

4.4 Discussion

The results showed a significant increase in density of both the ECs and BM marker in cases of PSD compared to PSND in the hippocampus. Such increases in L_v may be a reactive response, whereby increases of the perfusion surfaces between blood and brain result in remodelling of microvessels within a hypoxic environment. Such an interpretation does not refute the notion that microvessel structural changes reflect increased tortuosity in CA1 of PSD subjects or that this affect maybe the result of atrophy of the hippocampus.

Furthermore, significant increases in L_v were observed in AD cases compared to controls, PSND and VaD in the CA1 region. Additionally, AD L_v was found to be significantly increased compared to PSND and PSD in the EC. These findings are consistent with previous studies showing increased L_v using COL4 staining in CA1 of AD cases, compared to controls (Schwartz et al., 2010) and also in the temporal cortex (Richard et al., 2010). There were no differences observed in the CA2 region or DLPFC, suggesting that the increases in L_v are regionally-specific and attributed to increased vulnerability caused by AD pathology or hypoxic injury in the CA1(Leifer and Kowall, 1993). This appears to corroborate previous findings (Cavaglia et al., 2001) indicating selective reduction in capillary density within the CA1 but not CA3 region, albeit after acute ischemic insults.

SMA immunoreactivity in CA1 and CA2 was also measured, predominately to assess status of vascular smooth muscle cells within perforating branches of the hippocampal arterioles. Differences in SMA were found in CA1, with increased L_v in AD compared to controls and VaD but no differences were apparent in PSND and PSD. Relatively few SMA containing profiles were found in the hippocampus, which is largely perfused by capillaries, therefore, the probability of a random intersection between an arteriole and the probe was greatly reduced, conferring greater CE values than our targeted value and suggesting potential error within these results.

Consistent with previous work (Kalaria and Kroon, 1992, Kalaria and Hedera, 1995), GLUT1 immunostaining was not continuous along the vessel, unlike COL4. However, the vessel could be visualised with haematoxylin and intersections were recorded between the vessel and the sphere when another nearby portion of the vessel was positively stained for GLUT1. This irregular staining of ECs along the vessel is likely due to cellular damage either as a result of disease (Kalaria and Hedera, 1995) or as a result of processing post

mortem, which could not be ascertained in this study. It is possible the GLUT1 negative profiles seen only with haematoxylin reflected reduced GLUT1 protein in cases with dementia (Kalaria and Harik, 1989, Simpson et al., 1994, Liu et al., 2009). Though, no specific group could notably be seen to have difference in the percentage of GLUT1 staining of vessels and it should be recalled that the L_v results relate to the structural aspects of the microvasculature rather than a reflection of vessel functionality.

As with the earlier two dimensional analysis, the results were converted so that a ratio of GLUT1 to COL4 could be evaluated. Unlike the two dimensional analysis, which found a significant decrease in the ratio of PSND and AD compared to controls in the CA1, stereological analysis showed no significant difference between groups, with the majority of data suggesting a 1:1 ratio of GLUT1 compared to COL4. This suggests that there may be naturally occurring degrees of both vascular proliferation and degeneration in each group. According to this ratio, if angiogenesis has occurred it was at an earlier time point or that the increase in L_v is caused by another factor i.e. atrophy. It had been suggested that VaD vasculature maybe degenerating as a result of vascular diseases such as arteriolosclerosis, although a significant loss in ratio was not observed in these results. However, other studies have suggested a loss of vessels in leukoaraiosis lesions in the frontal cortex, comparing deep WM and cortex of VaD cases compared to corresponding regions of controls (Brown et al., 2007).

Following on from the hippocampal results, two cortical regions were analysed (EC and DLPFC), to discover if the effect found was specific to the CA1. Similar to hippocampal results, significant increases were observed in AD L_v in the EC; however these differences were found when compared to PSND and PSD and not with controls or VaD. Furthermore, a significant decrease in L_v was found in PSND cases compared to controls. No significant

differences were found between any of the groups in the DLPFC. These results again show an increase in the microvascular density in AD, however interestingly unlike in the CA1 there is significance with the PSD cases. Therefore it was suggested that if a process such as atrophy was the cause for the increase of L_v in the hippocampus then similar findings would be expected in the EC (Devanand et al., 2007). This might suggest that the increase in PSD L_v is either specific to the CA1 or that an increase in microvascular density begins in the hippocampus and develops later in the EC as the disease progresses.

Previous neuroimaging studies suggested that medial temporal lobe atrophy occurs in AD (Frisoni et al., 2010) in PSD (Firbank et al., 2007) as well as in VaD (van de Pol et al., 2011). Therefore, it could be argued that the observed increase in L_v in AD and PSD may result from hippocampal atrophy or tissue shrinkage.. Results from AD, increases in both the CA1 and EC may confirm this but the significant decrease in PSD compared to AD opens this issue to further debate. For these reasons we would then have expected to see an increase in VaD. While it is plausible, it is unlikely that the significant differences in L_v result from artefacts introduced during processing of the post-mortem tissue, as all the tissues were treated in a standardized manner from the same location, allowing valid comparisons to be made. In a related study (Gemmell et al., 2012), hippocampal neuronal volumes in CA1 were decreased across all demented groups including the majority of the cases studies here, suggesting similar alterations should be found in all groups. Although, atrophy could not be specifically measured in these cases, as MRI scans were not performed on all cases and therefore this confounding factor cannot be completely ruled out

Notably, a significant decrease was found in PSND cases compared to controls in the EC. An initial hypothesis had been that subjects who had maintained cognitive function into their elder years would have conserved their microvasculature. Therefore a significant finding

between these two groups is unexpected, Although we find a significant difference with our control group, there is much greater understanding of the PSND group through the clinical analysis of the CogFAST study. Control subjects have little background information and have been designated as being non demented following pathological assessment and follow up interviews with family members. In reality a number of unknown factors may be present within this group.

No significant difference was observed in the DLPFC between groups. The DLPFC was selected as it is known to be particularly vulnerable to cerebrovascular disease.. Unpublished work from within this group has shown that using the same technique as the Gemmell 2012 study, a significant loss of neuronal volume, in the DLPFC in the same cases was found. Therefore, it would have been expected to see an increase in the groups similar to those found in the CA1 region. There is evidence of atrophy of the frontal lobe in line with reduced function including working memory, episodic memory and strategic organisation and planning (Stuss, 2013) and in normal ageing (Nyberg et al., 2010)..

A technical limitation in this study was the lack of availability of the whole reference volume (the hippocampus, EC and DLPFC) for cutting and sampling. The tissue was obtained from predefined blocks, cut and stored some time previously and, due to the precious nature of the tissue, only one block per case was available for sampling. As a result of these inherent issues, we were unable to estimate volume of the structure analysed, meaning it was not possible to convert L_v into total length per structure (i.e. the hippocampus). As L_v is a measure that relies on the relationship between the numerator (in this case, the number of vessel intersections) and the denominator (the background neuronal tissue), this leaves open the possibility of 'reference trap' bias from tissue shrinkage. If we assume that the density of a component within a structure acts as a proxy for its total number, we must also make the

assumption that the reference volume of the structure itself must remain unchanged across the groups measured. Though we cannot rule out the effects of shrinkage, all sections were processed identically and assessment of section thickness revealed no significant differences between groups making group effects unlikely.

Our results are consistent with previous findings for L_v in AD cases (Schwarz, 2010 and Richard 2010), a previous study was able to sample the entire hippocampus and found no significant difference in overall capillary length of AD cases compared to controls (Bouras et al., 2006). Future analysis is required using full stereological assessment, including complete sampling of the hippocampus and cortical regions measured here as well as other regions, providing valuable information to determine the volume of structures and as a result the full length of all the vasculature can be measured.

In summary, results showed increased microvascular L_v in PSD and AD within the hippocampus compared to all other groups in the CA1. Analysis showed differential microvascular changes, as significant differences were not found in the CA2. Additionally, significant increases were found in AD compared to PSND and PSD in the EC, whilst no significant difference was found in the DLPFC. The observed increased L_v in CA1 of PSD cases compared to PSND, with no significant difference in other regions, whilst they exhibit similar but minimal neurodegenerative pathology, suggests that the described microvascular changes are a possible marker of hypoperfusion. Although, there were no significant differences in L_v between PSND and VaD subjects, it is possible that the microvasculature attributes are similar in PSD and AD in the hippocampus but remarkably different mechanisms appear to occur in VaD. However, specifically there does not appear to be a loss of vasculature in VaD cases. Increasing L_v may be an indicator of new microvascular loops via angiogenic processes (Biron et al., 2011), increased twisting of existing profiles (Brown

and Thore, 2011) or a consequence of inflammation (Akiyama et al., 2000). However, the possibility of atrophy affecting the results to produce false positives cannot be ruled out especially as the complete structure was not analysed.

Chapter 5 Angiogenic markers in the hippocampus

5.1 Introduction

Subsequent to the findings in chapter 4, which showed an increase in vessel density in PSD compared to PSND, it was important to establish the reasons which had led to this increase. One possibility is that atrophy of the hippocampus, a well established process in dementia, may have pulled all the inherent structures closer together giving a false positive result (Kalaria, 1996). To determine if this was the issue we required information with respect to the rates of atrophy. Unfortunately, in the cases of AD and VaD, this cannot be performed as a number of cases had been donated to the NBTR and not recruited as part of a study, and regarding the CogFAST study not all cases had been subjected to MRI scans. Alternatively, the entire hippocampal structure could have been analysed using stereology to perform volume analysis via the Cavalieri's principle (Highley et al., 2001, Andersen and Pakkenberg, 2003), which would have allowed a volume measurement for the AOI or brain region for all cases, although it would not have established the rate of atrophy and just indicated volume differences in tissue. As mentioned in chapter 4, due to the limitations of working in a brain bank environment, the entire structure was not available for analysis.

Another possibility was that the vessels had lost their structure and had developed increased tortuosity and therefore vessel density appeared elevated due to increasing twisting of the vessels. This could be further investigated by viewing thicker sections of tissue, approximately 100 μm, to visualise a greater depth of vasculature structure (Brown et al., 2007). However, human brain is a finite and precious resource and hence there are issues with ethical approval, especially when technical limitations may apply.

Alternatively, angiogenesis either prior to or at the time of death may have been responsible for the increase in vessel density as a compensatory mechanism alleviating reduced CBF and hypoperfusion (Schuff et al., 2009, Firbank et al., 2011) or as the result of neuroinflamation (Akiyama et al., 2000, Naldini and Carraro, 2005). Previous literature has suggested an increase in angiogenic markers in AD compared to controls. The possibility of being able to explain the increase in L_v as a result of other mechanisms i.e. atrophy and tortuosity, have a number of limitations. Therefore, it was decided to investigate angiogenic markers to identify possible increases in expression in PSD and AD cases compared to PSND, VaD and controls.

One of the first identified growth factors found to play a role in angiogenesis was vascular endothelial growth factor (VEGF) and is required as a stimulus for angiogenic sprouting (Dvorak et al., 1995, Adair and Montani, 2010). The VEGF family consists of five separate molecules, A-E, in addition VEGF-A exists in several isoforms. VEGF's bind differently to three high affinity receptors (VEGFR) and are responsible for different functions including the survival of ECs, vessel permeability and function. However, specific binding of VEGF to VEGFR-2 appears essential for the formation of ECs, proliferation, migration and survival. Whereas, VEGFR-1 may act as a dummy receptor and negatively regulate or contain over expression of VEGF. General knockout of VEGF-A and VEGFR2 are lethal due to deficiency in vessel formation (Shalaby et al., 1995), whereas VEGFR-1 knockouts show an overgrowth of immature vessels (Fong et al., 1995). VEGF is expressed by neurons, astrocytes and hypoxic tumour cells. It is then secreted from these cells and binds to receptors on nearby vessels therefore initiating the angiogenic process via sprouting of new vessels as described in chapter 1. VEGF also plays a role in a number of other mechanisms including neuronal outgrowth, cell survival (Carmeliet and Storkebaum, 2002) and a role in vessel permeability(Nagy et al., 2008).

The process of angiogenesis has long been associated with hypoxia. The discovery of the hypoxia inducible transcription factors (Hif), has given molecular reinforcement to this theory. Hif's are present in the promoter regions of hypoxia inducible genes (Acker and Acker, 2004). Currently, three members of the Hif family have been identified, however only the functional and importance of Hif 1 and 2 are fairly well understood. Hif 1 and 2 α subunits are regulated in an oxygen dependent process, these subunits bind to the respective β subunits to form heterodimers, which can transactivate genes by binding to the hypoxia response element [HRE, (Plate, 2006)].

In the presence of oxygen, Hif-1 is rapidly degraded by ubiquitination (Wang et al., 1995, Jiang et al., 1996), however under hypoxic conditions hydroxylation by Hif-prolylhydroxylases is inhibited. As a result Hif-1 accumulates in cells and is translocated into the nucleus and activates target genes on the transcriptional level via binding to the HRE (Carrero et al., 2000, Semenza, 2001). It is estimated that 2 to 3% of all genes include HRE, including VEGF. Therefore, Hifs can induce a complex cellular response by transcriptional up-regulation of a large variety of genes in hypoxic conditions. Hifs are upregulated in the brain post ischemia (Wiener et al., 1996). A number of other studies have shown that Hif-1α is upregulated in both vessels isolated from AD cases and in Tg mouse models of AD (Grammas et al., 2006, Peers et al., 2009, Ogunshola and Antoniou, 2009, Grammas et al., 2011).

Integrins are a family of multi adhesion molecules of a variety of ECM proteins including fibronectin and degraded collagen (Tarui et al., 2001). Integrins are composed of non-covalently associated α and β subunits, 15 α and 8 β subunits have been identified (Brooks, 1996). The integrin molecules have been shown to be involved in a number of processes in addition to adhesion including migration, proliferation and cell survival suggesting their role

is more diverse than the initial suggestion that they are the glue that binds cells to the extra cellular matrix (Brooks, 1996). When ECs become activated during angiogenesis, they lead to the upregulation of specific integrin receptors including α5β3, leading to protease secretion and cell activation (Rege et al., 2005). Proteases degrade the underlying BM and form a route for sprouting or migrating ECs (Liekens et al., 2001). Integrin α5β3 has previously been used to measure angiogenesis in a number of different conditions including cortical stroke, myocardial infarctions and retinopathy (Kalinowski et al., 2008, Lahdenranta et al., 2007, Wei et al., 2001).

Previous studies have suggested there is evidence of all three angiogenic markers described above expressed in post mortem tissue from human AD cases (Kalaria et al., 1998, Grammas et al., 2006, Desai et al., 2009). The Desai study used IHC analysis of integrin α5β3 in the hippocampus, midfrontal cortex, substania nigra, globus pallidus and locus coeruleus in post mortem tissue from the religious order study (Bennett et al., 2006). All areas except the globus pallidus exhibited increased integrin immunoreactivity in AD cases compared to controls.

Whilst, previous studies appear ambivalent on the issue of angiogenesis in cases of AD (Paris et al., 2004b, Vagnucci and Li, 2003), a few have suggested that there is increased expression of growth factors and hypoxic regulators in AD cases including VEGF, tumour necrosis factor (TNFα), interleukins, angiopoietin-2 and Hif-1α, possibly associated with neuroinflamation and damage to the brain (Kalaria and Kroon, 1992, Zetterberg et al., 2004, Thirumangalakudi et al., 2006, Fernando et al., 2006). Numerous studies have shown angiogenesis is active in the penumbra region shortly after a stroke (Krupinski et al., 1994,Hayashi et al., 2003).

The investigations here were undertaken to establish if expressions of Hif-1α, Integrin α5β3 and VEGF, as significant markers of angiogenesis, are increased in cases of PSD and AD compared to other groups, which may explain the increase in L_v density (Chapter 4). Although, the increase in L_v may also be explained by atrophy of the tissue and therefore no change may be observed in immunoreactivity. It is hypothesised that there will be an increase in immunoreactivity of these angiogenic markers in both AD and PSD cases compared to other groups.

5.2 Materials and Methods

5.2.1. Subjects

To confirm angiogenic marker expression, antibodies were first tested in tissue sections containing a glioblastoma. The case was a 75 year old male; the cause of death was bronchocarcinoma and the PM delay was 48hrs. The tumour was located in the frontal cortex from coronal levels 7-13 of the Newcastle Brain Map ((Perry, 1993) see materials and methods chapter). The tissue block that sections were cut from was between level 7 and 8. The tumour is clearly visible in Figure 5.1

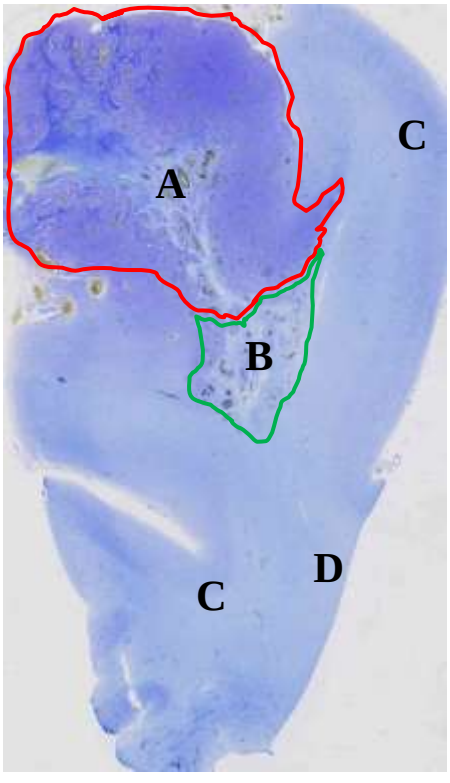


Figure 5.1 Nissl stain of frontal cortex containing glioblastoma. Glioblastoma located in section A. The growing metastases region is shown in area B. Cortical regions are shown as C and WM as D. Image was taken on a flatbed scanner at a resolution of 9,600 dpi as are all scans of sections from this case.

IHC staining was then performed on hippocampal sections of tissue from the same cohort as those used in chapter 4, as a pilot experiment for all markers and to allow for potential further analysis in relation to L_v results. In the case of Hif-1α extra cases were added for further analysis. The extended Hif-1α cohort can be seen in Table 5.1.

	Controls	**PSND**	**PSD**	**VaD**	**AD**
Total number of controls or cases analysed	13	10	10	11	10
Age, y (range)	81.8 (72-98)	86 (81-94)	87.9 (80-96)	87.6 (76-97)	84.9 (76-91)
PMD, h (±2SEM)	38.6 (12.4)	49.2 (24.4)	43.56 (17.8)	41.3 (12.4)	64 (11.3)
Fixation Time, wks (±2SEM)	10.4 (1.8)	9.0 (3)	7.9 (2.4)	9.0 (2.2)	8.2 (0.9)

Table 5.1. Demographic details for the increased cohort analysed for Hif-1α. No significant difference was found between age, PM delay or fixation time.

5.2.2. Immunohistochemistry, image capture and analyses

Standard methodology was used for staining of VEGF and Hif-1α on 10 μm-thick sections, heat induced epitope retrieval was used and anti-mouse IgG antibodies were used against both antibodies as secondaries. Subsequently, sections were stained with DAB. Both antibodies were incubated with the slides over night at 4˚C, VEGF at 1:100 (Santa Cruz Biotechnology Inc, JH121) and Hif-1α at 1:2000 (Abcam, UK, AB16066).

The integrin IHC staining was performed following protocol provided by Prof William Hendry, Rush Medical School, Chicago. Thirty μm-thick sections were stained for analysis to increase probability of immunoreactivity. Similar HIER methodology was used as for

other antibodies except before antigen retrieval; sections were quenched in 10% methanol peroxide for 30 minutes. Sections were incubated with 1:100 of antibody (Abcam, UK, AB7166), 5% horse serum and 1% bovine serum albumin in TBS-T overnight at 4˚C. Sections were stained with DAB for 4 minutes and counterstained with haematoxylin.

Hif-1α immunoreactivity intensity was measured from images at 20x magnification in vessels within the CA1; this was performed using Image Pro software measuring IOD as previously described. A semi-quantative scaling system was used to highlight differences in immunoreactivity quantity between groups (immunoreactivity per area of vessels may lead to bias of results as shown in chapter 3). The hippocampus was the primary area of assessment, however the surrounding cortex and WM were also observed for changes. The scoring scale was; 0) no immunoreactivity, 1) only immunoreactivity within and around large vessels, 2) moderate amount of immunoreactivity including most large vessels in hippocampus and a small proportion of microvasculature, 3) large proportion of all types of vessels with positive immunoreactivity in two distinction areas i.e. hippocampus and cortex or WM and 4) majority of vessels stained with antibody in multiple areas i.e. hippocampus, cortex and WM.

Immunoreactivity intensity was analysed for integrin α5β3 within the hippocampus (similar to Desai et al., 2009). Images were taken at 40x magnification. Additionally, due to the low numbers of positive integrin vessels within the section, the total number of vessels with positive immunoreactivity per section could be readily determined. To calculate the number of positive vessels per mm^2 the hippocampal area was recorded for each case. High resolution scans were taken of all the sections stained with integrin, the image was cropped so that each image was 400 mm^2. Using the Image Pro software it was possible to calculate the number of pixels in 400 mm^2. Subsequently, each hippocampus from the scanned image was outlined, using the freehand tool, to calculate total number of pixels for each individual

hippocampus. Using both pieces of information, the area of each hippocampus was calculated.

Similarly to Hif-1α analysis, a blind semi-quantitative analysis was performed on VEGF sections. A scoring system between 0 and 3 was created to indicate increasing immunoreactivity between cases. Scoring was defined as 0) no immunoreactivity, 1) few weakly stained neurons, 2) stronger neuronal immunoreactivity intensity and quantity or evidence of staining in oligodendricytes and 3) a large number of strongly stained cells in multiple regions.

5.3 Results

5.3.1 Hypoxia inducible factor 1α

5.3.1.1 Immunoreactivity in glioblastoma case

The Hif-1α antibody was shown to express predominantly in the vasculature of the glioblastoma tissue (Figure 5.2A). Immunoreactivity was specific to the vasculature and little or no staining was noted in the surrounding cells. Furthermore, the immunoreactivity appeared to be regionally specific, strong staining was noted in the metastasis region with a high percentage of vessels, both large and small, being clearly stained (Figure 5.2 C). Alternatively, there appeared to be less immunoreactivity within the middle of the tumour region (Figure 5.2 B). Also noted was the variation of immunoreactivity in different cortical and WM regions on the sections. The cortical area nearest to the expanding tumour showed strong immunoreactivity of the vasculature (Figure 5.2 D), whilst another cortical region on the same section showed no staining at all, although vessels are clearly visible (Figure 5.2 F). The same phenomenon is found in the WM, with areas near to the expanding tumour

showing increasing staining (Figure 5.2 E) and those further away showing no staining (Figure 5.2 G).

By gross examination, it was clear that the antibody was staining both the vessel and cells within the vessel, possibly erythrocytes or lymphocytes (Figure 5.3 A and B). In some cross sectional images of large vessels, there was no clear immunoreactivity of cells within the vessel (Figure 5.3 C) and in some cases a mix of strong and no immunoreactivity of blood cells (Figure 5.3 D). However, it is more difficult to ascertain which constituent is being stained in the microvasculature. This suggests there was a varying degree of immunoreactivity of blood cells within the vessel. However, entire vessel lengths appeared stained with the antibody, (Figure 5.2) unlike the discontinuous form of staining observed in GLUT1, which is known to stain endothelium and erythrocytes. This suggests the antibody is reactive in additional vessel components or blood constituents. Furthermore, as previously shown the immunoreactivity appears to be regionally specific which would suggest it is not erythrocytes that were reacting with the antibody. Although, in unperfused tissue it cannot be guaranteed that only the vessel is being stained, this antibody appeared to be a good marker for displaying regions of hypoxia.

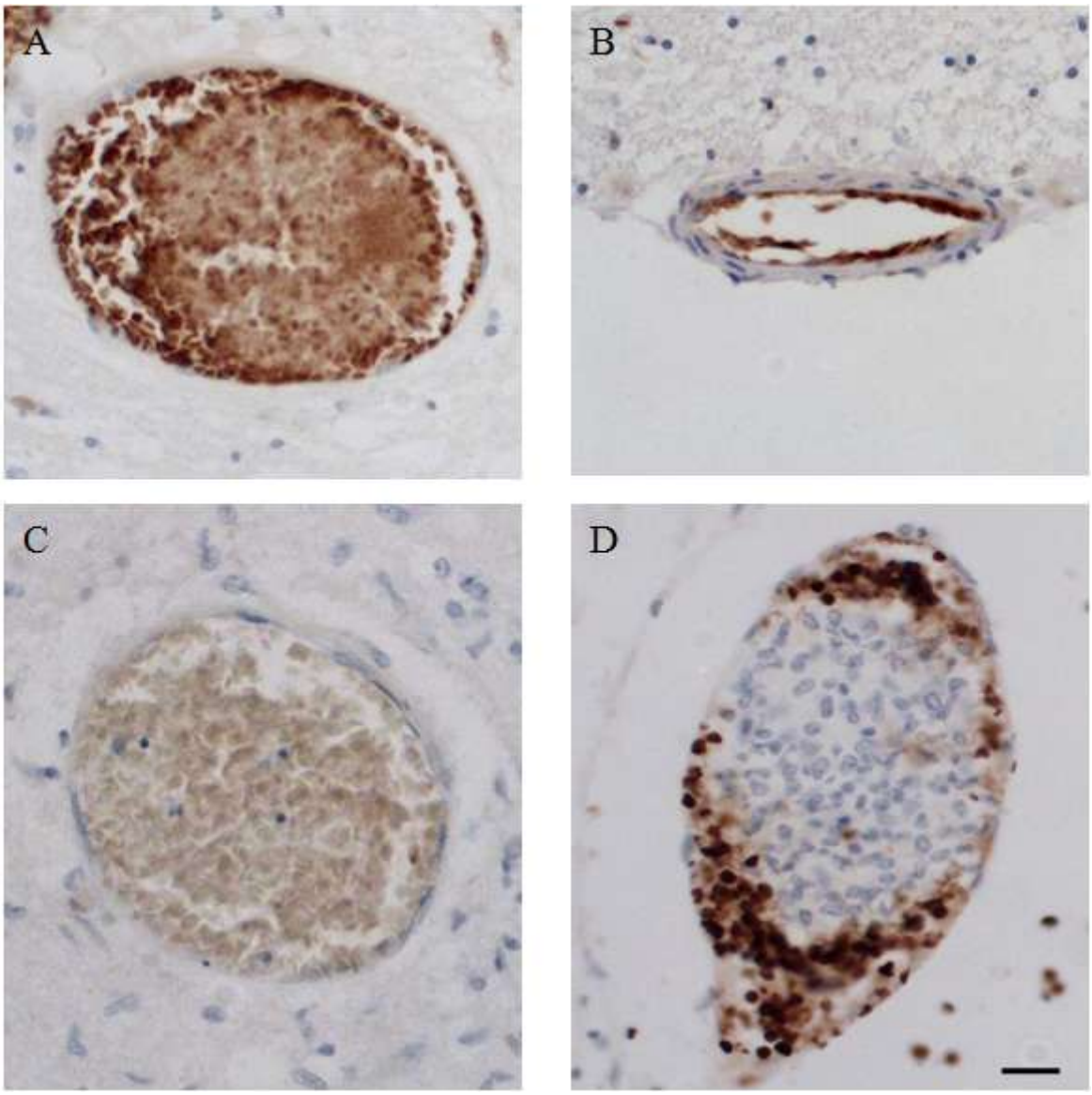


Figure 5.3 A selection of cross sectional vessels stained with Hif-1α, all images taken from the glioblastoma tissue. Image A shows staining through the vessel including possible vasculature components (ECs) and also staining of blood cells. B shows possible staining of ECs with no blood cells in the lumen. C shows no staining of either blood cells or the vascular components and Image D shows staining of possible vasculature components and blood cells in the nearby area, however there is no staining of cells at the centre of the lumen. Mag bar = 20 μm

5.3.1.2 HIF-1α immunoreactivity in human hippocampus

Grammas et al (2011) found a significant increase of Hif-1α in a Tg mouse model of AD and vessels isolated from AD cases (Grammas 2006). Analysis here tested whether a similar finding could be made in our human post mortem tissue by measuring staining intensity, as

was measured in the CA1 from the animal model experiment. However, no significant differences were found between groups (Kruskal-Wallis test, p=0.520, Figure 5.4).

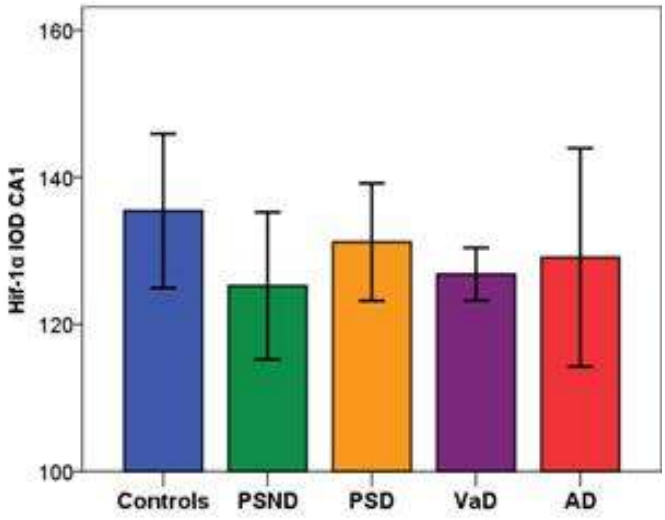


Figure 5.4 Mean immunoreactivity intensity (IOD) for Hif-1α in the CA1

Correlations were performed to check for significance against age, PM delay and fixation time. There were no relationships with age or PM delay. However, a strong negative correlation was found with increasing fixation time. Cases with a fixation time greater than 18 weeks were excluded from the analysis and a trend towards a significant increase in immunoreactivity was found in AD cases compared to VaD using ANOVA and Dunnett T3 post hoc test ($p \leq 0.075$).

As a result of these findings in the L_v stereology cohort, cases were added to increase the number to a minimum of 10 per group with all cases having a fixation time below 18 weeks. An ANOVA of the new result showed that there were no significant differences between cases (p=0.141, figure 5.5). There was also no significant correlation with age, PM delay or fixation time in the new cohort.

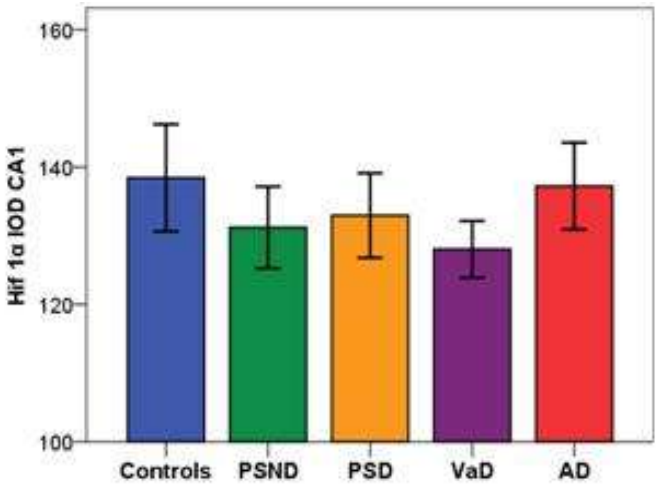


Figure 5.5 Staining intensity of Hif-1α in CA1 vessels of extended cohort. No significant difference between groups

Within the hippocampus varying quantities of staining were noted and there appeared to be significantly more vessels stained in the CA1 and CA4 compared to other regions such as CA2 and CA3, taking relative sizes into consideration. Furthermore, cases within the same group where seen to have significant staining of vasculature in the CA1 region and in other cases there was no staining present at all. In the cases where there is limited immunoreactivity in the CA1 subfield, it often appeared that there was staining in the WM tracks both in the hippocampus including the alveus, stratum radiatum and lacunosum regions, and additionally immunoreactivity in WM in nearby regions, whilst cortical regions were absent of all staining.

As there was significant variation in immunoreactivity between cases, a basic semi quantitative scoring system was devised to ascertain if this variation was specific to any group. As described in the materials and methods section the scoring system ranged from 0-4, with an increasing score given for increasing immunoreactivity. The entire hippocampus was observed as a whole and the surrounding areas were also considered for immunoreactivity.

A Kruskal-Wallis test was used to test for significance of the immunoreactivity quantity scale between the groups. Significant decreases in both PSND and VaD were seen compared to controls when using a Mann-Whitney test (p=0.046 and p=0.028 respectively). The PSND group was significantly decreased to the PSD group (p=0.050) and the VaD group showed a strong trend toward significance (p=0.051). No significant difference was observed with AD (Figure 5.6).

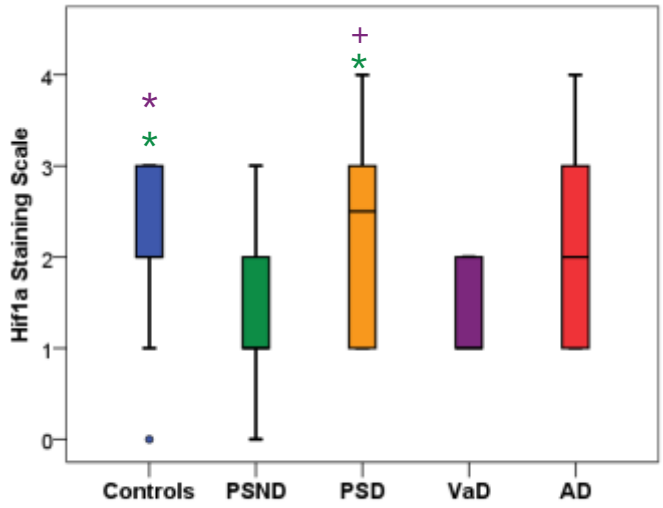


Figure 5.6 – Boxplot to show the difference in scoring of the Hif-1α scale for staining quantity. The line across the boxplot indicates the median value for each group. Circle in the control column indicates a data point that was an outlier from the rest of the data.

5.3.1.3 HIF-1α immunoreactivity in a perfused animal model of ischemia

To establish if the staining observed in human cases was in the vascular matrix, as well as cells within the vessels, the Hif-1α antibody was tested in perfused baboon sections. Baboon tissue was available due to an on-going collaboration the group has with the Primate Research Facility, Nairobi, Kenya and was sectioned coronally. The baboons were used as an acute stroke model having undergone a two vessel occlusion procedure. The animals were culled after the procedure at different time points, we selected sections so that a time course, post procedure, could be observed. Tissue from animals culled at the following time points were used 1, 3, 7, 14, 28 days and a sham experiment animal. An entire coronally sectioned baboon brain could be mounted onto a large glass slide (75 mm x 50 mm).

All sections showed there to be a significantly reduced quantity of immunoreactivity in comparison to the human tissue using the same IHC procedure. No staining was observed in the hippocampus or the cortex. However, immunoreactivity was observed in a number of vessels especially in sections from animals culled soon after the vessel occlusion procedure (1d, 3 d and 7d) and was mainly located in the basal ganglia region. Immunoreactivity was prominently seen in the trans-sectional cut large vessels; however some small vessels could also be clearly seen with immunoreactivity (Figure 5.7).

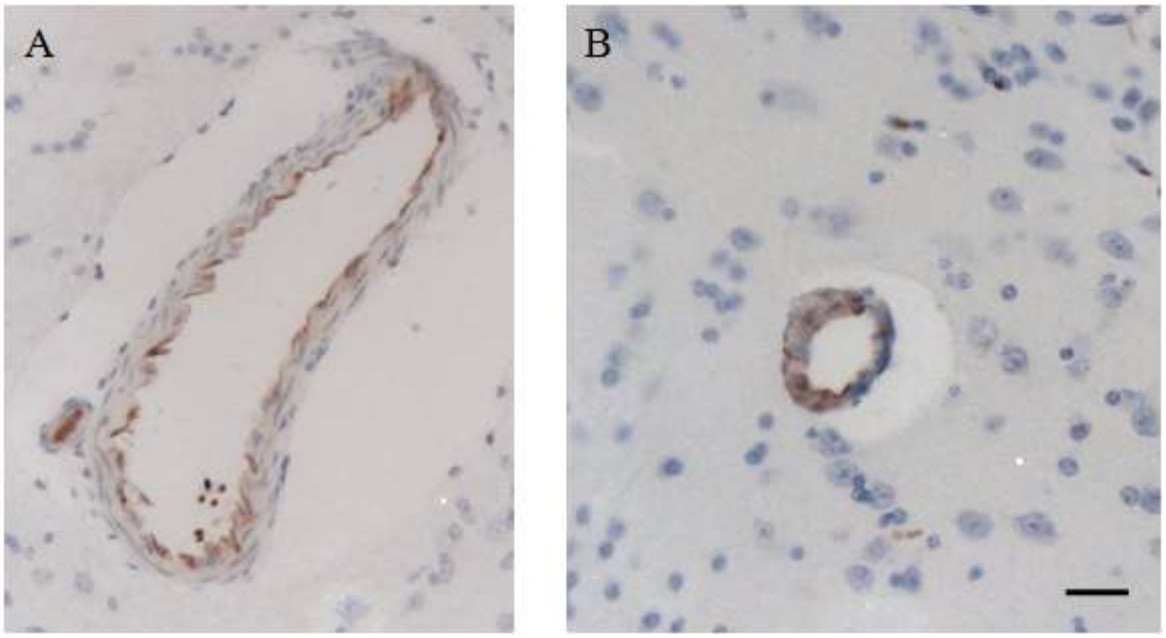


Figure 5.7 Hif-1α immunoreactivity in vessels located in baboon tissue. Image A shows staining of ECs in an artery. Image B shows staining throughout vessel wall, possibly of an arteriole. Mag bar = 20 μm

5.3.2 Integrin

5.3.2.1 Integrin immunoreactivity in glioblastoma case

Integrin immunoreactivity showed variable staining across the glioblastoma case (Figure 5.8A). Like Hif-1α a notable increase in immunoreactivity were observed in the metastasis region. In the established tumour region, there appeared to be possible weak non specific staining within vessels and staining appeared to be strongest in vessels cut trans-sectionally

but it was less clear to see staining of the vessels in this region (Figure 5.8 B). Specific immunoreactivity is noted in the metastasis region, particularly of vessels, some staining looked to be of both vasculature structure and cellular contents within the vessel. Immunoreactivity was seen in both large and small vessels (Figure 5.8 C). In an unaffected cortical region, limited immunoreactivity could be seen, although vessels could be clearly observed (Figure 5.8 D). Staining could be seen along the length of the vessel, in differing amounts and was not continuous (Figure 5.8 E). Although, staining was often weak and difficult to associate it was significant in comparison to negative control staining in a serial section. Images were examined by Professor William Hendey at the Rush Medical School, Chicago who was the correspondent author on the Desai 2009 paper. He agreed that this was similar staining to what they had found. However he did suggest some weak staining maybe found on platelets, activated t-cells and polymorphonuclear neutrophils.

Limited immunoreactivity was found in the hippocampus from the majority of cases. However staining was apparent but often weak and limited to a small number of vessels. Stained vessels could be seen in close proximity to other vessels where no immunoreactivity was observed. The majority of vessels showed random immunoreactivity at different parts along the vessel. With the limited immunoreactivity it was difficult to be sure if immunoreactivity was purely from the vessel structure or if there was some non specific staining of vessel contents (Figure 5.9).

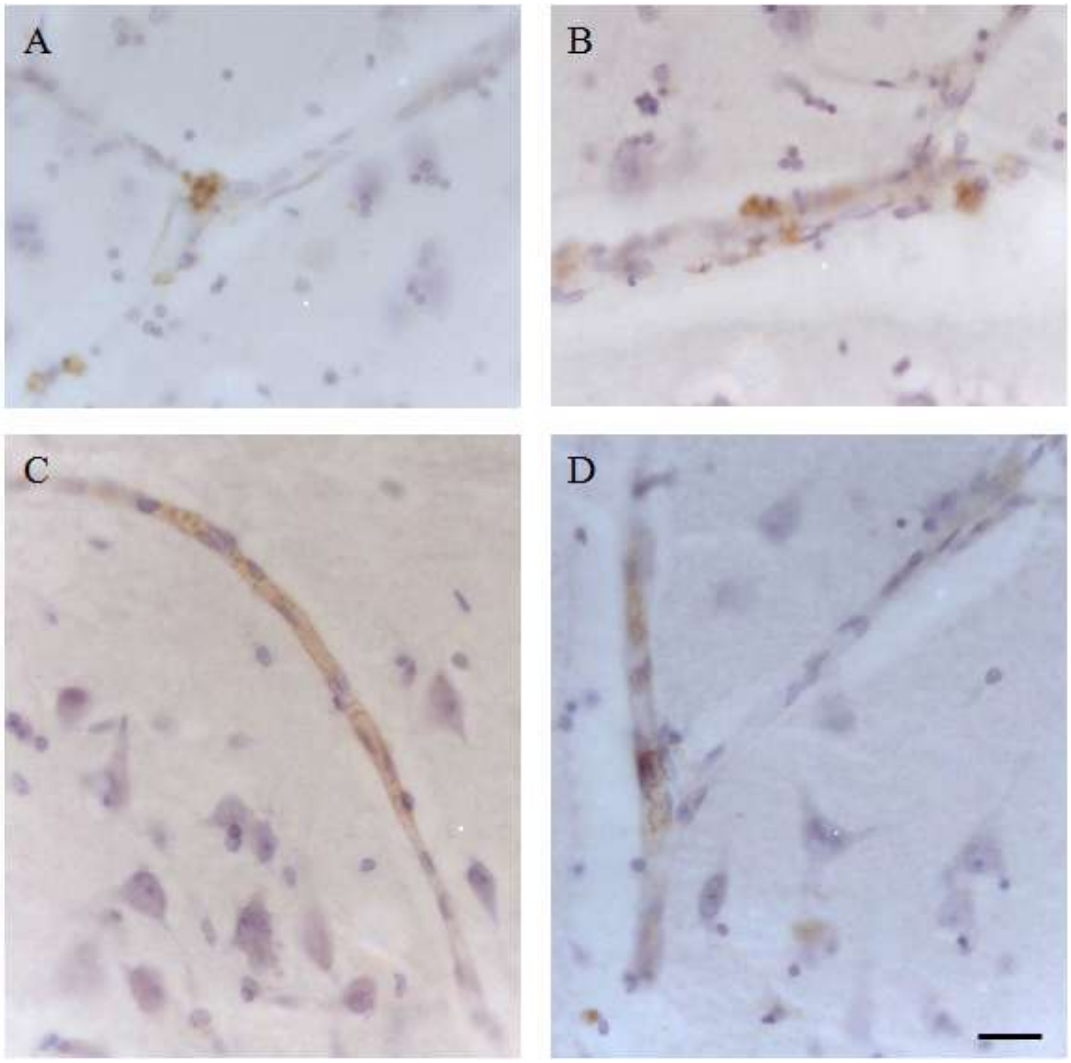


Figure 5.9 Shows examples of integrin immunoreactivity. Image A and B show possible specific immunoreactivity at locations along the vessel, which may suggest the location of possible sprouting angiogenesis. Image C and D, showing immunoreactivity more continual along the vessel, although D appears to show more immunoreactivity in one branch of vessel compared to an adjoining branch. Both C and D suggest more staining of possible blood cells. Mag bar = 40 μm for A-E

5.3.2.2 Integrin immunoreactivity in human hippocampus

To compare with previous findings (Desai et al., 2009) integrin reactivity intensity in all the groups was measured. Previous studies found an increase in intensity in the hippocampus as well as a number of other additional regions in AD cases compared to controls. In this analysis there was no significant difference between groups using ANOVA (p=0.392). Although AD showed the greatest intensity of staining, the variation between subjects was too great to reach significance for this number of cases (Figure 5.10).

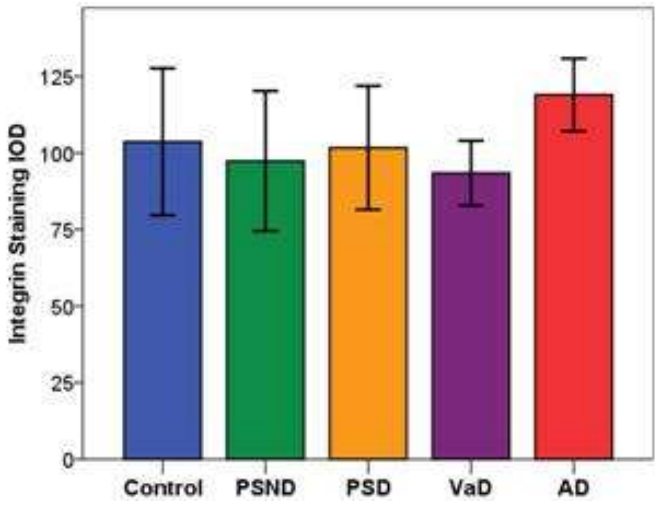


Figure 5.10 – Staining intensity of integrin in hippocampus. No significant difference between groups.

The number of vessels stained with integrin was then counted per case in the hippocampus. No significant difference was found between groups using ANOVA analysis (p=0.545).Cases were reanalysed and the number of positive vessels were measured as a factor of the total area of the hippocampus. When the cases were reanalysed, a strong trend toward a significant difference was found between groups (Kruskal-Wallis test, p=0.052). The Mann-Whitney U test showed significant increase in AD cases compared to PSND (p=0.011), PSD (p=0.037) and VaD (p=0.018, Figure 5.11).

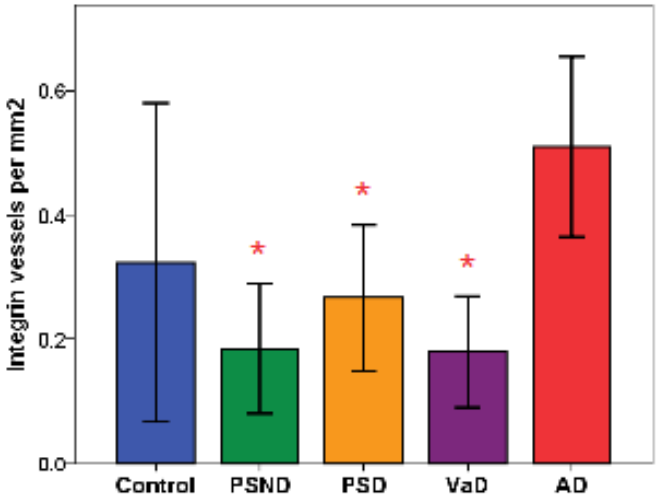


Figure 5.11 – Number of vessels stained with integrin per mm^2 of hippocampal tissue

5.3.3 Vascular Endothelial Growth Factor

5.3.3.1 VEGF immunoreactivity in glioblastoma case

Examination showed variable immunoreactivity across the tissue (Figure 5.12 A). Staining was seen in a number of the cells in the centre of the tumour (Figure 5.12 B); however they were fairly weak and limited in number. The immunoreactivity at the metastasis region was considerably more noticeable especially in cells surrounding large vessels (Figure 5.12 C). There was no clear background staining in the parenchyma around the tumour region and therefore staining appeared specific to cells. There was some immunoreactivity of cells within large vessels. A nearby cortical region, apparently unaffected by the tumour showed no signs of VEGF staining within neuronal cells or on vasculature (Figure 5.12 D). Although, some immunoreactivity was noticeable in the WM, a number of oligodendrocyte cell bodies strongly express the VEGF stained antibody (Figure 5.12 E). These variations in immunoreactivity suggests that the VEGF antibody was specific to the tumour region and in areas active in angiogenesis.

5.3.3.2 VEGF immunoreactivity in human hippocampus

Generally, there was very little specific immunoreactivity of VEGF within the hippocampus. The staining was mainly found in the pyramidal cells of CA2 and occasionally oligodendrocytes in the surrounding WM region. Unusual staining was noted in a control case, where there appeared to be more specific immunoreactivity along the length of vessels in the WM region. It is possible that this is non specific staining of cells within the vessel; however this effect was not observed in any other region within this case. Non neuronal cells, possibly astrocytes and microglia, did not appear to express VEGF either.

A scoring system was performed to try to establish if there were differences between the groups. The scale ranged from 0-3 but no case scored more than 2, therefore highlighting the limited amount of immunoreactivity across all groups. Only six cases were analysed from each group as a pilot study to establish if there was significant expression of VEGF in the hippocampus. A Kruskal-Wallis test was used to compare the scores between groups, no significant difference was found in any group ($p=0.847$). No correlation was found for age, PM delay or fixation time.

5.4 Discussion

The angiogenic markers selected for analysis play a pivotal role in angiogenic mechanisms. Immunoreactivity of all markers in the glioblastoma case suggests that all selected markers are present in regions active in the angiogenic process. Clearly, staining of all markers in the hippocampus were markedly reduced in comparison to the glioblastoma region, especially integrin and VEGF, which would suggest that at the time of death, angiogenesis is limited in these regions compared to a glioblastoma case, if occurring at all.

Nonetheless, significant staining of Hif-1α was observed in the hippocampus. The initial finding from the stereology cohort suggested there was no significance difference in staining intensity between the groups. However, further analysis showed there was a significant correlation with increased fixation time. When cases with a fixation greater than 18 weeks were removed, analysis showed a trend toward a significant increase in AD cases compared to VaD. Additional cases were added to the pilot study, so that a minimum of ten cases were included in each group. However, with these additional cases, no significant difference was found between groups for staining intensity.

It was also noted that there was a significant variation in the quantity of vessels positive for Hif-1α immunoreactivity between cases. Whilst it would be preferable to have a fully quantative assessment of the amount of Hif-1α or protein quantity by western blotting or assessment on the amount of vessels stained with Hif-1α as a possible indication of expression. Previous work in this thesis has shown that % per area values of string like or tubular structures may generate bias within the data (chapter 3 and 4), therefore ideally, stereological analysis would be performed, unfortunately there was not time within this project to perform such analysis. Nevertheless, with such a high degree of variation between cases a semi quantative scale was employed to initially investigate differences. There was a significant decrease in PSND cases compared to PSD, a strong trend toward decrease was also reported for VaD compared to PSD. There was a significant decrease in PSND and VaD cases compared to controls, whilst PSD showed no significant difference to controls. There was no significant difference with any group compared to AD.

An issue with Hif-1α staining, as clearly seen in cross sectionally cut large vessels of the glioblastoma case, was that the antibody stained a number of blood cells within the vessel as well as possibly the vasculature itself. Therefore any expression observed may not have been

specific for the vessel or area. The positive cells could be a type of blood cell, we could not confirm which type of cells these might be, although some cells did appear to have a nucleus suggesting that some are not erythrocytes. However, immunoreactivity recorded in the glioblastoma section appeared to be in regions where high levels of angiogenesis may be expected. Therefore, the staining may suggest that, although the staining is exclusively from the vasculature and not the surrounding neuronal cells, these regions are hypoxic. In small vessels it is often difficult to tell if staining is isolated to the intravascular blood cells or the microvasculature.

To try to address this issue, staining was performed in a perfused animal model to observe staining of the vasculature. The antibody was unsuitable for rodent tissue, but was expected to work in non-human primates. Fortunately, we had access to baboon tissue due to the group's collaboration with the Kenyan Primate Research Group. Immunoreactivity was observed within the vasculature predominately found in the basal ganglia region in animals culled shortly after the completion of the two vessel occlusion procedure (1, 3 and 7 days post-surgery). Staining was significantly reduced in comparison to that found in the human study, suggesting that the majority of staining found in human tissue is the result of intravascular blood cells. It has previously been suggested that there is a significant increase in Hif-1α in both human AD cases and Tg mouse models. It is believed that Hif-1α may play a role in increasing the production of Aβ (Peers et al., 2009, Ogunshola and Antoniou, 2009, Grammas et al., 2011,). Our result is limited by the fact that blood cells appear to also be specific to the Hif-1α antibody and therefore new targets should be investigated.

To further determine if angiogenesis was occurring in the hippocampus and to try to explain the increase in L_v of vasculature in AD and PSD reported in chapter 4, Integrin α5β3 was investigated in these groups, which followed on from previous literature, Desai et al., 2009.

The Desai study, found a significant increase in staining intensity of positive integrin vessels in multiple regions from AD post mortem brains, but not in the globus pallidus, an area not associated with AD pathology (Brilliant et al., 1997). Our study did not find a similar significant increase in the hippocampus in any group, however this was a study only in a small number of cases and there may not have been the appropriate power of this study to find significance.

Additionally, the number of positive vessels was investigated in each section and the initial analysis suggested there was no significant difference between groups. However, when results were normalised with respect to size of individual hippocampi, there was a significant increase in positive vessels per mm^2 in AD cases compared to PSND, PSD and VaD. This was in keeping with the finding in the Desai paper, which also found a significant increase in the number of vessels stained in the hippocampus of AD cases.

Immunoreactivity in the hippocampus was limited in both intensity and quantity and may therefore be non-specific. We observed variation in staining of vessels in both hippocampus and glioblastoma cases (Figure 5.9). Reviewing the literature by Desai, suggests that only longitudinal vessels were analysed to reduce any bias caused by edge effects. Images from this publication appear to exhibit strong phase contrast and therefore any alterations in intensity maybe debatable. Whilst, the analysis to determine differences in positive vessels per mm^2 suggests an increase in AD, there is a risk that vessels may have been missed in analysis and a more stringent form of determining quantity of integrin would be required. Also, as previously suggested any increase in microvasculature in the hippocampus of AD cases may be a result of increased hippocampal atrophy, which would affect the number of vessels counted. In the Desai publication, this would seem a distinct possibility, as in all the regions analysed it was only the hippocampus which showed a significant increase in vessel

number. However, taken with the L_v results from this thesis, it is of interest that an increase is number of vessels is not observed in PSD and are similar to both PSND and VaD. This would suggest that angiogenesis was not occurring at the time of death in this group.

VEGF was also analysed, as a central growth factor involved in angiogenesis (Waltenberger et al., 1994). Although there was clearly some positive immunoreactivity in the glioblastoma case (Figure 5.12), little specific immunoreactivity was seen in any hippocampus. No significant difference was seen between cases using a semi quantative score. Other studies have previously reported an increase in VEGF in both AD and VaD cases (Tarkowski et al., 2002, Chiappelli et al., 2006, Thirumangalakudi et al., 2006) and it may be a potential additional biomarker in the diagnosis of AD (Craig-Schapiro et al., 2011). We believe our findings may be compromised by technical limitations. VEGF is a soluble growth factor excreted by cells; it is likely that a large proportion is lost in the fixation process of the tissue. A more optimal target to analyse would be the VEGFR2, which as previously described is key to initiating angiogenesis (Waltenberger et al., 1994). VEGFR dimerise to form either homo or heterodimers of the receptors. Dimerization is accompanied by autophosphorylation, this allows the receptor to recruit interacting proteins and induce the activation of signalling pathways (Olsson et al., 2006). Although P-VEGFR2 antibodies are available, discussions were held with suppliers to ascertain if these antibodies would work in fixed human tissue. They informed me that in their experience, the antibodies work in fixed HUVEC cells; however they have had no successful trials in fixed human tissue and are unaware of any other antibodies that would provide such staining.

There are a number of limitations within the experiments in this chapter and other techniques would be required to fully elucidate changes in the angiogenic mechanism within these cases. Possibilities would include the use of RT-PCR array to measure changes in a number of

specific angiogenic genes in the tissue or optimising extractions of microvessels from tissue for mRNA and protein analysis by removing vessels from the tissue either by laser microdisection (Kaneko et al., 2011) or separating vessels from tissue via a filtration procedure and then resuspending in a specific buffer (Grammas, 1991). Unfortunately, at this time we have been unable to test any of these possible methodologies and they would be key experiments to future analysis. Furthermore, as shown in chapter 1, for angiogenesis to occur a wide variety of proteins play key and important roles and therefore a number of other markers include angiopoieten, erythropoietin and matrix metalloproteases should be investigated before conclusions are made.

Pathological angiogenesis is known to occur in a number of diseases and in this situation the vessel fails to form properly and deliver oxygen and glucose to the areas where it is required; this mechanism better describes vascular remodelling than the mass formation of new blood vessels (Carmeliet and Jain, 2000). A similar process maybe occurring in both AD and PSD cases as a result of a hypoxic environment possibly caused by neuroinflamation or hypoperfusion, leading to abnormal vessel formation and remodelling. Whilst vessel damage, known to occur, in VaD is caused by disease of the vessel itself and hence the vessel may be unable to respond to stimuli, which may explain some of the differences between the findings in these groups. Aβ has been implicated in causing angiogenesis, which may explain results in AD. Significant increases in Aβ are observed in PSND compared to PSD, suggesting this should not explain the increase in microvascular density observed in PSD. Furthermore, PSD and VaD have nearly identical pathological characteristics, however often strikingly different results for microvasculature morphology. In this study it has not been possible to establish whether there is disruption of the blood brain barrier or alteration to the greater vascular structure and these issues will need to be further investigated in future experiments. It is

possible that PSND cases have maintained functionality of their vessels, therefore the initial hypoxic environment, described for PSD, does not occur.

Although it is difficult to conclude from the findings in this chapter that angiogenesis is occurring in either AD, PSD or both, there are some possible indications; increased number of Hif-1α vessels in PSD compared to PSND and VaD, and an increased number of integrin vessels in AD hippocampus. A number of additional studies would need to be performed to arrive at an objective conclusion regarding angiogenesis, given that this is a very controversial argument compared with some previous findings although does agree with others. If correct, increases in microvascular density may be caused by neuroinflamation, previously reported in AD with an increase in a number of inflammatory markers are also increased including TNFα, IL1β, IL-6, and IL-8 (Naldini and Carraro, 2005, Akiyama et al., 2000) or as a consequence of hypoperfusion. However, the issue of atrophy still needs to be addressed to ascertain the affect that this is having on results for vascular density. From these studies, we find no clear evidence of a regenerative vascular mechanism occurring in PSND, as initially hypothesised, suggesting that the reason these cases can maintain cognitive ability is not due to an ability of vascular reserve.

Chapter 6 Hippocampal neuronal progenitor cell markers in post stroke cases and other age related dementias

6.1. Introduction

Over the last decade, a number of studies have demonstrated the existence of multipotent neural progenitor cells, as described in Chapter 1. These cells are specifically located in neurogenic niches in adult mammal and human brains (Eriksson et al., 1998), one of these being the SGL of the DG. Within the hippocampus neurogenesis is responsible for the production of new excitatory granule cells (Kempermann et al., 1998) and these newly formed granular cells connect via their axons to the CA3 subfields and are incorporated into the hippocampal tri-synaptic circuitry (Carlen et al., 2002). We wanted to identify if there was an increase in the neurogenesis mechanism in PSND subjects which may explain why they were able to maintain their cognitive function post stroke compared to PSD subjects.

It has been well established that the hippocampus is important for the process of learning and memory (see Chapter 1) and both these attributes are possibly linked to active neurogenesis (Saxe et al., 2006, Zhao et al., 2008, Deng et al., 2010). Human studies have suggested that there is an increase in neurogenesis in dementia, including both VaD and AD (Ekonomou et al., 2011, Jin et al., 2004b) and PS (Jin et al., 2006, Marti-Fabregas et al., 2010). However, others have suggested that developing AD may impair the process of sustained neurogenesis (Crews et al., 2010, Li et al., 2009, Boekhoorn et al., 2006). Whilst, Perry et al (Perry et al., 2012) showed that there is an alteration in neurogenic stages dependent on NFT burden. Significant loss of MSI1 and increasing nestin and PSAnCAM immunoreactivity with increasing Braak stages were reported. Furthermore, animal models of AD i.e. over

expressing APP Tg mice, have suggested increased neurogenesis (Lopez-Toledano and Shelanski, 2007, Rohe et al., 2008) whilst others reported the process decreased (Verret et al., 2007, Hamilton et al., 2010). It has also suggested that the decrease occurred before the onset of pathology in these models (Hamilton et al., 2010).

A large number of studies have indicated that lifestyle may increase neurogenesis. This has been measured in animal studies which have shown increased expression of neurogenic markers in rodents exposed to environmental enrichment, exercise and therapeutic treatments such as anti-depressants (Kempermann et al., 1997, van Praag et al., 1999, Boldrini et al., 2009). Whilst, neurogenesis has been shown to be down regulated as a result of ageing and a changing microenvironment, predominantly caused by local immune signalling specifically cytokines (Villeda et al., 2011).

Although, current findings as described above are inconclusive with respect to the beneficial effect of neurogenesis, especially in elderly human studies, it has raised the expectation as a potential new therapeutic target. This is especially relevant in a field where there are currently no treatments and recent clinical trials continually end in failure.

This study explored endogenous neurogenesis within the SGL and GCL in the DG in post mortem tissue. We hypothesised an increase in neurogenic expression, by measuring both the immunoreactivity quantity and intensity of previously established markers of neurogenic cells, in the PSND group compared to PSD. The theory being that an increase in neurogenesis would explain the difference in cognitive outcome and may be an indication of an increase in potential reserve in cases of PSND as the new cells would strengthen the circuitory in the hippocampus. Other demented subgroups were also investigated including AD and VaD, as well as age matched controls.

6.2. Materials and Methods

6.2.1 Subjects

	Controls	PSND	PSD	VaD	AD
Total number of controls or cases analysed	10	22	13	11	11
Age, y Mean (±2SEM)	80.8 (3.9)	83.8 (1.7)	86.7 (3.2)	84.8 (4.0)	83.3 (4.2)
PMD, h Mean (±2SEM)	27.8 (7.1)	43.4 (13.2)	44.9 (19.8)	38.4 (11.74)	58.6 (15.8)

Table 6.1 Demographics for cases used in neurogenesis analysis. The range of fixation for all cases was 4 to 40 weeks. No significant difference was found between age, PMD and tissue fixation.

6.2.2 Immunohistochemical Procedures

Ten µm thick sections were incubated with antibodies against neuronal stem and lineage proteins. These included Musashi 1 (MSI1; anti-rabbit polyclonal, 1:1000, Chemicon) an RNA binding protein, a marker of early stage progenitor cells (Okano et al., 2005). Nestin (anti-rabbit, 1:600, Chemicon) a large intermediate filament protein expressed during development and found in neural precursor cells (Lendahl et al., 1990). Polysiated neuronal cell adhesion molecule (PSAnCAM; anti-mouse, 1:800, Millipore) previously characterised in neurogenesis (Seki and Arai, 1991) and is found on migratory neuronal cells in the DG. PSAnCAM has been suggested to overlap with doublecortin (DCX) during particular stages of the neurogenesis mechanism. DCX is a microtubule associated protein also found in migratory neuronal cells (Meyer et al., 2002). However, DCX is also found in neuronal

determined progenitor cells and also allows identification of cells further advanced in development to a neuronal fate (DCX; anti-goat, 1:1000, Santa Cruz USA). Hu C/D, officially ELAV like [(embryonic lethal, abnormal vision *Drosophila*), anti-mouse, 1:1500, Invitrogen UK] binds to mRNA in specific AU rich untranslated domains. Hu, has been described as an early neuronal marker and suggested to control the timing of the switch between precursor cell proliferation and neuronal differentiation (Wakamatsu and Weston, 1997, Marusich et al., 1994, Magavi and Macklis, 2002, Jin et al., 2004b). Each antibody was selected so that we could attempt to analyse all stages of neurogenesis. Where these proteins are active can be seen in Figure 6.1. All antibodies underwent heat induced epitope retrieval (HIER). Antibodies were visualised using DAB (DCX and Hu) or nickel-DAB (MSI1, Nestin and PSA-nCAM).

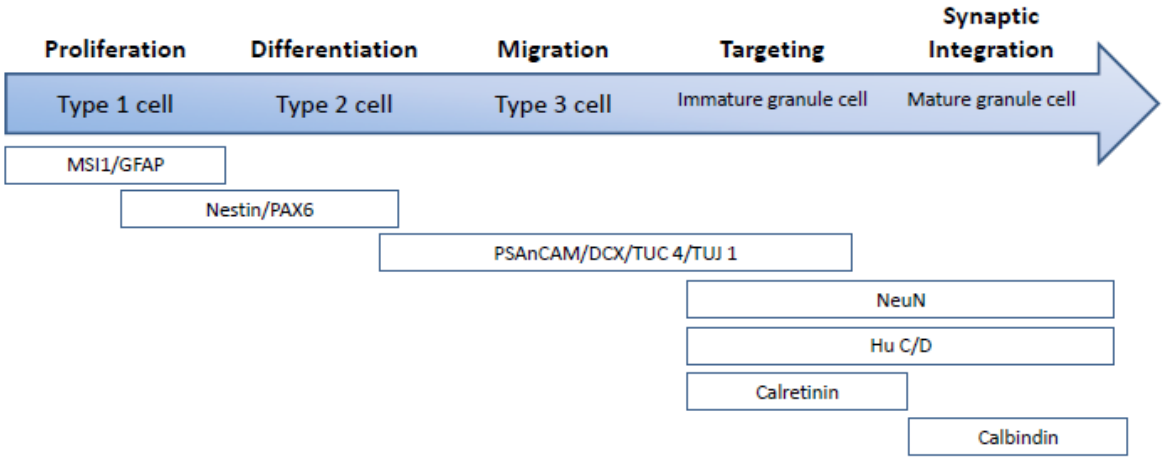


Figure 6.1 Neurogenesis markers expressed in relation to different stages of neurogenesis process. Adapted from (von Bohlen Und Halbach, 2007)

6.2.3 Image capture, analysis and statistical analysis

Images were acquired at x10 magnification for MSI1 and Hu C/D with 5 images of the DG being taken from each case. An x20 magnification was used for Nestin, PSA-nCAM and DCX, with 10 images of the DG taken. Image analysis was performed measuring both

staining quantity (% per area) and intensity (integrated optical density) in an AOI, SGL and GCL respectively.

The Shapiro-Wilk test was used to analyse normality of data. Staining quantity datum was parametric for Nestin in both regions, all other data was non parametric. Staining intensity data was parametric in the SGL for Nestin, DCX and Hu C/D and in the GCL for PSAnCAM and DCX, all other data was non parametric. Parametric data means were compared using ANOVA and Tukey post-hoc analysis was then performed. Non parametric data was analysed using the Kruskal-Wallis test and significance between groups was assessed using the Mann-Whitney U test.

6.3. Results

MSI1 staining was found in a number of granule cells in the SGL and to a lesser extent in the GCL, cell bodies were stained but no axonal extensions were observed as is reported in type 1 cells (Figure 6.2A). Nestin immunoreactivity is located in the cytoplasm of cells in the SGL and GCL, however there appears to be a high amount of possible no specific staining in the DG as most cells express some degree of staining. Staining was also seen in neuronal cells in the CA4 region (Figure 6.2B). PSAnCAM labelled granule cells both in the SGL and GCL, to a similar equal amount in each region. Staining appears to be granular in the cytoplasm (Figure 6. 2C). DCX staining was cytoplasmic in the granule cells and was often found in both SGL and GCL regions; although staining was generally weaker than the other markers (Figure 6.2D). Hu C/D is expressed in a high number of granule cells, with varying expression within the cytoplasm, a limited number of cells strongly expressed Hu. There were significantly more strongly expressing cells located in the GCL layer compared to the SGL (Figure 6.2E). No correlation was found with age, post mortem delay or fixation time to give any reason why processing would explain variation in staining quantity or intensity.

6.3.1 Staining quantity analysis

Percentage per area staining for MSI1 in both the SGL and GCL regions showed no significant differences between the groups (Kruskal Wallace test, p=0.463 and p=0.274, respectively). However, the Mann-Witney U test showed a significant decrease in the AD group compared to the two PS groups, PSND and PSD (p=0.039 and p=0.048 respectively) in the GCL (Figure 6.3).

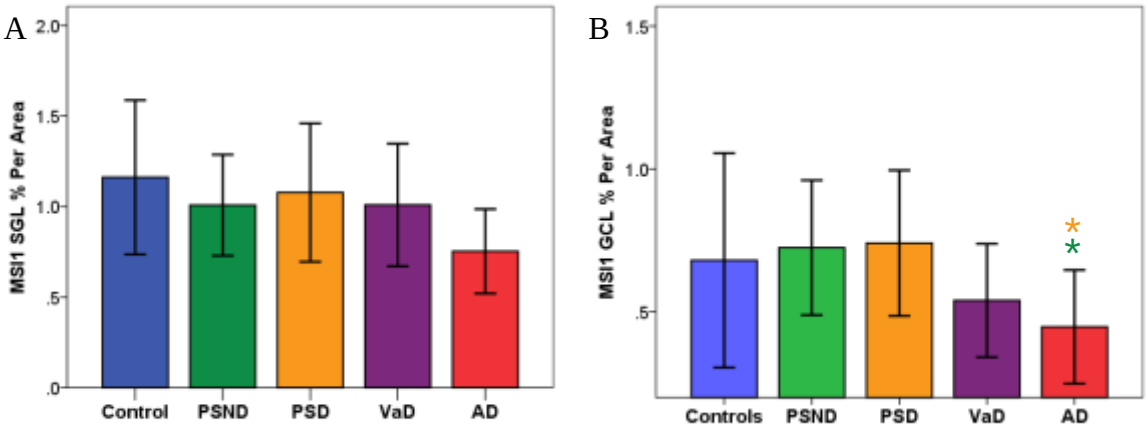


Figure 6.3 MSI1 staining quantity for SGL (A) and GCL (B).

There was no significant difference between any of the groups when stained for nestin (ANOVA, $p \geq 0.05$). Nevertheless, in both regions, AD cases expressed the highest immunostaining with a mean value 60% greater than controls and a general trend of increasing nestin staining within all the demented groups compared to controls in both regions. Statistical significance was not reached, due to the high degree of staining variation within the groups (Figure 6.4).

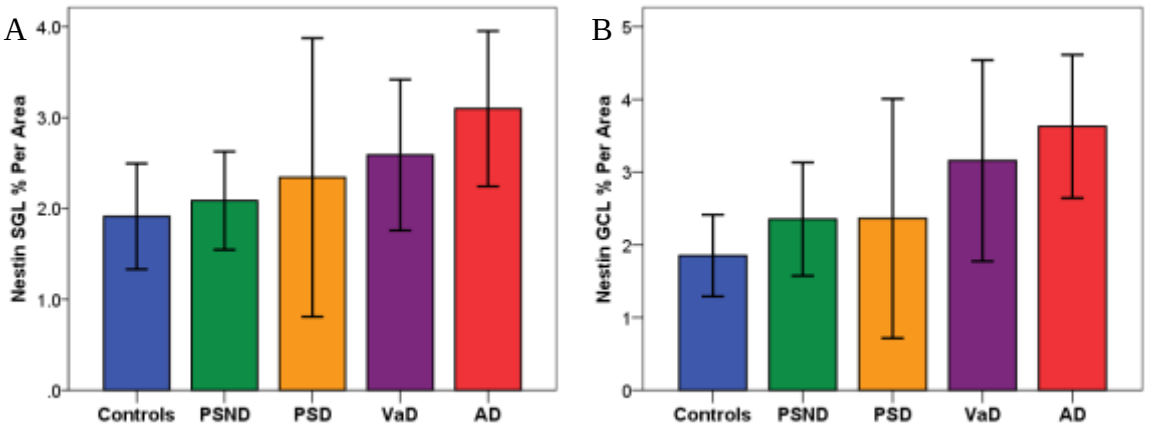


Figure 6.4 Nestin staining quantity in SGL (A) and GCL (B)

A decrease in staining quantity of PSAnCAM was observed in the SGL in PSD and AD compared to controls; p=0.005 and p=0.028 respectively. Additionally, significant increases of the VaD group compared to PSD (p=0.014) and AD (p=0.025) were also shown. There was a trend towards a significant increase in staining quantity of the PSND group compared to PSD (p=0.062) (Figure 6.5 A). Moreover in the GCL, significance was found between groups (Kruskal Wallis test, p=0.048). VaD were significantly increased compared to both PSND and AD expression (p=0.028 and p=0.018), whilst a trend towards a significant decrease was observed in PSD and AD compared to controls (p=0.064 and p= 0.070 Figure 6.5 B).

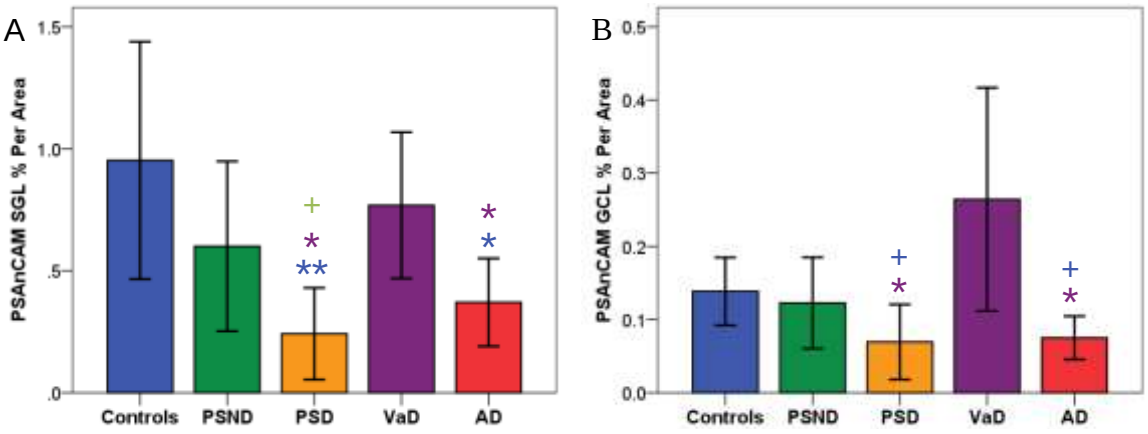


Figure 6.5 PSAnCAM staining quantity in SGL (A) and GCL (B)

DCX data indicated there were no significant differences, in both the SGL and the GCL (p=0.559 and p=0.347 respectively). However, a significant increase was observed when VaD cases were compared to PSND in GCL (p=0.048). A further lack of significant difference is caused by the large variance within each group with a number of cases having a high staining quantity whilst others cases in the same group had showed almost no staining at all (Figure 6.6).

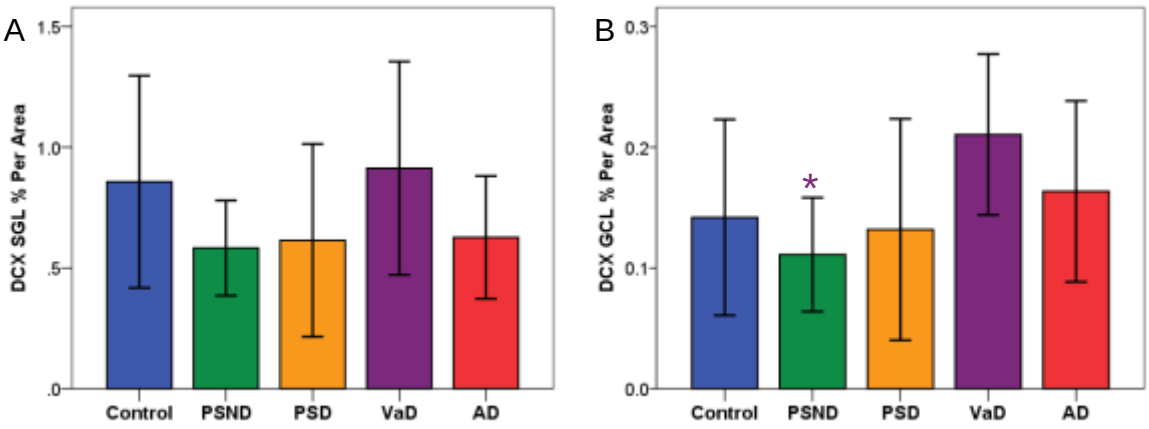


Figure 6.6 DCX Staining quantity in SGL (A) and GCL (B)

There were no significant differences between the groups with Hu C/D quantity in the SGL (Kruskal-Wallis test, p=0.097), although a significance decrease in VaD cases was observed compared to PSND (p=0.012) (Figure 6.7 A). There were significant reductions in GCL in PSD (p=0.05), VaD (p=0.017) and a trend towards reduced staining quantity in AD (p=0.057) compared to PSND (Figure 6.7 B).

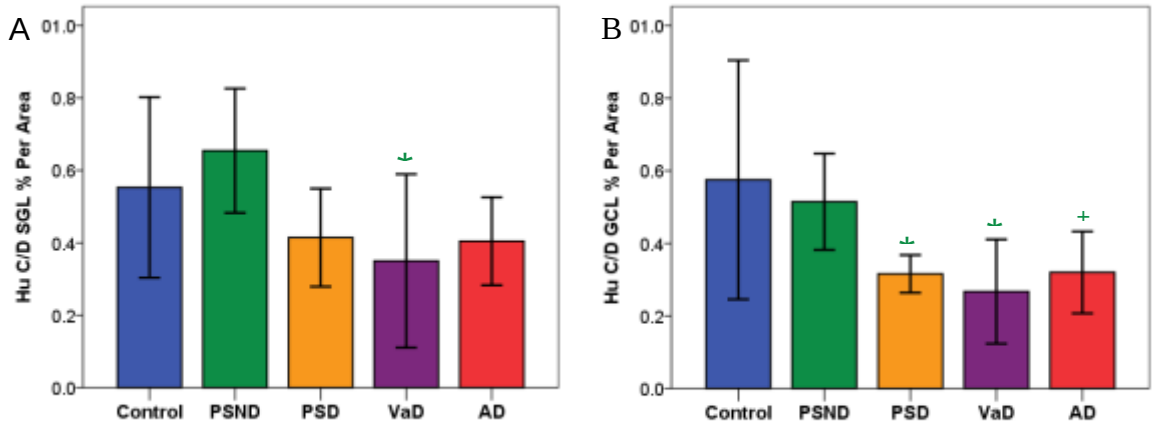


Figure 6.7 Staining quantity of Hu C/D in the SGL (A) and GCL (B)

To compare how the expression of markers differed between across the neurogenesis mechanism in the groups and regions, the levels of expression were normalised to controls (Figure 6.8). The analysis showed that in the SGL most of the markers have a reduced quantity compared to the controls, except for Nestin. PSD and AD groups showed that there is a large decrease in expression between Nestin and PSA-nCAM compared to controls. Furthermore, PSND is the only group that shows an increase in expression between DCX and Hu C/D staining. In the GCL layer, VaD, across all the markers except MSI1 and Hu C/D, has an increased quantity compared to controls and the majority of other groups, and is distinctly different from SGL expression. In both regions PSND and AD follow a similar pattern i.e. same increases and decreases across markers compared to other conditions; however AD shows the greatest difference from mean control value. PSND does not differ drastically from the mean control value.

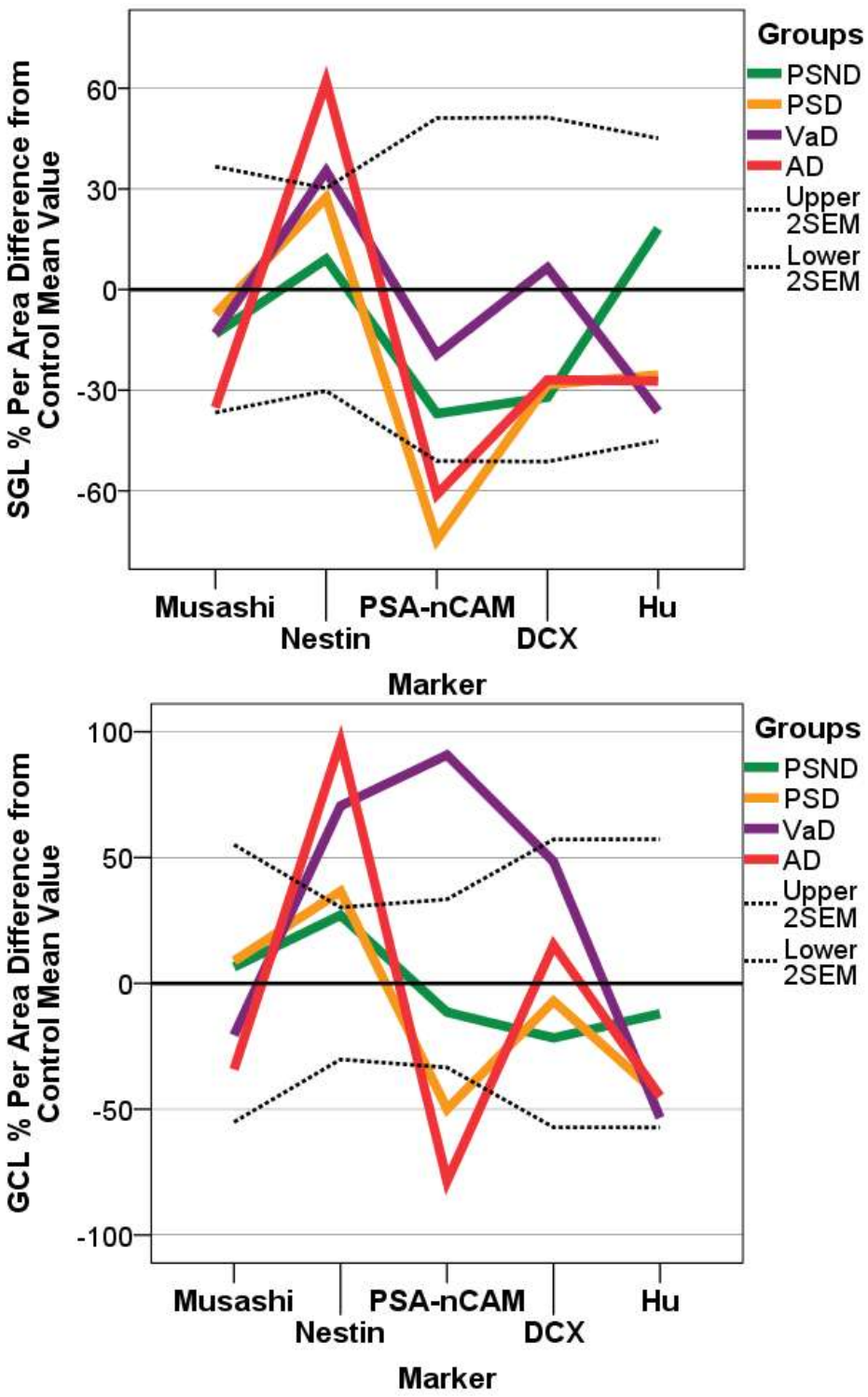
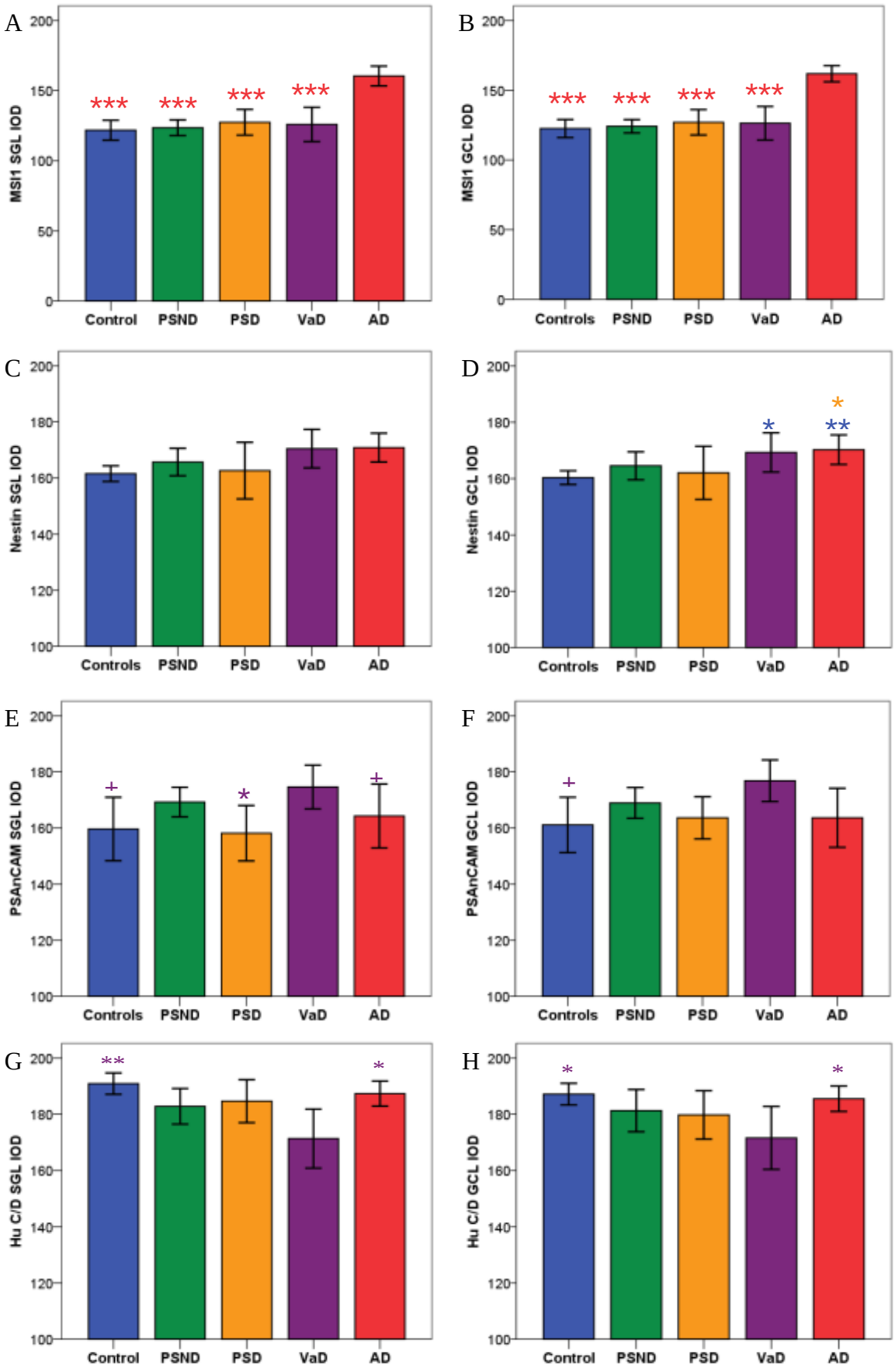


Figure 6.8 Staining intensity of each marker as an expression in comparison to the mean control staining quantity. Dotted lines indicate the upper and lower 2SEM for the control group in each marker.

6.3.2 Staining Intensity

Both SGL and GCL MSI1 staining intensity was significantly different between groups for both regions (Kruskal-Wallis $p \leq 0.001$), significant increases were shown in AD compared to all other groups (Mann-Whitney test, $p \leq 0.001$, Figure 6.9 A and B). There was no significant difference between groups for Nestin in the SGL (ANOVA and Tukey post hoc test). In the GCL, there was significant increases observed compared to controls for VaD and AD ($p=0.044$ and $p=0.003$ respectively). There was also a significant increase in AD compared to PSD ($p=0.041$, Figure 6.9 C and D). PSAnCAM showed a significant increase in intensity in VaD compared to PSD ($p=0.018$) and a trend toward significant increase compared to controls ($p=0.056$) and AD ($p=0.075$). There was a trend towards a significant increase in VaD compared to controls in the GCL ($p=0.058$, Figure 6.9 E and F). There was no significant difference in DCX intensity in either region (data not shown). There were significant differences in staining intensity of Hu C/D in SGL between groups (ANOVA $p=0.004$), the VaD group was significantly reduced; controls ($p=0.002$) and AD ($p=0.02$). Concurrently, the VaD group was also significantly reduced in GCL compared to the same groups ($p=0.027$, $p=0.031$ respectively, Figure 6.9 G and H).

Figure 6.9 Difference in staining intensity between groups for all markers in both the SGL and GCL. MSI1 (A and B), Nestin (C and D), PSAnCAM (E and F) and Hu C/D (G and H). *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ and + $p \leq 0.075$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6.3.3 Lifestyle factors affecting neurogenesis

Specific lifestyle factors have previously been suggested to have an involvement in the regulation of neurogenesis (Kempermann et al., 1997, van Praag et al., 1999, Boldrini et al., 2009). Due to the prospective nature of the CogFAST study, further analysis was possible to be conducted in these groups in an attempt to explain any variabity in results in neurogenic markers. Factors investigated included psychometric testing scores for diagnosis of dementia including MMSE and CAMCOG, time from stroke to death, number of cardiovascular risk factors, history of smoking, alcohol intake, pharmacological treatment for cardiovascular and CNS conditions and APOE epsilon type.

For most neurogenic markers, two consistent findings were observed throughout. There were significant increases in per area staining in individuals who had been prescribed antidepressant treatment or who had a history of smoking. Approximately 20% of the CogFAST cases investigated (n=5) were on antidepressant treatment, therefore numbers maybe a limiting factor in this analysis. Furthermore, we do not have any additional information on the specific antidepressants taken by each case. However, the data suggested possible trends or considerations that need to be taken into consideration in future analysis of neurogenic markers and supports previous findings in both animal and man studies (Malberg et al., 2000, Boldrini et al., 2009).

With regard to MSI1, a high trend towards a significant increase was observed in the GCL with cases which had been on antidepressant therapy ($p=0.053$). However, no significance was found in the SGL ($p=0.152$). In cases where the patient was an ex-smoker and had stopped within the last 6 months, there was a significant increase in SGL expression ($p=0.041$), but no significance within the GCL. Significant trends toward an increase in staining intensity were found with antidepressant treatment in both SGL and GCL ($p=0.061$

and p=0.053). Additionally, in cases of current smokers a trend toward significant increase was observed in SGL for intensity (p=0.054).

PSAnCAM staining quantity also showed increases with antidepressant treatment and smoking. A trend towards significant increase was observed in patients on antidepressant treatment in GCL (p=0.079). Whilst individuals who had previously smoked showed a significant increase in both SGL (p=0.013) and GCL (p=0.043). Ex-smokers who had stopped in the last 6 months showed significant increases in SGL (p=0.011). Staining intensity was significantly increased in the SGL with antidepressant treated cases (p=0.044), ex-smokers in the last six months (p=0.009) and previous smokers (p=0.013).

Similar findings are found in DCX analysis with significant increases in % PA with antidepressant treatment in SGL (p=0.039) and a trend towards significance in GCL (p=0.07). Ex-smokers who had quit within last 6 months showed a significant increase in DCX % PA in both regions; SGL (p=0.003), GCL (p=0.049). No significant change was seen in intensity relating to antidepressant treatment and smoking.

Significant increases were found in staining quantity for Hu C/D in cases with antidepressant therapy in SGL (p=0.012) and GCL (p=0.000). Whilst ex-smokers had a significantly increased quantity as opposed to non-smokers in the GCL (p=0.008) and a trend towards significance in the SGL (p=0.073). Smoking was the only group found to have a significant effect on staining intensity, with significant increases in ex-smokers in both SGL and GCL (p=0.007 and 0.009 respectively). Previous smokers who had quit more than six months prior to the start of the study also showed an increase in GCL (p=0.036).

Nestin was the only marker that did not show significant difference for antidepressant treatment or with previous smokers. However, there was a significantly decrease in quantity

of immunoreactivity in cases who had been prescribed antiplatelet treatment, in the GCL and a trend toward similar significance in SGL (p=0.035 and p=0.065) and a significant decrease in intensity in the same cases in GCL (p=0.035) and a trend toward significant decrease in the SGL (p=0.056). Additionally, trends towards significance increases in the SGL area of cases taking anticoagulant treatment (p=0.068) and also in the GCL in cases prescribed hypnotic treatment (p=0.052).

Other factors that were found to have an effect included a trend toward decrease in PSAnCAM intensity in hypertensive cases. A significant decrease was found in DCX in the GCL in cases who had hypertension (p=0.02) and for DCX intensity, there was a significant decrease in cases who were prescribed treatment for lipid lowering in both SGL and GCL (p=0.037 and 0.024). Whilst, there was a significant increase in intensity in cases on anticoagulant or anticonvulsant treatment (p=0.027 and 0.008 respectively). Furthermore, a significant increase in Hu C/D was found in cases who were prescribed anticonvulsant treatment, SGL (p=0.055), GCL (p=0.005).

There was no other significant difference or correlation with other factors from the data with these markers inclusive of MMSE and CAMCOG scores antemortem or the change in score from baseline.

6.4. Discussion

This work constituted the first attempt to demonstrate whether PSD subjects could be distinguished from the PSND group with respect to neural progenitor cell markers. Only the Hu C/D expression was significantly greater in PSND subjects compared to PSD, which might suggest a possible increase in the number of progenitor cells converting to mature

granule cells. A trend toward a significant increase ($p \leq 0.075$) was also recorded in PSAnCAM in PSND compared to PSD.

Results involving other groups showed significant decreases in immunostaining in the SGL of PSAnCAM cells in both PSD and AD subjects compared to controls and VaD. Additionally, a significant decrease of Hu C/D positive cells were observed for VaD compared to PSND in SGL. More significant increases of staining quantity were observed in GCL layer; MSI1 in PSND and PSD cases compared to AD, PSAnCAM in VaD group compared to PSD and AD. DCX in VaD cases compared to PSND and finally Hu C/D in PSND compared to PSD and VaD. Trends towards significant decreases were also observed in PSAnCAM for PSD and AD compared to controls and with Hu C/D in AD compared to PSND.

Furthermore, immunoreactivity for all the markers was demonstrated as a percentage difference from controls for both SGL and GCL (Figure 6.8 A and B). This allowed trends across the neurogenesis mechanism for the different groups to be visualised. This data suggests altered neurogenic expression occurs for different markers in distinctive conditions. PSND for all markers shows little alteration in parallel to the control group. PSD group emulates a similar yet lesser arrangement to AD in both SGL and GCL, which may suggest a similar mechanism. Overall, especially in the GCL, VaD cases show an increase in staining quantity (nestin, PSAnCAM and DCX) compared to other groups, which is in keeping with previous literature (Ekonomou et al., 2011, Ekonomou et al., 2012) however expression promptly decreases between DCX to Hu C/D. Although, we believe that this is the first time Hu C/D has been measured in VaD cases and may suggest some specific mechanism within VaD cases as an effect of disease progression limiting Hu C/D expression (see chapter 7).

Staining intensity was also measured; significant differences were seen including MSI1 significantly increased in AD compared to all groups in both SGL and GCL regions. Nestin expression was significantly increased in GCL for both VaD and AD cases compared to controls; moreover AD was significantly increased compared to PSD. PSAnCAM showed a significant increase in VaD compared to PSD and trends towards significance with controls and AD in SGL. Furthermore, in the GCL a trend towards increase in PSAnCAM intensity was seen for VaD cases compared to controls. A significant reduction in intensity was observed in Hu C/D in VaD cases compared to controls and AD in both the SGL and GCL. However, there are often additional factors that can cause differences in immunoreactivity intensity and therefore this data will not be discussed further but was added to be in keeping with previous studies, which found significant increases in intensity in demtia groups (Ekonomou et al., 2011, Perry et al., 2012, Ekonomou et al., 2012).

Collectively, these data suggest that there is considerable variation between the groups with different markers and does suggests neurogenesis is occurring in these cases even though they are all aged above 75. The decrease in Hu C/D quantity in demented groups may suggest a higher number of progenitor cells are unable to convert into mature granular cells compared to non clinically demented cases and this difference may be linked to cognitive performance (Shors et al., 2001). Although, this cannot be proven without additional clinical data from VaD, AD groups and age matched controls. However, a technical limitation of this study is that as neurogenesis is evident but limited especially with increasing age. Mean scores are often very low for all groups, expecially DCX. Therefore, conclusions must be made with caution and the likelihood of finding significant differences are reduced and those that are found maybe caused by non specific background staining.

Further analysis of the PS cases only have shown that certain lifestyle and clinical factors can alter quantity of neurogenic markers irrelevant of the clinical condition of the subject. It was commonly found that both smoking and antidepressants significantly increased expression of all neurogenic markers analysed. Although this study was not designed to investigate these factorsfurther these issues need to be considered in any future neurogenesis analysis when selecting cases. It has been previously established that antidepressants may increase neurogenesis (Malberg et al., 2000, Santarelli et al., 2003, Boldrini et al., 2009). The mechanism of increasing neurogenesis, by taking serotonin reuptake inhibitors (SSRIs), is through to involve cell signalling with the 5-HT_{1A} receptor (Santarelli et al., 2003). Other antidepressant treatments have also been shown to increase neurogenic activity i.e. serotonin-noradrenaline reuptake inhibitors (Malberg et al., 2000). Although, the mechanism by which these drugs have an effect on neurogenesis has not yet been fully deduced. However, it is believed that an increase in brain derived neurotrophic factor (BDNF) may play an important role (Chadwick et al., 2011). Unfortunately, details were not recorded for each individual case's antidepressant treatment for the CogFAST study.

Simultaneously, nicotine has been reported to improve learning and memory (Whitehouse and Kalaria, 1995). A randomised blinded test using nicotine patches showed an increase in reaction times, attention time and long term memory (Newhouse et al., 2012).However, other studies have shown that the direct injection of nicotine into rodent hippocampus is toxic and directly reduced the process of neurogenesis in a dose dependent manner (Shingo and Kito, 2005). Although a number of other chemicals from cigarette smoking could also be responsible for alterations in hippocampal expression or if the subjects were on nicotine replacement therapy, as a number of them having recently quit smoking. Our current study was unable to establish if other lifestyle factors, such as physical exercise, cognitive

stimulation or level of education would have effect on neurogenesis, as this information was not recorded as part of the initial study.

There is an increase in immunoreactivity of PSAnCAM and DCX in VaD cases, but a significant loss of expression in Hu C/D. This may suggests a potential initial upsurge in the neurogenesis process for groups clinically diagnosed with dementia; however the process maybe arrested before reaching completion. Possibly due to dysregulation of a cell signalling process, such as NOTCH which has previously been implicated in the neurogenesis process (Ables et al., 2011, Breunig et al., 2007, Imayoshi and Kageyama, 2011). Alternatively, a decrease in cell survival in these groups could be due to disruption of integration of new cells as a result of endogenous and exogenous factors (Danzer, 2008).

Previous studies have shown varied alterations in neurogenic expression in both animal models and human post mortem tissue from studies of dementia. It has been proven that ischemia triggers increased neurogenesis, using a number of the same markers described here, especially in the SVZ compared to the SGL (Liu et al., 1998, Darsalia et al., 2005, Jin et al., 2006). Neurogenesis as a response to ischemia requires a complicated mix of transcription factors including NF-κB, CREB, Sox2 and Hif-1α (Zhang et al., 2011). There are no previous studies that have analysed a time course to identify how long the increase in neurogenesis lasts post ischemic incident. The cases in the CogFAST study had on average life expectancy of 60 months PS, therefore speculation regarding initial neurogenesis in response to ischemia is not covered by this analysis. This study is more dependent on the long term and possibly microenvironment which may lead to an increase in neurogenesis and if it responsible for differences in clinical outcome.

There are limitations to this study, it has been well documented that neurogenesis is significantly reduced with increasing age (Shetty et al., 2011, Wang et al., 2011), although there is evidence of neurogenesis in human subjects over the age of 70 (Eriksson et al., 1998). No significant correlation was found with the age of the cohort compared to immunoreactivity in this study. The significance of age needs to be fully understood in neurogenesis especially in human studies and as described before, some markers showed very little immunoreactivity. Additionally, neurogenesis occurs in two specific niches, the SGL and the SVZ and it has been suggested that increased neurogenesis may occur in the SVZ compared to SGL post ischemia (Darsalia et al., 2005). Whilst these two niches appear to have different and varied roles with neuronal progenitor cells, it would be of great interest, to be able to investigate differences in the SVZ in these cases. The SVZ is an area that has previously been shown to be active in human for neurogenesis PS and in VaD cases (Marti-Fabregas et al., 2010, Ekonomou et al., 2011).

It was not possible to analyse more neurogenic markers in view of time constraints. However a marker was selected for every stage of the neurogenesis process in this study, from proliferation to conversion into mature granular cells. Other markers may have included SOX-2, a transcription factor than can be used as a marker of differentiating cells (Steiner et al., 2006), TUC-4, a marker for axonal growth (Jin et al., 2004b) and additional post mitotic markers calretenin (Llorens-Martin et al., 2006) and calbindin, found in all mature granule cells (Baimbridge, 1992).

However, fundamentally all these markers are endogenous protein markers and act as surrogate markers for neurogenesis. They have numerous roles and are expressed differently in various cell types but have been confirmed in other studies for presence with BrdU. DCX has been shown to be quite accurate with 90% co-labelling with BrdU (Rao and Shetty,

2004). Ki-67, which is expressed in the nucleus of all cells within the cell cycle, has been previously used as a marker for dividing cells (Kee et al., 2002). Although there are limitations, these are still the best techniques we currently have available to study neurogenesis in human subjects. A common marker for neurogenesis used in animal models, is the nucleotide analogue, BrdU, which labels dividing cells at the time when injected and from that point on every daughter cell of those cells incorporates some of the marker. However, due to ethical and moral concerns regarding BrdU and possible carcinogenic effects, its use in humans has mainly been prohibited (Cooper-Kuhn et al., 2002). Although,it was used in the pioneering study to establish adult human neurogenesis (Eriksson et al., 1998). There are also limitations with BrdU, including the normal use of fluorescence to allow for double labelling, the presence of autofluorescence from lipofuschin and RBCs can mask the tag's expression on progenitor cells. BrdU may also identify cell death and DNA repair mechanisms (Cooper-Kuhn et al., 2002)

In conclusion, in this study we found little difference in neurogenic expression between PSND and PSD cases. Significant increases were observed with the neuronal marker Hu C/D and a trend toward a significant increase in PSAnCAM in PSND compared to PSD. Increases were observed in markers of proliferation, differentiation and migration for VaD in comparision to other groups. While there seems to be a significant loss in both PSD and AD compared to both VaD and age matched controls for these markers. Furthermore, there is an increase in Hu C/D in PSND cases compared to all dementia groups. Additionally significant increases in expression were associated with antidepressants and a history of smoking, which may have affected the overall outcome. However, technical limitations, such as low immunoreactivity across the DG means conclusions have to be made with caution and further studies are required.

Chapter 7 Hippocampal expression of the RNA binding protein Hu C/D

7.1 Introduction

Following on from analysis using Hu as a marker for the development of mature granule cells in neurogenesis (Chapter 6), we noticed that there was variation of immunoreactivity of Hu C/D across the entire hippocampus. Particularly noticeable was the strong immunoreactivity of pyramidal neurons in the CA2 regions of all sections and the loss of similar expression specifically within the CA1 of most cases. Furthermore, it was noted that in the CA1 region there was a graded difference in staining between neurons, some stained very strongly whilst others had no staining at all.

Hu proteins are a family of RNA binding proteins (RBP) that are homologous with the embryonic lethal abnormal vision (ELAV) protein found in *Drosophila* and are an important protein for neurological development. Hu is one of the earliest expressed proteins in mature neurons (Marusich et al., 1994) and has been used as marker for neurogenesis to identify the development of new mature granule cells (Wakamatsu and Weston, 1997, Magavi and Macklis, 2002, Jin et al., 2004b). Over expression of Hu in undifferentiated PC12 cells was shown to initiate differentiation even in the absence of nerve growth factor (Akamatsu et al., 1999, Kasashima et al., 1999). There are four isoforms of Hu, A to D. Whilst, B, C and D are primarily expressed in neurons, isoform A (also referred to as HuR) is ubiquitously expressed in all tissues. These proteins are encoded by different genes and are present in the cell as multiple splice variants (Perrone-Bizzozero and Bird, 2013).

As well as being involved in development, Hu proteins exist in mature neurons. Experiments have shown that HuD levels are increased in the hippocampus after learning and memory

tasks in mice (Quattrone et al., 2001, Pascale et al., 2004). Interference of Hu inhibits performance of mice in the radial arm maze experiment, which tests spatial learning and memory (Bolognani et al., 2007). Recent analysis of some of the mRNA targets of Hu, have been shown to be involved in the process of LTP (Bolognani et al., 2010). One of Hu's most studied target mRNAs is that of growth associated protein (GAP) -43. Analysis has shown that there is an increase of both Hu and GAP-43 in response to peripheral nerve damage, where they co-localise in cytoplasmic granules in axons and growth cones during synaptogenesis (Anderson et al., 2003, Smith et al., 2004). It has been suggested that Hu plays a key role in neuronal development, plasticity and regeneration.

Recent immunoprecipitation and microarray studies have suggested that Hu may have more than 600 mRNA targets in the mouse brain (Bolognani et al., 2010). Some of the most studied targets include:

Target	**Reference**
c-fos	Chung, 1996 (Chung et al., 1996)
VEGF	(King, 2000)
Hif-1α	(Masuda et al., 2009)
Tau	(Aranda-Abreu et al., 1999)
Glycogen Synthase Kinase (GSK)-3β	(Tiruchinapalli et al., 2008)
AChE	(Deschenes-Furry et al., 2003)
Growth associated protein (GAP) 43	(Anderson et al., 2003)

Table 7.1 mRNA targets of Hu and references of studies

A role for Hu has recently been demonstrated in the progression of AD, where it is found to be significantly decreased in the hippocampus of human AD cases (Amadio et al., 2009). One of Hu's mRNA targets is the α-secretase, ADAM10, which is also down regulated in AD. When APP is cleaved by an α-secretase within the Aβ domain of APP, non-pathogenic

fragments of APP are produced (Buoso et al., 2010). It has been suggested that Hu may regulate ADAM10 by post translational regulation (Colciaghi et al., 2002, Amadio et al., 2009). Whilst a number of other proteins involved in AD pathogenesis e.g. β-site APP cleaving enzyme (BACE1) are increased, but have also been reported to be a target of Hu (Perrone-Bizzozero and Bird, 2013).

In this chapter, Hu expression in the human hippocampus was initially investigated using density analysis performed by Image Pro software and then by stereological neuronal counts. As mentioned previously with the variation of immunoreactivity across the hippocampus and due to its potential role in neuronal plasticity and maintenance it is hypothesised that increases in immunoreactivity could identify cells or individual cases with greater neuronal maintenance. Therefore this marker may reveal pyramidal neurons which are undamaged by ischemic injury and pathology or could suggest loss of functional circuitry in the hippocampus. Furthermore, individual isoforms of Hu, C and D, were investigated by both brightfield and fluorescent microscopy to determine if there was regional variation in these isoforms.

7.2 Methodology

7.2.1 Subject Cohort

For analysis of staining density and intensity performed by the previously described methodology using Image Pro software (Mediacybernetics, USA), sections previously used for Hu C/D analyses of the DG were reused here. For demographic information please refer to Chapter 6. However, additional stereological analysis were performed on a separate cohort, see table 7.2 for details.

	Control	PSND	PSD	VaD	AD
Total number of controls or cases analysed	7	8	8	7	8
Age, y Mean (range)	78.0 (72-91)	83.4 (81-87)	87.1 (80-98)	85.4 (76-90)	84.3 (70-91)
PMD, h Mean (± 2SEM)	34.0 (16.7)	49.0 (21.26)	43.6 (19.5)	37.0 (10.9)	60 (24)

Table 7.2 Demographics of groups including sample number, age and post mortem delay. The time period (weeks) of tissue fixation was in the range of 4-40 weeks for all cases.

7.2.2 Hu C/D Immunohistochemistry

IHC was performed as described previously in chapter 6 for Hu C/D on 10-μm-thick sections and the same methodology was followed for 30-μm-thick sections for stereological analysis, using DAB as the chromogen. For stereological analysis fifteen serial sections were cut from a block of hippocampal tissue and every fifth section available was selected so that three were analysed per case as previously described in chapter 4. Similar, IHC methodology was used for single Hu isoform– HuD (Millipore, UK, 1:400, anti-rabbit, 4˚C overnight) and HuC (Bioss, USA, 1:1000, anti-rabbit, 4˚C overnight). Serial 10 μm-thick sections were used for analysis to compare staining differences

Immunofluorescence double label staining combining Hu C/D and either Hu D or Hu C was performed on 6 μm-thick sections. Standard double labelling IHC was used. Single Hu isoforms (1:100) were labelled with anti-rabbit IgG Dylight 549 (red) and Hu C/D (1:375) was labelled with anti-mouse IgG Dylight488 (green). Slide mounted sections were incubated in 0.5% Sudan black for 30 seconds to reduce auto fluorescence and were then mounted with coverslips using Vectashield (Vector Labs, UK).

7.2.3 Image capture, % per area immunoreactivity and intensity analysis

Sections were viewed with a Zeiss AX10 brightfield microscope at 5x magnification. Images were captured with a Lumenera Infinity 2 camera in all areas of the hippocampus (CA1-4 fields and subiculum). Regions were detectable by the morphometric differences of subfields created by neurons in CA fields and subiculum, which were visible due to haematoxylin counterstain. The images were analysed using Image Pro–Plus 4 software to assess staining density (Mediacybernetics, USA).

7.2.4 Stereological analysis

Stereological analysis was used to provide additional data with relation to the differing expression of Hu C/D in CA1 and CA2. Using a Zeiss Axiolab microscope with a motorised stage (Prior Scientific, UK) connected to a computer with Stereologer 2000 software installed (Stereologer, WV, USA), live images of the desired reference space were visualised on the screen via a JVC camera.

The reference space was identified using 5x magnification with CA1 and CA2 being easily distinguishable due to the differentiation in pyramidal layer structure and the significant variation in Hu C/D staining. The software produces a uniformly spaced grid within the desired reference area, as previously described, approximately fifty frames were targeted for accurate analysis of the CA1 and thirty frames for CA2. The optical dissector method was used to determine neuronal density (Sterio, 1984); each dissector box had a depth of 18 μm to allow for guard volume and shrinkage of tissue during processing. A number of dissector probes were selected to allow for the quantification of several different parameters from the same image including; all Hu positive stained neurons, strongly stained neurons only, weakly stained neurons only and absent Hu staining. A pilot study was performed to confirm that the mean coefficient of error was acceptable (Gundersen and Jensen, 1987). As with length,

number had to be expressed as density due to the fact the total structure was unable to be analysed.

7.2.5 Confocal microscopy

Fluorescent slides with either antibody raised against Hu C or Hu D and double labelled with the antibody with Hu C/D were imaged with confocal microscopy, which was provided by the Bio-imaging Suite at Newcastle University. Further details relating to confocal microscopy can be found in chapter 2.

7.3 Results

A gross examination of the sections showed there was varied immunoreactivity of neuronal cells across the hippocampus in all cases. The general trend observed was that of a marked loss of Hu immunoreactivity in the pyramidal cells of the CA1 compared to the CA2 region. However, this decrease appeared to be more evident in VaD cases. Reduced of Hu C/D staining does not appear to be caused by neuronal cell loss, as haematoxylin identified a number of pyramidal neuronal cells present without Hu C/D staining (Figure 7.1).

The antibody was very specific to neuronal cells, with strong immunoreactivity found within the CA subfields and cortex. The strongest immunoreactivity was observed in the CA2/CA3 region. In a number of incidences both nucleus and cytoplasm stained strongly. In all cases the soma was clearly identifiable and from several of the large pyramidal cells found in this region a proportion of the axon was visible. In the CA1 there is a clear loss of immunoreactivity in all cases compared to the CA2. However in some cases that loss is more significant than others (Figure 7.2).

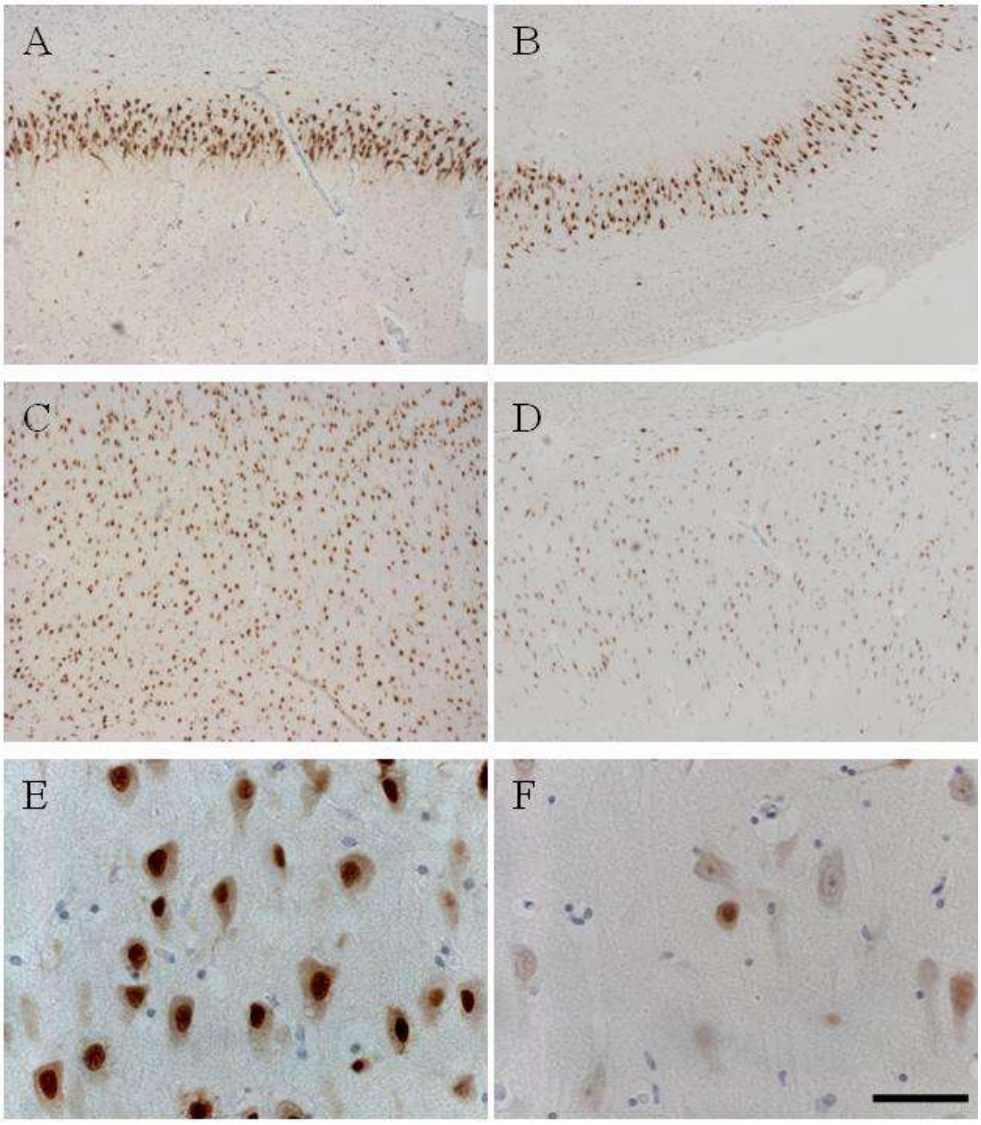

Figure 7.2 Images of Hu C/D immunoreactivity in CA1 and CA2 of a case with high (PSND) and low (VaD) immunoreactivity in CA1. Images A and B show staining in CA2, whilst C and D display staining in CA1. E and F show staining in the same CA1 region as the corresponding image above at higher magnification. Sections counterstained with haematoxylin. Mag bar = 400 μm in A-D and 50 μm in E and F

Hu C/D is expressed in either both the nucleus and cytoplasm of the neuron or just the cytoplasm. To ensure that this was not due to post mortem delay or inappropriate fixation, immunostaining of biopsy tissue sections from the neocortex was performed. It was verified that immunostaining for Hu C/D showed that large neuronal cells had staining in both the nucleus and cytoplasm. Additionally, the material was fixed for differing lengths of time; short fixation (3 months) and long fixation (19 months). Both samples showed significant neuronal Hu C/D staining (Figure 7.3).

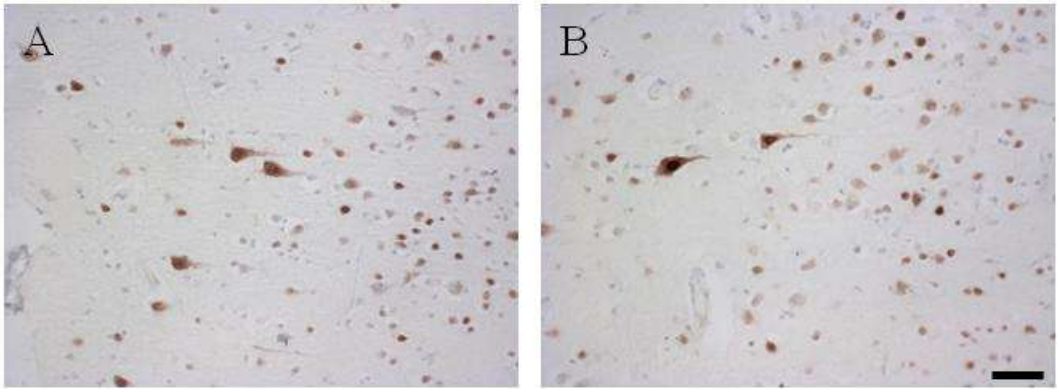

Figure 7.3 Hu C/D staining of cortex from the same material fixed for different durations. Image A shows tissue that was fixed for a long term (19 months) and B shows tissue fixed for short term (3 months). Mag bar = 50 μm

7.3.1 Hu C/D staining density by per area measurement

Significant differences in Hu C/D % per area were observed in the CA1 region between the groups (Kruskal-Wallis test p=0.010). PSND cases significantly differed from the demented groups; PSD (p=0.04), VaD (p=0.001) and AD (p=0.034). There were also differences in the CA4 region (ANOVA p=0.012), where VaD was decreased in % per area compared to PSND (p=0.007), PSD (p=0.028) and controls (p=0.030). Similarly, there were differences between the groups in CA3 (ANOVA p=0.020), where VaD % per area was significantly reduced compared to controls (p=0.043) and a trend toward significance for PSD (p=0.057) and PSND (p=0.075). Finally, in the subiculum, although significance was not found collectively between groups (Kruskal-Wallis p=0.176), the Mann-Whitney U test showed a

significant decrease in % per area in VaD compared to PSND (p=0.027). There were no significant differences apparent in the CA2 region (Figure 7.4 A-E).

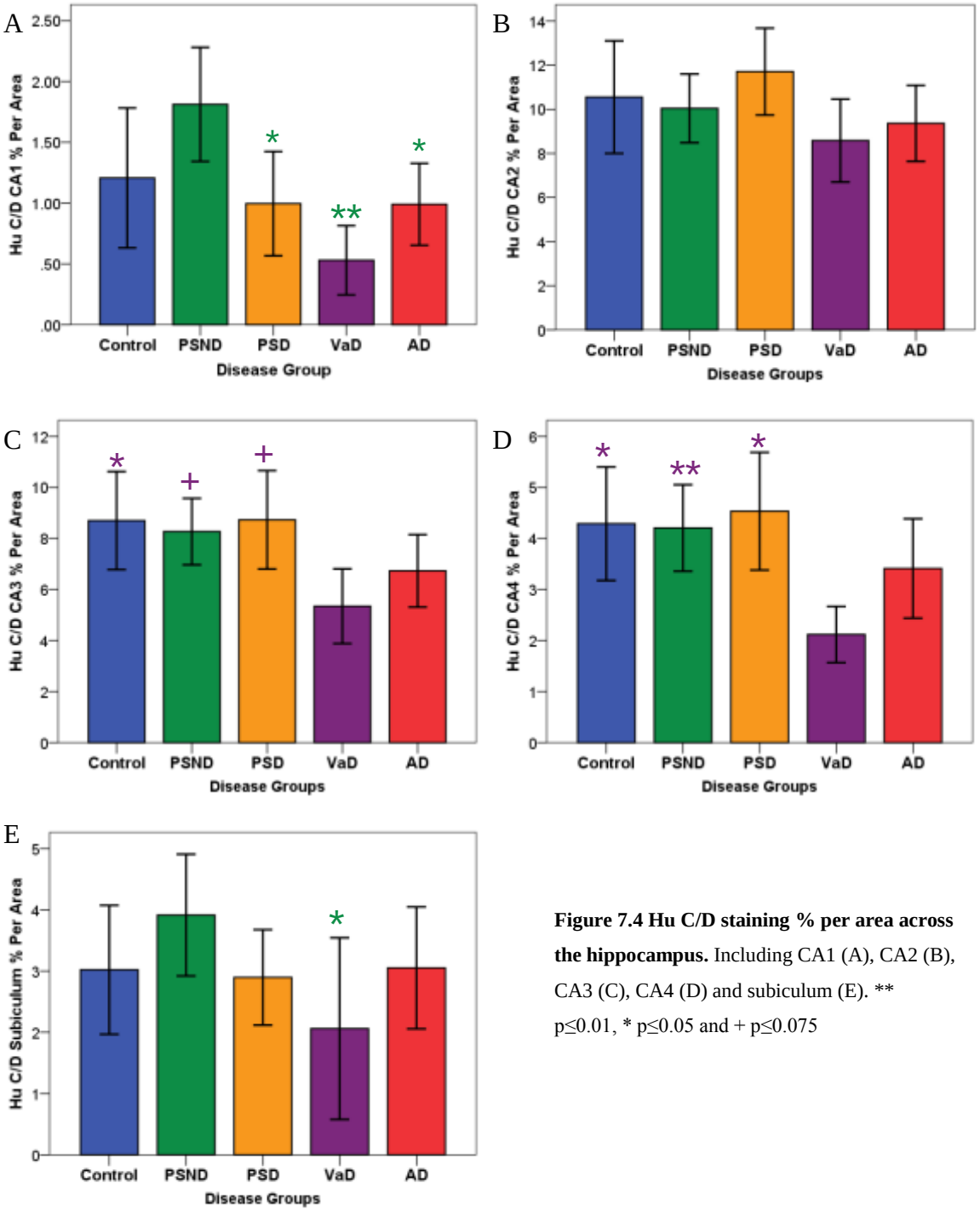

Figure 7.4 Hu C/D staining % per area across the hippocampus. Including CA1 (A), CA2 (B), CA3 (C), CA4 (D) and subiculum (E). ** p≤0.01, * p≤0.05 and + p≤0.075

To verify the % per area data changes in Hu C/D expression cell counts were included in the analysis. This probe counts all complete shapes selected by parameters for analysis. Shapes can be any size or orientation and therefore neuronal cells will be counted with all background staining. As Hu C/D appears to be a very specific marker for neuronal cells, this program will provide a reasonable indication of the number of neuronal cells stained with Hu C/D. To confirm these finding a correlation was drawn between the staining density data and the staining count. It was expected a strong correlation would be found between the two parameters and would show the number of positive stained cells increased with the % per area.

Significant correlations were found with Pearson's correlation in CA1 (ρ=0.932, p=0.000), CA4 (ρ=0.722, p=0.000) and subiculum (ρ=0.678, p=0.000). However, no significant correlation was found in either CA2 (ρ=0.196, p=0.144) or CA3 (ρ=0.129, p=0.343, Figure 7.5).

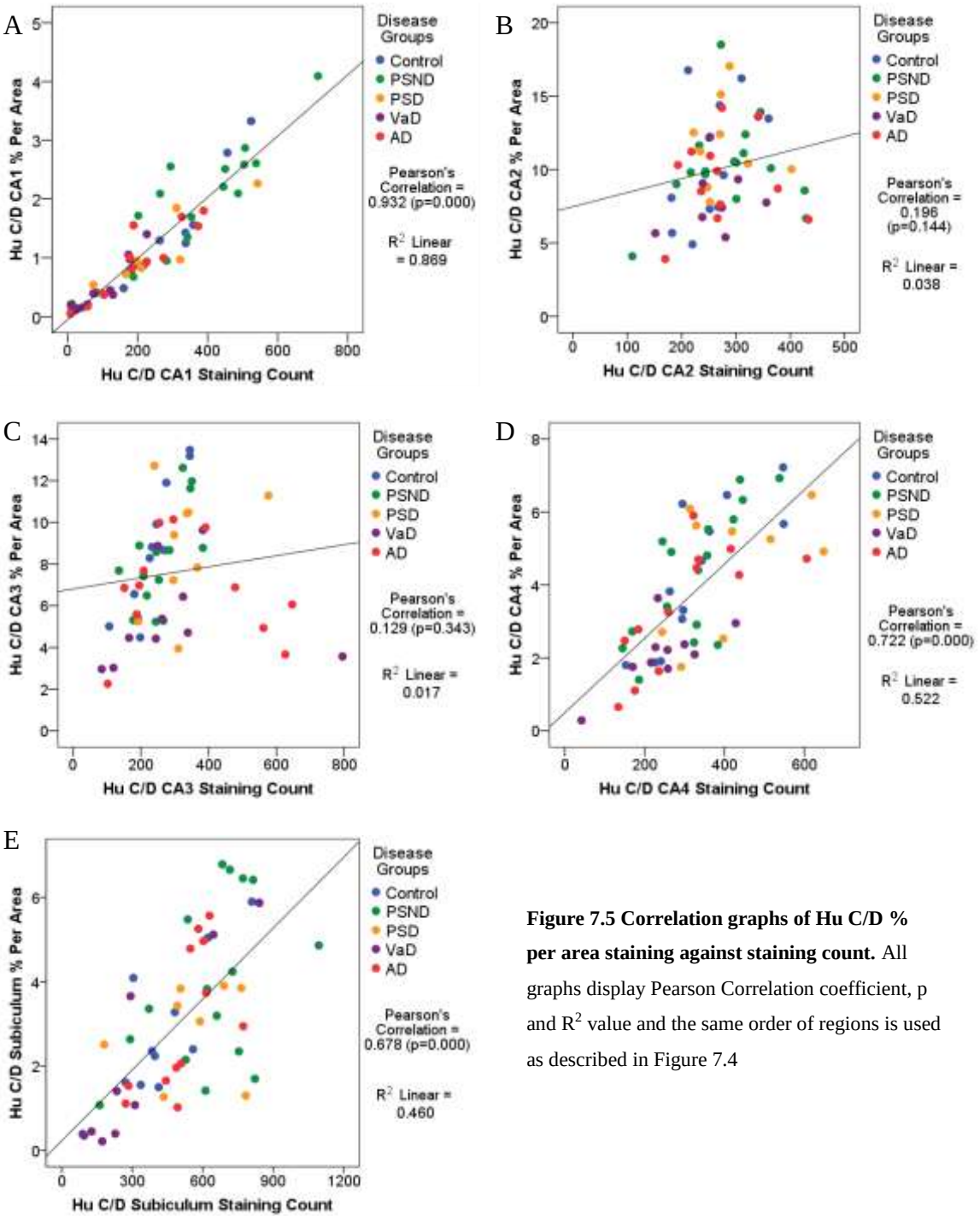

Figure 7.5 Correlation graphs of Hu C/D % per area staining against staining count. All graphs display Pearson Correlation coefficient, p and R^2 value and the same order of regions is used as described in Figure 7.4

Since, staining density appeared to be differentially distributed across the fields of the hippocampus the data were reanalysed to perform a two way ANOVA, looking for the interaction between groups and regions.

Analysis showed that there was significant differences in expression in groups when data was taken as a whole i.e. for all regions combined mean ($p \leq 0.01$, $F=11.6$). There was an even greater significance in regional variation ($p \leq 0.01$, $F=32.6$). This result confirmed that certain regions of the hippocampus expressed Hu C/D stronger than others (Figure 7.6). The graph shows that across all regions the VaD cases have reduced immunoreactivity compared to all other groups. Another interesting finding is that the PSD group has the highest % per area mean in CA2 region. Furthermore, all other groups follow a similar patern but we see that at all stages AD cases are less compared to controls, PSND and PSD.

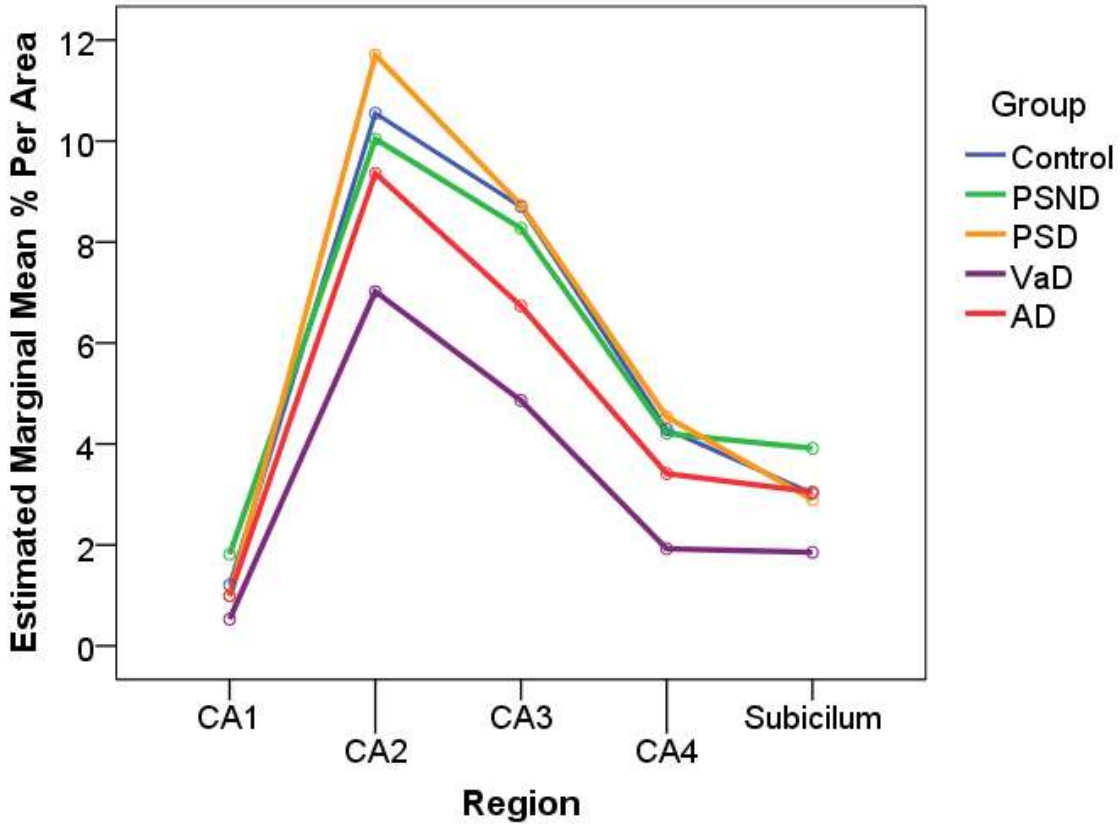

Figure 7.6 % Per Area Hu C/D estimated marginal mean for each group across all the regions of the hippocampus. Figure shows variation between each region, with the peak shown for CA2 and CA3 region. Figure shows that a similar profile is shown for all groups, but in all regions VaDshows the lowest mean of all groups. PSND has the highest mean in CA1 and a subicilum region, PSD has the highest mean in CA2 and both PSD and controls show the highest mean in CA3. AD sits between the cluster of controls, PSND and PSD which are usually higher mean results but is never as low as VaD

However, no significance was found for the interaction between the regions and groups (p=0.485, F=0.975). The analysis suggested that 66% of the difference was responsible due to the region while the different groups accounted for 14.5%. When individual pairwise comparisons were performed to look at the interaction a number of significant different factors were found and are displayed in table 7.3

CA1	None
CA2	VaD significant difference with all groups ($p \leq 0.01$, except AD p=0.016). AD significant with PSD (p=0.22).
CA3	VaD significant differences with all groups ($p \leq 0.01$) except AD (p=0.053). AD significant with controls (p=0.042) and trend toward significance with PSD (p=0.051)
CA4	VaD significant differences with all groups ($p \leq 0.01$) except AD
Subiclium	VaD significantly different from PSND (p=0.028)

Table 7.3 Significant results from pairwise comparisions of the interaction between Hu C/D immunoreactivity resulting from region and groups. These results show that the differences between results seen in Figure 7.6 are not equal for all results, therefore suggesting at specific points the difference of Hu C/D immunoreactivity is not only resulting from the location. Results are based on the estimated marginal means for each group

When the interaction was analysed one group verses another, only PSD and VaD showed a significant interaction between groups and region (p=0.032 and F=2.77), suggesting that the increase in expression in PSD differences between each region is not just dependent on the expression of the individual region.

No significant correlation was found to affect immunoreactivity with factors such as age, post mortem delay or fixation time.

7.3.2 Stereological assessment of density of Hu C/D stained neurons

Stereological analysis was performed as an alternative method to investigate the findings of % per area immunoreactivity of Hu C/D, which was increased in PSND subjects compared to dementia groups in the CA1 and no difference in the CA2. It was believed this technique would have an advantage as it would produce an actual neuronal count of positive Hu C/D neurons instead of a percentage of the total area. This analysis also allowed for greater interpretation of differences in the number of neurons with different cell staining compared to previous result for each case, as stereological analysis could be arranged to calculate the number of neurons within groups of the varying degrees of staining intensity.

Statistical analysis showed there were no significant differences in the CA1 region, between groups, of Hu C/D neuron density (ANOVA p=0.374). Additionally, there was no significant differences between groups with the number of strongly stained Hu C/D neurons density in the CA1 (Kruskal-Wallis p=0.079). Further analysis with the Mann-Whitney U test showed that there was a significant decrease in VaD cases compared to controls (p=0.035), PSD (p=0.028) and AD (p=0.028) but no PSND. There was no significant difference in CA1, between the groups, with neurons with no Hu C/D staining (Kruskal-Wallis p=0.090). However, there was a significant increase in VaD subjects compared to PSND (p=0.028), PSD (p=0.037) and AD (p=0.015) when tested by the Mann-Whitney test. In all variations of the number of neurones analysed for Hu C/D staining in the CA2 region there was no significant difference (Figure 7.7)

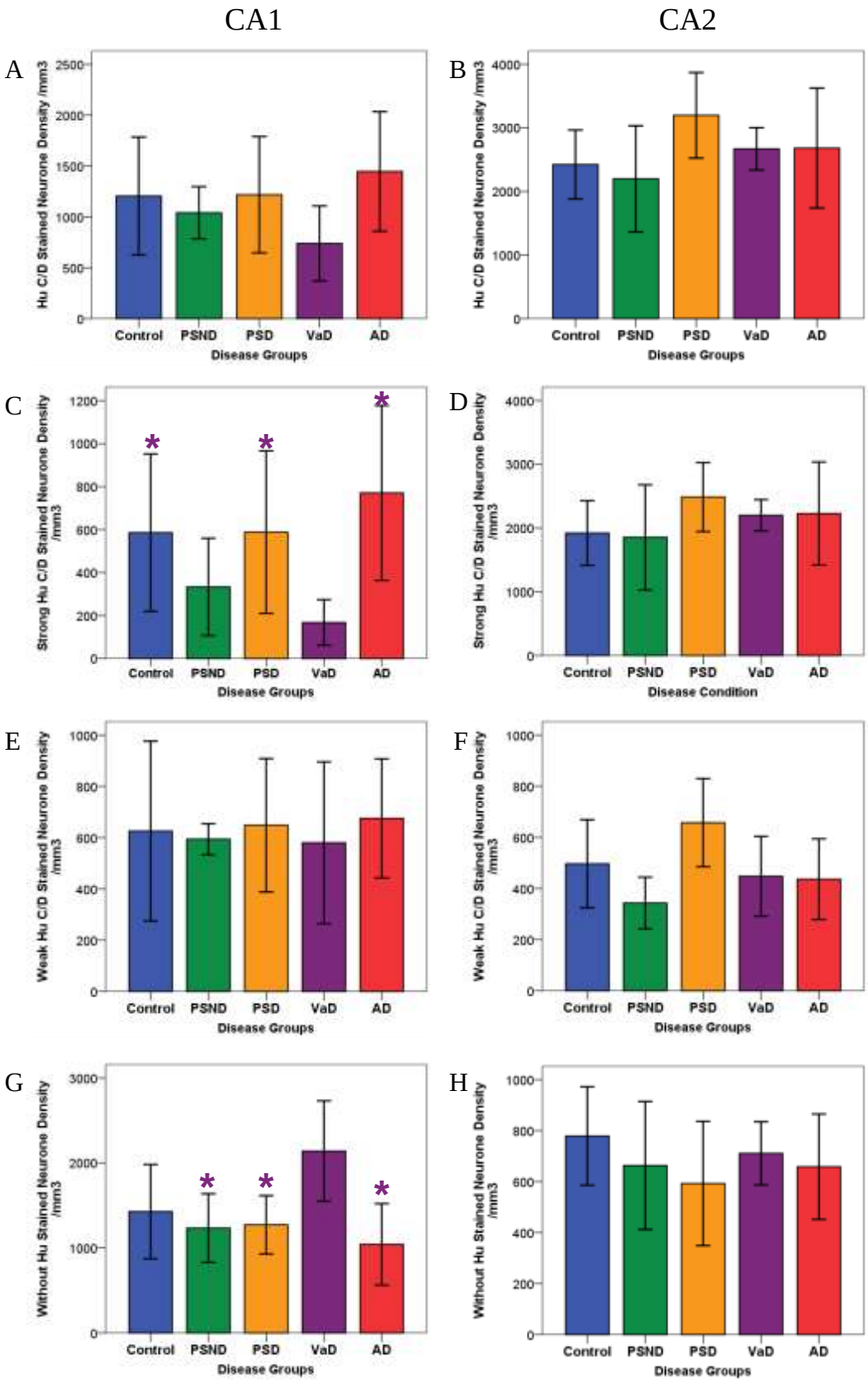

Figure 7.7 Stereological neuron density analysis of Hu C/D staining in both CA1 and CA2. All Hu C/D staining density (A and B), strong staining (C and D), weak staining (E and F) and neurons without Hu C/D staining (G and H).

7.3.3 Hu C/D and single Hu isoforms

7.3.3.1 Brightfield analysis

Examination of the sections stained with single Hu isoforms using DAB with a brightfield microscope clearly shows that there was significantly less staining in the neuronal cells compared to the Hu C/D antibody across all regions (Figure 7.8). However there appeared to be an increase in Hu D staining in most neuronal cells compared to Hu C especially seen in CA2 (Figure 7.8 B and J) and the DG (Figure 7.8 D and L). Although it could be suggested that there is a little more staining of Hu C in CA1 (Figure 7.8 A and I). However, there may be greater background staining in Hu D, especially notable in CA2. It also noticeable that unlike Hu C/D, these antibodies only stain neuronal cytoplasm and nuclear staining is absent. The staining of the cytoplasm appears more granular and forms in clusters unlike the Hu C/D staining which appeared continuously throughout the cell. As described in the gross examination of Hu C/D the staining emanates from the cell body and often axons can be clearly seen but axons are not visible with either of the single isoform staining of Hu. Furthermore, the single isoform types appeared to be recognising more non neuronal type cells, with increased staining of possible astrocytic and glia cells, particularly noticeable in CA4 (Figure 7.8 C and K) although this wasn't confirmed.

7.3.3.2 Double label fluorescent confocal imaging

Confocal images of the hippocampal regions CA1, CA2, CA4 and DG show dual staining of either single Hu isotope with the Hu C/D antibody. The fluorescent immunostaining emphasised the greater staining of Hu C/D in all regions compared to the single isoform. Also confirmed that the single isoforms stained in clusters unlike the C/D antibody which stained all of the cytoplasm and the nucleus. It is possible to also see staining of non neuronal cells stained with the single isoform in the CA4. Other than in the DG, which appears to have more Hu D staining, it is difficult to see differences between the single isoforms. However, in both types there is not very strong immunoreactivity of the single isoforms and further analysis would be required (Figure 7.9).

7.4 Discussion

The major finding from this study was that the neuronal marker Hu varied across the hippocampus, with the highest expression seen in the CA2 and CA3 regions, where almost every pyramidal cells strongly expressed the protein compared to the CA1. There was also a variation of staining within the CA1, with some neurons strongly expressing the protein whilst others neurons were not stained at all. Hu expression appears to be significantly reduced in VaD cases compared to other groups across all regions of the hippocampus (Figure 7.6) and PSD and AD are also both significantly reduced in the CA1 compared to PSND. Hu has been suggested to be involved, through its role as an RNA binding protein, in neuronal maintenance and plasticity. Therefore, Hu staining variation may indicate neurons that are unimpaired. It has been previously reported that, in general, the CA1 hippocampal region is more vulnerable to damage than CA2 by both hypoxia and Alzheimer type pathology (Leifer and Kowall, 1993) and also the CA1 plays an important role in the preparation of memories for storage in the cortex.

Initial analysis using to measure staining % per area density showed that in the CA1 region there was a significant increase in PSND cases compared to all dementia groups. Additionally significant decreased was also observed in VaD cases compared to controls, PSND and PSD in CA3 and CA4, and with PSND in the subiculum. Strong correlations were also observed between the staining density and the number of objects measured in CA1, CA4 and subiculum. This suggests that in these regions with raised staining density have an increasing number of neurons stained with Hu C/D due to the strong specificity of the antibody. Therefore the reduction in staining density, as seen in VaD subjects, could indicate loss of some neuronal functions within these regions, as neurons are still visibly present. There was no significant correlation observed in the CA2 and CA3 region between counts

and staining density, however there is a high degree of overlap of cells in these regions and therefore the software can not differentiate between individual cells. It could be argued that the decrease in % per area results is due to neurodegeneration; however haematoxylin staining clearly shows a number of neuronal cells present without Hu staining.

As there appeared to be significant differences in immunoreactivity between the different regions, further analysis was performed across regions using a two way ANOVA to investigate the interaction between groups and regions. The anlysis confirmed the loss of immunoreactivity in VaD across all regions. However, there was no significance in the interaction between groups and varying regions. The largest variation in immunoreactivity is based on the different regions and is independent of the groups involved (66% effect caused by regions). However when a single group was compared to another a significant interaction was found between PSD and VaD and would suggest differences between these groups is more than just dependent on the region being analysed.

The analysis also allows us to oversee the different expression in all the regions analysed (Figure 7.4) Some of the other differences of note are in all groups the CA2 has the highest expression in addition the expression in the subiclium in the PSD group is the only one to maintain a similar level to CA4. Therefore, suggesting it has increased density compared to what is usually expected in other groups, although it is not significantly increased compared to other disease groups. Furthermore, there is clearly a large increase in expression in all cases in the CA2, approximately 10 fold compared to CA1, as Hu C/D has been suggested to be involved with maintainance and plasticity does this marker confirm previous understandings of the CA2 being undamaged and could similar levels of expression also be found in CA1 at either a younger age or without disease?

To try to better understand the differential expression of Hu C/D staining in the CA1 and CA2 region, stereological analysis was performed. It was envisaged that this technique would allow us to make quantitative counts of the number of Hu positive cells in different groups, to confirm the initial findings with density analysis by Image Pro software. Similar to the concept described in Chapter 4 regarding L_v, this study counted neurons through a known thickness, as the whole hippocampus could not be analysed results were expressed as neuronal density (neurons/mm^3). This technique allowed multiple neuron density measurements to be made of the exact same live image, by using the same probe technique a number of different times. It also allowed the pre-setting of different groups dependent on neuronal staining including; all neurons stained with Hu C/D, strongly stained neurons, weakly stained neurons and those without staining were analysed.

Analysis showed that there were no significant differences in groups for all variations of Hu C/D stained neurons in either the CA1 or CA2 region..However, there was a significant decrease in strongly stained neurons in VaD compared to controls, PSD and AD cases in the CA1. There was also a significant increase in the number of neurons without Hu C/D staining in VaD cases compared to PSND, PSD and AD in the CA1. No significance was found in any variation of staining in the CA2. Although these results are not quite comparable with the % per area results, suitable differences were being measured. The % per area study was measuring overall expression and the stereological analysis was counting the number of neurones.However, we believe that a number of technical issues may have added to these differences. The major concern with the stereological technique is that whilst an antibody may look very specific at low magnification when it is analysed for % per area analysis, the software analyses only what it determines to be above the threshold level, which is manually set. However, when the magnification was increased to 40x, most neurons appeared to have

some degree of staining and the subjectivity of the stereological analysis made it difficult to know when counting neurons if staining is specific or background. This may have a knock on effect on other neurons staining hence results are very subjective when determining category assignment for individual neurons. However, the stereology results did confirm that there was a significant loss of Hu C/D in VaD compared to all other groups.

We also tried to determine how the expression of Hu C/D was composed by using brightfield and then fluorescent dual staining with the C/D antibody with antibodies raised against single Hu isoforms. Immunoreactivity was much reduced in and less specific for both the single isoforms compared to the dual antibody. We also found that expression was only cytoplasmic and granular for the single isoform. However, slight variations that were noticed between the two singular isoforms included a possible increase in Hu D in the DG and CA2. Further analysis is needed to be performed of these single isoforms and possible alternative antibodies should be tested before any full conclusions can be made.

To our knowledge, this is the first time that the neuronal marker Hu C/D has been analysed in this way in cases of dementia and linked as a possible marker of neuronal maintenance to a specific condition. It has been reported that there is a reduction in Hu C/D in human AD brain tissue using immunoblot analysis, however it was not specified if a particular region was affected (Amadio et al., 2009) and often the antibody can be used as a general neuronal marker and the study may be suggesting neuronal degeneration. Hu has been associated with learning and memory in the hippocampus from previous animal studies using both spatial discrimination tasks (Quattrone et al., 2001) and the Morris water maze (Pascale et al., 2004). When a Tg mouse model was developed over expressing HuD in the forebrain region, the animals with reduced levels of the transgene performed better compared to those with greater expression on similar tests. The authors of the paper speculate these unexpected findings may

results from the continual expression of Hu D and that for true memory storage there may have to be restricted temporal and spatial expression (Bolognani et al., 2007). These previous studies would suggest that the protein may have some role to play in memory and with our additional data, demonstrating the significant loss in cases of VaD, there may now be an additional reason to investigate this relationship further.

Previous analysis from the group using sections from the CogFAST study has suggested that in the CA1, the significant difference in PSND and PSD cases is there neuronal volume and not their density (Gemmell et al., 2012). It could therefore be speculated that the loss of neuronal volume maybe linked with the expression of Hu C/D, although this hypothesis could not be tested at this time. However if this was found to be the case it would suggest a possible link between neuronal maintenance in PSND cases and a possible molecular mechanism involvement. The Gemmell study also found that there was a significant decrease in neuronal volume of VaD and AD cases compared to PSND and no significant difference was found with controls, which are very similar to the results we found for Hu C/D expression. However, any conclusions made with Hu should be tapered due to its function as a major RNA binding protein and it is therefore likely to have numerous functions throughout the brain.

Future analysis of interest regarding Hu C/D would be to investigate whether the difference between CA1 and CA2 is found throughout life or if this reduction is caused with increasing age and impairment to the cell. Is the reason that there is strong stain in the CA2 linked to the fact that their axons have only short distances to travel and are unaffected by damage? Do the cells that weakly stain with Hu show colocalisation with intracellular pathology including NFTs or α-synucelin in DLB subjects? If these findings are subsequently established can electrophysiological techniques be applied and delineate differences in the excitability

between cells in the CA1 which have significant Hu C/D staining and those without. Is there a link between the expression of Hu C/D and neuronal volume and could this explain a molecular mechanism that regulates neuronal volume, possibly linked through increased synaptogenesis of these neurons? Furthermore is this finding in the hippocampus specific to dementia or do differences of expression affect specific regions of the brain associated with varying neurological conditions. Associations have been found between Parkinson's disease (PD) and Hu D, linking the age of onset of the disease with the expression of Hu D (Li et al., 2002, Hicks et al., 2002, Noureddine et al., 2005). Furthermore, a microarray study of mRNA in the prefrontal cortex of schizophrenic cases revealed that Hu D levels are increased and that GAP-43 expression was strongly co-regulated with Hu D (Hakak et al., 2001).

In conclusion, there was significant variation of the RNA binding protein, Hu C/D in the human hippocampus. Specifically a significant amount is located in the CA2/3 region especially compared to the CA1, but also CA4 and subiculum. Contrasting data may suggest that the loss of Hu in the CA1 is more significant in individuals who have suffered with dementia; especially in cases of VaD. However further investigation is required to confirm this. If so the loss of this protein may affect functionality of the region and circuitry, therefore having an effect on clinical outcomes. Alternatively, this marker may emphasis the notion that pyramidal cells in CA1 are abundantly different from neighbouring CA2 pyramidal cells and should be treated as entirely different entities.

Chapter 8 General Discussion

8.1 Introduction

Previous studies have shown that cognitive outcome in elderly subjects may not solely be dependent on clinical events such as a stroke or upon the degree of pathology (Katzman et al., 1988, Ince, 2001, Nelson et al., 2009, Price et al., 2009). This has led to the theories of both brain and cognitive reserve (Satz, 1993, Stern, 2002). These theories suggest that either through physiological differences, such as greater numbers of neurones, or the ability of enlisting compensatory processes an individual with greater reserve is able to accommodate equivalent lesions and not exhibit cognitive impairment.

This project was designed to investigate post mortem brain tissues from the CogFAST project, a prospective study in elderly post stroke survivors. The CogFAST study monitored the participants over time with annual clinical assessments and approximately 25% of subjects developed dementia over 5 years whilst others maintained cognitive function (Allan et al., 2011). This study specifically focused on uncovering evidence for angiogenesis and neurogenesis as an attempt to identify possible mechanisms that may explain why some subjects were able to maintain cognitive function whilst acquiruing similar burdens of pathology. The study also compared AD and VaD cases to identify if similar mechanisms played a role in either post stroke survivors.

The main findings from this study were:

- Increase in microvascular density in the CA1 in PSD and AD subjects compared to PSND, VaD and age matched controls. A significant increase of L_v in AD subjects in the EC compared to PSND and PSD.
- Increase in diameter of vasculature in PSND compared to PSD and all other groups in the CA1
- Differential expression of neurogenic markers for different stages of the neurogenesis mechanism in the DG. Significant increase in VaD subjects in PSAnCAM and DCX compared to PSD and AD cases and a significant decrease of Hu C/D immunoreactivity in all demented groups compared to PSND.
- Increase in Hu C/D expression in pyramidal cells in the CA1 of PSND compared to dementia groups, a possible marker of neuronal maintenance and plasticity. A significant decrease in VaD in the CA3, CA4 and subiculum compared to a number of groups including controls and PSND.

Collectively, studies including L_v in CA1, the quantity of neurogenic markers in the DG and expression of Hu C/D in neurones in the hippocampus demonstrated similar results between PSD and AD subjects, and both groups often exhibited significant differences against VaD subjects, although, 75% of PSD subjects that came to autopsy met the criteria for a diagnosis of VaD (Allan et al., 2011). This suggests a different underlying feature in PSD subjects, who did not meet classic pathological criteria for AD. These subjects could have been unmasking other degenerative mechanisms different from AD triggered by the stroke. In sporadic AD subjects, it has not been established what might initiate disease progression, although vascular lesions have been shown to be additive to the severity of dementia in cases of AD (Snowdon et al., 1997, Premkumar et al., 1996). More recent thoughts on the Aβ

cascade have suggested that it may be initiated by vascular injury or even ischemia, which may be initiated years before clinical symptoms occur (Kalaria et al., 2012). Is it possible that the PSD subjects, who showed no cognitive impairment prior to their stroke, had not suffered ischemic incidents earlier in life? This is probably unlike a high proportion of the sporadic AD subjects who may have suffered from a primary trigger event, possibly ischemia. As a result of their advanced age, the PSD subjects, cognitive decline is more rapid and has further reduced the threshold for dementia. If this is the case why are they differentiated from VaD subjects, who are closely linked with strokes at this stage of their lives, could it be genetic or possibly the location and severity of the stroke? Or is it that they are likely to be a mixed form of both types of dementia, as often occurs in the very old (Jellinger and Attems, 2010), even though they did not meet criteria for AD diagnosis. Although we identified a number of cases which would be categorised as having mixed dementia, the majority of cases were pathologically equivalent to VaD and there was no difference between results in the experiments performed in this thesis for PSD of those cases pathologically categorised as either being predominately VaD or mixed dementia.

8.2 Increase in vascular density in PSD and AD

The study has suggested that vascular density is increased in PSD compared to PSND in CA1. L_v was also increased in PSD compared to controls and VaD. Additionally, AD L_v was increased to all these groups except PSD in the CA1. Furthermore, AD L_v was significantly increased in the EC compared to PSND and PSD. There were no changes between the groups in either the CA2 of the hippocampus or the DLPFC.

These results may suggest that angiogenesis is or has occurred in subjects with increased microvascular density and this might be the result of neuroinflammation, particularly with

AD subjects, which has been shown to show an increase in inflammatory markers and cytokines (Akiyama et al., 2000, Naldini and Carraro, 2005). Alternatively, vascular impairment in PSD and AD, which is known to lead to a reduction in CBF (Firbank et al., 2011, Schuff et al., 2009) and therefore a regional hypoxic environment is created which can cause angiogenesis, as suggested by other studies (Biron et al., 2011, Meyer et al., 2008, Cameron et al., 2012). We speculate that microvessels in VaD subjects are unable to respond to similar stimuli and although there is a known reduction in CBF in this group (van de Pol et al., 2011), the vessels are not stimulated to grow.

Although, the results showed increased microvessel density, without knowing specific tissue atrophy in each case, then it cannot be concluded that the increase in L_v is a direct result of angiogenesis or if this is the only reason for the increase. It was not possible to measure the entire length of vasculature in the AOI, due to unavailability of the whole structure with the confines of working in a brain bank environment.

Although, analysis of the EC confirmed the increase in L_v in AD subjects, no such increase was observed in PSD subjects, which might suggest that microvascular changes begin in the hippocampus and progress to the cortical regions or that medial temporal atrophy is increasing L_v in AD subjects but this is not the case in PSD subjects. If the increase in L_v was simply the result of atrophy then similar findings would be expected in the EC for PSD. In another region studied, DLPFC, there were no significant differences in L_v in any group and therefore it is proposed that the increases in vessel density could be regionally specific, although additional regions would need to be investigated. DLPFC is another region that is known to be affected by atrophy in cases with dementia (Rajah and D'Esposito, 2005). The frontal lobe is known to have high amounts of Alzheimer type pathology (Rowe et al., 2007)

and it is a region associated specifically with VaD (Xi et al., 2011). Therefore we had expected to find significant difference between groups.

This study, however, advanced previous findings regarding vessel density in cases of dementia, as this is the first study to have investigated VaD and PS subjects. Previous studies using the spherical probe technique in human tissue have been limited to AD (Schwartz et al., 2010, Richard et al., 2010) or schizophrenia cases (Kreczmanski et al., 2009, Kreczmanski et al., 2005). With regard to AD, previous studies confirm similar findings with an increase compared to controls in both the hippocampus and temporal lobe. However, all of these studies only measured the L_v of the basement membrane marker COL4; this study has also investigated the changes in a marker of the endothelial layer, the functional compartment of the vessel, GLUT1 and changes in artery and arterioles (SMA), to give a complete profile of the microvasculature in the hippocampus. We also found a significant increase in AD and PSD for L_v of GLUT1 and although increase were found in AD for SMA concerns regarding the reliability of data were raised. However, another study using a different technique, but by sampling the entire hippocampus, found no significant difference in overall length of vasculature (Bouras et al., 2006) and a study of WM in VaD cases suggested a decreased microvascular density compared to comparable regions in controls (Brown et al., 2007).

To try to establish if angiogenesis was responsible for the increase in vessel density, three key pro-angiogenic markers were assessed to look for variation in expression. While, significant increases of immunoreactivity were observed in both PSD and AD for Hif 1α and integrin respectively, confirming similar findings reported previously (Grammas et al., 2006, Desai et al., 2009); there was no change between any groups in VEGF expression and no significant changes against controls. Additional studies with more markers will be required to

confirm the presence and relevance of these angiogenic proteins in relation to the increasing microvascular density results.

8.3 Increase in vessel diameter in PSND

We determined that there was an increase in vessel diameter in the PSND subjects relative to both the PSD subjects and age matched controls. This suggests blood vessels in these subjects are adaptable to their environment and more responsive to hypoperfusion. The vessels were analysed collectively as part of their individual group and then analysis was performed comparing all data group by group. This allowed standard statistical analysis and the results to be plotted using a cumulative frequency graph with increasing vessel diameter, facilitating the visualisation of the distribution of vessels with increasing diameter in each group (Figure 3.11). The analysis showed that whilst the majority of vessels analysed were capillaries, approximately 6-8 μm in diameter, PSND subjects had an increase in the number of vessels with a diameter between 8 and 12 μm compared to other groups.

To our knowledge, this is the first time vessel diameter has been analysed in VaD or PS subjects. There have been several reports suggesting vessel diameter is decreased in AD subjects compared to controls (Bouras et al., 2006, Bell and Ball, 1981) and there is also an increase in BM thickening (Mancardi et al., 1980, Wyss-Coray et al., 2000). A number of studies have suggested Aβ may play a direct role in increasing vasoconstriction (Niwa et al., 2001). The vasoconstrictor endothelin-1 (ET-1) may also play a role in both AD and VaD (Palmer et al., 2012, Palmer et al., 2010). Furthermore, inhibiting angiotensin II, a potent vasoconstrictor, with pharmacological treatments has been suggested to improve cognitive function in AD and VaD by ameliorating hypotension. However, the angiotensin II mechanism is also involved with inhibiting the release of acetylcholine and upregulation of

inflammatory response (Kehoe and Passmore, 2012, Wharton et al., 2012). AD subjects are less likely to have been prescribed angiotensin II receptor blockers, which may affect vessel diameter and AChE release (Davies et al., 2011).

Although vessels were not selected at random using computer software, we analysed a high proportion of vasculature in the CA1 per slide, which was designed to reduce bias in the analysis. A minimum of 100 vessels, from approximately 30 images, were analysed per case, with all available vessels measured per image. This was performed to ensure no vessel was measured twice or large vessels ignored and that the total CA1 was sufficiently analysed.

8.4 Differential expression of neurogenic markers across different stages of mechanism

Using established markers for neurogenesis including MSI1, nestin, PSAnCAM, DCX and Hu C/D, this study investigated the status of neurogenic markers across the entire neurogenesis mechanism from proliferation to maturation of granule cells in the DG. The analysis of the quantity of immunoreactivity of these markers suggested that there were significant increases in markers such as PSAnCAM and DCX in VaD subjects, similar to previous findings (Ekonomou et al., 2011) and in Hu C/D in PSND compared to all dementia groups. The proliferation marker, MSI1, showed a significant decrease in AD in the GCL. This might not suggest a decrease in proliferation as this process is found exclusively in the SGL, but MSI1 may still stain cells that are migrating into the GCL. Conflicting evidence also showed changes in the intensity of immunoreactivity, this analysis was performed to confirm findings in previous literature (Perry et al., 2012, Ekonomou et al., 2011, Ekonomou et al., 2012). Conflicting results could be caused by differences in agonal factors and may explain large variation in % per area results.

Collectively these results did not show the presence of a significant increase in neurogenesis in PSND compared to PSD as we had hypothesised. Although a significant increase was found in immunoreactivity of Hu C/D and a strong trend towards an increase was found in PSAnCAM in PSND compared to PSD. These findings suggest that there is not a complete loss of neurogenesis in the DG in the elderly and there appears to be a significant increase in VaD. We could not link these findings to changes in cognitive impairement. However, there could be a significant loss of cells at the conversion stage of progenitor into mature granule cells, as shown by the Hu C/D observations. Furthermore, we find that for different diseases there are dissimilar neurogenesis peaks suggesting that there is an arrest in the mechanism as a result of a cell signalling dysfunction, possibly caused by NOTCH (Ables et al., 2011, Breunig et al., 2007, Imayoshi and Kageyama, 2011). Alternatively there could be a decrease in cell survival (Danzer, 2008).

In the PS groups, we were also able to confirm that irrespective of cognitive function, particular life style choices and treatments could have an effect on the expression of neurogenic markers similar to previous findings. Our results indicate that patients on anti-depressant treatments showed an increase in expression consistent with findings reported previously (Malberg et al., 2000, Santarelli et al., 2003, Boldrini et al., 2009). We also found that patients who had a history of smoking but had recently quit also had an increase in expression of neurogenic markers.

While, all human neurogenesis studies are limited without the use of the proliferating marker BrdU, which cannot be used in human studies for ethical reasons (Cooper-Kuhn et al., 2002). These surrogate markers are well defined for this form of analysis and have been shown to provide highly accurate results in relation to BrdU (Rao and Shetty, 2004). We therefore believe that these results may suggest differences in the neurogenesis mechanism but further

analysis is required as some results produced very low amounts of % per area and may therefore increase the likelihood of error.

8.5 Increase in PSND for marker of neuronal plasticity and maintenance

Having used Hu C/D, a marker to investigate neurogenesis, we subsequently discovered compelling differences in immunoreactivity with this antibody across the entire hippocampus. Firstly, we discovered that this antibody was an excellent neuronal marker and did not appear to stain any glial cells or the vasculature. Secondly, differential expression was noted within the different hippocampal subfields particularly between the CA2 and CA1 regions. Strong immunoreactivity was seen in all pyramidal neurones in the CA2 and weak but variable staining was observed in the CA1 (Image 7.2). Quantitative analysis, by % per area, of these regions showed that not only was there a significant difference between regions but there was a significant decrease in per area results of all dementia groups in the CA1 region compared to PSND. Further analysis of other regions including the CA3, CA4 and subiculum, showed that in all regions % per area were significantly reduced in VaD subjects compared to all groups except AD. A two way ANOVA showed that there was not significant evidence of a relationship between different groups and regions.

To investigate these initial results further, we analysed sections using stereological methodologies, similar to those previously described for microvessel density in the CA1 and CA2. However, an appropriate probe was utilised to perform counts of neurons; the disector probe (Sterio, 1984, West et al., 1991). A significant reduction in the number of neuronal cells with Hu C/D immunoreactivity was reported for VaD in the CA1 compared to all groups except PSND, whilst no significant difference was reported in CA2. Significant increases were reported in VaD for neurones with no Hu C/D staining in the CA1. We

believe that limitations of stereological analysis could be caused by the variation in immunoreactivity of the antibody. It was discovered that the subjective nature of analysis made it difficult to determine if a cell should be counted, limited staining may show a loss of protein or peak to background ratios may be difficult to differentiate. To accurately address the differences in Hu C/D expression in the CA1, different techniques are required.

This finding is particularly interesting as Hu has previously been associated with learning and memory (Quattrone et al., 2001, Pascale et al., 2004, Bolognani et al., 2007) and is also involved in physiological alterations including LTP (Bolognani et al., 2010) and synaptogenesis (Anderson et al., 2003, Smith et al., 2004). Therefore, it is believed to play a key role in maintenance and plasticity of neurones and as a result it may be a marker of neuronal reserve. It is particularly of note that we observed a significant loss in numerous regions in the VaD hippocampus and leads us to speculate that this protein could be related to vascular impairment.

8.6 Future Directions

8.6.1 Short term studies

With this study, we have established key protocol for measuring changes in the quantity of string like morphological structures such as microvessels and axons, a facility that was previously not available on site. While a number of regions have been investigated so far, it would be worthwhile to investigate additional regions within the hippocampus and alternative brain regions. These regions should include the CA4, a region previously reported to have had reduced vascular density compared to other CA fields and would make an important comparison to CA1 results. Additionally, WM regions should be investigated as the involvement of vascular changes are well established, inclusive of demyelination and

increasing perivascular spaces (Roman et al., 1993). The quantitative assessment of microvessel density may confirm vessel degeneration in this region and would provide quantifiable comparison against future cases. Further investigation is required in regions outside the temporal lobe such as the basal ganglia and thalamus, which are the sites of lesions often linked with VaD (Van der Werf et al., 2003, Hachinski et al., 2006). A study analysing CogFAST subjects previously reported a significant increase in lacunes located in the putamen being associated with cognitive decline (Lopes et al., 2011). Additionally, various areas in the frontal lobe need to be investigated, as this region has previously been associated with VaD (Xi et al., 2011, Rowe et al., 2007) and we therefore might expect to find significant loss of microvessels. So far we have analysed the DLPFC, which showed no significant difference between groups.

One reason for speculation that there could be an increase in L_v was due to possible increased neuroinflammation, which has previously been reported in AD (Akiyama et al., 2000, Naldini and Carraro, 2005). This hypothesis needs to be tested by measuring and accurately quantifying markers of inflammation, inclusive of CD45 and CD68, which identify haematopoietic cells and macrophages respectively, and GFAP to identify changes in astrocytes and compare them with results for Lv.

The other key focus of this study has been to try to establish changes in expression of neurogenic markers in the DG, which may explain difference in cognitive test scores between PSND and PSD. All the markers used have previously been reported as suitable neurogenic markers in human post mortem tissue and our group has had previous experience working with these particular markers (Johnson et al., 2011, Ekonomou et al., 2011) . However, additional markers would strengthen these findings and particular markers of interest would include Sox2, a transcription factor expressed in the early stages of neurogenesis similar to

MSI1 and nestin (Steiner et al., 2006). Tuc 4 is a protein expressed between PSAnCAM and DCX (Jin et al., 2004). Calretenin and calbindin are both post mitotic markers similar to Hu C/D and could confirm an increase in PSND subjects (Llorens-Martin et al., 2006, Baimbridge, 1992). As shown in Chapter 1, there is evidence that neurogenesis in the SVZ of lateral ventricles, the only other location shown to be a neurogenic niche in humans to date, is more active both with increasing age and after ischemia (Darsalia et al., 2005). It would therefore be of interest to analyse this region and compare levels of expression with the hippocampus. Furthermore in future it would be important to look at the possibility of using stereology to accurately define the differences in expression of these markers as has been performed previously in animal studies (Jinno, 2011)., We have already shown the difficulties associated with this approach, when using IHC, as variation in staining can introduce potential bias to the results. Due to the time consuming nature of stereology and potential issues with staining counts, it has been important to elucidate these initial finding from IHC using markers in the DG. This will be beneficial to any future work as the precision of stereology will be of great benefit to identify minimal changes between groups.

The present study has suggested that there may be an increase in the expression of Hu C/D in the CA1 region of PSND subjects compared to other dementia subjects. In the short term, it would be ideal to test both protein and RNA expression by Western blot protocols and real time PCR, particularly in the CA1 region, which would confirm the expression levels in these groups. It would also be of interest to discover if the variable staining in the CA1 correlates with specific pathology, especially intracellular, including NFTs in AD and α-synuclein in DLB subjects. If this hypothesis, proposing that reduced Hu C/D immunoreactivity is associated with pathology, is correct it may confirm the use of Hu C/D as a marker of neuronal maintenance.

8.6.2 Long term studies

This study suggests that our initial hypothesis was incorrect and there is no increase in microvascular density in PSND subjects to suggest a form of vascular reserve or loss of density in dementia subjects. Our results do however suggest that the vasculature in PSND may be more adaptable to its environment, as seen in results from vessel diameter analysis.

Thus, a new hypothesis may be formulated: that PSND subjects are able to reduce damage to their vasculature thereby maintaining healthy blood flow and neurovascular unit function (Sagare et al., 2012). Therefore, it would be worthwhile to investigate BBB integrity markers, such as ZO-1(Fiala et al., 2002), and also other markers of the neurovascular unit including pericytes (Bell et al., 2010). The sclerotic index (SI) could be measured from arterioles and arteries in a number of regions as an additional marker of vessel integrity (Craggs et al., 2013).

It has also been shown that when vascular degeneration occurs, ECs are lost first and then subsequently the basement membrane is broken down by enzymes. However, before this can occur the vessel loses structure and is referred to as a string vessel (Brown, 2010). A large number of string vessels were observed in 30 μm thick sections stained with COL4 but these vessels were excluded from the L_v analysis. Therefore, it is proposed that string vessels could be quantified, as a separate entity, in order to determine group differences, as an indication of vascular degeneration. Additional analysis could be performed using the stereological technique to quantify the degree of CAA with respect to vascular density and as an indication of impairment (Weller et al., 2008).

One of the major findings from this study was the increase in vascular density of PSD subjects, similar to that found in AD. It was speculated that this result may have been a false

positive caused by either regional atrophy or microvessels losing supportive structures and therefore increasing in tortuosity. Firstly, if the entire hippocampal structure could be measured by stereological analysis this would primarily remove the need to give results as L_v and entire microvascular length results could be given, removing the issue of the reference trap previously discussed. Secondly, this would remove any question of variation for changes along the length of the hippocampal structure. We controlled for this factor as much as possible by sampling from the approximate same location in each subject; adjacent to anterior pole of the lateral geniculate body in the coronal plane, however this can never be as accurate as sampling the entire tissue. In a previous study, which analysed the entire hippocampal structure in AD subjects and controls, no significant difference was found in total length but a decrease in vessel diameter was also reported in AD cases. However, this study used an alternative technique for measuring length compared to the spherical probe approach used here (Bouras et al., 2006).

Another possible reason which may explain the increase in L_v was the possibility that vascular structure is lost and therefore increasing tortuosity. To examine this possibility the vascular structure must be re-analysed using thicker sections. Previous studies have cut celloidin sections at 100 μm thickness and stained with alkaline phosphatase (Brown et al., 2009) but our sections are cut from paraffin wax embedded blocks. However, a new technique has recently been developed, CLARITY, which has been used for neuronal and axonal imaging in an entire mouse brain (Chung et al., 2013). The tissue retains its structure due to a hydrogel based support built in to the tissue to allow the subsequent removal of lipids, which have in the past prevented imaging to be performed through thick tissue sections. This technique could be used in future studies with applications in post-mortem tissue.

While this study attempted to identify differences in expression of three key angiogenic proteins, there are many more that also play a considerable role in the process (Chapter 1 table 1.1). IHC has many advantages it is difficult to perform analysis of many multiple targets. It can be time consuming and there can be issues with antibodies and their reaction with the formalin fixative. Therefore, a complete analysis of angiogenic markers is required and could be performed by RT-PCR assay. As has been shown there is a high degree of convergence of angio- and neurogenesis (Palmer et al., 2000) and therefore results from this type of study may also give an indication of the presence of neurogenesis or an environment where the neurogenesis mechanism may have greater chance of being initiated and completing. However, additional assays can also be performed independently to identify specific genes which are pro-neurogenic including epidermal growth factor, fibroblast growth factor 2 and Noggin if tissue is available (Grimm et al., 2009, Zechel et al., 2010, Bonaguidi et al., 2008).

Finally, there was an increase in the expression of Hu C/D in PSND and age matched control subjects. Hu C/D may be a potential marker of cognitive reserve as it is known to play a significant role in synaptogenesis (Smith et al., 2004) and has also been suggested to play a significant role in learning and memory (Quattrone et al., 2001, Pascale et al., 2004). However, Hu has also been reported to have over 600 other targets in the mouse (Bolognani et al., 2010) and it will be important, if this is a potential marker for cognitive reserve to determine which of these downstream targets are being affected. This will require further analysis to determine the differentiation between Hu C and Hu D expression in the hippocampus and how these two isoforms both affect targets. It was also noted in analysis that there was variable immunoreactivity in the CA1 region in all groups and it would be of interest to determine if those cells that strongly express Hu either have a different signalling

role or axonal targets within the hippocampus and whether there is an alteration in the electrophysiology from cells with reduced or no immunoreactivity of Hu C/D.

The study was unable to categorically conclude differences in regenerative mechanisms between PSND and PSD. It is still vital that we identify what might be causing some subjects to developing dementia and others to maintain cognitive function after a stroke. The CogFAST study has already shown there to be no significant difference between lesion locations, size or history of repeated ischemic incidents (Allan et al., 2011). Although, more lesions were reported in the putamen by an MRI study using PSD cases (Lopes et al., 2011). The study also showed that there was no significant difference in APOE genotype; the widely accepted genetic risk factor for AD, between the groups. However, in light of new genome wide association studies of both AD and VaD it would be worthwhile to investigate changes in expression in genes identified in these types of studies and also from future research (Seshadri et al., 2010, Schrijvers et al., 2012).

8.7 Conclusions

The experiments described in this thesis were designed to establish whether there was an increase in regenerative mechanisms, particularly angiogenesis and neurogenesis, in PSND compared to PSD subjects in order to explain the deviation in clinical outcome between the demented and non-demented groups Additional disorders were studied including VaD and AD to look for possible differences in disease mechanism. In this regard, the findings from this study made several contributions:

- In chapter 3, we investigated the vascular density and vessel diameter. A significant increase in vessel diameter was found in PSND subjects compared to PSD and age matched controls, which may suggest that the microvasculature in this group is more

adaptable to the surrounding environment. Microvasculature density was significantly increased in AD subjects. However, concerns regarding vessel size differences and orientation lead to questions being raised concerning bias of data and lead to the investigation of alternative methodologies for a more accurate assessment of string-like structure length.

- The experiments in chapter 4 directly built on the issues raised in chapter 3. A stereological based technique using a spherical probe was used to accurately determine the L_v of vasculature in the CA1, CA2, EC and DLPFC. Increases were found in both PSND and AD subjects compared to all other groups in the CA1, furthermore a significant increase was found in AD compared to PSND and PSD. There were no significant differences in either the CA2 or DLPFC.
- The main aims of chapter 5 were to attempt to measure some of the key angiogenic markers including Hif 1α, Integrin $\alpha_5\beta_3$ and VEGF. Whilst there were some significant differences for both Hif 1α and Integrin $\alpha_5\beta_3$ questions surrounding issues with immunoreactivity, particularly with VEGF, meant no conclusive evidence to support the theory of angiogenesis increasing vessel density.
- Analysis in chapter 6 describes experiments concerning the expression of neurogenic markers within the DG. Using previously established immunohistochemical markers for neurogenesis, some significant changes in quantity of markers were reported particularly increases in VaD subjects for PSAnCAM and DCX. Although, a significant decrease was observed in the mature granule cell marker Hu C/D for all dementia groups compared to PSND and this may suggest a decrease in converting from the neuronal progenitor cells into mature granule cells. Additionally, some

lifestyle factors were confirmed to be involved with up regulation of neurogenesis including the use of anti-depressants.

- In chapter 7, we investigated the expression of Hu C/D, a possible marker of plasticity and maintenance of neuronal cells, in the CA fields and subiculum. Initial standard 2D analysis suggested that there was a significant increase in the number of pyramidal cells in the CA1 expressing Hu in PSND subjects compared to all dementia subjects and there were significant decreases in VaD subjects in CA3, 4 and subiculum compared to a number of groups including age matched controls, PSND and PSD. Due to its potential role in a number of regenerative mechanisms, Hu C/D may be a marker of brain or cognitive reserve in the CA1; the key region for preparing memories for storage in the cortex. Further quantitative analysis using stereology was less conclusive, although confirmation of Hu C/D expression loss was found in VaD. Further analysis attempted to establish if different isoforms (C and D) were responsible for changes in immunoreactivity in different regions. Using confocal fluorescent double label imaging of single isoform antibodies with Hu C/D, we were unable to report any notable difference between expressions.

Collectively these results suggest there may be an increase in vessel density, a representative measure of angiogenesis in PSD compared to PSND subjects. However, vessels in the CA1 are on average wider in PSND than PSD and this might suggest these vessels are more adaptable to their environment and may also indicate an equivalent perfusion area in both groups.

However, there is little evidence of variation in progenitor cells within the DG to explain difference in cognitive decline between the two PS subgroups. Although, Hu C/D expression in the granular layer suggests fewer progenitor cells are developing into

mature granule cells in PSD cases, these results need further confirmation. It should also be noted that there was a trend towards a significant increase in PSAnCAM immunoreactivity in PSND compared to PSD.

There is a suggestion of a possible increase in Hu C/D expression in the CA1, which may indicate increased neuronal maintenance in PSND subjects. This supports previous findings in a related study, regarding neuronal volume (Gemmell et al., 2012).

Results from these studies also found a number of similarities between PSD and AD and significant differences with VaD, which may suggest PSD cases are following a similar pathway to AD mechanisms without expressing the same pathology. In general, the results presented in this thesis do not unequivocally establish that PSND subjects have increased regenerative mechanisms, which might have explained the reason these cases were able to maintain their cognitive function. It is now hypothesised that the established inherent neurones and microvessels are more adaptable than originally thought and have increased functionality and maintenance in PSND subjects compared to those in the PSD group.

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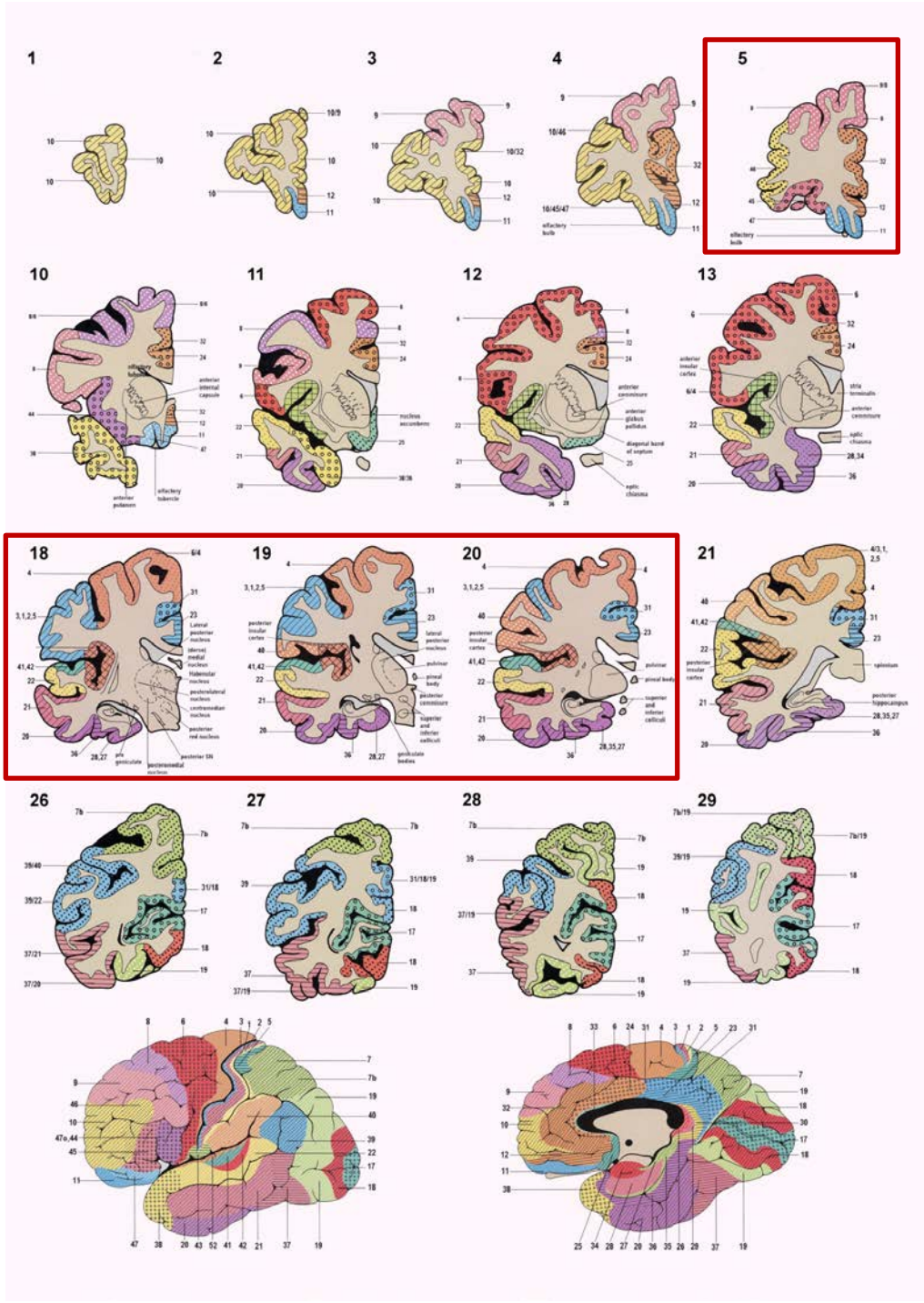
Figure 2.1 Newcastle Brain Map. Schematic representation of coronal slices from human brain, cut anterior to posterior. Large bold numbers indicate coronal levels of the brain. Brodmann areas are colour coded and numbered on each level. Sections were cut from blocks taken from these levels. Levels 5-6 for DLPFC and levels 18-20 for hippocampus and EC. Coronal levels used are highlighted in red, on the map.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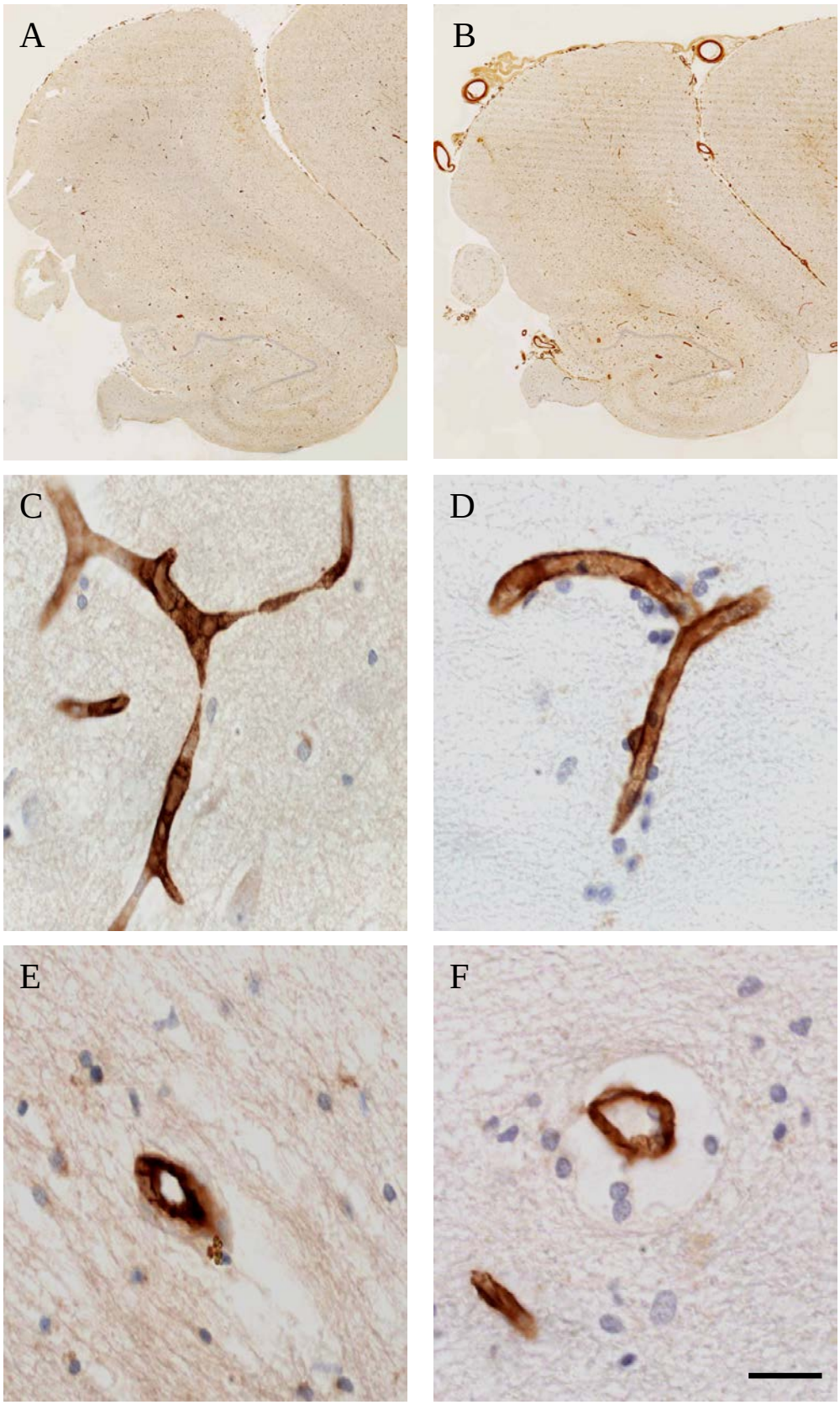


Figure 3.2 Shows GLUT1 (A,C and E) and COL4 (B, D and F) staining. Images A and B show high resolution scanned images of stained sections. Whilst image C and E show a longitudinal and transverse sectional cut of a GLUT1 stained vessel. D and F show similar vascular profiles stained with COL4. Images A and B scanned on flatbed scanner at 9,600 dpi. Images C-F taken at 40x magnification Mag bar = 20 μm for images C- F.

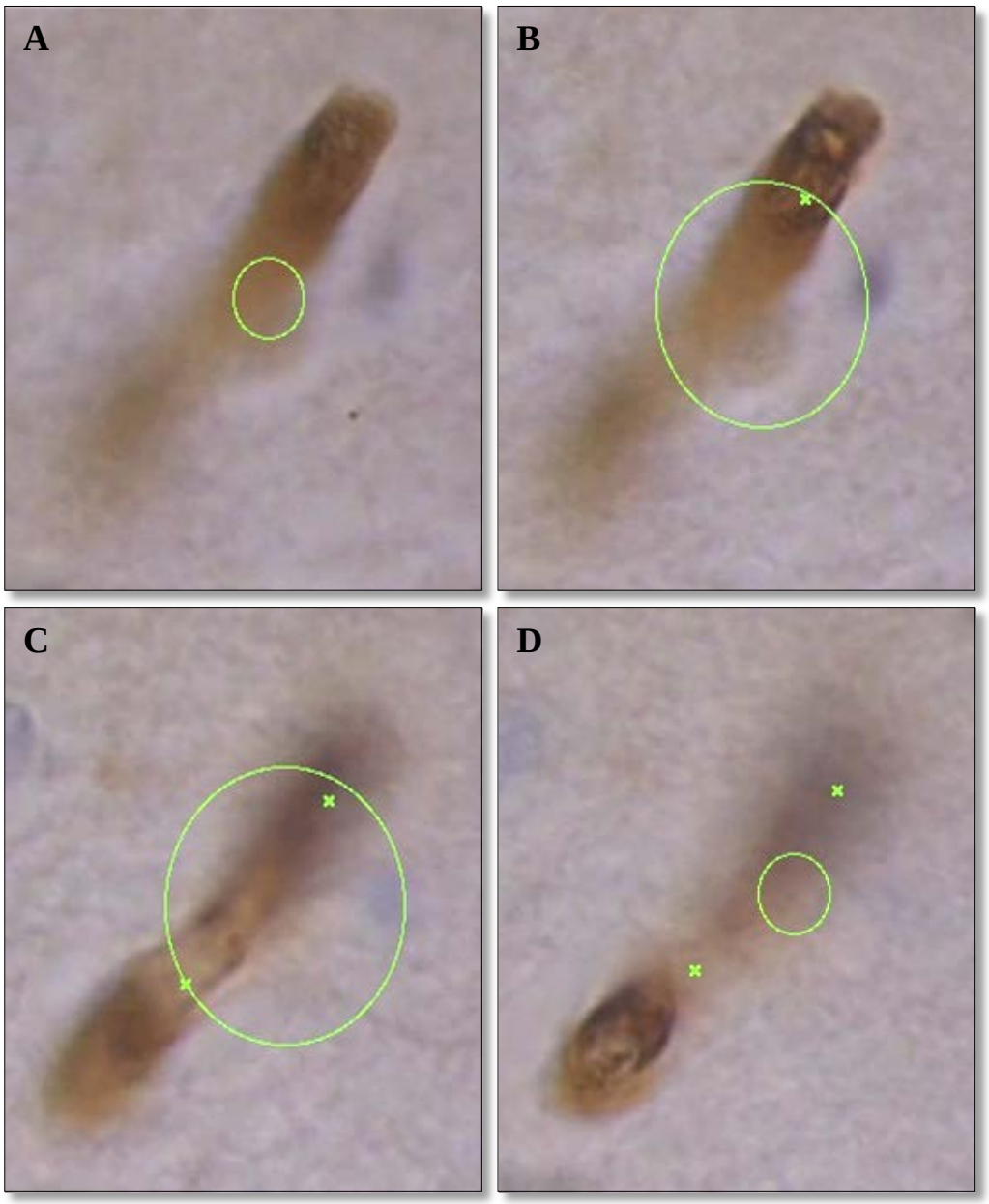


Figure 4.1 Measurement of vasculature using spherical probe technique.
Moving down through the z axis (A-D) the vessel comes into focus and as a focused region crosses the green circle parameter probe, a count is recorded. The spherical probe appears as concentric circles through the entire z axis. A new circle appears for every 0.5 µm traversed through the z axis.

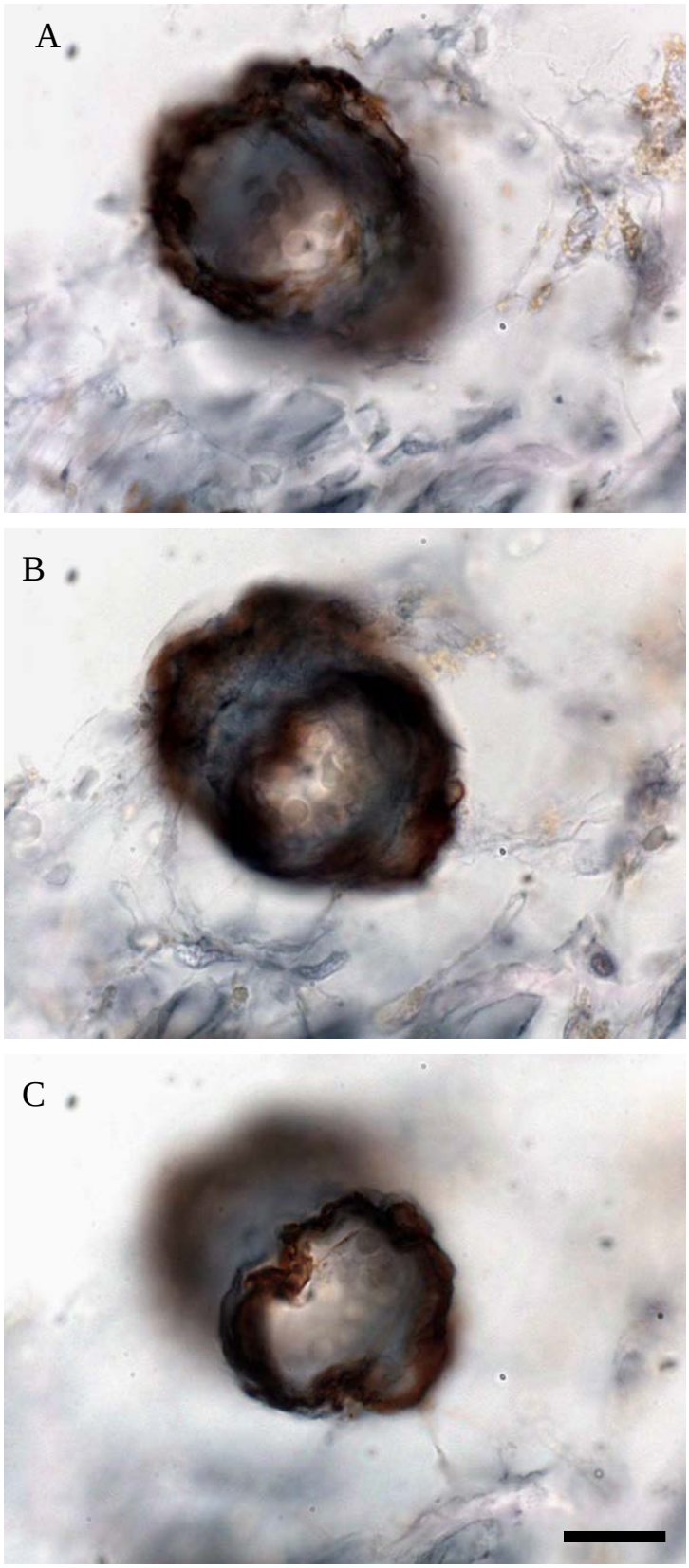


Figure 4.2 Arteriole Staining of SMA (DAB) and COL4 (Vector Grey).

Shows staining along the vessel by moving through the z axis. Top of section (A), showing SMA staining at the upper aspect of the vessel. Middle of section (B), showing basement membrane of the vessel and base of section (C), showing SMA staining in the perimeter of the vessel. Mag bar = 25 µm

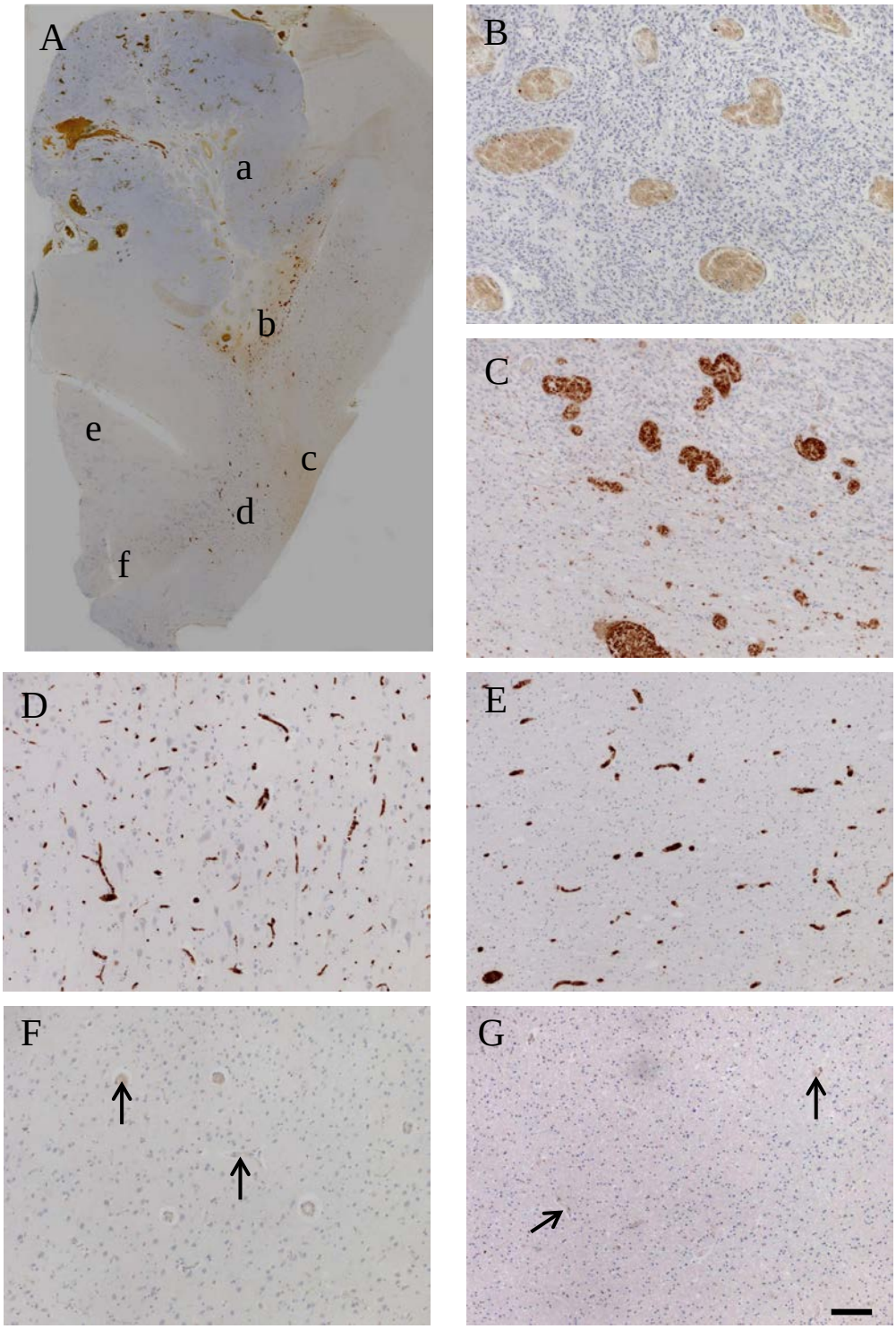
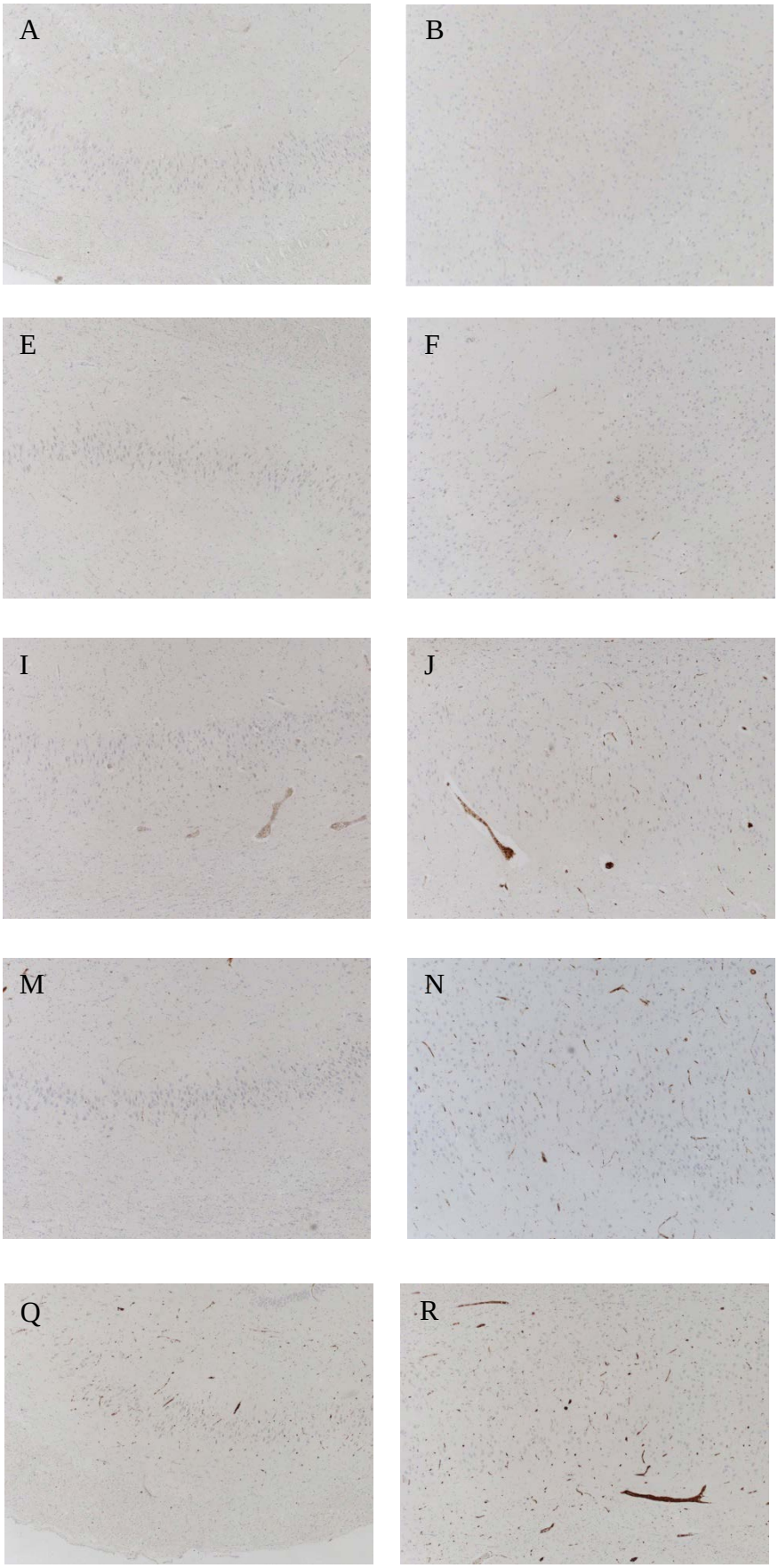


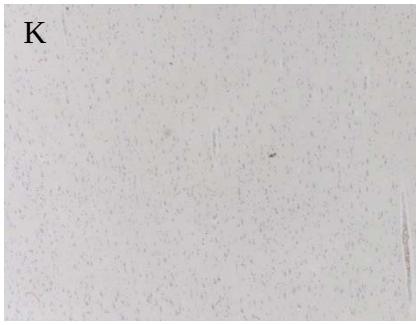
Figure 5.2 Hif-1α immunoreactivity of glioblastoma section. Image A shows a scan of the entire section, highlighting the regional variation in staining across the section..B shows staining in the centre of the tumour region (a) and C the metastasis region (b). Immunoreactivity can be clearly observed in both large and small vessels. D and E show immunoreactivity in nearby cortical and WM regions to the tumour respectively. Images F and G show that immunoreactivity is not consistant in all cortical regions and WM, as those that are distant from the tumour express no clear staining, although vessels have been highlighted with the use of arrows. Images B-G, mag bar = 100 μm

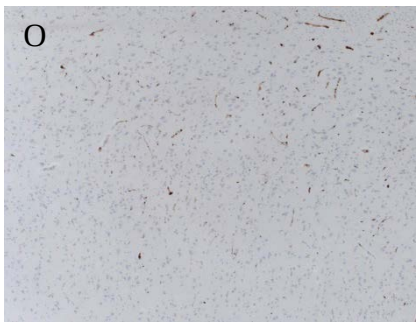
Figure 5.6 Hippocampal expression of Hif-1α , varying expression dependent on semi quantative staging. Images A-D, Stage 0 showing no staining in hippocampus. Images E-H, Stage 1, minor staining in the CA1 and WM. Images I-L Stage 2 display some staining in large vessels in CA1 and WM. Images M-P, Stage 3 show more numerous staining of microvessels in CA1, cortex and WM. Images Q-T, Stage 4 show staining of all vessel types in all regions, with numerous microvessels showing immunoreactivity for antibody.

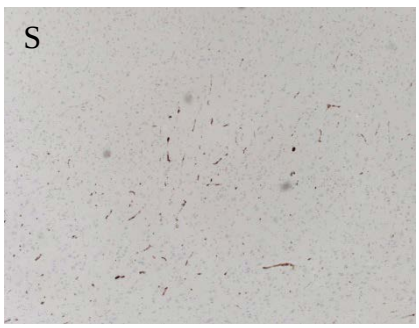
CA2 CA1

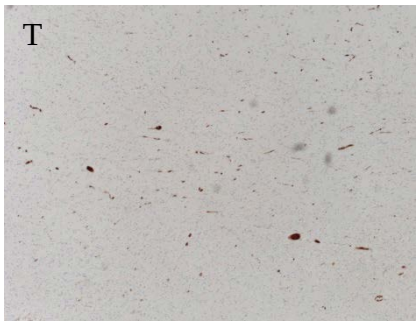


Cortex WM









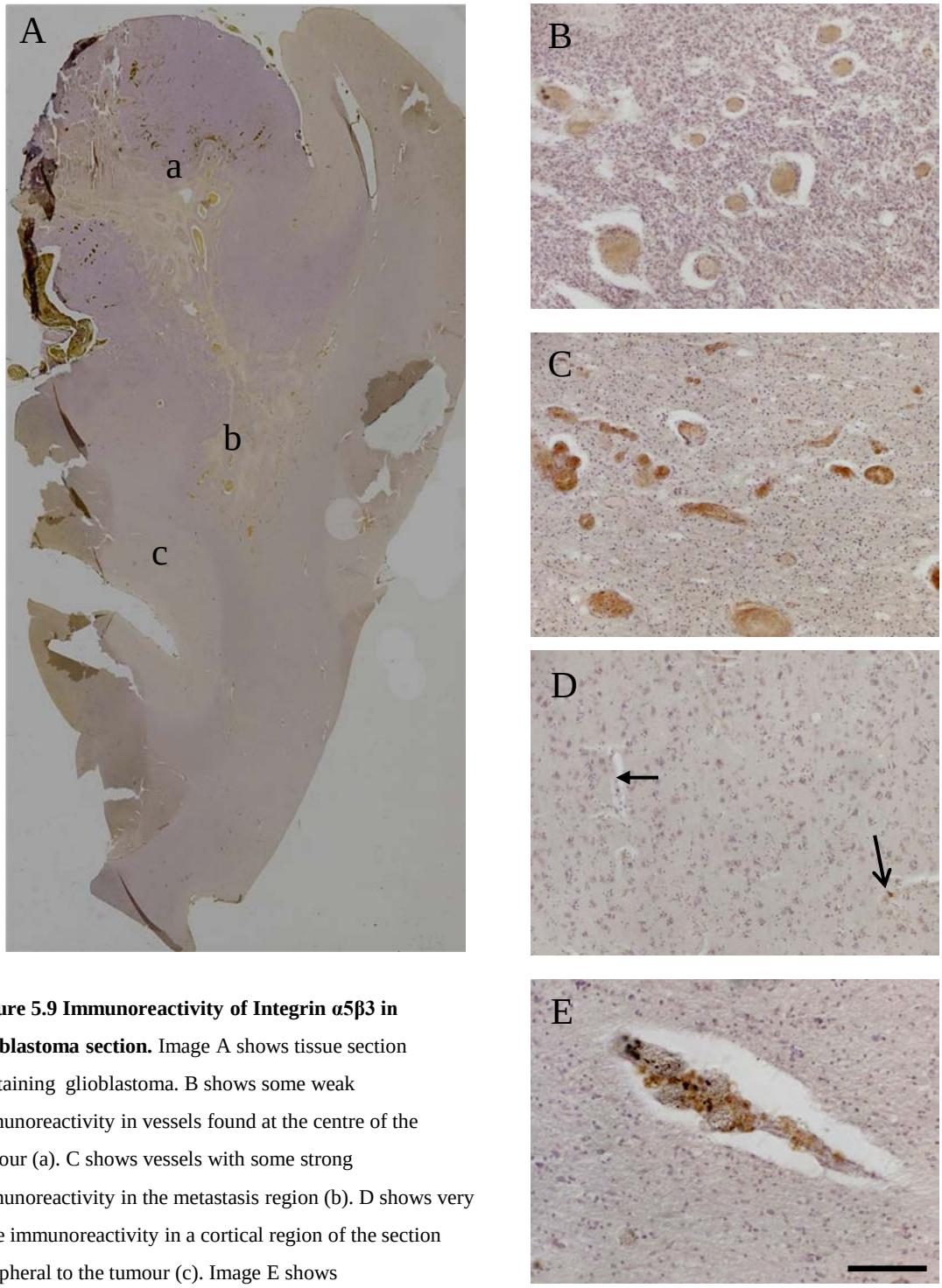


Figure 5.9 Immunoreactivity of Integrin α5β3 in glioblastoma section. Image A shows tissue section containing glioblastoma. B shows some weak immunoreactivity in vessels found at the centre of the tumour (a). C shows vessels with some strong immunoreactivity in the metastasis region (b). D shows very little immunoreactivity in a cortical region of the section peripheral to the tumour (c). Image E shows immunoreactivity along varying parts of the vessel length, mag bar = 40 μm for images B,C and 20 μm for D, E.

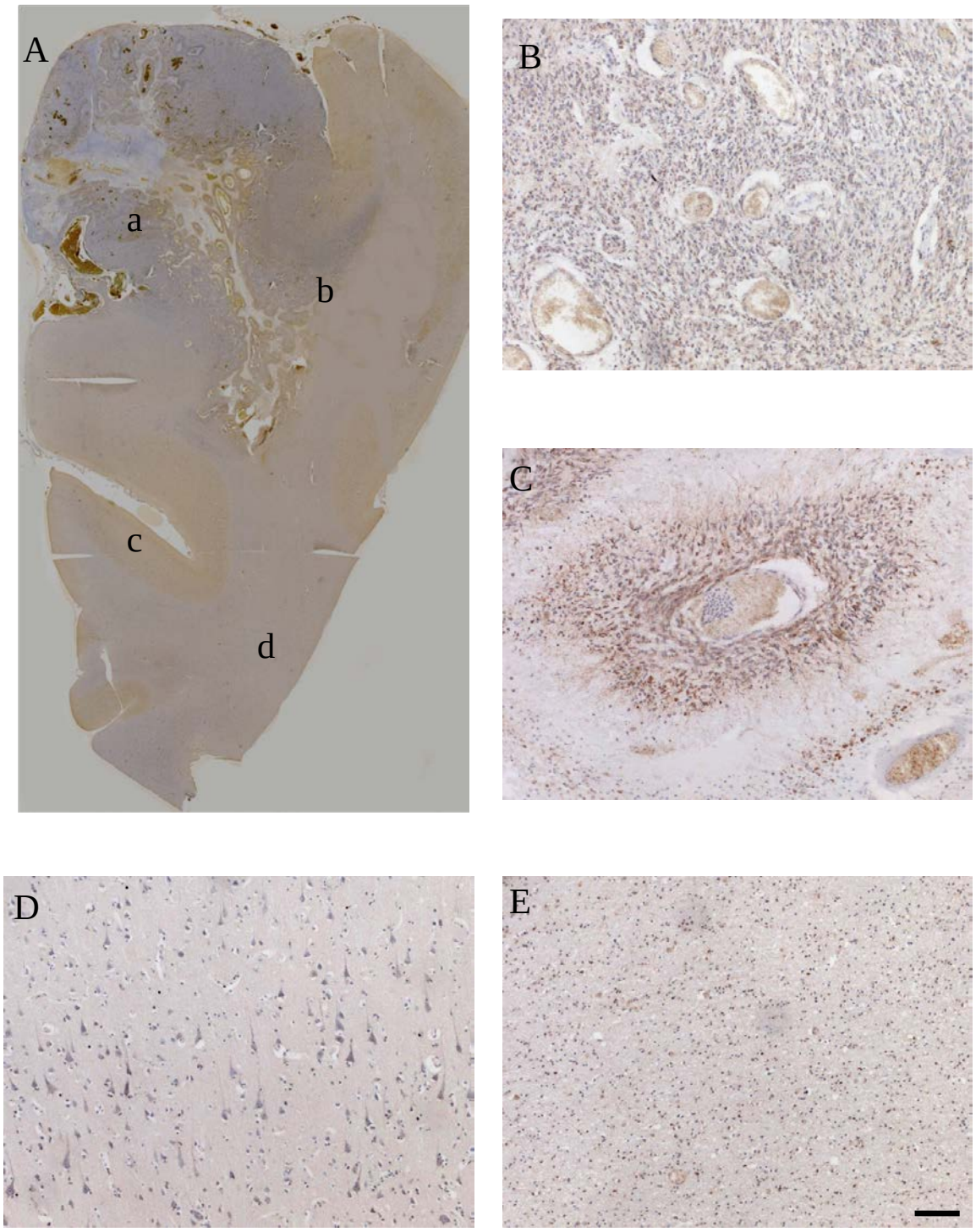


Figure 5.13 Immunoreactivity of VEGF in glioblastoma case. Image A shows a scanned image of the glioblastoma section and the letters correspond to the location of the other figure images. B shows little immunoreactivity at the centre of the tumour, with no clear staining of either cells or vasculature (a). C shows tumour cells surrounding a vessel in the metastasis region and tumour cells show strong immunoreactivity to the VEGF antibody with some possible staining also in nearby blood cells within vessels (b). D shows no immunoreactivity within a cortical region, which is distant from the tumour (c). E shows WM away from the expanding metastatis region, however some oligodendrocytes do strongly express the antigen. Mag bar = 100 μm

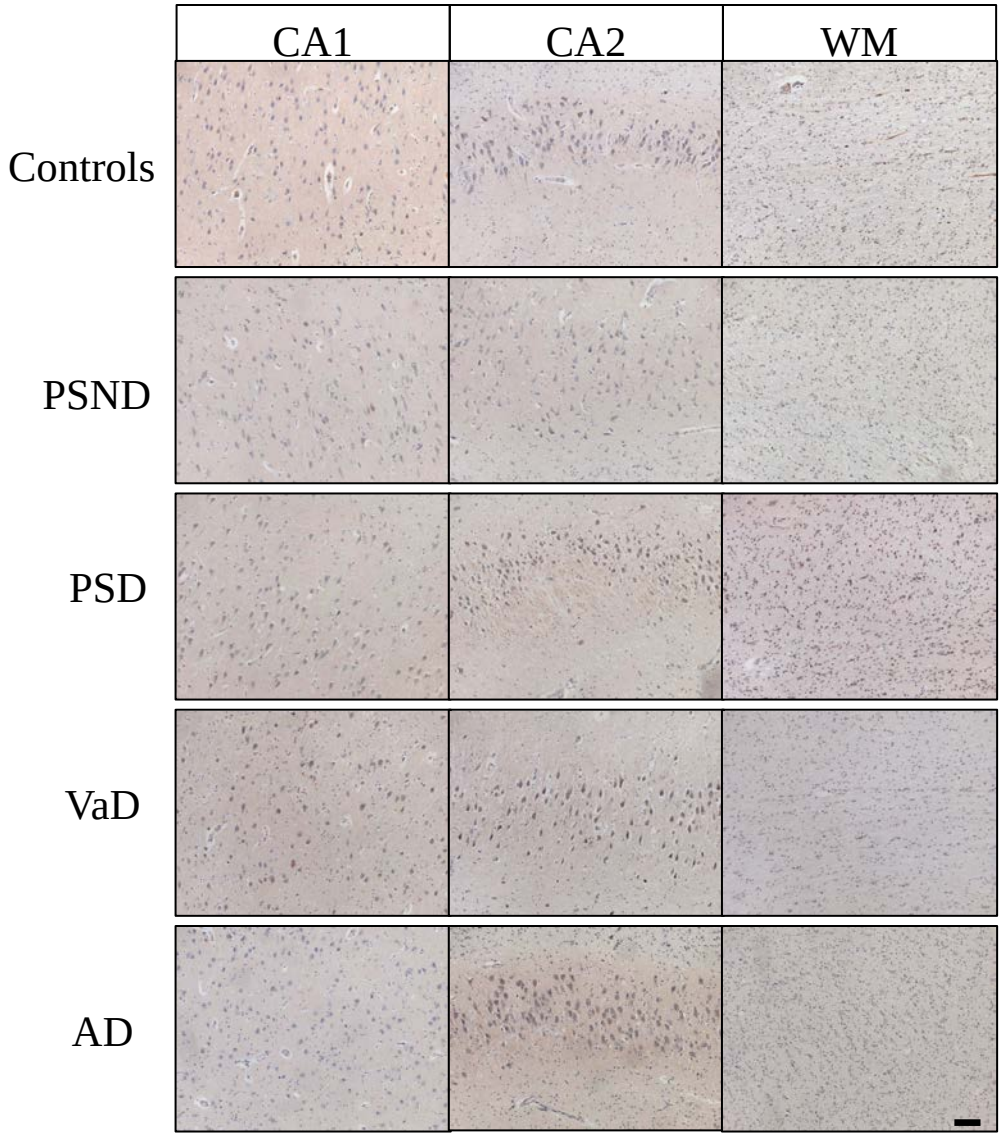


Figure 5.14 Images to show average immunoreactivity in the CA1, CA2 and nearby WM regions for all groups analysed. Immunoreactivity is limited in hippocampal tissue, when there is expression it appears to be strongest either in the CA2 or oligodendrocytes in the WM. Control WM shows possible staining of vessels, however this was a rare find. Mag bar = 100 μm.

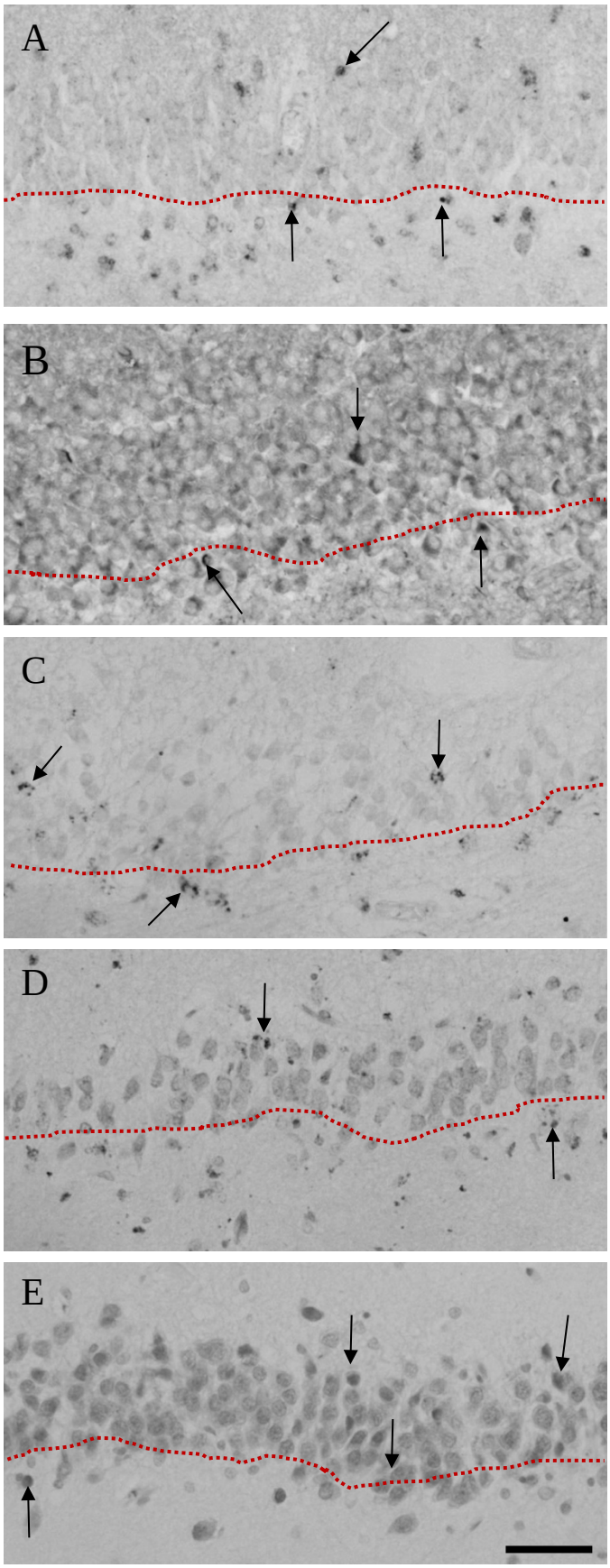


Figure 6.2 Neurogenic markers in the DG. Immunoreactivity with different neurogenic markers; MSI1 (A), nestin (B), PSAnCAM (C), DCX (D) and Hu C/D (E). Red line distinguishes approximate GCL with SGL. Arrows are used to highlight positive cells. Mag bar = 50 μm

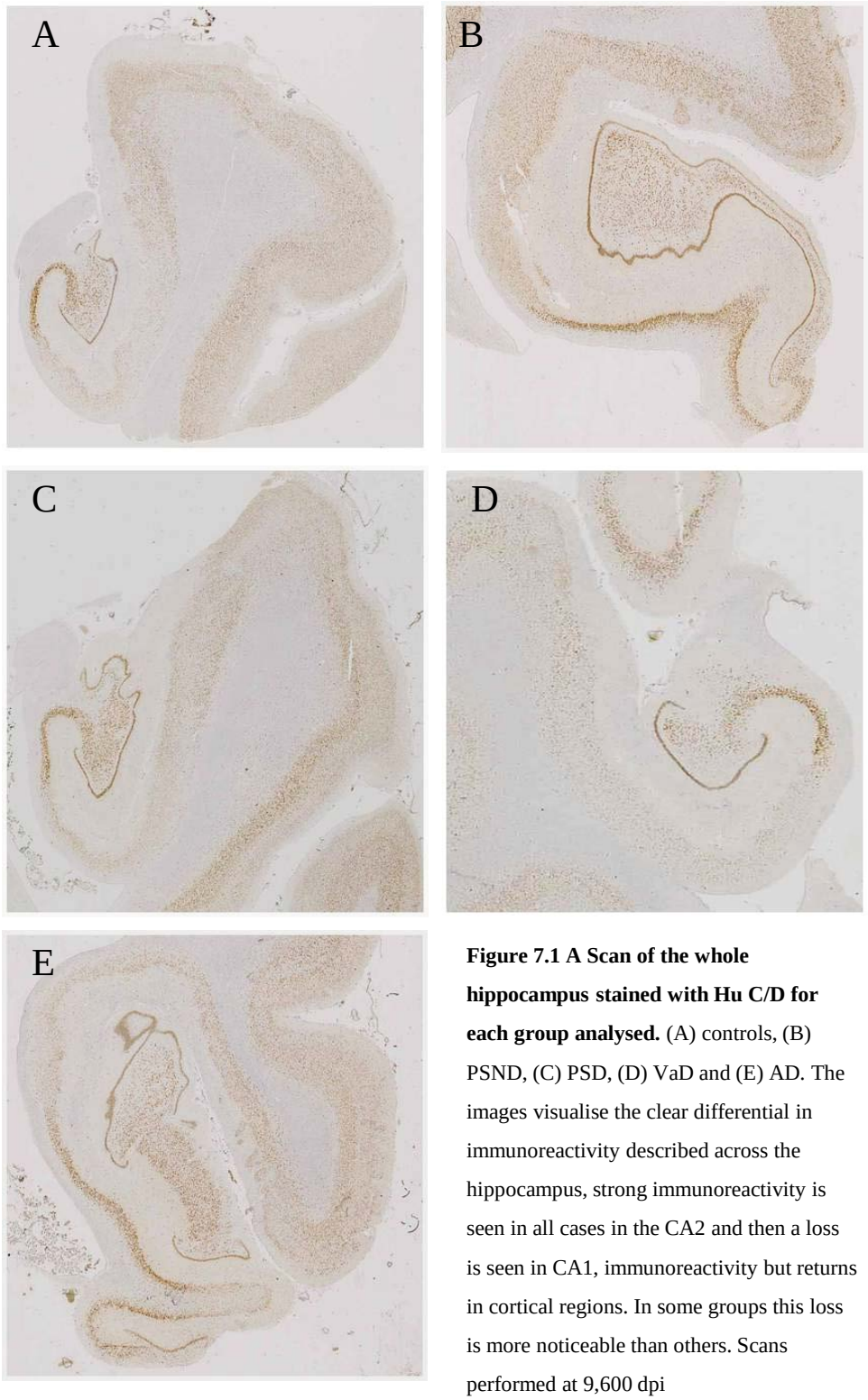


Figure 7.1 A Scan of the whole hippocampus stained with Hu C/D for each group analysed. (A) controls, (B) PSND, (C) PSD, (D) VaD and (E) AD. The images visualise the clear differential in immunoreactivity described across the hippocampus, strong immunoreactivity is seen in all cases in the CA2 and then a loss is seen in CA1, immunoreactivity but returns in cortical regions. In some groups this loss is more noticeable than others. Scans performed at 9,600 dpi

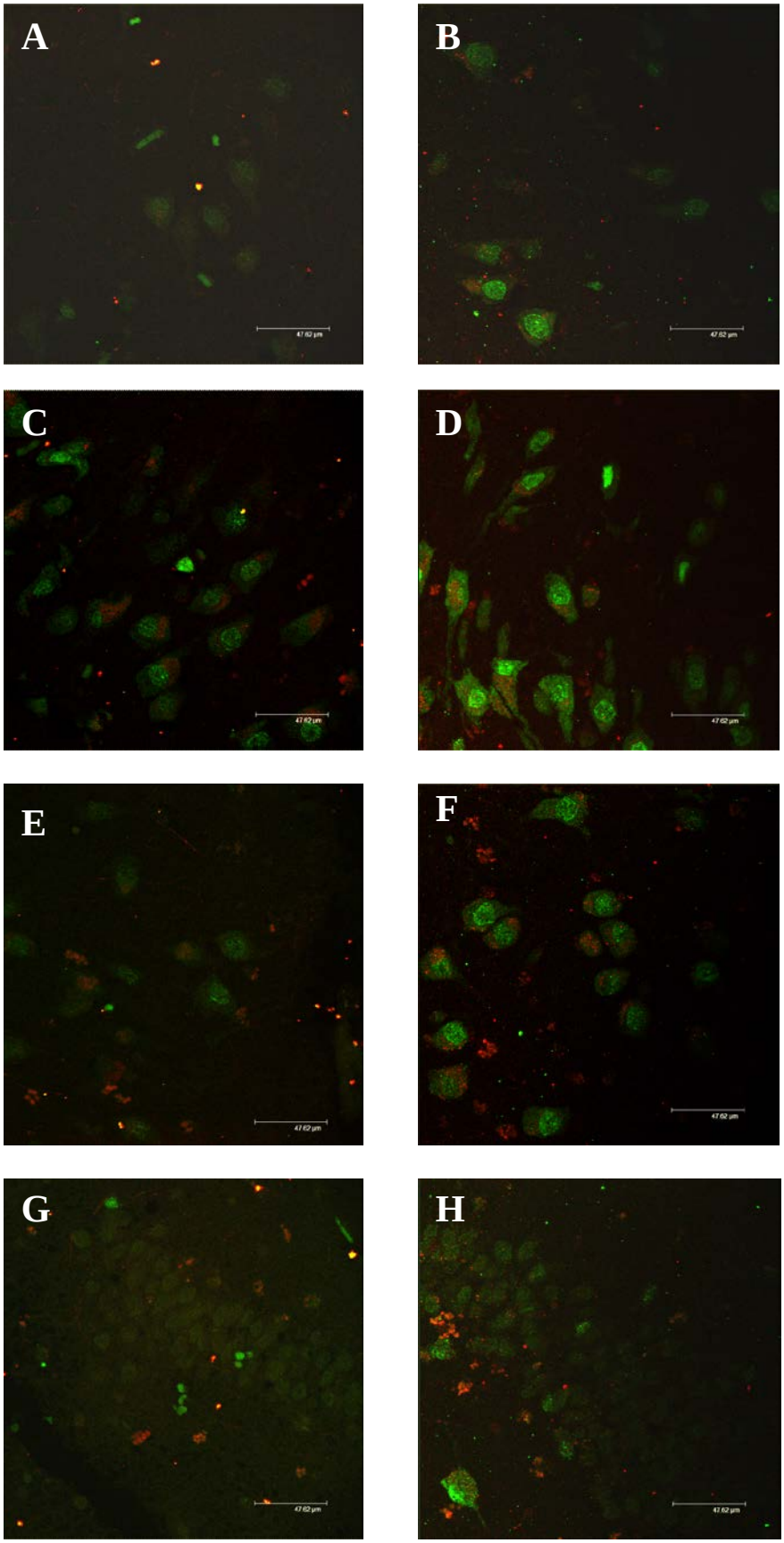


Figure 7.9 Double fluorescent labeling of Hu C or Hu D with Hu C/D. Images A,C,E and G show staining with Hu C and B,D,F and H shows immunoreactity with HuD. A and B show the CA1, C and D the CA2, E and F the CA4 and G and H the DG.

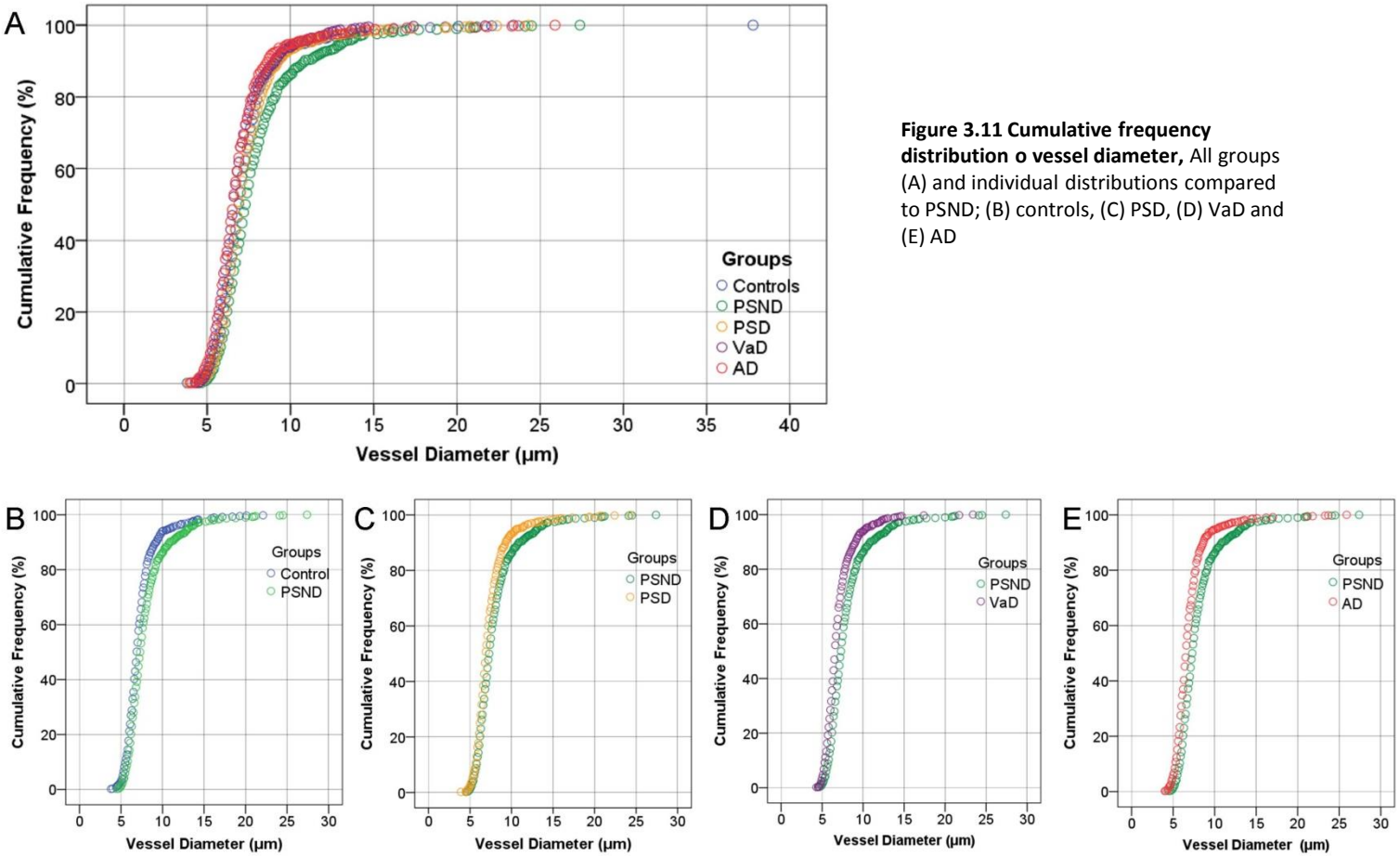


Figure 3.11 Cumulative frequency distribution o vessel diameter, All groups (A) and individual distributions compared to PSND; (B) controls, (C) PSD, (D) VaD and (E) AD

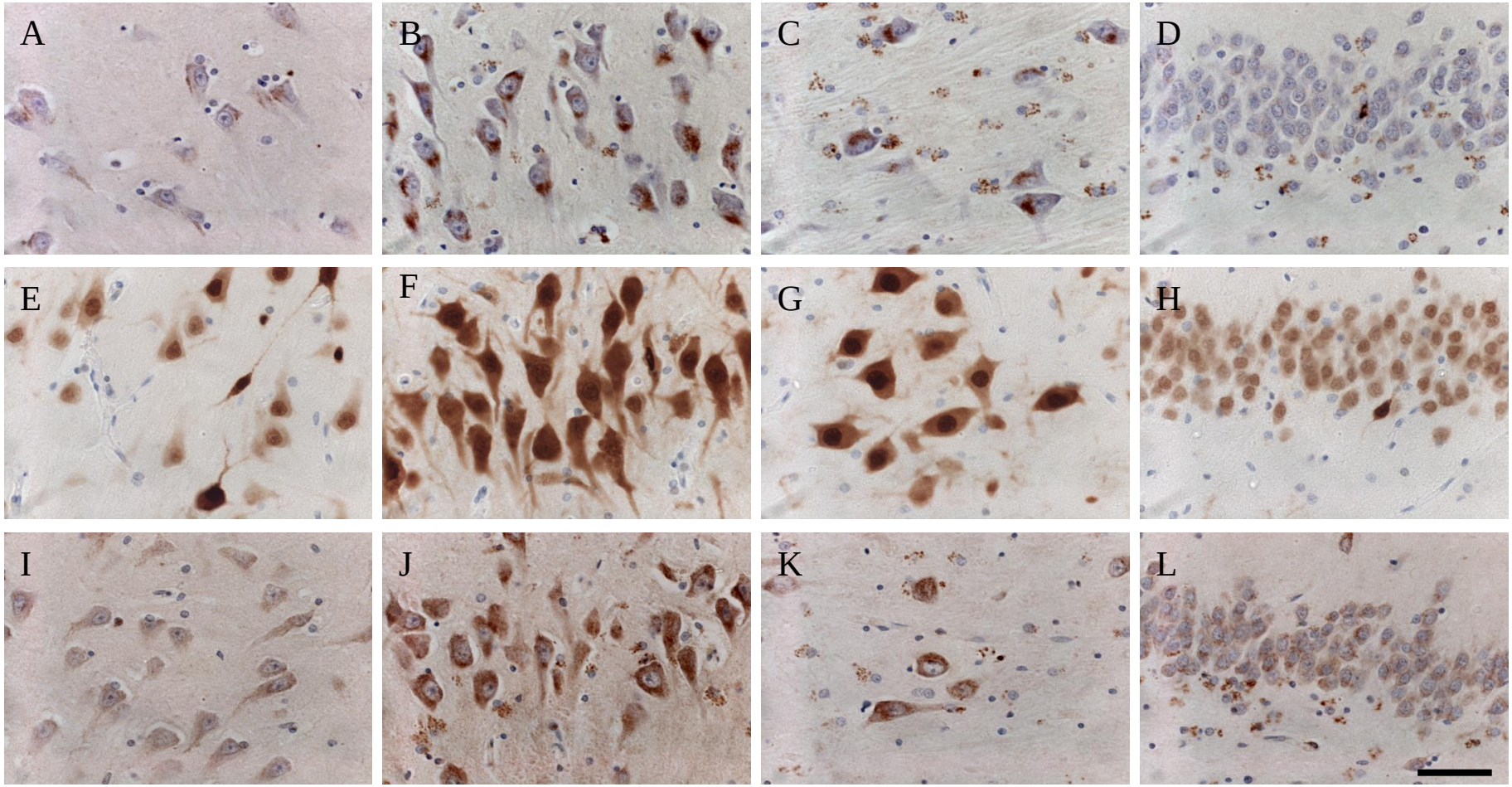


Figure 7.8 Showing varying staining of Hu C, C/D and D from the same subject in the hippocampus. Images horizontally, left to right, in the same order CA1, CA2, CA4 and DG. Image ordered vertically Hu C (top row), Hu C/D (middle row) and Hu D (bottom row). Mag bar = 50 μm