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“Take the option you can learn from and don’t be afraid to fail. Continue to be strong, because you created the ability to do what you do. And you can always rebuild.”

Brent Murray
1957-2005
Plasticity in the Human Alzheimer’s Disease Brain

Helen C. Murray

Abstract

Alzheimer’s disease (AD) is a progressive neurodegenerative disease and the leading cause of dementia. The characteristic symptoms of AD result from cortical atrophy that follows the spread of β-amyloid and tau aggregates through the brain. The earliest regions affected in AD are areas of high plasticity such as the hippocampus and entorhinal cortex, where new neurons are born (neurogenesis) and mature cells undergo structural and synaptic modification throughout life. It is thought that impaired plasticity in these regions may contribute to cortical atrophy and development of symptoms. There is currently only a partial understanding of the mechanisms that contribute to human brain plasticity and how they are affected in AD.

This thesis examined two mechanisms of plasticity in post-mortem human brain tissue from control and AD cases using immunohistochemistry. The first section of the thesis examined neuronal proliferation in the sub-granular zone (SGZ) and sub-ventricular zone (SVZ) neurogenic niches using cell counting methods on full tissue sections and tissue microarrays. The results of this quantification revealed that the number of proliferating cells in the SGZ and SVZ was unaltered in AD cases. However, there was an increase in the number of PSA-NCAM+ immature granule cells in the SGZ. This work indicates that neuronal proliferation is unaltered in AD, but changes may occur in the structural maturation of progenitor cells.

The second section of this thesis examined the distribution of PSA-NCAM expression in the human brain. PSA-NCAM is a membrane bound glycoprotein that mediates cell migration and structural plasticity through the regulation of cell-cell adhesion. PSA-NCAM distribution and function is commonly described in the SGZ and SVZ of the adult human brain, but there is limited evidence of its expression outside these regions. In this study, PSA-NCAM was found to be widely expressed throughout the adult human brain. Furthermore, PSA-NCAM+ cells were found in the caudate nucleus and cerebellum, two structures considered devoid of PSA-NCAM in the rodent brain. PSA-NCAM distribution was conserved throughout the AD brain except the entorhinal cortex, where PSA-NCAM staining load was significantly reduced and inversely correlated with tau load. These results indicate that PSA-NCAM-mediated plasticity is an important mechanism that is reduced in a region of the AD brain severely affected by aggregate pathology and associated with memory symptoms. Further characterisation of PSA-NCAM+ cells throughout the brain revealed an interneuron phenotype was predominant. An investigation of entorhinal cortex PSA-NCAM+ interneuron populations showed these cells were not selectively preserved or degenerated in AD. Overall this thesis has contributed key insights into neuronal proliferation and PSA-NCAM-mediated plasticity in the control and AD human brain.
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Chapter 6: Distribution of PSA-NCAM in Normal, Alzheimer's and Parkinson's disease Human Brain

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### Certification by Co-Authors

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- that the candidate wrote all or the majority of the text.

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<tr>
<td>Aβ</td>
<td>Beta-amyloid</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
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<td>BrdU</td>
<td>5-bromo-2′-deoxyuridine</td>
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<td>CA1</td>
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<tr>
<td>GCL</td>
<td>Granule cell layer</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acid protein</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase 3-beta</td>
</tr>
<tr>
<td>HP</td>
<td>Hippocampus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse-radish peroxidase</td>
</tr>
<tr>
<td>IOD</td>
<td>Integrated optical density</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule-associated protein 2</td>
</tr>
<tr>
<td>MAPT</td>
<td>Microtubule-associated protein tau</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MCM2</td>
<td>Minichromosome maintenance complex component 2</td>
</tr>
<tr>
<td>MTG</td>
<td>Middle temporal gyrus</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal nuclear antigen</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2a</td>
</tr>
<tr>
<td>PSA-NCAM</td>
<td>Polysialic acid neural cell adhesion molecule</td>
</tr>
<tr>
<td>PST</td>
<td>ST8SiaIV (sialyltransferase enzyme)</td>
</tr>
<tr>
<td>RMS</td>
<td>Rostral migratory stream</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SFG</td>
<td>Superior frontal gyrus</td>
</tr>
<tr>
<td>SGZ</td>
<td>Sub-granular zone</td>
</tr>
<tr>
<td>SM</td>
<td>Sensory-motor cortex</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>STX</td>
<td>ST8SiaII (sialyltransferase enzyme)</td>
</tr>
<tr>
<td>SVZ</td>
<td>Sub-ventricular zone</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TSA</td>
<td>Tyramide signal amplification</td>
</tr>
<tr>
<td>TUC-4</td>
<td>TOAD [Turned On After Division]/ Ulip/CRMP - 4</td>
</tr>
<tr>
<td>VC</td>
<td>Visual cortex</td>
</tr>
</tbody>
</table>
Chapter 1. General Introduction

Alzheimer’s disease (AD) is characterised by severe neurodegeneration and cognitive impairment. AD is particularly debilitating as there are only few treatments available that temporarily improve symptoms with varied effectiveness (reviewed by Nygaard, 2013). With an increasing prevalence of AD due to a worldwide aging population, there is a desperate need for strategies to alleviate symptoms, slow disease progression or delay disease onset. It has been estimated that delaying the onset of AD by 5 years would prevent up to 50% of cases (Brookmeyer et al., 1998; Wilson et al., 2011; Alzheimer Association, 2016). Therefore, AD prevention would significantly reduce the burden of disease for patients, caregivers, and public health systems.

Neuroplasticity refers to the brain’s ability to adapt and change throughout adult life and was described as the mechanism underlying learning and memory function as early as 1890 (reviewed by Berlucchi and Buchtel, 2009). Structural and molecular processes involved in neuroplasticity are hypothesised to maintain cognitive function during the preclinical phase of AD when pathological changes begin (Mufson et al., 2015). Therefore, reduced plasticity may contribute to AD symptom development and enhancement of endogenous brain plasticity is considered a promising strategy to delay disease onset (Lazarov and Marr, 2010; Rodríguez and Verkhratsky, 2011; Winner et al., 2011). However, fundamental understanding of the disease mechanism and the processes involved in functional compensation is lacking. Mechanisms of plasticity have been studied extensively in the rodent brain, but their role in the adult human brain is less defined. It remains to be determined which mechanisms of plasticity, if any, are promising targets for prevention of human AD and whether disease pathology affects their function.

In this thesis, two major processes involved in plasticity were examined in human brain tissue: neuronal proliferation and the remodelling of cell structure and connections. These two mechanisms of plasticity occur abundantly in brain regions that are severely affected by AD pathology and related to early AD symptoms (Winner et al., 2011). Impaired neuronal proliferation and structural plasticity may contribute to the development of AD symptoms, but enhancing these processes may also have therapeutic potential. Neuronal proliferation is hypothesised to have regenerative potential and increased structural plasticity is thought to maintain memory function (Mikkonen et al., 1999b; Diaz Brinton and Ming Wang, 2006). However, before plasticity can be investigated as a therapeutic treatment, it is necessary to determine whether endogenous levels of neuronal proliferation, and mechanisms of structural plasticity are affected by AD pathology.

Whether AD pathology affects neuronal proliferation in the human brain is currently unclear. New neurons are produced in the two neurogenic niches of the adult brain: the sub-granular zone (SGZ)
and sub-ventricular zone (SVZ) (reviewed by Curtis et al., 2012). Proliferation in these two regions of the human AD brain has been studied previously, but there is no clear consensus whether it is altered (Boekhoorn et al., 2006; Crews et al., 2010; Perry et al., 2012; Gomez-Nicola et al., 2014; Ekonomou et al., 2015). This lack of agreement can be attributed to the use of different quantification methods and proliferation markers between studies. Determining whether neuronal proliferation is affected in AD is crucial to assess whether it contributes to symptom onset or has regenerative potential. However, proliferation is only one, albeit important, process required for neuron production. Progenitor cell migration and maturation are also crucial and no study has yet examined these processes in the human brain. This thesis examined the extent of SGZ and SVZ proliferation in the AD brain using cell counting techniques to address the controversy in the field. A technique to assess progenitor cell structural maturation was also developed.

The remodelling of cell structure and synaptic connections is mediated by polysialylated neural cell adhesion molecule (PSA-NCAM), a membrane-bound glycoprotein that regulates cell-cell interactions (Theodosis et al., 1999; Eckhardt et al., 2000). PSA-NCAM has been extensively characterised in the rodent brain but little is known about its distribution in the adult human brain (Bonfanti, 2006). To date, PSA-NCAM has only been characterised in the medial prefrontal cortex, hippocampus and entorhinal cortex of the human brain (Mikkonen et al., 1999b; Varea et al., 2007b). It is also unclear whether only specific cell types utilise PSA-NCAM-mediated plasticity. This thesis investigated the distribution of PSA-NCAM in wider brain regions and used fluorescent double-labelling to characterise PSA-NCAM+ cell phenotypes in each region.

PSA-NCAM-mediated plasticity could provide valuable insight into the AD mechanism as pathology first accumulates in regions of high plasticity (Arendt et al., 1998). PSA-NCAM has been examined in one study of the human AD brain where it was found to be increased in the hippocampus (Mikkonen et al., 1999b). This study did not examine wider brain regions, despite extensive atrophy. Therefore, it remains to be determined whether PSA-NCAM is altered throughout the human AD brain. It is also unclear whether PSA-NCAM+ cells are preferentially preserved or degenerated in AD. These concepts were investigated in this thesis by quantifying PSA-NCAM staining load throughout the human brain in control and AD cases.

Overall, this thesis addresses fundamental questions about plasticity in the human brain. By examining the extent of neuronal proliferation and PSA-NCAM-mediated plasticity in AD it can be determined whether a reduction in these mechanisms contribute to symptom development. This study also provides valuable insight to whether enhancement of plasticity could aid in maintaining cognitive function and delaying disease onset.
Chapter 2. Literature Review

2.1 Alzheimer’s disease

Alzheimer’s disease (AD) is the leading cause of dementia in a worldwide aging population. In a 2015 estimate, 46.8 million people were living with dementia worldwide and this number is expected to increase by 9.9 million cases each year. By these estimates the number of people suffering from dementia will double every 20 years (Alzheimer’s Disease International, 2015). This sharp increase is due to a demographic transition toward an older worldwide population as a result of increased life expectancies. The cognitive deficits that occur as a result of AD pathology are debilitating for both patients and caregivers and place an incredible burden on elderly care systems. Globally, the cost of dementia care was an estimated US $818 billion in 2015, a 35% increase from 2010 (Alzheimer’s Disease International, 2015). From these sobering statistics, it is clear that AD is one of the major health care issues of modern times.

The risk of developing AD increases significantly with age. AD can be classified as either early onset (EOAD) or late onset (LOAD) depending on the age at which symptoms first appear. LOAD is determined by onset over 65 years old and is the most common form of the disease. EOAD can occur between 30 and 65 years old but is far less prevalent (Bekris et al., 2010). It is unclear how genetic and/or environmental factors may influence disease onset but the complexity of AD suggests it is likely to be multi-factorial. A clear genetic link has not been established for the majority of AD cases that are hence classed as sporadic. Familial AD accounts for just 10% of cases and has only been identified in EOAD (Bekris et al., 2010).

Of its many symptoms, AD is renowned for causing severe memory deficits. These deficits present as a slow, progressive decline in episodic memory from pre-clinical to late stage disease. Initially the memory deficits are classed as mild cognitive impairment (MCI): memory impairment beyond that expected for the person’s age but not so severe that it affects daily life (Minati et al., 2009). It begins as a subtle anterograde episodic memory deficit coupled with a temporally-graded retrograde episodic memory deficit. That is, the patient has difficulty forming new memories and recalling old memories. Memories of older events are preserved relative to recent events, but as the disease progresses, older memories are steadily lost with the most recent affected first (Jones et al., 2006; Minati et al., 2009). In addition to episodic memory disruption, short term memory and semantic memory (ideas and concepts) becomes progressively impaired (Small et al., 2003). Interestingly, implicit memory (the ability to learn a motor skill and retain it) remains relatively intact (Dick et al., 1995).
In addition to debilitating memory deficits, AD patients also suffer from cognitive symptoms, which include language deficits and impaired executive functions. Most language deficits are related to the progressive loss of semantic memory, but articulatory and phonological deficits increase with disease progression and mutism is common in severe cases (Minati et al., 2009). Early in the disease, many AD patients experience anosmia (loss of smell) and executive functions such as inhibition, task-switching, information manipulation and attention shifting are impaired (Serby et al., 1991; Collette et al., 1999; Devanand et al., 2000; Baddeley et al., 2001). Emotional and behavioural disturbances such as agitation, irritability, anxiety and apathy are also common. As the disease progresses, patients tend to become socially isolated and withdrawn. They become increasingly confused and aggressive, with the tendency to wander and perform aberrant movements and vocalisations. Patients are also known to experience delusions, hallucinations, dysphoria and euphoria (Mega et al., 1996; Minati et al., 2009).

Diagnosis of AD is based upon clinical history, neurological examination and cognitive testing. Therefore, the clinical onset of the disease is largely dependent on when the patient and their family distinguish the symptoms of MCI from the perceived deficits associated with normal aging. A confirmed diagnosis of AD is only possible by post-mortem histopathological assessment (Bekris et al., 2010). The histological criteria for diagnosis have changed very little from the initial description in 1906 by Alois Alzheimer, a German psychiatrist and neuropathologist. The presence of cortical atrophy, extracellular β-amyloid plaques and intracellular tau tangles at levels above normal aging are the key pathological features required for an AD diagnosis (Braak and Braak, 1991; Montine et al., 2012).

### 2.1.1 AD Pathology

#### 2.1.1.1 Atrophy

The most profound macroscopic pathology of AD is severe progressive cortical atrophy, although sub-cortical structures such as the amygdala and thalamus are also affected (Pini et al., 2016). The pattern of neurodegeneration is mainly conserved between AD cases and follows the deposition of pathological aggregates formed by β-amyloid and tau (Braak and Braak, 1991). Medial temporal structures including the entorhinal cortex and hippocampus are first affected. This atrophy is thought to result in a cortical disconnection from the hippocampus that underlies the early impairment of episodic memory (Nadel and Hardt, 2011). The hippocampal structure is not uniformly affected. Entorhinal cortex and CA1 pyramidal cells are susceptible to disease pathology and degenerate, while the CA3 pyramidal cells and dentate gyrus granule cells are relatively preserved (Morrison and Hof, 1997).
As the disease progresses, the parietal cortex degenerates, followed by the frontal cortex. Atrophy of these regions likely results in the cognitive and emotional impairments of AD (Pini et al., 2016). Regions such as the primary motor and sensory cortices, the visual cortex, striatum and cerebellum are relatively spared until late stages of the disease. The preservation of motor regions may be linked to the preservation of implicit memory (de Jong et al., 2011). Overall, this pattern of degeneration is likely to underlie the progression of AD symptoms. However, the exact mechanism of cell death and the contribution of β-amyloid and tau aggregates is still unclear.

2.1.1.2 Amyloid plaques

The abnormal abundance of extracellular β-amyloid plaques is a pathological hallmark required for AD diagnosis. Plaques predominantly accumulate in the parenchyma of the grey matter, although intracellular plaques and vascular deposition is also common (Finder and Glockshuber, 2007; Puzzo et al., 2015). The plaques are formed by the aggregation of 40 – 42 amino acid β-amyloid peptides (Aβ40/Aβ42), which are cleavage products of the transmembrane amyloid precursor protein (APP). The function of full length APP is unclear although it has been implicated in neuron survival, neurite outgrowth and memory function (Perez et al., 1997; Puzzo et al., 2011). APP is initially cleaved by either α- or β-secretase in the endoplasmic reticulum, resulting in two different cleavage pathways (Figure 2.1A). Cleavage by α-secretase initiates a non-amyloidogenic pathway that produces a soluble extracellular fragment (s-APPα) and a membrane bound C-terminal fragment of 83 amino acids (C83). Subsequent cleavage of C83 by γ-secretase forms an intracellular peptide (amyloid intracellular domain, AICD) and a small peptide referred to as P3. The amyloidogenic pathway is initiated by β-secretase cleavage of APP that produces a different sized soluble extracellular fragment (s-APPβ) and membrane bound C-terminal fragment (C99). C99 is further cleaved by γ-secretase, forming an AICD and either the 40 or 42 amino acid Aβ peptide (LaFerla and Oddo, 2005; Puzzo et al., 2015). The production of Aβ42 therefore requires both β-secretase and γ-secretase. Typically, Aβ42 comprises less than 10% of Aβ peptides produced and is the primary component of the extracellular amyloid plaques (Thinakaran and Koo, 2008). An overproduction of Aβ42 is thought to lead to plaque formation as this isotype is more prone to oligomerisation. Amyloid oligomers form protofibrils that extend into full fibrils and aggregate into large diffuse plaques (Figure 2.1B), Finder and Glockshuber, 2007). Mutations in APP and the presenilin proteins that form the catalytic sub-unit of γ-secretase result in increased Aβ42 production and have been linked to rare familial EOAD (Bekris et al., 2010). However, mounting evidence suggests plaques are unlikely to be the central pathology of the disease mechanism. Plaques can be relatively abundant in the brain of cognitively normal subjects and plaque load does not correlate with the extent of cognitive impairment in AD (Drachman, 2014).
An alternative hypothesis is related to the normal function of Aβ42. In normal physiological circumstances, Aβ42 production is upregulated by neuronal activity and thought to act via negative feedback to decrease neuronal activity. If Aβ42 production becomes unregulated, its aggregation into extracellular plaques may instead be a protective mechanism (Puzzo et al., 2015).

![Diagram of APP and Aβ42 cleavage and aggregation](image)

**Figure 2.1 Cleavage of APP and aggregation of Aβ42**

(A) APP can be cleaved by either α-secretase or β-secretase, leading to non-amyloidogenic or amyloidogenic cleavage respectively. Aβ42 is formed by the sequential cleavage of β-secretase and γ-secretase. (B) Aβ42 monomers can oligomerise and form protofilaments which subsequently form fibrils that aggregate into plaques. Adapted from LaFerla and Oddo, 2005; Finder and Glockshuber, 2007; Minati et al., 2009; Coppieters, 2015. Abbreviations: APP, amyloid precursor protein; AICD, amyloid intracellular domain; s-APP, soluble amyloid precursor protein; C83/C99 carboxy-terminal 83/99 amino acids; Aβ, amyloid beta.
2.1.1.3 Tau tangles

The presence of neurofibrillary tangles (NFT) is the second histopathology required for post-mortem diagnosis of AD. These intracellular inclusions are composed of paired helical filaments formed from hyperphosphorylated microtubule-associated protein tau (MAPT or tau). In its normal physiological role, unphosphorylated tau is involved in the assembly and stabilisation of microtubules (Figure 2.2A). The ability of tau to bind to microtubules is regulated by kinases and phosphatases which control tau phosphorylation (Figure 2.2B). When phosphorylated by glycogen synthase kinase 3 beta (GSK3β), tau is unable to bind microtubules. Dephosphorylation by protein phosphatase 2a (PP2A) removes this inhibition. In AD, increased levels of active GSK3β combined with a decrease in PP2A activity leads to abnormally high amounts of phosphorylated tau protein. This has both a loss of function effect, where microtubules become destabilised, and a gain of function effect due to the accumulation of NFTs. Hyperphosphorylated tau proteins form oligomers that are subsequently cleaved at the C and N termini, leaving a protease-resistant core (Figure 2.2C). This core can bind additional tau proteins, forming paired helical filaments and initiating an autocatalytic aggregation of tau into NFTs (Figure 2.2A, Harrington, 2012; Kolarova et al., 2012). The NFTs themselves have a toxic effect by disrupting local cellular processes and signalling. Unlike amyloid plaques, NFT load correlates closely with synapse loss and symptom progression.

![Figure 2.2](image)

**Figure 2.2** Formation of tau tangles
(A) Unphosphorylated tau functions to stabilise microtubules. (B) The phosphorylation of tau by glycogen synthase kinase 3 beta (GSK3β) inhibits its ability to bind to microtubules. Protein phosphatase 2a (PP2A) can remove this inhibition through dephosphorylation. (C) In AD, an upregulation of phosphorylated tau leads to the formation of oligomers that undergo proteolytic cleavage. This results in a protease-resistant oligomeric core. (D) The oligomeric core binds additional phosphorylated and unphosphorylated tau, forming protease-resistant neurofibrillary tangles (NFT) that accumulate inside neurons. Adapted from Harrington, 2012; Kolarova et al., 2012.
severity (Arriagada et al., 1992; Harrington, 2012). High NFT load is also always accompanied by
cognitive deficits and the deposition of NFTs can precede amyloid pathology by up to 20 years
(Duyckaerts, 2011). Furthermore, evidence suggests tau may mediate Aβ42 toxicity through
interaction at synapses (Ittner et al., 2010). These factors suggest tau is an important contributor
and possibly the cornerstone of the degeneration mechanism in AD.

2.1.1.4 Pathological staging of AD

Alone, none of the pathological features described above are unique to AD, but together they
represent a characteristic pathology specific to AD. For each case, the extent and severity of this
pathology is assigned a grade based on criteria outlined by Braak and Braak (1991). Brains that
are mildly affected by plaques and tangles are assigned a Grade I or II. In these cases the pathology
is largely isolated to the entorhinal cortex and below the threshold expected to cause neurological
symptoms (Braak and Braak, 1991). Grade I and II pathology is considered indicative of the
normal aging process.

Grade III and IV represents more severe pathology. Plaque and tangle pathology is observed in the
limbic regions, the entorhinal cortex is more severely affected and mild changes are observed in
the hippocampus. Macroscopic changes are not detectable at stage III and neocortical degeneration
is not obvious, therefore Grade III cases are not assigned an AD diagnosis. Mild cognitive
impairment is often apparent in Grade IV cases as the transfer of information between the
neocortex and the hippocampus is impaired (Braak and Braak, 1995). Grade IV therefore
represents mild dementia.

Grade V and VI pathology is used to confirm the clinical diagnosis of AD. In addition to pathology
in the temporal and limbic regions that is present in lower grades, Grade V and VI cases show
severe neocortical atrophy and aggregate load (Braak and Braak, 1991). Sub-cortical structures
such as the amygdala, thalamus, striatum, and substantia nigra are also affected. These grades are
indicative of end stage AD and the pathology is accompanied by severe dementia.
2.2 Human neurogenesis

The ability of the mammalian brain to produce new neurons throughout adult life (a process referred to as neurogenesis) is a relatively new concept. The field of adult neurogenesis has grown upon a foundation of challenging preconceived ideas about the brain. This is evidenced from the early discovery of multipotent precursors to the only recent acceptance of neurogenesis in the adult human brain. The history of the field illustrates how new technology and perseverance contributed to these landmark findings. An understanding of the neurogenic niches and the process of neuronal maturation reveals how adult neurogenesis occurs in two regions severely affected by AD pathology from early in the disease.

2.2.1 History of human neurogenesis

The notion that the adult brain does not produce new neurons or repair itself was held throughout most of medical history. The lack of mitotic activity in the adult brain fuelled the theory that the production of neurons ceased after development. In turn, it was believed that a dead neuron could not be replaced. Ramon y Cajal summarised this idea in his landmark paper in 1931:

“...In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree...” (Ramon y Cajal, 1931, translated 1991).

It would be another 13 years until this concept was challenged. By examining the origins of ependymal brain tumours, Globus and Kuhlenbeck showed mitotic activity of cells in the subependymal plate (now commonly referred to as the sub-ventricular zone) of the adult brain. They further proposed that this region contained multipotent precursors with the capacity to differentiate into both neurons and glia (Globus and Kuhlenbeck, 1944). This provided some of the first evidence of proliferative potential in the human brain. Further evidence emerged with the development of radioactive $[H^3]$thymidine analogues that were injected into an adult animal and incorporated into cellular DNA during mitosis. In the early 1960s, simultaneous studies emerged demonstrating the presence of cellular proliferation in the adult mouse and rat brain using this technology (Smart, 1961; Altman, 1962). In 1961 Smart et al. described the presence of $[H^3]$thymidine$^+$ neuroglia in the cortex, olfactory bulb and hippocampus of adult mice. Then in 1962 Altman and Das described the use of this technology to examine proliferation kinetics in rats after brain trauma. The presence of these thymidine analogues in glia and cortical neurons provided further conclusive evidence of neuronal proliferation in the adult mammalian brain. Altman and Das went on to show the presence of postnatal neurogenesis in the hippocampus, caudate nucleus

Despite the mounting evidence for adult neurogenesis in the mammalian brain, a strong stigma remained around the idea of neurogenesis in the adult human brain. This was fuelled by a primate study where \(^{\text{[H}^3\text{]thymidine}^{+}\) cells were identified in the caudate nucleus and hippocampus of juvenile rhesus monkeys but not adults. It was not until 1998 when Eriksson demonstrated hippocampal neurogenesis in the adult human brain that popular opinion swayed. In this landmark study, a thymidine analogue called BrdU was administered to five cancer patients aged between 57 and 72 years. They survived between 16 and 781 days and upon post-mortem analysis, in each case BrdU\(^{+}\) cells were identified in the hippocampus (Eriksson et al., 1998). This provided conclusive evidence that cellular proliferation occurred in the human brain. The field has since exploded with animal studies investigating the kinetics and neuronal fate of these proliferative cells as well as their migratory patterns and potential functional relevance. Inevitably the next challenge will be determining whether their findings translate to adult neurogenesis in the human brain.

In the years following Eriksson’s work, proliferation in the adult human sub-ventricular zone (SVZ) was also demonstrated (Curtis et al., 2003). Curtis et al. demonstrated the presence of this proliferative layer in post-mortem human brains and identified a 3-fold increase in proliferation in the Huntington’s disease brain. This provided further evidence that adult neurogenesis is a feature conserved between species and the first indication that the process can be altered in human neurodegenerative disease. By this time, it had been well documented that in the rodent brain SVZ neuroblasts migrate along a pathway known as the rostral migratory stream (RMS) to the olfactory bulb where they replace olfactory neurons. However, the apparent absence of this pathway in the human brain remained a strong counter-argument to the evidence of adult human SVZ neurogenesis. Opposition to this concept was finally quashed in 2007 when Curtis et al. found the human RMS by assessing mitotic cell marker labelling in sagittal sections of the human post-mortem brain (Curtis et al., 2007b). When corrected for orientation due to bipedal differences, the similarity of the human SVZ and RMS to that of the rodent brain finally provided conclusive evidence that neurogenesis occurred in the adult human brain.

The potential for adult neurogenesis to function as an endogenous repair mechanism for the brain makes it an exciting therapeutic target for brain injury and neurodegenerative disease. While there is evidence that SVZ proliferation is increased in Huntington’s disease and stroke patients, it is unclear whether these cells have a functional contribution to disease recovery. To understand
whether adult neurogenesis has therapeutic potential in Alzheimer’s disease it must first be determined whether the endogenous process is affected by the disease mechanism. This was the first objective of this thesis.

2.2.2 The neurogenic niches of the human brain

From the early work investigating mammalian neurogenesis, it emerged that proliferative neuronal precursors were concentrated in two distinct regions of the adult brain. The first of these regions is the sub-granular zone (SGZ) of the hippocampus. Neuronal precursors in this thin layer proliferate and migrate into the overlying granule cell layer where they mature and integrate into the learning and memory circuitry. The second region is the SVZ overlying the caudate nucleus and bordering the lateral ventricle. Neuronal precursors in this region migrate a much larger distance along the RMS to the olfactory bulb where they replace interneurons involved in olfaction. An understanding of how neuronal precursors mature in these two neurogenic niches provided neurochemical markers that can be used to detect them in the human brain. Furthermore, the location and function of neuronal precursors are relevant to the symptomatology of AD.

2.2.2.1 The hippocampus and neurogenesis in the sub-granular zone

The hippocampus is a highly plastic structure in the mammalian brain. The dynamic firing of the hippocampal circuitry in response to its variable inputs is considered a key component of the learning and memory network. The hippocampus lies within the temporal lobe and receives input from the cortical layers of the entorhinal cortex. These two regions are heavily affected by AD pathology and their degeneration contributes to the memory impairment that occurs in the disease.

The hippocampal circuitry comprises a trisynaptic loop that functions to integrate and consolidate new episodic memories. Afferent input from the entorhinal cortex is carried along the perforant pathway. These axons traverse the subiculum and synapse with granule cells in the dentate gyrus and pyramidal cells in the CA3 area (Figure 2.3A). The dentate gyrus is the central segment of the hippocampal formation and contains a dense band of granule cells. Immediately inferior to this granule cell layer (GCL) is a thin layer where proliferative precursors reside, known as the SGZ (Li et al., 2009; Duvernoy et al., 2013).

The axons of the dentate gyrus granule cells are called mossy fibres. They travel through the hilus toward the CA3 region where they synapse on the dendrites of pyramidal cells (Figure 2.3B). The axons of the CA3 pyramidal cells form the schaffer collateral projections which extend out to the CA1 region (Figure 2.3C). The CA1 neurons then project back to the entorhinal cortex (Figure 2.3D). In this way sensory information received by the entorhinal cortex is conducted through the
hippocampal circuit where it may be incorporated into new or existing memory networks or analysed relative to existing memory before returning to the entorhinal cortex (Li et al., 2009; Duvernoy et al., 2013).

The learning and memory processes mediated by this circuitry are dependent upon plasticity. A combination of long-term potentiation (LTP), long-term depression (LTD) and synaptic remodelling are involved in this plasticity. SGZ neurogenesis is thought to contribute to this plasticity by providing a reservoir of highly adaptable immature granule cells (Kempermann, 2011).

**Figure 2.3** The circuitry of the hippocampus
(A) Input from the entorhinal cortex travels via the perforant pathway through the subiculum to the granule cell layer and CA1 region. (B) The axons of the DG granule cells are referred to as mossy fibres and project to the CA3 pyramidal cells. (C) The CA3 axons are known as the Schaffer collateral projections and synapse with the CA1 pyramidal cells. (D) CA1 axons project back to the subiculum and the deep layer V of the entorhinal cortex. Adapted from Li et al., 2009. Abbreviations: DG, dentate gyrus; CA1, Cornu Ammonis area 1; CA3, Cornu Ammonis area 3; SUB, subiculum; EC, entorhinal cortex; SGZ, sub-granular zone; GCL, granule cell layer; IML, inner molecular layer; OML, outer molecular layer.
Current understanding of SGZ progenitor cell maturation is predominantly based on rodent studies as it is not possible to follow the maturation of individual progenitors in the human brain. The following discussion of SGZ neurogenesis is based on a comprehensive review of this process in the mouse brain by Kempermann, 2011 unless otherwise stated.

Adult neurogenesis in the SGZ occurs in four main stages; these include a proliferative precursor phase, an early survival phase, a post-mitotic maturation phase and a late survival phase. Each stage is characterised by certain cell morphologies and specific protein marker expression patterns (Figure 2.4). These markers are discussed further in section 2.2.3.

The precursor phase is a proliferative stage that produces a population of neuronal progenitors. Animal studies indicate that these progenitor cells arise from a subset of cells with radial glia-like morphology and protein expression patterns, referred to as type 1 cells. These cells reside in the SGZ with an apical dendrite that extends through the granule cell layer and branches in the inner molecular layer (Li et al., 2009; Kempermann, 2011; Mu and Gage, 2011). Type 1 cells express the astrocytic marker GFAP and the stem cell markers, nestin and Sox-2. Recent evidence suggests these cells divide asymmetrically by budding from the cell body to produce type 2 neural precursor cells (Namba et al., 2009). Type 2 cells have characteristic precursor morphology including small cell bodies with irregularly shaped nuclei and short horizontal processes. These cells are further classified into two subtypes. Type 2a cells have a similar neurochemical profile to type 1 cells, expressing GFAP, nestin and Sox-2, but lack the radial glia morphology. Conversely, type 2b cells do not express the glial marker GFAP and express additional markers specific to proliferation and neuronal lineage, such as NeuroD, Prox1, doublecortin, proliferating cell nuclear antigen (PCNA), Ki-67 and minichromosome maintenance complex 2 (MCM2).

From here the cells move into an early survival phase that signals the end of their proliferative capacity. In the rodent brain this proliferation phase generates a surplus of cells and a large proportion of the newly produced cells are eliminated prior to maturation and differentiation. These cells retain proliferation markers such as doublecortin but no longer express proliferation markers such as PCNA, nestin and Sox-2. They begin the process of neuronal differentiation and start migrating into the granule cell layer. Type 3 cells express PSA-NCAM, a key protein involved in migration, and receive excitatory GABA input from interneurons to support dendritic growth. Despite this migratory phenotype, adult born granule cells do not migrate far from the SGZ. The majority migrate no further than the inner third of the GCL (Kempermann et al., 2003). Furthermore, there is no evidence that progenitor cells from the SGZ migrate to any other areas of the brain in health or disease (Kempermann, 2011).
Once the immature granule cells are established in the GCL and committed to the neuronal lineage they enter a post-mitotic maturation phase. Functional differentiation occurs with the growth of axons, dendrites and the formation of synaptic connections. These type 4 cells continue to express migration markers such as PSA-NCAM and neuronal lineage markers such as NeuroD and Prox1 but now also express mature neuronal markers such as NeuN and differentiation markers such as calretinin and TUC-4. The population of immature granule cells continues to be culled during this phase of maturation.

The final stage of development is the late survival phase, in which type 5 cells undergo a period of fine tuning. By this stage the full dendritic tree has developed and the new granule cells receive glutamatergic input from the perforant pathway. Axon targeting occurs as the cells become integrated into the hippocampal pathway. This is critical for survival as cells without the correct excitatory connections are culled. Dendritic pruning also occurs and the GABA input from local interneurons becomes inhibitory. These cells no longer express developmental markers such as doublecortin, PSA-NCAM and calretinin, but are now indistinguishable from mature granule cell neurons and express calbindin (Kempermann, 2011).

Figure 2.4  Neurochemical markers expressed during adult hippocampal neurogenesis
SGZ progenitor cells express distinct neurochemical markers at each stage of neuronal maturation. Adapted from Ming and Song, 2005; Kempermann, 2011. Abbreviations of cell layers: ML, molecular layer; GCL, granule cell layer; SGZ, sub-granular zone; HIL, hilus.
Interestingly, the type 3, 4 and 5 immature granule cells have a lower threshold for LTP induction and higher LTP amplitude than the older mature granule cells (Schmidt-Hieber et al., 2004; Ge et al., 2007). These unique excitatory properties could be a mechanism that allows new granule cells to integrate into the hippocampal circuitry (Ge et al., 2007). The production of a highly plastic population of granule cells may also be an important function of adult neurogenesis, in addition to maintaining the granule cell population during aging. The distinctive properties of newborn granule cells likely permit them unique functions, at least for a certain time (Li et al., 2009). While the exact purpose of this immature granule cell population is unknown, hippocampal neurogenesis is thought to contribute to the detection and processing of novel stimuli and/or the temporary storage of information (Kempermann, 2002; Leuner et al., 2006). Whatever their specific purpose may be, the excitability of immature granule cells undoubtedly contributes to the overall plasticity of the hippocampal circuit. In turn, alterations to adult hippocampal neurogenesis due to AD pathology may have a profound effect on this plasticity and overall hippocampal function. This concept forms the basis for the first results chapter of this thesis.

2.2.2.2 Neurogenesis in the sub-ventricular zone

The SVZ is located on the lateral wall of the lateral ventricle and lies between the ciliated ependymal cell layer and the caudate nucleus. The SVZ in the adult human brain is a remnant of the embryonic SVZ which forms from a proliferative hub known as the lateral ganglionic eminence during brain development (Globus and Kuhlenbeck, 1944). Compared to the SGZ, the SVZ produces far more neural precursors in the adult human brain (Low et al., 2011; Curtis et al., 2012).

The SVZ of the adult human brain has a layered structure (Figure 2.5). Layer I consists of a single layer of ciliated ependymal cells that form the border of the lateral ventricle. Beneath this a cell-deficient ‘gap’ layer consisting of ependymal extensions and astrocytic processes, referred to as layer II. This layer is a feature of the human and non-human primate SVZ, but is absent in rodents. Layer III contains migrating neuroblasts (type A cells) which are surrounded by astrocytic (type B) cells. Layer IV consists of dense myelin fibres which form the boundary of the SVZ. This layer is also absent in rodents (Doetsch et al., 1997; Curtis et al., 2005b; Quiñones-Hinojosa et al., 2006).

SVZ neuroblasts (type A cells) are produced from type C transient amplifying cells (Figure 2.6). These type C cells are less numerous and reside deep in layer III, close to the myelin of layer IV. In the rodent brain, type C cells are produced by proliferating type B astrocytic cells that line layer III of the SVZ (Doetsch et al., 1999). However, it has not yet been possible to confirm this in the human SVZ. The type C cells have a high proliferative capacity and either produce type A cells or revert back to type B cells. The ratio of these three cell types (A: B: C) in the human SVZ is 1:
3: 1, indicating that the number of astrocytic type B cells far exceeds the transient amplifying type C cells or type A neuroblasts (Curtis et al., 2007b).

SVZ neuroblasts migrate a considerable distance compared to their counterparts in the SGZ. In the rodent brain, the majority of SVZ neuroblasts migrate to the olfactory bulb (Altman, 1969; Lois and Alvarez-Buylla, 1994). However, these cells have also been shown to migrate to the underlying caudate nucleus in response to stroke or growth factor injection (Benraiss et al., 2001; Arvidsson et al., 2002; Parent et al., 2002; Yamashita et al., 2006). From the SVZ, olfactory bulb-

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**Figure 2.5  Anatomy of the adult human SVZ**

The SVZ borders the lateral ventricle (LV) and has a layered structure. In the human SVZ Layer I consists of ependymal cells while layer II is a cell-deficient gap layer. Neuroblasts (type A cells) and astrocytic type B cells are located in layer III. Transient amplifying type C cells are located deeper within this layer. Layer IV consists of myelin fibres and forms the border between the SVZ and the underlying caudate nucleus (not shown). Adapted from Curtis et al., 2007a, 2012.
destined neuroblasts migrate ventrally along the SVZ. At the ventral boundary of the lateral ventricle the cells move in a caudo-ventral direction along the underside of the caudate nucleus. This path is referred to as the descending limb of the RMS. At the most ventral aspect of the caudate nucleus, the neuroblasts turn rostrally and migrate towards the olfactory bulb. This section of the RMS is referred to as the rostral limb (Curtis et al., 2007b; Kam et al., 2009). In the human brain, neuroblasts migrate in small clusters along the RMS. The regulation of this migratory process is poorly understood but PSA-NCAM is thought be an integral component. By insulating the neuroblasts from neighbouring cells and the extracellular matrix, PSA-NCAM facilitates this long-distance migration and prevents premature differentiation. The neuroblasts begin functional differentiation into olfactory interneurons once they reach the olfactory bulb. Approximately 95% of the cells that reach the bulb are thought to become granule cells while the other 5% become either dopaminergic or GABAergic periglomerular neurons (Bédard and Parent, 2004; Curtis et al., 2009).

The exact contribution of adult born interneurons to olfactory function remains elusive. Olfactory stimulation has been shown to influence the number of adult born olfactory neurons in the rodent brain suggesting these cells have an activity dependent function (Petreanu and Alvarez-Buylla, 2002; Rochefort et al., 2002). Furthermore, the survival of adult born olfactory neurons is enhanced by odor discrimination tasks, suggesting these cells may be involved in olfactory learning (Alonso et al., 2006). Whether these functions are conserved in the human brain is undetermined. A recent study using C\textsuperscript{14} dating showed the human olfactory bulb had very little postnatal neuronal turnover (Bergmann et al., 2012; Ernst et al., 2014). It is possible that the majority of SVZ neuroblasts are eliminated during integration or that few actually reach the olfactory bulb. In either case, it is not possible to predict the amount of olfactory bulb neurogenesis.
from the amount of SVZ proliferation (Bergmann et al., 2015). This is an important consideration when determining how SVZ neurogenesis may be altered in disease. SVZ proliferation and olfactory bulb neurogenesis is particularly relevant to AD as a large proportion of patients experience anosmia (loss of smell) prior to the manifestation of other clinical symptoms (Devanand et al., 2000).

2.2.3 The limitations and challenges of studying human neurogenesis

The quantification of neurogenesis in the human brain is fraught with challenges. The inability to use retroviral transfection on post-mortem tissue and the ethical considerations regarding the use of thymidine analogues make the identification of neural precursors difficult. As such, studies investigating human neurogenesis are limited to the use of neurochemical markers. Choosing the correct neurochemical marker is an important consideration. To obtain reliable results, markers need to have stable expression and a relatively long half-life since post-mortem delay of human tissue is often variable and extensive. Every marker has specific limitations that should also be considered. The method used to quantify these markers is particularly important as not all produce meaningful data and some can obscure the true effect.

2.2.3.1 Markers of proliferation and neuronal maturation

Since most proliferation markers indicate only certain phases of the mitotic cycle, it is critical to understand the physiological basis of the marker to make valid inferences from its quantification. It is important to note that some markers can also be expressed by mature cells undergoing DNA repair. In addition, proliferation markers only indicate cells that were mitotically active at the time of death, so it is not always possible to determine whether these cells would have followed a neuronal or glial lineage. This implies the proliferating cell population does not necessarily represent the neural progenitor cell population and the proportion of mitotically active cells that will successfully differentiate and mature into neurons cannot be predicted. As such, the quantification of proliferating cells should not be extrapolated to infer the net amount of neurogenesis. The assessment of markers specifically expressed by immature neurons is currently the best method to assess neurogenic output in post-mortem human tissue, although this too has limitations. Commonly used markers of proliferation and maturation in the human brain include the following:

Nestin

Nestin is an intermediate filament expressed by progenitor cells and a commonly used marker of proliferation. However, its immunoreactivity is not particularly specific as blood vessels also label
for nestin (Palmer et al., 2000). Furthermore, there is evidence that mitotically active precursors can be nestin-negative and astrocytes without neurogenic potential can be nestin-positive (Kukekov et al., 1997; Wilhelmsson et al., 2006; Kempermann, 2011). This lack of specificity makes nestin a questionable marker for use in human tissue. Despite this, it has been used to assess proliferation by several studies (Ziabreva et al., 2006; Perry et al., 2012; Ekonomou et al., 2015).

**PCNA**

PCNA is a cyclin protein associated with DNA polymerase δ and is expressed during the G1 and S phase of mitosis. It is commonly used as a marker of proliferation in human studies as it has a relatively long half-life and can be reliably detected in cases with long post-mortem delay (Bravo and Macdonald-Bravo, 1987; Hall et al., 1990; Curtis et al., 2003; Low et al., 2011). PCNA does have certain limitations that should be considered. Immunoreactivity can produce variable staining intensities depending on the mitotic stage of the cell at time of death (Kempermann, 2011). PCNA also only labels cells that were mitotically active at the time of death, essentially providing a snapshot of proliferation at this timepoint. Lastly, PCNA can be expressed by mature cells undergoing DNA repair, so co-labelling with mature neuronal markers should be performed to ensure the analysis is representative of progenitor cells (Tomasevic et al., 1998). Overall, PCNA is a reliable marker of proliferation in human neurogenic niches provided these factors are considered.

**Musashi-1**

Musashi-1 is an RNA-binding protein that functions to maintain the stem cell phenotype by regulating protein expression (Katz et al., 2014). Musashi-1 is commonly used to identify proliferative cells in the human neurogenic niches, although in-vivo characterisation of the protein is lacking and it is unknown whether expression can be reactivated in mature cells. There is also evidence to suggest Musashi-1 is expressed by non-proliferating cells outside the neurogenic niches (Maslov et al., 2004). Therefore, quantification of proliferation using musashi-1 should be interpreted with caution. If used in conjunction with other proliferation markers it can be a useful marker of human neurogenesis.

**Ki67**

Ki67 is a cell cycle-associated protein expressed during G1, S, G2 and M phases of mitosis. It is the broadest cell cycle antigen, although its exact function is unknown (Endl and Gerdes, 2000; Zacchetti et al., 2003; Kempermann, 2011). Ki67 is considered a specific and reliable marker of mitotic cells. Its use on human post-mortem tissue can be problematic due to its short half-life of
one hour and susceptibility to proteolytic cleavage (Endl and Gerdes, 2000; Zacchetti et al., 2003). Despite this, Ki67 has been used to label proliferating cells in previous studies of neurogenesis in human AD (Boekhoorn et al., 2006; Gomez-Nicola et al., 2014).

**Doublecortin**

Doublecortin is a microtubule-associated protein involved in migration through neurite extension (Meyer et al., 2002). Doublecortin is transiently expressed during neuronal maturation so it is commonly used to identify type 2b, type 3 and type 4 immature granule cells in the SGZ and type A cells in the SVZ (Kempermann, 2011). However, it is not a specific marker of immature neurons as it is also expressed by mature cells outside the neurogenic niches (Liu et al., 2008). Therefore, when used in studies of neurogenesis, doublecortin needs to be assessed specifically within the neurogenic niche. Doublecortin immunohistochemistry labels the full dendritic structure of a cell which can be useful for morphological studies. Doublecortin is susceptible to degradation during extended post-mortem delays so it is not an ideal marker for quantification using human tissue (Boekhoorn et al., 2006; Monje et al., 2007).

**PSA-NCAM**

PSA-NCAM is a membrane bound glycoprotein that regulates migration of progenitor cells. Like doublecortin, PSA-NCAM is transiently expressed by immature type 3 and 4 granule cells in the SGZ and by type A cells in the SVZ (Seki and Arai, 1991a; Curtis et al., 2007b; Kam et al., 2009; Kempermann, 2011). PSA-NCAM has also been identified in non-neurogenic regions of the rodent brain (reviewed by Bonfanti, 2006) so when used to study neurogenesis it must be assessed specifically within the neurogenic niches. As PSA-NCAM can be reliably labelled in post-mortem tissue using immunohistochemistry, it is a good marker for studying human neurogenesis. PSA-NCAM will be discussed further in section 2.3.

**β-III tubulin**

β-III tubulin is an isotype of tubulin found in the cytoskeleton of immature neurons in vitro. The in vivo characterisation of β-III tubulin is lacking so it is unknown whether β-III tubulin expression can be switched back on in mature neurons or if other cell types express it. Therefore, β-III tubulin is not an appropriate marker for use in human tissue, although it has been used in a previous study of neurogenesis in the human SGZ and SVZ (Perry et al., 2012).
Calretinin

Calretinin is a calcium binding protein used to identify post-mitotic immature granule cells in the SGZ. Calretinin is transiently expressed by immature granule cells and is replaced by calbindin once these cells become fully mature (Brandt et al., 2003). Outside of the GCL calretinin is expressed by interneurons, so it can only be used as a maturation marker within the SGZ neurogenic niche. Despite its stable expression and specificity, calretinin has only been used in one previous study of SGZ neurogenesis in the human AD brain (Gomez-Nicola et al., 2014).

NeuN

Neuronal Nuclei (NeuN) is a neuron-specific nuclear protein, although a phosphorylated form of the protein is found in the cytoplasm of neurons (Lind et al., 2005). NeuN is a stable and reliable marker of neurons in human tissue. Co-labelling of NeuN with a maturation marker such as PSA-NCAM is often used to indicate the neuronal lineage of immature cells. It should be noted that some neuronal cells do not express NeuN, such as cerebellar Purkinje cells and mitral cells in the olfactory bulb (Mullen et al., 1992).

2.2.3.2 Quantification of cell proliferation in the human brain

To ensure biologically relevant data is obtained, the method used to quantify progenitor cells labelled using immunohistochemistry is crucial. The relatively low abundance of progenitor cells in the two neurogenic niches and the heterogeneity of their distribution makes conventional stereological quantification difficult. For example, almost the entire SGZ would need to be analysed to obtain an adequate coefficient of error and satisfy the requirements of the stereology method (Kempermann, 2011). Instead, many studies of human neurogenesis use a simplified cell counting system that involves analysing the neurogenic niche in a number of evenly spaced sections from throughout the structure (Eriksson et al., 1998; Curtis et al., 2005a; Boekhoorn et al., 2006; Low et al., 2011; Ekonomou et al., 2015).

Normalisation of the raw cell counts to a reference parameter is another important consideration and is crucial for a meaningful interpretation of the counts. The number of progenitor cells per tissue section can give an indication of abundance; however, it does not account for the different sizes of the neurogenic niche between sections and between cases. The number of progenitor cells per unit length of the SGZ/SVZ is a commonly presented parameter. This is a reasonable indication of abundance as it accounts for different sized niches and the length of the niche can be accurately determined. Similarly, progenitor cells per unit area or volume is a useful measure, although the area of the neurogenic niches is anatomically difficult to define. This is particularly an issue for
the SGZ, as no anatomical landmark distinguishes this region from the GCL. The SGZ is classically defined in the rodent brain as a three nuclei wide zone that includes the most inferior layer of cells in the GCL and a two nuclei wide band into the hilus (Eriksson et al., 1998; Kempermann, 2011). This is a rather arbitrary definition that is complicated in the human brain as the GCL is not sharply delineated from the hilus. Therefore, cell counts are often normalised to the area of the entire GCL (Boekhoorn et al., 2006; Lucassen et al., 2010; Low et al., 2011). In contrast, the human SVZ is a distinct area, delineated from the caudate nucleus by a layer of myelin. The myelin layer is considered a boundary zone; however, as type C cells often infiltrate this layer, it should perhaps be included in the measurement of SVZ area (Curtis et al., 2005b, 2012). Different interpretations of these anatomical definitions between studies can have a significant effect on the resulting conclusion.

Another option is normalisation to total cells within the neurogenic niche. This too is plagued by the issue of delineating the SGZ and SVZ. Furthermore, this measurement may not be biologically relevant as it is unclear whether the number of non-progenitor cells in the niche is influenced by the amount of proliferation, for example if more progenitor cells attract additional glial support. Comparing to the total number of cells in the destination structure (GCL of the hippocampus or olfactory bulb) is also flawed as it is unknown what proportion of progenitor cells reach these structures, migrate elsewhere or are culled. Furthermore, in neurodegenerative diseases such as AD, accumulation of activated astrocytes and microglia can also confound total cell counts.

While cell counting methods and different types of normalisation all have limitations, they provide a far more relevant measurement of proliferation than other available methods. The measurement of integrated optical density (IOD) is one such example. This quantification method examines the mean average grey value of immunoreactivity in a region of interest. The intensity of immunoreactivity is then used as an indication of antigen abundance. This quantification method has several critical flaws. Firstly, it is not able to discriminate between true progenitor cell staining and non-specific staining, which makes IOD measurement prone to false-positives and overestimation. This is a problem for human tissue as non-specific staining due to post-mortem delay and fixation is often unavoidable and also, proliferation markers such as nestin can label blood vessels or astrocytes (see section 2.2.3.1 above). Secondly, automated analysis of IOD often does not account for the intensity of background staining which can vary considerably between cases and again result in overestimation. Lastly, IOD quantification is particularly flawed if applied to immunoperoxidase labelling. In this staining method, the brown precipitate that labels the antigen scatters light, therefore the relationship between the amount of antigen and the intensity of staining is not linear (van der Loos, 2008). In summary, IOD is not a reliable quantification
method for human neurogenesis, although it has been presented in previous studies using human tissue (Li et al., 2008; Perry et al., 2012).

The above discussion illustrates that quantification of progenitor cells in studies of human neurogenesis is a difficult task. The choice of neurogenesis markers, the cell counting method and the method of normalisation are all important considerations for this research. Comparison of common neurogenesis markers indicates that some are more suitable for use in human tissue than others. PCNA, PSA-NCAM and calretinin seem to be reliable markers of proliferation and maturation in the human brain based on current evidence, provided their specific limitations are considered. Since no method of normalisation for cell counts is clearly superior, good practice would be to present all methods to form a comprehensive conclusion.

2.2.4 Neurogenesis in the AD brain

SGZ neurogenesis has been extensively examined in a variety of transgenic mouse models of AD. These studies provide conflicting conclusions about whether proliferation is altered, with different models indicating SGZ BrdU+ cells are increased, decreased or unchanged (Table 1). There is even disagreement between studies using mouse models with the same mutations (Haughey et al., 2002b; Krezymon et al., 2013). Comparison across a range of studies provides a general consensus that SGZ differentiation is impaired in AD mouse models (Table 1). SVZ proliferation is less commonly investigated in AD mouse models and is affected differently in each model (Table 2). Differentiation of SVZ progenitors in the olfactory bulb is infrequently examined in AD mouse models. Overall, AD mouse models provide no clear indication of how pathology affects adult neurogenesis.

When comparing neurogenesis in AD mouse models to the human disease, several factors should be considered. Firstly, mouse models are based on familial forms of AD and possess combinations of APP, presenilin and MAPT mutations, yet most human AD cases are sporadic with no identified genetic cause. Secondly, no transgenic model fully replicates the complex pathology of human AD. APP and presenilin mutations increase the Aβ42: Aβ40 ratio or the overall production of both Aβ species and MAPT mutations increase the hyperphosphorylation of tau (Cai et al., 1993; Eckman et al., 1997; Nilsberth et al., 2001; Herl et al., 2009). However, even in the triple transgenic model that encompasses all three mutations, there is an absence of the characteristic neuronal loss that occurs in human AD (Oddo et al., 2003; Schaeffer et al., 2011). Therefore, it is difficult to reconcile conclusions from mouse models with those of human AD. Thirdly, studies of neurogenesis in AD mouse models often use BrdU to detect proliferating cells and doublecortin to detect differentiated cells. These markers are not commonly used for human studies as BrdU
administration is accompanied by ethical concerns and doublecortin labelling is sensitive to post-mortem delay. It should also be noted that BrdU labels cells that have proliferated in the time since its administration, while cell cycle markers used in human studies (such as PCNA) only indicate proliferation at the time of death. Lastly, the age of mice used in these studies (3 – 18 months) is not necessarily representative of sporadic human AD onset (> 65 years). Proliferation is far higher in the young brain compared to the aged brain and proliferation in the neurogenic niches of the aged mouse brain is far more extensive than that of the human brain. Therefore, it is likely that proliferation in the mouse brain and human brain are affected differently by AD pathology. Together, these factors suggest that an understanding of how AD pathology affects neurogenesis is best obtained from post-mortem human tissue.

The investigation of SGZ neurogenesis in the human AD brain has so far provided contradictory conclusions. Increased SGZ neurogenesis in the AD brain was first described by Jin et al., 2004b due to the detection of increased doublecortin and PSA-NCAM on western blots of hippocampus homogenate. This study was fundamentally flawed as the analysis was not isolated to the SGZ and both PSA-NCAM and doublecortin are expressed by non-proliferating cells in the wider hippocampus. Furthermore, western blotting has limited quantitative power and it is not possible to discriminate between glial and neuronal proliferation. The conclusion from Jin et al., 2004b was subsequently contradicted by Boekhoorn et al., 2006. This study quantified proliferation in the hippocampus using cell counts and found the increase described by Jin et al., 2004b was due to glial proliferation in the CA1 region of the hippocampus. In the GCL, the number of Ki67+ cells/mm² was unchanged in AD cases, therefore suggesting SGZ proliferation was unaltered in AD.

Other studies that examined SGZ proliferation using cell counts have produced further conflicting results. In contrast to Boekhoorn et al., 2006, a study by Gomez-Nicola et al., 2014 found an increase in Ki67+ and calretinin+ cells/mm² of the GCL, suggesting increased SGZ proliferation and maturation in AD. Conversely, Ekonomou et al., 2014 found that the number of PCNA+ cells/mm of the SGZ was unchanged in AD cases. Notably, this study normalised PCNA cell counts to SGZ length rather than area. Finally, Crews et al., 2010 specifically limited their counts to the SGZ area and described a decrease in doublecortin+ cells/mm². As previously stated, the use of doublecortin for human post-mortem studies is problematic due to its sensitivity to post-mortem delay (Boekhoorn et al., 2006; Monje et al., 2007). Comparing these three studies is difficult as they each use slightly different methods of normalisation for their cell counts.
Two other studies of human SGZ neurogenesis used IOD to quantify proliferating cell markers. Li et al., 2008 measured IOD of MAP2 isoforms and described a decrease in progenitor cell maturation while Perry et al., 2012 found a decrease in musashi-1 IOD, but an increase in nestin and PSA-NCAM IOD. As previously discussed, IOD is not a reliable indication of proliferating cell number and is easily confounded by non-specific staining, which may explain these disparate findings (van der Loos, 2008). Overall, the studies that have so far investigated SGZ proliferation offer no clear consensus as to whether the process is upregulated, downregulated or unchanged in AD.

There are very few studies of SVZ neurogenesis in human AD and these also do not provide a clear conclusion. Only Ekonomou et al., 2014 provides quantification using cells counts, which indicate the number of nestin+ cells/mm of the SVZ are unaltered in AD cases. Two further studies provide conflicting results regarding musashi-1 and nestin immunoreactivity. Ziabreva et al., 2006 found musashi-1 staining load was decreased in the AD SVZ but nestin was increased, while Perry et al., 2012 measured IOD and found SVZ nestin was increased while musashi-1 was unchanged. These results are difficult to reconcile due to the disparate methods of quantification. Furthermore, nestin is not an ideal marker for SVZ proliferation as it also labels cells associated with blood vessels, which are abundant in the SVZ (Palmer et al., 2000). From these limited studies, it is apparent that further investigation of SVZ proliferation in human AD is necessary.
Table 1: SGZ neurogenesis in AD mouse models

<table>
<thead>
<tr>
<th>Reference</th>
<th>Mouse Model</th>
<th>Age</th>
<th>Proliferation</th>
<th>Maturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Jin et al., 2004)</td>
<td>APP&lt;sub&gt;(Swe,Ind)&lt;/sub&gt; mice</td>
<td>12M</td>
<td>↑ BrdU</td>
<td>↑ DCX</td>
</tr>
<tr>
<td>(López-Toledano and Shelanski, 2007)</td>
<td>APP&lt;sub&gt;(Swe,Ind)&lt;/sub&gt; mice</td>
<td>3M</td>
<td>↑ BrdU, Ki67</td>
<td>↑ BrdU*/βIII tubulin*</td>
</tr>
<tr>
<td>(Kanemoto et al., 2013)</td>
<td>APP&lt;sub&gt;(Swe,Ind)&lt;/sub&gt; mice</td>
<td>5W</td>
<td>↑ BrdU</td>
<td>↔ BrdU*/NeuN*</td>
</tr>
<tr>
<td>(Haughey et al., 2002b)</td>
<td>APP&lt;sub&gt;(Swe)&lt;/sub&gt; mice</td>
<td>12M</td>
<td>↓ BrdU</td>
<td>↓ BrdU*/PSA-NCAM*</td>
</tr>
<tr>
<td>(Krezymon et al., 2013)</td>
<td>APP&lt;sub&gt;(Swe)&lt;/sub&gt; mice</td>
<td>3M, 5M, 12M</td>
<td>↑ BrdU</td>
<td>↓ BrdU*/DCX*</td>
</tr>
<tr>
<td>(Donovan et al., 2006)</td>
<td>APP&lt;sub&gt;(Ind)&lt;/sub&gt; mice</td>
<td>12M</td>
<td>↓ BrdU</td>
<td>↓ DCX</td>
</tr>
<tr>
<td>(Wen et al., 2004)</td>
<td>PS-1 mutant mice</td>
<td>3M</td>
<td>↔ BrdU</td>
<td>↓ BrdU*/βIII tubulin* ↔ PSA-NCAM</td>
</tr>
<tr>
<td>(Wang et al., 2004)</td>
<td>PS-1 mutant mice</td>
<td>3M</td>
<td>↓ BrdU</td>
<td>-</td>
</tr>
<tr>
<td>(Chevallier et al., 2005)</td>
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<td>3M</td>
<td>↑ BrdU</td>
<td>↔ BrdU*/NeuN*</td>
</tr>
<tr>
<td>(Chen et al., 2008)</td>
<td>PS-1, PS-2 KO mice</td>
<td>7 – 9M</td>
<td>↑ BrdU</td>
<td>↓ DCX</td>
</tr>
<tr>
<td>(Verret et al., 2007)</td>
<td>APP&lt;sub&gt;(Swe)&lt;/sub&gt;/PS-1 mutant mice</td>
<td>6M</td>
<td>↔ Ki67</td>
<td>↓ BrdU*/NeuN*        ↓ BrdU*/DCX*</td>
</tr>
<tr>
<td>(Zhang et al., 2007)</td>
<td>APP&lt;sub&gt;(Swe)&lt;/sub&gt;/PS-1 mutant mice</td>
<td>9M, 18M, 24M</td>
<td>↓ MCM2</td>
<td>↓ DCX</td>
</tr>
<tr>
<td>(Niidome et al., 2008)</td>
<td>APP&lt;sub&gt;(Swe)&lt;/sub&gt;/PS-1 mutant mice</td>
<td>9M</td>
<td>↔ PCNA</td>
<td>-</td>
</tr>
<tr>
<td>(Demars et al., 2010)</td>
<td>APP&lt;sub&gt;(Swe)&lt;/sub&gt;/PS-1 mutant mice</td>
<td>2M</td>
<td>↓ BrdU</td>
<td>↓ BrdU*/DCX*</td>
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<tr>
<td>(Hamilton and Holscher, 2012)</td>
<td>APP&lt;sub&gt;(Swe)&lt;/sub&gt;/PS-1 mutant mice</td>
<td>3M, 5M, 10M, 15M</td>
<td>↓ BrdU</td>
<td>↓ DCX</td>
</tr>
<tr>
<td>(Faure et al., 2011)</td>
<td>APP&lt;sub&gt;(Swe, Lon)&lt;/sub&gt;/PS-1 mutant mice</td>
<td>6M</td>
<td>↓ Ki67</td>
<td>↓ DCX</td>
</tr>
<tr>
<td>(Rodriguez et al., 2008)</td>
<td>APP&lt;sub&gt;(Swe, tau)&lt;/sub&gt;/PS-1 mutant mice</td>
<td>3 – 12M</td>
<td>↓ Histone H3</td>
<td>-</td>
</tr>
<tr>
<td>(Hamilton et al., 2010)</td>
<td>APP&lt;sub&gt;(Swe, tau)&lt;/sub&gt;/PS-1 mutant mice</td>
<td>11M, 18M</td>
<td>↓ BrdU, Ki67</td>
<td>↓ DCX</td>
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</table>
Table 2: SVZ neurogenesis in AD mouse models

<table>
<thead>
<tr>
<th>Reference</th>
<th>Mouse Model</th>
<th>Age</th>
<th>Proliferation</th>
<th>Maturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Haughey et al., 2002a)</td>
<td>APP&lt;sub&gt;(Swe)&lt;/sub&gt; mice</td>
<td>12M</td>
<td>↓ BrdU</td>
<td>-</td>
</tr>
<tr>
<td>(Jin et al., 2004)</td>
<td>APP&lt;sub&gt;(Swe,Ind)&lt;/sub&gt; mice</td>
<td>12M</td>
<td>↑ BrdU, DCX</td>
<td>-</td>
</tr>
<tr>
<td>(Niidome et al., 2008)</td>
<td>APP&lt;sub&gt;(Swe)/PS-1&lt;/sub&gt; mutant mice</td>
<td>9M</td>
<td>↔ PCNA</td>
<td>-</td>
</tr>
<tr>
<td>(Rodríguez et al., 2009)</td>
<td>APP&lt;sub&gt;(Swe)/tau/PS-1&lt;/sub&gt; mutant mice</td>
<td>3–12M</td>
<td>↓ Histone H3</td>
<td>-</td>
</tr>
<tr>
<td>(Hamilton et al., 2010)</td>
<td>APP&lt;sub&gt;(Swe)/tau/PS-1&lt;/sub&gt; mutant mice</td>
<td>11M, 18M</td>
<td>↓ BrdU, Ki67</td>
<td>↓ Mash-1</td>
</tr>
</tbody>
</table>

Abbreviations: APP, amyloid precursor protein; PS-1, presenilin-1; M, months; W, weeks; BrdU, bromodeoxyuridine; DCX, doublecortin; MCM2, minichromosome maintenance complex 2.

Note: APP<sub>(Swe)</sub> mutation K670N M671L causes increased Aβ42 and Aβ40 production and amyloid pathology. APP<sub>(Ind)</sub> mutation V717F and APP<sub>(Lon)</sub> mutation V717I increase Aβ42/Aβ40 ratio. Various PS-1 mutations result in altered γ-secretase activity and Aβ42/Aβ40 production.
Chapter 2

2.3 The role of PSA-NCAM in neurogenesis and structural plasticity

PSA-NCAM is highly expressed during brain development and progressively down-regulated during adolescence and adulthood. In the adult brain, PSA-NCAM is predominantly referred to as a mediator of progenitor cell migration in the SGZ and SVZ. However, PSA-NCAM is also expressed by mature cells outside the neurogenic niches to facilitate structural plasticity. This PSA-NCAM-mediated structural plasticity has been extensively examined in the rodent brain, but there is limited evidence of its distribution or function in the human brain. It is also unknown whether PSA-NCAM-mediated plasticity is affected in neurodegenerative conditions such as AD. This section will outline the structure and function of PSA-NCAM, which is mainly derived from rodent studies. It will also examine the current understanding of PSA-NCAM-mediated plasticity in the control and AD human brain.

2.3.1 Structure and function of PSA-NCAM

Neural cell adhesion molecule (NCAM) is a widely-expressed glycoprotein found on the outer membrane of cells throughout the central nervous system. Alternative splicing of the NCAM gene produces three different sized isoforms of NCAM. The 140 and 180 kDa isoforms have unique cytoplasmic domains while the 120 kDa isoform lacks this domain entirely (Edelman and Crossin, 1991). The extracellular region of all three isoforms contains five immunoglobulin (Ig1-5) and two fibronectin (FN1-2) domains (Figure 2.7A). NCAM proteins from neighbouring cells dimerise through adhesion of their Ig1 and Ig2 domains (Kasper et al., 2000). A proline-rich domain between Ig5 and FN1 forms a flexible hinge that can modify the orientation of the dimerisation and control the distance between membranes (Johnson et al., 2004).

The homophilic adhesion of NCAM proteins can be regulated by addition of polysialic acid (PSA) to the Ig5 domain (Figure 2.7A - B). The polysialylation of NCAM regulates its dimerisation through steric inhibition of the Ig1 and Ig2 binding sites (Yang et al., 1994; Johnson et al., 2004). PSA is a large hydrated polyanion consisting of between 8 and 100 sialic acid (α2-8-linked N-acetylneuraminic acid) monomers (Mühlenhoff et al., 1998). PSA is almost exclusively found on NCAM, so commercially available antibodies that recognise 8 – 14 sialic acid monomers allow the specific identification of PSA-NCAM in tissue.

PSA is constructed and added to NCAM by two sialyltransferases found in the Golgi apparatus: ST8SiaIV (PST) and ST8SiaII (STX). NCAM140 and 180 isoforms are commonly polysialylated, however NCAM120 is mostly devoid of PSA (Oltmann-Norden et al., 2008). The polysialylated NCAM is secreted from the Golgi apparatus in vesicles which fuse with the outer cell membrane.
Cell surface expression of PSA-NCAM can therefore be regulated by its synthesis and degradation. Cell activity can regulate the transcription of sialyltransferases and PSA-NCAM exocytosis (Kiss and Rougon, 1997; Bonfanti, 2006). Removal from the cell surface by endocytosis is mediated by integrins and can be increased by NMDA receptor-mediated calcium currents (Bouzioukh et al., 2001; Monzo et al., 2013). Endocytosis of PSA-NCAM can also be inhibited by insulin or insulin-like growth factor-1 (IGF-1) (Monzo et al., 2013). Lastly, PSA can be cleaved from NCAM by a bacterial endosialidase (EndoN) in experimental conditions, but it is unclear whether endogenous sialidases cleave PSA in vivo (Vimr et al., 1984).

The ability of PSA-NCAM to disrupt cell-cell adhesion by steric inhibition is the basis of its many functions. Once insulated from its surroundings by PSA-NCAM, a cell can undergo processes such as migration, synaptic reorganisation, and axon-dendrite growth and remodelling (Hu et al., 1996; Seki and Rutishauser, 1998; Theodosis et al., 1999; Eckhardt et al., 2000; Dityatev et al., 2004). PSA-NCAM therefore mediates the growth and remodelling of cell structure, a process known as structural plasticity. These processes are critical for the maturation of cells during brain development and adult neurogenesis. However, outside the neurogenic niches, many mature cells in the adult brain are dynamic and frequently undergo dendritic or synaptic remodelling mediated by PSA-NCAM (Bonfanti, 2006). This structural plasticity may be required for specific cell functions. For example, PSA-NCAM facilitates the synaptogenesis and spine enlargement that occurs during LTP in hippocampal neurons (Dityatev et al., 2004; Gascon et al., 2007; Guirado et al., 2014). Alternatively, PSA-NCAM-mediated plasticity can be regulated by cell activity or neurological injury (Muller et al., 1996; Bonfanti, 2006).

PSA-NCAM can also affect the activation of nearby receptors via steric inhibition. The large anionic structure of PSA can block dimerisation of neighbouring receptors or block soluble factors from binding their corresponding receptor (Figure 2.7C, D reviewed by Bonfanti, 2006). Conversely, PSA can enhance activation of nearby receptors by sequestering soluble factors, thereby enhancing their availability to the receptor (Figure 2.7E reviewed by Schnaar et al., 2014). Therefore, PSA-NCAM is capable of modulating receptor signalling.
Figure 2.7  Structure and function of PSA-NCAM
(A) NCAM is a transmembrane protein containing five immunoglobulin (Ig) domains and two fibronectin (FN) domains on its extracellular region. Polysialic acid (PSA) is a large hydrated polyanion that is added to the Ig5 domain. (B) Cell–cell adhesion is mediated by dimerisation of NCAM proteins on neighbouring membranes. Addition of PSA to NCAM leads to steric inhibition that prevents NCAM adhesion. This facilitates processes such as cell migration, synaptic reorganisation, and axon-dendrite growth and remodelling. PSA-NCAM can also inhibit dimerisation of other adhesion proteins (C) or receptors (not shown). PSA-NCAM can regulate cell signalling by blocking soluble factors from binding their respective receptor (D) or sequestering soluble factors which increases their availability to the receptor (E). Adapted from Bonfanti, 2006; Schnaar et al., 2014.
2.3.2 Distribution of PSA-NCAM in the adult mammalian brain

The distribution of PSA-NCAM has been extensively described in both neurogenic and non-neurogenic regions of the rodent brain (reviewed by Bonfanti, 2006). In the human brain, however, studies of PSA-NCAM have largely been limited to the hippocampal formation, allocortex and neurogenic niches. In both the rodent and human hippocampus, PSA-NCAM is abundant in the SGZ where it is expressed by type 3 and 4 immature granule cells (Seki and Arai, 1991a; Bonfanti et al., 1992; Ní Dhúill et al., 1999). PSA-NCAM is also expressed in the hilus, subiculum and CA regions of the hippocampus where it is thought to facilitate structural plasticity of the memory circuitry (Mikkonen et al., 1999b; Nacher et al., 2002). A rodent study has previously shown that PSA-NCAM is predominantly expressed by interneurons in these hippocampal regions (Nacher et al., 2002).

PSA-NCAM is also highly expressed in the allocortical regions of both the human and rodent brain. This includes the entorhinal, perirhinal and piriform cortices of the temporal lobe. The entorhinal cortex is a highly plastic region that sends and receives input from the hippocampus and is heavily affected by AD pathology. Entorhinal cortex PSA-NCAM expression is layer specific, with rodent studies showing cells concentrated in layer II (O’Connell et al., 1997; Fox et al., 2000; Bonfanti, 2006; Gómez-Climent et al., 2008). Previous human studies have described radial orientation of PSA-NCAM+ neurites predominantly in layer II (Mikkonen et al., 1998, 1999b; Arellano et al., 2002). The entorhinal cortex receives olfactory input from the piriform cortex which also has strong PSA-NCAM expression in layer II of its trilaminar structure. Piriform cortex PSA-NCAM+ cells have so far been described in the mouse, rat, lizard, cat, guinea pig and squirrel monkey brain (Seki and Arai, 1991b; Bonfanti et al., 1992; Bernier et al., 2002; Xiong et al., 2008; Luzzati et al., 2009; Varea et al., 2011; Rubio et al., 2016).

In the neocortex of the rodent brain, PSA-NCAM expression is generally considered to be scarce (Seki and Arai, 1991b; Bonfanti, 2006). However, PSA-NCAM+ cells have been described in the rat medial prefrontal cortex and throughout the cerebral cortex of the cat, guinea pig and lizard brain (Ramirez-Castillejo et al., 2002; Varea et al., 2005, 2011; Xiong et al., 2008). In the human brain, PSA-NCAM has been observed in the medial prefrontal cortex (Varea et al., 2007b). These cells were determined to be interneurons based on fluorescent double-labelling experiments (reviewed by Nacher et al., 2013). An examination of widespread cortical PSA-NCAM in the adult human brain is lacking from current literature.

In the SVZ neurogenic niche of both humans and rodents, PSA-NCAM is expressed by migrating neuroblasts (Bonfanti and Theodosius, 1994; Doetsch et al., 1997; Curtis et al., 2007b; Kam et al.,
In the underlying caudate nucleus, PSA-NCAM expression appears to be species-specific. Rodent studies describe PSA-NCAM+ neuropil in the striatum rather than cell soma (Bonfanti et al., 1992; Szele et al., 1994; Nacher et al., 2010). However, in the rabbit and primate brain, striatal PSA-NCAM+ cells have been identified and characterised as adult born medium spiny projection neurons (Bédard et al., 2006; Luzzati et al., 2006). PSA-NCAM expression in the adult human striatum has so far only been examined by western blot (Tong et al., 2011; Ernst et al., 2014).

Lastly, species-specific differences in PSA-NCAM expression are also apparent in the cerebellum. This complex structure is regarded as static and immutable in adulthood which is supported by the total absence of PSA-NCAM in mouse and rat cerebellum (Bonfanti et al., 1992; Dusart et al., 1999). However, in the rabbit cerebellum, chains of PSA-NCAM+ cells are found to persist into adulthood (Ponti et al., 2006). Whether PSA-NCAM-mediated plasticity exists in the adult human cerebellum has not yet been described.

2.3.3 Altered PSA-NCAM in the human Alzheimer’s disease brain

PSA-NCAM distribution and abundance have only been investigated in the hippocampus of human AD cases. This sole study by Mikkonen et al., 1999b found optical density of PSA-NCAM immunoreactivity was increased in the molecular layer of the AD dentate gyrus. They also showed an increase in PSA-NCAM+ cells per unit length of the SGZ and a disorganisation of PSA-NCAM processes in the CA1, subiculum and entorhinal cortex of AD cases. Together these results suggest PSA-NCAM expression is upregulated or dysregulated within the AD hippocampus. It was not determined whether these changes correlated with severity of AD pathology.

Several lines of evidence suggest that PSA-NCAM-mediated structural plasticity may be altered in wider brain regions throughout AD. Firstly, regions that are particularly vulnerable to tau NFTs are also those with high plastic capacity such as the hippocampus and limbic system. Non-plastic areas such as the primary motor/sensory cortices and cerebellum are relatively spared from AD pathology until late stage disease (Braak and Braak, 1995; Arendt et al., 1998). Altered PSA-NCAM could be a cause or consequence of NFT accumulation and a contributing factor to dysfunction of plastic regions. Secondly, insulin has recently been shown to increase cell surface expression of PSA-NCAM (Monzo et al., 2013). Since there is growing evidence of insulin dysregulation in AD, a concurrent dysregulation of PSA-NCAM-mediated plasticity may also occur. This could have a profound effect on processes that require structural plasticity.

From the literature presented in section 2.3, it is apparent that the role of PSA-NCAM in structural plasticity extends further than the neurogenic regions. Furthermore, the distribution of PSA-
NCAM and its role in adult brain plasticity is poorly understood in non-neurogenic regions of the human brain. There is a strong possibility that PSA-NCAM-mediated plasticity may be affected by AD pathology and contribute to disruption of memory processes. An investigation of PSA-NCAM expression in the control and AD human brain would address these gaps in the literature and provide an understanding of the brain’s capacity for plasticity in neurodegenerative disease.
2.4 Aims of this thesis

The above summary of neurogenesis and PSA-NCAM-mediated plasticity demonstrates that these two processes are well characterised in the rodent brain, but an understanding of their role in the adult human brain is lacking. Mechanisms of plasticity are vital for brain function and adaptability, so an appreciation of their specific abundance in the human brain is necessary to understand the potential effect of their dysregulation. From the literature presented, it is possible that disruptions to neurogenesis and/or structural plasticity may contribute to symptom progression in AD.

This thesis involved two main aims. The first aim was to determine whether neuronal proliferation is altered in the neurogenic niches of the human AD brain. The second aim was to investigate the distribution and phenotype of PSA-NCAM+ cells in the human brain and determine if this is altered in AD. To address these two key aims, four objectives were established:

1. To investigate whether SGZ neuronal proliferation and maturation is altered in human AD using reliable markers and robust quantification methods.

2. To investigate whether SVZ proliferation is altered in human AD using a quantification method that accounts for the different SVZ sub-regions.

3. To examine the distribution of PSA-NCAM in the human brain and determine whether it is altered in AD.

4. To characterise PSA-NCAM+ cell phenotype in each brain region and investigate whether the proportion of PSA-NCAM+ interneurons is altered in the AD entorhinal cortex, a region severely affected by AD pathology.

Overall this thesis sought to further the understanding of neuronal proliferation and PSA-NCAM-mediated plasticity in the human brain and investigate whether these processes are altered in AD.
Chapter 3. General Methods

3.1 Human Tissue Processing

3.1.1 Post-mortem tissue processing

The human post-mortem brain tissue used for this project was obtained from the Neurological Foundation of New Zealand Douglas Human Brain Bank at the University of Auckland. The tissue was donated to the Brain Bank with family consent and its use for these studies was approved by the University of Auckland Human Participants Ethics Committee. The control cases had no history of cognitive impairment and cause of death was unrelated to any neurological condition. Control cases selected for this study had a maximum Braak stage of II as determined by independent pathological analysis, corresponding to β-amyloid plaque and tau NFT changes consistent with that of normal aging (Braak and Braak, 1995). The AD cases selected for this study had a minimum Braak stage of IV and a clinical history of dementia with no evidence of other neuropathology.

3.1.1.1 Fresh frozen tissue processing

The post-mortem brain processing protocol has been published in detail by Waldvogel et al., 2007. Fresh frozen tissue was used for western blotting as fixation has been shown to reduce the effectiveness of protein extraction. Donated brains were obtained at autopsy and the left hemisphere was immediately dissected into blocks representing functional regions of the brain (Waldvogel et al., 2007). These blocks were frozen using CO2 powder, wrapped in tinfoil and stored at -80°C. For western blotting the frozen blocks were cut into 30-µm sections and the region of interest was scraped from the slide using a razor blade and collected in an Eppendorf tube. This tissue was stored at -80°C until protein extraction was performed.

3.1.1.2 Formalin-fixed frozen tissue processing

Formalin-fixed frozen tissue was used for free-floating immunohistochemistry staining as fixation preserves tissue integrity and cellular morphology. The right hemisphere of each brain was processed in this manner. Phosphate-buffered saline (PBS) containing 1% sodium nitrite was first perfused through the cerebral arteries to dilate the vasculature and clear blood from the brain. The hemisphere was then perfused with 15% formalin in 0.1 M phosphate buffer and then immersed in this same solution overnight. Following fixation, the brain was dissected into functional blocks in the same manner as the fresh hemisphere. These fixed blocks were subsequently cryoprotected by immersion in 20% sucrose for one week and 30% sucrose for three weeks before being frozen.
using CO₂ powder, wrapped in tinfoil and stored at -80°C. For free-floating immunohistochemical staining, 50-µm-thick coronal sections were obtained from each block using a freezing sliding microtome (Microm, HM450) and stored at 4°C in PBS containing 0.1% sodium azide. Solutions for post-mortem tissue processing are outlined in Table 3.

3.1.1.3 Paraffin-embedded formalin-fixed tissue processing

Paraffin-embedded formalin-fixed tissue was used for the independent pathological assessment of each case and also for the construction of tissue microarrays. During dissection of the formalin-fixed hemisphere, 1-cm-thick tissue blocks were taken to be embedded in paraffin (detailed by Waldvogel et al., 2007). This processing was carried out by the Histology Laboratory at the University of Auckland, School of Medical Sciences. Briefly, tissue blocks were dehydrated in a series of ethanol solutions, cleared in xylene and embedded in paraffin wax. For immunohistochemistry, paraffin-embedded tissue was cut into 7-µm-thick sections using a rotary microtome (Leica Biosystems RM2235) and mounted onto Superfrost-Plus charged slides (Menzel-Glaser) using a 37 °C water bath (Leica Biosystems, H1210).

3.1.2 Construction of tissue microarray

Tissue microarrays allow the analysis of small tissue samples from multiple cases on a single section. The SVZ tissue microarrays used in this study were prepared by Claire Lill (Research technician, CBR, University of Auckland) using paraffin embedded formalin-fixed SVZ samples obtained from control and AD cases. As illustrated in Figure 3.1, 2 mm diameter cores were extracted from the dorsal, middle, and ventral aspects of the SVZ and inserted into a recipient paraffin block. Sections of 7 µm thickness were cut using a microtome and mounted onto slides in preparation for immunoperoxidase staining.

![Figure 3.1 Schematic representation of tissue microarray construction](image)

(A) Cores were extracted from the dorsal, middle and ventral SVZ from a paraffin embedded block of CN. (B) These cores were inserted into a recipient paraffin block creating a tissue microarray block of cores from multiple cases. (C) The tissue microarray block was cut into single sections and mounted onto slides.
### Table 3: Solutions for human post-mortem brain processing

<table>
<thead>
<tr>
<th>Solution</th>
<th>Ingredient</th>
<th>Quantity</th>
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<tbody>
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<td>0.4 M Phosphate buffer pH 7.4</td>
<td>Disodium hydrogen phosphate dihydrate</td>
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</tr>
<tr>
<td>1.0 L</td>
<td>Sodium dihydrogen phosphate dehydrate</td>
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<td></td>
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</tr>
<tr>
<td>10x Phosphate buffered saline (PBS) pH 6.8</td>
<td>Potassium dihydrogen phosphate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>1.0 L</td>
<td>Disodium hydrogen phosphate dihydrate</td>
<td>14.4 g</td>
</tr>
<tr>
<td></td>
<td>Sodium chloride</td>
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</tr>
<tr>
<td></td>
<td>Potassium chloride</td>
<td>2 g</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>1x PBS + 1% sodium nitrite</td>
<td>10x PBS</td>
<td>100 mL</td>
</tr>
<tr>
<td>1.0 L</td>
<td>Sodium nitrite</td>
<td>10 g</td>
</tr>
<tr>
<td></td>
<td>MilliQ water</td>
<td>Make to 1.0 L</td>
</tr>
<tr>
<td>15% Formalin in 0.1 M phosphate buffer</td>
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</tr>
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<td>1.0 L</td>
<td>Formalin (containing 37% formaldehyde)</td>
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</tr>
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<td></td>
<td>MilliQ water</td>
<td>600 mL</td>
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<tr>
<td>20% Sucrose cyroprotectant</td>
<td>0.4 M Phosphate buffer</td>
<td>250 mL</td>
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<td>Sodium azide</td>
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<td>1.0 L</td>
<td>Sodium azide</td>
<td>1 g</td>
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<tr>
<td></td>
<td>MilliQ water</td>
<td>Make to 1.0 L</td>
</tr>
</tbody>
</table>
3.2 **Immunohistochemistry**

3.2.1 **Immunoperoxidase Staining of Free-Floating Tissue**

Immunoperoxidase staining uses antibodies in conjunction with a peroxidase enzyme to label a protein of interest with a stable chromogenic product that can be visualised using brightfield microscopy (Figure 3.2A). Sections to be stained in this manner were first treated with the appropriate antigen retrieval procedure as optimised for each antibody (Table 4). Endogenous peroxidase activity was then blocked by immersion in a solution of 50% methanol and 1% H$_2$O$_2$ for 20 minutes at room temperature (RT). The sections were incubated in primary antibody for 72 hours on a rocking platform at 4°C. A biotinylated secondary antibody specific to the primary antibody species was then applied for 24 hours at RT. This was followed by ExtrAvidin®-Peroxidase (E2886, Sigma; diluted 1: 1,000) for 4 hours at RT. Finally, a solution of 3,3-diaminobenzidine chromogen (DAB) in 0.1 M phosphate buffer and 0.01% H$_2$O$_2$ was used to produce a stable brown stain. In some instances, 0.04% Nickel Ammonium Sulfite was added to the solution to produce a black stain rather than a brown stain for greater contrast (section 7.3.1). Primary antibodies, secondary antibodies and ExtrAvidin®-Peroxidase were diluted in a blocking buffer of PBS containing 1% sera from the species in which the secondary antibody was raised and 0.04% merthiolate. Sections were washed in PBS-T (3 x 10 minutes) between each step. The sections were mounted onto glass slides using a 0.5% gelatin solution and left to dry for two days prior to being dehydrated in an ascending series of alcohol (75% to 100%) then cleared in xylene. Coverslips were applied using DPX mounting media (06522, Sigma). Solutions for immunohistochemistry are outlined in Table 7.

3.2.2 **Cresyl violet counterstain**

Cresyl violet labels the nissl bodies present in cells and was used in this study as a counterstain to delineate regions of interest. The cresyl violet procedure was carried out in conjunction with immunoperoxidase staining as well as independently on unstained sections mounted on glass sides using 0.5% gelatin. Sections were dried at RT for two days prior to staining. Once dry, the sections were first washed in distilled H$_2$O for 5 minutes before being dehydrated in an ascending series of alcohol to xylene (5 minutes each in 75%, 85% and 95% ethanol, 3 x 10 minutes in 100% ethanol, and 10 minutes in xylene). The xylene solution turned cloudy if the sections were not adequately dehydrated. In this situation, the sections were returned to a fresh solution of 100% ethanol before being returned to xylene. Once cleared, the sections were rehydrated through the same alcohol series in reverse order. After a 5-minute wash in distilled H$_2$O, the sections were immersed in  

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38
cresyl violet stain for 15 minutes. Sections were then dehydrated and coverslipped as described above in section 3.2.1.

### 3.2.3 Immunofluorescence Staining of Free-Floating Tissue

Immunofluorescent staining uses fluorescent dye-conjugated antibodies to label a protein of interest (Figure 3.2B). Due to the unique excitation properties of different dyes, it is possible to label multiple proteins on a single tissue section using this method. Sections were first treated with the appropriate antigen retrieval procedure as optimised for each antibody (Table 4). The sections were then incubated in a cocktail of the appropriate primary antibodies for 72 hours on a rocking platform at 4°C. A cocktail of Alexa Fluor® dye-conjugated secondary antibodies specific to the primary antibody species was then applied for 24 hours at RT (Table 6). From this step, sections were protected from light. Lastly sections were incubated in Hoechst (#33342, Molecular Probes diluted 1:20,000 in PBS) for 20 minutes to counterstain cell nuclei. As for immunoperoxidase staining, sections were washed in PBS-T between each step and antibodies were diluted in a blocking buffer of PBS containing 1% sera from the species in which the secondary antibody was raised and 0.04% merthiolate. PBS was used to mount the sections onto glass slides. Coverslips were applied using ProLong® Gold AntiFade mounting media (P36930, Life Technologies) and the edges sealed using nail polish.

Certain antibodies (as indicated in Table 5) required tyramide signal amplification (TSA) for optimum staining. This method increases fluorescent staining using peroxidase activation of a tyramide-conjugated fluorescent dye (Figure 3.2C). For these sections, antigen retrieval and primary antibody incubation was carried out as described for standard immunofluorescence staining above. A secondary antibody cocktail was prepared containing a biotinylated antibody specific to the primary antibody requiring amplification as well as the Alexa Fluor® dye-conjugated antibody appropriate for the non-amplified primary antibody. This cocktail was applied for 24 hours at RT and the sections protected from light. ExtrAvidin®-Peroxidase (E2886, Sigma; diluted 1:1,000) was subsequently applied for 4 hours at RT. Sections were then incubated in Alexa Fluor® 488 Tyramide (T20948, ThermoFisher) diluted in amplification buffer (T20950, ThermoFisher) and 0.0015% H₂O₂ for 15 minutes. Lastly the sections were counterstained with Hoechst (as above), mounted, coverslipped and sealed as described in the standard immunofluorescence protocol above.
Figure 3.2  Schematic illustration of immunohistochemistry methods used in this project.
(A) Immunoperoxidase labelling produces a stable brown stain visible by bright field microscopy. (B) Immunofluorescence directly labels a bound primary antibody with a fluorophore that can be visualised by excitation at a specific wavelength. Use of different fluorophores allows labelling of multiple antigens on a single tissue section. (C) Immunofluorescence signal can be enhanced using TSA, where a peroxidase enzyme is used to activate a tyramide-conjugated fluorophore.

3.2.4 Immunoperoxidase Staining of Paraffin-Embedded Tissue

Paraffin-embedded tissue sections were first heated for one hour at 60°C on a heating block. The paraffin wax was then removed by immersion in xylene (2 x 30 minutes) and the sections rehydrated in an alcohol series (2 x 10 minutes in 100% ethanol, 5 minutes each in 95%, 85% and 75% ethanol, and 3 x 5 minutes in distilled H2O). Antigen retrieval as optimised for each antibody was performed using a pressure cooker as outlined in Table 4. To reduce non-specific binding of the secondary antibody, the sections were incubated in a 10% solution of sera from the species in which the secondary antibody was raised. Primary antibodies were diluted in PBS-T containing 1% serum of the species in which the secondary antibody is raised. The primary antibody solution was added to the sections in a humidified slide chamber to prevent evaporation and incubated for 24 hours at 4°C. A biotinylated secondary antibody specific to the primary antibody species was then applied for 4 hours at RT. This was followed by ExtrAvidin®-Peroxidase (E2886, Sigma; diluted 1: 1,000) for 1 hour at RT. Finally, a solution of DAB in 0.1 M phosphate buffer and 0.01%
H₂O₂ was used to produce a stable brown stain. Sections were washed in PBS (3 x 10 minutes) between each step in this protocol. After the DAB incubation, the sections were dehydrated in an ascending series of alcohol to xylene and coverslipped using DPX mounting media (06522, Sigma).

### 3.2.5 Immunofluorescence Staining of Paraffin-Embedded Tissue

Heating, dewaxing, antigen retrieval and serum blocking was performed on paraffin-embedded tissue sections as for immunoperoxidase staining (section 3.2.4). The appropriate primary antibody combinations were diluted in PBS-T containing 1% serum of the animal from which the secondary antibody is derived. The primary antibody solution was added to the sections in a humidified slide chamber to prevent evaporation and incubated overnight at 4°C. A cocktail of Alexa Fluor® dye-conjugated secondary antibodies specific to the primary antibody species was then applied for 4 hours at RT (Table 6). From this step, sections were protected from light. Lastly sections were incubated in Hoechst (#33342, Molecular Probes, diluted 1:20,000 in PBS) for 10 minutes to counterstain cell nuclei.

For PCNA and PSA-NCAM fluorescent labelling, tyramide signalling amplification was required. This was performed as described in section 3.2.3 with the biotinylated secondary applied for 4 hours, ExtrAvidin®-Peroxidase applied for one hour and the Alexa Fluor® 488 Tyramide solution added for 10 minutes.
Table 4: Antigen retrieval procedures used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antigen Retrieval</th>
<th>Recipe</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium citrate buffer pH 4.6</td>
<td>0.1 M citric acid monohydrate 0.1 M sodium citrate dihydrate</td>
<td>Free-floating section immersed in 1 mL of buffer per cm² of well then heated to 100°C using a microwave. Solution left to cool at RT for one hour.</td>
</tr>
<tr>
<td>Sodium citrate buffer pH 6.0</td>
<td>10 mM tri-sodium citrate dehydrate 0.05% Tween 20</td>
<td>Paraffin-embedded sections immersed in solution then heated to 121°C in a pressure cooker and left to cool for two hours at RT</td>
</tr>
<tr>
<td>Tris-EDTA buffer pH 9.0</td>
<td>10 mM Tris base 1 mM Ethylenediaminetetraacetic acid (EDTA) 0.05% Tween 20</td>
<td>Free-floating section immersed in 1 mL of buffer per cm² of well then heated to 100°C using a microwave. Solution left to cool at RT for one hour. Paraffin-embedded sections immersed in solution then heated to 121°C in a pressure cooker and left to cool for two hours at RT</td>
</tr>
<tr>
<td>Formic acid pH 2.3</td>
<td>99% Formic Acid</td>
<td>Free-floating sections incubated in formic acid for 3 minutes at RT</td>
</tr>
<tr>
<td>Antigen</td>
<td>Company</td>
<td>Catalogue Number</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------------</td>
<td>------------------</td>
</tr>
<tr>
<td>β-amyloid</td>
<td>DAKO</td>
<td>M0872</td>
</tr>
<tr>
<td>Calretinin</td>
<td>Swant</td>
<td>7697</td>
</tr>
<tr>
<td>Calretinin</td>
<td>Millipore</td>
<td>AB1550</td>
</tr>
<tr>
<td>Calbindin</td>
<td>Swant</td>
<td>CB38a</td>
</tr>
<tr>
<td>DARPP32</td>
<td>Millipore</td>
<td>AB10518</td>
</tr>
<tr>
<td>Enkephalin</td>
<td>Seralab</td>
<td>-</td>
</tr>
<tr>
<td>GAD65/67</td>
<td>Sigma</td>
<td>G5163</td>
</tr>
<tr>
<td>GFAP</td>
<td>Sigma</td>
<td>G3893</td>
</tr>
<tr>
<td>GFAP</td>
<td>Millipore</td>
<td>AB4674</td>
</tr>
<tr>
<td>NeuN</td>
<td>Millipore</td>
<td>ABN78</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>Sigma</td>
<td>N9528</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>Synaptic</td>
<td>195004</td>
</tr>
<tr>
<td>PCNA</td>
<td>Santa Cruz</td>
<td>sc7907</td>
</tr>
<tr>
<td>PCNA</td>
<td>Santa Cruz</td>
<td>sc56</td>
</tr>
<tr>
<td>PSA-NCAM</td>
<td>DSHB</td>
<td>5a5</td>
</tr>
<tr>
<td>PSA-NCAM</td>
<td>Millipore</td>
<td>MAB5324</td>
</tr>
<tr>
<td>Tau</td>
<td>DAKO</td>
<td>A0024</td>
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</table>

*TSA amplification required
Table 6: Secondary antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Conjugate</th>
<th>Isotype</th>
<th>Company</th>
<th>Catalogue Number</th>
<th>Immunoperoxidase Dilution</th>
<th>Immunofluorescence Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-mouse</td>
<td>biotin</td>
<td>IgG</td>
<td>Sigma</td>
<td>B7264-2ML</td>
<td>1:1000</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse</td>
<td>biotin</td>
<td>IgG</td>
<td>Abcam</td>
<td>AB98673</td>
<td>1:1000</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit</td>
<td>biotin</td>
<td>IgG</td>
<td>Sigma</td>
<td>B7389-2ML</td>
<td>1:1000</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse</td>
<td>Alexa fluor 594</td>
<td>IgG</td>
<td>ThermoFisher</td>
<td>A11032</td>
<td>-</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit</td>
<td>Alexa fluor 488</td>
<td>IgG</td>
<td>ThermoFisher</td>
<td>A11034</td>
<td>-</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit</td>
<td>Alexa fluor 594</td>
<td>IgG</td>
<td>ThermoFisher</td>
<td>A11037</td>
<td>-</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-chicken</td>
<td>Alexa fluor 594</td>
<td>IgG</td>
<td>ThermoFisher</td>
<td>A11042</td>
<td>-</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-chicken</td>
<td>Alexa fluor 647</td>
<td>IgG</td>
<td>ThermoFisher</td>
<td>A21449</td>
<td>-</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-guinea pig</td>
<td>Alexa fluor 594</td>
<td>IgG</td>
<td>ThermoFisher</td>
<td>A11076</td>
<td>-</td>
<td>1:500</td>
</tr>
<tr>
<td>Donkey anti-goat</td>
<td>Alexa fluor 594</td>
<td>IgG</td>
<td>ThermoFisher</td>
<td>A11055</td>
<td>-</td>
<td>1:500</td>
</tr>
<tr>
<td>Donkey anti-rabbit</td>
<td>Alexa fluor 647</td>
<td>IgG</td>
<td>ThermoFisher</td>
<td>A21207</td>
<td>-</td>
<td>1:500</td>
</tr>
<tr>
<td>Donkey anti-chicken</td>
<td>Alexa fluor 488</td>
<td>IgG</td>
<td>Jackson ImmunoResearch</td>
<td>703-545-155</td>
<td>-</td>
<td>1:500</td>
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Table 7: Immunohistochemistry solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
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<tbody>
<tr>
<td>1x PBS pH 7.4</td>
<td>10x PBS stock</td>
<td>100 mL</td>
</tr>
<tr>
<td>1.0 L</td>
<td>MilliQ water</td>
<td>900 mL</td>
</tr>
<tr>
<td>1x PBS + 0.2% triton (PBS-T)</td>
<td>10x PBS stock</td>
<td>100 mL</td>
</tr>
<tr>
<td>1.0 L</td>
<td>Triton X-100</td>
<td>2.0 mL</td>
</tr>
<tr>
<td></td>
<td>MilliQ water</td>
<td>898 mL</td>
</tr>
<tr>
<td>Peroxidase blocking solution</td>
<td>Methanol</td>
<td>5 mL</td>
</tr>
<tr>
<td>10 mL</td>
<td>30% hydrogen peroxide</td>
<td>333 µL</td>
</tr>
<tr>
<td></td>
<td>MilliQ water</td>
<td>4.67 mL</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>Animal sera corresponding to host of secondary antibody</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>(for antibody dilutions)</td>
<td>1x PBS-T</td>
<td>99 mL</td>
</tr>
<tr>
<td>0.5% DAB solution (10x stock)</td>
<td>3,3 diaminobenzidine (DAB) powder</td>
<td>0.5g</td>
</tr>
<tr>
<td>100 mL</td>
<td>MilliQ water</td>
<td>100 mL</td>
</tr>
<tr>
<td>0.05% DAB solution + 0.01% hydrogen peroxide in 0.1M</td>
<td>0.5% DAB solution (10x stock)</td>
<td>1 mL</td>
</tr>
<tr>
<td>phosphate buffer (working solution)</td>
<td>1% hydrogen peroxide</td>
<td>100 µL</td>
</tr>
<tr>
<td>10 mL</td>
<td>0.4M phosphate buffer</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>0.5% Gelatin + 0.05% chromic potassium sulfate</td>
<td>MilliQ water</td>
<td>6.4 mL</td>
</tr>
<tr>
<td>350 mL</td>
<td>Porcine gelatin</td>
<td>1.75 g</td>
</tr>
<tr>
<td></td>
<td>Chromic potassium sulfate</td>
<td>0.175 g</td>
</tr>
<tr>
<td></td>
<td>MilliQ water</td>
<td>350 mL</td>
</tr>
</tbody>
</table>
3.3 Golgi Staining

Golgi staining was optimised to analyse the structure of immature granule cells. The Rapid GolgiStain kit from FD NeuroTech was used for Golgi staining as per the manufacturer’s instructions. An overview of the method is presented here and specific optimisations for human tissue and fluorescent co-labelling are presented in Chapter 4 of this thesis.

3.3.1 Silver impregnation

The impregnation solution, a 1:1 mixture of solution A and B from the Rapid GolgiStain kit, was prepared at least 24 hours prior to use to allow excess precipitate to settle. As human brain donation is unpredictable, this solution was made monthly. Tissue to be stained was obtained at the time of the relevant hemisphere dissection. As such, fresh tissue was collected the day of the brain donation while fixed tissue was perfused, then immersion fixed for approximately 24 hours. Blocks 5 - 10 mm in thickness were added to a falcon tube containing 5 mL of impregnation solution so that the block was entirely immersed. The tubes were balanced on the lid to keep the block flat, and protected from light during the staining process. The impregnation solution was replaced after the first 24 hours and the block was left to stain for 3 weeks.

After the impregnation period the block was transferred to solution C from the Rapid GolgiStain kit and incubated at 4°C for one week. The solution was replaced after the first 24 hours.

3.3.2 Sectioning and developing

The Golgi-stained block was cut into 100-μm-thick sections using a freezing sliding microtome (Microm, HM450). Each section was mounted directly onto a 3% gelatin-coated slide with care taken not to bend or fold the tissue. These slides were protected from light and left to dry for 72 hours. For development of the stain, the slides were first rinsed in Milli-Q water for 5 minutes before immersion in the developer solution comprised of 2 parts water, 1 part solution D, and 1 part solution E from the Rapid GolgiStain kit. The slides were then washed in Milli-Q water four times for 5 minutes each. At this stage, it was possible to counterstain cell nuclei using cresyl violet as per section 3.2.2. If no counterstain was to be used, the sections were dehydrated in an ascending series of alcohol from 75% to 100%, cleared in xylene and a coverslip was applied using DPX mounting media (06522, Sigma).
3.4 Western Blotting

In this project, western blot analysis of protein expression was carried out on samples from fresh-frozen post-mortem human tissue for the validation of PSA-NCAM antibodies in Chapter 6.

3.4.1 Protein extraction

Protein extraction was performed on fresh-frozen post-mortem human tissue collected from the region of interest as outlined in section 3.1.1.1. Briefly, 0.2 g of tissue was washed with ice cold PBS and centrifuged at 100 rpm for 1 minute to remove excess blood. This step was repeated until blood was sufficiently removed. Tissue was added to a microfuge tube with a small number of glass beads and enough tissue homogenisation buffer (150 mM sucrose, 15 mM Hepes, 60 mM KCl, 5 mM EDTA, 1 mM EGTA) containing protease inhibitor (1183613001, Roche, 1 tablet per 10ml buffer) to make 1 mL final volume. Homogenisation was performed using a bullet blender (BBX24B, Next Advance). Triton X100 was added to the sample (final concentration 1%) and the samples were incubated on ice for a further hour. Lastly the homogenate was centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatant was extracted and stored at -80°C prior to electrophoresis.

3.4.2 Western blotting

Electrophoresis and immuno-staining of the blot was performed as described in Monzo et al., (2012) using Invitrogen systems and buffers according to manufacturer’s instructions.

A BioRad DC protein assay (1500-011, BioRad) was first used to determine the protein concentration in each sample. For the experiments in this study, each sample included 10 µg of protein homogenate, 4x sample buffer and 10x sample reducing agent to a final volume of 20 µl. The samples were denatured at 62°C for 10 minutes using a hotplate prior electrophoresis. They were then loaded into pre-cast NuPAGE® Bis-Tris gels (NP0336, Novex-ThermoFisher Scientific) along with protein standards (LC5925, Seeblue plus and LC5602, Magic Mark XP, BioRad) for molecular weight assessment. Proteins were resolved by electrophoresis for 90 minutes at 150V using the Xcell SureLock Mini Cell and 1x NuPAGE MOPS SDS running buffer (NP0001) supplemented with antioxidant (NP0301).

Proteins were subsequently electrotransferred onto a polyvinylidene difluoride membrane (PVDF, Hybond-P; Amersham) using an Xcell Blot Module. The gel was gently removed from the cassette, overlayed with the PVDF membrane and inserted into a blotting sandwich of filter paper and blotting pads, pre-soaked in 1x
NuPAGE transfer buffer (NP0061) supplemented with antioxidant. The complete sandwich was secured into the blot module and filled with transfer buffer. The outer segment of the module was packed with ice to maintain consistent protein migration. The module was run at 30V for 90 minutes.

Once blotting was complete, the membrane was blocked for one hour using 5% skim milk diluted in tris-buffered saline containing 0.1% triton (TBS-T). Primary antibodies specific to the protein of interest (Table 8) were diluted in 5% bovine serum albumin in TBS-T and applied to the membranes overnight on an orbital shaker at 4°C. Species-specific secondary antibodies conjugated to horse-radish peroxidase (HRP) were diluted in 5% skim milk in TBS-T and applied for 3 hours on an orbital shaker at RT. Finally, the membrane was incubated in Amersham ECL Prime Western blotting detection reagent (RPN2236, GE Healthcare) for 5 minutes at RT. Excess reagent was removed and the chemiluminescent signal was visualised by using the BioRad ChemiDoc MP system.

Table 8: Primary antibodies used for western blotting

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Company</th>
<th>Catalogue Number</th>
<th>Host Species</th>
<th>Isotype</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA-NCAM</td>
<td>DSHB</td>
<td>5A5</td>
<td>Mouse</td>
<td>IgM</td>
<td>1:200</td>
</tr>
<tr>
<td>PSA-NCAM</td>
<td>Millipore</td>
<td>MAB5324</td>
<td>Mouse</td>
<td>IgM</td>
<td>1:3000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Abcam</td>
<td>AB9485</td>
<td>Rabbit</td>
<td>IgG</td>
<td>1:3000</td>
</tr>
</tbody>
</table>

Table 9: Secondary antibodies used for western blotting

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Conjugate</th>
<th>Isotype</th>
<th>Company</th>
<th>Catalogue Number</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-mouse</td>
<td>HRP</td>
<td>IgM</td>
<td>Serotec</td>
<td>STAR86P</td>
<td>1:3000</td>
</tr>
<tr>
<td>Donkey anti-rabbit</td>
<td>HRP</td>
<td>IgG</td>
<td>Sigma</td>
<td>NA934V</td>
<td>1:3000</td>
</tr>
</tbody>
</table>

Abbreviation: HRP, horse-radish peroxidase
Chapter 4. Cell Proliferation and Neurogenesis in the Alzheimer’s Disease Hippocampus

4.1 Introduction

The hippocampus is critically involved in memory function, the loss of which is a hallmark symptom of AD. Specific areas of the hippocampus become atrophied in AD and the region is one of the most severely affected by accumulation of β-amyloid plaques and intracellular tau tangles (Braak and Braak, 1991). As SGZ neurogenesis is thought to contribute to memory function, altered neurogenic capacity due to AD pathology may contribute to the memory impairment that occurs in the disease (Lazarov and Marr, 2010; Rodríguez and Verkhratsky, 2011; Winner et al., 2011).

Opinion is divided as to whether neurogenesis is altered in the human AD brain. Initial work in the field suggested proliferation was increased in the AD hippocampus (Jin et al., 2003). However, this study has since been criticised for drawing conclusions from qualitative analysis of fluorescent staining. Furthermore, increases detected by western blot analysis represent global hippocampal proliferation and cannot be specifically attributed to neuronal proliferation which is of therapeutic significance. Subsequent studies investigated this idea further and found an increase in overall hippocampal proliferation was largely due to astrocytic and microglial proliferation in the CA regions and that SGZ neuronal proliferation was unchanged (Boekhoorn et al., 2006; Ekonomou et al., 2015). This controversy highlights some important methodological considerations within the field. To obtain a meaningful assessment of neurogenic capacity it is necessary to use a reproducible quantification method that specifically identifies the appropriate cell type in the region of interest.

In the assessment of hippocampal neurogenic capacity, the proliferative cell population is often the focus. However, the number of proliferating cells does not necessarily indicate net neurogenic output; that is, the number of adult-born neurons reaching maturity (Kempermann, 2011). A few recent studies have examined markers of neuronal maturation in the human SGZ and present conflicting results. For example, Li et al., 2008 used IOD rather than cell counts and found a progenitor cell marker (MAP2c) to be unchanged in the SGZ of AD cases but markers of immature neurons to be decreased (MAP2a and MAP2b). This suggests maturation of adult-born neurons is impaired in AD. Conversely, Perry et al., 2012 used the same quantification method, but different cell markers. This study found a decrease in progenitor cells (Musashi1), but an increase in migratory cells (PSA-NCAM and doublecortin) and no change in overall post-mitotic immature
neurons (β-III tubulin). These results instead suggest that proliferation is impaired in AD, yet a population of early post-mitotic immature neurons is increased, then presumably culled to result in no net change in neurogenic output. These discordant conclusions highlight the importance of the markers used to label the progenitor and immature granule cell populations. These markers should allow the clear identification of individual cells and be unaffected by factors such as post-mortem delay that can confound the measurement of true population size.

From the current literature, it is apparent there is no clear consensus that neuronal proliferation or maturation is affected in AD. This can be attributed to the diverse methods of quantification and choice of cell markers that make it difficult to meaningfully compare the results of different studies. In this chapter, cell proliferation and neurogenesis in the human AD brain was assessed using an established cell counting method that produces a robust and reproducible estimate of cells expressing the marker of interest. Although labour and time intensive, manual cell counting provides the most reliable estimate of the neurogenic cell population specifically within the SGZ. Distinct phases of hippocampal neurogenesis were investigated using markers that consistently label individual cells in human tissue and are not affected by post-mortem delay. Proliferation, early maturation and late maturation phases of hippocampal neurogenesis were quantified using PCNA, PSA-NCAM and calretinin respectively (Figure 4.1). These data were also compared to the hippocampal β-amyloid and tau load to investigate whether hippocampal neurogenesis changes with disease progression. Together these results present a comprehensive analysis of neurogenic capacity in the AD hippocampus.

Another consideration is whether healthy development of new neurons occurs in AD. Markers of proliferation and maturation can provide an indication of cellular maturity, but don’t provide detail about the structural development of the cell. A key aspect of neuronal maturation is the correct growth of dendrites and synaptic spines. These properties are very difficult to measure in adult human post-mortem tissue due to the density of cells in the granule cell layer and the inability to specifically label immature neuron structure using fluorescent protein transfection. As such, no study has yet provided a structural analysis specifically of immature granule cells in the adult human hippocampus. Furthermore, there is evidence to suggest AD pathology could affect structural maturation. Previous studies have comprehensively described decreased spine density and dendrite length of granule cells in the human AD hippocampus, along with an increased size of the remaining synaptic contacts and aberrant dendritic sprouting (Scheibel and Tomiyasu, 1978; de Ruiter and Uylings, 1987; Scheff and Price, 2003, 2006). These studies examined granule cell structure using Golgi staining, a technique that uses metal impregnation to label the entire structure of a small proportion of cells in the tissue. This method allows clear visualisation of the entire
neuronal structure including dendritic processes and synaptic spines without obstruction from neighbouring cells. However, it is limited by the inability to predict which cells will be labelled or determine the neurochemical phenotype of a labelled cell. Therefore, this study attempted to pair the Golgi-staining technique with immunofluorescence to specifically study the structure of new-born granule cells. While this endeavour was ultimately unsuccessful, thorough optimisation of the Golgi method is presented in this chapter. These results outline the optimal tissue processing methods for Golgi-staining human brain tissue and examine factors that affect compatibility of Golgi-staining and immunohistochemistry in human tissue.

Figure 4.1  Neurochemical markers used for quantification of SGZ neurogenesis. PCNA labels proliferative neuroblasts while PSA-NCAM and calretinin label early and late post-mitotic immature granule cells respectively.
4.2 Methods Overview

4.2.1 Human tissue selection

Fixed-frozen hippocampi from fourteen neurologically normal controls and eleven AD cases were used in this chapter as outlined in Table 10. Tissue was prepared and sectioned as outlined in section 3.1.1.

The control cases used for this study had no history of neurological disease or treatment and pathological analysis excluded any neuropathology. These cases ranged from 63 to 88 years old with an average age of 75.3 ± 8.3 years and an average post-mortem delay of 15.5 ± 5.6 hours. Independent pathological analysis determined that any β-amyloid plaque changes in these cases was consistent with that of normal aging (Braak and Braak, 1995).

The AD cases used in this study were examined by an independent pathologist to confirm AD neuropathology. Widespread presence of plaques and tangle load was determined and neuropathological severity was assessed based on the Braak scoring system (Braak and Braak, 1995). All cases used in this study had a Braak stage between IV and VI, indicative of pathological AD. Cases ranged in age from 69 to 85 years old with an average age of 77.3 ± 5.3 years and an average post-mortem delay of 12.0 ± 6.1 hours.

<table>
<thead>
<tr>
<th>Case Number</th>
<th>Age</th>
<th>Sex</th>
<th>Post-mortem Delay (hr)</th>
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</thead>
<tbody>
<tr>
<td>H109</td>
<td>81</td>
<td>Male</td>
<td>8</td>
</tr>
<tr>
<td>H122</td>
<td>72</td>
<td>Female</td>
<td>9</td>
</tr>
<tr>
<td>H137</td>
<td>77</td>
<td>Female</td>
<td>12</td>
</tr>
<tr>
<td>H169</td>
<td>81</td>
<td>Male</td>
<td>24</td>
</tr>
<tr>
<td>H181</td>
<td>78</td>
<td>Female</td>
<td>20</td>
</tr>
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<td>H186</td>
<td>68</td>
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<td>H196</td>
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<td>Male</td>
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<td>H229</td>
<td>88</td>
<td>Female</td>
<td>17</td>
</tr>
<tr>
<td>H237</td>
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<td>Male</td>
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</tr>
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<td>H238</td>
<td>63</td>
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</tr>
<tr>
<td>H241</td>
<td>76</td>
<td>Female</td>
<td>12</td>
</tr>
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</table>
4.2.2 Immunoperoxidase staining and analysis of proliferation markers

DAB immunoperoxidase staining was carried out on 50 µm-thick coronal sections of the central 2 cm of the hippocampal formation, referred to as block HP2 (Waldvogel et al., 2007), from the control and AD cases indicated in Table 10, as per the protocol described in section 3.2.1. Ten sections from each case, spaced 200µm apart, were stained using PCNA (sc7907, Santa Cruz), PSA-NCAM (5a5, DSHB) and calretinin (7697, Swant) primary antibodies as indicated in Table 5. A no-primary antibody control was included for each staining condition and these sections showed no immunoreactivity. Cresyl violet counterstaining was performed as per section 3.2.2 to allow accurate measurement of SGZ length and GCL area, and to reduce false-positive counts. This counterstain was not performed for the calretinin sections as the GCL could be easily distinguished by lack of background staining alone (Figure 4.2A’) and false-positive counting was minimal as neurites were visible on calretinin+ cells. The GCL of each section was imaged using a Nikon E800 microscope mounted with a colour camera (MBF Bioscience DV-47d) at 20x objective. The acquired images were automatically stitched using StereoInvestigator 10 software (MBF Bioscience). Measurements and proliferation marker quantification were carried out using ImageJ v1.46. The entire length of the SGZ was measured and counted in the coronal plane. In those sections where the SGZ was observed at multiple sites, all sites were analysed. SGZ length was measured along the inferior boundary of the GCL as determined by cresyl violet staining (Figure 4.2A). GCL area was measured by tracing around the dense band of cresyl violet labelled cells (Figure 4.2B). For calretinin labelled sections, the GCL was identified by the absence of background staining (Figure 4.2A’). For each section, background cresyl violet staining density measurements were taken randomly from a 2.5 µm² area of the GCL in triplicate using the rectangular selection tool and averaged. A positively-labelled DAB cell was required to have a
density measurement at least 50 points darker than the background cresyl violet average for that section (0 = black, 255 = white). The number of DAB labelled cells was analysed per unit length and per unit area for each section.

Figure 4.2  Illustration of SGZ length and GCL area measurement
(A) SGZ length was determined by measuring the inferior border of the GCL (red). (A’) Calretinin labelled sections were not counterstained with cresyl violet as the GCL was able to be identified by lack of immunoreactivity. (B) GCL area was measured by tracing the the dense band of cresyl violet labelled nuclei (yellow), except for the calretinin quantification where the GCL was identified by the lack of immunoreactivity.
4.2.3 Immunoperoxidase staining and analysis of pathological aggregates

DAB immunohistochemistry using β-amyloid (M0872, DAKO) and tau (A0024, DAKO) primary antibodies (outlined in Table 5) was performed on hippocampal sections from the cases described above, as per the protocol outlined in section 3.2.1. For each case, six sections were stained per marker with a 200 µm spacing. No cresyl violet counterstaining was performed due to the method of quantification. Sections were imaged using the Nikon E800 microscope described above equipped with StereoInvestigator 10 software (MBF Bioscience). A series of at least 15 random images per section were obtained across the region of interest using a 10x objective for β-amyloid and a 20x objective for tau. All imaging parameters and microscope settings were kept constant throughout imaging to ensure unbiased assessment of staining between sections. Staining load was quantified using MetaMorph high-throughput image analysis software (version 7.8.0, Molecular Devices) as described by Narayan and Dragunow (2010). This software detects the area of DAB staining on an image using the ‘count nuclei’ algorithm which applies threshold parameters (minimum object area of 50 µm² and minimum object grey value intensity above background of 50 points) to detect staining relative to local background (Figure 4.3A, B). This analysis produced a measurement of ‘average percentage area of image stained’ as an indication of pathological aggregate load (Figure 4.3C).

Images were excluded from the analysis if the tissue border was visible to ensure an equal area of tissue was assessed per image. Both the hippocampus and entorhinal cortex were analysed for each case.

Figure 4.3 Summary of metamorph aggregate load analysis
(A) Using MetaMorph high-throughput image analysis software (version 7.8.0, Molecular Devices) the images were first converted to grayscale. (B) Threshold parameters were applied to the grayscale image to detect immunoreactivity relative to background. This allows for specific analysis of the stained regions only. (C) The thresholded area was then measured by the software to determine the percentage area of the image stained.
4.2.4 Statistical analysis

Statistical analysis was performed using the GraphPad Prism (version 6.05) statistics and graphing package. Due to the small number of PCNA+ cells in the SGZ, a normal distribution was not obtained for this data set, therefore a non-parametric Mann-Whitney test was performed to examine differences between control and AD cases. The PSA-NCAM and calretinin data sets had a normal distribution as determined by the Shapiro-Wilk normality test and met the equality of variance assumption as per the F-test. Therefore, a parametric unpaired t-test was performed on these data to test for a significant difference between control and AD cases. Values are presented in this section as mean ± standard deviation with statistical significance set at P ≤ 0.05.

The non-parametric Spearman’s correlation coefficient was used to investigate the relationship between PCNA+ cell counts and β-amyloid load or tau. This analysis was carried out for PSA-NCAM+ and calretinin+ cell counts using the Pearson’s correlation coefficient. For this study, two factors were considered to be correlated if a P value ≤ 0.05 was obtained. The Spearman’s or Pearson’s r-value has been reported for all comparisons.

4.2.5 Immunofluorescent characterisation of immature sub-granular zone cells

Free floating fluorescent triple labelling was performed to check for a neuronal or glial phenotype in the cells expressing the proliferation markers of interest. Permeabilisation and antigen retrieval steps were carried out as for DAB immunoperoxidase staining. Combinations of primary antibodies from different host animals were used to triple-label the progenitor cell markers (PCNA, PSA-NCAM and calretinin) with NeuN (ABN78, Millipore) and GFAP (AB4674, Abcam) as indicated in Table 5. Alexa-fluor dye-conjugated secondary antibodies of an appropriate host species were used to label the bound primary antibodies except those for PCNA and PSA-NCAM where TSA amplification was performed as described in section 3.2.3. A Hoechst counterstain was also applied to all sections. Fluorescent sections were imaged using an Olympus FV1000 confocal microscope with a 60x oil immersion lens.

4.2.6 Optimisation of Immuno-Golgi staining

4.2.6.1 Optimisation of human tissue processing for Golgi-staining

Golgi-staining was optimised for human tissue with the intent to specifically assess the structure of immature granule cells by pairing the technique with fluorescence immunohistochemistry. The mechanism of Golgi-staining is not well defined, despite several investigations throughout its 100+ years of popularity in anatomical brain research. There are two popular modifications of traditional
Golgi-staining that are commonly used in modern studies. This study used the FD RapidGolgi stain kit which is based on the Golgi-Cox method of staining where a solution of potassium dichromate and mercuric chloride results in the formation of white mercuric chloride deposits on neurons. These deposits nucleate and spread through the cytosol and across the cell membrane, essentially encrusting the cell. This crust is converted to mercuric sulfide upon alkali treatment (Ramón-Molina, 1970; Chan-Palay and Palay, 1972; Stean, 1974; Narayanan et al., 2014; Rosoklija et al., 2014). Intrinsic and fixative-derived di-sulfide bonds within the tissue are thought to be the source of sulfur for this blackening reaction. Mercuric chloride is also suggested to preferentially bind sulfur residues on membrane-bound proteins (Stean, 1974; Rosoklija et al., 2014). Golgi-Cox staining is reportedly a reliable and consistent method that results in more complete dendritic staining with a very clear background and is relatively insensitive to differences in post-mortem delay (Buell, 1982).

An alternative method is Rapid Golgi (not to be confused with the inconveniently named FD RapidGolgi staining kit) which uses osmium tetroxide or paraformaldehyde in the impregnation solution as a fixative and silver nitrate in the stain developer to form a silver dichromate precipitate (Buell, 1982). This method is preferred for prenatal or infant brains which do not stain well using Golgi-Cox, presumably due to low sulfur content at this developmental stage (Stean, 1974; Rosoklija et al., 2003). Rapid Golgi has been reported to preferentially stain degenerating neurons in human tissue and therefore was not used in this study (Buell, 1982). While the Rapid Golgi method can successfully stain neurons in tissue fixed for as long as 3.5 months, the effect of fixation duration on Golgi-Cox staining is surprisingly unreported in the literature.

Therefore, the optimal human tissue fixation and storage processes required for Golgi-staining were first determined by applying the FD RapidGolgi staining kit procedures (outlined in section 3.3) to blocks of hippocampus tissue that had undergone different stages of processing (Table 11).
Table 11: Methods of tissue processing used to test Golgi-staining

<table>
<thead>
<tr>
<th>Method</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>No fixation, cryoprotection or freezing</td>
</tr>
<tr>
<td>Short-term fixed</td>
<td>Perfusion fixed and immersed in formalin for 2 days.</td>
</tr>
<tr>
<td>Medium-term fixed</td>
<td>Perfusion fixed and immersed in formalin for 2 weeks.</td>
</tr>
<tr>
<td>Long-term fixed</td>
<td>Immersion fixed for 7 years</td>
</tr>
<tr>
<td>Fixed-sucrosed</td>
<td>Perfusion fixed and immersed in formalin for 2 days.</td>
</tr>
<tr>
<td></td>
<td>Cryoprotected in 20% and 30% sucrose solution.</td>
</tr>
<tr>
<td>Fresh-frozen</td>
<td>No fixation or cryoprotection.</td>
</tr>
<tr>
<td>Fixed-frozen</td>
<td>Perfusion fixed and immersed in formalin for 2 days.</td>
</tr>
<tr>
<td></td>
<td>Cryoprotected in 20% and 30% sucrose solution.</td>
</tr>
</tbody>
</table>

Single section Golgi-staining was also optimised to allow testing of different Immuno-Golgi paradigms. Briefly, 100 μm sections of short-term (2 day) fixed hippocampus were cut using a vibratome to avoid freezing the tissue prior to staining. The section was sandwiched between two glass coverslips and sealed on the corners using a dot of superglue. This process helps the section impregnate evenly and reduces the crystal formation which usually occurs on the outside of a tissue block. The section was then immersed in the impregnation solution for 10 days with the initial solution replaced after the first 24 hours. After impregnation, the section was removed from the coverslips and either processed for fluorescent immunohistochemistry as per section 3.2.3 or developed and dehydrated as per section 3.3.2.

4.2.6.2 Immuno-Golgi staining variations

The immunofluorescent labelling of Golgi-stained sections was first attempted using the protocols outlined by Spiga et al., 2011 and Pinto et al., 2012. Two variations were also tested as outlined in Table 12. Short-term fixed tissue was used for all Immuno-Golgi testing and fluorescent immunohistochemistry was performed for PSA-NCAM and calretinin as per the method outlined in section 4.2.5 above. These markers label post-mitotic immature neurons and therefore the maturation phase of interest. Triton was omitted from all solutions used in the immunofluorescence protocol to limit membrane disruption and prevent degradation of the Golgi-stain.
Table 12: Immuno-Golgi variations tested in this study

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Golgi stain → Develop → Immunofluorescence (Spiga et al., 2011; Pinto et al., 2012)</td>
<td>Block Golgi impregnation, sectioning and stain development followed by immunofluorescence</td>
</tr>
<tr>
<td>2. Golgi stain → Immunofluorescence → Develop</td>
<td>Block Golgi impregnation and sectioning followed by immunofluorescence and then stain development</td>
</tr>
<tr>
<td>3. Immunofluorescence → Golgi stain → Develop</td>
<td>Immunofluorescence followed by single section Golgi impregnation and stain development.</td>
</tr>
</tbody>
</table>
4.3 Results

4.3.1 No significant change to number of PCNA$^+$ proliferating cells in the sub-granular zone of AD cases

PCNA is a nuclear antigen and was used to identify mitotically active cells in the SGZ. As expected, PCNA$^+$ nuclei co-labelled with Nissl stained cell soma and were located in the SGZ and lower third of the GCL in the hippocampus (Figure 4.4A). PCNA$^+$ nuclei were round and approximately 8 µm in diameter, which is consistent with previous reports (Low et al., 2011; Gomez-Nicola et al., 2014).

A mean of 2.5 ± 2.7 cells/section and 1.3 ± 1.6 cells/section was counted for control and AD cases respectively (Figure 4.4B). This difference was not statistically significant (P = 0.3505). To obtain a more standardised estimate of PCNA$^+$ cell count the SGZ length was measured for each section by tracing the inferior border of the GCL. Normalising the data in this way accounts for differences in the length of the SGZ which occur along the coronal plane of the hippocampal structure and between individuals. Using this method, a mean of 0.25 ± 0.29 cells/mm SGZ and 0.08 ± 0.09 cells/mm SGZ was obtained for control and AD cases respectively (Figure 4.4C). Again, this difference was not statistically significant (P = 0.2996).

The PCNA$^+$ cell count was also normalised to the area of the GCL to account for differences in GCL size (Figure 4.4D). No significant difference was found between the two groups when the data were analysed in this manner (mean of 2.5 ± 2.9 cells/mm$^2$ GCL for controls and 0.8 ± 0.8 cells/mm$^2$ GCL for AD, P = 0.1908).

Sections from more rostral and caudal regions of the hippocampus were also analysed to ensure the averages obtained from the HP2 region of the hippocampus are representative of the entire structure. The counts from these regions were satisfactorily similar, indicating that the amount of proliferation is uniform throughout the hippocampal structure.

Overall it was apparent that although proliferation in the human SGZ is a rare event, there is considerable variability between individuals.
**SGZ PCNA Quantification**

Figure 4.4  PCNA quantification in the SGZ
(A) A PCNA+ cell nucleus (arrow) in the SGZ of the hippocampus imaged with a 20x objective. Scale bar = 20 µm. The number of SGZ PCNA+ cells was analysed per section (B), per unit length SGZ (C) and per unit area GCL (D). No significant difference was detected between Alzheimer’s disease or control cases for any of these analyses.
4.3.2 Increase in PSA-NCAM+ cells in the sub-granular zone of AD cases

PSA-NCAM is a membrane-bound glycoprotein expressed by cells undergoing migration or structural remodelling. PSA-NCAM+ cells were located in the SGZ and GCL as expected, although cells were also observed within the hilus and CA regions of the hippocampus (investigated further in Chapter 6 and 7). SGZ cells showed clear membrane localisation of PSA-NCAM along the cell soma with processes extending up through the GCL. Cells had elongated soma 10 - 20µm in diameter and a bipolar structure characteristic of hippocampal granule cells (Figure 4.5A).

The number of PSA-NCAM+ cells was highly variable, even between individuals within a group. There was no significant difference in the number of SGZ PSA-NCAM+ cells per section between control and AD cases (Figure 4.5B, average 27.6 ± 17.8 cells/section for controls and 33.8 ± 15.3 cells/section for AD, P = 0.3654). However, when normalised to SGZ length, AD cases had significantly more PSA-NCAM+ cells compared to controls (Figure 4.5C, average 2.2 ± 1.0 cells/mm SGZ for controls and 3.6 ± 1.6 cells/mm SGZ for AD, P = 0.0158). Interestingly, when standardised to the GCL area this increase was not statistically significant (Figure 4.5D, average 27.8 ± 13.8 cells/mm² GCL for controls and 38.7 ± 21.2 cells/mm² GCL for AD, P = 0.1348).

Since these disparate results could reflect a difference in GCL size, the dimensions of the layer were examined. There was no statistically significant difference in the average GCL area between the two groups (data not shown, 1.03 ± 0.49 mm² for controls and 1.03 ± 0.38 mm² for AD, P = 0.6092). The GCL width was derived from the measurements of GCL area and SVZ length per section and averaged for each case. From this analysis, GCL width was larger in AD cases compared to controls (Figure 4.5E, average 84.4 ± 15.0µm for controls and 100.7 ± 19.3µm for AD, P = 0.0254). In turn, this infers SGZ length was smaller for AD cases. However, averaging the length across all sections within a case resulted in a mean SGZ length that was not significantly different compared to controls (Figure 4.5F, 12.1 ± 5.1 mm for controls, 10.2 ± 3.7 mm for AD, P = 0.3048). Despite this, the analysis suggests the GCL was thicker and shorter in the AD cases compared to controls, so the increased number of PSA-NCAM+ cells/mm SGZ may be masked by a simultaneous increase in GCL thickness.
Figure 4.5  
PSA-NCAM quantification in the SGZ.
(A) PSA-NCAM+ cells (arrows) in the SGZ of the hippocampus imaged with a 20x objective. Scale bar = 50 µm. There was no significant difference in the number of PSA-NCAM+ cells per section (B) or per unit area of the GCL (D) between AD and control cases. However, there was a significant increase in the number of PSA-NCAM+ cells per unit length SGZ in AD cases (C). This disparate result is likely indicative of variable GCL dimensions. The GCL was significantly thicker in AD cases (E) and although mean SGZ length was smaller in AD cases, this change was not statistically significant. (F).
4.3.3 No significant change to number of immature calretinin$^+$ cells in the sub-granular zone of AD cases

Immature granule cells express the calcium binding protein calretinin prior to their integration into the hippocampal circuitry. As they become electrophysiologically mature, calretinin expression is replaced by calbindin (Kempermann, 2011). Calretinin$^+$ cells had a similar structure and distribution to PSA-NCAM, with cells visible in the GCL, SGZ and deep within the hilus of the hippocampus. SGZ calretinin$^+$ cells had a bipolar structure, with long apical dendrites extending into the GCL. These cells showed dense calretinin staining throughout the soma and processes (Figure 4.6A).

There was no significant difference in the number of calretinin$^+$ cells per section between the control and AD cases (Figure 4.6B, average $61.0 \pm 18.9$ cells/section for controls and $48.11 \pm 21.2$ for AD, $P = 0.1324$). Similarly, when standardised to the SGZ length no statistically significant difference was observed (Figure 4.6C, average of $5.0 \pm 1.5$ cells/mm SGZ for controls and $5.2 \pm 1.9$ cells/mm SGZ for AD, $P = 0.6784$). This was also true when analysed relative to GCL area (Figure 4.6D, average of $64.5 \pm 20.6$ cells/mm$^2$ GCL for controls and $66.8 \pm 24.8$ cells/mm$^2$ GCL for AD, $P = 0.8010$).

Overall the number of SGZ calretinin$^+$ cells was remarkably similar between AD and control cases. This suggests there is no change in the number of calretinin$^+$ immature neurons in the AD SGZ.
Figure 4.6  Calretinin quantification in the SGZ
(A) Calretinin+ cells in the SGZ (arrows) imaged with a 20x objective. Scale bar 50 µm. There was no significant difference in the number of calretinin+ cells/section (B), calretinin+ cells/mm SGZ (C) or calretinin+ cells/mm² GCL (D).
4.3.4 No correlation between proliferation marker cell counts and B-amyloid plaque load

β-amyloid plaques are heavily implicated in AD pathology and are a determinant in the pathology grading system, thus it was of interest to investigate whether amyloid plaque load was correlated with neurogenic marker expression. Some plaques were observed in the hippocampus and entorhinal cortex of control cases, but within the bounds of normal for age (Figure 4.7A, B). AD cases presented with a high hippocampal and entorhinal plaque load. Hippocampal plaques were particularly abundant throughout the CA1, CA2 and CA3 regions and present around the GCL and hilus (Figure 4.7C). The entorhinal cortex showed numerous small plaques throughout the superficial cortical layers and larger diffuse plaques in the deeper cortical layers (Figure 4.7D).

β-amyloid load was quantified as average percent area stained per image. Hippocampal β-amyloid load was assessed as well as entorhinal cortex β-amyloid load since this region is one of the earliest affected in AD and often shows more severe amyloid pathology.

No significant correlation was detected between β-amyloid load and any of the three cell markers quantified in this study for control or AD cases. This was true for both the per unit length and per unit area analysis of each cell specific marker (Figure 4.8).
Figure 4.7  β-amyloid staining in the hippocampus and entorhinal cortex
Control cases showed low amounts of β-amyloid immunoreactivity in the hippocampus (A). β-amyloid plaques were often observed in the subiculum (A) and entorhinal cortex of controls (B), however at much lower levels than that of AD cases. β-amyloid plaques were particularly abundant throughout the CA1, CA2 and CA3 regions of the AD hippocampus, with some plaques also located around the GCL and hilus (C). The entorhinal cortex of AD cases showed very high numbers of β-amyloid plaques (D). Abbreviations: SUB, subiculum; H, hilus. Scale bars: 500 μm (A, C); 200 μm (B, D).
Figure 4.8
Correlation of β-amyloid load with PCNA, PSA-NCAM and calretinin.
Hippocampal (HP) and entorhinal cortex (EC) amyloid load was compared with PCNA⁺ (A-D), PSA-NCAM⁺ (E-H) and calretinin⁺ (I-L) cell number per unit length and per unit area. No significant correlation was detected between amyloid load in either region with any of these three markers.
4.3.5 Inverse correlation between PSA-NCAM+ cell number and tau load

Aggregated hyperphosphorylated tau protein is another pathological marker of AD and an important factor in the disease grading system. Tau aggregate load also correlates strongly with disease symptom severity (Wilcock and Esiri, 1982; Arriagada et al., 1992), therefore the correlation between tau load and PCNA+, PSA-NCAM+ and calretinin+ cell counts was investigated to determine if any changes occurred with disease progression.

Similar to β-amyloid, control cases had little or no tau aggregates in the hippocampus and entorhinal cortex (Figure 4.9A, B) while AD cases presented with very high tau loads. In AD cases the tau aggregates were abundant throughout the CA1, CA2, CA3 and subiculum of the hippocampus with numerous aggregates also located in the hilus (Figure 4.9C). The entorhinal cortex of AD cases showed aggregates accumulated in layers II, III and V, consistent with the location of neuronal cell soma (Figure 4.9D).

An inverse correlation was observed between hippocampal tau load and the number of PSA-NCAM+ cells/mm SGZ for both control and AD cases, however, only the control group correlation was statistically significant (Figure 4.10E, \( r = -0.6107, P = 0.0266 \)). An inverse correlation was also observed between hippocampal tau load and the number of PSA-NCAM+ cells/mm² GCL for both control and AD cases. In this instance the correlation was statistically significant for both groups (Figure 4.10F, control: \( r = -0.6394, P = 0.0186 \). AD: \( r = -0.8367, P = 0.0086 \)).

No significant correlation was detected between hippocampal tau load and number of PCNA+ cells (Figure 4.10A-B) or calretinin+ cells (Figure 4.10I-J) for either control or AD cases. Furthermore, entorhinal cortex tau load showed no significant correlation with PCNA+ (Figure 4.10C-D), PSA-NCAM+ (Figure 4.10G-H) or calretinin+ (Figure 4.10K-L) cell counts for control or AD groups when analysed per unit length or area.

Overall, the lack of correlation with either PCNA+ or calretinin+ cell numbers suggests tau pathology or disease severity has a minimal effect on the overall neurogenic capacity of the region. The inverse correlation between hippocampal tau load and PSA-NCAM expression in both control and AD groups suggests PSA-NCAM expression could be negatively impacted by tau aggregation. It could also indicate PSA-NCAM expression declines with disease progression.
Figure 4.9  Tau aggregate staining in the hippocampus and entorhinal cortex.
(A) Control cases showed low amounts of tau immunoreactivity in the CA1 area, CA3 area and subiculum of the hippocampus. (B) Some aggregates were also observed in the entorhinal cortex of controls. (C) An extremely large amount of tau aggregates were present throughout the CA1 and CA3 regions of the AD hippocampus. Tau aggregates were also abundant around the GCL and hilus. (D) The entorhinal cortex of AD cases had very high numbers of tau aggregates, particularly in layer II/III and V. Abbreviations: SUB, subiculum; H, hilus. Scale bar 500 μm (A, C); 200 μm (B, D).
Cell Proliferation and Neurogenesis in the Alzheimer's Disease Hippocampus

Figure 4.10
Correlation of tau load with PCNA, PSA-NCAM and calretinin
Hippocampal (HP) and entorhinal cortex (EC) tau load was compared with PCNA+ (A-D), PSA-NCAM+ (E-H) and calretinin+ (I-L) cell number per unit length and per unit area. Hippocampal tau load was inversely correlated with PSA-NCAM+ cell number per unit length for control cases (E) and per unit area for control and Alzheimer’s disease cases (F). No significant correlation was detected between hippocampal tau load and PCNA+ (A, B) or calretinin+ (I, J) cell number per unit length or area for either condition. There was also no significant correlation between entorhinal cortex tau load and any of the cell specific markers (PCNA, PSA-NCAM or calretinin) in the per unit length or per unit area analysis for either condition (C, D, G, H, K, L).
4.3.6 No correlation between proliferation marker cell counts and age, post-mortem delay or gender

To determine whether case specific factors affected the detection of proliferating or maturing cells, the correlation of PCNA⁺, PSA-NCAM⁺ and calretinin⁺ cell numbers with age, post-mortem delay and gender was examined.

It has been well documented that proliferation declines with age (Amrein et al., 2011; Spalding et al., 2013). Therefore, to ensure the quantification of proliferation was not confounded by differences between the age of the cases, the correlation between these two factors was assessed. No significant correlation was detected between age and SGZ PCNA⁺, PSA-NCAM⁺ or calretinin⁺ cell numbers per unit length or area in either control or AD cases (Figure 4.11A, D, G).

Extended post-mortem delay may result in tissue degradation and impaired antibody binding however no significant correlation was detected between SGZ PCNA⁺ or calretinin⁺ cell number per unit length or per unit area (Figure 4.11B, H). Conflicting correlations were identified between PSA-NCAM⁺ cell number and post-mortem delay when control and AD cases were assessed separately (Figure 4.11E. r = -0.5663, P = 0.0347 and r = 0.6480, P = 0.0311 respectively) however when both groups were assessed together there was no significant correlation between PSA-NCAM⁺ cell number and post-mortem delay (r = -0.0357, P = 0.8656). Therefore, it is unlikely that the counts were affected by tissue degradation due to differences in post-mortem delay between cases.

All cases used in this study were gender matched and no difference in SGZ PCNA⁺, PSA-NCAM⁺ or calretinin⁺ cell numbers were detected per unit length or area between males and females in either the control or AD group (Figure 4.11 C, F, I).
Case Factor Correlation Analysis

Figure 4.11 Correlation between cell marker counts and case factors.

(A-C) No significant correlation was detected between PCNA+ cell counts and either age (A), post-mortem delay (B) or gender (C) of the cases. (D-F) Additionally no correlation was detected between PSA-NCAM+ cell counts and age (D) or gender (F). A significant correlation was detected between post-mortem delay and PSA-NCAM cells for AD cases however when compared across the entire case list no correlation was detected (E). Calretinin+ cell counts also showed no correlation with age (G), post-mortem delay (H) or gender (I). The correlations with cell counts per unit length are presented in this figure for clarity however the same conclusions were obtained for the analysis of counts per unit area.
4.3.7 PSA-NCAM\(^{+}\) and calretinin\(^{+}\) cells in the sub-granular zone have a neuronal lineage

Immunofluorescent triple-labelling of the cell specific markers with the neuronal marker NeuN and glial marker GFAP was performed to ensure the cell counts obtained were representative of neuronal and not glial proliferation in the SGZ. NeuN is expressed in late stage type 4 immature granule cells while GFAP is expressed by type 1 radial glia cells but is absent in type 2 neural precursors (Kempermann, 2011). GFAP expression is maintained throughout adult gliogenesis (Steiner et al., 2004). Therefore, neural precursors in the SGZ would be expected to express NeuN if they are in a late post-mitotic phase, but would not express GFAP at all.

PCNA\(^{+}\) cells did not co-label with either NeuN or GFAP. The absence of GFAP excludes a glial lineage (Figure 4.12A). As such, it can be concluded that the PCNA\(^{+}\) cell counts are representative of neuronal proliferation in the SGZ.

Many PSA-NCAM\(^{+}\) (Figure 4.12B) and calretinin\(^{+}\) (Figure 4.12C) cells in the SGZ co-labelled with NeuN, however, the intensity of the NeuN labelling was often much lower than that of the surrounding granule cells. There were also PSA-NCAM\(^{+}\) and calretinin\(^{+}\) cells that did not co-label with NeuN. This is expected as PSA-NCAM and calretinin can be expressed by type 3 post-mitotic cells while NeuN expression occurs later in late stage type 4 post-mitotic cells (Kempermann, 2011). No GFAP co-labelling was observed on any PSA-NCAM\(^{+}\) or calretinin\(^{+}\) cells so it is reasonable to assume these markers label a population of type 3 and 4 immature granule cells in the SGZ that are not yet mature enough or have just begun to express NeuN. The cell counts for these two markers are therefore likely to represent neuronal rather than glial maturation.

Figure 4.12 Co-localisation of PCNA, PSA-NCAM and calretinin with mature glial and neuronal markers. (A) Arrowheads indicate the location of a PCNA\(^{+}\) cell in the SGZ underlying the GCL. PCNA did not co-localise with NeuN (A\(_{3}\)) or GFAP (A\(_{4}\)) indicating that a neuronal or glial lineage has not yet been established in these cells. (B) Arrowheads indicate the location of a PSA-NCAM\(^{+}\) cell in the SGZ. PSA-NCAM\(^{+}\) cells co-localised with NeuN (B\(_{3}\)) but not GFAP (B\(_{4}\)) indicating a neuronal lineage. For reasons unknown, GFAP staining produced a non-specific speckle only on sections where TrisEDTA antigen retrieval was used. (C) Arrowheads indicate the location of calretinin\(^{+}\) cells in the SGZ. As for PSA-NCAM\(^{+}\), calretinin\(^{+}\) cells co-localised with NeuN (C\(_{3}\)) but not GFAP (C\(_{4}\)), indicating a neuronal rather than a glial lineage. Scale bars = 10µm. Images are 2D projections of confocal z-stacks.
4.3.8 Optimisation of Golgi staining for structural analysis of immature granule cells

The therapeutic enhancement of neurogenesis would depend on both the rate of neurogenic output and the healthy growth and integration of new neurons into the hippocampal circuitry. This study has so far presented evidence that neurogenic output is unaltered in AD. In this section, immunogolgi-staining was optimised to investigate the structure of immature SGZ cells in AD. If successful, this technique would provide an indication of whether the growth and maturation of adult-born granule cells is normal in AD.

Presented here is an overview of the findings from the pilot experiments where the optimal post-mortem tissue processing method of Golgi-staining was determined. Different sequences of Golgi-staining and fluorescent immunohistochemistry were then tested to allow the specific identification of Golgi-labelled immature cells. While these optimisations were ultimately unsuccessful in producing fluorescently-labelled Golgi-stained granule cells, this work provides a foundation for future optimisation and outlines several important considerations regarding the applicability of antibodies for this purpose.

4.3.8.1 Length of tissue fixation affects quality of Golgi-staining

Formalin fixation maintains tissue integrity for immunohistochemistry so its effect on Golgi-staining was first investigated. Comparison of the Golgi-staining from fresh tissue and short-term fixed tissue (2 days) showed far more non-specific Golgi-stain crystals in the fixed tissue (Figure 4.13A, B). However, the quality of the neuronal staining was similar with minimal fragmentation of processes and good visibility of spines. Fresh tissue was extremely delicate and difficult to process, resulting in fewer good quality sections compared to fixed tissue. As the non-specific crystals did not adversely affect the ability to distinguish individual granule cells and fresh tissue would be unlikely to withstand the immunohistochemistry procedure, subsequent optimisation was performed using fixed tissue.

Tissue from the Neurological Foundation Douglas Human Brain Bank is stored at -80°C. Successful Golgi-staining of this frozen tissue would allow tissue stored in the Brain Bank to be used for this study and therefore enable adequate matching of age, gender and post-mortem delay for control and AD groups. Prior to freezing, the fixed tissue blocks were cryoprotected in sucrose. To investigate whether this process affected the Golgi-stain, a block of cryoprotected short-term fixed tissue was tested. The quality of the Golgi-stain was comparable to untreated short-term fixed tissue (Figure 4.13C). As the cryoprotection treatment did not affect Golgi-staining, fixed-frozen and fresh-frozen tissue from the human brain bank was tested. This tissue was first thawed in PBS prior to immersion in the impregnation solution. Frozen tissue only produced non-specific
Golgi-stain crystals and no discernible neuronal staining (Figure 4.13F, G). The inability to use frozen tissue together with the highly irregular and scarce supply of fresh tissue meant that case-matching was not possible given the time constraints of this study.

The effect of fixation length was also investigated to determine if tissue that had been previously fixed but not frozen could be used for Golgi-staining. This would enable tissue to be obtained from other brain banks where it is stored in formalin rather than frozen. A 2-week fixation duration appeared to have a negative effect on the number of granule cells labelled by the Golgi-stain, although the quality of the dendrite and spine staining was still adequate for analysis (Figure 4.13D). Long term fixation was investigated using a brain that had been stored in formalin for 7 years. No discernible neuronal staining was observed in this tissue (Figure 4.13E). From these results, it can be concluded that short-term fixation of fresh post-mortem tissue produces optimal Golgi-staining for analysis of granule cell structure and spine density as well as tissue preservation for fluorescent co-labelling.
Figure 4.13 Comparison of block Golgi staining fresh tissue and short, medium and long term fixed tissue

(A) Fresh tissue produced successful labelling of granule cells in the GCL with some crystallisation. (A') Clear branches and dendritic spines are visible at higher magnification. (B) Short-term fixed tissue also produced good labelling of granule cells however there is much more crystallisation evident in the hilus and molecular layer. (B') Branching and spines are still clear at higher magnification. (C-C') A similar quality of staining was obtained for tissue that short-term fixed and cryoprotected in sucrose. (D) Medium term fixation resulted in a poorer quality of staining with far fewer granule cells observed, however spine quality is still good for those labelled (D'). (E-E') Long term fixed tissue did not produce any granule cell staining with only large clumps of crystallisation observed. (F, G) Tissue that had been frozen at -80°C prior to staining did not produce any granule cell staining. Images (A-C) are of tissue from the same case (HC150). Images (A-G) are a 2D projection of a brightfield z-stack due to the thickness of the section, scale bar: 100 μm. Images (A'-E') are a single z-plane, scale bar: 20 μm.
4.3.8.2 Optimisation of Immuno-Golgi Technique

Three different methods were attempted to fluorescently label Golgi-stained neurons. This immuno-Golgi technique would allow the identification of immature Golgi-stained granule cells and permit an analysis of spine density and branching structure to determine if healthy neuronal development occurred in AD. These pilot studies aimed to reach a balance between preservation of the Golgi-stain and sufficient antibody penetration for clear fluorescent labelling. Calretinin and PSA-NCAM antibodies were used to compare the compatibility of two different immunohistochemistry methods with Golgi-staining. PSA-NCAM is a membrane-bound glycoprotein that required antigen retrieval and tyramide signal amplification for strong immunofluorescence. Conversely, calretinin is a cytoplasmic protein and strong fluorescent signal was achieved without antigen retrieval or signal amplification in 50 μm-thick sections. Unfortunately, none of the three methods in combination with these antibodies achieved a standard that would permit detection of Golgi-stain fluorescent co-labelling.

Previous studies published successful immuno-Golgi staining of rat brain when fluorescent immunohistochemistry was performed after the Golgi-stain had been developed (Spiga et al., 2011; Pinto et al., 2012). This approach was attempted first, but only yielded a reasonable result for the PSA-NCAM antibody. That is, fluorescent labelling of PSA-NCAM was achieved although it was hard to distinguish due to very high background fluorescence (Figure 4.14B). The Golgi-stain degraded during the fluorescent labelling, although some neuronal structures were visible. Presumably this was due to the antigen retrieval procedure as the Golgi-stain was well preserved in the calretinin labelled sections that did not undergo this process (Figure 4.14C, D). However, as calretinin labelling was unsuccessful, it is possible that antigen retrieval may be required for adequate antibody penetration in thick tissue (Figure 4.14D).

The second method involved fluorescent labelling on undeveloped Golgi-stained sections to assess the effect of the developer on the fluorescent signal. Fluorescent labelling for PSA-NCAM was achieved on the Golgi-stained tissue prior to development of the stain, suggesting the antibody penetrated the tissue reasonably well (Figure 4.15A). Unfortunately when developed, the Golgi-stain had entirely degraded with no neuronal structures observed. The background fluorescence also increased, making it difficult to accurately identify PSA-NCAM labelled cells (Figure 4.15B). Some calretinin immunoreactivity was observed on the undeveloped sections, although this was mostly confined to the processes rather than the neuronal cell soma required (Figure 4.15C). Once developed, this immunoreactivity was undetectable due to increased background fluorescence (Figure 4.15D). The Golgi-stain on the calretinin section was not as severely degraded as the PSA-NCAM section although fewer neurons were observed compared to the control section from Figure
4.14C. The Golgi-stain was also more degraded than those sections where fluorescent labelling was performed after the Golgi stain was developed (method 1 seen in Figure 4.14B, C). From these experiments, it appeared the development process improved the preservation of the Golgi-stain during the fluorescent labelling procedure. It was also interesting that the undeveloped Golgi-stain auto-fluoresced on the section processed for calretinin (Figure 4.15C). This did not occur on the PSA-NCAM section most likely due to the severe degradation of the Golgi-stain.

From these first two attempts, it appeared the Golgi-stain was not well preserved during the immunohistochemistry procedures. Therefore, for method 3 a single section Golgi-stain protocol was attempted on sections after fluorescent-labelling for PSA-NCAM or calretinin. This single section method produced good Golgi-staining on 100 µm-thick sections (Figure 4.16B”). PSA-NCAM labelling produced a clear fluorescent signal in 100 µm-thick sections whilst calretinin labelling was successful but had high background (compared to that observed on 50 µm-thick sections in Figure 4.12C). The single-section Golgi-staining was not successful on either the PSA-NCAM (Figure 4.16B) or calretinin (Figure 4.16D) labelled sections compared to the unlabelled control section (Figure 4.16B”) and no co-labelling could be assessed. Interestingly, the single section Golgi-staining on fluorescently labelled tissue only produced blood vessel labelling, particularly on the calretinin section (Figure 4.16D). The fluorescent PSA-NCAM signal was well preserved during Golgi-staining (Figure 4.16A’, B’) and was far superior to the labelling that was performed after Golgi-staining (seen in Figure 4.14B, Figure 4.15A), despite increased background of the Hoechst staining. The fluorescent calretinin signal could not be detected after development of the Golgi-stain (Figure 4.16D’).

In summary, none of the three immuno-Golgi combinations produced staining of sufficient quality for analysis. It appears method 1, where fluorescent labelling was performed after the tissue had been Golgi-stained and developed provided the best balance between Golgi-stain preservation and clarity of fluorescent labelling (for the PSA-NCAM sections at least). This method would be the best to pursue for future studies.
Immuno-Golgi method 1: Golgi-stain, develop then fluorescent immunohistochemistry

(A, C) The block Golgi-stain successfully labelled granule cells in the hippocampus. (A’, C’) Further magnification of a Golgi-stained neuron. (B, B’) Subsequent fluorescent immunohistochemistry for PSA-NCAM did produce positive staining, however, the background signal was very high which made the PSA-NCAM signal hard to distinguish. (D, D’) Calretinin staining did not appear to be successful with only high background labelling observed. The Golgi-stain was better preserved after labelling for calretinin compared to PSA-NCAM.

Figure 4.14
Figure 4.15

**Immuno-Golgi method 2: Golgi-stain, fluorescent immunohistochemistry, then develop**

(A, A’) PSA-NCAM labelling was successful after Golgi-staining. Cells could be distinguished from background fluorescence; however, the labelling process appears to have degraded the Golgi stain. (B, B’) After development, the background fluorescence increased and it was apparent no Golgi-stained neurons were present. (C, C’) When the tissue was labelled for calretinin the undeveloped Golgi-stain could be observed but no calretinin+ cells. (D, D’) Golgi-staining was preserved during this staining method however after development the background fluorescence was too high to accurately detect even Hoechst staining.
Figure 4.16

Immuno-Golgi method: fluorescent immunohistochemistry, Golgi-stain then develop

(A) PSA-NCAM labelling was excellent in 100 μm sections prior to Golgi-staining. (A’) GCL PSA-NCAM cell soma are clearly labelled. (B) The single section Golgi-stain method did not produce any neuronal staining compared to the control section (B” inset); however, the fluorescent signal was well preserved (B’). (C) The 100 μm section labelled reasonably well for calretinin with cells visible in the GCL (C’). Hoechst labelling had high background. (D) Only Golgi-staining of blood vessels was achieved on this section. Background fluorescence was also too high to detect and calretinin signal after Golgi-staining (D’).
4.4 Discussion

The data presented in this chapter examine the process of hippocampal granule cell proliferation and maturation using a robust quantification method that specifically targets the SGZ. This is an important feature as many conflicting results in the field of AD neurogenesis can be attributed to disparate quantification methods. Although time consuming, direct counting of labelled cells in the SGZ is the only reliable and reproducible method that avoids the confounding effect of changes in glial proliferation and maturation which occur in the wider hippocampus. Furthermore, inconsistency between studies are likely attributed to the presentation of either cell number normalised to SGZ length or GCL area. The PSA-NCAM results presented in this chapter question the relevance of normalisation to GCL area and highlight how different methods of normalisation can produce different conclusions in studies of human neurogenesis.

4.4.1 Proliferation in the SGZ

Using the proliferation marker PCNA, it was determined that the number of proliferating cells was unchanged in the AD SGZ. This finding is in agreement with other human tissue studies that investigated proliferation in AD using cell counts (Boekhoorn et al., 2006; Ekonomou et al., 2015). Furthermore, no significant correlation was detected between PCNA⁺ cell counts and either β-amyloid or tau load in the hippocampus, suggesting proliferation is unaffected by AD pathology. Together these results indicate the proliferative capacity of SGZ neurogenic region is maintained in AD and is unlikely to contribute to memory impairment.

The estimate of PCNA⁺ cell number also highlights the rarity of SGZ proliferation, even in the control adult brain. This observation has been noted by previous human tissue studies where equivalent estimates have been presented using the same or similar counting methods (Boekhoorn et al., 2006; Lucassen et al., 2010; Low et al., 2011). Fluorescent triple labelling was used to confirm specific labelling of progenitor cells as PCNA can also be expressed by adult neurons undergoing DNA repair (Tomasevic et al., 1998). As PCNA only labels progenitor cells that were mitotically active around 24 hours prior to death, SGZ PCNA⁺ cells were not expected to co-label with neuronal differentiation markers (Bravo and Macdonald-Bravo, 1987; Hall et al., 1990). Indeed, none of the SGZ PCNA⁺ cells co-labelled with the neuronal marker, NeuN so these cells were determined to be progenitor cells rather than adult neurons undergoing DNA repair. SGZ PCNA⁺ cells also did not co-label with GFAP, which is retained by glial precursors in adult hippocampal gliogenesis (Steiner et al., 2004). Therefore, it can be concluded that the PCNA quantification is truly representative of the SGZ neural progenitor cell population. The rare
occurrence of these progenitors suggests that SGZ proliferation is not likely to be an effective mechanism for cell replacement in AD. However, it is important to note that proliferation is only one measure of neurogenic capacity and the proportion of progenitors that survive, differentiate and mature is perhaps a better measure of net neurogenesis (Kempermann, 2011). For this reason, PSA-NCAM and calretinin were also analysed as markers of post-mitotic immature neurons and Golgi-staining was optimised so structural maturation could be assessed.

4.4.2 Maturation of new-born neurons in the SGZ

The analysis of SGZ PSA-NCAM+ cells revealed a significant increase per unit length of the SGZ in AD cases compared to controls. However, this difference was not significant when normalised to GCL area. Further investigation revealed AD cases had a thicker and shorter GCL, indicating the increased number of PSA-NCAM+ cells per unit length of the SGZ was masked when normalised to GCL area. While the increase in GCL thickness may be a disease-related change, it is more likely to be an artefact of hippocampal dissection. That is, the central 2 cm of an AD hippocampus may not represent the same coronal level as that of controls due to atrophy of the overall structure.

This result also questions the relevance of normalisation to GCL area. While the area normalisation allows comparison to previous studies, it does not indicate the density of labelled cells in the actual neurogenic niche. That is, since the SGZ is defined as a three cell nuclei-wide band including only the most inferior layer of granule cells, total GCL area encompasses only small part of the neurogenic niche and a large non-neurogenic area (Eriksson et al., 1998; Kempermann, 2011). Normalisation to SGZ area would be the most biologically relevant reference area to determine progenitor cell density; however, it is difficult to accurately delineate SGZ area in the human brain. Since adult-born granule cells do not migrate further than the bottom third of the GCL, perhaps a more meaningful and accurate normalisation for future studies would be area or total granule cell count in this portion of the GCL (Kempermann et al., 2003). With the above factors considered, the increased number of PSA-NCAM+ cells per unit length of the SGZ is the more biologically relevant result from this quantification.

Fluorescent double-labelling illustrated that the increase in PSA-NCAM+ cells was not due to glial migration or maturation as all SGZ PSA-NCAM+ cells were devoid of GFAP. The majority of PSA-NCAM+ cells co-labelled with NeuN, but some did not. As NeuN is expressed by late stage post-mitotic neurons when PSA-NCAM expression begins to decrease, this observation supports the immature phenotype of SGZ PSA-NCAM+ cells (Kempermann, 2011). Overall, the increase in PSA-NCAM+ cells per unit length suggests a greater number of immature neurons are
undergoing structural maturation and migration in the AD hippocampus. However, the magnitude of this increase indicates it may not be a change of biological significance. The increased PSA-NCAM+ cell number in AD cases is agreement with previous literature, however, as these studies analysed optical density of staining rather than cell counts, the degree of change is difficult to compare (Mikkonen et al., 1999b; Perry et al., 2012).

Naturally, one could surmise than an increase in PSA-NCAM+ cell number serves to replace cells lost during disease progression. However, since neuronal proliferation (detected by PCNA expression) and the overall population of surviving adult born granule cells (detected by calretinin expression) was not increased in AD this explanation seems unlikely. Instead, the increased number of PSA-NCAM+ cells in AD could be a compensatory mechanism for the memory decline produced by AD pathology. Electrophysiological studies of post-mitotic immature granule cells revealed them to be highly adaptable, exhibiting increased LTP that has been linked to facilitation of learning and memory (Gould et al., 1999; Shors et al., 2001; Schmidt-Hieber et al., 2004; Ge et al., 2007). Once the granule cells mature, this level of plasticity declines and they become functionally indistinguishable from the older established cells (Laplagne et al., 2006; Stone et al., 2011). Therefore, maintaining a larger population of highly plastic immature granule cells may facilitate learning and memory more so than increasing the proportion reaching full maturity. During this plastic maturation phase vast numbers of immature cells are eliminated by apoptosis as they undergo synaptic integration into the hippocampal circuitry (Kempermann, 2011). PSA-NCAM has the unique ability to maintain cells in this transitional immature state as it prevents NMDA receptor activation required for integration by steric inhibition of neurotransmitter binding (Hammond et al., 2006; Ge et al., 2007). Therefore, sustained PSA-NCAM expression could facilitate the maintenance of a larger population of highly plastic immature granule cells that may have a compensatory effect on memory function. As PSA-NCAM prevents the synaptic integration necessary for survival, this increased population of immature cells does not necessarily lead to a net increase in neurogenesis as reflected by the calretinin+ cell number.

While AD cases showed significantly more PSA-NCAM+ cells than controls, a significant, inverse correlation between PSA-NCAM+ cell number and hippocampal tau load was observed in both groups. As tau load strongly correlates with disease progression and symptom severity, these data indicate that patients with more severe AD symptoms have less PSA-NCAM+ cells (Arriagada et al., 1992; Bierer et al., 1995). It is possible that as AD pathology progresses, the number of highly plastic immature granule cells declines, which would reduce their compensatory effect and potentially contribute to increased symptom severity. The inverse correlation detected in the control group suggests that this effect is not limited to the AD brain, with higher levels of age-
related tau pathology linked to reduced PSA-NCAM-mediated plasticity. However, in the normal brain, the compensatory effect may be sufficient to prevent manifestation of symptoms. It would be worthwhile to examine PSA-NCAM+ cell number in early and late stage AD as well as low and high tau pathology controls to investigate this phenomenon further.

Calretinin immunoreactivity was used as a marker of post-mitotic immature granule cells in the last phase of their maturation. Calretinin is a calcium binding protein transiently expressed by immature granule cells during synaptic integration. During this phase, large numbers of calretinin+ cells are eliminated in an activity-dependent manner (Kempermann, 2011). Analysis of calretinin+ cells indicated that immature granule cell number was not altered in AD cases when analysed per unit length or area. It is likely that a large number of early post-mitotic cells are eliminated during this phase of calretinin expression which results in a population of late phase post-mitotic cells of similar size to that seen in controls.

In conclusion, this study used a highly reproducible counting method to specifically assess the number of adult-born immature granule cells in AD. The analysis of PCNA+, PSA-NCAM+, and calretinin+ SGZ cells illustrates that net neurogenic output (the number of progenitor cells reaching full maturity) is not altered in AD. This study also detected an increased PSA-NCAM+ cell population that was inversely correlated to hippocampal tau load. While this may not be of biological significance, it could represent a compensatory response to impaired memory function in AD. Further functional assessment would be required to determine if an increased population of PSA-NCAM+ immature granule cells can improve memory and learning in AD models.

4.4.3 Optimisation of Golgi-staining in human tissue

The second aim of this study was to optimise immuno-Golgi staining to investigate the structural maturation of adult-born granule cells. Using software such as Neurolucida (MBF Bioscience), the structure of these cells could be traced and parameters such as dendrite length, branching complexity, and synaptic spine size and density could be investigated. Several studies have identified aberrant branching and decreased spine density of granule cells in AD (Scheibel and Tomiyasu, 1978; de Ruiter and Uylings, 1987; Scheff and Price, 2003, 2006) so the analysis suggested here would provide an indication of whether structural maturation of SGZ cells is also impaired.

A comprehensive comparison of how different tissue fixation and storage processes affect Golgi-Cox staining has not been done in human tissue. In order to obtain quality Golgi-staining, several tissue fixation methods were first tested. Optimum Golgi-staining of human tissue provides
detailed labelling of a cell structure including dendritic processes and synaptic spines. Ideally at least 20 granule cells would be labelled for each tissue section to provide a reasonable sample size for analysis. An individual cell should be clearly distinguishable from neighbouring labelled cells and unobscured by non-specific crystallisation. Overall, the dentate gyrus of a Golgi-stained hippocampus section should be intact with minimal cracks or tears in the tissue.

Different limitations were encountered for each fixation method. Although fresh tissue produced good staining of hippocampal granule cells, the brittle tissue resulted in cracked sections which limited the overall sample size for analysis. Short-term fixed tissue also produced good staining of granule cells but was marred by large numbers of non-specific crystals. This could be due to mercuric chloride deposits forming along formalin cross-links, which then nucleate during the impregnation process and become blackened by sulfur in the surrounding tissue during alkali treatment (Narayanan et al., 2014).

Frozen tissue and long term fixed tissue produced large non-specific crystals and were therefore incompatible with Golgi-staining. The only study to so far claim successful Golgi-staining of frozen human post-mortem tissue is questionable as it did not present any neuronal structures, rather non-specific crystals amongst a network of processes (Melendez-Ferro et al., 2009). The presence of these crystals suggested the freezing or defrosting process impaired the neuronal deposition of mercuric chloride rather than its development to a black product. In general, increased fixation duration also had a negative effect on Golgi-staining, with far fewer cells labelled in 2-week fixed tissue compared to 2-day fixed tissue and no cells labelled in 7-year fixed tissue. This is the first study to examine the effect of fixation duration on human tissue Golgi-Cox staining. The incompatibility of frozen and long-term fixed tissue with Golgi-staining limited the study of immature granule cell structure to fresh or fixed tissue as it is received. As the non-specific crystallisation did not obscure labelled granule cells, short-term fixed tissue was the preferred processing method due to superior tissue integrity.

4.4.4 Optimisation of Immuno-Golgi staining for analysis of immature neuron structure

Golgi-staining alone cannot distinguish between mature and immature granule cells, so it was necessary to optimise a fluorescent immuno-Golgi protocol to specifically investigate structural maturation of immature granule cells. To date, fluorescent co-labelling of Golgi-stained tissue has only been reported using rat brain tissue (Spiga et al., 2011; Pinto et al., 2012). When translating the technique to human tissue, the aim was to preserve the quality of Golgi-stain during the immunohistochemistry procedure and achieve a strong fluorescent signal.
Several difficulties were encountered in all three of the staining combinations tested in this study. In general, the factors that improved the fluorescent labelling had a negative impact on Golgi-stain preservation. Heat-induced antigen retrieval is one such factor. The Golgi-stain was more degraded on the sections labelled for PSA-NCAM where antigen retrieval was performed compared to calretinin sections which were not treated. The inability to use heat-mediated antigen retrieval prohibits the use of antibodies such as PSA-NCAM which require this treatment to unmask the antigen in formalin fixed tissue. It would be worthwhile to investigate different antigen retrieval methods such as enzymatic digestion, which reportedly does not affect Golgi-staining in human tissue (Buller and Rossi, 1993). While no studies have investigated the concept, the disassembly of formalin cross-links using acid/base treatment and heat could potentially dislodge mercuric chloride deposits of the Golgi-stain. Since the Golgi-stain was best preserved when developed prior to PSA-NCAM labelling, the conversion to mercuric sulfide likely stabilises the deposits.

Another factor that improved the fluorescent signal but degraded the Golgi-stain was the use of triton detergent in immunohistochemistry solutions. This detergent was omitted when fluorescent labelling was performed after Golgi-staining as it degraded the Golgi-stain (Levine et al., 2013). However, the lack of triton instead had a detrimental effect on antibody penetration as calretinin labelling was far stronger when triton was used (method 3) compared to when it was omitted (method 1 and 2). Other factors such as longer antibody incubation and agitation periods may compensate for the lack of triton and could be tested in future studies (Spiga et al., 2011).

Lastly, auto-fluorescence of the Golgi-stain affected the detection of fluorescent signal from the antibodies. This issue has also been previously reported by Spiga et al., 2011. Golgi-stain development after fluorescent labelling (method 2 and 3) increased the auto-fluorescence far more than when the Golgi-stain was developed first (method 1). Since fluorescently labelled cells could not be detected and the Golgi-staining was not successful when developed after immunohistochemistry, these two methods were not pursued further.

From these optimisations, the fluorescent labelling of developed Golgi-stained tissue (method 1) proved the most viable method. However, the issues of auto-fluorescence and Golgi-stain degradation were not adequately resolved to provide a combination of fluorescent labelling and Golgi-staining of sufficient quality for analysis. With further optimisation, successful immuno-Golgi could be used to identify immature granule cells in the human SGZ and assess whether their structural development is altered in AD.
4.5 Summary

This chapter provided an analysis of PCNA+, PSA-NCAM+, and calretinin+ cell number in the SGZ of AD and control cases using a reproducible counting method. This provided a focused examination of SGZ neurogenesis which concluded that net neurogenic output is not altered in AD. A significant increase in PSA-NCAM+ cell number per mm SGZ suggests an increase of highly plastic immature granule cells in AD. This is hypothesised to represent a compensatory response to impaired memory function, although further verification is required. PSA-NCAM+ cell number was also inversely correlated with hippocampal tau load, suggesting this response declines with disease progression. Collectively, this illustrates that a key aspect of adult human brain plasticity is preserved throughout AD and is unlikely to contribute to the development of memory deficits.

It remains to be determined whether SGZ progenitor cells successfully mature and integrate into the hippocampal circuitry in AD. This chapter attempted to investigate the structural maturation of SGZ progenitor cells using immuno-Golgi. The optimal tissue processing methods for Golgi-staining human brain tissue was examined and different methods of combining Golgi-staining with fluorescent immunohistochemistry in human tissue was investigated. Short-term fixed tissue produced the best compromise between tissue integrity and quality of Golgi-staining. While immuno-Golgi was not optimised to a standard that permits the analysis of immature granule cell structure, several important considerations were identified. From this work, fluorescent labelling after development of the Golgi-stain was found to be the most promising method. These findings will hopefully facilitate successful optimisation of immuno-Golgi and analysis of immature granule cell structure in the future.
Chapter 5. Cell Proliferation in the Alzheimer’s Disease Sub-Ventricular Zone

5.1 Introduction

Of the two neurogenic niches in the adult human brain, the SVZ produces far more neural progenitor cells than the SGZ of the hippocampus (Low et al., 2011; Curtis et al., 2012). Surprisingly, the SVZ has largely been neglected from the study of neurogenesis in the human AD brain. The human SVZ produces progenitor cells that migrate along the RMS to the olfactory bulb where they replace neurons involved in olfaction (Curtis et al., 2007b). A current hypothesis in the literature is that reduced SVZ proliferation may contribute to AD symptoms, since anosmia (loss of smell) occurs early in the disease (Devanand et al., 2000; Lazarov and Marr, 2010; Rodríguez and Verkhratsky, 2011; Winner et al., 2011; Curtis et al., 2012). However, it is unclear from previous studies whether SVZ proliferation is indeed affected in AD.

Only three studies using human tissue have examined SVZ neurogenesis in AD, each producing a contrasting conclusion using different quantification methods. Most recently, Ekonomou et al., 2014 presented cell counting data normalised to SVZ length, which indicated no change in nestin+ cells. This suggests proliferation is unchanged in the AD SVZ. Conversely, Perry et al., 2012 found nestin integrated optical density (IOD) was increased in the AD SVZ but musashi-1 IOD was unchanged. A different conclusion again was presented by Ziabreva et al., 2006 using these same markers. A nine-fold decrease in musashi-1 staining load was detected in the AD SVZ but nestin load was increased. These findings are difficult to reconcile as they use the same markers, but different quantification methods and produce conflicting conclusions.

These disparate conclusions again highlight the importance of using a reliable and reproducible quantification method that specifically identifies the progenitor cells to obtain a meaningful assessment of neurogenic capacity. The use of IOD to quantify immunoperoxidase labelling is flawed by its inability to distinguish cellular staining from non-specific background or blood vessel staining. Similarly, the quantification of staining load is not a reliable indication of cell number. The number of proliferating cells in a given area and along a given length of the neurogenic niche are the relevant measurements of interest when quantifying neurogenic capacity. Thus, manual cell counting is undoubtedly the most reliable method of quantification and was used in this chapter. Given the amount of proliferation per unit length SVZ and per unit area of the SVZ are both biologically relevant measures in this region, both sets of data were obtained.
This study also accounted for potential regional changes along the SVZ by assessing dorsal, middle and ventral sub-regions individually. The potential for a dorso-ventral gradient in proliferation has not previously been accounted for in studies of the AD SVZ. PCNA and PSA-NCAM were used to identify SVZ progenitor cells as they clearly label individual cells and are not affected by post-mortem delay of the tissue. PCNA labels cells that were mitotically active 24 hours before death and PSA-NCAM labels cells undergoing structural plasticity or migration in the SVZ (Bravo and Macdonald-Bravo, 1987; Hall et al., 1990; Bonfanti and Theodosis, 1994). These two markers identify SVZ cells in similar phases of neurogenesis and therefore both give an indication of proliferative capacity.

In line with previous studies, areas of the SVZ that included blood vessels were excluded from this analysis (Curtis et al., 2005a; Ekonomou et al., 2015). However, the effect of blood vessels on local proliferation was also evaluated by analysing the density of PCNA+ cells across the entire SVZ. This assessment revealed several important considerations that affect quantification of SVZ proliferation. Overall, this chapter presents a reliable assessment of proliferation in the AD SVZ, investigates the distribution of proliferative cells across the SVZ and considers factors to further improve the quantification method.
5.2 Methods

5.2.1 Tissue Microarray Construction

As outlined in section 3.1.2, the SVZ tissue microarrays used for this study were prepared by Claire Lill (Research technician, CBR, University of Auckland) using paraffin-embedded formalin-fixed SVZ tissue. The control cases used for these tissue microarrays had no history of neurological disease or treatment and pathological analysis excluded any neuropathology. Control cases ranged from 35 to 98 years old with an average age of 68.7 ± 17.4 years and an average post-mortem delay of 20.2 ± 10.6 hours (Table 13). Pathological analysis also determined that any β-amyloid plaque changes in these cases were consistent with that of normal aging (Braak and Braak, 1995).

The AD cases used for the tissue microarrays were examined by an independent neuropathologist to determine AD pathology. Widespread presence of plaques and tangle load was confirmed and all cases had a Braak stage between IV and VI, indicative of pathological AD (Braak and Braak, 1995). AD cases ranged in age from 62 to 85 years old with an average age of 75.3 ± 7.1 years and an average post-mortem delay of 15.5 ± 14.3 hours (Table 13).

Briefly, cores with a 2 mm diameter were extracted from the dorsal, middle and ventral aspects of the SVZ from each case and inserted into a recipient paraffin block. Tissue microarray sections 7 µm in thickness were cut using a microtome and mounted onto slides in preparation for staining.

<table>
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<th>Case Number</th>
<th>Age</th>
<th>Sex</th>
<th>Post-mortem Delay (hr)</th>
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<tr>
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<td>87</td>
<td>F</td>
<td>11</td>
</tr>
<tr>
<td>H152</td>
<td>79</td>
<td>M</td>
<td>18</td>
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5.2.2 Immunoperoxidase staining and analysis of proliferation markers

Immunoperoxidase labelling of tissue microarray sections was performed as outlined in section 3.2.4. Three sections from the control and AD tissue microarrays were labelled for PCNA (sc7907, Santa Cruz) and PSA-NCAM (MAB5324, Millipore). A cresyl violet counterstain was performed as per section 3.2.2 to allow accurate measurement of SVZ area and reduce false-positive cell counts. The cores were imaged with a 20x objective using the MetaSystems VSlide scanning system including a fully motorised Zeiss Axio Imager Z2 microscope and MetaCyte acquisition software. Measurements and proliferation marker quantification were carried out using ImageJ v1.46. A 500 µm length of SVZ from each core was selected for quantification using the segmented line tool and the area of this selection was determined using the polygon selection tool. Regions for analysis were selected away from blood vessels as these structures confound the measurement of SVZ area. For each core, background cresyl violet density measurements were taken randomly from a 0.01 mm² area of the caudate in triplicate using the rectangular selection tool and averaged. A cell was determined to be positively labelled for DAB if it had a grey value at least 60 points darker than the averaged cresyl violet background measurement for that core (0 = black, 255 = white). The number of DAB labelled cells was analysed per unit length and per unit area for each core. The data were averaged across all sections for each core and analysed per sub-region. The data were also averaged for all cores within a case across all three sections and analysed as overall SVZ counts per case.
5.2.3 Immunoperoxidase staining and analysis of pathological aggregates

Two sections from each tissue microarray were labelled for β-amyloid (M0872, DAKO) and tau (A0024, DAKO) as described in section 3.2.4. No cresyl violet counterstaining was used for this analysis; however, due to the total absence of background staining in the β-amyloid labelled sections, a coloured background was applied to determine the boundaries of each core. This was achieved by incubating the stained sections in tartrazine (also known as yellow food colouring E102) and washing in xylene prior to coverslipping. Cores were imaged as described above on the MetaSystems VSlide scanning system. A series of 5 random images were obtained from the region of caudate nucleus on each core using Adobe Photoshop CS6. A grid of 1000 x 1000 pixels was randomly superimposed on the stitched image of the core and the area of the grid boxes extracted into separate image files. The staining load of each image was quantified using MetaMorph high-throughput image analysis software (version 7.8.0, Molecular Devices) as described in section 4.2.3. The ‘count nuclei’ algorithm was used to threshold the staining relative to local background (minimum object area of 50 µm² and minimum object grey value intensity above background of 50 points). This analysis produced a measurement of ‘average percentage area of image stained’ as an indication of pathological aggregate load.

5.2.4 Statistical analysis

Statistical analysis was performed using the GraphPad Prism (version 6.05) statistics and graphing package. The assumption of equality of variance was met for the PCNA and PSA-NCAM data sets as per the F-test, but a normal distribution was not obtained as determined by a Shapiro-Wilk normality test. Therefore, non-parametric Mann-Whitney U tests were performed to detect significant differences in proliferation marker counts between the two groups. A normal distribution was obtained for the SVZ width data set so a parametric unpaired t-test was performed for this data. All values are presented in this section as mean ± standard deviation with statistical significance set at P ≤ 0.05.

A non-parametric Spearman’s correlation coefficient was used to investigate the relationship between β-amyloid load, tau load and proliferation marker counts. The correlation between proliferating cell counts and case factors (age, gender and post-mortem delay) was also investigated. A parametric Pearson’s correlation coefficient was used to investigate the relationship between β-amyloid load, tau load and SVZ width. For this study, two factors were considered to be correlated if a P value ≤ 0.05 was obtained. The Spearman’s or Pearson’s r value has been reported for all comparisons.
5.2.5 Immunofluorescent characterisation of immature sub-ventricular zone cells

Fluorescent triple labelling of paraffin-embedded caudate nucleus sections (outlined in section 3.2.5) was used to check for a neuronal or glial phenotype in the SVZ cells expressing PCNA or PSA-NCAM. Permeabilisation and antigen retrieval steps were carried out as for DAB immunoperoxidase staining. Combinations of primary antibodies from different host animals were used to triple-label PCNA (sc7907, santa cruz) and PSA-NCAM (MAB5324, Millipore) with NeuN (ABN78, Millipore) and GFAP (AB4674, Abcam) as indicated in Table 5. Alexa-Fluor dye-conjugated secondary antibodies of an appropriate host species were used to label the bound primary antibodies except those for PCNA and PSA-NCAM where TSA amplification was performed as described in section 3.2.3. A Hoechst counterstain was also applied to all sections. Fluorescent sections were imaged using an Olympus FV1000 confocal microscope with a 60x oil immersion lens.

5.2.6 Analysis of proliferation variability across the sub-ventricular zone

To assess the effect of blood vessels on SVZ proliferation, PCNA+ cell density was investigated across the entire SVZ of two control and two AD cases (indicated on Table 13). These four cases had also been included on the tissue microarrays so the regions where the cores had been taken could be determined. For this analysis, paraffin-embedded sections of caudate nucleus were labelled for PCNA using immunoperoxidase labelling (section 3.2.4) with a cresyl violet counterstain (section 3.2.2). The sections were imaged with a 20x objective using the MetaSystems VSlide scanning system described above. All measurements and quantification was performed using ImageJ v1.46. As for the cores, background cresyl violet density measurements were taken randomly from a 0.01 mm² area of the caudate in triplicate using the rectangular selection tool and averaged. The entire length of the SVZ was subdivided into 200 µm segments, irrespective of blood vessel location, using the segmented line tool. For each segment the area of the SVZ was also determined using the polygon selection tool. The blood vessel lumen was excluded from this area measurement. PCNA labelled cells were counted using the cell counter plug-in. A cell was determined to be positively labelled for DAB if it had a grey value at least 60 points darker than the averaged cresyl violet background measurement (0 = black, 255 = white). The number of PCNA+ cells per 200 µm segment was then normalised to the exact length and area of that segment. A heat map of PCNA+ cell density was then generated in Adobe Illustrator CS6 by drawing a path around each 200 µm segment which was coloured according to its density value.
5.3 Results

5.3.1 No significant change to number of PCNA$^+$ cells in the sub-ventricular zone of AD cases.

PCNA$^+$ nuclei were predominantly observed in layer III of the SVZ (Figure 5.1A-B, arrows). PCNA$^+$ ependymal cells were also observed in layer I; however, these cells were excluded from the quantification analysis as they are not considered to contribute to the pool of migrating precursor cells that eventually differentiate into olfactory interneurons (Chiasson et al., 1999). This same layer-specific distribution of PCNA$^+$ cells was observed in all three sub-regions assessed (dorsal, middle, and ventral SVZ).

To determine the mean PCNA$^+$ cell number for the overall SVZ in each case, the data from all cores pertaining to that case were averaged. From this analysis, there was no significant change in PCNA$^+$ cells per unit length in AD cases compared to controls (Figure 5.1C, control mean = 16.7 ± 11.6 cells/mm SVZ, AD mean = 15.4 ± 8.8 cells/mm, $P = 0.7495$). There was also no difference when the PCNA$^+$ cell count was normalised to SVZ area (Figure 5.1D, control mean = 166.2 ± 96.9 cells/mm$^2$ SVZ, AD mean = 191.5 ± 116.2 cells/mm$^2$ SVZ, $P = 0.5593$).

The mean of each SVZ sub-region was also analysed independently to determine if changes in proliferation were restricted to certain regions of the SVZ in AD cases. All three sub-regions showed no change in PCNA$^+$ cell number when normalised to either SVZ length or area (Figure 5.1C – D).

For the dorsal SVZ sub-region, no significant difference was detected between control and AD cases. When normalised to SVZ length, the dorsal region of AD cases had a mean of 12.9 ± 21.9 cells/mm SVZ compared to 6.1 ± 3.3 cells/mm SVZ for controls (Figure 5.1C, $P = 0.4221$). When normalised to SVZ area AD cases had a mean of 68.5 ± 37.6 cells/mm SVZ compared to 125.4 ± 96.8 cells/mm SVZ for controls (Figure 5.1D, $P = 0.3100$).

No change in PCNA$^+$ cell number was detected in the middle SVZ sub-region with a mean of 11.3 ± 10.1 cells/mm SVZ for control cases and 11.2 ± 4.8 cells/mm SVZ for AD cases (Figure 5.1C, $P = 0.3475$). The same was true when normalised to SVZ area with a mean of 143.3 ± 90.2 cells/mm$^2$ SVZ in control cases and 180.5 ± 94.4 cells/mm$^2$ SVZ in AD (Figure 5.1D, $P = 0.3235$).

Lastly the ventral SVZ sub-region appeared to have the most PCNA$^+$ cells of all the three regions. However, it showed the same result as the dorsal and middle sub-regions with no change in PCNA$^+$ cell number for AD cases. AD cases had a mean of 24.4 ± 15.4 cells/mm SVZ compared to 28.7
± 25.1 cells/mm SVZ for controls. (Figure 5.1C, P = 0.6562). When analysed per unit area, the same conclusion was obtained. AD cases had a mean of 294.3 ± 200.4 cells/mm² SVZ compared to 223.4 ± 151.2 cells/mm² SVZ in controls (Figure 5.1D, P = 0.4001).

The overall distribution of PCNA+ cells across the SVZ was investigated for control and AD groups separately by comparing the means from each sub-region using a one-way ANOVA test. For the control group the number of PCNA+ cells per unit length was significantly different across the sub-regions. Post-hoc tests revealed the ventral region had more PCNA+ cells/mm SVZ than the dorsal and middle sub-regions (Figure 5.2A. Mean difference ventral vs dorsal = 17.7 cells/mm SVZ, P = 0.0404. Mean difference ventral vs middle = 17.3 cells/mm SVZ, P = 0.0404). There was no significant difference between dorsal and middle sub-regions for control cases (P = 0.9531). This distribution was also observed for AD cases, with the ventral region showing the most PCNA+ cells/mm SVZ (Figure 5.2A. Mean difference ventral vs dorsal = 20.2 cells/mm SVZ, P = 0.0047. Mean difference ventral vs middle = 13.8 cells/mm SVZ, P = 0.0341).

The distribution of PCNA+ cells per unit area showed no significant difference between the sub-regions for control groups (Figure 5.2, P = 0.0761). In the AD group, there was a significant difference only between the ventral and dorsal sub-regions (Figure 5.2B. Mean difference ventral vs dorsal = 228.4 cells/mm² SVZ, P = 0.0178).

It is apparent from these results that SVZ PCNA+ cell density varies along the dorso-ventral axis of the SVZ and normalisation to SVZ length and area can provide different conclusions. Overall, the comparison between control and AD groups suggests proliferative capacity is unchanged in the SVZ of the AD brain. This analysis has highlighted that the proliferative capacity of the SVZ is variable between individuals within a group and also within the structure of the SVZ itself.
Figure 5.1  PCNA Quantification in the SVZ

(A-B) PCNA was observed as a nuclear stain in layer III of the SVZ (arrows). Several ependymal cells in layer I also show PCNA immunoreactivity. (C) No significant difference in PCNA⁺ cell number was detected between AD cases and controls when normalised to SVZ length. This was also true for each sub-region (dorsal, middle ventral). (D) No significant difference was observed between AD and control cases when PCNA⁺ cell number was normalised to SVZ area. Again, this was true for the dorsal, middle and ventral SVZ sub-region when analysed separately. Abbreviations: CN, caudate nucleus. Scale bars: 100 µm (A), 20 µm (B).
Figure 5.2  Distribution of PCNA⁺ cells across the dorso-ventral axis of the SVZ
(A) For both control and AD cases the ventral sub-region had significantly more PCNA⁺ cells/mm SVZ than the dorsal and middle sub-regions. (B) The ventral sub-region of AD cases had significantly more PCNA⁺ cells/mm² SVZ than the dorsal sub-region, but not the middle. No significant difference was detected per unit area for controls.
5.3.2 Increase in PSA-NCAM+ cells in the AD sub-ventricular zone reflects a change in sub-ventricular zone thickness

PSA-NCAM+ cells were observed in layers III and IV across all sub-regions of the SVZ (dorsal, middle and ventral) as illustrated in Figure 5.3A - B (arrows).

The PSA-NCAM+ cell numbers from all cores within a case were averaged to determine the mean cell count for the overall SVZ of that case. For this overall SVZ analysis there was no significant difference in the number of PSA-NCAM+ cells per unit length between AD cases (mean of 15.6 ± 8.4 cells/mm SVZ) and controls (mean of 12.2 ± 5.7 cells/mm SVZ, P = 0.1426, Figure 5.3C). However, a significant increase was detected when PSA-NCAM+ cell number was normalised to the SVZ area with a mean of 104.8 ± 52.4 cells/mm² SVZ for controls compared to a mean of 161.4 ± 77.5 cells/mm² SVZ for AD cases (Figure 5.2G, P = 0.0420).

For the dorsal sub-region, the PSA-NCAM+ cell number was not significantly different in AD cases (mean = 13.8 ± 5.9 cells/mm SVZ) compared to controls (mean of 11.9 ± 7.6 cells/mm SVZ, Figure 5.3C, P = 0.5027). The same was found when counts were normalised to SVZ area, with a mean of 146.1 ± 65.0 cells/mm² SVZ for AD cases and 97.7 ± 66.8 cells/mm² SVZ for controls (Figure 5.3D, P = 0.0513).

The middle sub-region reflected the findings of the overall SVZ analysis. No significant difference was detected between AD cases (16.5 ± 8.3 cells/mm SVZ) and controls (12.3 ± 6.0 cells/mm SVZ, Figure 5.3C, P = 0.1716) when analysed per unit length. There was a significant increase in PSA-NCAM+ cell number for AD cases compared to controls when analysed per unit area (Figure 5.3D; control mean = 121.8 ± 64.7 cells/mm² SVZ, AD mean = 205.9 ± 96.9 cells/mm² SVZ, P = 0.0204).

The ventral sub-region showed no significant differences between AD and control cases for the per unit length analysis (Figure 5.3C; control mean = 14.4 ± 8.4 cells/mm SVZ, AD mean = 10.9 ± 4.2 cells/mm SVZ, P = 0.4698) or the per unit area analysis (Figure 5.3D; control mean = 101.8 ± 59.7 cells/mm² SVZ, AD mean = 139.4 ± 101.6 cells/mm² SVZ, P = 0.3638).

The significant increase in PSA-NCAM+ cells per unit area, but not per unit length may reflect a change in the dimensions of the SVZ. This was also noted in the quantification of SGZ PSA-NCAM+ cells (section 4.3.2). To investigate this further, SVZ width was compared between AD and control cases.
Figure 5.3  PSA-NCAM quantification in the SVZ

(A) PSA-NCAM immunoreactivity was observed in layer III and the border of layer IV of the SVZ. (B) PSA-NCAM+ cells had an elongated shape characteristic of migrating cells (arrows). (C) Overall there was no significant difference in the number of PSA-NCAM+ cells/mm SVZ between control and AD cases. This was also true for each sub-region (dorsal, middle ventral). (B) When normalised to SVZ area, a significant increase in PSA-NCAM+ cells/mm² was detected in AD cases, specifically in the middle sub-region. However, no significant difference was detected in either the dorsal or ventral sub-regions. Abbreviations: CN, caudate nucleus. Scale bars: 100 µm (A), 20 µm (B). SVZ width is reduced only in the middle sub-region of AD cases.
5.3.3 SVZ width is reduced only in the middle sub-region of AD cases

The increase in PSA-NCAM$^+$ cells per unit area, but not per unit length, suggests SVZ width may be altered in AD cases. SVZ width was calculated using the length and area measurements obtained during cell counting and averaged for each core. As the regions that contained large blood vessels were excluded from the counting analysis, the width measurements do not reflect the presence of these structures. As the main purpose of this analysis was to determine the width of the layers where neural progenitors reside, only the combined width of layers II and III was used (Figure 5.4A).

Overall no significant difference in SVZ width was detected between control and AD cases (average width $110 \mu m \pm 16 \mu m$ for controls and $99 \mu m \pm 28 \mu m$ for AD, $P = 0.2352$, Figure 5.4B). When individual sub-regions were analysed, a significant decrease was detected in the middle SVZ region only (average width $97 \mu m \pm 27 \mu m$ for controls and $76 \mu m \pm 20 \mu m$ for AD, $P = 0.0396$, Figure 5.4B). This reflects a 21.7% reduction in width, a relatively small fold change which is unlikely to have any major functional significance.

No significant difference between AD and control cases was detected in the dorsal sub-region (average width $110 \mu m \pm 31 \mu m$ for controls and $109 \mu m \pm 41 \mu m$ for AD, $P = 0.9257$, Figure 5.4B) or the ventral sub-region (average width $128 \mu m \pm 31 \mu m$ for controls and $96 \mu m \pm 40 \mu m$ for AD, $P = 0.0700$, Figure 5.4B).

The increased number of PSA-NCAM$^+$ cells per unit area detected in the middle sub-region of AD cases therefore reflects a reduction in SVZ width in this region. These results suggest similar numbers of progenitor cells are present along the SVZ in AD compared to controls, even in areas where the SVZ is thinner.
Figure 5.4  SVZ width analysis

(A) SVZ labelled for PCNA and counterstained with Nissl. The SVZ is comprised of four layers, only the width of layers II – III was used for this analysis. (B) When analysed overall there was no significant difference in SVZ width between control and AD cases. When assessed by sub-region, SVZ width was significantly reduced in the middle sub-region. No significant difference was detected in the dorsal or ventral sub-regions. Abbreviations: CN, caudate nucleus. Scale bar: 100 μm (A).
5.3.4 No correlation between proliferation markers and AD pathology

β-amyloid and tau loads were assessed in the region of caudate nucleus underlying the SVZ in each core. Some plaques were observed in the caudate nucleus of control cases, although this was deemed within the normal limits of aging by independent pathological analysis (Figure 5.5A). Far more β-amyloid plaques were observed in the caudate nucleus of the AD cases, but none where observed in the SVZ itself (Figure 5.5B). The amyloid load was quantified as average percent area stained per image.

No significant correlation was detected between β-amyloid load and either PCNA⁺ cell number, PSA-NCAM⁺ cell number or the SVZ width. Only the per unit length analysis of PCNA and PSA-NCAM has been presented in Figure 5.6, however this result was also true for the per unit area analysis of these markers.

Tau immunoreactivity was very low in the cores from control cases (Figure 5.7A), but the black intracellular deposits were present in the SVZ and abundant in the caudate nucleus of cores from AD cases (Figure 5.7B arrows).

No significant correlation was detected between tau load in the caudate nucleus immediately inferior to the SVZ and PSA-NCAM⁺ cell number. This was true for both the per unit length and per unit area analysis of PSA-NCAM. A positive correlation was found between PCNA⁺ cells/mm SVZ and tau load in the dorsal sub-region of AD cases only (Figure 5.8B, Spearman’s r = 0.8095, P = 0.0218). However, this was not detected in the per unit area analysis (data not shown, Spearman’s r = 0.5952, P = 0.1322) nor in any other sub-region. Therefore, the relevance of this correlation is difficult to interpret. Similarly, a positive correlation was found between SVZ width and tau load in the middle sub-region of control cases only (Figure 5.8K, Pearson’s r = 0.6340, P = 0.0268). While statistically significant, this is a relatively weak correlation.

Overall these results indicate there is unlikely to be a biologically relevant interaction between SVZ proliferating cells and AD pathological aggregates.
**Figure 5.5  β-amyloid staining in the SVZ and caudate nucleus**

(A) Control cases showed some β-amyloid immunoreactivity in the caudate region of the cores, but substantially less than that of AD cases (B). β-amyloid plaques appeared as dark diffuse patches of staining in both control (A’) and AD cores (B’). No β-amyloid plaques were observed within the SVZ of either control (A’’) or AD cases (B’’).
Figure 5.6
Correlation graphs of proliferation markers and SVZ width with caudate nucleus β-amyloid load
No significant correlation was detected between caudate nucleus β-amyloid load and either PCNA+ (A) or PSA-NCAM+ (E) cell number per unit length SVZ for either control or AD cases. This finding was consistent across all three SVZ sub-regions (dorsal, middle, ventral) for PCNA (B-D) and PSA-NCAM (F-H). There was also no significant correlation between caudate nucleus β-amyloid load and SVZ width (I) for either control or AD cases. Again, this was consistent across the three SVZ sub-regions (J-L).
Figure 5.7  Tau staining in the SVZ and caudate nucleus

(A) Control cases showed very low amounts of tau immunoreactivity in the caudate region of the cores. (A’) High magnification image illustrating the high background staining but absence of tau immunoreactivity in a control case. (A”) The SVZ had much lower background staining than the overlying caudate. (B) AD cases showed relatively more tau immunoreactivity, characterised by black intracellular deposits (B’ arrowhead). (B”) A number of punctate tau deposits were observed in the SVZ of AD cases. The overall reduction in tissue integrity of AD cores compared to controls is also illustrated in this comparison. Abbreviations: CN, caudate nucleus; SVZ, sub-ventricular zone. Scale bars: 200 μm (A, B), 50 μm (A’, B’).
Cell Proliferation in the Alzheimer's Disease Sub-Ventricular Zone

Figure 5.8
Correlation graphs of proliferation markers and SVZ width with caudate nucleus tau load

No significant correlation was detected between caudate nucleus tau load and either PCNA+ (A) or PSA-NCAM+ (E) cell number per unit length SVZ for either control or AD cases. This finding was consistent across all three SVZ sub-regions (dorsal, middle, ventral) for PCNA (B-D) and PSA-NCAM (F-H).

There was a positive correlation between caudate tau load and SVZ width for control cases (I), however this was only significant in the middle sub-region (K) and the correlation coefficient ($r = 0.6340$) suggests this is a weak correlation. This positive correlation was not observed for AD cases.
5.3.5 No correlation between proliferation markers and age, post-mortem delay or gender

As for the SGZ analysis, it was important to confirm that case-specific factors did not affect the detection of proliferating or maturing cells in the paraffin embedded tissue. Only the case factor correlations for the per unit length analysis have been presented in this section as the conclusions for the per unit area analysis were the same.

No significant correlation was detected between age and PCNA$^+$ cell counts (Figure 5.9A) or SVZ width (Figure 5.9G). PSA-NCAM$^+$ cell counts did show a significant positive correlation with age for control cases only (Figure 5.9D, Spearman’s $r = 0.6235$, $P = 0.0336$). This was a weak correlation but it was detected when both AD and control groups were assessed together (data not shown, Spearman’s $r = 0.4826$, $P = 0.0169$) and in the per unit area analysis for control cases (data not shown, Spearman’s $r = 0.7400$, $P = 0.0051$). The possibility that PSA-NCAM immunoreactivity may be influenced by age should be taken into account when interpreting this data.

No significant correlation was detected between post-mortem delay and either PCNA$^+$ cell counts (Figure 5.9B), PSA-NCAM$^+$ cell counts (Figure 5.9E) or SVZ width (Figure 5.9H). Lastly, PCNA$^+$ cell counts (Figure 5.9C), PSA-NCAM$^+$ cell counts (Figure 5.9F) and SVZ width (Figure 5.9I) were not significantly different between males and females for either the control or AD groups. These data indicate that post-mortem delay and gender had no obvious effect on the detection of PCNA, PSA-NCAM or on SVZ width.
Figure 5.9  Correlation between case factors and PCNA\textsuperscript{+} cell counts, PSA-NCAM\textsuperscript{+} cell counts or SVZ width

(A-B) No correlation was detected between PCNA\textsuperscript{+} cell counts and either age or post-mortem delay when analysed for the SVZ overall. (C) Additionally, no difference between males or females was detected for either the control or AD group. (D) There was a correlation between PSA-NCAM\textsuperscript{+} cells/mm SVZ and age for control cases only. This was a weak correlation ($r = 0.6235$) and was not detected in the per unit area analysis (not shown). (E) No correlation was detected between PSA-NCAM\textsuperscript{+} cell counts and post-mortem delay. (F) No difference in mean PSA-NCAM\textsuperscript{+} cells/mm was detected between males and females for either the control or AD group. Lastly there was no correlation between SVZ width and age (G) or post-mortem delay (H). Similarly, no difference in SVZ width was detected between males and females in either AD or controls (I).
5.3.6 Fluorescent co-labelling of PCNA/PSA-NCAM with NeuN & GFAP

Immunofluorescent triple-labelling of PCNA and PSA-NCAM with the mature neuron marker NeuN and glial marker GFAP was performed to ensure the cell counts obtained were representative of neuronal and not glial proliferation. The type A SVZ neuroblasts are produced by type C transient amplifying cells which are in turn derived from type B glial cells (Doetsch et al., 1999). GFAP expression is lost during the conversion from type B to type C cells and the type A neuroblasts remain in a precursor state until they reach the olfactory bulb where differentiation cues are received (Eriksson et al., 1998; Curtis et al., 2003; Ming and Song, 2005). Therefore, SVZ neuroblasts identified by PCNA and PSA-NCAM expression would not be expected to co-label with neuronal or glial markers.

PCNA and PSA-NCAM triple labelling showed no co-labelling with either NeuN or GFAP (Figure 5.10), indicating neither a neuronal or glial lineage had been determined in these cells. From this result, it can be determined that the cell counts presented in this chapter are unlikely to be the result of glial proliferation or migration.
Figure 5.10 Fluorescent characterisation of PCNA and PSA-NCAM cells in the SVZ

(A) Arrowheads indicate the location of a PCNA+ cell doublet in the SVZ (inset). These cells are presumably dividing. PCNA (A2) did not co-localise with NeuN (A3) or GFAP (A4) indicating that a neuronal or glial lineage has not yet been established in these cells. (B) Arrowheads indicate the location of a PSA-NCAM+ cell in the SVZ (inset). PSA-NCAM (B2) also did not co-localise with NeuN (B3) or GFAP (B4). Scale bar: 10 μm (A, B), 2 μm (inset).
5.3.7 Blood vessels appear to influence local proliferation in the SVZ

The quantification method used in this study excluded regions where blood vessels were present to reduce the variability caused by these structures. This has been the practice in previous studies of human SVZ proliferation (Curtis et al., 2005a; Ekonomou et al., 2015). However, during the cell counting procedure it was noted that blood vessels appeared to increase the local SVZ thickness and cell density, although this effect was not quantified. This observation suggested blood vessels may influence local progenitor proliferation and questions the validity of their exclusion from the analysis. It is possible that the variability of proliferation across the SVZ is an important feature in itself and it would not be captured by the sub-sampling method used in this study.

To address this possibility, the PCNA+ cell density per unit length and per unit area was assessed across the dorsal to ventral aspect of the SVZ in 200 μm segments. These data were obtained for four of the cases used to construct the tissue microarray and values were converted to a heat map to visually compare the distribution.

5.3.7.1 Blood vessels influence local PCNA+ cell density

In general, the number, size, and location of blood vessels in the SVZ appeared to vary across the four cases. However, all the cases examined had a large vessel in the middle sub-section and clusters of smaller vessels in the ventral sub-section. Whether this is a conserved feature of the SVZ would need to be examined with additional cases.

It was observed that the number of PCNA+ cells/mm SVZ increased in the presence of a blood vessel (Figure 5.11C, C’). This fluctuation was also observed when counts were normalised to SVZ area but was not always increased (Figure 5.11C”, Figure 5.13C”). In the presence of a blood vessel the SVZ either bifurcated around the vessel (Figure 5.13C) or deviated toward the ventricular border (Figure 5.11C). This resulted in an increase or decrease in SVZ width, respectively, which affected the per unit area analysis.

From these observations, it appears blood vessels influence the local density of PCNA+ cells. As vascular degeneration is a well-reported feature of AD and many neurodegenerative diseases, this may be an important consideration when assessing SVZ proliferation in disease states. These observations also highlight the complexity of SVZ structure and dynamics. By isolating quantification to areas devoid of blood vessels it is not possible to assess local changes in SVZ proliferation and migration that may occur due to vascular degeneration. Conversely, the inclusion of areas containing blood vessels introduces a large amount of variability that could mask any
broad changes. Therefore, a comprehensive analysis of full SVZ tissue sections is necessary to provide a full picture of SVZ anatomy and variability.

5.3.7.2 SVZ regions without blood vessels show uniform PCNA+ cell density

Areas of the SVZ devoid of blood vessels appeared to have a more uniform PCNA+ cell distribution compared to those areas with blood vessels. However, relatively large fluctuations of 10 - 20 cells per unit length and 100 cells per unit area still occurred between the segments (Figure 5.12C). This variability is a feature of the SVZ that would not have been captured by the sub-sampling method used presently and in other studies of SVZ proliferation (Curtis et al., 2005a; Low et al., 2013). It does explain the variability observed even between sections from the same core, as cell density could be very different depending on where the sub-sample was taken.

Again, this observation points to proliferation being highly variable along the SVZ length. While sub-sampling regions for quantification provides a meaningful assessment of any overall change in proliferation, such an effect would have to be large to overcome the amount of variability in the analysis. Unfortunately, sub-sampling would not identify any subtle changes in distribution, although whether these would have any functional relevance is uncertain.

5.3.7.3 Multiple blood vessels have a cumulative effect on PCNA+ cell density

From qualitative observation, larger vessels did not appear to have a larger effect on local proliferation. However, clusters of blood vessels seemed to produce a larger effect in terms of magnitude and extent of the local change (Figure 5.13C). That is, a large increase in proliferation was noted near clusters of blood vessels and the region of increased density appeared to extend further than that of a single vessel where the change was quite local. This effect was particularly noted in the ventral aspect (Figure 5.11A - B, Figure 5.12A - B) but not exclusively (Figure 5.13A - B dorsal region).

The abundance of blood vessels in the ventral region raises questions regarding effective sub-sampling of the ventral tissue microarray cores. With so many blood vessels along this portion of the SVZ, it may not be possible to sample an unaffected area. This could confound the comparison to dorsal and middle samples.

5.3.7.4 The length and area normalisation can produce vastly different conclusions

Lastly, the full SVZ analysis revealed conclusions can differ greatly depending on the type of normalisation used. AZ71 (Figure 5.14A - C) presents a striking example of how PCNA+ cell number per unit length is comparable to other cases, while the per unit area analysis shows a very high PCNA+ cell density. This issue was also apparent in the quantification of PSA-NCAM where
a significant difference was only detected per unit area due to local decrease in width of the middle sub-region (section 5.3.2).

Since both methods of normalisation provide valid yet sometimes different conclusions about the proliferating cell density, both results should be presented in any analysis of SVZ proliferation.
Figure 5.11  Heat map of PCNA⁺ cell density along the SVZ of case AZ68
Each colour block illustrates the density of cells/mm (A) or cells/mm² (B) along a 200 μm section of SVZ. Regions of the SVZ that were included on the tissue microarray are indicated. There is an obvious variability in the distribution of PCNA⁺ cells along the SVZ, even within the sub-regions designated dorsal, middle and ventral. There is an apparent increase in PCNA⁺ cell density in the vicinity of blood vessels (marked by grey circles). This is highlighted in the magnified images (C - C”). Scale bars: 200 μm (A, B), 50 μm (C - C”).
Figure 5.12  Heat map of PCNA\(^+\) cell density along the SVZ of case H181
Each colour block illustrates the density of PCNA\(^+\) cells/mm (A) or cells/mm\(^2\) (B) along a 200 μm section of SVZ. Regions of the SVZ that were included on the tissue microarray are indicated. As observed in Figure 5.11, there is variability in the distribution of PCNA\(^+\) cells along the SVZ and an increase in PCNA\(^+\) cell density around blood vessels (marked by grey circles). (C – C") PCNA\(^+\) cell density is low and more uniform in regions further from blood vessels. Scale bars: 200 μm (A, B), 50 μm (C - C").
Cell Proliferation in the Alzheimer’s Disease Sub-Ventricular Zone

Figure 5.13  Heat map of PCNA⁺ cell density along the SVZ of case H160
Each colour block illustrates the density of PCNA⁺ cells/mm (A) or cells/mm² (B) along a 200 µm section of SVZ. Regions of the SVZ that were included on the tissue microarray are indicated. Again, considerable variability in PCNA⁺ cell number is observed across the SVZ when assessed per unit length or area. (C – C”) Clusters of blood vessels appear to have a larger and more widespread effect on PCNA⁺ cell number than single vessels alone. Scale bars: 200 µm (A, B), 50 µm (C - C”).

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Figure 5.14  Heat map of PCNA+ cell density along the SVZ of case AZ71
Each colour block illustrates the density of PCNA+ cells/mm (A) or cells/mm² (B) along a 200 μm section of SVZ. Regions of the SVZ that were included on the tissue microarray are indicated. Again, the variability of PCNA+ cell density and effect of blood vessels can be seen in this case however there is also a remarkable difference between the density of PCNA+ cells when analysed per unit length (A) and per unit area (B). This case had a very thin SVZ, which produces a value of cells per mm comparable to other cases, but a highly variable number of cells/mm² (C). Scale bars: 200 μm (A, B), 50 μm (C - C’).
5.4 Discussion

This chapter assessed overall and regional proliferation in the AD SVZ using an established method of sub-sampling, the efficiency of which was enhanced by using tissue microarrays (Curtis et al., 2005a; Low et al., 2013). The PCNA+ and PSA-NCAM+ cell counts presented in this chapter provide an overview of proliferation in areas of the SVZ devoid of blood vessels and indicate that proliferation is largely unaffected in the AD SVZ. This work constitutes the most robust analysis of proliferation in the AD SVZ to date, and provides clarity in a field previously marred by methodological shortcomings. The results of the PSA-NCAM analysis highlight the importance of presenting cell counts normalised to both SVZ length and area. The investigation of PCNA+ cell density across the entire dorso-ventral axis challenges a key assumption of common SVZ quantification methods, that proliferation dynamics are uniform across the SVZ structure. This work demonstrates that the variable nature of the SVZ should be accounted for in future quantification studies.

5.4.1 SVZ proliferation in AD

Using a sub-sampling method of cell counting it was determined that PCNA+ cell number was unchanged in AD cases when normalised to both SVZ length and area. This finding agrees with two of the previous human studies that found similar markers of proliferation were unchanged in AD when analysed by cell counts per mm SVZ and IOD (Perry et al., 2012; Ekonomou et al., 2015). Admittedly any change in SVZ proliferation would need to be large and robust to overcome the natural variability in proliferative capacity that was observed between individuals. However, it can also be argued that such a change would be necessary to be functionally significant in the disease process. A strength of the current study was the separate analysis of sub-regions along the dorsal to ventral axis. While there was no change in PCNA+ cell number between AD and control groups in any sub-region, the ventral sub-region of both groups contained significantly more cells per unit length than the dorsal or middle sub-region. This finding was also noted in a previous study of SVZ proliferation in Huntington’s disease and indicates proliferation is not uniform across the length of the SVZ (Curtis et al., 2005a). This is a feature which should be considered in any quantification of SVZ proliferation, but has not been accounted for in previous studies of human AD.

The analysis of SVZ PSA-NCAM+ cells revealed a significant increase in the middle sub-region of AD cases, only when normalised to SVZ area. These data highlight the importance of presenting cell counts normalised to both length and area as different conclusions can be obtained from each
method. The increase in PSA-NCAM* cells only when normalised to area indicated a likely change in SVZ width, which was apparent in the middle sub-region upon further examination. This finding suggests the absolute number of PSA-NCAM* cells in the middle sub-region is unchanged, however these cells are moving through a thinner SVZ in AD cases. The functional relevance of a thinner SVZ with no difference in overall proliferative capacity is unclear. These results would need to be confirmed using a method that more accurately identifies the different SVZ layers such as hematoxylin and eosin histological staining.

Together the data from the PCNA and PSA-NCAM analysis show proliferation is largely unaffected in the AD SVZ. The lack of correlation between cell counts and β-amyloid or tau load also indicated SVZ neurogenesis was not affected by local pathology and did not vary with disease severity. This supports previous studies that have shown tau and Aβ42 have no effect on SVZ proliferation in the older rodent brain (Sotthibundhu et al., 2009; Hong et al., 2011).

The validity of PCNA and PSA-NCAM as markers of SVZ progenitor cells was supported by the lack of GFAP co-labelling in the fluorescent staining analysis. The lack of correlation between cell counts and post-mortem delay, age or gender reaffirms that case factors did not confound this quantification. Overall, this analysis provides convincing evidence that reliable markers of SVZ proliferation are unchanged in regions devoid of blood vessels in AD.

5.4.2 Considerations for future SVZ quantification methods

It was apparent during the analysis that the SVZ is a highly variable structure. While this study is the first to account for changes along the dorso-ventral axis of the AD SVZ, future studies could also assess the rostro-caudal axis. A previous study of the human SVZ showed PCNA* cell density did not differ significantly along the rostro-caudal axis in the normal brain (Curtis et al., 2005a). However, this finding should be verified in AD cases and with additional markers. The use of tissue microarrays did not permit analysis of this axis in the current study.

The current method and those used in previous studies deliberately omit areas that include blood vessels in order to reduce the variability introduced by these structures. However, additional investigations in this chapter suggest that blood vessels influence local PCNA* cell number. Thus, rather than being excluded from analysis, their impact on local progenitor proliferation warrants further attention.

Blood vessels are an important component of the neurogenic niche and have a complex interaction with SVZ progenitor cells. In addition to their primary function of cycling nutrients and waste, blood vessels can also regulate proliferation through endothelial secretion of pro-neurogenic
factors and availability of blood-borne neurogenic substances (extensively reviewed by Goldman and Chen, 2011; Licht and Keshet, 2015). The clustering of precursor cells near blood vessels has previously been described in the rat SGZ and mouse SVZ (Palmer et al., 2000; Shen et al., 2008; Tavazoie et al., 2008). Studies of the mouse SVZ have shown progenitor cells contact vessels at sites where astrocyte end-feet and pericytes are absent. This is a unique feature of the SVZ vasculature that provides direct access to nutrients and pro-neurogenic factors from the blood (Tavazoie et al., 2008). With such a specialised vascular neurogenic niche, it is logical that PCNA+ cell density is variable near blood vessels in the human SVZ. While SVZ vascular degeneration has not been investigated in the human brain, it is highly plausible that such changes could affect proliferation in discrete locations along the structure. These changes would not have been detected using the sub-sampling method in this study as regions including vessels were excluded from the analysis. Future work should examine this effect in the human SVZ more thoroughly. Such an endeavour would need to account for individual blood vessel size, clustering of vessels and the integrity of the blood brain barrier in the SVZ. It is likely that these factors would be affected in AD, as vascular degeneration is a prominent feature of the disease pathology: specific regions of the AD brain show reduced blood vessel density and abberant coiling of vessels, resulting in hypoperfusion (Fischer et al., 1990; Buée et al., 1997; Bell and Zlokovic, 2009; Miyakawa, 2010).

Lastly, the results presented in this chapter have highlighted the necessity of presenting cell counts normalised to both SVZ length and area. Both the sub-sampling results from PSA-NCAM and the qualitative assessment of full SVZ PCNA distribution revealed the overall conclusion can differ between these two types of normalisation. This suggests the results from both data sets should always be considered together to provide a complete summary of SVZ proliferation.

In conclusion, this work has shown that the quantification of SVZ proliferation in AD could be further improved by analysing the density of proliferating cells across full SVZ sections. By assessing the full SVZ, the data that was obtained from the tissue microarray method could be acquired as well as additional relevant data. That is, a reliable assessment of SVZ proliferation in regions devoid of blood vessels could be performed that accounts for the dorso-ventral gradient. Additionally, the distribution of proliferation markers could be determined across the entire SVZ to examine whether the variability of proliferation is altered in AD and if blood vessels have a measurable effect. In this way, an analysis of the full SVZ would provide an even more comprehensive understanding of SVZ proliferation in the AD human brain.
5.5 Summary

Overall the sub-sampling analysis of PCNA and PSA-NCAM using tissue microarrays provided reliable and reproducible results demonstrating that SVZ proliferation is unchanged in AD. This study was the first analysis of SVZ proliferation in AD to account for differences in proliferation across the dorso-ventral axis. Analysis of PCNA+ cell distribution along the entire SVZ revealed proliferation was highly variable, particularly in areas close to blood vessels. This challenges an underlying assumption of previous SVZ quantification methods, namely that cell proliferation is uniform across the entire SVZ structure. Furthermore, these results propose that the full SVZ should be investigated in future studies so the effect of blood vessels can be considered.
Chapter 6. Distribution of PSA-NCAM in Normal, Alzheimer’s and Parkinson’s Disease Human Brain

6.1 Introduction

For the investigation of SGZ and SVZ neurogenesis presented in Chapters 4 and 5, PSA-NCAM was used as a marker of immature neurons and neuronal plasticity. During the imaging of these sections it was noted that PSA-NCAM+ cells were not limited to the neurogenic regions, but were also present throughout the hippocampus and caudate nucleus. PSA-NCAM expression has been documented outside the neurogenic regions in several rodent studies (reviewed by Bonfanti, 2006); however, there is limited evidence for its wider distribution in the adult human brain. Expression in non-neurogenic regions suggests PSA-NCAM has an important role in the structural and synaptic plasticity of cell types other than immature neurons in the adult human brain (Rutishauser, 1996; Gascon et al., 2007).

PSA-NCAM is highly expressed throughout the embryonic and juvenile mammalian brain and is involved in a range of developmental processes including cell migration, axon/dendrite growth and remodelling, and synaptic reorganisation (Hu et al., 1996; Seki and Rutishauser, 1998; Theodosis et al., 1999; Eckhardt et al., 2000; Dityatev et al., 2004). The polysialylation of NCAM is thought to mediate these processes by disrupting cell-cell adhesion through steric inhibition of binding sites (Yang et al., 1994; Johnson et al., 2005). This property is also thought to mediate neuronal plasticity in the adult brain by facilitating the remodelling of synapses and neuronal structure, a concept referred to in this study as structural remodelling. Emerging evidence suggests this is a process that may be affected in disease states (Ernst et al., 2014).

Previous quantification of PSA-NCAM in the human AD and Parkinson’s disease (PD) brain has been limited to the regions with most severe disease pathology. An increase in PSA-NCAM was detected in previous studies of the AD hippocampus (Mikkonen et al., 1999b) while no change in PSA-NCAM+ cell number was detected in the substantia nigra of PD patients compared to controls (Yoshimi et al., 2005). However, since broad atrophy is observed throughout the human brain in these diseases, it was of interest to determine whether PSA-NCAM expression is altered more broadly in other brain regions. While AD is the primary focus of this thesis, the results of the PD analysis have been presented in this chapter as a neurodegenerative disease contrast group.

This study explored the widespread distribution of PSA-NCAM in the adult human brain. The distribution was also investigated in neurodegenerative diseases by quantifying PSA-NCAM load
in selected regions of the control, AD and PD disease brain. The work presented in this chapter provides a comprehensive overview of the degree of PSA-NCAM-mediated structural plasticity in the aged human brain and whether this is affected in AD or PD.
6.2 Methods

6.2.1 Human tissue selection

Fixed-frozen brain tissue from eleven neurologically normal controls, ten AD and eight PD cases was used in this chapter as outlined in Table 14. Tissue was prepared and sectioned as outlined in section 3.1.1. The brain regions examined in this study included the middle temporal gyrus (MTG), superior frontal gyrus (SFG), hippocampal formation (HP), sensory-motor cortex (SM), caudate nucleus (CN), substantia nigra (SN), visual cortex (VC) and cerebellum (CB) (Figure 6.1).

As for all post-mortem tissue used for these studies, the control cases had no history of neurological abnormalities and cause of death was unrelated to any neurological condition. Independent pathological analysis confirmed the absence of other neuropathology and determined any amyloid pathology was normal for age (average age: 71 ± 11.2 years, range: 48 – 83 years; average post-mortem delay: 15.3 ± 5.2 hours, range: 8 – 24 hours). The AD cases selected for this study had a clinical history of dementia with no evidence of other neuropathology. Independent pathological analysis was carried out to determine pathological diagnosis and disease severity. All AD cases selected for this study had a minimum Braak stage of IV indicative of late stage AD (average age: 79.4 ± 7.5 years, range: 68 – 94 years; average post-mortem delay: 10.2 ± 6.1 hours, range: 4 – 21
Chapter 6

hours). All PD cases selected for this study had a clinical diagnosis of idiopathic PD with no evidence of other neuropathology (average age: 83 ± 4.7 years, range: 78 – 91 years; average post-mortem delay: 10.1 ± 8.2 hours, range: 4 – 25 hours).

Table 14: Human cases used to assess PSA-NCAM distribution in the brain

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6.2.2 Immunoperoxidase staining for PSA-NCAM and pathological aggregates

Four evenly spaced sections were selected from each region of the above cases to be stained for each of the antibodies used in this study. DAB immunoperoxidase staining was performed for PSA-NCAM (5A5, DSHB and MAB5324, Millipore), β-amyloid (M0872, DAKO) and tau (A0024, DAKO) as described in section 3.2.1. The appropriate antigen retrieval protocol and dilutions were carried out for each antibody as described in Table 5. A no-primary antibody control was included for each staining condition and these sections showed no immunoreactivity. Immunoperoxidase staining of calbindin (CB38a, Swant) and enkephalin (Seralab) was also performed on sequential sections of caudate nucleus to investigate the striosome location relative to PSA-NCAM immunostaining.

6.2.3 Cresyl violet delineation of cortical layers

A single unstained section from each of the regions assessed was processed for cresyl violet labelling as outlined in section 3.2.2. Stitched images of these sections were acquired using a Nikon Ni-E microscope equipped with Nikon DS-Ri2 camera and motorised stage. These images were overlaid with the corresponding PSA-NCAM sections to enable accurate identification of cortical layers for analysis.

6.2.4 Immunofluorescence and microscopy for cellular localisation assessment

To identify the cellular localisation of PSA-NCAM, free floating fluorescent immunohistochemistry was performed on sections of cerebellum as per the tyramide signal amplification protocol outlined in section 3.2.3. PSA-NCAM primary antibody (5A5, DSHB), biotinylated goat anti-mouse IgM secondary antibody (AB98673, Abcam) and Alexa Fluor-488 conjugated tyramide (T20948, ThermoFisher) were used for this staining. An Olympus FV1000 confocal microscope with a 20x objective was used to obtain a z-stack through a PSA-NCAM+ cell. Reflection confocal microscopy was also used to obtain a z-stack through DAB immunoperoxidase labelled PSA-NCAM+ cells using a 40x and 100x objective.

6.2.5 Quantification of immunoreactivity and statistical analysis

Imaging was carried out on a Nikon E800 microscope equipped with a motorised stage and stereology software (StereoInvestigator 7; MBF Bioscience). For quantification of PSA-NCAM immunoreactivity, the area of interest was first delineated. In cortical regions PSA-NCAM showed a layer specific distribution, therefore it was necessary to quantify the staining in each cortical layer separately as an overall cortical analysis is unlikely to identify any layer specific differences.
Layer V was selected as the region of interest for the cortical regions due to its high density of PSA-NCAM staining. The hippocampus and caudate nucleus are comprised of distinct functional regions which were separated for analysis. The hippocampus was sub-divided into the dentate gyrus and CA1 regions and the caudate nucleus into dorsal, middle and ventral sub-regions. A sample of 15 random images throughout each region of interest was acquired using a 20x objective. All imaging parameters and microscope settings were kept constant throughout imaging to ensure unbiased assessment of staining between sections. The same protocol was used for the analysis of β-amyloid and hyperphosphorylated tau; however, a 10x objective was used for imaging due to object size.

Immunoreactivity was quantified using MetaMorph high-throughput image analysis software (version 7.8.0, Molecular Devices). PSA-NCAM staining was quantified using the ‘neurite outgrowth’ algorithm, (threshold parameters: minimum object area 20 µm², minimum object grey value intensity above background = 20). For this study, we configured the data log to record total area of staining (presented here as ‘average percentage area of image stained’ as an indication of PSA-NCAM load) and neurite integrated optical density. This is a unique method of PSA-NCAM quantification with several advantages over cell count or optical density measurements. Traditional cell counting would not capture any changes due to fibrous PSA-NCAM staining which was predominant in many brain regions. Alternatively, classical optical density analysis does not discriminate background staining, which can alter drastically between individual human brains due to post-mortem delay or fixation and could subsequently affect the quantification. The MetaMorph analysis avoids these issues by specifically analysing areas with positive immunoreactivity.

Analysis of β-amyloid and tau was also carried out using this method as described in section 4.2.3. The ‘count nuclei algorithm’ (minimum object area 50 µm², minimum object grey value intensity above background = 50) was applied to detect aggregate staining. The data log was configured to record total area of staining, producing a measurement of ‘average percentage area of image stained’ as an indication of pathological aggregate load.

Statistical analysis was performed using GraphPad Prism (version 6.05). The data set from each region of interest was first tested for a normal distribution using the Shapiro-Wilk normality test. For regions that met the required assumptions of normal distribution and equality of variance a one-way ANOVA was performed with Tukey’s post-hoc comparisons. If these assumptions were not met, a Kruskal-Wallis test was performed with Dunn’s post-hoc comparisons. The statistical significance of correlated data was assessed using the Pearson correlation coefficient for data that
met the assumptions for parametric testing or a Spearman’s correlation coefficient for the data that did not. Results are presented with standard deviation and statistical significance set at $p < 0.05$.

### 6.2.6 Western blot confirmation of PSA-NCAM antibody specificity

Confirmation of PSA-NCAM antibody specificity was carried out using western blot analysis of grey matter dissected from sections of fresh frozen MTG. Protein extraction was carried out as described for post-mortem tissue in section 3.4.1. The supernatant fraction of the sample was resolved by electrophoresis and transferred onto a PVDF membrane as described in section 3.4.2. The membrane was probed for both PSA-NCAM antibodies (5A5, DSHB and MAB5324, Millipore) and GAPDH (AB9485, Abcam) as a loading control as per Table 8. The appropriate species-specific HRP conjugated antibodies were applied (Table 9) and the membrane was visualised by chemiluminescence using ECL prime detection reagent (RPN2232, Amersham, GE Healthcare) and the BioRad ChemiDoc MP system.
6.3 Results

6.3.1 Antibodies are specific for PSA-NCAM

Immunohistochemistry was performed using two IgM antibodies from different manufacturers to verify the specificity of staining for PSA-NCAM. DSHB5A5 is described as binding to an epitope of the polysialylglycan chain of NCAM while MAB5324 is specifically described as binding to PSA with more than 10 monomers (Dodd et al., 1988). The same distribution of PSA-NCAM immunoreactivity was observed for both antibodies in each of the regions assessed, despite different levels of background staining (Figure 6.2A, B). No staining was observed in the respective negative control section (Figure 6.2C). Pre-adsorption controls are another mechanism of testing antibody specificity where blocking peptides are added to an antibody dilution prior to incubation on the tissue section. The expected result for a specific antibody would be a lack of positive staining on the tissue section due to the peptide blocking all available antigen binding sites. These controls were not performed in the current study as they have been extensively provided by previous studies using these specific PSA-NCAM antibodies as per the Journal of Comparative Neurology Antibody Database (Dodd et al., 1988; Bradford et al., 2010; Choi et al., 2010).

Using western blotting it was confirmed that both antibodies label proteins in the 200-220 kDa range (Figure 6.2D). Post-translational addition of different PSA chain lengths to the NCAM protein results in variable molecular weights within this range, which is consistent with previous reports of PSA-NCAM size (Mikkonen et al., 1999b; Monzo et al., 2013). As low background is required to accurately threshold an image using the MetaMorph software, the DSHB5A5 antibody was used to label sections used for quantification of PSA-NCAM load.
Figure 6.2  Validation of PSA-NCAM antibody staining
Brightfield images of PSA-NCAM staining in the caudate nucleus of a control case, aged 48 years, using PSA-NCAM antibody DSHB5A5 (A) and MAB5324 (B). Both antibodies produce the same pattern of immunoreactivity in all regions despite differences in background staining. As the staining protocol was the same for both PSA-NCAM antibodies, one section where the primary antibody was omitted served as the control for both. No immunoreactivity was observed in this condition (C). Scale bar = 100 µm. (D) Western blot of MTG homogenate illustrating that both the DSHB5A5 and MAB5324 antibodies labelled PSA-NCAM in the 200 – 220 kDa range. GAPDH was probed as a loading control. Abbreviation: NC, negative control.
6.3.2  PSA-NCAM distribution in the adult human brain

PSA-NCAM immunoreactivity was primarily observed in the cytoplasm and along the membrane of cell bodies and processes throughout the regions assessed. None of the PSA-NCAM$^+$ cells identified had glial morphology and the distribution observed was consistent between control, Alzheimer’s and Parkinson’s disease cases.

6.3.2.1  Cortical Regions

PSA-NCAM was abundant in the entorhinal cortex (Figure 6.3A), middle temporal gyrus (Figure 6.3B) and superior frontal gyrus (Figure 6.3C). In these regions, PSA-NCAM staining was characterised by a dense network of processes concentrated in cortical layers II-III and V. PSA-NCAM$^+$ cell somata were also concentrated in these layers although sparsely located in the other cortical layers as well. The elongated somata were approximately 10-15 µm in diameter at their widest point and had a complex arborisation. This morphology does not match that of the pyramidal projection neurons located in these cortical regions. Interestingly, the intensity of PSA-NCAM staining was noticeably varied between cells and even varied along the length of a single process or cell soma. The sensory-motor cortex (Figure 6.3D) contained several PSA-NCAM$^+$ cell soma throughout layers II/III and V; however, this region appeared largely to consist of background staining. For this reason, the sensory-motor cortex was not included in the quantification analysis.
Figure 6.3 PSA-NCAM immunoreactivity in the control human cortical regions and cresyl violet stained sister sections

In the entorhinal cortex (A’), middle temporal gyrus (B’) and superior frontal gyrus (C’) PSA-NCAM was observed in layers II-III and V as confirmed by cresyl violet stained sister sections (A, B, C). Higher magnification revealed staining of processes and cell somata (arrows) in each of these regions (A”, B”, C”). The sensory-motor cortex (D) shows staining in layer V however, while some cell somata were identified (arrow), this staining largely appeared to be background (D”). Scale bars: 500 µm (A-D; A”–D”), 100 µm (A”–D”).
6.3.2.2 Cerebellum

PSA-NCAM immunoreactivity was sparse in the cerebellum. Typically, only 3 - 5 large PSA-NCAM+ cells were identified in the Purkinje layer on each 50 µm-thick, full coronal section of cerebellum (Figure 6.4A). These PSA-NCAM+ cells appeared to be Purkinje cells due to their location and morphology. They often appeared to be partially labelled, with a dendritic tree visible but no cell soma or vice versa. Due to the size of these cells, it is likely that the partial labelling is due to the Purkinje cell being cut during sectioning, rather than selective expression of PSA-NCAM along the cell structure.

A small number of faintly stained PSA-NCAM+ cells were identified throughout the granule cell layer and molecular layer of the cerebellum. These cells had a bipolar structure and extended horizontally across the layer (Figure 6.4B). The molecular layer also showed a large amount of punctate staining, possibly representing staining of parallel fibres (Figure 6.4A). Due to the low density of immunoreactivity, cerebellar PSA-NCAM load was not quantified in this study.

The identification of PSA-NCAM immunoreactivity in the adult human cerebellum was unexpected as this structure has been described as devoid of PSA-NCAM in the rodent brain (reviewed by Bonfanti, 2006). These results provide the first evidence of cerebellar PSA-NCAM+ cells in the adult human brain.

The large size and infrequent occurrence of PSA-NCAM+ Purkinje cells made them an ideal cell to confirm the membrane localisation of PSA-NCAM immunoreactivity. By obtaining a confocal z-stack of a fluorescently labelled Purkinje cell, the membrane localisation of PSA-NCAM on the cell soma was confirmed (Figure 6.5A). These images also illustrate that PSA-NCAM immunoreactivity could be mistaken as cytoplasmic if the top or bottom of the cell soma was present in a section visualised using standard brightfield microscopy. A Z-stack of an immunoperoxidase labelled Purkinje cell using reflection confocal microscopy further demonstrates this membrane localisation of PSA-NCAM on cell somata and dendrites (Figure 6.5B, C).
Figure 6.4 PSA-NCAM immunoreactivity in the control human cerebellum
PSA-NCAM⁺ cells were sparsely distributed throughout the layers of the cerebellum. (A) PSA-NCAM⁺ cells in the Purkinje layer (PL) were infrequently observed in the cerebellum and often were partially stained, likely due to the plane of sectioning. (B) A small number of faintly stained PSA-NCAM⁺ cells were located throughout the granular layer (GL) and molecular layer (ML) of the cerebellum. Scale bars: 500 µm (A, B), 100 µm (A’, B’).
Figure 6.5  Cellular localisation of PSA-NCAM on Purkinje cells in the control human cerebellum

(A) Confocal images of a z-plane through the center (A) and bottom surface (A’) of a fluorescently labelled Purkinje cell illustrating that PSA-NCAM is located on the cell membrane but can appear cytoplasmic depending on the visible surface of the membrane. (B-B’) Reflection confocal microscopy of a immunoperoxidase labelled Purkinje cell soma illustrating the membrane localisation of PSA-NCAM in different focal planes. (C) Reflection confocal microscopy of an immunoperoxidase labelled Purkinje cell dendritic tree and a higher magnification (C’) illustrating the membrane localisation of PSA-NCAM on Purkinje cell dendrites. Scale bars: 100 μm (A, A’), 5 μm (B, B’, C’), 50 μm (C).
6.3.2.3 Visual Cortex

A distinct band of staining was observed on the Line of Gennari in the visual cortex. As this layer represents a dense band of myelinated fibres, the PSA-NCAM immunoreactivity in this area was mostly fibrous with high background (Figure 6.6A). Small PSA-NCAM$^+$ cells, with somata measuring 5 - 10 µm in diameter, were randomly orientated throughout cortical layers II - III and V - VI in the visual cortex. These cells were either bipolar or multipolar and showed variable intensity of PSA-NCAM immunoreactivity along their structure.

6.3.2.4 Substantia Nigra

A dense network of processes was observed throughout the substantia nigra. PSA-NCAM was identified on processes in the pars compacta and pars reticulata (Figure 6.6B). PSA-NCAM$^+$ fibre staining was also observed in the region between the SNc and the red nucleus. This is consistent with reports of nigral PSA-NCAM expression by Yoshimi et al. (2005). Substantia nigra PSA-NCAM load was not quantified in this study due to the conflicting signal from black neuromelanin pigment produced by the dopaminergic neurons this region.
Figure 6.6 PSA-NCAM immunoreactivity in the control human visual cortex and substantia nigra

(A) A distinct band of staining was observed on the Line of Gennari in the visual cortex (layer IV, arrows). At higher magnification, it was apparent that this was mostly fibrous staining (not shown) however PSA-NCAM+ cells were sparsely located in layers II-III and V-VI of the visual cortex (A’). (B’) PSA-NCAM+ processes were observed in the substantia nigra (arrows), in the vicinity of dopaminergic neurons that express black neuromelanin pigment. Scale bars: 500 µm (A, B), 100 µm (A’, B’).
6.3.2.5 Hippocampal Formation

PSA-NCAM was heavily expressed throughout all sub-regions of the hippocampus. The CA1 area had a complex distribution of PSA-NCAM immunoreactivity (Figure 6.7B). A number of immunoreactive cell somata were observed in the stratum pyramidale and stratum moleculare as well as a network of PSA-NCAM+ processes which spanned the stratum radiatum (Figure 6.7A). These cells ranged in size and showed either bipolar or stellate morphology (Figure 6.7A). PSA-NCAM+ fibres were also orientated perpendicularly through the stratum oriens.

The expression of PSA-NCAM by SGZ cells has been well characterised and is consistent with the observations from Chapter 4 (Mikkonen et al., 1999b). PSA-NCAM+ cells in this region possessed a single apical process that extended through the overlying granule cell layer and into the molecular layer of the dentate gyrus (Figure 6.7C). Immunoreactive cell somata were elongated and varied between 10 - 15 µm in diameter. PSA-NCAM+ cells were also observed in the molecular layer of the dentate gyrus and ranged in size with either bipolar or stellate morphology.

A range of PSA-NCAM+ cells were observed in the hilus (CA4) of the hippocampus (Figure 6.7D). Large multipolar cells were the most abundant subtype. These cells were 20 - 25 µm in diameter with multiple dendrites projecting from the cell soma, often extending toward the GCL. A number of large bipolar cells were also observed throughout the hilus. These cells often projected perpendicularly toward the GCL; however, some were orientated parallel to the GCL. Small stellate cells approximately 5 - 10 µm in diameter were also observed. Hilar PSA-NCAM+ cells were clustered close to the GCL, consistent with the location of the polymorphic (or plexiform) layer interneurons. The intensity of PSA-NCAM staining was strikingly different between cells with many even showing variability along their cell membrane. It was especially common to observe darker patches of staining along the circumference of the cell soma (Figure 6.7D arrows).

No difference in PSA-NCAM+ cell staining or morphology was observed in the GCL or CA1 between controls (Figure 6.7E, F) and AD cases (Figure 6.7G, H) in the hippocampus.
Figure 6.7  PSA-NCAM immunoreactivity in the control human hippocampus

(A) An overview of hippocampal PSA-NCAM staining demonstrating the presence of immunoreactive cells in the CA1 region (B), SGZ (C) and hilus (D). A variety of cell morphologies are observed in each region. Distinct differences in staining intensity are visible along a membrane and between cells (D, arrows).  

(E - H) Nissl counterstained PSA-NCAM* cells in the SGZ (E, G) and CA1 region (F, H) from an AD and control case illustrating the similarity of cell morphology between conditions. Abbreviations: SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SM, stratum lacunosum moleculare; GCL, granule cell layer; H, hilus. Scale bars: 500 µm (A), 100 µm (B, C, D), 20 µm (E - H).
### 6.3.2.6 Striatum

Small multipolar PSA-NCAM$^+$ cells were abundant throughout the caudate nucleus and putamen (Figure 6.8A). PSA-NCAM expression was observed in layers III - IV of the SVZ, consistent with the paraffin-embedded tissue staining described in Chapter 5 and with previous literature (Curtis et al., 2005b; Dieriks et al., 2013). The location and morphology of these cells was consistent with that of migrating precursor cells (Figure 6.8B) (Curtis et al., 2005b; Dieriks et al., 2013).

A large number of PSA-NCAM$^+$ cells were observed throughout the caudate nucleus. These cells had a small soma with a diameter of 5 - 15 µm and one or two main processes that exhibited a simple branching structure (Figure 6.8C). Several spine-like swellings were observed along these processes. The morphology of these cells appears very similar to the calretinin$^+$ cells with polymorphous spines described by Revishchin et al. (2010).

As for the cerebellum, the observation of PSA-NCAM$^+$ cells in the striatum was unexpected as previous studies in rodents have not identified cellular immunoreactivity in this region (reviewed by Bonfanti, 2006). As such, further characterisation of striatal PSA-NCAM$^+$ cell phenotype and distribution was carried out using fluorescent double labelling. This work will be presented in the following chapter. When the distribution of striatal PSA-NCAM$^+$ cells was examined, it was noted that these cells appeared to be clustered throughout the striatum (Figure 6.9A). By labelling sequential caudate nucleus sections with the striosome markers enkephalin and calbindin, it was determined that the PSA-NCAM$^+$ cells were largely limited to the matrix compartment of the striatum. Very few cells were present in the striosome compartments and there was a complete absence of cells in the annular zone (centre) of the striosomes (Figure 6.9B-D). This matrix-specific localization was also observed in AD and PD cases.
Figure 6.8  PSA-NCAM immunoreactivity in the control human striatum
(A) PSA-NCAM$^+$ cells were identified throughout the caudate nucleus. (B) PSA-NCAM$^+$ cells were identified along the SVZ (arrow) in agreement with our previous findings using paraffin embedded tissue (Chapter 5). (C) PSA-NCAM$^+$ cells in the caudate nucleus had a small soma and simple branching structure, inconsistent with that of striatal projection neurons. Abbreviation: SVZ, sub-ventricular zone. Scale bars: 200 µm (A) 50 µm (B - C).
Figure 6.9 PSA-NCAM immunoreactivity in the striosome-matrix compartments of the control human striatum
(A) An overview of the striatum illustrating the patchy distribution of PSA-NCAM immunoreactivity. This corresponded to the striosome-matrix organisation of the striatum. (B) PSA-NCAM$^+$ cells were absent from the striosome compartments in the caudate nucleus. The striosome specific marker, enkephalin (C) and the matrix specific marker, calbindin (D), were used to delineate the striosome compartments. Abbreviations: CN, caudate nucleus; P, putamen; M, matrix; S, striosome. Scale bars: 2 mm (A), 100 μm (B - D)
6.3.3 Decreased PSA-NCAM in the Alzheimer’s disease entorhinal cortex

Quantification of PSA-NCAM load in each of the regions assessed in this study revealed that PSA-NCAM expression is highly variable between cases within a condition. Overall there was no significant difference in PSA-NCAM load between control, AD and PD for the middle temporal gyrus, superior frontal gyrus, caudate nucleus or hippocampal sub-regions. A significant decrease in PSA-NCAM load was observed in the AD entorhinal cortex relative to PD and controls (mean ± SD, AD: 0.29% ± 0.34%, PD: 2.04% ± 1.02%, control: 1.76% ± 1.45%, P = 0.020). Analysis of mean neurite integrated optical density (IOD) showed this same trend. A significant decrease was detected in the AD entorhinal cortex IOD relative to PD and controls (mean ± SD, AD: 2647 ± 1996, PD: 14435 ± 9085, control: 9066 ± 8901, P = 0.035). No difference was observed between control, AD and PD cases in the other regions assessed (Figure 6.10A, B). This decreased entorhinal cortex PSA-NCAM was macroscopically visible in layers II-III and V (Figure 6.10C - E).

Interestingly, the control cases were clearly divided into sub-groups with high or low entorhinal PSA-NCAM load (Figure 6.10F). Using correlation analysis, it was determined that this variation did not correlate with age, gender, post-mortem delay, brain weight, duration of tissue storage, β-amyloid or tau load (not shown).

As the entorhinal cortex provides input to the GCL and receives input from the CA1 region of the hippocampus, the relationship between PSA-NCAM loads was investigated in these regions. A significant positive correlation was found between entorhinal cortex PSA-NCAM load and both GCL and CA1 PSA-NCAM load (Figure 6.10G) in AD cases only (EC-GCL: r = 0.9880, P ≤ 0.0016. EC-CA1: r = 0.8143, P = 0.0485). A significant positive correlation was also observed between CA1 and GCL PSA-NCAM load. Again, this correlation was limited to the AD cases only (CA1-GCL: r = 0.8586, P = 0.0063). This indicates a possible link between the PSA-NCAM load in these three regions of AD cases. While positive correlations were observed between these factors for control and PD cases, none of these relationships were statistically significant.
6.3.4 PSA-NCAM load is inversely correlated with tau load in the entorhinal cortex of Alzheimer’s disease cases

Entorhinal cortex PSA-NCAM load was inversely correlated with tau load in the same region (Figure 6.10H, $r = -0.9441, P = 0.0046$,) and GCL tau load (not shown, $r = -0.9147, P = 0.0106$,) of AD cases only. The inverse correlation between entorhinal cortex PSA-NCAM and tau load is illustrated in Figure 6.10I - L. No significant correlation was detected between PSA-NCAM load and tau load for either control or PD cases. There was also an inverse correlation between CA1 PSA-NCAM load and CA1 tau load for AD cases (not shown, $r = -0.9131, P = 0.0110$).

As for tau, β-amyloid load was assessed in control, AD, and PD cases. For all three conditions, there was no relationship between the β-amyloid load and PSA-NCAM load in the entorhinal cortex, middle temporal gyrus, granule cell layer or CA1 region.
Figure 6.10 Quantification of PSA-NCAM load and IOD in the control, AD and PD brain
(A) There is a large amount of variability in PSA-NCAM load between cases within a condition and between regions. PSA-NCAM load was significantly decreased in the entorhinal cortex of AD relative to PD and controls. (B) Similar trends were observed in the analysis of neurite integrated optical density with a significant decrease in the AD entorhinal cortex. This reduction in PSA-NCAM is evident in layers II/III and V of the AD entorhinal cortex (D) in comparison with control (C) and PD (E). Interestingly, control cases showed either high or low PSA-NCAM loads in the entorhinal cortex, while the distributions of AD and PD cases were more uniform (F). A significant correlation was observed between PSA-NCAM load in the entorhinal cortex, CA1 and GCL regions in AD cases only (G) as well as a significant inverse correlation between PSA-NCAM load and tau load in the entorhinal cortex (H). This inverse correlation is illustrated (I - L). Scale bars: 100 µm (C - E, I - L).
6.4 Discussion

This study presents the first widespread investigation of PSA-NCAM distribution in the post-mortem human brain. As a protein with a well-documented role in the regulation of cell-cell interaction (Yang et al., 1994; Johnson et al., 2005), it can be hypothesised that the expression of PSA-NCAM is indicative of a cell undergoing structural plasticity, synaptic remodelling or migration. Additionally, PSA-NCAM expression may also represent regulation of synaptic integration due to its insulative properties, as well as regulation of cell signalling and neurotransmission by mediating receptor binding (reviewed by Bonfanti, 2006; Nacher et al., 2013). As such, the presence of PSA-NCAM+ cells in discrete layers and sub-compartments throughout many brain regions suggests that structural and synaptic remodelling in the human brain is widespread, yet specific. The findings from this work also emphasise that PSA-NCAM mediated plasticity is maintained in most regions of the elderly normal, AD, and PD brain as well as highlighting some important differences between PSA-NCAM distribution in the human and rodent brain.

6.4.1 Distribution of PSA-NCAM in the adult human brain

Previous studies of PSA-NCAM expression in the human brain have largely focused on the SVZ and SGZ neurogenic niches, while the global distribution of PSA-NCAM in the adult rodent brain has been extensively described (Bonfanti, 2006). The pattern of PSA-NCAM immunoreactivity detected in the SVZ and SGZ in this chapter is in line with that described by numerous studies of both human and rodent neurogenesis (Seki and Arai, 1991a; O’Connell et al., 1997; Mikkonen et al., 1999b; Bonfanti, 2006; Dieriks et al., 2013). Notably, it was observed that PSA-NCAM was not limited to these thin neurogenic belts but was also widely expressed in the surrounding structure. The diverse range of PSA-NCAM+ cell types observed throughout all hippocampal sub-regions emphasizes that PSA-NCAM-mediated plasticity is not limited to the neurogenic SGZ, but is a feature of the entire hippocampal structure. This diverse hippocampal expression of PSA-NCAM closely matches that described in rodent and human studies of the region (Fox et al., 1995; O’Connell et al., 1997; Mikkonen et al., 1999b; Ni Dhúill et al., 1999; Nacher et al., 2002).

The PSA-NCAM distribution observed in the substantia nigra also matches that reported by a previous human study (Yoshimi et al., 2005). Neurogenesis in the nigra is a subject of debate with conflicting mouse studies offering evidence for and against the presence of newly generated dopaminergic neurons (Lie et al., 2002; Zhao et al., 2003; Frielingsdorf et al., 2004). The observation of PSA-NCAM immunoreactive fibres throughout the region supports the proposition
of structural neuronal plasticity in the nigra. However, as PSA-NCAM expression does not seem to be limited to newly generated cells, these findings are not conclusive support for the occurrence of nigral neurogenesis.

The identification of cerebellar PSA-NCAM expression was very interesting due to the complete absence of immunoreactivity described in the rodent brain (Bonfanti et al., 1992; Dusart et al., 1999; Ponti et al., 2006). However, a report of PSA-NCAM+ cell chains in the subpial layer of the rabbit cerebellum has suggested this may not be the case in all mammals (Ponti et al., 2006). Indeed, the results from this study show robust PSA-NCAM immunoreactive cells in the granule layer and Purkinje layer. This finding highlights an important species-specific difference in PSA-NCAM-mediated plasticity in the adult brain.

The distribution of PSA-NCAM observed in the human cortical regions showed both similarities and differences to that seen in rodent studies and previous reports in the human brain. The laminar distribution of PSA-NCAM+ cells and processes observed is similar to that described in the human medial prefrontal cortex (Varea et al., 2007b), rat medial prefrontal cortex (Varea et al., 2005) and cat cerebral cortex (Varea et al., 2011). The strongest immunoreactivity was seen in layer V with a dense network of PSA-NCAM+ processes and cell somata. Interestingly the pattern of immunoreactivity observed in the superior frontal and middle temporal gyri illustrates a more extensive network of PSA-NCAM+ cells and processes than that described by a previous study of the human medial prefrontal cortex (Varea et al., 2007b). However, the pattern of immunoreactivity observed in the sensory-motor cortex does match this description, with a much lower density of PSA-NCAM+ cells and processes compared to the other cortical regions examined. This highlights an important difference in the capacity for structural remodelling across the human neocortex, possibly in an activity dependent manner.

The results of this work also indicate some differences in entorhinal cortex PSA-NCAM immunoreactivity compared to previous reports. Early studies of the rodent brain identified the piriform cortex as the only temporal lobe structure to express PSA-NCAM (Seki and Arai, 1991b; Bonfanti et al., 1992). However, more recent studies have comprehensively described high PSA-NCAM expression in the entorhinal cortex of rodent, cat and human brain (O’Connell et al., 1997; Mikkonen et al., 1998; Fox et al., 2000; Arellano et al., 2002; Nacher et al., 2010; Gómez-Climent et al., 2011; Varea et al., 2011). The staining in this study also shows the entorhinal cortex is an area of high PSA-NCAM immunoreactivity. However, dense labelling of randomly orientated processes and cells was observed in layers II - III and V, which contrasts with the occasional cell bodies and vertically orientated processes described in previous studies of the human entorhinal
cortex (Mikkonen et al., 1999b; Arellano et al., 2002). The functional relevance of these cortical PSA-NCAM+ cells can only be surmised from current data. Previous studies investigating the neurochemical phenotype of these cells suggest they are likely to be interneurons and this hypothesis is investigated further in the following chapter.

It is uncertain whether the distribution of PSA-NCAM observed in this study represents constitutive or transient expression. Previous studies indicating pharmacologically induced alterations in number of PSA-NCAM+ cells in the rat brain suggest PSA-NCAM expression can be externally influenced (Maćkowiak et al., 2005; Varea et al., 2007a; Castillo-Gómez et al., 2008). Such evidence suggests PSA-NCAM expression is likely to be a more dynamic process, but does not exclude the potential for constitutive expression in certain interneuron subgroups. It is possible that PSA-NCAM expression represents a mechanism to regulate interneuron signaling through structural remodeling or receptor activation. It has also been suggested that PSA-NCAM may insulate interneurons from the surrounding environment, creating a reservoir of cells that could be integrated into the local circuitry after PSA-NCAM is down-regulated (Gómez-Climent et al., 2011).

A further point of debate is the origin of PSA-NCAM+ cells in the aged brain. There is limited evidence for the presence of adult-born PSA-NCAM+ cells in the rat piriform cortex using BrdU co-labelling (Shapiro et al., 2007). However, several studies have determined that PSA-NCAM+ cells in the piriform cortex of many species are doublecortin+/BrdU+, suggesting they are immature cells originating from fetal development rather than adult neurogenesis. It is hypothesized that these cells were produced during development but left the mitotic cell cycle and appear to be in a dormant phase prior to final neuronal differentiation (Gómez-Climent et al., 2008; Luzzati et al., 2009; Rubio et al., 2016). The fetal origins of PSA-NCAM+ cells in the neocortex of cat, guinea pig, rabbit, mouse, rabbit and lizard have also been described (Luzzati et al., 2009; Gómez-Climent et al., 2011; Varea et al., 2011). While such a conclusion cannot be reached for the PSA-NCAM+ cells observed in this study, investigating this concept should certainly be the subject of future investigations.

6.4.2 PSA-NCAM+ cells in the human striatum

Another species-specific difference observed was an abundance of PSA-NCAM+ cells in the striatum of all cases examined. This was an unexpected finding due to the distinct lack of striatal PSA-NCAM+ cell bodies described in the adult mouse and rat (reviewed by Bonfanti, 2006). While PSA-NCAM+ neuropil has been described in 3-month-old mice and punctate PSA-NCAM immunoreactivity described in 25-day-old rats, these studies did not detail the presence of distinct
PSA-NCAM$^+$ cell bodies in adult animals (Szele et al., 1994; Nacher et al., 2010). Striatal PSA-NCAM$^+$ cells have been detected in rabbits and primates, although these studies attributed this expression to the maturation and migration of new striatal projection neurons (Bédard et al., 2006; Luzzati et al., 2006). As the size and morphology of the striatal PSA-NCAM$^+$ cells seen here do not match that of the large striatal projection neurons, this seems an unlikely explanation for the current study. The PSA-NCAM$^+$ cells observed in the human striatum appear similar to a population of immature calretinin$^+$ striatal interneurons described in mice (Dayer et al., 2005; Revishchin et al., 2010a, 2010b). However, the large number of PSA-NCAM$^+$ cells observed is well in excess of current striatal neurogenesis estimates (Ernst et al., 2014). Further characterisation using fluorescent double-labelling will be presented in the following chapter to support the hypothesis that striatal PSA-NCAM$^+$ cells in the adult human brain are a mature subtype of calretinin$^+$ interneurons that may be undergoing synapse formation or structural remodelling.

The distinct absence of PSA-NCAM$^+$ cells in the striosome compartments suggests that PSA-NCAM-mediated plasticity in the striatum is limited to specific functional circuitry. However, the functional relevance of these cells and their matrix specific localisation is unclear. The matrix predominantly receives input from sensorimotor and association cortices while the striosomes are primarily connected to the limbic network (Tippett et al., 2006; Crittenden and Graybiel, 2011). Together these sub-compartments coordinate motor patterns based on environmental input and past experiences (Graybiel, 2008). As such it can only be hypothesised that the distribution of PSA-NCAM in the striatum is reflective of a structure that performs specific adaptive motor functions.

6.4.3 Decreased PSA-NCAM immunoreactivity in the Alzheimer’s disease entorhinal cortex is inversely correlated with tau load

Quantification of PSA-NCAM load revealed a large amount of variability between individuals. This variability could represent fluctuations in PSA-NCAM expression throughout aging or an inherent difference in PSA-NCAM-mediated plastic capacity between individuals (Murphy and Regan, 1998). Nevertheless, these results demonstrate that PSA-NCAM expression is conserved in most regions of the aged brain in neurologically normal and disease conditions.

An exception is the AD entorhinal cortex where a significant decrease in layer V PSA-NCAM load was detected compared to control and PD cases. PSA-NCAM load in the entorhinal cortex and hippocampus was also inversely correlated with tau load, indicating that AD cases with more tau aggregates in these regions have relatively less PSA-NCAM.
This decrease is contradictory to previous reports of increased hippocampal PSA-NCAM detected in studies investigating hippocampal neurogenesis (Mikkonen et al., 1999b). The underlying cause of decreased entorhinal cortex PSA-NCAM in AD is unclear. It is unlikely to be the result of cortical atrophy alone as no significant changes in PSA-NCAM load were identified in areas such as the middle temporal gyrus and hippocampus, regions that were severely atrophied in AD cases. It is possible that these findings conflict with previous reports due to disparate quantification methods. Where the study by Mikkonen et al. (1999) measured optical density of the overall staining in the region, optical density measurements in this study exclude local background and relate only to immunoreactive processes and cell somata. Furthermore, quantification of PSA-NCAM load in this study indicates the area occupied by immunoreactive soma and processes rather than cell number.

No significant change in PSA-NCAM load was detected in the hippocampal GCL between AD, PD or control cases. These findings support the results from Chapter 4 which indicate SGZ neuronal plasticity is unaltered in AD. As previously discussed, current literature predominantly suggests there is no change in SGZ proliferation in AD, yet an increase in overall hippocampal proliferation has been reported in AD and presymptomatic PD due to microglial rather than neuronal proliferation (Boekhoorn et al., 2006; Doorn et al., 2014; Marlatt et al., 2014). These studies agree that changes to overall hippocampal proliferation are unlikely to represent changes in neurogenesis, particularly in the SGZ. Yet they propose an interesting idea relevant to the current study: while no significant change in PSA-NCAM load was identified in the many brain regions examined, it is possible the types of cells expressing PSA-NCAM could be altered. This concept is further investigated in Chapter 7.

The inverse correlation with tau load but not β-amyloid load suggests intracellular tangles may play a role in reducing PSA-NCAM-mediated plasticity or conversely, that a reduction in PSA-NCAM-mediated plasticity may predispose cells to tangle formation. Arendt et al., 1998 provides some evidence for the former idea with the hypothesis that regions of high cellular plasticity could be predisposed to tangle formation. Thus, the degeneration of PSA-NCAM+ cells may be more pronounced in the entorhinal cortex than in other regions due to its highly plastic nature. This hypothesis would require further investigation.

Another possibility is that PSA-NCAM expression is affected by increased long-term depression thought to occur in the AD hippocampus (Peineau et al., 2007; Hooper et al., 2008). PSA-NCAM has an important role in the process of activity-dependent spine remodelling and synaptic plasticity (Theodosis et al., 1999; Eckhardt et al., 2000; Dityatev et al., 2004; Gascon et al., 2007; Guirado
et al., 2014; Castillo-Gómez et al., 2016). Therefore, the reduction in synaptic activity that occurs in the AD hippocampus could result in a downstream decrease in activity-dependent PSA-NCAM expression in the entorhinal cortex layer V. This hypothesis points to PSA-NCAM-mediated plasticity as a possible cause of the spine retraction observed in AD. Consequently, a decrease in PSA-NCAM could result in reduced compensatory capacity of the region and an increase in neuropathology such as tau tangles.

### 6.5 Summary

This chapter has detailed a widespread yet specific distribution of PSA-NCAM in the aged human brain. The detection of PSA-NCAM in regions such as the cerebellum and caudate nucleus matrix compartment highlight important species-specific differences in PSA-NCAM-mediated plastic capacity. Further characterisation of the cell types expressing PSA-NCAM is necessary to determine the functional relevance of these differences and is investigated in Chapter 7. The significant decrease in PSA-NCAM observed in the AD entorhinal cortex was inversely correlated with tau load. These findings suggest PSA-NCAM expression is affected by AD pathology in a highly specific manner. Lastly, the widespread distribution of PSA-NCAM+ cells provides evidence that while PSA-NCAM labels immature neurons in the neurogenic niches, outside these regions it appears to be a marker of mature cells undergoing structural plasticity. This study emphasizes that PSA-NCAM expression, and therefore structural neuronal plasticity, is conserved in the neurologically normal aged human brain but may be altered in neurodegenerative disease.
Chapter 7. Neurochemical Characterisation of PSA-NCAM$^+$ cells in the Normal and Alzheimer’s Disease Human Brain

7.1 Introduction

In Chapter 6, PSA-NCAM was revealed to be abundantly expressed throughout the adult human brain and not restricted to the SVZ and SGZ. While PSA-NCAM is traditionally used as a marker of immature neurons in the neurogenic niches, it is also a marker of structural plasticity (Theodosis et al., 1999; Eckhardt et al., 2000; Dityatev et al., 2004). As many of the regions that expressed PSA-NCAM are not considered neurogenic, it appears that many mature brain cells undergo structural plasticity throughout adult life. This chapter aimed to identify the specific type of cells that express PSA-NCAM to gain a better understanding of how plasticity occurs in different brain regions.

The neurochemical phenotype of PSA-NCAM$^+$ cells has previously been examined in the hippocampus and neocortex of the rat brain (Nacher et al., 2002; Varea et al., 2005; Gómez-Climent et al., 2011). In the human brain however, only PSA-NCAM$^+$ cells in the medial prefrontal cortex have been characterised (Varea et al., 2007b). Using fluorescent double labelling of molecular markers, both the rat and human studies found PSA-NCAM was expressed by mature interneuron sub-types in these regions. As the size and morphology of PSA-NCAM$^+$ cells described in Chapter 6 did not match that of astrocytes, microglia, oligodendrocytes or projection neurons, an interneuron phenotype seemed likely and was the focus of the characterisation in this chapter.

Different interneuron phenotypes can be characterised using cell morphology, physiological properties, and molecular features. These molecular features include, but are not limited to, the specific expression of neurotransmitters, transcription factors, calcium binding proteins and receptors (Ascoli et al., 2008). As it was not possible to determine the physiological properties of PSA-NCAM$^+$ cells in human post-mortem tissue, morphological and molecular features relevant to the interneuron sub-types in each particular region were used in this characterisation study.

The distinction between neurons and glia was determined using NeuN, a nuclear protein specific to mature neurons, and GFAP, a structural filament specific to glia (Mullen et al., 1992). Additional markers were used to further distinguish PSA-NCAM$^+$ cell phenotype in each brain region. PCNA was used to determine whether PSA-NCAM$^+$ cells had proliferative capacity, as PCNA labels cells that were mitotically active in the 24 hours prior to death (Bravo and
Inhibitory GABAergic signalling, which is typical of interneurons, was determined using glutamate decarboxylase (GAD), an enzyme involved in GABA synthesis. A range of interneuron-specific markers were also investigated. Calbindin, calretinin and parvalbumin are calcium binding proteins expressed by different interneuron subtypes. In cortical regions, interneurons do not appear to express more than one calcium binding protein, so these three markers identify distinct interneuron sub-types (Miettinen et al., 1992; Andressen et al., 1993; del Rio and DeFelipe, 1996). Neuropeptide Y is a neurotransmitter selectively used by a subset of interneurons and was also used to distinguish interneuron sub-types (Raghanti et al., 2013). In the striatum, the distribution of calbindin, calretinin and parvalbumin is more complex but expression is still limited to distinct cell populations. Calbindin is primarily expressed by medium spiny projection neurons while calretinin and parvalbumin are selectively expressed by a range of striatal interneurons. DARPP-32, a protein phosphatase inhibitor, was also used as a marker of medium spiny neurons in the caudate nucleus (Cicchetti et al., 2000; Tepper and Bolam, 2004; Tepper et al., 2010). Co-labelling analysis of PSA-NCAM with these cell-specific markers provided an indication of PSA-NCAM+ cell phenotype in each brain region.

The second aim of this work was to investigate the underlying cell phenotype responsible for the decreased entorhinal cortex PSA-NCAM in AD cases. From the results of the qualitative characterisation analysis, PSA-NCAM+ interneuron sub-types were quantified in neuron-rich layers II and V of the entorhinal cortex from control and AD cases. Since specific neuronal populations can be susceptible to disease pathology, it was hypothesised that particular PSA-NCAM+ interneuron sub-types may be affected in AD. Overall this analysis was aimed at investigating whether the decreased entorhinal PSA-NCAM load could reflect a specific loss of interneurons in this region.
7.2 Methods

7.2.1 Human tissue selection

Fixed-frozen brain tissue from seven neurologically normal controls and six AD cases was used for the quantification of PSA-NCAM+ interneurons in the entorhinal cortex as outlined in Table 15. Four of the control cases were used for the qualitative characterisation of PSA-NCAM+ cells in different brain regions (indicated on Table 15). Tissue was prepared and sectioned as outlined in section 3.1.1, except for the cerebellum where 100 μm sections were cut rather than 50 μm to increase the probability of observing a PSA-NCAM+ cell. PSA-NCAM+ cells were characterised in eight different brain regions. These included the middle temporal gyrus (MTG), superior frontal gyrus (SFG), caudate nucleus (CN), cerebellum (CB), entorhinal cortex (EC), and the CA1 region, granule cell layer (GCL) and hilus of the hippocampus (Figure 7.1).

![Brain regions used for PSA-NCAM+ cell characterisation](image)

**Figure 7.1** Brain regions used for PSA-NCAM+ cell characterisation
Cortical regions included the entorhinal cortex, middle temporal gyrus and superior frontal gyrus. Sub-cortical structures (indicated by black arrow) included the caudate nucleus and hippocampus (sub-divided into GCL, CA1 and hilus regions). Cerebellar PSA-NCAM+ cells were also characterised.

As for the post-mortem tissue used in previous chapters, the control cases had no history of neurological abnormalities and cause of death was unrelated to any neurological condition. Independent pathological analysis confirmed the absence of other neuropathology. The average
age of control cases was 72 ± 14 years old and ranged from 48 – 88 years. The average post-mortem delay was 18.6 ± 5.1 hours with a range of 8 – 24 hours. The AD cases selected for this study had a clinical history of dementia with no evidence of other neuropathology. Independent pathological analysis was carried out to determine pathological diagnosis and disease severity. All AD cases selected for this study had a minimum Braak stage of IV indicative of AD. The average age of AD cases was 76.5 ± 5.7 years old and ranged from 69 – 84 years. The average post-mortem delay was 10.4 ± 5.4 hours with a range of 4.5 – 18 hours.

<table>
<thead>
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<tbody>
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</tr>
<tr>
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<td>48</td>
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</tr>
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<td>H181*</td>
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</tr>
<tr>
<td>H186</td>
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</tr>
<tr>
<td>H229</td>
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<td>Female</td>
<td>17</td>
</tr>
<tr>
<td>H242*</td>
<td>61</td>
<td>Male</td>
<td>19.5</td>
</tr>
</tbody>
</table>

* cases used for qualitative characterisation of PSA-NCAM* cells in different brain regions.

<table>
<thead>
<tr>
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<th>Sex</th>
<th>Post-mortem Delay (hr)</th>
<th>Braak Stage</th>
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<tbody>
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<td>VI</td>
</tr>
<tr>
<td>AZ81</td>
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</tr>
<tr>
<td>AZ102</td>
<td>84</td>
<td>Female</td>
<td>14.5</td>
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</tr>
</tbody>
</table>

### 7.2.2 Immunofluorescent co-labelling of PSA-NCAM and characterisation markers

Free floating fluorescent double labelling was performed to assess the neurochemical properties of PSA-NCAM* cells. For qualitative characterisation of PSA-NCAM* cells, two sections from each brain region (specific cases identified in Table 15) were double labelled for PSA-NCAM and the cell marker of interest. For the quantification of PSA-NCAM* interneurons in the entorhinal cortex, three sections from each case, with a spacing of 200 μm, were double labelled for PSA-
NCAM and each of calbindin, calretinin and parvalbumin. Immunofluorescent staining was carried out as described in section 3.2.3. Tris-EDTA pH 9.0 antigen retrieval was used to unmask the PSA-NCAM epitope for antibody binding. The sections were then incubated in the appropriate primary antibody combinations of PSA-NCAM (5A5, DSHB) with either NeuN (ABN78, Millipore), GFAP (AB4674, Abcam), GAD65/67 (G5163, Sigma), calretinin (CR7697, Swant), calbindin (CB38a, Swant), neuropeptide Y (N9528, Sigma), parvalbumin (194004, synaptic systems) or DARPP32 (AB10518; Millipore) for 72 hours. Tyramide signal amplification was required for PSA-NCAM, so biotinylated goat anti-mouse IgM secondary antibody (AB98673 Abcam) and an Alexa Fluor® 594-conjugated secondary antibody relevant to the corresponding cell marker species (detailed in Table 6) were added to the sections for 24 hours. ExtrAvidin®-Peroxidase (E2886, Sigma), Alexa Fluor® 488 Tyramide (T20948, ThermoFisher) and Hoechst (#33342, Molecular Probes) were applied as outlined in section 3.2.3. Finally, sections were mounted onto slides using PBS and coverslipped using ProLong® Gold AntiFade mounting media (P36930, Life Technologies).

For the qualitative characterisation of PSA-NCAM+ cells, the sections were imaged using an Olympus FV1000 confocal microscope with a 60x oil immersion lens. Imaging for the quantification of PSA-NCAM+ interneurons in the entorhinal cortex is detailed in section 7.2.4 below.

7.2.3 Immunoperoxidase co-labelling of PSA-NCAM with PCNA

Tyramide signal amplification was required for both PSA-NCAM and PCNA fluorescent labelling. However, sequential immunohistochemistry using this protocol was repeatedly unsuccessful for unknown reasons. Therefore, to investigate whether PSA-NCAM+ cells also expressed PCNA, immunoperoxidase double labelling was performed using DAB and DAB-nickel. Sections were labelled for PSA-NCAM using DAB as described in section 6.2.2 to produce a brown stain. A second antigen retrieval using citrate buffer pH 4.6 was then performed and the immunohistochemistry protocol was repeated using PCNA primary antibody (sc7907, Santa Cruz), however the DAB was supplemented with 1% nickel solution to give a black stain. Sections were mounted, dehydrated and coverslipped as outlined in section 3.2.1.

7.2.4 Quantification of PSA-NCAM+ interneurons in the entorhinal cortex.

Stitched images from Layer II and V of the entorhinal cortex were acquired for each section using a 20x objective on a Nikon Ni-E microscope equipped with Nikon DS-Ri2 camera and motorised stage. The ‘grab large image free shape’ tool on the NiS Elements software was used to define the
region of interest. The 4D acquisition module was then used to acquire and stitch the tile images for each channel independently. This process produced a large stitched image of entorhinal cortex layer II and V for each section containing all three channels.

Measurements and cell counts were performed using ImageJ v1.46. The area of the cortical layer was first defined and measured using the Hoechst channel (Figure 7.2A). Using the cell counter plug-in, PSA-NCAM+ cells within the delineated area were first marked using a coloured dot (Figure 7.2B). This was done with only the PSA-NCAM and Hoechst channels overlaid. A PSA-NCAM+ cell was only marked if the cell soma was labelled and a Hoechst+ nucleus was observed. The marker was placed on the nucleus of the PSA-NCAM+ cells. The 594 channel (containing either calbindin, calretinin or parvalbumin interneuron labelling) was then overlaid with the Hoechst channel and the process repeated for these cells using a different coloured dot (Figure 7.2C). Finally, co-labelling was determined by identifying nuclei that had both coloured dots. This was verified by overlaying all three channels to observe the co-labelled cell (Figure 7.2D). A third coloured dot was placed on the co-labelled cell and the total number of each cell population (PSA-NCAM+ cells, interneurons, and co-labelled cells) was determined. The proportion of total PSA-NCAM+ cells that were co-labelled has been presented as well as the proportion of total interneurons that were co-labelled.

Statistical analysis was performed using the GraphPad Prism (version 6.05) statistics and graphing package. Due to the small sample size for both control and AD groups, a normal distribution was not obtained for this data set as determined by the Shapiro-Wilk normality test. Therefore, a non-parametric Mann-Whitney test was performed to examine whether there was a significant difference between control and AD cases. Values are presented in this section as mean ± standard deviation with statistical significance set at P ≤ 0.05.
Neurochemical Characterisation of PSA-NCAM+ cells in the Normal and Alzheimer’s Disease Human Brain

Figure 7.2  Quantification of co-labelled PSA-NCAM+ cells in the entorhinal cortex
PSA-NCAM and calbindin co-labelling in layer II has been used as an example. (A) The layer was first delineated using the Hoechst channel only. (B) The PSA-NCAM channel was then overlaid and PSA-NCAM+ cells (white arrows) were marked using a coloured dot (not shown). (C) The PSA-NCAM channel was removed and the calbindin channel overlaid. Calbindin+ cells (yellow arrows) were marked using a different coloured dot (not shown). (D) Finally, all three channels were overlaid and cells with both coloured dots (white and yellow arrow) were marked as co-labelled with a third coloured dot. In this way, the population of PSA-NCAM+ cells, calbindin+ cells and co-labelled cells per mm² were determined for layer II and V.
7.3 Results

7.3.1 PSA-NCAM$^+$ cells are not in a mitotic cell cycle

PCNA$^+$ cells were observed in the entorhinal cortex, middle temporal gyrus, superior frontal gyrus, caudate nucleus, and the GCL, hilus and CA1 regions of the hippocampus (Figure 7.3A - G). No PCNA$^+$ cells were observed in the cerebellum (Figure 7.3H). PCNA$^+$ cells showed variable intensities of DAB labelling which is likely related to the phase of mitosis at the time of death (Kempermann, 2011). No PSA-NCAM$^+$ cells were observed to co-label with PCNA in any of the regions examined. This suggests PSA-NCAM$^+$ cells in these regions are not mitotically active; however, it does not exclude the possibility of an immature phenotype.
Figure 7.3 Co-labelling of PSA-NCAM and PCNA in the adult human brain
No co-labelling of PSA-NCAM (black arrows) and PCNA (white arrows) was observed in the hippocampal granule cell layer (A), hilus (B) or CA1 region (C). Nor was co-labelling observed in the entorhinal cortex (D), middle temporal gyrus (E), superior frontal gyrus (F) or caudate nucleus (G). No PCNA labelling was observed in the cerebellum (H). Scale bars: 20 μm
7.3.2 PSA-NCAM⁺ cell phenotype differs between brain regions

7.3.2.1 Hippocampus: granule cell layer

As described in sections 4.3.2 and 6.3.2.5, PSA-NCAM⁺ cells were primarily located in the SGZ of the hippocampal GCL. These cells had an elliptical soma with a single apical process that extended up through the granule cell layer. Most of the PSA-NCAM⁺ cells observed in this region co-labelled with NeuN, indicating a neuronal phenotype (Figure 7.4A). The intensity of the NeuN labelling was often less than that of surrounding granule cells and some PSA-NCAM⁺ cells showed an absence of NeuN co-labelling. This variable NeuN staining may reflect PSA-NCAM⁺ cells in an earlier stage of maturation as NeuN is expressed by late stage 4 immature granule cells, while PSA-NCAM is expressed as early as stage 3 of maturation (refer to Section 4.1, Figure 4.1). The morphology of the SGZ PSA-NCAM⁺ cells and absence of GFAP staining supports the notion that they are not of a glial lineage. Their immature phenotype is further supported by co-labelling with GAD (Figure 7.4C), an indication of inhibitory neurotransmission which is characteristic of immature granule cells prior to full maturity (Gutiérrez et al., 2003; Cabezas et al., 2013). A proportion of PSA-NCAM⁺ cells in the GCL also co-labelled with calretinin, a calcium binding protein transiently expressed during granule cell maturation. As they become fully mature, granule cells switch from calretinin expression to calbindin, so the GCL demonstrates abundant calbindin expression. Co-labelling of PSA-NCAM with calretinin, but not with calbindin further indicates an immature phenotype.

PSA-NCAM⁺ cells within the GCL did not co-label with parvalbumin and neuropeptide Y, which are markers of GCL interneurons. This indicates PSA-NCAM⁺ cells in the GCL region are unlikely to be interneurons (Figure 7.4F, G). Overall this co-labelling analysis suggests PSA-NCAM⁺ cells in the hippocampal GCL are immature granule cells.
Figure 7.4 Fluorescent co-labelling of granule cell layer PSA-NCAM cells and characterisation markers
Green borders indicate markers that were found to co-label with PSA-NCAM while red borders indicate markers that were not found to co-label with PSA-NCAM. GCL PSA-NCAM+ cells co-labelled with the mature neuronal marker NeuN (A), inhibitory cell marker GAD (C) and interneuron marker calretinin (E). No PSA-NCAM+ cells were found to co-label with the glial marker GFAP (B) or the interneuron markers calbindin (D), parvalbumin (F) or neuropeptide Y (G). Scale bars: 20 μm
7.3.2.2 Hippocampus: CA1 region

PSA-NCAM* cells were located throughout the stratum pyramidale, stratum radiatum and stratum lacunosum moleculare layers of the CA1 region. A range of morphologies were observed, including medium sized multipolar cells, large bipolar cells with long processes, and small spherical cells with small processes (presented in section 6.3.2.5). PSA-NCAM* cells in the CA1 region co-labelled with NeuN but not GFAP, indicating these cells are neurons rather than glia (Figure 7.5A, B). Co-labelling with GAD suggests these PSA-NCAM* cells are inhibitory and therefore likely to be interneurons rather than the excitatory pyramidal projection neurons (Figure 7.5C). To determine whether different types of interneurons expressed PSA-NCAM, co-labelling with calbindin, calretinin, parvalbumin and neuropeptide Y was investigated.

The distribution of these four interneuron sub-types in the CA1 region matched that from previous publications (reviewed by Freund and Buzsáki, 1996). Calbindin* interneurons were predominantly located in the stratum radiatum and stratum lacunosum moleculare layers and had bipolar or multipolar structures. PSA-NCAM* calbindin interneurons of both morphologies were observed in these layers (Figure 7.5D). Calretinin mostly labelled small multipolar interneurons in the stratum lacunosum moleculare layer and some large bipolar interneurons in the stratum pyramidale. PSA-NCAM was expressed by these larger calretinin* interneurons (Figure 7.5E). Parvalbumin* interneurons were abundant throughout all layers of the CA1 region. Large or medium sized multipolar cells were common in all layers. Large bipolar cells were also found in the stratum radiatum with long processes that extend down through the stratum lacunosum moleculare. PSA-NCAM was expressed by both types of parvalbumin* interneurons (Figure 7.5F). Very few neuropeptide Y interneurons were present in the CA1 region, although PSA-NCAM was observed on small spherical neuropeptide Y* cells in the stratum lacunosum moleculare (Figure 7.5G).

This pattern of co-labelling indicates PSA-NCAM is expressed by a wide variety of inhibitory interneurons in the CA1 region of the hippocampus.
Figure 7.5 Fluorescent co-labelling of CA1 PSA-NCAM cells and characterisation markers
Green borders indicate markers that were found to co-label with PSA-NCAM while red borders indicate markers that were not found to co-label with PSA-NCAM. CA1 region PSA-NCAM+ cells co-labelled with the mature neuronal marker NeuN (A), inhibitory cell marker GAD (C) and interneuron markers calbindin (D), calretinin (E), parvalbumin (F) and neuropeptide Y (G). No PSA-NCAM+ cells were found to co-label with the glial marker GFAP (B). Scale bars: 20 μm
7.3.2.3 Hippocampus: hilus

Hilar PSA-NCAM+ cells were predominantly located close to the GCL in a region known as the polymorphic (or plexiform) layer, although some cells were observed deeper within the hilus. The hilar PSA-NCAM+ cells tended to be large with either bipolar or multipolar structures. These cells co-labelled with NeuN but not GFAP, indicating a neuronal rather than a glial phenotype (Figure 7.6A, B). Co-labelling with GAD indicates these cells are likely to be inhibitory interneurons similar to those in the CA1 region (Figure 7.6C). Therefore, co-labelling with calbindin, calretinin, parvalbumin and neuropeptide Y was investigated to determine if different interneuron sub-types preferentially expressed PSA-NCAM.

The distribution of these four interneuron sub-types in the hilus was similar to that described in previous publications (reviewed by Freund and Buzsáki, 1996). Calbindin+ interneurons in the hilus were large bipolar shaped cells located in the polymorphic layer. A small proportion of these cells co-labelled with PSA-NCAM (Figure 7.6D). Neuropeptide Y+ interneurons were abundant in the hilus and both bipolar and multipolar cells were observed in plexiform layer. Most of the PSA-NCAM+ cells in the hilus co-labelled with neuropeptide Y (Figure 7.6G). Parvalbumin+ interneurons were also abundant in the polymorphic layer and deeper hilar layers; however, none of these cells were observed to co-label with PSA-NCAM (Figure 7.6F). Similarly, bipolar and multipolar calretinin interneurons located throughout the hilus did not co-label with PSA-NCAM (Figure 7.6E).

These findings suggest only particular sub-types of hilar interneurons express PSA-NCAM.
Hippocampus - Hilus

Figure 7.6 Fluorescent co-labelling of hilar PSA-NCAM cells and characterisation markers
Green borders indicate markers that were found to co-label with PSA-NCAM while red borders indicate markers that were not found to co-label with PSA-NCAM. Hilar PSA-NCAM+ cells co-labelling with the mature neuronal marker NeuN (A), inhibitory cell marker GAD (C) and interneuron markers calbindin (D), and neuropeptide Y (G). No PSA-NCAM+ cells were found to co-label with the glial marker GFAP (B) or the interneuron markers calretinin (E) and parvalbumin (F). Scale bars: 20 μm
7.3.2.4 Middle temporal gyrus and superior frontal gyrus

PSA-NCAM+ cells were located in layers II-III and V of the middle temporal gyrus and superior frontal gyrus. Most of these cells had a multipolar morphology although some bipolar cells were observed. Like the other brain regions examined, PSA-NCAM+ cells in these cortical regions showed co-labelling with NeuN but not GFAP which indicates a neuronal rather than glial lineage (Figure 7.7A, B, Figure 7.8A, B). These cells also co-labelled with GAD, indicating an inhibitory phenotype (Figure 7.7C, Figure 7.8C). Together the GAD and NeuN co-labelling point toward an interneuron phenotype for these cortical PSA-NCAM+ cells. Co-labelling with calbindin, calretinin, parvalbumin and neuropeptide Y was examined to investigate which interneuron sub-types expressed PSA-NCAM.

The distribution of these four interneuron sub-types in the middle temporal gyrus and superior frontal gyrus was similar to that described in previous publications of neocortical interneurons (reviewed by Raghanti et al., 2010, 2013). Calbindin+ interneurons were predominantly located in layer II-III and mostly had a multipolar morphology, although some large bipolar cells were present in this layer. Some small spherical cells were also scattered throughout layer V. PSA-NCAM was expressed by the multipolar calbindin+ interneurons in layer II-III and the small spherical cells in layer V (Figure 7.7D, Figure 7.8D). Calretinin+ cells were found mostly in layer II-III and had variable morphologies including bipolar, multipolar and small spherical cells. Few calretinin+ cells were present in layer V. PSA-NCAM was mainly expressed by the small spherical cells in layer II (Figure 7.7E, Figure 7.8E). Parvalbumin labelled bipolar and multipolar cells in layers III, IV and V. None of these cells were observed to co-label with PSA-NCAM (Figure 7.7F, Figure 7.8F). Neuropeptide Y+ interneurons were mostly seen in the white matter with bipolar or multipolar morphology. Very few cells were seen in layer II and V, although cell processes were observed. No PSA-NCAM+ neuropeptide Y interneurons were observed in the cortical grey matter (Figure 7.7G, Figure 7.8G).

Overall, PSA-NCAM was expressed by only two of the three interneuron sub-types predominantly found in the cortical grey matter. This suggests PSA-NCAM expression is specific to certain interneuron sub-types.
Neurochemical Characterisation of PSA-NCAM+ cells in the Normal and Alzheimer’s Disease Human Brain

**Figure 7.7** Fluorescent co-labelling of middle temporal gyrus PSA-NCAM cells and characterisation markers

Green borders indicate markers that were found to co-label with PSA-NCAM while red borders indicate markers that were not found to co-label with PSA-NCAM. Middle temporal gyrus PSA-NCAM+ cells co-labelled with the mature neuronal marker NeuN (A), inhibitory cell marker GAD (C) and interneuron markers calbindin (D) and calretinin (E). No PSA-NCAM+ cells were found to co-label with the glial marker GFAP (B) or the interneuron markers parvalbumin (F) and neuropeptide Y (G). Scale bars: 20 μm
**Figure 7.8** Fluorescent co-labelling of superior frontal gyrus PSA-NCAM cells and characterisation markers

Green borders indicate markers that were found to co-label with PSA-NCAM while red borders indicate markers that were not found to co-label with PSA-NCAM. Superior frontal gyrus PSA-NCAM+ cells co-labelled with the mature neuronal marker NeuN (A), inhibitory cell marker GAD (C) and interneuron markers calbindin (D) and calretinin (E). No PSA-NCAM+ cells were found to co-label with the glial marker GFAP (B) or the interneuron markers parvalbumin (F) and neuropeptide Y (G). Scale bars: 20 μm
7.3.2.5 Caudate nucleus

PSA-NCAM* cells were observed in the matrix compartment of the caudate nucleus. These cells had a small soma and one or two main processes that exhibited a simple branching structure. As PSA-NCAM* cells have not previously been described in the human or animal caudate nucleus, the phenotype of these cells was of great interest. Caudate nucleus PSA-NCAM* cells co-labelled with NeuN but not with GFAP, indicating a neuronal rather than a glial phenotype (Figure 7.9A, B). Surprisingly, these cells did not co-label with GAD which indicates they are not GABAergic (Figure 7.9C). Caudate nucleus PSA-NCAM* cells are therefore unlikely to be medium spiny projection neurons (MSNs) or inhibitory striatal interneurons, which both use GABA signalling.

To confirm this hypothesis, additional medium spiny neuron markers and striatal interneuron markers were investigated. Calbindin and DARPP-32 were used as markers of medium spiny neurons, while calretinin, parvalbumin and neuropeptide Y were investigated as markers of different striatal interneuron sub-types (Cicchetti et al., 2000; Tepper and Bolam, 2004; Tepper et al., 2010). Striatal interneurons are commonly defined by their morphology and neurochemical phenotype. In general, two main populations exist: cholinergic giant aspiny interneurons which are identified by the expression of choline acetyltransferase (ChAT) and GABAergic medium aspiny neurons of which there are three main sub-types. The first type express GAD and parvalbumin and comprise the majority of GABAergic striatal interneurons. The second type express GAD, somatostatin, and neuropeptide Y as well as nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), which suggests these cells may also use nitric oxide signalling. The third type express calretinin and show a range of sizes and morphologies which indicate further classification may be required (Cicchetti et al., 2000; Tepper and Bolam, 2004; Tepper et al., 2010). As the PSA-NCAM* cells found in the striatum did not show the size or morphology of the giant aspiny interneurons, co-labelling with ChAT was not investigated (DiFiglia et al., 1976; Cicchetti et al., 2000).

No PSA-NCAM* cells in the caudate nucleus co-labelled with calbindin (Figure 7.9D) or DARPP-32 (Figure 7.9H) which indicates they are unlikely to be medium spiny neurons. Furthermore, the PSA-NCAM* cells did not co-label with parvalbumin (Figure 7.9F) or neuropeptide Y (Figure 7.9G) suggesting they are also unlikely to be medium aspiny interneurons pertaining to these sub-types. The only marker that did co-label with striatal PSA-NCAM* cells was calretinin (Figure 7.9E), however the lack of GAD co-labelling suggests these cells may not be a medium aspiny interneuron sub-type. Overall these results suggest caudate nucleus PSA-NCAM* cells are unlikely to be any of the commonly identified striatal cell types and may represent an uncharacterised type of striatal calretinin* interneurons.
Neurochemical Characterisation of PSA-NCAM+ cells in the Normal and Alzheimer’s Disease Human Brain

Caudate Nucleus

NeuN

GFAP

GAD

Calbindin

Calretinin

Parvalbumin

Neuropeptide Y

DARPP-32
7.3.2.6 Cerebellum

The identification of PSA-NCAM$^+$ cells in the Purkinje layer of the human cerebellum was another novel finding in this study. These PSA-NCAM$^+$ cells had large cell soma in the Purkinje layer and an extensive dendritic tree which suggested they were likely to be cerebellar Purkinje cells. To verify this, PSA-NCAM co-labelling with common Purkinje cell markers was investigated. Calbindin is considered to label all Purkinje cells, while parvalbumin is expressed by a proportion of the total Purkinje cell population (Fortin et al., 1998; Whitney et al., 2008). As Purkinje cells are also GABAergic, GAD was used as a marker (Voogd et al., 1996). NeuN is not expressed by Purkinje cells so this marker was not examined (Mullen et al., 1992).

The results of the co-labelling analysis did not produce clear evidence that the PSA-NCAM$^+$ cells are Purkinje cells. While some PSA-NCAM$^+$ cells were found to co-label with calbindin (Figure 7.10A), others showed a clear absence of calbindin co-labelling (Figure 7.10B). Since calbindin is considered to be the primary marker of human cerebellar Purkinje cells, this suggests at least some of the PSA-NCAM$^+$ cells are not Purkinje cells (Fortin et al., 1998; Whitney et al., 2008). The GAD co-labelling analysis further supports this notion, whereby some PSA-NCAM$^+$ cells expressed GAD (Figure 7.10C) and others did not (Figure 7.10D). Furthermore, no PSA-NCAM$^+$ cells were found to co-label with parvalbumin which is expressed by a sub-set of Purkinje cells in conjunction with calbindin (Figure 7.10E) (Fortin et al., 1998).

Overall this co-labelling analysis provides a surprising conclusion. A proportion of cerebellar PSA-NCAM$^+$ cells do not show co-labelling with characteristic Purkinje cell markers, despite having a morphology and location that are considered specific to these cells.
Figure 7.10 Fluorescent co-labelling of PSA-NCAM<sup>+</sup> cells in the cerebellar Purkinje cell layer with characterisation markers
Green borders indicate markers that were found to co-label with PSA-NCAM while red borders indicate markers that were not found to co-label with PSA-NCAM. Some Purkinje cell layer PSA-NCAM<sup>+</sup> cells co-labelled with calbindin (A) while others did not (B). Similarly, some Purkinje cell layer PSA-NCAM<sup>+</sup> cells co-labelled with GAD (C) while others did not (D). None of the PSA-NCAM<sup>+</sup> cells were found to co-label with parvalbumin (E). Scale bars: 20 μm.
7.3.2.7 Entorhinal cortex

PSA-NCAM* cells were predominantly located in layers II/III and V/VI of the entorhinal cortex. These cells had small cell soma and either a bipolar or multipolar morphology, although some small unipolar cells were also identified in layer II. The entorhinal cortex is unique in that it lacks the layer of cells where layer IV is situated in other cortical regions. This region is referred to as lamina dissecans, but its boundaries are poorly defined from layer III and V (Insausti et al., 1995).

PSA-NCAM* cells showed co-labelling with NeuN but not GFAP, indicating a neuronal rather than glial lineage (Figure 7.11A, B). PSA-NCAM* cells also co-labelled with GAD, indicating an inhibitory phenotype (Figure 7.11C). Entorhinal PSA-NCAM cells are therefore likely to be interneurons rather than the excitatory pyramidal cells. Co-labelling with calbindin, calretinin, parvalbumin and neuropeptide Y was examined to investigate which interneuron sub-types expressed PSA-NCAM (Tuñón et al., 1992; Mikkonen et al., 1999a).

Calbindin* interneurons were predominantly located in layers II-III of the entorhinal cortex but were found in layers V and VI as well. These cells had a range of medium sized bipolar and multipolar morphologies. PSA-NCAM co-labelled with calbindin* cells of both morphologies throughout all cortical layers (Figure 7.11D). Calretinin* interneurons were concentrated in layer II-III with very few scattered throughout layer V. Most of these cells had a radially orientated bipolar structure. PSA-NCAM only co-labelled with calretinin* interneurons in layer II. The co-labelled cells were small and spherical, some with a single process (Figure 7.11E). This suggests that only a specific sub-type of calretinin interneurons express PSA-NCAM.

Parvalbumin* interneurons were mostly absent from layer II compared to layer III where they were densely clustered. Some cells were scattered throughout layer V. Bipolar and multipolar cells were observed and co-labelling with PSA-NCAM was seen for both morphologies in layer III and V (Figure 7.11F). Neuropeptide Y* interneurons were mostly located in the white matter of the entorhinal cortex with some cells on the border of layer VI. None of the cells in the grey matter were found to co-label with PSA-NCAM (Figure 7.11G).

These results indicate PSA-NCAM is expressed by three different interneuron sub-types within the entorhinal cortex and within these sub-types only particular cells may express PSA-NCAM. This is evidenced by the calretinin* interneurons where only PSA-NCAM* cells in layer II showed co-labelling.
Figure 7.11 Fluorescent co-labelling of entorhinal cortex PSA-NCAM+ cells and characterisation markers

Green borders indicate markers that were found to co-label with PSA-NCAM while red borders indicate markers that were not found to co-label with PSA-NCAM. Entorhinal cortex PSA-NCAM+ cells co-labelled with the mature neuronal marker NeuN (A), inhibitory cell marker GAD (C) and interneuron markers calbindin (D), calretinin (E) and parvalbumin (F). No PSA-NCAM+ cells were found to co-label with the glial marker GFAP (B) or the interneuron marker neuropeptide Y (G). Scale bars: 20 μm.
7.3.3 Quantitative analysis of PSA-NCAM\(^{+}\) interneuron populations in the control and AD entorhinal cortex

A major finding from the PSA-NCAM quantification in Chapter 6 (section 6.3.3) was the significant decrease in staining load in the entorhinal cortex of AD cases. It was hypothesised that this reduction could be due to degeneration of specific neuronal sub-types in the AD entorhinal cortex. The fluorescent co-labelling analysis in this region (section 7.3.2.7) indicates that three different interneuron sub-types (identified by calbindin, calretinin and parvalbumin) express PSA-NCAM. In this section, PSA-NCAM co-labelling with these three interneuron markers was quantified to investigate if a loss of PSA-NCAM expression from specific interneuron populations underlies the decrease in staining load detected in AD cases. This analysis focussed on layers II and V which could be clearly determined by Hoechst labelling in both control and AD cases. These two layers also had the densest PSA-NCAM immunoreactivity.

### 7.3.3.1 Calbindin

As outlined in section 7.3.2.7, PSA-NCAM\(^{+}\) cells were concentrated in layer V with some cells in layers II-III and VI. Conversely, calbindin\(^{+}\) cells were predominantly located in layers II-III with some cells in layer V and VI (Figure 7.12A). This distribution was also observed in AD cases.

Quantification of the cell populations in layer II showed no significant difference in calbindin\(^{+}\) cells between AD and control cases (Figure 7.12B, mean for AD cases: 72.2 ± 36.4 cells/mm\(^2\), mean for control cases: 104.1 ± 47.0 cells/mm\(^2\), \(P = 0.2949\)). There was a significant decrease in the number of PSA-NCAM\(^{+}\) cells in layer II of AD cases (Figure 7.12B, mean for AD cases: 13.6 ± 4.0 cells/mm\(^2\), mean for control cases: 21.5 ± 4.5 cells/mm\(^2\), \(P = 0.0221\)) as well as a significant decrease in the number of co-labelled PSA-NCAM\(^{+}\)/calbindin\(^{+}\) cells (Figure 7.12B, mean for AD cases: 3.4 ± 1.8 cells/mm\(^2\), mean for control cases: 6.6 ± 2.3 cells/mm\(^2\), \(P = 0.0350\)). However, when the number of co-labelled cells was normalised to the total PSA-NCAM or calbindin cell population, no significant difference was identified between AD cases and controls (Figure 7.12C, percent co-labelled PSA-NCAM\(^{+}\) cells = 25.3 ± 13.4\% for AD and 30.1 ± 8.5\% for controls, \(P = 0.3660\). Figure 7.12D, percent co-labelled calbindin\(^{+}\) cells = 5.8 ± 3.9\% for AD and 7.2 ± 4.1\% for controls, \(P = 0.5064\)). If PSA-NCAM\(^{+}\) calbindin interneurons were specifically degenerated in the AD entorhinal cortex, the proportion of calbindin\(^{+}\) interneurons expressing PSA-NCAM and the proportion of PSA-NCAM\(^{+}\) cells expressing calbindin would both be reduced. In contrast, an increase in these proportions compared to controls would suggest PSA-NCAM\(^{+}\) calbindin interneurons are preserved.
Layer V also did not show a significant difference in calbindin$^+$ cells between AD and control cases (Figure 7.12E, mean for AD cases: 25.8 ± 5.8 cells/mm$^2$, mean for control cases: 29.4 ± 7.0 cells/mm$^2$, P = 0.3660). There was a significant decrease in the number of PSA-NCAM$^+$ cells in layer V of AD cases which supports the findings from the staining load analysis in Chapter 6 (Figure 7.12E, mean for AD cases: 16.3 ± 2.7 cells/mm$^2$, mean for control cases: 25.3 ± 5.7 cells/mm$^2$, P = 0.0012). The number of co-labelled PSA-NCAM$^+$/calbindin$^+$ cells in layer V was not significantly different between AD and controls (Figure 7.12E, mean for AD cases: 1.7 ± 1.9 cells/mm$^2$, mean for control cases: 1.3 ± 1.2 cells/mm$^2$, P = 0.9289). This was also true when the number of co-labelled cells was normalised to the total PSA-NCAM or calbindin cell population. controls (Figure 7.12F, percent co-labelled PSA-NCAM$^+$ cells = 10.9 ± 13.7% for AD and 5.1 ± 4.6% for controls, P = 0.4936. Figure 7.12G, percent co-labelled calbindin$^+$ cells = 6.8 ± 6.9% for AD and 3.9 ± 3.3% for controls, P = 0.5047).

Overall these data suggest that a decrease in layer II PSA-NCAM$^+$ calbindin interneurons likely contributes to the overall reduction of PSA-NCAM$^+$ cells in this layer. However, this does not seem to be the case for layer V as the number of co-labelled cells per mm$^2$ was not altered in AD. Since the proportion of co-labelled cells in the overall PSA-NCAM$^+$ and calbindin$^+$ cell populations was not altered in either layer, it seems PSA-NCAM$^+$ calbindin interneurons are not preferentially preserved or lost in AD.
Figure 7.12  Quantification of PSA-NCAM⁺/calbindin⁺ interneurons in the entorhinal cortex of control and AD cases
(A) Calbindin⁺ interneurons (red) were located throughout layers II-III and V-VI of the entorhinal cortex. PSA-NCAM (green) was densely distributed through layer V with some cells and processes present in layers II, III and VI.
(B) There was a significant decrease in the number of PSA-NCAM⁺ cells/mm² (PSA⁺) and co-labelled PSA-NCAM⁺/calbindin⁺ (PSA⁺/CB⁺) cells in layer II of AD cases. There was no significant difference between the number of calbindin⁺ cells/mm² (CB⁺) in layer II of control and AD cases. The proportions of PSA-NCAM⁺ cells expressing calbindin (C) and calbindin⁺ cells expressing PSA-NCAM (D) was also not significantly altered in layer II. (E) In agreement with PSA-NCAM load quantification (Chapter 6), there was a significant decrease in the number of PSA-NCAM⁺ cells/mm² in layer V of AD cases, although there was no change in layer V calbindin⁺ cells/mm² or co-labelled PSA-NCAM⁺/calbindin⁺ cells. (F) There was no significant difference in the proportion of PSA-NCAM⁺ cells expressing calbindin (F) and calbindin⁺ cells expressing PSA-NCAM (G) between control and AD cases.
7.3.3.2 Calretinin

As described above, PSA-NCAM was concentrated in layer V with some cells in layers II-III and VI. The highest density of calretinin+ interneurons was in layer II with some cells in layer III and scattered throughout layer V (Figure 7.13A). This distribution was also observed in AD cases.

Consistent with the above results (section 7.3.3.1), the sections analysed for the calretinin analysis showed a significant decrease in the number of PSA-NCAM+ cells/mm² was found in layer II of AD cases compared to controls (Figure 7.13B, mean for AD cases: 8.4 ± 4.4 cells/mm², mean for control cases: 15.5 ± 5.5 cells/mm², P = 0.0350). However, no significant difference in calretinin+ cells/mm² was detected between AD and control cases for this layer (Figure 7.13B, mean for AD cases: 240 ± 105 cells/mm², mean for control cases: 154 ± 38 cells/mm², P = 0.1807). Nor was there a difference in the number of co-labelled cells, although very few co-labelled cells were detected in both AD and control cases (Figure 7.13B, mean for AD cases: 0.9 ± 1.6 cells/mm², mean for control cases: 1.7 ± 1.5 cells/mm², P = 0.1690). When the number of co-labelled cells was normalised to the total PSA-NCAM or calretinin cell population, no significant difference was identified between AD cases and controls (Figure 7.13C, percent co-labelled PSA-NCAM+ cells = 6.4 ± 10.9% for AD and 11.2 ± 9.1% for controls, P = 0.2774. Figure 7.13D, percent co-labelled calretinin+ cells = 0.25 ± 0.45% for AD and 1.0 ± 0.8% for controls, P = 0.0699).

A significant decrease in PSA-NCAM+ cells was also found in layer V of AD cases, again consistent with the PSA-NCAM quantification in section 7.3.3.1 (Figure 7.13E, mean for AD cases: 12.6 ± 2.7 cells/mm², mean for control cases: 22.5 ± 5.8 cells/mm², P = 0.0047). No significant difference in calretinin+ cells/mm² was detected between AD cases and controls for layer V (Figure 7.13E, mean for AD cases: 25.0 ± 4.8 cells/mm², mean for control cases: 24.2 ± 9.7 cells/mm², P = 0.7308). Interestingly, no PSA-NCAM+ cells in layer V were found to co-label with calretinin.

Overall these results suggest that layer II PSA-NCAM+ calretinin interneurons are rare and are not preferentially preserved or lost in AD. The lack of co-labelling in layer V of both control and AD cases suggests the expression of PSA-NCAM by calretinin+ interneurons is layer specific.
Figure 7.13  Quantification of PSA-NCAM⁺/calretinin⁺ interneurons in the entorhinal cortex of control and AD cases

(A) Calretinin⁺ interneurons (red) were abundant throughout layers II and III and sparse throughout layer V and VI of the entorhinal cortex. (B) Analysis of the cell populations indicated there was a significant decrease in the number of PSA-NCAM⁺ cells/mm² (PSA⁺) of layer II in AD cases while the number of calretinin⁺ cells (CR⁺) and co-labelled PSA-NCAM⁺/calretinin⁺ cells (PSA⁺/CR⁺) was not significantly altered. The proportions of PSA-NCAM⁺ cells expressing calretinin (C) and calretinin⁺ cells expressing PSA-NCAM (D) was also not significantly altered in layer II. (E) There was also a significant decrease in the number of PSA-NCAM⁺ cells/mm² in layer V of AD cases, although there was no change in number of calretinin⁺ cells/mm². (F) Interestingly no co-labelled PSA-NCAM⁺/calretinin⁺ cells were identified in layer V of the entorhinal cortex.
7.3.3.3 Parvalbumin

Parvalbumin$^+$ interneurons were located in layer III which was not included in this quantification as it was difficult to distinguish from lamina dessicans. Some parvalbumin$^+$ interneurons were present in layers II and V where PSA-NCAM$^+$ cells were most concentrated (Figure 7.14A).

The number of parvalbumin$^+$ cells/mm$^2$ was not significantly different between AD and control cases in layer II of the entorhinal cortex (Figure 7.14B, mean for AD cases: $21.3 \pm 10.6$ cells/mm$^2$, mean for control cases: $30.6 \pm 12.7$ cells/mm$^2$, $P = 0.1807$). Again, AD cases showed a significant decrease in PSA-NCAM$^+$ cells/mm$^2$ compared to control cases (Figure 7.14B, mean for AD cases: $6.1 \pm 1.8$ cells/mm$^2$, mean for control cases: $12.6 \pm 5.2$ cells/mm$^2$, $P = 0.0140$). Interestingly, no co-labelled PSA-NCAM$^+$/parvalbumin$^+$ cells were identified in AD cases. This resulted in a significant decrease in the number of co-labelled cells between AD cases and controls (Figure 7.14B, mean for AD cases: 0 cells/mm$^2$, mean for control cases: $1.6 \pm 1.9$ cells/mm$^2$, $P = 0.0047$), as well as a significant difference in the proportion of total PSA-NCAM and parvalbumin cell populations that were co-labelled (Figure 7.14C, percent co-labelled PSA-NCAM$^+$ cells = 0% for AD and 10.2 $\pm$ 8.9% for controls, $P = 0.0047$. Figure 7.14D, percent co-labelled parvalbumin$^+$ cells = 0% for AD and 5.2 $\pm$ 5.9% for controls, $P = 0.0047$).

In layer V, the number of parvalbumin$^+$ cells/mm$^2$ was also not significantly different between AD and controls (Figure 7.14E, mean for AD cases: $23.8 \pm 14.3$ cells/mm$^2$, mean for control cases: $22.2 \pm 6.2$ cells/mm$^2$, $P = 0.8357$). As for the previous data sets, AD cases showed a significant decrease in PSA-NCAM$^+$ cells/mm$^2$ compared to controls in layer V (Figure 7.14E, mean for AD cases: $21.7 \pm 6.3$ cells/mm$^2$, mean for control cases: $10.8 \pm 3.9$ cells/mm$^2$, $P = 0.0047$). Co-labelled PSA-NCAM$^+$/parvalbumin$^+$ cells were identified in layer V of both control and AD cases, but no significant difference was observed (Figure 7.14E, mean for AD cases: $1.3 \pm 1.6$ cells/mm$^2$, mean for control cases: $1.4 \pm 1.0$ cells/mm$^2$, $P = 0.4697$). Furthermore, there was no significant difference in the proportion of total PSA-NCAM and parvalbumin cell populations that were co-labelled (Figure 7.14F, percent co-labelled PSA-NCAM$^+$ cells = $9.5 \pm 8.4$% for AD and $7.5 \pm 7.0$% for controls, $P = 0.7010$. Figure 7.14G, percent co-labelled parvalbumin$^+$ cells = $4.4 \pm 3.9$% for AD and $6.2 \pm 4.9$% for controls, $P = 0.4697$).

Overall these results suggest that co-labelled PSA-NCAM$^+$ parvalbumin interneurons are rare in layer II of the entorhinal cortex, even in control cases. The decreased proportion of co-labelled cells in the total PSA-NCAM and parvalbumin populations in AD cases suggests PSA-NCAM$^+$ parvalbumin interneurons may be preferentially lost in AD. However, these cells do not seem to be specifically affected in layer V.
Figure 7.14  Quantification of PSA-NCAM+/parvalbumin+ interneurons in the entorhinal cortex of control and AD cases

(A) Parvalbumin+ interneurons (red) were primarily located in layer III although many could also be found in layers II, V and VI of the entorhinal cortex. (B) There was a significant decrease in the number of PSA-NCAM+ cells/mm² (PSA+) in layer II of control and AD cases. Interestingly there were no co-labelled PSA-NCAM+/parvalbumin+ cells (PSA+/PV+) in layer II of AD cases and hence a significant decrease in this co-labelled population was observed. This also resulted in a significant decrease in the proportions of PSA-NCAM+ cells expressing parvalbumin (C) and parvalbumin+ cells expressing PSA-NCAM (D) in layer II of AD cases. (E) Again, a significant decrease in the number of PSA-NCAM+ cells/mm² was identified in layer V of AD cases, although there was no change in layer V parvalbumin+ cells/mm² or co-labelled PSA-NCAM+/parvalbumin+ cells/mm². (F) There was also no change in the proportion of PSA-NCAM+ cells expressing parvalbumin (F) and parvalbumin+ cells expressing PSA-NCAM (G) in layer V of AD cases.
7.4 Discussion

The widespread distribution of PSA-NCAM throughout the human brain (chapter 6) suggests cells in many brain regions undergo structural plasticity. To determine the functional relevance of this plasticity the phenotype of PSA-NCAM+ cells was examined in different brain regions. Co-labelling of PSA-NCAM with different cell specific markers provided evidence of a mature interneuron phenotype in most regions. However, the phenotype of caudate nucleus and cerebellum PSA-NCAM+ cells remains unclear. Finally, three PSA-NCAM+ interneuron populations were quantified in the entorhinal cortex of AD and control cases to investigate whether the decreased PSA-NCAM load in this region of the AD brain is due to loss of specific interneuron sub-types.

7.4.1 PSA-NCAM+ cells are likely to be interneurons in most brain regions

PSA-NCAM is traditionally used as a marker of immature neurons in the neurogenic niches of the adult human brain. However, many PSA-NCAM+ cells were identified outside the neurogenic niches and the number far exceeds estimates of adult proliferation in these regions (Spalding et al., 2005; Bhardwaj et al., 2006; Ernst et al., 2014). Therefore, PSA-NCAM+ cells in non-neurogenic regions are unlikely to be immature neurons, and may instead be mature cells undergoing structural remodelling. This study found PSA-NCAM+ cells were not mitotically active as they did not co-label with PCNA in any of the brain regions assessed. While the lack of PCNA co-labelling does not exclude an immature phenotype, co-labelling of PSA-NCAM with NeuN in all regions except the cerebellum indicates that a mature neuronal phenotype is more likely.

The fluorescent co-labelling analysis in this chapter (summarised in Table 16 below) showed PSA-NCAM+ cells also express interneuron makers. PSA-NCAM was predominantly expressed by interneurons in all regions assessed, other than the cerebellum where the phenotype of PSA-NCAM+ cells was unclear. This study presents the first widespread assessment of PSA-NCAM neurochemical phenotype in the adult human brain. Previously studies that investigated PSA-NCAM+ cell phenotype focused only on the non-neurogenic regions of the rat hippocampus and the medial prefrontal cortex of rat and human brain (Nacher et al., 2002; Varea et al., 2005, 2007b; Gómez-Climent et al., 2011). The medial prefrontal cortex studies agree that PSA-NCAM+ cells have an interneuron phenotype and described co-labelling with GAD, calbindin, parvalbumin, neuropeptide Y and somatostatin, but not with calretinin (reviewed by Nacher et al., 2013). In contrast, the current study found cortical PSA-NCAM+ cells did co-label with calretinin (although infrequently), that no cells co-labelled with neuropeptide Y, and that parvalbumin co-labelling was
only seen in the entorhinal cortex (Table 16). These disparate results suggest the plasticity of different interneuron populations may vary across the cortical regions.

Characterisation of PSA-NCAM+ cells in the non-neurogenic regions of the human hippocampus suggested species-specific differences in PSA-NCAM+ cell phenotype may also exist. Hilar PSA-NCAM+ cells frequently co-labelled with GAD, calbindin and neuropeptide Y, but no calretinin co-labelling was observed. In the CA1 region, PSA-NCAM co-labelled with all the interneuron markers assessed. Conversely, in the hilus and CA1 region of the rat hippocampus, PSA-NCAM+ cells were only found to co-label with calretinin and GAD and not with parvalbumin, calbindin, somatostatin or neuropeptide Y (Nacher et al., 2002). From this comparison, it seems that a broader variety of interneurons may express PSA-NCAM in human hippocampus compared to the rat.

The phenotype of PSA-NCAM+ cells in the caudate nucleus was not clear from the co-labelling analysis in this study. The striatal PSA-NCAM+ cells were most likely interneurons based on their morphology and co-labelling with only NeuN and calretinin. However, since the majority of striatal calretinin+ interneurons are considered GABAergic, the lack of GAD co-labelling is conflicting. Striatal PSA-NCAM+ cells may belong to a relatively uncharacterised population of small calretinin+ interneurons. These cells were first described in the mouse striatum by Revishchin et al., 2010a, 2010b, and show a strikingly similar morphology to the PSA-NCAM+ cells observed in this study as well as the same matrix-specific localisation. Very little is known about these small calretinin+ interneurons, other than they do not co-label with mature or immature cell markers such as NeuN, GAD, ChAT, parvalbumin, calbindin, neuropeptide Y or nestin. They were also negative for PSA-NCAM in the mouse striatum, however as PSA-NCAM+ cells have not been described in the striatum of any other species to date, this does not necessarily exclude the phenotype. Since it is difficult to conclusively determine whether PSA-NCAM+ cells belong to this population of small calretinin+ interneurons, further characterisation using additional interneuron markers could be performed to eliminate known cell phenotypes. Such markers could include ChAT, somatostatin, tyrosine hydroxylase, cholecystokinin and vasoactive intestinal polypeptide.

The phenotype of cerebellar PSA-NCAM+ cells in the Purkinje layer was also perplexing. Their location and extensive dendritic tree strongly suggested a Purkinje cell phenotype. However, only a proportion of these PSA-NCAM+ cells showed expression of key Purkinje cell markers such as calbindin and GAD. This indicates that at least some of the cerebellar PSA-NCAM+ cells are not Purkinje cells. Another possibility are the candelabrum cells which are found in the same location and also have an extensive, although more basket-like, dendritic tree (Laine and Axelrad, 1994;
Crook et al., 2006). Very little is known about these cells other than they use both GABA and glycine as neurotransmitters. As there is no reliable marker for candelabrum cells, a future investigation of glycine expression would be the only way to determine if the calbindin-negative PSA-NCAM+ cells belong to this population of cerebellar interneurons.

Overall the phenotypic characterisation presented in this chapter indicates that PSA-NCAM is mainly expressed by interneurons in the human brain and this expression is not specific to a single interneuron sub-type. Furthermore, the interneuron populations that express PSA-NCAM vary across different brain regions and likely between species as well. Additional markers such as somatostatin and cholecystokinin could be used to further characterise PSA-NCAM+ cells into more specific interneuron subsets. Markers of excitatory neurotransmission should also be investigated to confirm PSA-NCAM is not expressed by pyramidal cells. Finally, a quantification of the different PSA-NCAM+ interneuron populations would provide a useful indication of plastic capacity for each interneuron sub-type in each brain region.

Table 16: Summary of qualitative PSA-NCAM co-labelling results

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<th>CR</th>
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* DARPP32 cells were also examined in the caudate nucleus but none were found to co-label with PSA-NCAM
7.4.2 Decreased PSA-NCAM in the entorhinal cortex of AD is not due to loss of specific interneuron sub-types.

The results from Chapter 6 revealed a decreased PSA-NCAM load specifically in the entorhinal cortex of AD cases. The fluorescent characterisation of entorhinal PSA-NCAM\(^+\) cells in section 7.3.2.7 indicated these cells mainly had an interneuron phenotype. It was therefore hypothesised that the decreased PSA-NCAM load could reflect a loss of interneurons in the entorhinal cortex. Since specific neuronal populations can be susceptible to disease pathology, particular PSA-NCAM\(^+\) interneuron sub-types may be affected. To investigate this hypothesis, PSA-NCAM co-labelling with calbindin, calretinin and parvalbumin was quantified.

From the data sets of all three markers, AD cases had significantly less PSA-NCAM\(^+\) cells in layer II and V of the entorhinal cortex. This result agrees with the decrease in layer V PSA-NCAM load that was presented in Chapter 6 and shows the alteration in entorhinal cortex PSA-NCAM extends to layer II as well.

The quantification of PSA-NCAM\(^+\) interneurons showed only layer II calbindin co-labelled cells and layer II parvalbumin co-labelled cells were significantly decreased in AD cases. Furthermore, there was no change in the proportion of total PSA-NCAM\(^+\) cells that were co-labelled, or total calbindin\(^+\), calretinin\(^+\) and parvalbumin\(^+\) cells that were co-labelled for either layer II and V. These results indicate that the loss of PSA-NCAM\(^+\) calbindin and parvalbumin interneurons likely contributes to the overall decrease in layer II PSA-NCAM\(^+\) cells, but none of the three PSA-NCAM\(^+\) interneuron sub-types were preferentially preserved or lost in layer II or V of AD cases. The complete absence of layer II parvalbumin co-labelled cells in AD cases is difficult to interpret. Since control cases only show an average of 1.6 co-labelled cells/mm\(^2\), the absence of co-labelled cells in AD may just reflect the low frequency of both cell types in this layer. The calretinin analysis also showed very low numbers of co-labelled cells in layer II. Furthermore, there was a complete absence of co-labelling in layer V, despite the relative abundance of both PSA-NCAM\(^+\) and calretinin\(^+\) cells in this layer. This finding suggests the expression of PSA-NCAM by calretinin\(^+\) interneurons is layer specific. It could also be specific to a particular sub-type of small calretinin\(^+\) interneurons specifically located in layer I and II of the entorhinal cortex (Wouterlood et al., 2000). Overall the co-labelling analysis suggests PSA-NCAM is expressed by layer specific sub-types even within a class of interneurons. Furthermore, all three PSA-NCAM\(^+\) interneuron sub-types in the entorhinal cortex seem to be relatively unaffected in AD. Future studies could compare the number of co-labelled interneurons to the total number of neurons in the entorhinal cortex to determine if PSA-NCAM\(^+\) interneurons are preserved relative to the overall neuron
population. The entorhinal cortex can also be divided into eight subfields along its rostro-caudal axis based on cytoarchitecture and projection patterns (Mikkonen et al., 1999a). Future studies could examine whether the proportion of PSA-NCAM+ interneurons is altered in each of these subfields, rather than in the overall structure.

Interestingly, the co-labelling analysis shows the combined proportions of PSA-NCAM+ calbindin, calretinin and parvalbumin interneurons only account for around 30-50% of total PSA-NCAM+ cells counted in layer II and 10-20% of total PSA-NCAM+ cells in layer V. Although this proportion probably varies between cases, it suggests there must be additional cell types expressing PSA-NCAM that weren’t identified in the co-labelling analysis. A quantification of PSA-NCAM+ cells that co-label with NeuN would be a useful indicator of whether these unidentified cells are likely to be neuronal.

The co-labelling analysis also included an assessment of the overall interneuron populations in control and AD. The density of calbindin+, calretinin+ and parvalbumin+ interneurons were unchanged in layer II and V of AD cases. Surprisingly, a comprehensive quantification of interneuron populations in the AD entorhinal cortex is lacking in the literature. For calbindin+ interneurons, data is limited to just two studies. One of which only qualitatively describes a decrease of calbindin+ cells (Mikkonen et al., 1999a). The other shows a decrease in the raw number of calbindin+ cells in layer II of AD cases, but does not normalise the calbindin+ cell count to total neurons or layer area (Thorns et al., 2001). Therefore, it is hard to determine how the results from the current study compare with previous data. Only parvalbumin+ interneurons have been adequately quantified in the entorhinal cortex, with a decrease observed in layer II of AD cases (Solodkin et al., 1996). This conflicts with the results from this chapter where no significant difference was identified. The data from this chapter also appears to be the first quantification of calretinin+ cell density in the entorhinal cortex of AD cases, as only qualitative studies have been previously published (Brion and Résibois, 1994; Mikkonen et al., 1999a). These results agree with the observations by Mikkonen et al., 1999a that the calretinin+ interneuron population is preserved in AD.

In conclusion, this quantification analysis has shown the overall decrease in entorhinal cortex PSA-NCAM is not solely due to the loss of PSA-NCAM+ interneurons. It has further identified a gap in current literature regarding the effect of AD pathology on entorhinal cortex interneuron populations. Additional PSA-NCAM characterisation is required to fully determine the cell types that contribute to loss of entorhinal PSA-NCAM load.
7.5 Summary

This chapter provides the first widespread assessment of PSA-NCAM neurochemical phenotype in the adult human brain. Fluorescent double-labelling showed PSA-NCAM is mainly expressed by mature interneurons in the adult human brain and this expression is not specific to a single interneuron sub-type. Different interneuron populations were found to express PSA-NCAM in different regions and comparison to previous rodent studies suggests the cell types expressing PSA-NCAM in each region can differ between species. Interestingly, PSA-NCAM+ cells in the caudate nucleus and cerebellum appear to be relatively uncharacterised interneuron sub-types. Further investigation will be required to validate this finding.

A quantification of PSA-NCAM+ interneuron populations in layer II and V of the entorhinal cortex showed these cells were not preferentially affected in AD. The loss of PSA-NCAM+ calbindin and parvalbumin interneurons does not account for the magnitude of the overall PSA-NCAM+ cell loss in the entorhinal cortex. A loss of these interneurons may contribute to the overall PSA-NCAM loss, but it is likely that other PSA-NCAM+ cell types are involved. The quantification analysis showed that the three PSA-NCAM+ interneuron sub-types investigated comprise only a proportion of total entorhinal cortex PSA-NCAM+ cells. Therefore, further characterisation is required to determine other cell types that may contribute to loss of entorhinal cortex PSA-NCAM+ cells.
Chapter 8. General Discussion

Alzheimer’s disease (AD) has a complex symptom profile related to the advancement of β-amyloid and tau aggregates through the brain. It is unclear exactly how these pathological aggregates contribute to neuronal degeneration, but the field is shifting from a β-amyloid-driven hypothesis to a tau-centred hypothesis. This shift follows evidence suggesting β-amyloid plaques may be a neuroprotective mechanism and evidence that tau load correlates with symptom severity, rather than β-amyloid (Arriagada et al., 1992; Harrington, 2012; Puzzo et al., 2015). Furthermore, phase 3 clinical trials of drugs that clear β-amyloid have consistently failed to provide any therapeutic benefit despite decreasing plaque load. As tau pathology accumulates long before clinical onset and cortical atrophy, the brain appears to have a considerable capacity to compensate for neuronal degeneration (Jack et al., 2010) (Morris, 2005; Sperling et al., 2011). This thesis examined whether neuronal proliferation and PSA-NCAM-mediated structural plasticity are affected in AD. The inverse correlation between PSA-NCAM and tau load in the hippocampal GCL (Chapter 4) and entorhinal cortex (Chapter 6) suggests tau may contribute to a loss of PSA-NCAM-mediated plasticity. However neuronal proliferation in the neurogenic niches appears to be unaffected by tau pathology (Chapter 4 and 5).

8.1 Neurogenesis in the Alzheimer’s disease brain

Neuronal proliferation in the SGZ and SVZ was found to be unaltered in AD cases, indicating this mechanism of plasticity is unlikely to contribute to AD symptoms. This conclusion contradicts the results of AD mouse models that show memory impairment and anosmia parallel a decline in SGZ and SVZ progenitor cell proliferation and/or maturation (Haughey et al., 2002a, 2002b; Wen et al., 2004; Donovan et al., 2006; Zhang et al., 2007; Verret et al., 2007; Chen et al., 2008; Demars et al., 2010; Hamilton et al., 2010; Faure et al., 2011; Hamilton and Holscher, 2012; Krezymon et al., 2013). However, mouse models do not replicate the neuronal degeneration or chronic nature of AD and results of proliferation studies have not been replicated using human post-mortem tissue (reviewed by Rodríguez and Verkhratsky, 2011; Winner et al., 2011). Therefore, the development of early AD symptoms appears to occur irrespective of preserved SGZ and SVZ proliferation.

There is a strong possibility that maturation of SGZ progenitor cells is impaired by tau pathology in AD. Llorens-Martín et al., 2016 recently proposed that hyperphosphorylation of tau impairs progenitor cell maturation, not through aggregation, but by destabilisation of developing cytoskeleton that leads to abnormal morphology. This is supported by numerous studies that show aberrant granule cell morphology in the human hippocampus (Scheibel and Tomiyasu, 1978; de
Ruiter and Uylings, 1987; Scheff and Price, 2003, 2006). The increased number of SGZ PSA-NCAM$^+$ cells in AD cases and the inverse correlation with hippocampal tau load also supports this hypothesis. A larger PSA-NCAM$^+$ cell population could be a mechanism of functional compensation (as discussed in section 4.4.2) or it could indicate a block in progenitor cell maturation. That is, progenitor cells may stall in this phase of maturation in AD as microtubule destabilisation impairs dendritic growth. As tau pathology becomes more severe, progenitor cells may be unable to structurally mature, leading to cell death and a relative decrease in PSA-NCAM$^+$ cells compared to earlier disease stages. In this thesis, immuno-Golgi staining was also performed to investigate whether immature granule cells develop abnormal dendrite and synaptic spine morphology in human AD. While no significant conclusion was obtained, dendritic morphology could be explored further by analysing PSA-NCAM$^+$ cells. Since SGZ PSA-NCAM labelling specifically identifies immature cells, assessing the dendritic structure of SGZ PSA-NCAM$^+$ cells could indicate whether this element of maturation is altered in AD.

The SGZ results indicate that the therapeutic potential of neurogenesis in AD may lie in manipulation of progenitor maturation rather than proliferation. The SGZ PCNA$^+$ cell quantification indicates memory symptoms occur despite normal proliferation. Furthermore, proliferation appears to be a rare event in the adult human hippocampus (Low et al., 2011). It is debateable whether such a low rate of proliferation provides any meaningful contribution to granule cell numbers, let alone has a regenerative effect in disease. Alternatively, increasing the proportion of immature granule cells may have therapeutic potential. Rather than neuronal replacement, these immature cells could offer a form of functional regeneration. As discussed in section 4.4.2, immature granule cells are highly adaptable and more readily undergo LTP that facilitates learning and memory processes (Schmidt-Hieber et al., 2004; Ge et al., 2007). These cells are hypothesised to contribute to the processing of novel stimuli and short-term memory, two processes severely affected during AD (Kempermann, 2002; Leuner et al., 2006). Increasing the population of immature granule cells by inhibiting further maturation may improve these processes and alleviate memory symptoms. However, if progenitor cell maturation is impaired by tau as discussed above, increasing the population of immature granule cells would only increase the proportion of abnormal cells. Although the effect of abnormal maturation is unknown, it would likely have a negative impact on cell function.

SVZ proliferation was unaltered in AD, indicating it is unlikely to contribute to AD symptoms. Tau and Aβ pathology was notably low in the SVZ compared to the underlying caudate nucleus. It is possible that this absence of pathology leaves the SVZ relatively spared from degeneration. However, as AD symptoms develop irrespective of preserved SVZ proliferation it is unlikely that
an upregulation of SVZ proliferation will have any therapeutic effect. The maturation of SVZ progenitor cells was not investigated in this thesis, however the process could be affected by microtubule destabilisation due to tau pathology and affect cell function. As SVZ progenitor cells migrate much further distances than SGZ progenitor cells, this process may also be affected by pathological aggregates. In addition to the RMS that leads to the olfactory bulb, SVZ progenitors can migrate into the caudate nucleus or along a lateral cortical stream to the neocortex. Although, these alternative migration pathways occur in response to ischemic events (Arvidsson et al., 2002; Jin et al., 2003; Yamashita et al., 2006). The abundance of progenitor cells in these pathways and their destination should be assessed in future studies to determine if AD pathology affects migration.

The observation that proliferating cell density is altered near blood vessels has consequences for AD. Available evidence suggests the SVZ contains specialised vasculature that may degenerate in AD (Fischer et al., 1990; Buée et al., 1997; Bell and Zlokovic, 2009; Miyakawa, 2010). If so, the SVZ may experience hypoperfusion as seen in the wider AD brain. Hypoperfusion could prevent an upregulation of proliferation in response to AD pathology. This hypothesis is based on observations that SVZ proliferation increases in Huntington’s disease, a neurodegenerative condition also characterised by pathological aggregates (Curtis et al., 2003). Alternatively, SVZ vessels could become leaky, leading to a greater availability of trophic factors (Ryu and McLarnon, 2009). If this is the case, the effect on overall SVZ proliferation is probably minor as no change was detected in this study. There is currently no specific evidence of SVZ vascular degeneration in human AD, so investigating these hypotheses would be a progression for the AD neurogenesis field. The effect of blood vessels on local SVZ proliferation should also be accounted for in future quantification studies. Blood vessel integrity can be assessed through fluorescent dye perfusion and immunohistochemical studies. The effect of blood vessels on proliferation would involve analysis of proliferating cell density across the entire SVZ on evenly spaced caudate nucleus sections from control and AD cases.

8.2 PSA-NCAM-mediated plasticity in the Alzheimer’s disease brain

As the quantification of SGZ and SVZ progenitor cells revealed neuronal proliferation was unaltered in the AD brain, plasticity was examined in wider brain areas. PSA-NCAM was observed in non-neurogenic regions during the progenitor cell quantification and further investigation revealed it was widely distributed throughout the adult human brain in control and AD cases. This suggests PSA-NCAM-mediated plasticity is an ongoing process throughout adult life and persists during neurodegenerative disease.
Specific differences in PSA-NCAM distribution between humans and other mammalian species suggest PSA-NCAM-mediated plasticity may be involved in processes unique to the human brain. The discovery of PSA-NCAM+ cells in the human cerebellum and caudate nucleus directly contrasts the results of rabbit and rodent studies (reviewed by Bonfanti, 2006). The cerebellum is considered by some to be an anatomically static structure and it shows no PSA-NCAM expression in rodents (Bonfanti, 2006). However, the identification of rare PSA-NCAM+ cells in the human cerebellum challenges this notion and suggests PSA-NCAM-mediated structural plasticity does occur, albeit very specifically in this region. The characterisation of cerebellar PSA-NCAM+ cells in Chapter 7 was inconclusive, but their complex dendritic structure suggests they are mature rather than developing cells. The purpose of such specific structural plasticity in the adult human cerebellum is unknown. The cerebellum has a complex somatotopic organisation, so the specificity of PSA-NCAM-mediated plasticity may be related to upstream or downstream connections (Rijntjes et al., 1999; Grodd et al., 2001). Similarly, PSA-NCAM+ cells in the human caudate nucleus could be linked to a unique process. The matrix-specific localisation of these cells suggests their function relates to sensorimotor input and structural plasticity may facilitate learning or adaptation of motor processes (Section 6.4.2).

The function of PSA-NCAM in non-neurogenic regions is most likely related to interneuron activity. Analysis of PSA-NCAM distribution and cell phenotype in this thesis suggest that a wide range of interneuron sub-populations express PSA-NCAM throughout the human brain (Chapter 7). The overview of PSA-NCAM+ cell distribution in this study likely represents a snapshot of dynamic PSA-NCAM expression. PSA-NCAM expression is regulated by neuronal activity in the rodent hippocampus and this may also be the case throughout the human brain (Muller et al., 1996; Theodosis et al., 1999). Two plausible functions of activity-dependent PSA-NCAM expression have previously been proposed: 1. as interneurons regulate the activity of local projection neurons, PSA-NCAM expression can potentially modify this interaction. PSA-NCAM-mediated remodelling of interneuron dendrites and synaptic spines can alter the physical connections between interneurons and projection neurons (Gómez-Climent et al., 2011; Nacher et al., 2013). 2. PSA-NCAM may insulate interneurons from neighbouring cells, preventing the formation of synapses and their regulatory role on projection neurons. This could create a reservoir of interneurons able to be recruited into the local circuitry when required (Gómez-Climent et al., 2011). Such a reservoir could contribute to functional compensation during the pre-clinical phase of AD.

It is unclear whether tau NFTs affect PSA-NCAM expression and function. The regions where tau and PSA-NCAM load were found to be inversely correlated in this study are also the first regions
to be affected by tau pathology in AD (Braak and Braak, 1995). In fact, tau aggregates first accumulate in regions of high plasticity and spread progressively to less plastic areas (Arendt et al., 1998). This suggests that regions of high plasticity are more vulnerable to NFT formation. A recent hypothesis suggests hyperphosphorylated tau can be transmitted between neurons in a prion-like manner (Clavaguera et al., 2009; de Calignon et al., 2012; Saiz-Sanchez et al., 2015). Neurons in regions of high plasticity may be more vulnerable to this transmission due to their dynamic connectivity with other neurons. It remains to be determined whether accumulation of tau in this manner leads to a reduction of PSA-NCAM or whether a loss of PSA-NCAM expression makes neurons susceptible to NFT accumulation. That is, NFTs may accumulate in the entorhinal cortex due to its highly plastic nature and cause a decrease in neuronal activity that leads to reduced PSA-NCAM expression. Or alternatively, PSA-NCAM may be neuroprotective based on its ability to insulate neurons from their environment. This insulation could temporarily prevent the prion-like transmission of NFTs and provide a reservoir of healthy interneurons to maintain cognitive function despite the accumulation of pathology in neighbouring cells. A reduction of PSA-NCAM for unknown reasons would therefore enhance susceptibility of the region to NFT formation. To investigate these two possibilities, PSA-NCAM should be examined along the pathway that tau aggregates spread through the brain. If PSA-NCAM is reduced in regions ahead of the aggregates it would indicate that impaired PSA-NCAM-mediated plasticity precedes aggregate formation and lend support to a neuroprotective mechanism.

The therapeutic potential of PSA-NCAM-mediated plasticity in AD cannot be determined from this study alone; however, it is likely that the specific reduction of entorhinal cortex PSA-NCAM contributes to memory symptoms. This hypothesis could be investigated further in AD mouse models. While no available model fully captures all aspects of AD pathology, the effect of decreased entorhinal cortex PSA-NCAM on memory function could be determined. The therapeutic potential of PSA-NCAM could be assessed by upregulating sialyltransferase activity or reducing PSA-NCAM internalization by endocytosis. If PSA-NCAM has a neuroprotective effect against AD pathology, then the therapeutic benefit of its upregulation may lie in the preclinical phase of AD. Enhancing PSA-NCAM expression early in the disease process may aid in maintaining cognitive function and delaying symptom onset, thereby reducing the prevalence of AD. Balanced PSA-NCAM activity is essential as overexpression could be detrimental to connectivity. Therefore, understanding the mechanisms that regulate PSA-NCAM activity and expression is vital and should be investigated further.
8.3 Concluding Remarks

This thesis demonstrates that two different mechanisms of plasticity persist in the human brain during adult life. Furthermore, these plasticity mechanisms are remarkably resilient to the severe degeneration and aggregate pathology that occurs in AD. SGZ and SVZ proliferation were unaltered in AD but it remains to be determined whether adult-born progenitor cells are able to structurally mature and become functional in an environment contaminated by disease pathology. This thesis also demonstrated that the revision of current analysis methods is crucial for comparison between human tissue studies examining neuronal proliferation. Furthermore, that advancements to current technology are required to address fundamental questions about the process of neurogenesis in the adult human brain. The investigation of PSA-NCAM-mediated plasticity in this thesis is the first of its kind using human tissue. The distribution and characterisation of PSA-NCAM+ cells proved this mechanism of plasticity to be abundant even in non-neurogenic regions of the brain. The specific reduction of PSA-NCAM in the entorhinal cortex indicates that further investigation of PSA-NCAM function would be highly relevant to the understanding of AD symptom development. Overall this thesis has contributed to the current knowledge of neuronal proliferation and PSA-NCAM-mediated plasticity in the normal and AD human brain.
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