Statement of Originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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Signature: ______________________________________

Date: ________________________________
Authorship Attribution Statement

Chapter 2 of this thesis is published as:


I was responsible for the design of the study along with CSW and KD. I also performed all experimental procedures, analysed the data and prepared the figures and manuscript.

Chapter 3 of this thesis has not yet been written for publication.

I was responsible for the design of the study along with Associate Professor Kay Double. I also performed all experimental procedures, analysed the data and prepared the figures and thesis chapter. This project was a collaboration with Associate Professor John Kwok at the Brain and Mind Centre, University of Sydney.

Chapter 4 of this thesis is published as:

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I was responsible for the interpretation of data previously collected and analysed by SV, and the preparation of the submitted figures and manuscript. I also collected and analysed additional data included in the manuscript. *SV and I are credited as co-first authors on this paper.*
In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

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As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Name: Associate Professor Kay Double

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Abstract

Neurogenesis persists in the adult human hippocampus and is hypothesised to contribute to hippocampus-based memory function and emotional regulation. It has been previously reported that neurogenesis declines in normal, healthy ageing as well as Parkinson’s disease dementia (PDD). This decline in neurogenesis with age and disease may therefore be a contributing factor to the development of dementia. Recently, the established understanding of human hippocampal neurogenesis has been questioned, necessitating new approaches for measuring and analysing human neurogenesis and how it may contribute to normal hippocampal function. This thesis aimed to determine if neurogenesis gene expression is altered in the aged and PDD hippocampus and factors which may contribute to these changes.

Qualitative polymerase chain reaction was used to quantify the expression of genes associated with various stages of neurogenesis from stem cell quiescence to neuronal maturation. Levels of hippocampal neurotransmitters and their metabolites were quantified using high performance liquid chromatography, and levels of hippocampal growth factors with enzyme-linked immunosorbent assay. Western blotting was used to examine expression of neurogenesis proteins.

Expression of neurogenesis-related genes specific to cellular proliferation and maturation decline in both age and PDD, in-line with previous findings at the protein level in humans and other species. In contrast to previous animal studies, genes associated with neuronal maturation were more highly expressed in the anterior hippocampus than the posterior. The dopamine metabolite 3,4-dihydroxyphenylacetic acid and serotonin metabolite 5-hydroxyindoleacetic acid were associated with the pathological changes in
neurogenesis, but not the regional variability. Protein levels of growth factors associated with normal hippocampal function were also altered in the PDD hippocampus, in the absence of any apparent neuronal loss.

These data suggest that neurogenesis gene expression declines in both ageing and PDD, in line with reports of reduced numbers of new neurons. Alterations in hippocampal neurotransmitters and growth factors may underlie these changes and contribute to an altered microenvironment that impacts the normal cognitive functions of the human hippocampus.
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First and foremost, I’d like to thank my supervisor, Associate Professor Kay Double for her guidance and support over the past four years. She has unhesitatingly shared with me not only her immense knowledge of neuroscience research, but her expert understanding of the jungle that is academia. Her guidance is and always will be appreciated.

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Poster Presentations:


Awards:

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2018 Australasian Neuroscience Society Travel Award

2017 University of Sydney Three Minute Thesis - Open Heat Winner and University Finalist

2016 Australasian Neuroscience Society Travel Award
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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>5-HIAA</td>
<td>5-Hydroxyindolacetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine, or Serotonin</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu ammonis</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CDNF</td>
<td>Cerebral dopamine neurotrophic factor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-Dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EOMES</td>
<td>Eomesodermin</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast growth factor 2</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein, isoform alpha</td>
</tr>
<tr>
<td>GFAPδ</td>
<td>Glial fibrillary acidic protein, isoform delta</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin-binding EGF-like growth factor</td>
</tr>
<tr>
<td>HPLC</td>
<td>Reverse-phase high performance liquid chromatography</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanillic acid</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IPO8</td>
<td>Importin 8</td>
</tr>
<tr>
<td>LB</td>
<td>Lewy body</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>MANF</td>
<td>Mesencephalic astrocyte-derived neurotrophic factor</td>
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<tr>
<td>MKI67</td>
<td>Ki67</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween®20</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PDD</td>
<td>Parkinson's disease dementia</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Qualitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>S100B</td>
<td>S100 calcium-binding protein B</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SNc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-box binding protein</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween®20</td>
</tr>
<tr>
<td>UBC</td>
<td>Ubiquitin C</td>
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</tbody>
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Chapter 1: Introduction
Advancing age is the leading risk factor in a range of disorders which place a significant strain on the health and wellbeing of sufferers. In many instances, these disorders lead to loss of independence, reduced quality of life and increased pressure on communities and families sharing the burden of disease. Dementia is most commonly associated with advancing age and is characterised by persistent deterioration of memory and cognitive ability, but may also cause emotional and mood disturbances as well as language and speech difficulties. Alzheimer’s disease (AD) and vascular dementia make up most cases where dementia is the main clinical feature, although there are a range of other disorders which feature dementia as a secondary symptom (Australian Institute of Health and Welfare, 2012). Over 400,000 Australians are currently living with dementia, which is projected to reach around 1 million by 2050 (Brown, Hasnata and La, 2017). In 2017, the direct (medical care, hospitalisation, pharmaceuticals etc.) and indirect (foregone income, lost productivity etc.) costs of dementia totalled AUD 14.67 billion (Brown, Hasnata and La, 2017), demonstrating the massive personal and economic burden of dementia.

Although it is classified as a neurodegenerative movement disorder, Parkinson’s disease (PD) is a disease in which dementia and cognitive decline is a prominent symptom. It is characterised mainly by a progressive degeneration of the nigrostriatal dopamine pathway, manifesting primarily as a movement disorder with symptoms including shuffling gait, tremor at rest, muscle rigidity and slowness of movement (Jankovic, 2008). A significant number of PD sufferers also develop dementia as a result of the disease, which is one of the main contributing factors towards decreased quality of life amongst sufferers (Leroi et al., 2012; Schrag, Jahanshahi and Quinn, 2000). Approximately 80% of PD sufferers have developed dementia by 20 years post-diagnosis (Hely et al., 2008), and the incidence of dementia in PD patients has been reported as two to six times higher than normal, age-matched controls.
(Aarsland et al., 2003; de Lau et al., 2005; Marder et al., 1995). Regardless of these findings, the aetiology and pathophysiology of dementia in PD is still largely unknown.

Perhaps the main reason for this is that the process of normal physiological ageing is also little understood, thus there is no reliable way of determining the differences in how a healthy brain ages in comparison to a diseased one. Although neurodegeneration and dementia also occurs as a result of normal physiological ageing (Australian Institute of Health and Welfare, 2012; Hindle, 2010), the differences in disease presentation and dementia prevalence amongst PD and normally aged brains indicates that there may be significant molecular differences in how the brain degrades in age and disease.

A promising avenue of research in both normal brain ageing and PD concerns the process of neurogenesis in the hippocampus. Neurogenesis, the generation of neurons from neural stem cells, is thought to be altered in both ageing and PD, although the exact mechanisms of this change and the reasons for them have been little studied in the human brain (Desplats et al., 2012; Knoth et al., 2010; Lugert et al., 2010). The hippocampus itself plays an important role in aspects of memory and cognition and thus, in conjunction with changes to neurogenesis in age and PD, may play a role in the declining memory and cognition which defines dementia (Clelland et al., 2009; Drapeau et al., 2003; Squire, 2009). This thesis investigates changes in neurogenesis in both the ageing and PD brain. Specifically, it examines changes in the expression of neurogenesis-related genes with age, as well as the regional variations in the expression of these genes along the length of the aged and PD hippocampus.
1.1 Neurogenic niches of the adult central nervous system

Stem cells in the embryonic and postnatal brain are crucial for the establishment of working neural circuits and the overall development of the functional central nervous system (CNS). During this period of development, stem cells proliferate, differentiate and mature into neurons, astrocytes and oligodendrocytes. This process is strictly regulated by a range of signalling pathways (Kanski et al., 2014). This process, while robust, is also transitory, with the majority of the developing brain structure established in utero (Casey, Giedd and Thomas, 2000). After birth, widespread neurogenesis declines to a baseline level by around two years of age (Casey, Giedd and Thomas, 2000), although animal studies suggest that neurogenesis in the adolescent brain remains more active than in their adult counterparts, reflective of greater neural plasticity in younger animals (Ahmed et al., 2007; Hwang et al., 2008).

As the CNS reaches adulthood neurogenesis is significantly reduced, leading to the early opinion that the adult brain is static and unchanging. It was not until the 1960’s that ongoing neurogenesis was discovered in the rodent brain (Altman and Das, 1965), followed by the discovery of adult neurogenesis in the human and primate brain in the 1990’s (Eriksson et al., 1998; Gould et al., 1999). These discoveries not only changed the prevailing opinion of an unchanging brain, they allowed researchers the opportunity to investigate the role of ongoing adult neurogenesis in a range of conditions including ageing, PD, AD and neuropsychiatric disorders such as depression and schizophrenia. Although there are several known areas of the CNS where adult stem cells can be found, only two areas of the human brain have been shown to contain neuronally differentiating stem cells, and thus can be considered true neurogenic niches. These two areas of the brain are the subventricular zone
(SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG), a region of the hippocampus.

1.1.1 The subventricular zone

The subventricular zone is located on the lateral wall of the lateral ventricles. The zone forms distinct layers in the human brain, which consist of ciliated ependymal cells which line the ventricle wall, as well as layers of specialised astrocytes and stem cells. It remains one of the most studied stem cell niches in both the human and rodent brain and has been studied in a range of disorders including PD, AD, schizophrenia and depression.

In the normal brain, stem cells in the SVZ undergo proliferation and differentiation into immature neurons. These neurons then migrate to the olfactory bulb via the rostral migratory stream (RMS), where they are integrated into the existing circuitry as interneurons (Lois and Alvarez-Buylla, 1994). The RMS has been well-described in the rodent brain, however the presence and function of the RMS in the human brain remains controversial within the literature (Curtis et al., 2007; Sanai et al., 2004). Recent evidence also suggests that SVZ-derived neurons can be found in the human striatum, which is adjacent to the neurogenic niche (Arvidsson et al., 2002; Ernst et al., 2014). This finding raises the possibility that immature neurons from the SVZ may have migratory potential greater than was previously thought, making it an important field of research into diffuse brain repair after injury or neurodegeneration.

1.1.2 The subgranular zone

The human hippocampus is a curved, roughly cylindrical structure located beneath the cortical surface of the medial temporal lobe. It consists primarily of two interconnected regions known as the cornu ammonis (CA; which is further divided into three subregions
known as CA1, CA2 and CA3) and the dentate gyrus (DG), which is the region where neurogenesis occurs (Eriksson et al., 1998).

The main cellular component of the DG are granule cells, identified morphologically by their small, granular cell bodies and dense organisation within the DG (Seri et al., 2004). These excitatory granule cells project to the CA3 region of the hippocampus, which subsequently projects to several other regions of the hippocampal formation (Markakis and Gage, 1999). Below this granule cell layer is the subgranular zone (SGZ). The SGZ, located between the granule layer and hilus of the DG, contains stem cells but also glial cells, blood vessels and other components necessary for the maintenance of the neurogenic niche (Aizawa et al., 2011; Riquelme, Drapeau and Doetsch, 2008). From the SGZ, stem cells proliferate and differentiate into newly formed immature neurons. The immature neurons subsequently integrate into the existing SGZ neural circuitry upon maturation (Fig. 1.1).
Stem cells in the hippocampus are located within the SGZ, the area directly below the granule neurons which form the DG, represented by the grey cells. Once stimulated, quiescent stem cells in the SGZ follow a pathway of proliferation, differentiation and maturation before being integrated into the existing neuronal circuitry of the DG approximately 4 weeks after neuronal differentiation (in rodent models [Aimone, Deng and Gage, 2014]). Adapted from Mathews et al. (2017).
1.2 Functions and modulation of neurogenesis in the adult hippocampus

Neurogenesis in the adult hippocampus has been established in both the rodent and human brains (Altman and Das, 1965; Eriksson et al., 1998; Priyadarshi et al., 2001). However, the mechanisms and role of neurogenesis itself is still incompletely understood, particularly in regard to pathological changes to neurogenesis in the diseased hippocampus.

1.2.1 Cognitive and affective functions of the hippocampus

The exact role of the hippocampus is yet to be fully elucidated, although human and rodent studies indicate that it plays an important role in learning, memory and emotion. In the human, some of the most important insight into hippocampal function comes from studies on patient H.M., who suffered persistent anterograde amnesia up until his death in 2008 after complete resection of both his left and right hippocampuses (Squire, 2009). Studies of H.M. revealed a distinct loss of long-term memory abilities as a result of his surgery, but with no corresponding loss of short-term memory capabilities (Corkin, 1984; Milner, Corkin and Teuber, 1968; Wickelgren, 1968). These studies, as well as many more conducted on H.M., gave the first indication that the hippocampus was crucial for the efficient processing of memory, and the conversion of short-term to long-term memory. Based on the data collected from patient H.M., further hippocampal studies have been conducted mainly in rodents in order to better understand the precise functions of the hippocampus in the mammalian CNS. From these studies, it has become clear that the hippocampus plays an important cognitive and emotional role in the CNS.

The hippocampus is involved in three distinct kinds of memory; spatial navigation, pattern separation and episodic memory. Spatial navigation involves the encoding and recall of objects in space and their location relative to the individual. In humans, subjects with injury
to the hippocampus report difficulty in orienting themselves with the world around them and remembering directions, places or objects in space (Bird, Vargha-Khadem and Burgess, 2008; Bohbot, Iaria and Petrides, 2004; Goodrich · Hunsaker et al., 2010; Graham, Barense and Lee, 2010). Rodents with hippocampal damage similarly have difficulty in recalling learned environments (Logue, Paylor and Wehner, 1997). This may be due to a decrease in or dysfunction of specialised “place” cells which fire reliably and selectively when an animal re-encounters a previously learned location (O'Keefe, 1979; O'Keefe and Dostrovsky, 1971). These cells provide compelling evidence that the hippocampus plays a critical role in spatial navigation, although the intricacies of place cells and spatial encoding in the human brain is less understood (Ekstrom et al., 2003; Krabbe et al., 2013).

Pattern separation refers to the process of categorising similar events into distinct and non-overlapping representations. In both humans and rodents, the hippocampal DG and CA3 regions are found to be largely responsible for this process (Bakker et al., 2008; Lacy et al., 2011). Interestingly, the DG connection to the CA3 tends to be quite sparse, suggesting a highly specific interaction between the two structures in providing clear resolution of memories (Amaral, Scharfman and Lavenex, 2007). The high specificity of these connections suggests that a large number of cells are needed to encode new patterns of stimuli; neurogenesis has thus been hypothesised as a major influence in this form of memory.

The hippocampus is also thought to play a role in the formation and recall of episodic memories; memories of autobiographical events and situations. This theory was first considered during studies of patient H.M., who had no recall of episodic events occurring after his surgery (Scoville and Milner, 1957). Anatomical studies have shown both afferent and efferent hippocampal connections to the entorhinal area and perirhinal and
parahippocampal cortices, associated with object and spatial recollection (Davachi, 2006; Pihlajamäki et al., 2004; Stern et al., 1996). These connections indicate that the hippocampus may participate in processing both object and spatial information, resolving it into complete recollections of events. Studies in humans and rodents also indicate that the hippocampus, while potentially responsible for processing episodic memories, likely projects these memories to other regions of the brain for long-term storage (Fortin, Wright and Eichenbaum, 2004; Rempel-Clower et al., 1996; Squire and Alvarez, 1995). This process of information acquisition and processing highlights the importance of the hippocampus in interacting with other brain regions in order to produce fully-formed episodic memories.

The hippocampus also contributes to emotional regulation and mood, although the mechanism by which it does this is less well understood. Lesions to the rodent hippocampus impairs conditioned fear responses and anxiety, suggesting that it plays a role in initiating or modulating these emotional responses (Bannerman et al., 2003; Kjelstrup et al., 2002; Trivedi and Coover, 2004). Somewhat conversely, disorders in humans such as depression, bipolar disorder, post-traumatic stress disorder and schizophrenia are associated with reductions in hippocampal volume (Bonne et al., 2008; Bremner et al., 2000; Cao et al., 2017; Csernansky et al., 2002; Neumeister et al., 2005; Vythilingam et al., 2005; Weiss et al., 2005). The cause of this atrophy is currently unknown, but may be related to reduced neurogenesis, impaired hippocampal metabolism (Hermens et al., 2015) or changes to hippocampal connectivity with other brain regions (Femenia et al., 2012).
1.2.2 Cognitive and affective functions of hippocampal neurogenesis

The cognitive and affective roles of the hippocampus are well-studied in both behavioural and imaging studies. However, it is far less established how neurogenesis may contribute to normal hippocampal function.

Given the clear importance of the hippocampus to cognitive function and memory, many studies have subsequently investigated the contribution of neurogenesis to this process. Of particular interest is the role of neurogenesis in the resolution of distinct memories. Ablation of neurogenesis in rodents via ionising radiation significantly affected pattern separation in several discriminatory memory tasks (Clelland et al., 2009; Creer et al., 2010; Raber et al., 2004; Tronel et al., 2012). In humans, the (presumed) ablation of neurogenesis with chemotherapy leads to cognitive deficits including impaired memory formation, suggesting a similar role of neurogenesis in the human hippocampus (Christie et al., 2012; Monje and Dietrich, 2012). On the other hand, enhancing neurogenesis via exercise, environmental enrichment, learning or genetic modification improves memory function in rodents (Aimone, Deng and Gage, 2011; Creer et al., 2010; Gould et al., 1999; Van Praag, Kempermann and Gage, 2000). While neurogenesis cannot be measured in living patients, it has nevertheless been demonstrated that similar activities also result in improved cognitive performance in humans (Erickson et al., 2011; Maher et al., 2017).

Importantly, alterations in neurogenesis are strongly associated with dementia disorders, and other neurodegenerative disease where dementia is a common symptom. Post-mortem studies in AD and PD (Boekhoorn, Joels and Lucassen, 2006; Gatt et al., 2019; Hoglinger et al., 2004), and mouse models of Huntington’s disease (Kohl et al., 2007; Simpson et al., 2011) all show significant reductions in neurogenesis. Reduced neurogenesis is also
associated with increasing age, which is itself the main risk factor for dementia (Knoth et al., 2010; Mathews et al., 2017; Spalding et al., 2005). These studies collectively suggest a crucial role of neurogenesis in maintaining cognitive health and implicates altered neurogenesis in the development of cognitive decline.

These studies indicate that neurogenesis contributes to memory function, but the mechanisms by which it does so is less understood. The hippocampus can be considered a “hub” of cognitive function; integrating complex series of sensory stimuli, forming distinct memories before sending those memories to other cortical areas for long-term storage (Battaglia et al., 2011). In this sense, new neurons in the hippocampus may contribute to creating new and distinct patterns of neuronal activation to represent each new piece of information encoded into memory. In rodents, learning tasks stimulate individual hippocampal granule cells, which fire in response to familiar sensory cues with high specificity (Quiroga et al., 2005). One study of human hippocampal function similarly demonstrated a specific pattern of neuronal activation upon encountering a repeated visual stimulus (Quiroga et al., 2005). Newly differentiated granule cells, being more excitable and plastic than established granule cells (Mongiat et al., 2009; Schmidt-Hieber, Jonas and Bischofberger, 2004), may therefore be responsible for encoding novel stimuli, integrating them with established neural networks yet remaining highly specific so that new stimuli can be effectively partitioned into distinct memories.

Apart from being involved in the assembly of new memories, it is also believed that hippocampal neurogenesis may play a role in the consolidation of these memories from short-term to long-term memory, as well as the retrieval of stored memories. Studies in rodents demonstrate that the hippocampus maintains a memory for a period of a few weeks.
(Anagnostaras, Maren and Fanselow, 1999), before the short-term memory is converted to a long-term memory in the neocortex (Bonnici et al., 2012; O'Reilly and Rudy, 2000; Wittenberg and Tsien, 2002). In one rodent study, ablation of hippocampal neurogenesis via irradiation extended the period of time that new memories were stored in the hippocampus (Kitamura et al., 2009), indicating that hippocampal neurogenesis contributes to the consolidation of memory and projection of these memories to long-term cortical storage. Similarly, ablation of neurogenesis reduced the ability to recollect both short- and long-term memories, indicating that newborn granule cells may also be involved in the retrieval of remote, stored memories in addition to the consolidation and storage of new ones (Goodman et al., 2010; Trouche et al., 2009).

The role of neurogenesis in affective function is less well-defined than its contributions to memory. It is known from both rodent and human studies that altered neurogenesis is implicated in a range of mood disorders such as depression, post-traumatic stress disorder and anxiety (Yun et al., 2016). Chronic stress and anxiety also negatively regulate stem cell proliferation and survival in rodents (Ferragud et al., 2010; Gould et al., 1992; Pham et al., 2003). On the other hand, antidepressant medications may positively regulate hippocampal neurogenesis in rodents and primates (Malberg et al., 2000; Perera et al., 2007), as well as in human studies of depression (Anacker et al., 2011; Boldrini et al., 2009). This indicates that hippocampal neurogenesis may be linked to mood regulation in the adult brain and can be actively modulated, although this effect is variable depending on the type of antidepressant used (Aimone, Deng and Gage, 2014).
1.2.3 Modulation of hippocampal function and neurogenesis

While there is evidence to suggest that declining hippocampal neurogenesis may contribute to cognitive changes and dementia, it remains uncertain what factors may be driving that change. Many studies in humans and rodents have suggested a range of potential modulators; growth factors and neurotransmitters are examples of neurogenic modulators which have potential translational applications for the regulation of hippocampal neurogenesis.

1.2.3.1 Neurotransmitters

The intrinsic circuitry of the hippocampus is largely glutamatergic, however a variety of extrinsic connections for other brain regions innervate the hippocampus also. Dopamine (DA), noradrenaline, acetylcholine and serotonin (5-HT) are four neurotransmitters which have modulatory effects on hippocampal neurogenesis.

Dopaminergic innervation of the hippocampus originates largely in the ventral tegmental area, with further inputs from the substantia nigra and locus coeruleus (Höglinger et al., 2014; Kempadoo et al., 2016; Scatton et al., 1980). Dopaminergic axons have been shown to associate closely with cells in the SGZ and DG (Hoglinger et al., 2004). As a result, DA appears to have a direct stimulatory effect on neurogenesis in the hippocampus, demonstrated by rodent studies (Salvi et al., 2016; Schlachetzki et al., 2016; Takamura et al., 2014; Winner et al., 2009). In humans, reduced DA levels in the hippocampus is associated with altered neurogenesis and memory dysfunction (Hall et al., 2014; Hoglinger et al., 2004; Kempadoo et al., 2016).

Noradrenergic innervation of the hippocampus originates from the locus coeruleus (Mongeau, Blier and De Montigny, 1997), another degenerating area of the brain in PD which
is also associated with cognitive decline. Depletion of noradrenergic innervation of the hippocampus reduces neurogenesis and impairs cognitive function in the hippocampus (Coradazzi et al., 2016; Hansen, 2017; Masuda et al., 2012). Conversely, activation of noradrenergic receptors in the hippocampus stimulates stem cell proliferation (Jhaveri et al., 2010).

Acetylcholine is an important neurotransmitter for cognitive function, and dysfunctional cholinergic innervation is suspected to contribute to the pathophysiology of AD. Cholinergic innervation of the hippocampus originates from the basal forebrain, which degenerates in both AD and PD (Hall et al., 2014; Whitehouse et al., 1982). Cholinergic denervation is associated with reduced neurogenesis (Kotani et al., 2008; Mohapel et al., 2005), as well as dysfunctional memory formation and retrieval (Tajik et al., 2016).

Lastly, neurons of the raphe nucleus innervate the hippocampus, supplying 5-HT. 5-HT plays a primary role in the affective functions of the hippocampus, regulating mood and emotional memory formation (Mahar et al., 2014; Zhang et al., 2013). It also positively regulates neurogenesis in the hippocampus, as evidenced by the action of serotonergic antidepressants on stem cell proliferation (Alenina and Klempin, 2015; Banasr et al., 2004).

**1.2.3.2 Growth Factors**

Growth factors are currently considered potentially restorative of damaged neurons in neurodegenerative diseases such as PD. In a broad sense, growth factors are secreted molecules which support neuronal function and survival and may be considered neuroprotective or neurorestorative of damaged neurons. Altered growth factors are found in both the PD and AD brain, and may contribute to the patterns of neurodegeneration seen in these diseases (Siegel and Chauhan, 2000). Current clinical investigations of growth factors
suggest that restoring these growth factors may promote cell survival in PD and AD (Nutt et al., 2003; Tuszynski et al., 2005; Whone et al., 2019), although currently the effectiveness of these treatment strategies is under debate (Sherer et al., 2006).

Growth factors such as brain-derived neurotrophic factor, glial cell-line derived neurotrophic factor (GDNF) and fibroblast growth factor 2 modulate hippocampal memory function in rodent models of cognitive decline (Bekinschtein et al., 2008; Kiyota et al., 2011; Revilla et al., 2014; Yoshimura et al., 2001; Zhang et al., 2014). In addition, these growth factors stimulate stem cell proliferation in the hippocampus (Chen et al., 2005; Rossi et al., 2006; Scharfman et al., 2005). Given that the safety of growth factor treatments, particularly GDNF, has been established in clinical trials in PD patients (Gill et al., 2003; Slevin et al., 2005), it may therefore be useful to consider them as potential treatments to restore hippocampal function and neurogenesis in cases of cognitive decline.

This chapter provides only a basic overview of the main neurotransmitters and growth factors that may play a role in the effective function of the hippocampus and hippocampal neurogenesis. Studies characterising these molecules, their distribution and their integrated effects on the hippocampal microenvironment would be beneficial for not only understanding hippocampal function in age and disease, but treatment strategies for cognitive decline and dementia.

1.3 Structural and functional differences along the length of the hippocampus

It is clear from existing animal studies that the hippocampus contributes to a range of tasks involving memory and mood. This wide range of hippocampal functions in animals suggest that there may be a logical organisation the hippocampus, with specific areas controlling different functions. One area of research describes these functional differences in
the hippocampus and hypothesise that the hippocampus is both structurally and functionally heterogeneous along its length.

1.3.1 Definition of the hippocampal longitudinal axis

The definition of the longitudinal axis is somewhat difficult when comparing the human and non-human hippocampus, largely due to macroscopic differences in the position of the hippocampus within the brain. The human hippocampus (Fig. 1.2) is located within the medial temporal lobe, sometimes considered an elaboration at the end of the cerebral cortex. On the other hand, the non-primate hippocampus, particularly the rodent hippocampus, is heavily curved with its ends instead positioned dorso-ventrally. With this in mind, the rodent dorsal hippocampus is equivalent to the human posterior hippocampus, and the rodent ventral hippocampus to the human anterior hippocampus.
Figure 1.2 Differences in basic hippocampal anatomy between rodents and humans.

The rodent hippocampus (A; hippocampus in red) is highly curved, with the ventral and dorsal poles being approximately the same size. On the other hand, the human hippocampus (B; hippocampus in red) is oriented along the anterior-posterior axis of the brain, with a large anterior pole and more slender tail. The length of the hippocampus is reported to be structurally and functionally heterogeneous in both rodents and humans. Adapted from Strange et al. (2014).
1.3.2 Structural dynamics along the length of the hippocampus

Anatomically the hippocampus consists of the DG and CA, with each layer of cells folded upon the other to create the distinctive cellular organisation found in all mammalian species. However, while the basic components of the hippocampus are consistent throughout, its overall structure and connectivity differs significantly along its length. First and foremost, the human and non-human primate hippocampus in particular is differentiated from other species by its shape. Other mammalian species have dorsal and ventral poles that are roughly equivalent in size, contrasting to the primates’ large anterior and tapered posterior poles (Fig. 1.2). Interestingly, the rodent and primate dorsal hippocampus contains a larger proportion of DG granule cells than in the ventral hippocampus (Amrein et al., 2015; Bekiari et al., 2015), a pattern that remains in the human posterior hippocampus regardless of its smaller overall volume compared to the anterior pole (Malykhin et al., 2010). This may be due to the increased proportion of place cells crucial for spatial memory and navigation in the dorsal hippocampus (Jung, Wiener and McNaughton, 1994), however this has yet to be confirmed in a human study.

In addition to the gross anatomical differences between the anterior and posterior hippocampus, the structural connectivity of the hippocampus differs along its longitudinal axis. The major input into the hippocampal circuitry originates in the entorhinal cortex; these afferent fibres are topographically organised in the rodent, with the medial region of the entorhinal cortex projecting to the ventral hippocampus while the lateral region projects to the dorsal hippocampus (van Groen, Kadish and Wyss, 2002). Other brain regions innervating the hippocampus such as the ventral tegmental area (VTA) innervate the entire structure, but with greater projection to the ventral than the dorsal hippocampus in rodents (Kempadoo et al., 2016; Rocchetti et al., 2015). Conversely, catecholaminergic fibres of the locus coeruleus
and cholinergic fibres of the septal nuclei project predominantly to the dorsal hippocampus (Amaral and Kurz, 1985; Kempadoo et al., 2016). Intrinsic hippocampal pathways in CA3 and DG mossy cells also tend to be largely divided; while these cell types are highly connected within the hippocampus this connectivity is largely between the ventral third and dorsal two thirds of the rodent hippocampus, with very little overlap between these two regions (Strange et al., 2014). Lastly, analyses of gene expression also demonstrate clear genotypic subdivisions of the rodent hippocampus along its length (Cembrowski et al., 2016; Dong et al., 2009).

1.3.3 Functional dynamics along the length of the hippocampus

Apart from the differences in the structure and connectivity of the hippocampal long axis, there are many studies which suggest that the function of the hippocampus is specialised along its length. A seminal study by Moser et al. (1993) demonstrated this, showing that lesions of the dorsal hippocampus impaired spatial navigation in the rodent whereas lesions of the ventral hippocampus showed no significant effects on navigation. The impairments were noted when as little as 25% of the dorsal half of the hippocampus was lesioned, providing a clear indication the functional role of the dorsal hippocampus in visuospatial cognition and memory. Human functional magnetic resonance imaging (fMRI) studies similarly suggest a role for the posterior hippocampus in spatial memory (Nadel, Hoscheidt and Ryan, 2013). Taxi drivers, who are required to memorise complex street patterns, have larger grey matter volume in the posterior hippocampus than either bus drivers or normal controls, neither of whom require the same degree of detailed spatial memory (Maguire, Woollett and Spiers, 2006; Woollett and Maguire, 2011). However, it has also been suggested that spatial memory is not completely hippocampus-dependent, but rather it is crucial for resolving fine details of spatial maps and the learning of new visuospatial information.
Similarly, several human imaging studies demonstrated the encoding of memories in humans preferentially activates the posterior hippocampus (Fernández et al., 1998; Stern et al., 1996).

In contrast to studies of the dorsal hippocampus, ventral hippocampal lesions in rodents have been linked to decreased anxiety and depression-like symptoms, suggesting a role of the ventral hippocampus in modulation of affective states (Bagot et al., 2015; Bannerman et al., 2003; Kheirbek et al., 2013; Trivedi and Coover, 2004). These findings are further reinforced by the known reciprocal connectivity between the amygdala and ventral hippocampus in rodents (Kishi et al., 2006; Risold and Swanson, 1996) and primates (Thierry et al., 2000), which are associated with mechanisms modulating fear, anxiety and social behaviours (Felix-Ortiz and Tye, 2014; McHugh et al., 2004; Zarrindast et al., 2008).

In human imaging studies, volumetric changes in the anterior hippocampus and amygdala are associated with anxiety, post-traumatic stress disorder and schizophrenia (Csernansky et al., 2002; Oler et al., 2010; Vythilingam et al., 2005). DG cells are also significantly reduced in the anterior, but not posterior, hippocampus in major depression and recovered by antidepressant treatment (Boldrini et al., 2013). Additionally, the total volume of the anterior hippocampus is selectively increased with electroconvulsive therapy for the treatment of depression (Joshi et al., 2016). Functional imaging studies in humans also demonstrate a cognitive role for the anterior hippocampus, specifically in encoding emotional memories, as opposed to the posterior hippocampus which encodes more neutral or factual memories (Dolcos, LaBar and Cabeza, 2004; Murty et al., 2010).

Studies in both animals and humans generally suggest a role of the anterior/ventral hippocampus in emotional regulation and memory encoding, while the dorsal hippocampus
plays a more important role in the more neutral spatial and episodic memory encoding (Strange et al., 2014). Neurogenesis persists along the length of the hippocampus; however, these findings suggest that the dynamics of neurogenesis may vary along this axis in response to the different functional roles along the length of the hippocampus.

1.3.4 Neurogenesis along the length of the hippocampus

While there are no studies quantifying adult neurogenesis along the length of the human hippocampus, neurogenesis has been quantified along the hippocampal longitudinal axis of several different species including the mouse (Jinno, 2011), rat (Bekiari et al., 2015; Snyder et al., 2009), canine (Ballesteros et al., 2015) and marmoset (Amrein et al., 2015). These studies found no difference in numbers of stem cells (Ballesteros et al., 2015) or proliferating cells in the hippocampus, whereas there was consistently higher expression of immature neuronal markers in the dorsal hippocampus of all of these species examined. (Amrein et al., 2015; Bekiari et al., 2015; Jinno, 2011; Snyder et al., 2009) These findings suggest that while the rate of stem cell proliferation is consistent throughout the hippocampus, the ultimate difference in neurogenesis along the length of the hippocampus a result of regional variations affecting cell survival and maturation (Piatti et al., 2011; Snyder and Cameron, 2012). It is currently unknown if the same neurogenesis “gradient” exists in the human hippocampus, however given the previously discussed differences in dentate gyrus anatomy (Malykhin et al., 2010) it is possible that neurogenesis also persists at a greater rate in the posterior human hippocampus.

1.3.5 Potential significance to dementia development and progression

Studies in animal models have shown convincing evidence to suggest that the hippocampus is both structurally and functionally heterogeneous, and that neurogenesis is
similarly organised in a topographically distinct manner. Recent data from human studies have also demonstrated that the structural and functional organisation of the human hippocampus may largely follow the same patterns as other species. Given this, neurogenesis along the length of the human hippocampus may also follow the established pattern seen in animal models, although no human study has been performed to confirm this hypothesis. Given that alterations in neurogenesis are significantly linked to cognitive change, it is prudent to establish firstly if these regional patterns of neurogenesis persist in the human hippocampus, and if alterations are region-specific and potentially contributory to dementia risk in ageing and disease.

1.4 Parkinson’s disease

Parkinson’s disease is the second most common neurodegenerative disease and is expected to affect more than 10 million people worldwide by 2030 (Dorsey et al., 2007). In Australia, PD affects approximately 1 in 340 individuals and represents a significant public health cost; in 2017 the total economic cost of PD was in excess of AUD 14 billion (Brown, Hasnata and La, 2017).

PD was first described in 1817 and characterised by the motor symptoms such as shuffling gait, tremor at rest, muscle rigidity and slowness of movement (Chiaravalloti et al., 2014). While the motor impairments that are ubiquitous in PD are the classical representation of the disease, a range of non-motor symptoms such as depression, mild cognitive impairment, sleep disturbances, hallucinations and dementia are also common (Chaudhuri, Healy and Schapira, 2006). Recent studies have indicated that these non-motor symptoms are among the leading factors in nursing home admission and the loss of quality of life amongst sufferers (Leroi et al., 2012; Schipper et al., 2014; Schrag, Jahanshahi and Quinn, 2000),
particularly since mainstream drug treatments for PD are aimed at alleviating motor, rather than non-motor, symptoms (Connolly and Lang, 2014).

1.4.1 Aetiology of Parkinson’s disease

The aetiology of PD remains unknown, and the vast majority of PD cases are considered idiopathic at this time. It is believed that a combination of both genetic and environmental factors contribute towards the development of PD (Wirdefeldt et al., 2011), although around 10% of patients report a positive family history of the disease, suggesting a stronger genetic link in some cases (Klein and Westenberger, 2012). Several gene loci, collectively called PARK, have been identified in human PD cases and are thought to influence the development of PD (Klein and Westenberger, 2012). On the other hand, environmental factors are also considered to play a significant role in the development of PD. Substances such as pesticides, solvents and some metals have been investigated as potential environmental triggers of PD (Bonaguidi et al., 2011; Goldman, 2014; Pezzoli and Cereda, 2013), although there has been no conclusive proof that any of these factors represent a causative agent of PD (Ballesteros et al., 2015).

1.4.2 Pathophysiology of Parkinson’s disease

In the 200 years since PD was first described, great lengths in research have gone towards describing its pathophysiology, in the hopes that effective treatments can be created to alleviate the burden of disease. Regardless of this, the pathophysiology of PD is yet to be fully characterised, and significant hurdles towards our understanding of PD still remain.

1.4.2.1 Dopaminergic cell loss in the substantia nigra

At its core, PD affects the dopaminergic neurons of the substantia nigra (SN), particularly the pars compacta subsection of the SN (SNC). The neuromelanin-rich,
dopaminergic neurons of the SNc project to the striatum, especially the motor centres of the putamen to form the nigrostriatal pathway, one of four major dopaminergic pathways of the CNS (Fuxe et al., 2006). The nigrostriatal pathway in turn is involved in the initiation and control of motor function in the brain (Deumens, Blokland and Prickaerts, 2002; Jeyasingham et al., 2001).

In PD the progressive death of dopaminergic neurons within the SNc leads to the ongoing degeneration and dysfunction of the nigrostriatal pathway (Damier et al., 1999; Gibb and Lees, 1991). DA levels in the striatum are significantly reduced, and the loss of neuronal communication between the two regions subsequently impairs the brain’s ability to produce smooth, purposeful movement. Clinically, this manifests as the severe and gradually worsening motor symptoms characteristic of PD. Importantly, it has been shown that the clinical symptoms of PD do not manifest until approximately 60% of the dopaminergic neurons of the SNc have been lost (Dauer and Przedborski, 2003). This represents a challenge in the treatment of PD, as a significant portion of the neuronal damage has occurred before the patient experiences any of the motor symptoms on which a diagnosis is made. DA levels in the striatum have been shown to be reduced before widespread neuronal loss in the SNc, indicating that cell death may be preceded by “dying off” of axons projecting from the SNc to the striatum long before clinical symptoms or neuropathological evidence of cell death (Gustafsson, Nordström and Nordström, 2015; Kordower et al., 2013). This process is also evident in incidental Lewy body disease, considered a prodromal form of PD (DelleDonne et al., 2008). Axonal degeneration preceding cell death may also explain the emergence of non-motor affective symptoms such as depression before the onset of motor symptoms, with human hippocampal studies demonstrating reduced levels of DA in the PD hippocampus.
(Scatton et al., 1983), as well as reduced numbers of dopaminergic axons innervating the hippocampus (Hoglinger et al., 2004).

1.4.2.2 Other areas of neuronal loss in PD

While dopaminergic cell loss is the most defining factor of PD pathophysiology, other brain regions also experience disease-related neuronal loss. Profound cellular loss of catecholaminergic neurons of the locus coeruleus is found in both demented and non-demented PD, as well as AD (Haglund et al., 2016; Zarow et al., 2003). The locus coeruleus provides the main source of noradrenergic innervation to many other brain regions and is implicated in the modulation of functions such as attention (Aston-Jones, Rajkowski and Cohen, 1999), emotion (Aston-Jones et al., 1996) and cognition (Mather and Harley, 2016). Rodent studies have demonstrated noradrenergic input to the hippocampus (Haring and Davis, 1985), and subsequently associated this with hippocampus-based memory (Kempadoo et al., 2016; Khakpour-Taleghani et al., 2009) as well as attention and motivation (Sara, 2009). Loss of these neurons in neurodegenerative diseases such as PD may therefore contribute significantly to the development of common non-motor symptoms such as depression, apathy and memory deficit.

The serotonergic raphe nuclei have also been found to experience cellular loss in PD (Halliday et al., 1990). As the major source of cerebral serotonin, projections from the raphe nuclei are believed to play a significant role in the regulation of mood and emotion (Baumann et al., 2002). The raphe nuclei project extensively to the hippocampus and amygdala in rodent models (Moore and Halaris, 1975), and dysfunctional neurotransmission to these regions is considered a major driving force in the development of affective disorders such as depression and anxiety (Mahar et al., 2014). Reduction of serotonergic cells and their projections in PD may therefore be reflected in the development of depression, which is amongst the most
common non-motor symptoms of PD. Given that the onset of depression in PD often precedes motor symptoms (Postuma et al., 2012), this may suggest that neuronal loss in the raphe nuclei and/or locus coeruleus may occur before dopaminergic cell death in the SNc. Post-mortem human studies have alternatively suggested that locus coeruleus neuronal loss is more extensive than in the SNc (Zarow et al., 2003), suggesting earlier or more acute neuropathology may underlie some premotor PD symptoms.

Lastly, the cholinergic basal forebrain is strongly associated with memory and cognition, and a major region of neuronal loss in AD. Similarly, loss of cholinergic neurons from the basal forebrain has been reported in post-mortem PD brains, particularly those with dementia. Basal forebrain lesion results in memory deficit in multiple species, as well as reduced innervation of the hippocampus. These studies are significant as they suggest that even without the severe hippocampal neuronal loss associated with AD, cholinergic input into the hippocampus has an important role in maintaining cognitive ability and may therefore be influential in the development of cognitive decline in PD and other neurodegenerative diseases.

It is important to note that all of the regions experiencing disease-related neuronal loss not only innervate the hippocampus, but their specific neurotransmitters have demonstrated a role in modulating hippocampal neurogenesis, as previously described in 1.2.3. This therefore suggests that denervation of the hippocampus in PD and other neurodegenerative diseases such as AD alter hippocampal neurogenesis, which in turn precipitates cognitive and emotional change. However, the dynamics of human hippocampal
innervation need to be characterised more fully to better understand how these varied brain regions contribute to neurogenesis and overall hippocampal function.

1.4.2.3 Protein aggregation and Lewy body pathology

Proteinopathy in PD has also been implicated as a factor in SNc cell death, as well as other aspects of PD pathology. Lewy bodies (LBs) are large, intracellular aggregates which form throughout many areas of the brain over the course of PD progression. LBs consist of a dense proteinaceous core surrounded by a halo of radiating protein fibrils, the major component of which is the protein α-synuclein (Kordower et al., 2013; Spillantini et al., 1997). The gene coding for α-synuclein, SNCA, has been subsequently implicated in autosomal dominant forms of PD (Nishioka et al., 2006; Singleton et al., 2003). It has been suggested that LB formation may be an indication of proteasomic dysfunction in the cells, with misfolded proteins aggregating as a result of proteasomes failing to clear them (Dennissen, Kholod and van Leeuwen, 2012; McNaught et al., 2001). It is also possible that LBs exert a toxic effect on the cells in which they accumulate (Power, Barnes and Chegini, 2015).

PD pathophysiology is multi-faceted and complex, likely involving a range of converging pathways and mechanisms. Better characterising the disease, particularly in humans, would be beneficial for developing new approaches for the treatment or curature of PD.

1.4.3 Parkinson’s disease with dementia

PD does not only encompass motor dysfunction; there are a host of non-motor symptoms which challenge the understanding and treatment of the disease. Of these, cognitive decline is a prominent and particularly destructive component of PD. Often mild cognitive impairment (MCI) is a feature of PD but generally does not significantly impact on
day-to-day life for the PD patient (Walker et al., 2015). Unfortunately, MCI in PD frequently leads to more severe cognitive deficits throughout the course of the disease, ultimately resulting in the onset of dementia with increasing age and duration of disease. Parkinson’s disease dementia (PDD) occurs in around 80% of PD patients by 20 years post-diagnosis (Hely et al., 2008), and the risk of developing PDD rises to 50% by ten years post-diagnosis (Williams-Gray et al., 2013).

The diagnosis of PDD is dependent on several criteria. First and foremost, the development of dementia must be within the context of previously diagnosed PD (Emre et al., 2007). This differentiates PDD from Lewy body dementia, in which the onset of dementia occurs prior to or concurrently with Parkinsonism (McKeith et al., 1996). Differentiating between these two is important in the ongoing treatment of both diseases, although there remains considerable confusion about the diagnostic criteria of PD, Lewy body dementia and AD which requires greater focus in clinical studies (Walker et al., 2015).

Apart from the timing of dementia onset, the degree of cognitive impairment in PDD needs to be firmly established before a diagnosis is made. Most importantly, PDD is differentiated from MCI in its ability to affect daily life for sufferers (Litvan et al., 2012). The common cognitive functions that are tested when diagnosing PDD and MCI include executive functioning, attention, free recall and visuospatial reasoning (Emre et al., 2007; Litvan et al., 2012). For a diagnosis of PDD, at least two of these cognitive functions must be affected to the point where it impairs the ability of the patient to carry out normal daily life (Emre et al., 2007).

Ultimately, PDD has a severe effect on patient quality of life, and has been cited in multiple studies as one of the most debilitating symptoms of the disease for both sufferers
and their families and caretakers (Leroy et al., 2012; Schipper et al., 2014; Schrag, Jahanshahi and Quinn, 2000). It is also a major contributing factor to nursing home admission amongst PD patients (Aarsland et al., 2000; Hobson and Meara, 2004). In 2014, $567.7 million was spent on the medical care of PD patients in Australia, of which 48% percent went towards residential aged care costs (Deloitte Access Economics, 2015). Therefore, roughly $272.5 million contributed to the care of PD patients who, with effective drug therapies specifically targeting PDD, may have been able to lead a more independent lifestyle without the need for full-time care. Clearly this impacts not only on the patient, but the healthcare system and society as a whole.

The difficulty in addressing PDD is that there has been comparatively little study of the cause and effects of cognitive decline in PD. Dementia in PD is still considered a secondary symptom of the disease, and it remains unclear what impact dopaminergic cell loss in the SNc has on the development of dementia. However, given that the majority of PD patients will develop dementia by 20 years post-diagnosis, it seems prudent that the underlying features of PDD should be better investigated in future.

1.5 Alterations in adult hippocampal neurogenesis in ageing and PD

Since the discovery of neurogenesis in the adult human brain, research in this field has focused on determining the properties of the process in both health and disease. A varied number of diseases and disorders have been studied in the context of human hippocampal neurogenesis, including neurodegenerative disorders and healthy ageing. Stem cell transplant therapies, while remaining a promising avenue for treatment of the dysfunctions associated with ageing and neurodegenerative disease, have thus far failed to be a silver bullet of brain repair. It is crucial therefore to investigate not only how our own endogenous stem cells react
in health and disease, but how these reactions might be manipulated, modified or exploited in order to produce novel treatments.

1.5.1 Ageing

The effect of normal ageing on hippocampal neurogenesis is an important yet understudied area of research. Without an understanding of how neurogenesis changes with age, there is no benchmark to determine what represents a normal or pathogenic change to neurogenesis in disease. Investigations of hippocampal neurogenesis and function generally utilise human post-mortem and rodent tissue, although advances in functional imaging allow a better understanding of the functional context in which hippocampal neurogenesis takes place during normal ageing.

It is well-established that age is the leading risk factor for cognitive decline (Bishop, Lu and Yankner, 2010). This has been noted in a range of both human and rodent studies showing a direct correlation between advancing age and memory and cognitive deficits (Bishop, Lu and Yankner, 2010; Salthouse, 2009; Schroeder and Salthouse, 2004). Structural MRI of aged patients show a marked decrease in hippocampal volume with age (Driscoll et al., 2009; Knoops et al., 2012). Declines in hippocampal blood flow and DG function have also been correlated with old age in human subjects (Ajmani et al., 2000; Graham, Barense and Lee, 2010; Kennedy et al., 2009; O'brien et al., 2010; Petersen et al., 2000; Raz et al., 2005; Toner et al., 2009), although an increase in hippocampal CA3 and DG function with age has also been shown in one study, suggesting possible links between hippocampal hyperactivity and functional deficit (Lacy et al., 2011). Post-mortem human studies have likewise confirmed a decrease in hippocampal volume and cell number correlated with age (Bobinski et al., 1999; Kaye et al., 1997). Conversely, increased hippocampal volume has been shown to correlate
with improved memory function, indicating that the decline in hippocampal volume in age may be associated to changes in cognitive function (Makizako et al., 2015; Mielke et al., 2012; Pohlack et al., 2014). Links between hippocampal atrophy, declining neurogenesis and cognitive change have not been proven, although several studies suggest that they may be strongly related. For example, exercise has been shown in multiple studies to increase hippocampal volume, neurogenesis and memory function in both rodents and humans, lending credence to the hypothesis that volumetric changes to the hippocampus in age may be reflective of both decreased neurogenesis and declining cognition (Erickson et al., 2011; Kohman et al., 2012; Makizako et al., 2015; Van Praag et al., 2005; Vukovic et al., 2012). On the other hand volume loss is not confined to the hippocampus, and the loss of both grey and white matter in the aged brain has been well-examined in human MRI studies (Driscoll et al., 2009; Good et al., 2002; Kennedy et al., 2009; Scahill et al., 2003). Since there is no method of imaging neurogenesis changes in the living brain however, the precise relationship between hippocampal volume and cognition has yet to be fully described in living patients. Further developments in the field of brain imaging will hopefully help to elucidate the functional relationship between hippocampal volume, neurogenesis and cognitive decline.

The inability to fully reconcile these factors in living patients means that the majority of investigations of neurogenesis and cognition are confined to studies of post-mortem brain tissue. Human, rodent and primate studies have been utilised for this purpose, and have consistently shown across species that the proliferation, differentiation and maturation of new neurons in the hippocampus is decreased with advancing age (Aizawa et al., 2011; Jinno, 2011; Knoth et al., 2010; Kuhn, Dickinson-Anson and Gage, 1996; Spalding et al., 2013; West, 1993). This, in turn, has been linked to decreases in learning and memory in aged rodents (Drapeau et al., 2003; Dupret et al., 2007; Dupret et al., 2008; Van Praag et al., 2005), further
lending credence to the idea that changes to neurogenesis in ageing may be a significant factor impacting cognitive ability. However, alternative conclusions by Bizon et al. (2003) and (2004) found that a reduction in hippocampal proliferation is not correlated with declining spatial learning ability in middle-aged or aged rats, but did not investigate changes to the differentiation and maturation of the proliferating cells. This may suggest that declining proliferation in the hippocampus is not the major cause of cognitive decline, but rather the failure of these cells to mature into functional neurons. Alternatively, hippocampal neurogenesis may influence some but not all aspects of hippocampus-based cognition, hypothesised previously by Shors et al (2002). These conflicting statements highlights the difficulty in interpreting the effect of neurogenic changes on cognition in old age, and represents an area in which further studies, especially human studies, are warranted.

The fact that adult neurogenesis occurs in the mammalian brain has been well-established, although rodent studies have shown that the rate of adult neurogenesis is significantly lower than neurogenesis occurring in infancy and early childhood (Akers et al., 2014; Ben Abdallah et al., 2010). Furthermore, the decline in human neurogenesis begins in early adulthood (Ben Abdallah et al., 2010; Knoth et al., 2010), while rates of dementia and other cognitive deficiencies do not increase rapidly until around the age of 65 (Lobo et al., 1999) (Australian Institute of Health and Welfare, 2012) and do not necessarily progress at the same rate amongst otherwise healthy, aged adults (Buter et al., 2008; Mortimer et al., 1992). This is important, as it suggests that changes to neurogenesis are not the sole cause of cognitive decline in age but may be just one of a host of factors which influence cognition and hippocampal function in old age. The ability to monitor changes to neurogenesis in humans over time is critical to our understanding of how these processes work, and the development
of suitable methods to investigate neurogenic changes in the hippocampus are required to further elucidate these ideas.

In the hippocampus, alterations in stem cell fate have been hypothesised to contribute towards decreased new neurons in the aged brain. Studies by Encinas et al. (2011) have suggested that stem cells within the DG have a finite ability to proliferate, eventually terminally differentiating into astrocytes and gradually depleting stem cell numbers over time. The idea of terminal astrocytic differentiation has been supported by other studies, although a consensus on the pathways leading to astrocytic differentiation is yet to be found (Bonaguidi et al., 2011; Lugert and Taylor, 2011). However, this hypothesis has not been confirmed in human studies, as methods to effectively trace stem cell fate in living patients have not yet been established. Although astrocyte numbers are reported to be unchanged in the human brain (Fabricius, Jacobsen and Pakkenberg, 2013; Pelvig et al., 2008), these studies have not included the hippocampus in their methodologies. To further elucidate on the idea of terminal astrocytic differentiation, studies of hippocampal gliogenesis need to be completed in human tissues, in order to better characterise the hippocampal microenvironment in old age.

It is accepted that age is the number one risk factor for the development of dementia, however it is still largely unknown how and why the brain degenerates in old age. The hippocampus, a hub for cognitive processes in the brain, has been shown to be impaired in multiple ways during normal ageing. Changes to neurogenesis in the hippocampus has been implicated in cognitive decline, although there is still little information on what factors may contribute to this change as we age. By further investigating the hippocampal microenvironment in age, a more complete picture of hippocampal function can be resolved,
allowing us greater insight into memory and cognition in both normal physiological ageing and disease.

1.5.2 Parkinson’s disease

The effect of PD on hippocampal neurogenesis has only recently come to prominence in the literature. In particular, the existing links between neurogenesis and cognitive ability have led to a raft of new studies investigating the effect of age, Alzheimer’s disease and other dementias on levels of hippocampal neurogenesis. Recently these studies have been extending to PD which, as previously described, often involves the development of PDD.

On a macroscopic level, the hippocampus is one of the brain areas that is most severely affected in PD. Human imaging and stereological analyses of PD hippocampuses have found a significant decrease in the volume of the PD hippocampus when compared to age-matched controls; most of these studies have shown greater atrophy in PDD patients than PD and controls (Bouchard et al., 2008; Camicioli et al., 2003; Kandiah et al., 2014), albeit with some exceptions (Joelving et al., 2006). Moreover, it has been shown that these changes can be a predictor of dementia in PD, potentially leading to better screening of patients for indicators of cognitive decline (Kandiah et al., 2014). While hippocampal volume loss is a definite feature of normal ageing, the elevated level of atrophy in the PD, and particularly the PDD brain may be indicative of hippocampal involvement in the development and advancement of dementia in PD.

The question that remains to be asked is how changes to the hippocampus may precipitate cognitive decline and dementia in PD. Given that the hippocampus is one of the few brain regions that continues to produce new neurons throughout life, it is thought that changes to hippocampal neurogenesis may have an impact on cognition in PD. This is further
supported by the known decline in hippocampal neurogenesis during normal ageing, as well as the links between hippocampal neurogenesis and memory, cognition and spatial awareness. It has been shown in several studies of both post-mortem human and rodent tissue that hippocampal neurogenesis is reduced as a result of dopamine depletion, which may be in part due to the degeneration of dopaminergic inputs to the hippocampus from the SNc (Gasbarri, Sulli and Packard, 1997; Hoglinger et al., 2004; Suzuki et al., 2010). This is also true of the human, primate and rodent SVZ, which is similarly innervated by the SNc and experiences reduced neurogenesis in PD (Baker, Baker and Hagg, 2004; Freundlieb et al., 2006; Hoglinger et al., 2004; Winner et al., 2009; Winner et al., 2006). Impaired olfaction is a common symptom of PD (Casjens et al., 2013), and since SVZ stem cells supply the olfactory bulb it may be possible that changes to neurogenesis in this region contribute towards this well-defined functional deficit in PD. Likewise, it may be possible that the specific functional deficit resulting from a similar decrease in hippocampal neurogenesis may relate to changes in memory and cognition noted in PD. This is suggested by Höglinger et al. who showed that stem cell proliferation in the human post-mortem hippocampus was more severely reduced in PDD than both PD and controls (Hoglinger et al., 2004). However, as the hippocampus has been consistently understudied in both PD- and neurogenesis-focused studies compared to the SVZ, the biological links between PD, hippocampal neurogenesis and cognitive decline require further definition and study.

DA depletion in the PD brain is one of the major pathological features of the disease and may have some influence over stem cell proliferation and differentiation in the hippocampus. The second major pathological feature of PD, Lewy bodies, may also have a similar influence over hippocampal neurogenesis and, in turn, cognitive decline and dementia in PD. Studies of both mouse and human embryonic stem cells have shown that aberrant
expression of α-synuclein impairs the proliferation and survival of stem cells (Crews et al., 2008; Schneider et al., 2007; Yamashita et al., 2006). Importantly, it has also been shown that this decrease in neurogenesis may be the result of α-synuclein acting in a detrimental manner on the expression of Notch1 signalling (Crews et al., 2008; Desplats et al., 2012), which is known to be involved in cell proliferation and survival signalling in the neurogenic regions of the post-natal hippocampus (Breunig et al., 2007). The effects of α-synuclein overexpression on neurogenesis has also been tested in vitro in adult rat hippocampal cells (Desplats et al., 2012), as well as in vivo in mouse hippocampuses and SVZ overexpressing human wild type α-synuclein (Crews et al., 2008; Desplats et al., 2012; Winner et al., 2004). All of these studies have suggested that the presence of aberrantly expressed α-synuclein results in changes to neurogenesis, both in the hippocampus and other neurogenic regions of the brain. These conclusions are of value to PD research as they indicate that LB pathology may precipitate changes to hippocampal neurogenesis, and thus contribute towards cognitive decline and dementia. However, these studies are also of greater importance to our understanding of familial PD, where specific alterations to SNCA have been previously determined. In sporadic PD, similar gene mutations are yet to be discovered, indicating that overexpression and/or aberrant expression of SNCA may or may not play a role in both LB formation and changes to neurogenesis. Further studies of human sporadic PD cases is thus clearly warranted, in order to determine the exact impact of α-synuclein, LBs and protein aggregation on the proliferation and maturation of stem cells, particularly in the hippocampus.

1.6 Current controversy in the study of human hippocampal neurogenesis

The study of neurogenesis in the human hippocampus has been hindered by the lack of appropriate techniques with which to quantify neurogenesis. To date, most studies of human hippocampal neurogenesis involve using immunohistochemical techniques to identify
and quantify new neurons (Boekhoorn, Joels and Lucassen, 2006; Eriksson et al., 1998; Knoth et al., 2010). Despite these significant studies quantifying neurogenesis, as well as others which utilise different techniques such as carbon dating (Spalding et al., 2005), dissenting views still remain within the literature. Dennis et al. (2016) quantified hippocampal neurogenesis across the human lifespan, concluding that while previous estimates of the rate of human neurogenesis were correct, these numbers were too low to have a substantial effect on hippocampus-based learning and memory. An immunohistochemical study by Sorrells et al. (2018) claimed that no hippocampal neurogenesis could be reliably detected in humans past adolescence, a view that has been since contested (Kempermann et al., 2018; Snyder, 2018; Tobin et al., 2019). Lastly, and conversely, Boldrini et al. (2018) concluded that human hippocampal neurogenesis not only existed but that it did not decline in ageing, contrary to alternate studies showing a clear age-related decline in neurogenesis (Knoth et al., 2010; Spalding et al., 2013; Moreno-Jinenez at al., 2019). These findings are in stark contrast to those in rodent models, which demonstrate a clear presence of adult hippocampal neurogenesis, as well as strong links between neurogenesis and hippocampal function.

Ultimately, these controversial studies challenge not only the current understanding that neurogenesis occurs in the human hippocampus, but also its functional relevance and how to best go about investigating it. In particular, although animal models remain a valuable tool in studying brain function, these studies highlight that the process of neurogenesis may proceed quite differently in humans and contribute in different ways to overall brain function. Future studies should begin to explore these areas in order to best understand how hippocampal neurogenesis contributes specifically to human brain function, and critically assess the usefulness of animal models for the study of human neurogenesis in ageing and disease.
1.7 Hypothesis and aims

PD is a devastating disease, not only due to motor symptoms but also to the cognitive deficits which lead to the highly debilitating PDD. Although there have been few studies, available evidence suggests that hippocampal neurogenesis may play an important role in maintaining cognitive ability. Likewise, studies have also suggested that hippocampal neurogenesis declines in both normal ageing as well as in PD. If this is the case, what factors exist in both ageing and PD which precipitate this decline? PD is characterised by a range of degenerative and dysfunctional cellular mechanisms, although the effect of these mechanisms on hippocampal neurogenesis has been little explored in human tissue and remains controversial in the literature.

This thesis investigates the changes to hippocampal neurogenesis over the course of both normal human ageing and in PDD, focusing on whether changes to neurogenesis are associated with either global or region-specific alterations in the endogenous microenvironment of the hippocampus, specifically in neurotransmitter levels within the hippocampus.

I hypothesise that the expression of genes associated with neurogenesis, and thus neurogenesis itself, will be decreased in both the aged and PDD hippocampus and will be regionally-distributed along the length of the hippocampus in agreement with previous findings in animals. I also hypothesise that the regional variability in neurogenesis along the length of the hippocampus will be differentially affected in PDD cases, with a preferential loss of neurons in the posterior hippocampus. These changes may be due to regional variability on modulatory factors such as neurotransmitters and growth factors.
Thus, the aims for this thesis are as follows:

1. To quantify neurogenesis gene expression in the hippocampus across the healthy adult human life span.
2. To quantify neurogenesis gene expression along the length of the healthy young, healthy aged and PDD hippocampus.
3. To quantify neurotransmitter levels along the length of the healthy young, healthy aged and PDD hippocampus.
4. To quantify growth factor levels in the hippocampus of PDD cases and age-matched controls.

1.8 Significance of study

This thesis represents the first study to quantify patterns of neurogenesis gene expression across the healthy ageing brain and PDD hippocampus. Similarly, it is the first study to investigate how these genes are distributed along the length of the hippocampus and how this is affected in healthy ageing and dementia. Given the role of hippocampal neurogenesis in memory and cognition, this knowledge may contribute to a better understanding of how dementia may develop in the PD brain, and what factors influence its high incidence in PD patients. This will ultimately contribute to a greater understanding of PD pathology and thus a better ability to create curative, rather than symptomatic, drugs for treating PD.

1.9 Conclusions

PD remains a highly debilitating disease, not only due to the characteristic motor symptoms, but also its strong association with cognitive decline and dementia. Hippocampal
neurogenesis is one area which may provide promising insight into cognitive change in PD but has been understudied in post-mortem human tissue. This thesis aims to better characterise changes to hippocampal neurogenesis in the ageing and PD brain, in the hope that this may contribute to better, more effective treatments for a facet of PD with particularly devastating effects on patients, families and societies.
Chapter 2: Hippocampal expression of cellular proliferation and neuronal maturation mRNA is reduced throughout the healthy adult life span

Evidence for reduced neurogenesis in the aging human hippocampus despite stable stem cell markers

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Summary

Reduced neurogenesis in the aging mammalian hippocampus has been linked to cognitive deficits and increased risk of dementia. We utilized postmortem human hippocampal tissue from 26 subjects aged 18–88 years to investigate changes in expression of six genes representing different stages of neurogenesis across the healthy adult lifespan. Progressive and significant decreases in mRNA levels of the proliferation marker Ki67 (MKI67) and the immature neuronal marker doublecortin (DCX) were found in the healthy human hippocampus over the lifespan. In contrast, expression of genes for the stem cell marker glial fibrillary acidic protein delta and the neuronal progenitor marker eomesodermin was unchanged with age. These data are consistent with a persistence of the hippocampal stem cell population with age. Age-associated expression of the proliferation and immature neuron markers MKI67 and DCX, respectively, was unrelated, suggesting that neurogenesis-associated processes are independently altered at these points in the development from stem cell to neuron. These data are the first to demonstrate normal age-related decreases at specific stages of adult human hippocampal neurogenesis.

Key words: cognition; doublecortin; healthy aging; hippocampus; Ki67; neurogenesis.

Introduction

Neurogenesis persists in the subgranular zone of the adult human hippocampus and arises from a pool of quiescent stem cells which, if appropriately stimulated, undergo proliferation and subsequent maturation into neurons. These new neurons are thought to play an important role in normal hippocampal function, particularly in the ongoing maintenance of hippocampus-dependent spatial and declarative memory (Aimone et al., 2014; Christian et al., 2014). It has therefore been hypothesized that alterations in hippocampal neurogenesis may be a contributing factor to cognitive decline and dementia. This is supported by observations that radiation-induced ablation of hippocampal neurogenesis in rats results in cognitive decline (Monje et al., 2002) and clinical evidence of cognitive changes in patients receiving radiation therapy and chemotherapy (Monje & Dietrich, 2012). Further evidence of a relationship between hippocampal neurogenesis and cognitive function is provided by disorders associated with cognitive decline, including Alzheimer's disease (Boekhoorn et al., 2006) and Parkinson's disease (Hoglinger et al., 2004) where stem cell proliferation and/or nascent neuron maturation is altered.

The birth of new neurons is a complex, multistep process, any stage of which could potentially be altered with age (Fig. 1). Reduced neurogenesis in the aged rodent hippocampus has been attributed to impaired proliferation and maturation of neuronal precursors (Rao et al., 2006). The two available studies of neurogenesis in the aging human hippocampus using immunohistochemical (Knott et al., 2010) and carbon dating (Spalding et al., 2013) techniques suggest that hippocampal neurogenesis is progressively reduced with age but employed markers that were either relatively restricted to a specific developmental stage or that broadly identified new cell birth (Fig. 1). To date, no studies have investigated the progression of neurogenesis through quiescence, proliferation, differentiation and maturation in the same hippocampal tissue over the healthy adult lifespan. Appreciating at which stage neurogenesis may fail with age in the human brain is critical to the development of strategies that aim to preserve neurogenesis and thus, cognitive function. Studies in aged rodents consistently report that interventions including exercise (Van Praag et al., 2005), environmental enrichment (Kempermann et al., 2002), inflammatory blockade (Monje et al., 2003; Ormerod et al., 2013; Speisman et al., 2013) or hormonal modulation (Montaron et al., 1999) are associated with both increased neurogenesis and improved cognition. The potential of similar interventions, such as exercise or cognitive training, in maintaining cognitive function in the healthy aged human brain is now recognized (Ballestero et al., 2015), although for technical reasons linking these findings to human neurogenesis is more difficult. Such approaches will, however, be most effective if an adequate pool of quiescent stem cells are available to be influenced for therapeutic benefit.

Despite these promising avenues of research, the effect of age upon the hippocampal stem cell population in the rodent brain remains a matter of debate and has not been explored in the human hippocampus. Hippocampal stem cells in the rodent brain were reported to possess no means of self-renewal (Encinas et al., 2011), suggesting that a stem cell deficit may underlie the observed progressive decline in neurogenesis with age (Walter et al., 2011; Andersen et al., 2014). In contrast, other studies reported that hippocampal stem cells in the rodent brain are capable of self-renewal (Suh et al., 2007) and that this ability persists throughout adulthood (Montaron et al., 1999; Hattiangady & Shetty, 2008; Bonaguidi et al., 2011), which may thus represent a tractable target for restoring age-related decreases in neurogenesis. In this study,
we aimed to investigate the effect of age upon multiple stages of neurogenesis by analysing changes to neurogenesis-associated gene expression across the healthy adult human lifespan.

Gene transcripts were chosen to estimate the proportion of cells at different stages of neurogenesis (Fig. 1, Table S2). Markers for stem cells (glial fibrillary acidic protein isoform delta; GFAPδ), cell proliferation (MKI67; MKI67), neuronal progenitor cells (eomesodermin; EOMES), immature neurons (doublecortin; DCX) and mature astrocytes (S100 calcium-binding protein B; S100B and glial fibrillary acidic protein; GFAP – although GFAP also marks stem cells) were quantified using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) from total RNA extracted from the hippocampus of 26 normal individuals aged 18–88 (Table S1, Data S1).

In this study, we found that expression of genetic markers for cellular proliferation (MKI67; p(26) = −0.577; P = 0.002, Fig. 2a) and neuronal maturation (DCX; p(26) = −0.617; P = 0.001, Fig. 2b) declined significantly with age. Immunohistochemistry studies in both humans and rodents report declining protein expression of these markers with age (Rao et al., 2006; Knoth et al., 2010), and we show here that these findings likely reflect transcriptional changes preceding protein synthesis. We found no significant relationship between our markers of proliferation and of immature neurons (MKI67 and DCX mRNAs) when controlling for age (partial correlation, r(23) = −0.162; P = 0.439), suggesting that the decline in expression of these genes represents independent changes. In contrast to MKI67 and DCX mRNAs, expression of the stem cell marker GFAPδ (p(26) = 0.227; P = 0.264, Fig. 2c) and the neuronal progenitor marker EOMES (p(26) = −0.067; P = 0.745, Fig. 2d) was unchanged with age. Expression of GFAP was significantly increased with age (p(26) = 0.486; P = 0.012; Fig. 2e), while S100B was unchanged with age (p(26) = 0.070; P = 0.734; Fig. 2f).

In order to explore the potential persistence of the hippocampal stem cell population with age, we investigated the expression of GFAPδ, an isoform of GFAP associated with quiescent stem cells (Roelofs et al., 2005). GFAPδ mRNA upregulation concurrent with GFAP mRNA upregulation has been reported (Roelofs et al., 2005) and has also been noted at the protein level in reactive astrocytes (Roelofs et al., 2005; Martinian et al., 2009). A partial correlation of age with GFAPδ expression, controlling for GFAP expression, however, found no relationship between these factors, (r(23) = −0.001; P = 0.995), demonstrating that expression of GFAPδ is independent of age despite the age-associated increase in GFAP. Our finding of consistent GFAPδ expression, coupled with significantly reduced proliferation, suggests therefore that the stem cell pool in the aging human hippocampus is not depleted over time. Rather, intra- or extracellular factors triggering or supporting the conversion of stem cells to rapidly dividing precursors may be progressively altered with age. While further studies quantifying stem cell numbers in the aging human hippocampus will be invaluable to confirm this hypothesis, here we report data suggesting that declining neurogenesis with age may not be the result of stem cell depletion in the human hippocampus. Rather, our data are consistent with specific
 mechanistic changes at two independent stages of neurogenesis which influence proliferation and neuronal maturation.

We also found that expression of DCX decreases significantly with age, consistent with previous studies of decreased DCX expression in the aged human subventricular zone (Weissleder et al., 2016). In our study in the hippocampus, the decline in DCX mRNA was accompanied by consistent expression of EOMES (Tbr2 in rodents), a transcription factor involved in neuronal fate decisions and expressed transiently in differentiating neurons in rodent studies (Hodge et al., 2012). These data suggest that neuronal progenitor cells, like quiescent stem cells, maintain a consistent population in which early neuronal specification is intact but where overall progenitor proliferation may be reduced. Reduced DCX expression, in conjunction with stable EOMES expression, suggests an unknown alteration in the hippocampal microenvironment specifically affecting the expression of genes influencing the successful maturation and migration of new neurons into the existing hippocampal circuitry. Alternatively, it may also indicate an uncontrolled exit from the immature neuronal state via early terminal differentiation or excessive cell death.

Our data suggest that the early and intermediate phases of neurogenesis (represented by GFAPs and EOMES) are unchanged, while the proliferation and early maturation stages are significantly reduced. We speculate that neurogenesis is altered in at least two distinct stages with age. The lack of any direct correlation between reduced cell proliferation and immature neuron markers supports the idea that the changes in neurogenesis at those stages may be the result of two separate physiological changes in the aging human brain.

Neuroinflammation is one such factor which may differentially alter distinct stages of neurogenesis, being increasingly chronic in the aging hippocampus (Barrientos et al., 2015) and previously shown to modulate neurogenesis in vivo rodent models (Ekdahl et al., 2003; Monje et al., 2003). While neuroinflammation is driven primarily by activated microglia, activated astrocytes have also recently been implicated in the regulation of the neuroinflammatory response (Jang et al., 2013). Experimentally, astrocyte activation is evidenced by increased GFAP mRNA (Nichols et al., 1993) and protein (David et al., 1997) in humans as well as morphological changes including hypertrophy and thickened radial processes (Rodríguez

Fig. 2 Alterations in neurogenesis gene expression and astrocytic activation state with age. Expression of marker Ki67 (MKI67) (a) and doublecortin (DCX) (b) was significantly reduced with age; in contrast, expression of glial fibrillary acidic protein isoform delta (GFAPδ) (c) and eomesodermin (EOMES) (d) did not vary with age. GFAP expression (e) significantly increased with age, but expression of S100B was unchanged (f), suggesting that the rate of astrogliogenesis was unvaried. Astrocyte morphology was examined using immunofluorescence in a subset of five cases as indicated in Table S1 (supporting information). Astrocytes within the hippocampus of younger individuals (g; representative image from an 18-year-old) exhibited typical astrocyte morphology with narrow, radiating processes and small cell bodies. Astrocytes within the hippocampus of older individuals (h; representative image from a 73-year-old) primarily presented with larger cell bodies strongly stained for GFAP and thick radiating processes, consistent with the morphology of activated astrocytes. Scale bar = 75 μm. Inset: magnified images of the field bound by the dotted box.
et al., 2014). Importantly, it has been suggested that the expression of GFAP influences the ability of new neurons to integrate into existing neural circuitry, with GFAP-knockout mice reporting higher rates of neurogenesis in the hippocampus (Larsson et al., 2004; Widenstradam et al., 2007). In our study, we found a significant increase in GFAP expression with age, consistent with previous studies. As GFAP is expressed in both astrocytes and stem cells, we also quantified the expression of the mature astrocyte marker S100B and found it to be unchanged with age, suggesting that the observed change is associated with increased astrocyte activation rather than astrogliaogenesis. This is supported by our observation that astrocyte morphology in our aged cases is consistent with that reported for activated astrocytes (Fig. 2h). Partial correlation analysis showed that neither MKI67 (r(23) = −0.204; P = 0.327) nor DCX (r (23) = −0.342; P = 0.094) expression was associated with GFAP expression when controlling for age. This suggests that age-related decreases in neurogenesis in the human hippocampus may not be a direct consequence of increased GFAP expression.

In this study, we provide data supporting the hypothesis that the stem cell population of the human hippocampus may be maintained throughout adult life. We also found that mRNA markers of proliferation and of nascent neurons are concomitantly, but potentially independently, reduced with age. Our data suggest that human hippocampal neurogenesis is altered at specific developmental stages in the aging human brain. We suggest that these stages may thus be appropriate points for the development of treatments which aim to restore neurogenesis and thus potentially support cognitive function.

Author contributions
KM contributed to the conception and design of this study, experimental procedures, statistical analysis and writing of the manuscript. KA and DB contributed to the experimental procedures and editing of the manuscript. CSW contributed to the conception and design of this study and editing of the manuscript. KD contributed to the conception and design of this study, statistical analysis and editing of the manuscript.

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Conflict of interest
No conflicts of interest have been reported by any of the authors involved in the publication of this study.

References


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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

Table S1. Case demographics for qRT-PCR and IF analysis.

Table S2. TaqMan probes used in qRT-PCR analysis.

Data S1. Experimental procedures.
Chapter 3: mRNA expression of neuronal maturation markers varies regionally along the long axis of the human hippocampus
3.1 Introduction

Alterations in hippocampal neurogenesis in animal models have been extensively studied, particularly at the protein level, although how these findings relate to human hippocampal neurogenesis is still being actively explored. As described previously in Chapter 1.3.4, studies in animal models suggest that the rate of neurogenesis varies along its length and that a greater number of new neurons are produced in the dorsal/posterior hippocampus than in the ventral/anterior. In addition, functional roles for the ventral-dorsal axis of the hippocampus in animal models has been suggested, as described in Chapter 1.3.3. In particular, the dorsal hippocampus appears to be more closely involved in episodic memory formation and spatial navigation, while the ventral hippocampus is more strongly associated with emotional- and fear-based learning. Given that the number of new neurons is reportedly higher in the dorsal hippocampus in animals, this may suggest role for neurogenesis in regulating memory and cognitive ability, with a potentially smaller role in emotional regulation.

Hippocampal neurotransmitters also have to potential to influence neurogenesis, as described in Chapter 1.2.3. Previous studies in animals suggest that the innervation of the hippocampus is unevenly distributed along its length, which may in turn contribute to the reported differences in the rate of new neuronal birth along its length. For example, serotonergic neurons have a reportedly denser distribution in the ventral rodent hippocampus (Gage and Thompson, 1980; Oleskevich and Descarries, 1990) while dopamine levels are significantly higher in the dorsal rodent hippocampus (Ishikawa, Ott and McGAUGH, 1982). The dorsal hippocampus similarly has a dense distribution of DA receptors (Fremeau et al., 1991), activation of which promotes spatial learning and memory formation (Kempadoo et al., 2016).
Human imaging studies suggest a role of the posterior hippocampus in regulating memory, particularly spatial navigation, and a greater number of DG cells are also reported in the posterior hippocampus. This may, in turn suggest a higher rate of neuronal birth in the posterior hippocampus, which until recently had not been investigated. Boldrini et al suggested in their 2018 study of healthy aged individuals that there was no regional variation in the number of new neurons present in the healthy human hippocampus, and that this number does not decline with age. These data contradict many previous findings, including those within this thesis (see Chapter 2). While the Boldrini study did quantify neuronal birth along the length of the hippocampus, no statistical analysis of regional variation in neurogenesis were made. It similarly did not investigate pathological changes to neurogenesis in cases of cognitive decline. It is therefore worthwhile to investigate these claims to better clarify the dynamics of human hippocampal neurogenesis in both ageing and disease.

This thesis chapter investigates the expression of neurogenesis genes, proteins, neurotransmitters and metabolites along the length of the hippocampus in young controls, aged controls and PDD cases. I hypothesise that neurogenesis gene expression will be highest in the posterior hippocampus, and that alterations in this expression will be most pronounced in PDD cases. I hypothesise that these changes will be associated with regional and pathological alterations in the distribution of serotonin and dopamine throughout the hippocampus.
3.2 Materials and Methods

3.2.1 Human post-mortem brain tissue

This study was approved by the Human Research and Ethics Committee at the University of Sydney (USyd HREC 2017/394). Post-mortem human tissue from 30 individuals was provided by the National Institutes of Health NeuroBioBank (Table 3.1). Three experimental groups were created based on age and pathological state; young controls (n=12), aged controls (n=9) and PDD (n=9). Control cases were free of significant neuropathology affecting the hippocampus at post-mortem examination. PDD cases had a neuropathological diagnosis of PD, as well as clinical symptoms of dementia at the time of death.

From each case, fresh-frozen tissue was provided from the anterior, middle and posterior hippocampus. The middle hippocampus was defined as the region at the coronal level of the lateral geniculate nucleus, while the anterior and posterior hippocampus was defined as the region 1-1.5 cm anterior and posterior to the middle hippocampus (Fig 3.1).

Tissue was supplied either as hippocampal tissue punches or as a coronal block containing the hippocampus. Tissue punches were pulverised on dry ice using a mortar and pestle and distributed randomly into centrifuge tubes with tissue weight between 50-100 mg. For coronal blocks, 50 µm sections were cut using a freezing microtome and distributed sequentially into centrifuge tubes with tissue weight between 50-100 mg.
Table 3.1 – Demographic information for all cases included in the experimental cohort.

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<th>Sex</th>
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<th>Brain pH</th>
<th>Brain Weight (g)</th>
<th>Cause of Death</th>
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Aged Controls

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</table>

**Parkinson's disease dementia**

<p>| | | | | | |</p>
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PMI – Post-mortem interval; RIN – RNA integrity number.

^ - PDD significantly lower than young controls (p=0.010) and aged controls (p=0.050). # - PDD significantly lower than young controls (p=0.027).
Figure 3.1 - Location of tissue sampled from the anterior, middle and posterior hippocampus.

From each individual, tissue was sampled from the anterior, middle and posterior hippocampus. The middle hippocampus was defined as the region at the coronal level of the lateral geniculate nucleus, as indicated by the vertical crosshair in B. The anterior and posterior hippocampus were defined as the regions 1-1.5 cm anterior or posterior of the middle hippocampus, as indicated by the vertical crosshairs in A and C. D, E and F depict the approximate macroscopic view of the hippocampus in the anterior, middle and posterior hippocampus, respectively. Adapted from (Mai, Paxinos and Voss, 2008).
3.2.2 Quantification of neurogenesis-related gene expression

3.2.2.1 RNA extraction

Tissue samples of 50-100 mg were pestle-homogenised in 1 mL TRIzol reagent (Thermo Fisher Scientific) and incubated at room temperature for 5 minutes to allow dissociation of the nucleotide complex. To initiate phase separation 200 µL of chloroform was added, vortexed vigorously and incubated for 3 mins before centrifuging for 15 mins at 12,000 xg (4°C). The upper aqueous phase was transferred into new centrifuge tubes and RNA precipitated using 500 µL isopropyl alcohol, incubated for 10 mins at room temperature and centrifuged for 10 mins at 12,000 xg (4°C). The resulting RNA pellet was washed 3x in 1 mL 75% ethanol and re-pelleted in a centrifuge for 5 mins at 7,500 xg (4°C) after each wash. The RNA pellet was air-dried for 5-10 mins and dissolved in 50 µL RNase-free water and stored at -80°C until further use. Optical density was read at 260 nm and 280 nm using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) to determine RNA concentration and purity. The $A_{260}/A_{280}$ ratio of all samples was between 1.9-2.0, suggesting little to no protein or DNA contamination.

3.2.2.2 cDNA synthesis

Complimentary DNA was synthesised using a SuperScript® III First-Strand Synthesis kit (Invitrogen, cat #18080-051) according to the manufacturer’s protocol. Briefly, 2 µg total RNA was combined with random hexamers and free nucleotides and incubated for 5 mins at 65° before placing immediately on ice for 1 min. 10 µL cDNA synthesis mix was added to each RNA sample, containing 10x reaction buffer, 25mM MgCl$_2$, 0.1 M Dithiothreitol, RNaseOUT™ RNase inhibitor and SuperScript® III reverse transcriptase in quantities defined in the manufacturers protocol. cDNA synthesis was initiated ay 50°C for 50 mins, followed by
reaction termination at 85°C for 5 mins. Samples were diluted 5x with RNase-free water to prevent the remaining reaction mix from inhibiting polymerase chain reaction amplification. Eight randomly chosen samples underwent an additional round of cDNA synthesis. These samples were pooled and serially diluted (3x) to create a standard curve to measure relative gene expression. Samples were stored at -20°C until further use.

3.2.2.3 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Relative levels of mRNA expression were measured using TaqMan® Gene Expression Assays (Applied Biosystems, California, USA). Exon-spanning probes for the genes GFAPD (glial fibrillary acidic protein isoform delta; stem cells), MKI67 (Ki67; cell proliferation), EOMES (eomesodermin; neuronal progenitors), DCX (doublecortin; immature neurons) and GFAP (glial fibrillary acidic protein isoform α; astrocytes and stem cells) were chosen to ensure appropriate capture of mature mRNA expression. These genes were chosen to accurately reflect the multiple stages of neurogenesis, as described in Chapter 2 (See also Appendix 1.2).

3.2.3 Relative quantification of neurogenesis-related protein expression

3.2.3.1 Case selection

A subset of five cases from each experimental group were randomly selected as representative cases for Western blotting analysis. An additional statistical analysis demonstrated that the cases selected demonstrated the same patterns of DCX and MKI67 gene expression as the whole cohort, and thus were considered a suitable subset of cases for further analysis.

3.2.3.2 Sample preparation

For protein extraction, tissue samples were homogenised using a motorised pestle homogeniser in 5x tissue weight of ice-cold PBS. An equal volume of 2x RIPA buffer (50 mM
Tris, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% SDS, 1% sodium deoxycholate; pH 8.0) was added and endogenous protease and phosphatase activity was inhibited using cOmplete™ protease inhibitor cocktail (Roche, Basel, Switzerland) and PhosSTOP™ phosphatase inhibitor (Roche, Basel, Switzerland). Samples were centrifuged for 15 mins at 15,000 xg (4°C) and the supernatant collected and stored at -80°C until further use. Total protein concentration was calculated using a Pierce™ bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer’s protocol.

3.2.3.3 Western Blot

Twenty micrograms total protein was loaded onto Mini-PROTEAN® TGX Stain-Free™ precast gels (Bio-Rad, California, USA). All samples were run in triplicate. Separated proteins were transferred onto polyvinyl difluoride membrane (Millipore, Massachusetts, USA) and total transferred protein determined using SYPRO™ Ruby Protein Gel Stain (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer’s protocol. Membranes were then blocked with 1% bovine serum albumin in Tris-buffered saline (20 mM Tris, 150 mM NaCl) with 0.1% Tween®20 (TBST) for 1 h at room temperature. Membranes were incubated with primary antibodies against DCX (1:1000; Abcam ab18723) and proliferating cell nuclear antigen (PCNA; 1:1000; Abcam ab18197) overnight at 4°C, washed with TBST and incubated with an appropriate horseradish peroxidase- (HRP) conjugated secondary antibody (α-Rabbit HRP; 1:5000; Bio-Rad 170-6515) for 2 h at room temperature.

Protein bands were visualised using enhanced chemiluminescence detection (Bio-Rad, California, USA) according to the manufacturer’s protocol and imaged using a ChemiDoc™ MP System (Bio-Rad, California, USA). Densiometric analysis of protein bands was completed using ImageLab™ Software v.6.0.1 (Bio-Rad, California, USA), normalising bands of interest to
total protein content visualised with SYPRO™ Ruby Protein Gel Stain. A loading control was run on each gel to confirm consistent gel loading, transfer and visualisation between gels.

3.2.4 Quantification of hippocampal dopamine, serotonin and metabolites

Reversed phase high performance liquid chromatography (HPLC) was used to quantify hippocampal levels of DA and its metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), as well as 5-HT and its metabolite 5-hydroxyindoleacetic acid (5HIAA). These neurotransmitters were chosen as levels of these neurotransmitters, their receptors, or innervation containing these neurotransmitters are reported to be reduced in the PD hippocampus (Ballanger et al., 2012; Hoglinger et al., 2004; Scatton et al., 1983). Given the primary hypothesised role of 5-HT in emotional regulation (Luo, An and Zhang, 2008; Mahar et al., 2014) and DA in cognition (Jokinen et al., 2009; Kempadoo et al., 2016; Silva et al., 2012), this suggests these neurotransmitters may be variably distributed along the length of the hippocampus.

Hippocampal tissue samples were sonicated (Branson Sonifier, Connecticut, USA) on ice in 10x volume of ice-cold homogenisation buffer (150 mM phosphoric acid and 500 µM diethylenetriaminepentaacetic acid). Homogenates were centrifuged for 25 mins at 18,000 xg (4°C) to pellet cellular debris. Supernatant was then filtered through Amicon® 3 kDa cut-off spin filters in a centrifuge for 90 mins at 14,000 xg (4°C). Known volumes of filtrate were lyophilised overnight at room temperature and concentrated by adding half of the original volume of homogenisation buffer to the dried samples. Samples were stored at -80° until further HPLC analysis. Total protein content of the homogenised samples was measured using a Pierce™ BCA assay according to the manufacturer’s protocol.
The HPLC system used consisted of a Shimadzu Prominence LC-20AD pump module (Shimadzu Corporation, Kyoto, Japan) fitted with a 150 x 4.6 mm, 5 µm diameter, reverse-phase C-18 column (Phoenemex, California, USA) maintained at 40°C. Mobile phase contained 87% (v/v) 0.01 M sodium phosphate monobasic buffer and 13% (v/v) methanol with 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.65 mM 1-octenesulfonic acid and 0.5 mM triethylamine (pH 2.81). Mobile phase was delivered at 1 mL/min, through a 0.22 µm vacuum disc filter. Samples were measured against a standard curve of known concentration created using commercially-prepared DA, DOPAC, HVA, 5-HT and 5-HIAA (Sigma-Aldrich, Missouri, USA).

Twenty microlitre sample injections were performed in duplicate with a Shimadzu Prominence SIL-20A autosampler (Shimadzu Corporation, Kyoto, Japan) with electrochemical detection by an electrochemical detector (Antec-Leyden, Zoeterwoude, Netherlands) with a glassy carbon electrode maintained at +0.82 V versus a silver-silver chloride reference electrode. Chromatogram analysis was completed using Shimadzu LabSolutions software v. (Shimadzu Corporation, Kyoto, Japan). Data was expressed in pg of the compound of interest per mg total protein. Data points appearing above or below the levels defined by the standard curve were removed before analysis.

3.2.5 Statistical analysis

All statistical tests were performed using IBM SPSS Statistics v24 (IBM Corporation, New York, USA). A Kruskal-Wallis H test was used to determine any significant differences in post-mortem interval (PMI), RNA integrity number (RIN), brain pH and brain weight between young, aged and PDD cases. A Mann-Whitney U test was used to confirm age matching between the aged and PDD groups. Parametric and non-parametric tests were performed for
all demographic data. However, due to the non-normal distribution of residuals for pH and brain weight (data not shown), non-parametric tests are reported for all data sets to maintain consistency. Significant results of the parametric tests remained significant in the corresponding non-parametric test.

Relative mRNA expression, DCX and PCNA protein expression and neurotransmitter levels were analysed using linear mixed modelling. Hippocampal region and group were entered as fixed factors, with a random intercept for each case to account for the repeated measurement of hippocampal region.

A hierarchical linear regression was subsequently used to examine a range of predictors for \textit{MKI67} and \textit{DCX}, to determine which factors best predict neurogenesis gene expression. For both genes, a three-block regression model was designed. The first block investigated the predictive effect of PMI and brain weight, both of which were found to be significantly lower in the PDD group. The second level included gene expression level of the genes preceding the predicted gene; \textit{GFAPD} was included in the \textit{MKI67} model and \textit{GFAPD}, \textit{MKI67} and \textit{EOMES} were included in the \textit{DCX} model. The last block included the DA metabolite DOPAC and 5-HT metabolite 5-HIAA. An additional two-block model was created to examine the predictive effect of the DA metabolite HVA and 5-HT on \textit{DCX} expression, when controlling for expression of \textit{GFAPD}, \textit{MKI67} and \textit{EOMES}.

Normality of the original data sets is not a requirement of the linear mixed model or linear regression, although residuals should be approximately normally distributed. To achieve this, dependent variables were log-transformed prior to statistical analysis, although data in its original form was used for the creation of graphs. Normal Q-Q plots were used to assess residuals, which were found to be approximately normally distributed for all analyses.
of log-transformed data (data not shown). Outliers were detected using Grubbs test, and
removed from the analyses of DCX protein (2 outliers removed) and PCNA protein (1 outlier
removed). No outliers were removed from the gene expression data set.

Significance level for all analyses was set at 0.05. A Sidak correction was used for all
post-hoc multiple comparisons where appropriate. Graphs were created in GraphPad Prism
7 (GraphPad Software Inc., California, USA).

3.3 Results

3.3.1 Case Demographics

Neither RIN or pH differed between experimental groups, although the PDD group had
significantly lower PMI ($\chi^2 (2) = 9.53; p=0.009$) and brain weight ($\chi^2 (2) = 7.39; p=0.025$) than
the young and aged controls (For pairwise comparisons see Table 3.1). Average age did not
differ between aged controls and PPD cases ($U=45.00; p=0.730$).

3.3.2 Expression of proliferation and maturation markers of neurogenesis are reduced in
PDD and vary along the length of the hippocampus

The gene expression data obtained from the qRT-PCR analysis can be found in Fig 3.2. This
data was used to form the linear mixed model upon which the statistical analysis of regional
and group-related differences in neurogenesis-related genes was based. Of the five genes
analysed, four were associated with changes in either hippocampal region or experimental
group on the expression of mature mRNA (See Table 3.2 for full summary). MKI67 varied with
group, exhibiting significantly lower expression in PDD cases than in young controls (Fig. 3.3a).
EOMES varied with hippocampal region (Fig. 3.3b), exhibiting higher levels of expression in
the anterior hippocampus, compared to the posterior region. DCX varied with both group (Fig.
3.3c) and hippocampal region (Fig. 3.3d). GFAP similarly exhibited changes with group (Fig.
3.3e) and region (Fig. 3.3f). In contrast, GFAPD did not vary with either group or region. There were no significant interactions between hippocampal region and group for any of the genes analysed.
Figure 3.2 - Raw qRT-PCR gene expression data analysed with a linear mixed model, categorised by region and group.

Figure legend next page.
Raw gene expression data obtained in the qRT-PCR analysis of the neurogenesis-related genes *GFAPD* (A), *MKI67* (B), *EOMES* (C), *DCX* (D) and *GFAP* (E). Note that due to the repeated measures design of the linear mixed model, the n for each group (young, aged and PDD) represents the same cohort of individuals throughout each of the regions. Columns represent mean ± SEM.
Table 3.2 – Statistical results of linear mixed modelling of neurogenesis gene expression with fixed effects of hippocampal region and group as well as interactions between region and group.

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</tbody>
</table>
mRNA expression of the proliferation marker *MKI67* (A) is significantly reduced in PDD, while the neuronal fate choice marker *EOMES* is expressed at significantly higher levels in the anterior than posterior hippocampus (B). The early neuronal marker *DCX* is expressed at significantly lower levels in PDD (C), as well as in the posterior hippocampus (D), sharing pathological expression patterns similar to *MKI67* and regional expression patterns similar to *EOMES*. *GFAP* also varied both pathologically (E) and regionally (F). Error bars = standard error of the mean (SEM). * = p<0.05; ** = p<0.01. *** = p<0.005. Sidak correction applied for all pairwise comparisons.
3.3.4 Hippocampal neurotransmitters and their metabolites vary with hippocampal region and pathology

The neurotransmitter and metabolite data obtained from the HPLC analysis can be found in Fig 3.4. This data was used to form the linear mixed model upon which the statistical analysis of regional and group-related differences in hippocampal neurotransmitters was based. Quantification of striatal DA in post-mortem human tissue has demonstrated considerably higher levels of DA, even in PD cases, than was able to be reliably detected in the post-mortem human hippocampus in this study (Kish, Shannak and Hornykiewicz, 1988; Kish et al., 2007). As a result, a number of data points from all experimental groups were excluded from the analysis due to DA levels below the limit of detection (Table 3.3). Of the remaining cases, hippocampal levels of DA did not show a significant main effect of either hippocampal region or group. The DA metabolite DOPAC exhibited a group effect, with significantly lower levels in PDD (Fig. 3.5a). HVA did not vary with group but with hippocampal region, with significantly higher levels present in the anterior and middle hippocampus compared with the posterior (Fig. 3.5b).

There was a significant effect of region on the levels of 5-HT, with the highest levels found in the anterior hippocampus (Fig. 3.5c). Regional levels of 5-HIAA also followed this pattern (Fig. 3.5d), although there was also a group effect, with levels highest in PDD cases (Fig. 3.5e). There were no significant interactions of region and group for any of the neurotransmitters or metabolites analysed. A standard curve for noradrenaline was also created, although no measurable noradrenaline was detected using the methodology described in Chapter 3.2.4.

A Spearman’s correlation was also run to examine the relationship between each neurotransmitter and its metabolites. DA correlated significantly with both DOPAC ($p=0.514$;...
p<0.001) and HVA (ρ=0.376; p=0.022), while 5-HT similarly correlated significantly with 5-HIAA (ρ=0.619; p<0.001).
Figure 3.4 - Raw qRT-PCR gene expression data analysed with a linear mixed model, categorised by region and group.

Figure legend next page.
Raw gene expression data obtained in the qRT-PCR analysis of the hippocampal neurotransmitter DA (A), its metabolites DOPAC (B) and HVA (C), 5-HT (D) and its metabolite 5-HIAA (E). Note that due to the repeated measures design of the linear mixed model, the $n$ for each group (young, aged and PDD) represents the same cohort of individuals throughout each of the regions. Columns represent mean ± SEM.
Table 3.3 - Statistical results of linear mixed modelling of levels of DA, 5-HT and their metabolites, with fixed effects of hippocampal region and group as well as interactions of region and group.

<table>
<thead>
<tr>
<th>NT/Metabolite</th>
<th>Data Points Excluded</th>
<th>Region</th>
<th>Group</th>
<th>Region*Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>Y = 17, A = 11, PDD = 10</td>
<td>$F_{(2,26)}=1.40$</td>
<td>$F_{(2,26)}=3.06$</td>
<td>$F_{(4,26)}=0.325$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p=0.264$</td>
<td>$p=0.069$</td>
<td>$p=0.859$</td>
</tr>
<tr>
<td>DOPAC</td>
<td>Y = 3, A = 3, PDD = 8</td>
<td>$F_{(2,45)}=0.53$</td>
<td>$F_{(2,27)}=6.14$</td>
<td>$F_{(4,45)}=0.517$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p=0.595$</td>
<td>$p=0.006$</td>
<td>$p=0.724$</td>
</tr>
<tr>
<td>HVA</td>
<td>Y = 7, A = 7, PDD = 5</td>
<td>$F_{(2,44)}=14.90$</td>
<td>$F_{(2,27)}=1.45$</td>
<td>$F_{(4,44)}=0.620$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p&lt;0.001$</td>
<td>$p=0.253$</td>
<td>$p=0.650$</td>
</tr>
<tr>
<td>5-HT</td>
<td>Y = 11, A = 0, PDD = 2</td>
<td>$F_{(2,45)}=9.95$</td>
<td>$F_{(2,25)}=0.611$</td>
<td>$F_{(4,45)}=0.537$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p&lt;0.001$</td>
<td>$p=0.551$</td>
<td>$p=0.709$</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>Y = 3, A = 5, PDD = 4</td>
<td>$F_{(2,44)}=3.39$</td>
<td>$F_{(2,28)}=6.18$</td>
<td>$F_{(4,44)}=0.281$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p=0.043$</td>
<td>$p=0.006$</td>
<td>$p=0.889$</td>
</tr>
</tbody>
</table>

NT – Neurotransmitter, Y – Young Controls, A - Aged Controls, PDD – Parkinson’s disease dementia
Full data set - Young n= 36; Aged n= 27; PDD n= 27
Figure 3.5 – Pairwise comparison of estimated marginal means for hippocampal neurotransmitters and metabolites showing a main effect of region or group.

Levels of DOPAC were significantly lower in PDD compared with young, but not aged, controls (A). On the contrary, levels of HVA varied regionally, with significantly higher levels found in the anterior hippocampus (B). Levels of 5-HT (C) and 5-HIAA (D) also followed the same regional pattern as HVA, and 5-HIAA was additionally found at significantly higher levels in PDD (E). Error bars = SEM. * = p<0.05; ** = p<0.01. *** = p<0.005. Sidak correction applied for all pairwise comparisons.
3.3.5 Dopamine and serotonin metabolites best predict alterations in neurogenesis gene expression

Analysis of the group-associated DCX regression model (Table 3.4) demonstrated that the first model (PMI and brain weight) did not significantly deviate the $R^2$ value from 0. However, the $R^2$ value of both the second ($\Delta R^2 = 0.133; p= 0.038$) and third models ($\Delta R^2 = 0.125; p= 0.012$) differed significantly from the previous model, suggesting that both previously expressed neurogenesis genes and group-associated metabolites predict DCX expression. In support of this, Model 3 (the full model) indicates that expression of GFAPD ($B=-0.190; p= 0.014$; Fig 3.6a) and DOPAC ($B=0.248; p= 0.003$; Fig 3.6b) levels are the most significant predictors of DCX expression.

An additional two-block model was run for DCX (Table 3.5), to account for metabolites that showed significant regional variation along the length of the hippocampus and to avoid multicollinearity suggested by the strong correlation between DA, 5-HT and their metabolites. The $\Delta R^2$ value of the first model differed significantly from 0 ($\Delta R^2 = 0.274; p< 0.001$), however the second model did not significantly alter the $R^2$ value of the first model ($\Delta R^2 = 0.045; p=0.180$). Like the group-associated regression model, DCX expression was significantly predicted by GFAPD ($B=-0.268; p= 0.001$) expression, and also by MKI67 ($B=0.144; p= 0.005$; Fig 3.6c) expression in the full model. Neither HVA ($B=0.205; p= 0.074$) nor 5-HT ($B=-0.025; p= 0.767$) were significant predictors of DCX expression.

Finally, a three-block model was designed to consider predictors of MKI67 expression, set up identically to the group-associated DCX model (Table 3.6). The first model, including PMI and brain weight did not show a significant change in $R^2$ from 0 ($\Delta R^2 = 0.025; p=0.478$), nor did the second model including GFAPD, the gene expressed prior to MKI67 ($\Delta R^2 = 0.008$;
p=0.469). The third model, including DOPAC and 5-HIAA revealed a significant change in $R^2$ ($\Delta R^2 = 0.259; p<0.001$), suggesting that one or both metabolites are significant predictors of MKI67 expression. In the full model, brain weight ($B=-2.709; p=0.018$), DOPAC levels ($B=0.528; p=0.005$; Fig. 3.6d) and 5-HIAA levels ($B=-0.484; p=0.006$; Fig 3.6e) were significant predictors of MKI67 expression.
Table 3.4 – Results of a three-block hierarchical linear mixed model to determine group-specific demographic, genetic and metabolic predictors of DCX expression.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
<td>B</td>
</tr>
<tr>
<td>PMI</td>
<td>0.201</td>
<td>0.126</td>
<td>0.087</td>
</tr>
<tr>
<td>Br. Weight</td>
<td>0.324</td>
<td>0.493</td>
<td>0.455</td>
</tr>
<tr>
<td>GFAPD</td>
<td>-0.174*</td>
<td>0.080</td>
<td>-0.190*</td>
</tr>
<tr>
<td>MKI67</td>
<td>0.103</td>
<td>0.052</td>
<td>0.026</td>
</tr>
<tr>
<td>EOMES</td>
<td>0.107</td>
<td>0.075</td>
<td>0.118</td>
</tr>
<tr>
<td>DOPAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HIAA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[
R^2 \quad 0.074 \quad 0.207 \quad 0.331
\]

\[
F \quad 2.269 \quad 2.811*\quad 3.679**
\]

\[
\Delta R^2 \quad 0.074 \quad 0.133 \quad 0.125
\]

\[
\Delta F \quad 2.269 \quad 3.013*\quad 4.847*
\]

Br. Weight = Brain weight. Total data points = 60. * = p<0.05; ** = p<0.01. *** = p<0.005
Table 3.5 – Results of a two-block hierarchical linear mixed model to determine region-specific genetic and metabolic predictors of DCX expression.

| Variable | Model 1 | | Model 2 | |
|----------|---------|-------------------|---------|
|          | B       | Std. Error        | B       | Std. Error |
| GFAPD    | -0.237*** | 0.072             | -0.268*** | 0.073      |
| MKI67    | 0.136**  | 0.050             | 0.144**  | 0.049      |
| EOMES    | 0.112    | 0.074             | 0.077    | 0.077      |
| HVA      | 0.205    |                   | 0.113    |             |
| 5-HT     | -0.025   |                   | 0.084    |             |

$R^2$ 0.274 0.319  
$F$ **7.053*** 5.058**  
$\Delta R^2$ 0.274 0.045  
$\Delta F$ **7.053*** 1.773  

Total data points = 60. * = p<0.05; ** = p<0.01. *** = p<0.005
**Table 3.6** – Results of a three-block hierarchical linear mixed model to determine group-specific demographic, genetic and metabolic predictors of MKI67 expression.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model 1</th>
<th></th>
<th></th>
<th></th>
<th>Model 2</th>
<th></th>
<th></th>
<th></th>
<th>Model 3</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
<td>B</td>
<td>Std. Error</td>
<td>B</td>
<td>Std. Error</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMI</td>
<td>0.285</td>
<td>0.307</td>
<td>0.357</td>
<td>0.326</td>
<td>0.116</td>
<td>0.295</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br. Weight</td>
<td>-1.324</td>
<td>1.204</td>
<td>-1.248</td>
<td>1.215</td>
<td>-2.709*</td>
<td>1.109</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFAPD</td>
<td></td>
<td>0.134</td>
<td>0.195</td>
<td>0.067</td>
<td>0.171</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPAC</td>
<td></td>
<td></td>
<td></td>
<td>0.528**</td>
<td>0.180</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HIAA</td>
<td></td>
<td></td>
<td></td>
<td>-0.484**</td>
<td>0.169</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.025</td>
<td>0.033</td>
<td></td>
<td>0.228</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F$</td>
<td>0.748</td>
<td>0.650</td>
<td></td>
<td>4.540**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta R^2$</td>
<td>0.025</td>
<td>0.008</td>
<td></td>
<td>0.259</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta F$</td>
<td>0.748</td>
<td>0.469</td>
<td></td>
<td>10.065***</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Br. Weight = Brain weight. Total data points = 61. * = p<0.05; ** = p<0.01. *** = p<0.005.
Figure 3.6 – Significant genetic and metabolic predictors of DCX and MKI67 expression across the experimental cohort

Of the two DCX regression models, GFAPD (A), DOPAC (B) and MKI67 (C) were significant predictors. In the MKI67 regression model, both DOPAC (D) and 5-HIAA (E) were significant predictors. It is interesting to note that in most significant regression models there is some clustering of young controls and PDD cases, with aged controls spanning across the entire dataset.
3.3.6 Protein expression of maturation markers do not follow the same pattern as the expression of the equivalent genes

Western blots were performed to examine the protein levels of DCX and PCNA across hippocampal regions and groups in a subset of cases. PCNA was substituted for KI67 in this study as previous work by Knoth et al. (2010) demonstrated that at the protein level KI67 did not co-localise well with DCX in adults over 38 years of age. This suggests poor translation of the MKI67 gene to protein, or that KI67 protein was particularly susceptible to degradation in aged cohorts. On the other hand, PCNA⁺DCX⁺ neurons were consistently found in adults up to 100 years of age. The protein data obtained from the Western blot analysis can be found in Fig 3.7. Using linear mixed modelling (Table 3.7) it was found that in contrast to gene expression studies, no main effects of group (Fig. 3.8a) or region (Fig. 3.8b) were found in levels of DCX protein. However, PCNA exhibited a significant group effect, with significantly lower protein levels in PDD cases compared with both young and aged controls (Fig. 3.8c). In agreement with the expression of the MKI67 gene, PCNA protein expression did not vary regionally (Fig. 3.8d). No significant interaction effects were reported for either DCX or PCNA.
Figure 3.7 - Raw Western blot protein expression data analysed with a linear mixed model, categorised by region and group

Raw protein expression data obtained in the Western blot analysis of the neurogenesis-related proteins PCNA (A) and DCX (B). Note that due to the repeated measures design of the linear mixed model, the n for each group (young, aged and PDD) represents the same cohort of individuals throughout each of the regions. Columns represent mean ± SEM.
Table 3.7 - Statistical results of linear mixed modelling of protein levels of DCX and PCNA, with fixed effects of hippocampal region and group as well as interactions of region and group.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Region</th>
<th>Group</th>
<th>Region*Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCX</td>
<td>$F_{(2,23)} = 0.38$</td>
<td>$F_{(2,13)} = 2.21$</td>
<td>$F_{(4,24)} = 0.427$</td>
</tr>
<tr>
<td></td>
<td>$p = 0.685$</td>
<td>$p = 0.151$</td>
<td>$p = 0.788$</td>
</tr>
<tr>
<td>PCNA</td>
<td>$F_{(2,23)} = 2.75$</td>
<td>$F_{(2,12)} = 5.45$</td>
<td>$F_{(4,23)} = 2.338$</td>
</tr>
<tr>
<td></td>
<td>$p = 0.084$</td>
<td>$p = \textbf{0.021}$</td>
<td>$p = 0.085$</td>
</tr>
</tbody>
</table>
Levels of DCX protein did not vary with group (A) or region (B). However, levels of PCNA protein are reduced in PDD (C) and unchanged with region (D), in agreement with the mRNA expression of fellow proliferation marker *MKI67*. Error bars = SEM. * = p<0.05; ** = p<0.01. *** = p<0.005. Sidak correction applied for all pairwise comparisons. Demonstrative immunoblot band images taken from the same cases for A and C; B and D.
3.4 Discussion

Neurogenesis appears to play a crucial role in memory and cognition in animal models, although in humans the importance of neurogenesis is more widely debated. While it has been believed for several decades that humans produce new hippocampal neurons throughout life, several new studies question the specifics of these long-held beliefs (Boldrini et al., 2018; Dennis et al., 2016; Sorrells et al., 2018). It is important, therefore, that the dynamics of human hippocampal neurogenesis are better explored, not only to better understand how neurogenesis functions in humans, but to analyse the suitability of animal models for modelling human hippocampal function.

Linear mixed modelling was used to determine if neurogenesis genes were regionally-associated in the human hippocampus, and if this regional variability is differentially affected in ageing, or in PDD. Of the neurogenesis-related genes examined, only EOMES and DCX demonstrated a regional pattern of expression. Contrary to animal studies at the protein level however (Chang et al., 2015; Jinno, 2011; Snyder et al., 2009), these genes were more highly expressed in the anterior, rather than the posterior, hippocampus. This pattern of gene expression may be due to several different cellular mechanisms. Firstly, it may simply reflect an overall greater rate of neurogenesis in the anterior hippocampus. Neurogenesis plays an important role in both emotion and cognition in animals and appears to have similarly important roles in these functions in humans (Bremner et al., 2000; Eisch and Petrik, 2012; Monje and Dietrich, 2012). More prevalent neurogenesis in the anterior hippocampus may suggest that, contrary to the hypothesis of this study, neurogenesis plays a more important role in emotional regulatory functions of the hippocampus, rather than in cognitive and memory functions in the human brain.
Alternatively, given that anatomically the human hippocampus is far less uniform in shape along its anterior-posterior axis than the rodent hippocampus (Strange et al., 2014), it is possible that the functional divisions of the human hippocampus are clustered towards the anterior hippocampal pole and thus are less distinct than those in the rodent, where clear emotional and cognitive divisions along the entire dorsoventral axis have been established (Bannerman et al., 2003; Kjelstrup et al., 2002; Moser, Moser and Andersen, 1993; Trivedi and Coover, 2004). This idea is, however, not supported by imaging data on human hippocampal function, which demonstrates similar functional divisions along the length of the hippocampus as seen in animal studies (Maguire, Nannery and Spiers, 2006; Maguire, Woollett and Spiers, 2006; Murty et al., 2010).

Lastly, since neither the stem cell marker GFAPD or proliferation marker MKI67 demonstrated similar regional patterns of expression, these data may reflect differences in the rate of differentiation and maturation of new neurons along the hippocampus. The reduction in both EOMES and DCX expression in the posterior hippocampus may therefore be either a reflection of reduced numbers of new neurons, or of new neurons passing through the maturation phase faster than that in the anterior hippocampus. Previous reports in rodents have found that new dorsal neurons mature faster than their ventral counterparts (Piatti et al., 2011; Snyder and Cameron, 2012), suggesting the rate of neuronal maturation in the posterior hippocampus may also vary in humans. Unfortunately, at this time this theory would be difficult to investigate, as it would largely depend on bromodeoxyuridine labelling or carbon dating techniques established by Spalding et al. to distinguish between fully-mature granule neurons born in adulthood and those established in childhood. While these methods have been used in humans previously (Eriksson et al., 1998; Spalding et al., 2005), they are not generally not used in post-mortem human studies as they rely on specific, but rare, events
such as nuclear bomb testing or on bromodeoxyuridine administration prior to a patient’s death; for most studies these are not practical methodological considerations.

Apart from the regional variation in the expression of DCX and EOMES, linear mixed modelling also demonstrated a reduction in the expression of MKI67 and DCX in PDD. This is consistent with previous studies in both animal models of PD and human post-mortem studies (Desplats et al., 2012; Hoglinger et al., 2004; Suzuki et al., 2010; Winner et al., 2009). Interestingly, while both MKI67 and DCX were reduced in aged controls compared to young controls, this difference was not significant. Several studies, including previous work by the featured in this thesis (see Chapter 2), have suggested a significant negative correlation between age and both cellular proliferation and maturation (Knoth et al., 2010; Spalding et al., 2013). The lack of significant reduction in this case may reflect the fact that the aged control group were chosen because they had no clinical evidence of cognitive decline or dementia and thus represent a pre-clinical stage between healthy brain function in young adulthood and the development of clinical signs of dementia, represented by the PDD group.

The fact that neurogenesis is further reduced in PDD suggests that this decline can and does become pathologically significant; further studies into the mechanism of neurogenesis decline is necessary to further delineate the “normal” decline in neurogenesis in healthy ageing from that associated with clinical disease.

Despite the regional and pathological changes to neurogenesis gene expression found in this study, no significant interactions of region and group were found for any of the genes examined. These data indicate that, regardless of an overall decline in neurogenesis with both age and PDD, there is no preferential decline in any region along the length of the hippocampus. This may explain why both dementia and depression are common non-motor
symptoms of PD, as the decline in hippocampal neurogenesis in PD appears to be global rather than regionally-specific. Moreover, some studies have reported that depression is a predictor of later cognitive decline (Pirogovsky-Turk et al., 2017; Stefanova et al., 2006), although see also Guo et al. (2019); this suggests that the two symptoms are linked, potentially both via alterations in hippocampal neurogenesis. However, the onset of these two symptoms is generally opposite, with depression commonly appearing in pre-clinical PD or in the early stages of disease (Pfeiffer, 2016; Postuma et al., 2012), while dementia onset may be years after the initial diagnosis of PD (Hely et al., 2008). The raphe nuclei also degenerate in PD (Halliday et al., 1990), and it has been suggested that neuronal dysfunction and LB pathology in this region may precede dopaminergic dysfunction (Braak et al., 2003; Hawkes, Del Tredici and Braak, 2010). This may therefore reflect the early onset of affective disorders before cognitive decline in later PD. Future studies investigating neurogenesis along the anterior-posterior axis of the human hippocampus may therefore find a preferential loss of neurogenesis in the anterior and/or posterior hippocampus when comparing depressed but non-demented PD cases with PDD cases and age-matched controls.

The functional and anatomical heterogeneity of the hippocampal formation has been established in human studies, and this study has determined that regional heterogeneity of neurogenesis gene expression is also present, particularly in genes expressed in the differentiation and maturation phases of neurogenesis. Regulatory factors driving neuronal maturation may be similarly heterogeneously distributed and thus result in the gradient of gene expression seen here. I hypothesised that neurotransmitters in the hippocampus may modulate this process. Both DA and 5-HT were considered potential regional regulators of neurogenesis, given their strong association with emotion and memory, regionally-varied
patterns of innervation and ability to modulate neurogenesis in animal models (Banasr et al., 2004; Winner et al., 2009).

DA levels were particularly low in this study, and several samples were excluded from each group due to low or undetectable levels of DA. While it is likely that some post mortem degradation of DA occurred prior to this study, all other neurotransmitters and metabolites were more consistently expressed in the hippocampus. The lack of measurable DA in this study therefore supports previous studies demonstrating the extremely low level of DA innervation of the human hippocampus (Gaspar et al., 1989; Torack and Morris, 1990), particularly in comparison to other species (Goldsmith and Joyce, 1994). This difference in DA innervation of the hippocampus may aid in understanding why animals, particularly rodents, have much higher baseline levels of neurogenesis than humans. Further studies elucidating on the changing innervation patterns between animals and humans would be useful in better understanding the role of neurotransmitters, particularly DA, in modulating hippocampal neurogenesis.

Both 5-HT and the DA metabolite HVA were regionally-distributed along the hippocampus reflecting that pattern that was observed for DCX, however neither 5-HT nor HVA significantly predicted DCX expression.

In contrast to 5-HT and HVA, DOPAC and 5-HIAA varied with group. DOPAC, like MKi67 and DCX, was expressed at its lowest level in the posterior hippocampus. The opposite was true of 5-HIAA, which was expressed at its lowest level in the anterior hippocampus and highest in the posterior hippocampus. Linear regression indicated that DOPAC was a strong predictor of DCX expression, while both DOPAC and 5-HIAA significantly predicted MKi67 expression. The reduction of DOPAC in the PDD group may result from decreased dopamine
innervation and release in the hippocampus, particularly given the strong correlation between DA and DOPAC in the entire cohort. The relationship between DOPAC and DCX expression is currently unknown, but may simply reflect decreased DA bioavailability in PD (Scatton et al., 1983), which cannot then modulate neurogenesis. Additional loss of ventral tegmental area neurons in PDD may also contribute significantly to the decrease in hippocampal DOPAC seen here (Hall et al., 2014). Importantly, DA has been hypothesised to play a role in the modulation of neuronal proliferation (Winner et al., 2009) and maturation (Aizawa et al., 2011), and these findings support this theory. In future studies, a more sensitive measure of DA content in the hippocampus could further elucidate on this point.

Unlike DOPAC, 5-HIAA levels are significantly increased in the PDD group. As 5-HT levels were unchanged in PDD, this may indicate that serotonergic modulation of neurogenesis in PD result from increased metabolism of 5-HT, which may in turn prevent the appropriate stimulation of stem cells in the hippocampus. This is consistent with studies investigating the effects of selective serotonin reuptake inhibitors, which increase 5-HT bioavailability, induce proliferation in the hippocampus (Banasr et al., 2004; Boldrini et al., 2009), reduce 5-HIAA levels in human CSF (De Bellis et al., 1993; Sheline, Bardgett and Csernansky, 1997) and animal extracellular fluid (Anderson et al., 2002; Petersen et al., 2000) and alleviate depressive symptoms. Ultimately, these results suggest that DA may positively modulate both the proliferation and maturation phases of neurogenesis, while the catalytic metabolism of 5-HT negatively regulates cell proliferation.

In addition to the metabolic regulators of neurogenesis described above, genetic regulation of neurogenesis was also considered. Specifically, linear regression was used to determine if genes expressed earlier in the progression of neurogenesis influenced the
expression of genes later in the progression of neurogenesis. GFAPD was not found to be a predictor of MKI67 expression, however both GFAPD and MKI67 expression predicted DCX expression. MKI67 and DCX were positively related, in contrast to previous findings presented in this thesis (see Chapter 2). GFAPD was, however, negatively associated with DCX, with higher GFAPD expression corresponding to lower DCX expression. As a marker of stem cell quiescence, the negative association between GFAPD and DCX suggests that increased quiescence may at least partly underlie reduced DCX expression with ageing and disease.

Western blotting was used to semi-quantitatively examine the expression of DCX and PCNA protein along the length of the hippocampus. Group and regional alterations in DCX gene expression were not replicated at the protein level, but PCNA protein levels were significantly decreased in PDD, consistent with mRNA expression of MKI67 and with previous unpublished data from our group (Zhang et al., 2011). The lack of regional and group changes in DCX protein may suggest that DCX gene and protein expression are not correlated; that is the amount of DCX expression does not predict its protein expression. This is contradictory with previous studies however, which clearly demonstrate reductions in DCX protein expression in human ageing cohorts (Boldrini et al., 2018; Dennis et al., 2016; Knoth et al., 2010) and animal models of PD (Kohl et al., 2016; Winner et al., 2004). The discrepancy seen in this study may have resulted from low sensitivity of the semi-quantitative Western blot method, particularly for proteins that are already sparsely-expressed such as DCX. Ideally, quantitative protein analysis methods and/or stereological studies such as those by Dennis et al. (2016) and Boldrini et al. (2018) can further elucidate on how neurogenesis gene expression translates to protein expression.
3.5 Conclusions

This study has demonstrated specific reductions in neurogenesis gene expression in PDD, consistent with previous studies. Additionally, late-stage neurogenesis genes associated with neuronal differentiation and maturation are regionally distributed along the length of the hippocampus in a pattern opposite to that reported in animal models. While both DA and 5-HT metabolism may play a role in the observed changes to neurogenesis gene expression, we found no relationship between neurotransmitters and the maturation gene DCX, regardless of strong regionally-associated levels of 5-HT. These data suggest denervation and reduced neurotransmitters in PDD are responsible for the observed declines in stem cell proliferation and maturation while other, as yet unidentified factors may influence the regional distribution of new-born neurons within the hippocampus.

Importantly, this study demonstrates that animal models, while a valuable resource for studying human disease, should not be assumed to accurately represent the human brain. This is particularly true of studies of neurogenesis, which show vast differences in the rate of new neuronal birth between rodents and humans (Cameron and McKay, 2001; Spalding et al., 2005) and thus call into question the role of neurogenesis in the adult human hippocampus. This study has further highlighted this difference with its finding of different regional patterns of neurogenesis between the human hippocampus and that reported in other species.

Ultimately, these findings are valuable for understanding the dynamics of human hippocampal neurogenesis in ageing and disease, and how both animal and post-mortem human data can, in tandem, help to better understand cognitive change in normal ageing and cognitive decline.
Chapter 4: Growth factor expression is altered in the Parkinson’s disease dementia hippocampus in the absence of neuronal loss


\textsuperscript{a}KJM and SV contributed equally to this work

See Appendix 3.3 for final published journal article.
Abstract

Growth factors can facilitate hippocampus-based learning and memory and are potential targets for treatment of cognitive dysfunction via their neuroprotective and neurorestorative effects. Dementia is common in Parkinson’s disease (PD) but treatment options are limited. We aimed to determine if levels of growth factors are altered in the hippocampus of patients with PD, and if such alterations are associated with PD pathology. Enzyme-linked immunoassays were used to quantify seven growth factors in fresh frozen hippocampus from ten PD and nine age-matched control brains. Western blotting and immunohistochemistry were used to explore cellular and inflammatory changes that may be associated with growth factor alterations. In the PD hippocampus, protein levels of the glial cell line-derived neurotrophic factor (GDNF) were significantly decreased, despite no evidence of neuronal loss. In contrast, protein levels of fibroblast growth factor 2 (FGF2) and cerebral dopamine neurotrophic factor (CDNF) were significantly increased in PD compared to controls. Levels of the growth factors epidermal growth factor (EGF), heparin binding epidermal growth factor (HB-EGF), brain-derived neurotrophic factor (BDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) did not differ between groups. Our data demonstrate changes in specific growth factors in the hippocampus of the PD brain, which potentially represent targets for modification to help attenuate cognitive decline in PD. This data also suggests that multiple growth factors and direction of change needs to be considered when approaching growth factors as a potential treatment for cognitive decline.
**Introduction**

In addition to movement changes, mild cognitive impairment is present in up to 40% of early-stage PD patients (Litvan et al., 2011; Pedersen et al., 2017; Walker et al., 2015), whilst frank dementia is present in 80% of patients 20 years post diagnosis (Hely et al., 2008). Treatment options for dementia in PD are limited (Cooney and Stacy, 2016; Goldman and Weintraub, 2015) but dementia is associated with decreased quality of life (Lawson et al., 2016; Leroi et al., 2012), increased incidence of nursing home admissions (Safarpour et al., 2015), increased carer burden (Martinez-Martin et al., 2015) and increased mortality (Levy et al., 2002) in this disorder. The aetiology of cognitive decline in PD is incompletely understood but is reported to be associated with Lewy body pathology (Kotzbauer et al., 2012), dopaminergic dysfunction (Hoglinger et al., 2004), cholinergic dysfunction (Hall et al., 2014; Irwin et al., 2017) and atrophy within the hippocampus (Delgado-Alvarado et al., 2016; Patel et al., 2017). This widely varying pathology suggests that endogenous factors which maintain hippocampal function may be altered in Parkinson’s disease.

Growth factors represent potential therapeutic agents for neurodegenerative disorders including PD. Glial cell line-derived neurotropic factor (GDNF), cerebral dopamine neurotrophic factor (CDNF), mesencephalic astrocyte-derived neurotrophic factor (MANF) and fibroblast growth factor 2 (FGF2, also referred to as basic FGF) restore and protect dopaminergic neurons in the substantia nigra and striatum of rodent and non-human primate models of PD (Cordero-Llana et al., 2015; Date et al., 1993; Garea-Rodriguez et al., 2016; Tomac et al., 1995). Therapies based on increasing the bioavailability of these factors in the brain may represent a novel approach to attenuate motor dysfunction in PD. GDNF therapy for movement dysfunction has progressed into clinical trials in PD and, while initial results were disappointing, improved drug delivery and treatment during early disease may enable
the translation of pre-clinical success (Bartus et al., 2016; Grondin et al., 2018; Olanow et al., 2015). Recent clinical trials of CDNF have been established to determine its safety and efficacy in attenuating nigrostriatal cell loss, although it is unknown if CDNF and other growth factors are also altered in other brain regions external to the basal ganglia in PD.

Emerging evidence suggests that cognitive dysfunction is associated with altered growth factors in the Alzheimer’s disease (AD) brain (Buchman et al., 2016; Connor et al., 1997; Gómez-Pinilla, Cummings and Cotman, 1990; Sampaio et al., 2017; Schindowski, Belarbi and Buee, 2008; Stopa et al., 1990; Weinstein et al., 2014). Studies of growth factors as candidate therapies in animal models of AD suggests these factors can reduce hippocampal pathology and restore memory function. For example, in rodent models hippocampal-targeted GDNF protect against AD-associated pathology (Revilla et al., 2014), while CDNF improves long-term memory in both aged mice and in an AD mouse model (Kemppainen et al., 2015). FGF2 gene therapy reduces hippocampal plaque load, stimulates neurogenesis and restores spatial learning in a mutant mouse model of AD (Katsouri et al., 2015; Kiyota et al., 2011). Together these data suggest that augmenting the growth factor microenvironment of the hippocampus can influence the development of abnormal pathology and associated hippocampus dysfunction in AD; this relationship may also be pertinent in PD, a disorder commonly associated with cognitive dysfunction. Here we quantified endogenous levels of growth factors and pathology in the hippocampus in PD compared with age-matched control cases to determine if growth factor support in this brain region is altered and associated with concomitant proteinopathy.
**Materials and Methods**

Growth factors GDNF, CDNF, MANF, FGF2, BDNF, epidermal growth factor (EGF) and heparin binding epidermal growth factor (HB-EGF) were chosen for inclusion in this study based on reports demonstrating these factors to be neuroprotective or neurorestorative for dopaminergic neurons (Chen et al., 2013; Cordero-Llana et al., 2015; Farkas and Kriegstein, 2002; Garea-Rodriguez et al., 2016; Iwakura et al., 2005; Jaumotte, Wyrostek and Zigmund, 2016; Shadfar et al., 2018), or that these factors support learning and memory in animal models of AD (Kemppainen et al., 2015; Kiyota et al., 2011; Oyagi et al., 2011; Revilla et al., 2014; Shao et al., 2013). Hippocampal levels of these factors were quantified in ten cases of PD and nine age-matched normal controls. Hippocampal α-synuclein load and glial cell protein markers were also quantified to determine if altered growth factor levels were associated with disease pathology.

**Tissue collection**

This project was approved by the University of New South Wales Human Research Ethics Advisory Panel. Ten PD and nine age-matched control human brains were sourced by the Sydney Brain Bank and NSW Tissue Resource Centre. PD subjects were followed prospectively prior to death and clinical data regarding the severity of movement disorder was quantified annually. Severity of synucleinopathy was quantified according to Braak staging for α-synuclein pathology (Braak et al., 2004) and clinical symptoms of movement dysfunction using Hoehn and Yahr scaling (Hoehn and Yahr, 1967). All PD cases had a clinical diagnosis of dementia and met pathological criteria for dementia according to Braak staging for neurofibrillary tangles (Braak and Braak, 1995) and CERAD plaque staging (Table 1) (Mirra et al., 1991). All PD cases were treated with levodopa, while other anti-parkinsonian medications prescribed for the cohort included entacapone (four cases), selegiline (two cases),
bromocriptine (two cases), cabergoline (two cases), pergolide (two cases), tolcapone (two cases) and amantadine (one case). Control cases revealed no clinical signs or symptoms of neurological or psychiatric disorders and neuropathological abnormalities were absent in all cases.

Brain tissues were prepared and sampled identically in all cases as previously described (Werry et al., 2010). For frozen tissues the head of the hippocampus, containing the cornu ammonis (CA) and dentate gyrus (DG), was dissected from a single coronal block located 0.5-1 cm anterior to the coronal block containing the lateral geniculate nucleus (Fig 1). Formalin-fixed, paraffin-embedded hippocampal tissue from the contralateral hemisphere to the frozen hippocampus was sampled from two cases for double immunofluorescence staining to characterise cell morphology.

Brain pH was determined by the NSW Tissue Resource Centre and Sydney Brain Bank upon collection of donated brains. Briefly, a 1-2 g segment of the lateral cerebellum was homogenized with a hand-held motorized homogenizer in 2.5 mL deionised water. A hand-held pH meter was then used to measure the pH of the homogenate and this reading recorded. Any pH readings below 5.0 or above 7.2 were re-measured to ensure accuracy.
Table 1 Demographic information for all cases included in the experimental cohort.

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<th>Storage time (wk)</th>
<th>Disease duration (y)</th>
<th>Hoehn and Yahr Scale</th>
<th>Braak α-synuclein Staging</th>
<th>Dementia Duration (y)</th>
<th>Dementia Severity&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Braak Tangle Staging</th>
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Values shown are mean ± SEM. PMD- Post Mortem Delay; F- female; M- male; PDD- Parkinson’s disease dementia. *p≤0.05 compared with control group. #Cases used for immunohistochemistry. ^Cerebrovascular accident was noted as cause of death, however pathologically there were no signs of infarction or significant vascular disease in brain tissues. For dementia severity 1=mild, 2=moderate and 3=severe.
**Figure 1** Sampling of hippocampus tissue for the quantification of hippocampal growth factors and semi-quantification of neuronal density

Diagnostic tissue sections used for cell density semi-quantification were sampled from the middle hippocampus at the coronal level indicated by the vertical crosshair (A). This is identified as the coronal level containing the lateral geniculate nucleus (abbreviated LG; B). Hippocampal tissue used for all other experimental procedures was sampled from the coronal level 0.5-1 cm anterior of the level indicated in A. Adapted from Mai et al. (2008).
ELISA

Frozen hippocampal tissue samples used to measure EGF, HB-EGF, FGF2 and BDNF were homogenized as previously described by Werry et al (Werry et al., 2010). BDNF levels were quantified in homogenates according to manufacturer protocol, while EGF, HB-EGF and FGF2 were quantified in supernatant following centrifugation at 14,000 xg for 30 min at 4°C. Tissues for measurement of CDNF, MANF and GDNF were pestle-homogenised in 10x volume of homogenization buffer (137 mM NaCl, 20 mM Tris-HCl, 2.5 mM EDTA, 1% NP-40, 10% glycerol, 0.5mM sodium orthovanadate and cOmplete™ Mini Protease Inhibitor Cocktail (Roche); pH 8.0) on ice and quantified from supernatant following centrifugation at 12,000 xg for 20 min at 4°C.

Protein concentrations in supernatant were measured using a bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA) and levels of growth factor levels normalized to total amount of sample protein extracted. Supernatant protein levels were quantified using ELISA and assayed in triplicate (GDNF in duplicate) according to the manufacturer’s protocol. For each factor, standard curves were run to identify a protein amount within the linear range of detection and ranged from 35-240 ug/sample. Acidification of tissue supernatant with 1 M HCl and re-neutralization with 1 M NaOH prior to analysis for GDNF (Okragly and Haak-Frendscho, 1997) was performed to increase detectability (Werry et al., 2010).

Protein levels for CDNF and MANF were quantified using a custom designed double-antibody sandwich ELISA. Plates were coated (anti-CDNF monoclonal antibody clone 7D6 (Icosagen, Tartu, Estonia) in 1 μg/ml, 0.05 M carbonate coating buffer (pH 9.6) or goat anti-MANF polyclonal antibody (RandD Systems, Minneapolis, MN, USA), 1μg/ml in 0.05 M carbonate coating buffer (pH 9.6)) and incubated overnight at 4°C. Plates were blocked using
3% BSA in PBS for CDNF or 1% casein in PBST (PBS containing 0.05% Tween® 20) for MANF, for 2 h at room temperature then samples added and incubated with shaking at 4°C overnight. Detection antibody horseradish peroxidase (HRP)-linked mouse anti-CDNF clone 6G5 (Icosagen, Estonia; 1:1000) or HRP-linked mouse anti-MANF clone 4E12 (Icosagen, Estonia; 1:1000) was added and incubated with shaking for 5 h at room temperature. Substrate solution (Duoset ELISA Development system, RandD Systems, USA) was added and incubated for 20 min before 1 M sulfuric acid was added.

**Immunohistochemistry**

Immunohistochemistry was performed to investigate cellular morphology associated with altered neurotrophic factor protein levels in the hippocampus. A single 20 μm, formalin-fixed section was sourced from a subset of cases (five PD cases, five control cases indicated in Table 1). Sections were rehydrated, then cleared before antigen retrieval was carried out in citrate buffer (pH 6; Fronine, Sydney, Australia) with 0.05% Tween® 20 in a water bath at 95°C for 30 min. Sections were cooled before quenching endogenous peroxidases with 3% hydrogen peroxide in PBS and then blocked (CDNF: 10% normal horse serum in PBST; GDNF: 0.25% casein in PBST; FGF2: 10% normal goat serum in PBST) and incubated with primary antibody (Appendix 3.1) overnight at 4°C. Sections were then incubated with biotinylated secondary antibody (Appendix 3.1) followed by Vector Elite Kit tertiary antibody complex (Vector Laboratories, Burlingame, CA, USA) before being visualized with 3,3’ diaminobenzidine (Sigma, USA), counterstained with cresyl violet, dehydrated and cover slipped with DPX mountant (VWR International Ltd, Radnor, PA, USA). Negative control sections were performed with primary antibodies omitted (data not shown).
**Immunofluorescence**

To identify cell types containing FGF, CDNF and GDNF, double immunofluorescence staining for astrocytic (gliarial fibrillary acidic protein; GFAP), microglial (ionized calcium-binding adapter molecule 1; Iba1) and neuronal (neuronal nuclei; NeuN) marker proteins using tyramide amplification was completed. Seven micron hippocampal sections from two representative cases (one control and one PD; Table 1) were deparaffinized in xylene and rehydrated through an ethanol gradient before antigen retrieval in citrate buffer (pH 6.0) for 30 mins at 95°C. Sections were washed 3 times in 50% ethanol before endogenous peroxidases were quenched with 0.3% hydrogen peroxide in phosphate buffered saline (PBS). Sections were incubated in blocking solution (0.5% casein, 1% bovine serum albumin and 0.05% Tween® 20 in PBS) before overnight incubation at 4°C in the first primary antibody (Table S1 [Appendix 2.1]) in blocking solution. Sections were then washed and incubated in appropriate HRP-conjugated secondary antibody (Table S1 [Appendix 2.1]) in blocking solution before incubation in 1:50 Cyanine 5 (Cy5)- Tyramide amplification reagent according to the manufacturer’s instructions (Perkin Elmer, Waltham, MA, USA). For fluorescent double staining, the protocol described above was repeated from peroxidase quenching in 0.3% hydrogen peroxide in PBS, using a second primary antibody and appropriate HRP-conjugated secondary antibody (Table S1). Due to low signal intensity sections stained for CDNF were further amplified via incubation in biotinylated anti-goat secondary antibody (1:1000, Vector Laboratories, USA) in blocking solution and Vector Laboratories ABC kit (1:500; both reagents) prior to tyramide amplification. Sections were then incubated in 1:50 Cyanine 3 (Cy 3)-Tyramide amplification reagents according to the manufacturer’s instructions (Perkin Elmer, USA). Sections were washed, incubated in DAPI and coverslipped with 80% glycerol in PBS. Negative control sections were performed with either one or both primary antibodies or
secondary antibodies omitted (data not shown). Acquisition of microscopy images was performed on a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany).

**Semi-quantitative Neuronal Density Measurement**

Single, slide-mounted sections (seven or ten microns thick; sampled from the coronal level of the lateral geniculate nucleus [Figure 1]) stained with hematoxylin and eosin were obtained from the NSW Tissue Resource Centre for all cases and were scanned on an Olympus VS 120 slide scanner (Olympus Corporation, Tokyo, Japan) using extended focus imaging to produce a clear image of the entire section for neuronal quantification. Three sampling squares were placed randomly on the scanned image within the CA1 (300x300 µm), hilus (200x200 µm) and DG (100x100 µm) regions using OlyVIA v.2.9.1 (Olympus Corporation, Tokyo, Japan). Within these defined regions of interest neurons were identified based on characteristic morphology; CA1 and hilus neurons exhibit pyramidal morphology with a large nucleus and a small, dense nucleolus while DG neurons are distinguishable by their ovoid, granular nucleus. Neurons were counted within each sampling square and neuronal density calculated using the sum of the neurons counted and the combined volume of each region of interest. This was standardized to a final measurement of neuronal number/mm$^3$ to normalize the differences in the area of the regions of interest and section thickness.

**Western Blots**

Glial cell protein markers (GFAP, IBA1 and human leukocyte antigen DR [HLA-DR]) and α-synuclein were quantified via western blotting to identify changes in cell populations and pathology respectively. Frozen hippocampal tissue were sonicated in 50 mM Tris-HCl (pH 7.5) buffer (10x volume) containing 125 mM NaCl, 5 mM EDTA disodium salt, 0.002% sodium azide, protease and phosphatase inhibitors (Roche, Germany) and supernatant collected following
centrifugation at 120,000 xg for 2.5 h at 4°C. Pellets were resuspended in buffer containing 5% SDS, sonicated and centrifuged at 100,000 xg for 40 min at 25°C to collect a supernatant termed the SDS fraction.

Equal amount of protein from supernatant (30 μg) and SDS fractions (Iba1 and HLA-DR: 15 μg; GFAP: 30 μg) from each sample were separated on XT Bis-Tris precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA) then transferred onto PVDF membrane (Millipore, USA). Membranes used for α-synuclein analysis were fixed with 0.4% PFA before immunoblotting. Membranes were blocked then incubated with specific primary antibodies (Table S2 [Appendix 2.2]) overnight at 4°C. Membranes were incubated with appropriate secondary antibodies (Table S2 [Appendix 2.2]) and then visualised using Clarity ECL detection (Bio-Rad, USA) according to the manufacturer’s protocol using a ChemiDoc MP Imagining System (Bio-Rad, USA). Band intensity was analysed by densitometry using Image Lab™ Software 4.1 (Bio-Rad, USA) and normalized to β-actin (Millipore, USA).

**Statistical Analysis**

Demographic characteristics, levels of growth factors, glial and Lewy pathology markers in the diagnostic groups were examined using one-way analysis of variance and analysis of covariance (SPSS Statistics 20.0, SPSS Inc., Illinois, USA). Relationships between levels of each factor of interest and brain tissue pH, storage time and post-mortem interval (PMI) were investigated separately in each diagnostic group using linear regression. Number of cases analysed for each factor are presented in all figures and tables as numbers varied with tissue availability. Additionally, two data points greater than two standard deviations from the mean were removed prior to analysis of CDNF levels. Relationships between growth factors, glial protein and pathology marker levels were investigated using linear regression.
Semi-quantitative analysis of neuronal density was examined using an independent t-test, with one data point greater than two standard deviations from the mean removed prior to the analysis of the hilus (Control $n=8$; PD $n=10$). Significance level was set at $p \leq 0.05$ for all analyses.
Results

Age, PMI and brain pH did not differ significantly between control and PD cases (Table 1). Regression analysis demonstrated that protein levels of all growth factors investigated were not associated with brain pH, PMI, age or storage time in either control or PD cases. Similarly, protein levels of all growth factors were not associated in PD with either dementia-related functional and neuropathological scores (dementia duration, dementia rating, Braak tangle stage or CERAD plaque stage) nor with movement disorder-related functional and neuropathological scores (disease duration, Hoehn and Yahr score or Braak α-synuclein stage). Freezer storage time was longer in the PD group (p=0.02; Table 1), however, levels of all proteins were independent of storage time in this group, suggesting no confounding effect. Levels of total α-synuclein quantified using immunoblotting were unchanged in the hippocampus in PD (F(1,18)=1.05, p=0.32), however, levels of phosphorylated α-synuclein (phosphorylated at serine 129: pS129), specifically associated with PD pathology were significantly increased in the PD hippocampus (F(1,17)=4.68, p=0.05; Fig. 3g), confirming disease-associated pathology in the hippocampus of the PD cases.

Protein levels of neuronally-expressed neurotrophic factors are altered in the PD hippocampus in the absence of an alteration in neuronal density

Hippocampal levels of GDNF protein were significantly decreased (19%) in PD, compared with age-matched controls (F(1,11)=5.55, p=0.04; Fig. 2A). In contrast, CDNF protein levels (F(1,11)=8.37, p=0.02; Fig. 2D) were significantly increased (41%) in the PD hippocampus, compared with age-matched controls. GDNF- and CDNF-immunopositive cells expressed morphology consistent with that of neurons within the hippocampus of controls (Figs. 2B and 2E) and PD cases (Figs. 2C and 2F), and double immunofluorescence with the neuron marker protein NeuN confirmed that these growth factors were exclusively found in NeuN+ cells in
both control and PD hippocampus (Figs. 2G and 2H). GDNF and CDNF staining was most marked in the neurons of the hilus and CA regions of the hippocampus. Less intense staining for CDNF was also observed in neurons in the granule cell layer of the DG, whereas no GDNF-positive cells were observed in DG granule neurons. Despite the significant alterations in hippocampal GDNF and CDNF protein levels observed here, overall density of neurons was unchanged in the hilus ($t_{(16)}=1.806; p=0.090; \text{Fig. 2I}$), DG ($t_{(16)}=-1.554; p=0.139; \text{Fig. 2J}$) and CA1 ($t_{(17)}=1.319; p=0.205; \text{Fig. 2K}$). These are consistent with previous quantitative measures of cell density in normal ageing (Harding et al., 1997; Šimić et al., 1997; West and Gundersen, 1990), as well as with reports of hippocampal atrophy (Camicioli et al., 2003) without neuronal loss (Harding, Lakay and Halliday, 2002; Joelving et al., 2006).
Figure 2 Alterations in the protein level of the neuronally-expressed growth factors GDNF and CDNF
GDNF protein levels were decreased (A) whilst CDNF (D) was increased within the Parkinson’s disease hippocampus. GDNF-positive cells were found in both controls (B; scale bar = 25 μm) and PD cases (C; scale bar = 25 μm) and had morphology consistent with neurons. CDNF-positive cells also had morphology consistent with neurons in controls (E; scale bar = 25 μm) and PD cases (F; scale bar = 25 μm). This was confirmed with double immunofluorescence labelling, demonstrating that both GDNF (G; scale bar = 10 μm) and CDNF (H; scale bar = 25 μm) co-localize with the neuronal marker NeuN. Despite being expressed in neurons, the alterations in both GDNF and CDNF could not be associated with changes in cellular density, with no apparent change to cell numbers in the hippocampal hilus (I), dentate gyrus (J) or CA1 (K). Data represented as individual values overlaying mean ± SEM.
**Hippocampal protein levels of FGF2 are increased in the PD hippocampus**

Hippocampal levels of FGF2 were significantly increased (40%) in the PD hippocampus ($F_{(1,17)}=7.47, \ p=0.01$; Fig. 3A) and FGF2-positive cells in both control (Fig. 2B) and PD (Fig. 2C) hippocampus were present in the hilus and all CA regions. Consistent with our previous finding that FGF2 is primarily present in glial cells, double immunofluorescence for FGF2 and cell type-specific protein markers demonstrated microglia (Iba1$^+$; Fig. 3D) and astrocytes (GFAP$^+$; Fig. 3E) strongly expressed FGF2, whereas only moderate staining was observed in neurons (NeuN$^+$; Fig. 3F). Activated microglia, strongly expressing HLA-DR, were observed in the PD hippocampus however, very few of these cells were observed to be FGF2-positive.

Levels of hippocampal EGF ($F_{(1,17)}=4.16, \ p=0.06$), HB-EGF ($F_{(1,17)}=0.12, \ p=0.73$), BDNF ($F_{(1,15)}=0.46, \ p=0.51$) and MANF ($F_{(1,13)}=0.28, \ p=0.61$) did not vary between PD and control cases.
Figure 3  Alterations in the protein level of the glial-expressed growth factor FGF2 and its association with glial protein markers

FGF2 was significantly increased in the PD hippocampus (A) and showed morphology consistent with neurons and glia in controls (B; scale bar = 25 μm) and PD cases (C; scale bar = 25 μm). Double immunofluorescence staining confirmed that FGF2 co-localized mainly with markers of microglia [ionised calcium binding adaptor 1 (Iba1); D; scale bar = 10 μm] and astrocytes [glial fibrillary acidic protein (GFAP); E; scale bar = 25 μm]. Some staining was also seen in neurons (NeuN; F; scale bar = 10 μm). Western blotting analysis (G) demonstrated that levels of GFAP (P = 0.495), Iba1 (P = 0.154), activated microglia marker, human leukocyte antigen-DR (HLA-DR; P = 0.559) and total α-synuclein (α-syn; P = 0.32) did not differ between PD and age-matched controls. However, levels of α-synuclein phosphorylated at serine 129 (pS129 α-syn) were significantly increased (P = 0.05) in the PD hippocampus. Levels of FGF2 protein were significantly associated with GFAP protein in both PD and controls (H; dashed
line) and this association remained when the PD cohort was analysed separately (H; grey line). Data represented as individual values with overlying mean ± SEM.
Association between growth factors, glial and pathology markers.

Levels of FGF2 and CDNF co-varied for the cohort as a whole ($r^2=0.559$, $p=0.003$), however this relationship reflected an association in the control ($r^2=0.938$, $p=0.001$), but not PD, cases ($r^2=0.118$, $p=0.451$). Levels of FGF2 and GDNF ($r^2=0.011$, $p=0.74$), and levels of GDNF and CDNF ($r^2=0.109$, $p=0.749$) were not associated. There were no further associations between CDNF and GDNF (Control $r^2=0.622$, $p=0.113$; PDD $r^2=0.024$, $p=0.771$) or FGF2 and GDNF (Control $r^2=0.486$ $p=0.124$; PDD $r^2=0.004$, $p=0.894$) when the cohorts were analysed separately.

As FGF2 is strongly expressed by glial cells and levels of this protein are increased in the PD hippocampus, levels of glial cell markers were investigated via immunoblotting (Fig. 3G). Total hippocampal levels of the astrocytic marker GFAP ($F_{1,15}=2.26$, $p=0.15$), microglial marker Iba1 ($F_{1,12}=0.36$, $p=0.56$) and activated microglial marker HLA-DR ($F_{1,17}=0.48$, $p=0.49$) were unchanged in PD compared with age-matched controls. FGF2 levels were, however, significantly associated with GFAP levels in the cohort as a whole ($r^2=0.34$, $p=0.02$) and this association was preserved when the PD cases were analysed separately ($r^2=0.44$, $p=0.05$; Fig. 3H). No association was found between any of the growth factors investigated and pS129 α-synuclein protein levels.
Discussion

While GDNF-based treatment strategies for attenuating movement dysfunction are the subject of current clinical trials (Dere et al., 2006; Gill et al., 2003; Slevin et al., 2005), a role for GDNF in other brain functions, such as cognition and memory, has been little explored. GDNF contributes to normal hippocampal development (Irala et al., 2016) and hippocampal GDNF levels are maintained throughout the healthy human lifespan (Werry et al., 2010), suggesting this protein may have an ongoing effect on the regulation of hippocampal function. In contrast to the healthy aged hippocampus, but similarly to PD striatum and substantia nigra (Mogi et al., 2001) we found a significant decrease in GDNF in PD.

GDNF co-localised exclusively to neuronal markers in immunofluorescence staining however we found no apparent neuronal loss in the hippocampal CA1, hilus and dentate gyrus, suggesting that a reduction in hippocampal GDNF in PD does not simply reflect the death of neurons containing this protein, but rather a potential reduction of GDNF per neuron and thus the involvement of other disease-related processes other than apoptotic or necrotic activation. Reduced synaptogenesis may underlie hippocampal atrophy in PD, which demonstrates an overall loss of volume without neuronal loss, as described in previous studies (Harding, Lakay and Halliday, 2002; Joelving et al., 2006). Previous rodent studies demonstrate a role of GDNF in driving axonal and dendritic sprouting in the hippocampus (Irala et al., 2016) and basal ganglia (Rosenblad, Kirik and Björklund, 2000), suggesting that it may also contribute to structural reorganisation and plasticity of hippocampal cells in addition to, or as a component of, neuroprotection or neurorestoration. Alterations in human synaptogenesis and plasticity are strongly associated with cognitive decline in humans (Jackson et al., 2017; Scheff et al., 2006). The observed reduction in GDNF in the hippocampus
in PD is therefore consistent with reduced neuroplasticity, whereby reduced GDNF contributes to cognitive decline by attenuating synaptic function.

It is also important to note that our finding of reduced GDNF in the hippocampus is not necessarily reflective of a pathological change affecting the hippocampus itself. GDNF is typically delivered to neuronal soma via retrograde transport (Tomac et al., 1995), therefore cells containing GDNF may be representative of either GDNF-receiving or GDNF-producing cells. Given that GDNF mRNA is present in the hippocampus (Imamura et al., 2005; Tanichi et al., 2018) we speculate that the neurons found to be immunopositive for GDNF in this study were producing rather than receiving GDNF. If so, reduced levels of GDNF may not only affect the hippocampus itself, but also other brain regions which innervate it and which may be dependent on receiving GDNF from hippocampal neurons. For example, the cholinergic basal forebrain and noradrenergic locus coeruleus directly innervate the hippocampus (Mesulam, 2013; Sara, 2009), contribute to cognitive function (Chalermpalanupap et al., 2017; Teipel et al., 2018) and degenerate in PD (Hall et al., 2014; Zarow et al., 2003). GDNF is also suggested to be neuroprotective of these cells (Pascual et al., 2008; Williams et al., 1996). Ultimately, these combined findings may indicate that alterations in GDNF affect hippocampal function via a reduction in endogenous neuroprotection as well as failing to adequately maintain connectivity between the hippocampus and other brain regions. Future studies into the role of GDNF on hippocampal function are needed to further elucidate alternative pathways via which GDNF may affect hippocampal function and therefore contribute to cognitive change in PD.

Together with the observed decrease in GDNF expression, we found significant increases in the expression of both the neuron-associated CDNF and glia-associated FGF2,
demonstrating for the first time that CDNF is present in hippocampal neurons. Both CDNF and FGF2 exhibit neuroprotective and neurorestorative properties for dopaminergic neurons (Airavaara et al., 2012; Bäck et al., 2013; Lindholm et al., 2007; Takayama et al., 1995; Zhu et al., 2015); the reported increase in both factors in this study may therefore reflect a similar function towards glutamate neurons in the hippocampus, evidenced by consistent neuronal density in PD cases compared with controls. CDNF is thought to modulate protein folding in the endoplasmic reticulum, preventing the accumulation of misfolded proteins which may then induce endoplasmic reticulum stress and stress-induced apoptosis (Huang et al., 2016; Voutilainen et al., 2015). Phosphorylated α-synuclein (Sugeno et al., 2008) and α-synuclein oligomers (Colla et al., 2012) are known inducers of endoplasmic reticulum stress, which has been well-characterised in the PD brain (Tsujii, Ishisaka and Hara, 2015; Wang and Kaufman, 2016). Here we found significantly increased levels of phosphorylated α-synuclein in the PD hippocampus, thus we speculate that increased levels of CDNF in this brain region reflects a protective response by these stressed hippocampal neurons. Interestingly, we found no significant difference in MANF levels between PD and control cases. Given that the structure and function of CDNF and MANF are closely related (Lindahl, Saarma and Lindholm, 2017), this may indicate an alternative role of CDNF in the progression of PD pathology, warranting further investigation in future studies.

We also found a significant increase in FGF2, in contrast to the PD midbrain (Mogi et al., 1996; Tooyama et al., 1993) or healthy aged hippocampus (Werry et al., 2010). FGF2 is involved in the regulation of astrocytic and microglial reactivity and neuroinflammation (Blanco-Alvarez et al., 2015; Yoshimura et al., 2001); here we observed a positive association between FGF2 and GFAP expression in the PD hippocampus which may be reflective of these mechanisms. However, we also observed consistent levels of microglial and cell reactivity
protein markers in PD and control cases, which does not support a marked inflammatory response in this brain region in our cases of advanced PD. The lack of gliosis in the hippocampus of PD patients would be consistent with previous reports of an attenuated inflammatory response in late stage PD (Doorn et al., 2014; Mirza et al., 1999; Song et al., 2009). Alternatively, increased levels of FGF2 are found in AD hippocampus and co-localise to senile plaques (Cummings, Su and Cotman, 1993; Kato et al., 1991), however we found no association between FGF2 levels and severity of either AD or PD proteinopathy in the PD hippocampus. Given that FGF2 is not strongly associated with glial reactivity markers in this study, we suggest that an alternate mechanism underlies the increase in FGF2 expression seen in this study. FGF2 is a modulator of long-term potentiation and synaptic plasticity in hippocampal neurons (Cuppini et al., 2009; Ishiyama, Saito and Abe, 1991; Zhao et al., 2007), both of which are important for learning and memory (Nicoll, 2017; Zhao et al., 2007). This is similar to the previously described role of GDNF, indicating that FGF2 may be upregulated as a compensatory response to buttress synaptic function in the absence of GDNF. This is supported by work demonstrating the importance of growth factors in combination in order to exert a neuroprotective effect (Jaumotte, Wyrostek and Zigmond, 2016).

An important factor to consider in this study is that there is currently no information available regarding the effect of drugs for the treatment of PD on levels of growth factors. L-DOPA in particular has a documented effect on rodent neurogenesis (Hoglinger et al., 2004), although there are currently no studies investigating the effect of Parkinsonian drugs on growth factor levels in humans. The predominance of L-DOPA as a first-line treatment of PD means that few, if any human subjects have been unexposed to L-DOPA, therefore no control group exists for comparison.
GDNF is reduced in the PD striatum and substantia nigra, and has demonstrated a strong potential as a treatment option to attenuate dopaminergic cell death. In this study we aimed to determine if this factor or others may similarly represent a tractable and effective treatment option for cognitive decline, which is a common and debilitating consequence of PD. We found significant reductions in levels of GDNF but increased levels of the CDNF and FGF2 in the PD hippocampus, in the absence of apparent neuronal loss. These data suggest that alterations in GDNF protein may contribute to cognitive decline in PD, although the lack of apparent neuronal loss suggests that hippocampal GDNF exerts its neuroprotective function differently to GDNF within the dopaminergic midbrain. Given that GDNF-based treatments are already in Phase 2 clinical trials, we suggest that these treatment options may also be suitable for further studies of GDNF supplementation for the management of cognitive decline in PD.
Chapter 5: General Discussion and Conclusions
5.1 Summary of Findings

In this thesis I have investigated the dynamics of hippocampal neurogenesis in human post-mortem tissue, with the goal of better understanding how neurogenesis is distributed along the length of the hippocampus, how neurogenesis is affected in normal, healthy ageing and PDD, and several factors which may contribute to these changes.

The first aim of the thesis was to quantify neurogenesis gene expression across the healthy human life span. To do this I used qRT-PCR, a sensitive and reliable method of gene expression quantification. I found specific declines in the genes \textit{MKI67} and \textit{DCX}, associated with stem cell proliferation and neuronal maturation, respectively. In addition, using immunofluorescence I found significant morphological changes to astrocytes, possibly reflective of a changed glial microenvironment associated with increasing age.

Altered neurogenesis is suggested to play a role in cognitive decline and the development of dementia (Christie et al., 2012; Kempermann, Wiskott and Gage, 2004; Monje and Dietrich, 2012). Since I found a significant decline in neurogenesis gene expression in normal ageing, itself the most common risk factor for the development of dementia (van der Flier and Scheltens, 2005), I chose to explore these changes in dementia. In this work I used PDD as a representative dementia disorder in which reduced neurogenesis has been demonstrated (Hoglinger et al., 2004). I aimed to explore neurogenesis along the length of the hippocampus, as previous studies have suggested that the hippocampus is structurally and functionally heterogenous (Kempadoo et al., 2016; Moser, Moser and Andersen, 1993; Strange et al., 2014; Trivedi and Coover, 2004) and that the rate of neurogenesis is not consistent along its length (Jinno, 2011; Lowe et al., 2015; Snyder et al., 2009). Therefore, the second aim of this thesis was to quantify neurogenesis gene expression along the length of the human hippocampus, in normal ageing and in dementia. I found significant regional
differences in the expression of the mid- to late-stage neurogenesis markers *EOMES* and *DCX*, suggesting that the differentiation and maturation of new neurons may proceed at different rates along the length of this hippocampus. These regional changes were consistent in healthy young controls, healthy aged controls and in PDD cases; no preferential loss of new neurons was found in the posterior hippocampus in PDD cases as hypothesised. Importantly, these regional differences were opposite to what has been previously reported in animal studies. I also found a reduction in the expression of *MKI67* and *DCX* in PDD compared to young controls. Aged controls in this instance appear to represent a mid-point in neurogenesis gene expression, with less expression than in young controls but more than PDD cases.

Given that neurogenesis gene expression is regionally variable, and is altered in normal ageing and in PDD, I wanted to explore regulatory factors which may contribute to these changes. The hippocampus is innervated by a range of brain areas, releasing a variety of neurotransmitters suspected to modulate neurogenesis (Banasr et al., 2004; Coradazzi et al., 2016; Mohapel et al., 2005; Winner et al., 2009). The third aim of this study was therefore to quantify levels of neurotransmitters along the hippocampus and in young, aged and PDD cases. I found a significant decline in the DA metabolite DOPAC and an increase in the 5-HT metabolite 5-HIAA in PDD. Both DOPAC and 5-HIAA were significant predictors of *MKI67* expression and DOPAC was also a significant predictor of *DCX* expression. Neither 5-HT nor the DA metabolite HVA were significant predictors of *DCX* expression, regardless of having a similar regional distribution along the length of the hippocampus.

Growth factors are also suspected to be neuroprotective, neurorestorative and strong regulators of hippocampus-based cognitive function (Kemppainen et al., 2015; Revilla et al., 2014; Zhang et al., 2014). In the fourth aim, I examined growth factor alterations in PDD, building on findings by a previous PhD candidate, Dr Sophie Virachit. GDNF was significantly
reduced in PDD, while CDNF and FGF2 were significantly increased. These changes were largely independent of pathological and neuroinflammatory changes in the PDD hippocampus, as well as in the absence of any major neuronal cell loss across multiple hippocampal subfields.

5.2 Significance of Findings

Chapter 2 explored hippocampal gene expression over the course of the healthy human life span. Independent declines in the proliferation marker MKI67 and DCX were found across the healthy human life span, in the absence of any change to the stem cell marker GFAPD. The decline in gene expression with age seen here is consistent with previous human post-mortem studies of neurogenesis using protein markers (Dennis et al., 2016; Knoth et al., 2010). This suggests that declining neurogenesis is a result of cellular or microenvironmental dysfunction affecting neurogenesis at the genetic level, rather than at the translational or post-translational level. The independence of the decline in MKI67 and DCX also suggest that two separate pathological events are influencing neurogenesis declines in ageing, rather than reduced DCX expression being a consequence of earlier declines in MKI67 expression.

Chapter 3 expanded on these findings by investigating the expression of the same subset of neurogenesis-related genes along the length of the hippocampus and in healthy young, healthy aged and PDD cases. Again, MKI67 and DCX expression was reduced in both normal ageing and PDD consistent with previous studies of cellular proliferation and maturation (Hoglinger et al., 2004). Interestingly the genes EOMES and DCX, both associated with neuronal differentiation and maturation, were found to be regionally distributed along the length of the hippocampus in a pattern opposite to that reported in animal studies (Jinno, 2011; Lowe et al., 2015; Snyder et al., 2009). Contrary to the hypothesis of this thesis, neurogenesis gene expression was lowest in the posterior hippocampus and was not regionally affected in cases with dementia. Neurotransmitters from various brain regions are
considered modulatory factors for neurogenesis. The neurotransmitters DA and 5-HT have been strongly associated with the cognitive and emotional roles of the hippocampus (Fremeau et al., 1991; Kempadoo et al., 2016; Luo, An and Zhang, 2008; Mahar et al., 2014; Silva et al., 2012), and I hypothesised that they may be regionally distributed along the length of the hippocampus, reflective of preferential roles in emotional regulation and learning (5-HT) and episodic and spatial memory (DA). While both 5-HT and the DA metabolite HVA were regionally-distributed in a pattern similar to DCX, neither significantly predicted DCX expression. The DA metabolite DOPAC and 5-HT metabolite 5-HIAA, however, were significant predictors in the decline in MKI67 and DCX expression seen in healthy ageing and in PDD. Hippocampal neurotransmitters do appear to regulate neurogenesis, but these changes are age- and pathology-specific, rather than having an overall impact on the regional distribution of neurogenesis along the length of the hippocampus. Other factors may influence these regional changes, however these are still unknown.

Chapter 4 considered the influence of growth factors as potential regulators of hippocampal function, particularly in cases of cognitive decline. This is of particular translational importance, as growth factors have already been investigated in clinical trials for the attenuation of dopaminergic cell loss in PD. While the results of these trials were disappointing, it is worthwhile to consider the applicability of these clinical trials to other brain regions which may benefit from growth factor therapies. GDNF was significantly decreased in PDD cases, while CDNF and FGF2 was increased. These factors have been previously associated with cognitive function in PD and AD (Kemppainen et al., 2015; Revilla

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1 Trial titles - AAV2-GDNF for Advanced Parkinson’s Disease; Clinical Study to Test the Safety of CDNF by Brain Infusion in Patients With Parkinson’s Disease; GDNF in ideopathic Parkinsons Disease. See references for more details.
et al., 2014; Zhang et al., 2014), or with attenuating cellular death in degenerating regions of the PD brain (Cordero-Llana et al., 2015; Garea-Rodriguez et al., 2016; Jaumotte, Wyrostek and Zigmond, 2016). No change to hippocampal cell number was seen, regardless of previous human imaging studies demonstrating a loss of total hippocampal volume in PD (Camicioli et al., 2003; Patel et al., 2017). This suggests that the changes in growth factors seen here may result from up- or down-regulation, rather than as a consequence of cellular loss. This study indicates that multiple growth factors may play a role in regulating hippocampal function but that complex compensatory mechanisms involving multiple growth factors is likely affecting hippocampal function in PDD.

Ultimately, this thesis has expanded on our current knowledge of human hippocampal neurogenesis in ageing and disease in several ways. For the first time it has been demonstrated that neurogenesis is altered at the genetic level in both ageing and in PDD; this decline appears to affect both the proliferation of stem cells and the maturation of neuroblasts independently. These changes may be at least partly attributable to alterations in hippocampal neurotransmitters and growth factors, particularly those originating for degenerating regions of the PD brain. Importantly, the results of this thesis suggest that the distribution of new neurons along the length of the hippocampus differs to that previously reported in other species. This highlights the importance of using human studies to confirm and clarify the results of animal studies, particularly when considering both neurogenesis and PD.
5.3 Experimental Strengths

Studies of neurogenesis have resulted in a large array of findings; however the function, purpose and regulation of neurogenesis is still uncertain. This is particularly true when considering human hippocampal neurogenesis, which has not been as extensively studied as in other species and remains controversial. This thesis exclusively utilises human post-mortem tissue throughout, thus offering a direct insight into the specifics of human hippocampal neurogenesis and its regulation. This is particularly important in studies of neurogenesis since animals (rodents in particular) produce vastly more new neurons daily (Cameron and McKay, 2001; Spalding et al., 2005) thus the significance of this difference to human neurogenesis requires further elucidation through post-mortem studies such as this. Similarly, human post-mortem studies are important for studies of PD as no completely representative animal models of PD exist. Ultimately studies such as these are a necessary stepping stone between animal and translational studies, making this work a valuable contribution to the current literature.

A major strength of this thesis is the quantification of multiple neurogenesis-related genes representing the various stages of neurogenesis. Many studies of neurogenesis investigate either proliferation of stem cells or neuronal maturation; this however offers a limited view of the many stages of neurogenesis, any of which could potentially be affected by ageing and disease. By examining multiple stages of neurogenesis, I have shown two independent changes in cell proliferation and maturation in age, giving a better overall understanding of how neurogenesis is affected in age. I have also shown these two genes to be reduced further in the PDD hippocampus, suggesting that this disease-associated decline in neurogenesis is continuous with the decline in neurogenesis associated with normal physical and cognitive ageing.
5.4 Experimental Limitations

In this thesis, mRNA expression of neurogenesis genes was measured in human post-mortem tissue. qRT-PCR is a sensitive and fully quantitative method which, unlike semi-quantitative methods such as Western blotting, limits variability within replicates and between experimental groups. However, measuring gene expression in the hippocampus does not necessarily accurately represent the translation of that gene, nor how many cells are expressing it. Additionally, general proliferation markers such as MKI67 may be indicative of proliferation of other cell types such as microglia and astrocytes. While these results and previous work generally agree that stem cell proliferation and maturation is reduced in age and PD, this cannot be conclusively demonstrated in this thesis. Stereological analysis would complement this gene expression data and be a valuable addition to our existing knowledge of human hippocampal neurogenesis. However, protein is sensitive to PMI, storage time and fixation method; DCX in particular is reported to be quickly degraded in human post-mortem tissue (Boekhoorn, Joels and Lucassen, 2006). On the other hand, mRNA is better able to withstand increased PMI and storage time if handled correctly (White et al., 2018). This was demonstrated in Chapter 3; neither MKI67 or DCX expression was significantly predicted by PMI. The results in this thesis show that a gene expression approach may be a promising method to reliably and reproducibly examine neurogenesis changes in the hippocampus, although further validation of this method is required in future studies to reconcile gene and protein expression in human post-mortem tissue.

The examination of hippocampal neurotransmitters in Chapter 3 could also be improved in future studies. Like qRT-PCR, HPLC is also a sensitive quantitative technique which provides reliable data. The standard curve that was created extended to the limit of detection for this technique. In many cases neurotransmitter levels fell below the limit of
detection, thus could not be reliably calculated from the standard curve. As a result, multiple data points were deleted from each experimental group, as shown in Table 3.3. Measuring neurotransmitters, particularly those with a sparse innervation of the hippocampus such as DA (Gaspar et al., 1989; Torack and Morris, 1990; Goldsmith et al., 1994), is therefore particularly difficult in human post-mortem tissue. However, the neurotransmitter levels noted in this thesis do not differ significantly to those previously described (Scatton et al., 1983; Semerdjian-Rouquier et al., 1981). While these results provide valuable preliminary data on patterns of hippocampal innervation, more sensitive quantitation methods of human hippocampal neurotransmitters, such as mass spectrometry or enzyme-linked immunosorbent assay, may provide more data for future analyses.

5.5 Future Directions

As discussed in Chapters 5.3 and 5.4, examining neurogenesis gene expression may be a reliable way of investigating age- and pathology-related changes in hippocampal neurogenesis, particularly in human tissue in which protein quality is often affected by long storage times and PMIs. This method does require further validation, however. It would be of interest to repeat these experiments using in situ hybridisation techniques to determine conclusively if changes in mRNA expression are truly a consequence of fewer cells expressing these genes, or rather a comparable number of cells expressing the genes at a lower rate. With this information, it could be definitively determined if gene expression is a valid method of investigating changes to neurogenesis. This would be of great benefit to future studies in human post-mortem tissue, which is of limited supply and difficult to obtain in quantities sufficient for stereological analysis, currently considered the gold standard for quantifying human neurogenesis. Also, the recent paper by Sorrells et al. (2018) has been controversial as it suggest that no human neurogenesis could be detected after adolescence. It also states
that the finding of reduced DCX-positive cells in the hippocampus is a consequence of DCX upregulation in microglia. Additional studies utilising immunofluorescence and/or in situ hybridisation should be attempted in future to either support or deny this assertion.

In Chapter 3 I quantified hippocampal neurotransmitters using HPLC. While the results of this analysis were promising, some data points were deleted as the transmitter or metabolite levels were too low to be reliably quantified. In future, it would be prudent to also use an alternate technique for quantifying these neurotransmitters, such as enzyme-linked immunosorbent assay. It would also be of interest to re-optimise the method to detect a wider range of neurotransmitters; in this instance our methodology was not well-optimised for detecting noradrenaline, for example. Further optimisation of these techniques will provide additional clarity to the distribution and role of neurotransmitters to hippocampal neurogenesis.

In Chapter 4, growth factors were examined as potential regulators of hippocampal function which may be altered in PDD. This study, however, was not able to link alterations in hippocampal growth factors to alterations in neurogenesis due to a lack of tissue availability. If a new cohort could be obtained with sufficient tissue, it would be of interest to quantify neurogenesis, neurotransmitter levels and growth factor levels in the same cohort, to better understand the interplay of all of these factors on the regulation of neurogenesis and overall hippocampal function.

5.6 Conclusions
The work presented in this thesis demonstrated that alterations in human hippocampal neurogenesis in both healthy ageing and PDD begin at the genetic level, rather than as a result of protein dysfunction. These alterations may be the result of specific pathological changes to hippocampal neurotransmitters and growth factors. It also demonstrates that the patterns
of neurogenesis which are accepted in animal models may not be representative of the human hippocampal microenvironment. These findings add important information to our understanding of the specific patterns of human neurogenesis. Ultimately, this work presents a novel method of quantifying neurogenesis in humans, adds to our knowledge of this process, and highlights the importance of careful interpretation and application of animal models in the study of human disease states.
Appendices
## Appendix 1.1 Demographic information for cases used in Chapter 2

<table>
<thead>
<tr>
<th>Case Number</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Cause of Death</th>
<th>PMI (hours)</th>
<th>pH</th>
<th>RIN</th>
<th>qRT-PCR</th>
<th>IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>Male</td>
<td>Cardiac failure</td>
<td>28.5</td>
<td>6.70</td>
<td>7.9</td>
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<td>x</td>
</tr>
<tr>
<td>2</td>
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<td>Trauma</td>
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<td>6.3</td>
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<td>x</td>
</tr>
<tr>
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<td>6.7</td>
<td></td>
<td>x</td>
</tr>
<tr>
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<td>Female</td>
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<td>x</td>
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<tr>
<td>5</td>
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<td>x</td>
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<tr>
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<tr>
<td>7</td>
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<tr>
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<tr>
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<td>x</td>
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<tr>
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</tr>
<tr>
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<td></td>
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<tr>
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<td>86</td>
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<td>Infection</td>
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<td>6.94</td>
<td>6.9</td>
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<tr>
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<td>9.0</td>
<td>6.36</td>
<td>6.2</td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

Average: 56.6 - - 30.2 6.62 6.73 - -

SEM (±): 3.69 - - 2.32 0.04 0.14 - -

PMI – post-mortem interval, RIN – RNA integrity number, qRT-PCR – Qualitative reverse transcriptase polymerase chain reaction, IF – Immunofluorescence, SEM – Standard error of the mean, * - Case excluded in calculation of mean ± SEM due to RIN <5.0
### Appendix 1.2 TaqMan probes used for gene expression analysis in Chapters 2 and 3

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>NCBI Reference Sequence</th>
<th>TaqMan Assay ID</th>
</tr>
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<tbody>
<tr>
<td>Glial fibrillary acidic protein, isoform delta</td>
<td>GFAP</td>
<td>NM_001131019.2</td>
<td>A1IJJYD</td>
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<tr>
<td>Ki67</td>
<td>MKI67</td>
<td>NM_002417.4</td>
<td>HS01032443_m1</td>
</tr>
<tr>
<td>Eomesodermin</td>
<td>EOMES</td>
<td>NM_001278182.1</td>
<td>HS00172872_m1</td>
</tr>
<tr>
<td>Doublecortin</td>
<td>DCX</td>
<td>NM_000555.3</td>
<td>HS00167057_m1</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein, isoform alpha</td>
<td>GFAP</td>
<td>NM_002055.4</td>
<td>HS00909233_m1</td>
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<tr>
<td>S100 calcium binding protein B</td>
<td>S100B</td>
<td>NM_006272.2</td>
<td>HS00902901_m1</td>
</tr>
<tr>
<td>Importin 8 (housekeeping gene)</td>
<td>IPO8</td>
<td>NM_001190995.1</td>
<td>HS00183533_m1</td>
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<td>TATA-box binding protein (housekeeping gene)</td>
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<td>Ubiquitin C (housekeeping gene)</td>
<td>UBC</td>
<td>NM_021009.5</td>
<td>HS00824723_m1</td>
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</table>
Appendix 1.3 Supplementary methods published with Chapter 2

Human post-mortem brain tissue

Post-mortem tissue from the anterior hippocampus was provided from 29 healthy individuals aged between 18 and 104 years (New South Wales Tissue Resource Centre, Sydney, Australia). Each case was free of significant neuropathology upon post-mortem examination. Fresh frozen tissue was collected from all 29, of which 26 were used in the final analysis. Additionally, formalin fixed paraffin embedded (FFPE) hippocampus tissue was also obtained from a subset of cases of the full cohort aged 18-88 (n=5), taken from the opposite brain hemisphere to the frozen tissue (Supplementary Table 1). This study was approved by the Human Research and Ethics Committee at the University of Sydney (USyd HREC 2015/584).

RNA extraction and cDNA synthesis

Fresh frozen tissue was supplied in 60 µm thick sections, from which the hippocampus was dissected and collected for RNA extraction. Briefly, 800 µL of TRIzol reagent (Thermo Fisher Scientific; 15596018) was added to 50-100 mg of tissue. The tissue and reagent were homogenised manually with a disposable pestle and incubated at room temperature for 5 minutes to allow dissociation of the nucleoprotein complex. 160 µL of chloroform was added to each sample and vortexed briefly to mix. After incubating for 3 minutes at room temperature, the samples were spun in a centrifuge at 12,000 xg (15 mins, 4°C) to facilitate phase separation. The aqueous phase was pipetted into a new tube and the RNA precipitated from solution by adding 500 µL of isopropanol and incubating for 10 minutes at room temperature. The RNA/isopropanol mixture was centrifuged a second time at 12,000 xg (10 mins, 4°C). The supernatant was poured off and the resulting RNA pellet resuspended in 500 µL of 75% ethanol. The tubes were then centrifuged a third time at 7500 xg (5 mins, 4°C). The supernatant was poured off and the RNA pellet allowed to air dry. The dry pellet was then
resuspended in 50 µL of RNase-free water and stored at -80°C until further use. RNA concentration and purity was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and RNA integrity number (RIN) by the Agilent 2100 Bioanalyser (Agilent Technologies, California, USA). Samples with a RIN less than 5.0 (n=3) were excluded from mRNA analysis due to poor RNA quality, the average RIN of the remaining cases used for all statistical analyses was 6.73 ± 0.14.

cDNA was synthesised using the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen; 18080400). 2 µg of RNA from each case was combined with 1 µL of both random hexamers and annealing buffer. RNase-free water was added to bring the final volume to 8 µL. Samples were incubated in a thermocycler at 65°C for 5 minutes before being placed immediately on ice for at least 1 minute. 10 µL of 2x First-Strand Reaction Mix was added, followed by 2 µL of ice-cold SuperScript III/RNaseOUT Enzyme Mix. Samples were then immediately incubated in a thermocycler for 5 mins at 25°C followed by 50 mins at 50°C. Synthesised cDNA was kept at -20°C for short-term and -80°C for long-term storage. A second round of cDNA synthesis was also completed using eight randomly-selected cases from the entire cohort. This cDNA was pooled and serially diluted (3x) to create standards against which the final mRNA concentration of each case was measured.

**Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)**

mRNA expression of neurogenesis and gliogenesis markers were measured using TaqMan Gene Expression Assays (Applied Biosystems; Supplementary Table 2). TaqMan probes were selected to measure the expression of the genes GFAPδ (glial fibrillary acidic protein isoform δ; stem cells), Mki67 (Ki67; cell proliferation), Eomes (eomesodermin; neuronal progenitors), DCX (doublecortin; immature neurons), GFAP (glial fibrillary acidic
protein isoform α; astrocytes and stem cells) and S100B (S100 calcium-binding protein B; mature astrocytes). Data were collected using an ABI Prism 7900HT fast real-time PCR system in a 384-well format and captured using sequence detector software (SDS version 2.4; Applied Biosystems). All measurements were made in triplicate, and relative quantities determined using a seven-point standard curve using the pooled and serially diluted cDNA described above. The expression of three housekeeping genes was also measured; importin 8 (IPO8), TATA binding protein (TBP) and ubiquitin C (UBC), with mRNA expression of the target genes normalised to the geomean of the housekeeping genes. This geomean was not significantly correlated with age in the hippocampus (r=-0.187; p=0.361).

**Immunofluorescence**

Immunofluorescence (IF) staining was performed on 10 µm thick FFPE sections to examine changes to the expression of GFAP in astrocytes. Briefly, slides were deparaffinised in xylene and taken through graded alcohol to water. Antigen retrieval was performed by incubating slides in 10mM citrate buffer (pH 6.0) at 95°C for 30 minutes, followed by 30 minutes cooling at room temperature. Slides were washed 3x in 50% ethanol before peroxidase quenching in 0.3% H₂O₂ in phosphate buffered saline (PBS; pH 7.4) for 30 mins. Slides were then blocked for 1 hour at room temperature in blocking buffer (0.5% casein, 1% bovine serum albumin, 0.05% Tween20 in PBS). Primary antibody (Ms α-Hu GFAP, 1:7000; Sigma, G3893) diluted in blocking buffer was applied and the slides incubated at 4°C overnight. Sections were washed 3x in PBST (PBS plus 0.1% Tween 20) before incubation with horseradish peroxidase-conjugated secondary antibody (α-Ms HRP, 1:1000; Chemicon, AP308P) for 2 hours at room temperature. After washing, a tyramide signal amplification kit was used, and the slides incubated with Cyanine 3 according to the manufacturer’s instructions (PerkinElmer, NEL752001KT). Nuclear counterstaining with 4’, 6-diamidino-2-
phenylindole (DAPI) was performed (1:750; 10 minutes) before coverslipping with 80% glycerol in PBS. Coverslipped slides were stored in the dark at 4°C.

Images of the stained sections were taken using a Zeiss LSM 710 confocal microscope, Zeiss Axiocam HRm camera and Zeiss ZEN v11.0 capture software (Zeiss, Oberkochen, Germany). Three randomly selected areas of the hippocampal hilus were photographed at 200x magnification for visual analysis.

**Statistical Analysis**

All statistical tests were performed using IBM SPSS Statistics v22. Normalised mRNA expression was analysed using Spearman’s rank correlation coefficient, with a confidence interval of 95%. The potential correlative relationship between two experimental variables, while controlling for a third experimental variable included as a covariate, were examined using partial correlation. This examined the effects of *MKI67* and *DCX* controlling for age and *GFAPδ* and age controlling for total *GFAP* expression. Graphs were created in GraphPad Prism 6 (GraphPad Software, California, USA).
Appendix 2.1 Demonstrative Western blot images for protein levels of DCX and PCNA measured in Chapter 3.

DCX (expected band 40kDa)

PCNA (expected band 29kDa)

SYPRO Ruby Total Protein Gel Stain
## Appendix 3.1 Primary and secondary antibodies used for immunostaining in Chapter 4

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary Antibody</th>
<th>Secondary Antibody (IHC)</th>
<th>Secondary Antibody (IF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF2</td>
<td>Rabbit Polyclonal, 1:1500 (SC-79, Santa Cruz Biotechnology, Dallas, TX, USA)</td>
<td>Biotinylated goat anti-rabbit IgG, 1:200 (Vector laboratories, Burlingame, CA, USA)</td>
<td>Goat anti-rabbit IgG-HRP conjugated, 1:1000 (SC-2030, Santa Cruz, Dallas, TX, USA)</td>
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<td>CDNF</td>
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<td>GDNF</td>
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<td>Biotinylated goat anti-rabbit IgG, 1:200 (Vector laboratories, Burlingame, CA, USA)</td>
<td></td>
</tr>
<tr>
<td>Iba1</td>
<td>Goat polyclonal, 1:500 (ab5076, Abcam, Cambridge, UK)</td>
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<td>Donkey anti-sheep/goat IgG HRP-conjugated, 1:1000 (AB324P, Millipore, Billerica, MA, USA)</td>
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<td>Mouse monoclonal, 1:4000 (G3893, Sigma-Aldrich, St. Louis, MO, USA)</td>
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<td>Goat anti-mouse, 1:1000 (AP308P, Millipore, Billerica, MA, USA)</td>
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</tbody>
</table>

CDNF: cerebral dopamine neurotrophic factor; FGF2: basic fibroblast growth factor; GDNF: glial-derived neurotrophic factor; HLA-DR: human leukocyte antigen-DR; Iba1: ionized ionised calcium binding adaptor molecule 1; NeuN: NEUronal Nuclei IgG; GFAP: glial fibrillary acidic protein; immunoglobulin G; HRP: Horse-radish peroxidase.
### Appendix 3.2 Primary and secondary antibodies used for Western blots in Chapter 4

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Cell Type</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
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<td>α-synuclein</td>
<td>Lewy pathology</td>
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</table>

GFAP: glial fibrillary acidic protein; HLA-DR: human leukocyte antigen-DR; Iba1: ionized ionised calcium binding adaptor molecule 1; IgG: immunoglobulin G; HRP: Horse-radish peroxidase.
Appendix 3.3 Published paper “Levels of glial cell line-derived neurotrophic factor are decreased, but fibroblast growth factor 2 and cerebral dopamine neurotrophic factor are increased in the hippocampus in Parkinson’s disease”, Virachit and Mathews et al.
Levels of glial cell line-derived neurotrophic factor are decreased, but fibroblast growth factor 2 and cerebral dopamine neurotrophic factor are increased in the hippocampus in Parkinson’s disease

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Keywords
- cerebral dopamine neurotrophic factor
- fibroblast growth factor 2
- glial cell line-derived neurotrophic factor
- hippocampus
- Parkinson’s disease.

Abstract
Growth factors can facilitate hippocampus-based learning and memory and are potential targets for treatment of cognitive dysfunction via their neuroprotective and neurorestorative effects. Dementia is common in Parkinson’s disease (PD), but treatment options are limited. We aimed to determine if levels of growth factors are altered in the hippocampus of patients with PD, and if such alterations are associated with PD pathology. Enzyme-linked immunosorbent assays were used to quantify seven growth factors in fresh frozen hippocampus from 10 PD and nine age-matched control brains. Western blotting and immunohistochemistry were used to explore cellular and inflammatory changes that may be associated with growth factor alterations. In the PD hippocampus, protein levels of glial cell line-derived neurotrophic factor were significantly decreased, despite no evidence of neuronal loss. In contrast, protein levels of fibroblast growth factor 2 and cerebral dopamine neurotrophic factor were significantly increased in PD compared to controls. Levels of the growth factors epidermal growth factor, heparin-binding epidermal growth factor, brain-derived neurotrophic factor and mesencephalic astrocyte-derived neurotrophic factor did not differ between groups. Our data demonstrate changes in specific growth factors in the hippocampus of the PD brain, which potentially represent targets for modification to help attenuate cognitive decline in PD. These data also suggest that multiple growth factors and direction of change needs to be considered when approaching growth factors as a potential treatment for cognitive decline.

INTRODUCTION
In addition to movement changes, mild cognitive impairment is present in up to 40% of early-stage Parkinson’s disease (PD) patients (49, 61, 85), while frank dementia is present in 80% of patients 20 years post diagnosis (28). Treatment options for dementia in PD are limited (16, 23) but dementia is associated with decreased quality of life (44, 45), increased incidence of nursing home admissions (67), increased carer burden (50) and increased mortality (46) in this disorder. The etiology of cognitive decline in PD is incompletely understood but is reported to be associated with proteinopathology (42), dopaminergic dysfunction (30), cholinergic dysfunction (26, 64) and atrophy within the hippocampus (19, 60). This widely varying pathology suggests that endogenous factors which maintain hippocampal function may be altered in PD.
Growth factors represent potential therapeutic agents for neurodegenerative disorders including PD. Gial cell line-derived neurotrophic factor (GDNF), cerebral dopamine neurotrophic factor (CDNF), mesencephalic astrocyte-derived neurotrophic factor (MANF), and fibroblast growth factor 2 (FGF2, also referred to as basic FGF) restore and protect dopaminergic neurons in the substantia nigra and striatum of rodent and non-human primate models of PD (17, 21, 81). Therapies based on increasing the bioavailability of these factors in the brain may, therefore, represent a novel approach to attenuate motor dysfunction in PD. Recent clinical trials of CDNF have been established to determine its safety and efficacy in attenuating nigrostriatal cell loss (13), although it is unknown if CDNF and other growth factors are also altered in other brain regions external to the basal ganglia in PD.

Additionally, GDNF therapy for movement dysfunction has progressed into clinical trials in PD (10–12), although no clinical benefit to GDNF therapy was reported in these studies. Notably, the subjects in these trials suffered from late-stage PD, while studies in rodents and primates suggest that earlier intervention and improved drug delivery results in better clinical outcomes (25, 63). Given that clinical signs of PD are suggested to occur only after significant nigral degeneration (5), the failure of these trials may therefore reflect a paucity of nigral cells on which GDNF can act. A more recent clinical trial has also found improved clinical PD symptoms after 80 weeks of GDNF therapy (91), compared to no significant improvement at 40 weeks (90). This also suggests that clinical trials of the efficacy of growth factors may need to consider a longer trial length in order to produce clinically significant results. Ultimately, while GDNF therapy may not be suitable for treatment of PD motor symptoms, it may still represent a promising treatment for other brain regions with different patterns of neuronal loss in PD.

Emerging evidence suggests that cognitive dysfunction is associated with altered growth factors in the Alzheimer’s disease (AD) brain (6, 15, 24, 68, 71, 76, 87). Studies of growth factors as candidate therapies in animal models of AD suggest these factors can reduce hippocampal pathology and restore memory function. For example, in rodent models hippocampal-targeted GDNF protects against AD-associated pathology (65), while CDNF improves long-term memory in a transgenic AD mouse model (40). FGF2 gene therapy reduces hippocampal plaque load, stimulates neurogenesis, and restores spatial learning in a mutant mouse model of AD (39, 41). Together these data suggest that augmenting the growth factor microenvironment of the hippocampus can influence the development of abnormal pathology and associated hippocampal dysfunction in AD; this relationship may also be pertinent in PD, a disorder commonly associated with cognitive dysfunction. Here, we quantified endogenous levels of growth factors and pathology in the hippocampus in PD compared with age-matched control cases to determine if growth factor support in this brain region is altered and associated with comitant proteinopathy.

**MATERIALS AND METHODS**

Growth factors GDNF, CDNF, MANF, FGF2, brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF) and heparin-binding epidermal growth factor (HB-EGF) were chosen for inclusion in this study based on reports demonstrating these factors to be neuroprotective or neurorestorative for dopaminergic neurons (9, 17, 20, 21, 34, 36), or that these factors support learning and memory in animal models of AD (40, 41, 58, 65, 94). Hippocampal levels of these factors were quantified in ten cases of PD and nine age-matched normal controls. Hippocampal α-synuclein load and glial cell protein markers were also quantified to determine if altered growth factor levels were associated with disease pathology.

**Tissue collection**

This project was approved by the University of New South Wales Human Research Ethics Advisory Panel. Ten PD and nine age-matched control human brains were sourced by the Sydney Brain Bank and NSW Tissue Resource Centre. PD subjects were followed prospectively prior to death and clinical data regarding the severity of movement disorder was quantified annually. Severity of synucleinopathy was quantified according to Braak staging for α-synuclein pathology (5) and clinical symptoms of movement dysfunction using Hoehn and Yahr scaling (29). All PD cases had a clinical diagnosis of dementia and met pathological criteria for dementia according to Braak staging for neurofibrillary tangles (4) and CERAD plaque staging (Table 1) (52). All PD cases were treated with levodopa, while other anti-parkinsonian medications prescribed for the cohort included entacapone (four cases), selegiline (two cases), bromocriptine (two cases), cabergoline (two cases), pergolide (two cases), tolcapone (two cases) and amantadine (one case). Control cases revealed no clinical signs or symptoms of neurological or psychiatric disorders and neuropathological abnormalities were absent in all cases.

Brain tissues were prepared and sampled identically in all cases as previously described (88). For frozen tissues, the head of the hippocampus, containing the cornu ammonis (CA) and dentate gyrus (DG), was dissected from a single coronal block located 0.5–1 cm anterior to the coronal block containing the lateral geniculate nucleus (Figure 1). Formalin-fixed, paraffin-embedded hippocampal tissue from the contralateral hemisphere to the frozen hippocampus was sampled from two cases for double immunofluorescence staining to characterize cell morphology.

Brain pH was determined by the NSW Tissue Resource Centre and Sydney Brain Bank upon collection of donated brains. Briefly, a 1–2 g segment of the lateral cerebellum was homogenized with a hand-held motorized homogenizer in 2.5 mL deionized water. A hand-held pH meter was then used to measure the pH of the homogenate and this reading recorded. Any pH readings below 5.0 or above 7.2 were re-measured to ensure accuracy.
Table 1. Demographic characteristics of PD and control cases.

<table>
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<tr>
<th>Age (y)</th>
<th>Sex</th>
<th>PMI (h)</th>
<th>Tissue pH</th>
<th>Storage time (wk)</th>
<th>Disease duration (y)</th>
<th>Hoehn and Yahr scale</th>
<th>Braak α-synuclein staging</th>
<th>Dementia duration (y)</th>
<th>Dementia severity</th>
<th>Braak tangle staging</th>
<th>CERAD plaque staging</th>
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<td>0</td>
<td>–</td>
<td>–</td>
<td>0</td>
<td>0</td>
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<td>17</td>
<td>5</td>
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<td>5</td>
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<tr>
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<td>5</td>
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<td>1</td>
<td>3</td>
<td>1</td>
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<td>168</td>
<td>14</td>
<td>5</td>
<td>V</td>
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<td>1</td>
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<td>90†</td>
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<td>5</td>
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<tr>
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<tr>
<td>79.9 ± 2.0</td>
<td>10</td>
<td>14.3 ± 3.4</td>
<td>6.5 ± 0.1</td>
<td>211.2 ± 18.9*</td>
<td>15 ± 1.4</td>
<td>–</td>
<td>–</td>
<td>2.8 ± 0.4</td>
<td>1.7 ± 0.9</td>
<td>–</td>
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</tr>
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</table>

Values shown are mean ± SEM.

Abbreviations: F = female; M = male; PD = Parkinson’s disease; PMI = post mortem interval.

*P < 0.05 compared with control group.

†Cases used for immunohistochemistry.

‡Cases used for immunofluorescence.

§Cerebrovascular accident was noted as cause of death, however pathologically there were no signs of infarction or significant vascular disease in brain tissues.

‖For dementia severity 1 = mild, 2 = moderate and 3 = severe.
Enzyme-linked immunosorbent assay (ELISA)

Frozen hippocampal tissue samples used to measure EGF, HB-EGF, FGF2 and BDNF were homogenized as previously described by Werry et al (88). BDNF levels were quantified in homogenates according to the manufacturer’s protocol, while EGF, HB-EGF and FGF2 were quantified in supernatant following centrifugation at 14,000 × g for 30 min at 4°C.

Tissues for measurement of CDNF, MANF and GDNF were pestle-homogenized in 10x volume of homogenization buffer (137 mM NaCl, 20 mM Tris-HCl, 2.5 mM EDTA, 1% NP-40, 10% glycerol, 0.5mM sodium orthovanadate and cOmplete™ Mini Protease Inhibitor Cocktail (Roche); pH 8.0) on ice and quantified from supernatant following centrifugation at 12,000 × g for 20 min at 4°C.

Protein concentrations in supernatant were measured using a bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA) and levels of growth factor levels normalized to the total amount of sample protein extracted. Supernatant protein levels were quantified using ELISA and assayed in triplicate (GDNF in duplicate) according to the manufacturer’s protocol. For each factor, standard curves were run to identify a protein amount within the linear range of detection and ranged from 35–240 μg/sample. Acidification of tissue supernatant with 1M HCl and re-neutralization with 1M NaOH prior to analysis for GDNF (57) was performed to increase detectability (88).

Protein levels for CDNF and MANF were quantified using a custom designed double-antibody sandwich ELISA. Plates were coated with anti-CDNF monoclonal antibody clone 7D6 (Icosagen, Tartu, Estonia) in 1 μg/mL, 0.05 M carbonate coating buffer (pH 9.6) or goat anti-MANF polyclonal antibody (R&D Systems, Minneapolis, MN, USA) in 1 μg/mL in 0.05 M carbonate coating buffer (pH 9.6) and incubated overnight at 4°C. Plates were blocked using 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for CDNF or 1% casein in PBST (PBS containing 0.05% Tween® 20) for MANF, for 2 h at room temperature then samples added and incubated with shaking at 4°C overnight. Detection antibody horseradish peroxidase (HRP)-linked mouse anti-CDNF clone 6G5 (Icosagen, Estonia; 1:1000) or HRP-linked mouse anti-MANF clone 4E12 (Icosagen, Estonia; 1:1000) was added and incubated with shaking for 5 h at room temperature. Substrate solution (Duoset ELISA Development system, R&D Systems, USA) was added and incubated for 20 min before 1M sulfuric acid was added. The plates were read using a POLARstar Omega microplate reader (BMG Labtech, Ortenberg, Germany).

Immunohistochemistry

Immunohistochemistry was performed to investigate cellular morphology associated with altered neurotrophic factor protein levels in the hippocampus. A single 20 μm, formalin-fixed section was sourced from a subset of cases (five PD cases, five control cases indicated in Table 1). Sections were rehydrated, then cleared before antigen retrieval was carried out in citrate buffer (pH 6; Fronine, Sydney, Australia) with 0.05% Tween® 20 in a water bath at 95°C for 30 min. Sections were cooled before quenching endogenous peroxidases with 3% hydrogen peroxide in PBS and then blocked (CDNF: 10% normal horse serum in PBST; GDNF: 0.25% casein in PBST; FGF2: 10% normal goat serum in PBST) and incubated with primary antibody (Table S1) overnight at 4°C. Sections were then incubated with biotinylated secondary antibody (Table S1) followed by Vector Elite Kit tertiary antibody complex (Vector Laboratories, Burlingame, CA, USA) before being visualized with 3,3’-diaminobenzidine (Sigma, USA), counterstained with cresyl violet, dehydrated and cover slipped with DPX mountant (VWR International Ltd, Radnor,
Growth factors in the Parkinson’s hippocampus

Virachit et al

Immunofluorescence

For further confirmation of cell types containing FGF, CDNF and GDNF, double immunofluorescence staining for astrocytic (glial fibrillary acidic protein; GFAP), microglial (ionized calcium-binding adapter molecule 1; Iba1) and neuronal (neuronal nuclei; NeuN) marker proteins using tyramide amplification was completed. Seven micron hippocampal sections from two representative cases (Table 1) were deparaffinized in xylene and rehydrated through an ethanol gradient before antigen retrieval in citrate buffer (pH 6.0) for 30 mins at 95°C. Sections were washed three times in 50% ethanol before endogenous peroxidases were quenched with 0.3% hydrogen peroxide in PBS. Sections were incubated in blocking solution (0.5% casein, 1% bovine serum albumin and 0.05% Tween® 20 in PBS) before overnight incubation at 4°C in the first primary antibody (Table S1) in blocking solution. Sections were then washed and incubated in appropriate HRP-conjugated secondary antibody (Table S1) in blocking solution before incubation in 1:50 Cyanine 5 (Cy5) Tyramide amplification reagent according to the manufacturer’s instructions (Perkin Elmer, Waltham, MA, USA). For fluorescent double staining, the protocol described above was repeated from peroxidase quenching in 0.3% hydrogen peroxide in PBS, using a second primary antibody and appropriate HRP-conjugated secondary antibody (Table S1). Due to low signal intensity, sections stained for CDNF were further amplified via incubation in biotinylated anti-goat secondary antibody (1:1000, Vector Laboratories, USA) in blocking solution and Vector Laboratories ABC kit (1:500; both reagents) prior to tyramide amplification. Sections were then incubated in 1:50 Cyanine 3 (Cy 3) Tyramide amplification reagents according to the manufacturer’s instructions (Perkin Elmer, USA). Sections were washed, counterstained with 4’,6-diamidino-2-phenylindole (DAPI) and coverslipped with 80% glycerol in PBS. Negative control sections were performed with either one or both primary antibodies or secondary antibodies omitted (data not shown). Acquisition of microscopy images was performed on a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany).

Semi-quantitative neuronal density measurement

Single, slide-mounted sections [7 or 10 μm thick; sampled from the coronal level of the lateral geniculate nucleus (Figure 1)] stained with hematoxylin and eosin were obtained from the NSW Tissue Resource Centre for all cases and were scanned on an Olympus VS 120 slide scanner (Olympus Corporation, Tokyo, Japan) using extended focus imaging to produce a clear image of the entire section for neuronal quantification. Three sampling squares were placed randomly on the scanned image within the CA1 (300 × 300 μm), hilus (200 × 200 μm) and DG (100 × 100 μm) regions using OlyVIA v.2.9.1 (Olympus Corporation, Tokyo, Japan). Within these defined regions of interest neurons were identified based on characteristic morphology; CA1 and hilus neurons exhibit pyramidal morphology with a large nucleus and a small, dense nucleolus while DG neurons are distinguishable by their ovoid, granular nucleus. Neurons were counted within each sampling square and neuronal density calculated using the sum of the neurons counted and the combined volume of each region of interest. This was standardized to a final measurement of neuronal number/mm³ to normalize the differences in the area of the regions of interest and section thickness.

Western blots

Glia1 cell protein markers [GFAP, IBA1 and human leucocyte antigen DR (HLA-DR)] and α-synuclein were quantified via Western blotting to identify changes in cell populations and pathology, respectively. Frozen hippocampal tissue was sonicated in 10× volume of 50 mM Tris-HCl (pH 7.5) buffer containing 125 mM NaCl, 5 mM EDTA disodium salt, 0.002% sodium azide, protease and phosphatase inhibitors (Roche, Germany). Supernatant was collected following centrifugation at 120 000 × g for 2.5 h at 4°C. Pellets were resuspended in buffer containing 5% SDS, sonicated and centrifuged at 100 000 × g for 40 min at 25°C to collect a supernatant termed the SDS fraction. Equal amount of protein from supernatant (30 μg) and SDS fractions (Iba1 and HLA-DR: 15 μg; GFAP: 30 μg) from each sample were separated on XT Bis-Tris precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA) then transferred onto PVDF membrane (Millipore, USA). Membranes used for α-synuclein analysis were fixed with 0.4% paraformaldehyde before immunoblotting. Membranes were blocked then incubated with specific primary antibodies (Table S2) overnight at 4°C. Membranes were incubated with appropriate secondary antibodies (Table S2) and then visualized using Clarity ECL detection (Bio-Rad, USA) according to the manufacturer’s protocol using a ChemiDoc MP Imaging System (Bio-Rad, USA). Band intensity was analyzed by densitometry using Image Lab™ Software 4.1 (Bio-Rad, USA) and normalized to β-actin (Millipore, USA).

Statistical analysis

Demographic characteristics, levels of growth factors, glial and Lewy pathology markers in the diagnostic groups were examined using one-way analysis of variance and analysis of covariance (SPSS Statistics 20.0, SPSS Inc., Illinois, USA). Relationships between levels of each factor of interest and brain tissue pH, storage time and post-mortem interval (PMI) were investigated separately in each diagnostic group using linear regression. Number of cases analyzed for each factor are presented in all figures and tables as numbers varied with tissue availability. Additionally, two data points greater than two standard deviations from the mean were removed prior to analysis.
of CDNF levels. Case numbers analyzed for each growth factor as therefore as follows: EGF (Control n = 9; PD n = 10), HB-EGF (Control n = 9; PD n = 10), FGF2 (Control n = 9; PD n = 10), GDNF (Control n = 6; PD n = 7), CDNF (Control n = 6; PD n = 7), BDNF (Control n = 7; PD n = 10) and MANF (Control n = 7; PD n = 8).

Relationships between growth factors, glial protein and pathology marker levels were investigated using linear regression. Semi-quantitative analysis of neuronal density was examined using an independent samples t-test, with one data point greater than two standard deviations from the mean removed prior to the analysis of the hilus (Control n = 8; PD n = 10). Significance level was set at $P \leq 0.05$ for all analyses.

**RESULTS**

Age, PMI and brain pH did not differ significantly between control and PD cases (Table 1). Regression analysis demonstrated that protein levels of all growth factors investigated were not associated with brain pH, PMI, age or storage time in either control or PD cases (data not shown). Similarly, protein levels of all growth factors were not associated in PD with either dementia-related functional and neuropsychological scores (dementia duration, dementia rating, Braak tangle stage or CERAD plaque stage) nor with movement disorder-related functional and neuropsychological scores (disease duration, Hoehn and Yahr score or Braak $\alpha$-synuclein stage). Freezer storage time was longer in the PD group ($P = 0.02$; Table 1), however, levels of all proteins were independent of storage time in this group, suggesting no confounding effect. Levels of total $\alpha$-synuclein quantified using immunoblotting were unchanged in the hippocampus in PD ($F_{(1,18)} = 1.05, P = 0.32$), however, levels of phosphorylated $\alpha$-synuclein, specifically associated with PD pathology, were significantly increased in the PD hippocampus ($F_{(1,17)} = 4.68, P = 0.05$; Figure 3G), confirming disease-associated pathology in the hippocampus of the PD cases.

**Protein levels of neuronally expressed neurotrophic factors are altered in the PD hippocampus in the absence of an alteration in neuronal density**

Hippocampal levels of GDNF protein were significantly decreased (19%) in PD, compared with age-matched controls ($F_{(1,11)} = 5.55, P = 0.04$; Figure 2A). In contrast, CDNF protein levels ($F_{(1,11)} = 8.37, P = 0.02$; Figure 2D) were significantly increased (41%) in the PD hippocampus, compared with age-matched controls. GDNF-immunopositive cells expressed morphology consistent with that of neurons within the hippocampus of controls and PD cases (Figure 2B and 2C). CDNF-immunopositive cells similarly showed neuronal morphology in controls and PD (Figure 2E and 2F). Double immunofluorescence with the neuron marker protein NeuN confirmed the presence of GDNF (Figure 2G) and CDNF (Figure 2H) within cells of a neuronal phenotype. GDNF and CDNF staining was most marked in the neurons of the hilus and CA regions of the hippocampus. Less intense staining for CDNF was also observed in neurons in the granule cell layer of the DG, whereas no GDNF-positive cells were observed in DG granule neurons. Despite the significant alterations in hippocampal GDNF and CDNF protein levels observed here, overall density of neurons was unchanged in the hilus ($t_{(18)} = 1.806; P = 0.090$; Figure 2I), DG ($t_{(18)} = -1.554; P = 0.139$; Figure 2J) and CA1 ($t_{(18)} = 1.319; P = 0.205$; Figure 2K). These data are consistent with previous quantitative measures of cell density in normal aging (27, 73, 89), as well as with reports of hippocampal atrophy (7) without neuronal loss in PD (27, 37).

**Hippocampal protein levels of FGF2 are increased in the PD hippocampus**

Hippocampal levels of FGF2 were significantly increased (40%) in the PD hippocampus ($F_{(1,17)} = 7.47, P = 0.01$; Figure 3A) and FGF2-positive cells with glial morphology were found in both control and PD hippocampus (Figure 3B and 3C). Consistent with our previous finding that FGF2 is primarily present in glial cells, double immunofluorescence for FGF2 and cell type-specific protein markers demonstrated microglia (Iba1$^+$; Figure 3D) and astrocytes (GFAP$^+$; Figure 3E) strongly expressed FGF2, whereas only moderate staining was observed in neurons (NeuN$^+$; Figure 3F). Activated microglia, strongly expressing HLA-DR, were observed in the PD hippocampus, however, very few of these cells were observed to be FGF2-positive.

Levels of hippocampal EGF ($F_{(1,17)} = 4.16, P = 0.06$), HB-EGF ($F_{(1,17)} = 0.12, P = 0.73$), BDNF ($F_{(1,19)} = 0.46, P = 0.51$) and MANF ($F_{(1,10)} = 0.28, P = 0.61$) did not vary between PD and control cases.

**Association between growth factors, glial and pathology markers**

Levels of FGF2 and CDNF co-varied for the cohort as a whole ($r^2 = 0.559, P = 0.003$), however this relationship reflected an association in the control ($r^2 = 0.938, P = 0.001$), but not PD, cases ($r^2 = 0.118, P = 0.451$). Levels of FGF2 and GDNF ($r^2 = 0.011, P = 0.74$), and levels of GDNF and CDNF ($r^2 = 0.109, P = 0.749$) were not associated. There were no further associations between CDNF and GDNF (Control $r^2 = 0.622, P = 0.113$; PD $r^2 = 0.024, P = 0.771$) or FGF2 and GDNF (Control $r^2 = 0.486 P = 0.124$; PD $r^2 = 0.004, P = 0.894$) when the cohorts were analyzed separately.

As FGF2 is strongly expressed by glial cells and levels of this protein are increased in the PD hippocampus, levels of glial cell markers were investigated via Western blotting (Figure 3G). Total hippocampal levels of the astrocytic marker GFAP ($F_{(1,10)} = 2.26, P = 0.15$), microglial marker Iba1 ($F_{(1,10)} = 0.36, P = 0.56$) and activated microglial marker HLA-DR ($F_{(1,17)} = 0.48, P = 0.49$) were unchanged.
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Figure 2. Alterations in the protein level of the neuronally-expressed growth factors GDNF and CDNF. GDNF protein levels were decreased (A) whilst CDNF (D) was increased within the Parkinson’s disease hippocampus. GDNF-positive cells were found in both controls (B; scale bar = 25 μm) and PD cases (C; scale bar = 25 μm) and had morphology consistent with neurons. CDNF-positive cells also had morphology consistent with neurons in controls (E; scale bar = 25 μm) and PD cases (F, scale bar = 25 μm). This was confirmed with double immunofluorescence labelling, demonstrating that both GDNF (G, scale bar = 10 μm) and CDNF (H; scale bar = 25 μm) co-localize with the neuronal marker NeuN. Despite being expressed in neurons, the alterations in both GDNF and CDNF could not be associated with changes in cellular density, with no apparent change to cell numbers in the hippocampal hilus (I), dentate gyrus (J) or CA1 (K). Data represented as individual values overlaying mean ± SEM.

Figure 3. Alterations in the protein level of the glial-expressed growth factor FGF2 and its association with glial protein markers. FGF2 was significantly increased in the PD hippocampus (A) and showed morphology consistent with neurons and glia in both controls (B; scale bar = 25 μm) and PD cases (C; scale bar = 25 μm). Double immunofluorescence staining confirmed that FGF2 co-localized mainly with markers of microglia [ionised calcium binding adaptor 1 (Iba1); D; scale bar = 10 μm] and astrocytes [glial fibrillary acidic protein (GFAP); E; scale bar = 25 μm]. Some staining was also seen in neurons (NeuN; F; scale bar = 10 μm). Western blotting analysis (G) demonstrated that levels of GFAP (P = 0.495), Iba1 (P = 0.154), activated microglia marker, human leukocyte antigen-DR (HLA-DR; P = 0.559) and total α-synuclein (α-syn; P = 0.32) did not differ between PD and age-matched controls. However, levels of α-synuclein phosphorylated at serine 129 (pS129 α-syn) were significantly increased (P = 0.05) in the PD hippocampus. Levels of FGF2 protein were significantly associated with GFAP protein in both PD and controls (H; dashed line) and this association remained when the PD cohort was analysed separately (H; grey line). Data represented as individual values overlaying mean ± SEM.
in PD compared with age-matched controls. FGF2 levels were, however, significantly associated with GFAP levels in the cohort as a whole ($r^2 = 0.34, P = 0.02$) and this association was preserved when the PD cases were analyzed separately ($r^2 = 0.44, P = 0.05$; Figure 3H). Total α-synuclein levels were not altered in PD, and no association was found between any of the growth factors investigated and either total or pS129 α-synuclein protein levels.

**DISCUSSION**

GDNF-based treatment strategies for attenuating movement dysfunction have been the subject of clinical trials (22, 43, 74), although the failure of these studies to produce clinical improvement has hampered the study of growth factors as potential treatments for PD. However, this does not preclude the use of growth factor-based therapies in other brain regions such as the hippocampus, which we report does not experience neuronal loss in PD and may, therefore, prove more responsive to growth factor therapies than the degenerating substantia nigra.

While the neuroprotective and neurorestorative effects on dopaminergic cells has been wellreported, GDNF in other brain functions, such as cognition and memory, has been little explored. GDNF contributes to normal hippocampal development (32) and hippocampal GDNF levels are maintained throughout the healthy human lifespan (88), suggesting this protein may have an ongoing effect on the regulation of hippocampal function. In contrast to the healthy aged hippocampus, we found a significant decrease in GDNF in PD.

GDNF co-localized exclusively to neuronal markers in immunofluorescence staining, however, we found no apparent neuronal loss in the hippocampal CA1, hilus and dentate gyrus, suggesting that a reduction in hippocampal GDNF in PD does not simply reflect the death of neurons containing this protein, but rather a potential reduction of GDNF per neuron and thus the involvement of other disease-related processes other than apoptotic or necrotic activation. Reduced synaptogenesis may underlie hippocampal atrophy in PD, which demonstrates an overall loss of volume (7) without neuronal loss (27, 37), as described in previous studies. Previous rodent studies demonstrate a role of GDNF in driving axonal and dendritic sprouting in the hippocampus (32) and basal ganglia (66), suggesting that it may also contribute to structural reorganization and plasticity of hippocampal cells in addition to, or as a component of, neuroprotection or neurorestoration. Alterations in synaptogenesis and plasticity are strongly associated with cognitive decline in rodents and humans (35, 70). The observed reduction in GDNF in the hippocampus in PD is, therefore, consistent with reduced neuroplasticity, whereby reduced GDNF contributes to cognitive decline by attenuating synaptic function.

It is also important to note that our finding of reduced GDNF in the hippocampus is not necessarily reflective of a pathological change affecting the hippocampus itself. GDNF is reported to be delivered to neuronal soma via retrograde transport (81), therefore, cells containing GDNF may be representative of either GDNF-receiving or GDNF-producing cells. Given that GDNF mRNA is present in the human hippocampus (31, 80) and specifically in hippocampal neurons in both rodents and humans (62, 72), it is likely that the neurons found to be immunopositive for GDNF in this study may be producing rather than receiving GDNF. If so, reduced levels of GDNF may not only affect the hippocampus itself, but also other brain regions which innervate it and which may be dependent on receiving GDNF from hippocampal neurons. For example, the cholinergic basal forebrain and noradrenergic locus coeruleus directly innervate the hippocampus (51, 69), contributing to cognitive function (8, 55) and degenerate in PD (26, 93). GDNF is also suggested to be neuroprotective of these cells (59, 92). Ultimately, these combined findings may indicate that alterations in GDNF affect hippocampal function via a reduction in endogenous neuroprotection as well as failing to adequately maintain connectivity between the hippocampus and other brain regions. Future studies into the role of GDNF on hippocampal function are needed to further elucidate alternative pathways via which GDNF may affect hippocampal function and therefore contribute to cognitive change in PD.

Together with the observed decrease in GDNF expression, we found significant increases in the expression of both the neuron-associated CDNF and glia-associated FGF2, demonstrating for the first time that CDNF is present in hippocampal neurons. Both CDNF and FGF2 exhibit neuroprotective and neurorestorative properties for dopaminergic neurons (1, 2, 48, 78, 97); the reported increase in both factors in this study may, therefore, reflect a similar function toward neurons in the hippocampus, evidenced by consistent neuronal density in PD cases compared with controls. CDNF is thought to modulate protein folding in the endoplasmic reticulum, preventing the accumulation of misfolded proteins which may then induce endoplasmic reticulum stress and stress-induced apoptosis (84, 96). Phosphorylated α-synuclein (77) and α-synuclein oligomers (14) are known inducers of endoplasmic reticulum stress, which has been well-characterized in the PD brain (83, 86). Here, we found significantly increased levels of phosphorylated α-synuclein in the PD hippocampus, thus we speculate that increased levels of CDNF in this brain region reflects a protective response by these stressed hippocampal neurons. Interestingly, we found no significant difference in MANF levels between PD and control cases. Given that the structure and function of CDNF and MANF are closely related (47), this may indicate an alternative role of CDNF in the progression of PD pathology, warranting further investigation in future studies.

We also found a significant increase in FGF2, in contrast to the PD midbrain (54, 82) or healthy aged hippocampus (88). FGF2 is involved in the regulation of astrocytic and microglial reactivity and neuroinflammation (3, 79); here, we observed a positive association between FGF2 and GFAP expression in the PD hippocampus which may be reflective of these mechanisms. However, we also observed consistent levels of microglial and cell reactivity protein markers in PD and control cases, which does not
support a marked inflammatory response in this brain region in our cases of advanced PD. The lack of gliosis in the hippocampus of PD patients would be consistent with previous reports of an attenuated inflammatory response in late stage PD (53, 75). Alternatively, increased levels of FGF2 are found in AD hippocampus and colocalize to senile plaques (18, 38), however we found no association between FGF2 levels and severity of either AD or PD proteinopathy in the PD hippocampus. Given that FGF2 is not strongly associated with glial reactivity markers in this study, we suggest that an alternate mechanism underlies the increase in FGF2 expression seen in this study. FGF2 is a modulator of long-term potentiation and synaptic plasticity in hippocampal neurons (33, 95), both of which are important for learning and memory (56, 95). This is similar to the previously described role of GDNF, indicating that FGF2 may be upregulated as a compensatory response to buttress synaptic function in the absence of GDNF. This is supported by work demonstrating the importance of growth factors in combination in order to exert a neuroprotective effect (36).

Despite initially disappointing clinical outcomes, GDNF has demonstrated potential as a treatment option to attenuate dopaminergic cell death. In this study, we aimed to determine if this factor or others may similarly represent a tractable and effective treatment option for cognitive decline, which is a common and debilitating consequence of PD. We found significant reductions in levels of GDNF but increased levels of CDNF and FGF2 in the PD hippocampus, in the absence of apparent neuronal loss. These data suggest that alterations in GDNF protein may contribute to cognitive decline in PD, although the lack of apparent neuronal loss suggests that hippocampal GDNF exerts its neuroprotective function differently to GDNF within the dopaminergic midbrain. Given that GDNF-based treatments are already in Phase 2 clinical trials, we suggest that these treatment options may also be suitable for further studies of GDNF supplementation for the management of cognitive decline in PD.

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CONFLICT OF INTEREST

MS holds stock in Herantis Pharma Ltd and Mobidaig Ltd. GMH holds stock in Cochlear Ltd and NIB Holdings. MS and PL are inventors of a patent owned by Herantis Pharma Ltd. MS is a consultant for Genecode Ltd and Amanarantus Ltd. MS is on the advisory boards for Heidelberg University Neuroscience Center, Aarhus University Dandrite Center, Helsinki Institute of Information Technology and Göttingen University Neuroscience Center. GMH is on the advisory board for the Danish Research Institute of Translational Neuroscience, Aarhus University. CSW is on the advisory board for Lundbeck Australia Pty Ltd and in collaboration with Astellas Pharma Inc., Japan.

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