Microvascular alterations and corpora amylacea progression in the *post-mortem* hippocampus of patients with obstructive sleep apnoea

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Cuicui Xu

27/06/2019
Publications

Manuscripts that are in preparation as a direct result of this thesis:


2. **Cuicui Xu**, Jessica E. Owen, Thorarinn Gislason, Bryndis Benedictsdottir, Jiming Ye, Stephen R. Robinson. Increased incidence of microvascular abnormalities in the CA1 region of the hippocampus in obstructive sleep apnoea (To be submitted to Circulation).


Conference abstract that has arisen as a direct result of this thesis:

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<td>Amyloid β</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>AHI</td>
<td>Apnoea-hypopnoea index</td>
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<td>ARIA</td>
<td>Automated retinal image analyser</td>
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<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<td>CA</td>
<td>Cornu ammonis</td>
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<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CoA</td>
<td>Corpora amylacea</td>
</tr>
<tr>
<td>CPAP</td>
<td>Continuous positive airway pressure</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GluT-1</td>
<td>Glucose transporter-1</td>
</tr>
<tr>
<td>IH</td>
<td>Intermittent hypoxia</td>
</tr>
<tr>
<td>n/N</td>
<td>Numbers (group sample size/Total sample size)</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
</tr>
<tr>
<td>ODI</td>
<td>Oxygen desaturation index</td>
</tr>
<tr>
<td>OSA</td>
<td>Obstructive sleep apnoea</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>SaO₂</td>
<td>Blood oxygen saturation (%)</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>VWF</td>
<td>Von-Willebrand factor</td>
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Abstract

Obstructive sleep apnoea (OSA) is a sleep disorder that involves frequent episodes of breathing cessation or significant decreases in airflow during sleep, leading to periods of blood oxygen desaturation. These episodes of intermittent hypoxia (IH) followed by reoxygenation are thought to induce oxidative stress and neuroinflammation. Patients with severe OSA exhibit neuropsychological deficits and impaired memory. Continuous positive airway pressure (CPAP) is the standard treatment for OSA patients, but not all cognitive symptoms can be reversed by this procedure, suggesting that severe OSA may permanently injure the brain. The CA1 region of the hippocampus is one of the first regions of the brain to suffer hypoxic injury. This vulnerability is supported by imaging studies showing that the volume of the hippocampus is reduced in people with severe OSA. Since it is not known why some parts of the hippocampus are selectively vulnerable to hypoxia, an investigation of the microvessels in these regions could provide new insights.

The first aim of the present study was to investigate the effects of OSA severity, regular CPAP use and advanced age on angiogenesis and microvascular remodelling in different regions of the hippocampus (Chapter 3). It was hypothesised that there would be significantly increased microvascular alterations in the high OSA group (oxygen desaturation index: ODI ≥ 20 events/h sleep) compared to the low OSA group (ODI < 20 events/h sleep). The hypothesis was tested by measuring capillaries that had been immunostained by either CD34, VWF, Claudin 5, GluT-1 or Collagen IV, in the fimbria, CA4, CA1, subiculum and collateral sulcus regions. Analyses were conducted on formalin-fixed, paraffin-embedded hippocampal tissues obtained at autopsy from 30 OSA patients. The results showed that angiogenesis did not occur in OSA; instead moderate-severe OSA was associated with a 10 – 25% increase in the mean diameters of capillaries in the fimbria and CA4 regions, suggesting that microvascular remodelling occurred in response to IH in OSA. These changes were not
reversed by CPAP treatment. The lack of adaptive microvascular changes in the CA1 region might be a factor in the selective vulnerability of this region to hypoxia.

The second aim was to systematically investigate the presence of morphological abnormalities in the microvessels of the hippocampus of OSA patients (Chapter 4). It was hypothesised that if microvascular abnormalities are caused by oxidative stress, they would be more prevalent in the high OSA group and advanced age group, while regular CPAP users would display fewer abnormalities. The results showed there were increased numbers of abnormal microvessels in the high OSA group in the CA1 region, but not in other parts of the hippocampus. It is speculated that increased numbers of abnormal microvessels in the CA1 region enhances its vulnerability to hypoxic injury and causes permanent degenerative changes that limit the ability of CPAP to reverse memory impairments.

Corpora amylacea (CoA) are often regarded as correlates of the ageing process, and their numbers have been reported to increase steadily after the age of 50. Despite the fact that CoA were first identified in the central nervous system (CNS) over a century ago, little is known about their spatial distribution in the hippocampus of aged OSA patients. The third aim was to determine the distribution and progression pattern of CoA in the hippocampus, and to assess how the density and size of CoA change with advancing age (Chapter 5). It was hypothesised that CoA would be concentrated in periventricular and subpial regions, and that their density and size would increase with age. The results revealed that while the size of CoA increases with age, the packing density does not. A distinct distribution pattern of CoA was observed by low-magnification scanning photomicrographs that began at the fimbria and then progressively spread along the pial surface of the hippocampal formation to more distant regions. This progression pattern did not correlate with age. This spatiotemporal sequence has not been reported previously, and the reasons for this pattern of spread are unknown.
The fourth aim of the present study was to investigate factors that may be associated with CoA formation (Chapter 6). Since CoA contain oxidised lipids and proteins, it has been speculated that they are markers of oxidative stress or the consequence of neurodegenerative processes. It was hypothesised that increased OSA severity would be associated with larger and more numerous CoA. The results confirmed that OSA severity was significantly correlated with the spatiotemporal distribution of CoA, as well as increased CoA density. However, as these correlations did not diminish with regular CPAP use, a role for hypoxia or oxidative stress was not supported. Furthermore, CoA were rarely observed in the CA1 region, despite this region being thought to experience the highest levels of hypoxia and oxidative stress. The data also failed to support neurodegeneration as a cause of CoA, as no correlations were found between CoA burden (distribution, density or size), and the burden of Amyloid β (Aβ) plaques, Tau+ neurofibrillary tangles, neuropil loss or demyelination.

This study advances our understanding of regional differences in the capacity of the hippocampal microvasculature to remodel in response to increasing severity of OSA and to ageing, and offers a new explanation for the selective vulnerability of the CA1 to hypoxia. This study also advances our understanding of the factors that contribute to the formation of CoA, showing that OSA severity is correlated with the spatial extent and numbers of CoA in the hippocampus, while patient age is correlated with their size. In contrast, oxidative stress and neurodegeneration now appear unlikely to contribute to the formation of CoA in the human hippocampus. It is recommended that larger studies be conducted, that include patients without OSA, so that the present findings can be confirmed and extended.
Chapter 1 Literature Review

1.0 Overall introduction

Obstructive sleep apnoea involves frequent cessations of breathing during sleep, leading to intermittent hypoxia and oxidative stress in the brain. Animal models of intermittent hypoxia have shown that the oxidative stress is associated with neuronal injury and cell death in the hippocampus. Hypoxia is a well-known stimulus for angiogenesis and vascular remodelling, whereas perturbations in cerebral blood flow and oxidative stress can be injurious to capillaries. Since all of these factors are thought to be present in OSA, it seems likely that severe OSA will be characterised by remodelling of the capillary network and pathological changes in the ultrastructure of the capillaries, particularly in sectors of the hippocampus that are known to be sensitive to hypoxia.

Although it is widely assumed that OSA injures the hippocampus via oxidative stress, direct evidence is lacking. In other conditions, such as brain ageing and Alzheimer's disease, oxidative stress has been associated with an increased burden of corpora amylacea in the hippocampus. These extracellular globules are composed of oxidised lipids and proteins, and are regarded as indicators of neuronal damage, particularly from oxidative stress.

This literature review focuses on relevant research to provide: i) a general introduction to OSA, including its diagnosis with polysomnography, and treatment with continuous positive airway pressure; ii) an overview of cerebral microvasculature adaptations (angiogenesis and vascular remodelling) that typically occur in response to hypoxia; iii) a summary of what is known about injury to the cerebral microvasculature in response to oxidative stress; and iv) a discussion of current views on the distribution and formation of corpora amylacea in the brain. Gaps in the current literature are identified and hypotheses are developed to guide the research presented in this thesis.
1.1 Obstructive sleep apnoea

Obstructive sleep apnoea is the most common type of sleep-disordered breathing. It is due to upper airway collapse, where the lumen of the pharyngeal airway is obstructed by the relaxation of the tongue and soft tissue during sleep (Dempsey, Veasey et al. 2010, Gislason and Sunnergren 2014). OSA patients are capable of controlling their upper airway dilators properly in wakefulness, yet lose control of these muscles during sleep, resulting in the collapse of the upper airway (Schwab, Pasirstein et al. 2003, Dempsey, Veasey et al. 2010). The lumen of the pharyngeal airway can also be narrowed by the craniofacial structure or by fat deposition in the walls of the throat (Jordan, McSharry et al. 2014). The reduced airflow can lead to blood oxygen desaturation, hypercapnia, recurrent snoring, choking or gasping, arousals from sleep, sleep fragmentation and sleep deprivation (Malhotra and White 2002, Azagra-Calero, Espinar-Escalona et al. 2012, Kielb, Ancoli-Israel et al. 2012). OSA sufferers often experience daytime sleepiness, morning headache, fatigue or tiredness, depression and anxiety (Kasai, Floras et al. 2012, Phillips and O’Driscoll 2013, Morsy Nesreen, Farrag Nesrine et al. 2019), which in turn reduce the quality-of-life (Punjabi 2008).

As is well known, a sufficient quantity and quality of sleep is critical for our physical, mental and emotional wellbeing. The frequent arousals and sleep fragmentation in OSA alter the sleep patterns, resulting in less deep sleep and more light sleep (Yaouhi, Bertran et al. 2009, Kim, Joo et al. 2016). OSA is recognised as a major public health problem, and individuals with OSA are more likely to have motor vehicle accidents (Leger, Bayon et al. 2012, Morsy Nesreen, Farrag Nesrine et al. 2019), reduced work productivity and work-related injuries (Hillman, Murphy et al. 2006, Morsy Nesreen, Farrag Nesrine et al. 2019). A meta-analysis reported a 2.43-fold increased risk of a car accident in OSA patients compared to controls (Tregear, Reston et al. 2009). Medical costs plus physical impairment not only place a
significant financial burden upon victims, but also on the entire national economy and healthcare system (Skaer and Sclar 2010, Tarasiuk and Reuveni 2013, Morsy Nesreen, Farrag Nesrine et al. 2019). The burden that OSA places on economic and healthcare system costs in Australia was estimated in a report commissioned by the Sleep Health Foundation in 2011 (Table 1.1); the direct total health system cost for OSA and associated conditions was estimated to be $408.5 million per year, while indirect financial costs associated with lost productivity, informal care and other costs of motor vehicle accidents amounted to $2.6 billion annually (Economics 2011).

**Table 1.1 Medical conditions and health costs attributed to OSA.**

<table>
<thead>
<tr>
<th>Attributed injury/illness</th>
<th>Proportion (%) of OSA prevalence</th>
<th>Proportion (%) of OSA health system cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroke</td>
<td>5.3%</td>
<td>6%</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>1.1%</td>
<td>–</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>3.6%</td>
<td>14%</td>
</tr>
<tr>
<td>Other cardiovascular diseases</td>
<td>–</td>
<td>24%</td>
</tr>
<tr>
<td>Depression</td>
<td>6.2%</td>
<td>31%</td>
</tr>
<tr>
<td>Motor vehicle accidents</td>
<td>4.3%</td>
<td>10%</td>
</tr>
<tr>
<td>Work-related injuries</td>
<td>0.6%</td>
<td>15%</td>
</tr>
</tbody>
</table>

Based on (Economics 2011).
OSA is often comorbidly associated with metabolic syndrome, which is characterised by hypertension, hyperglycaemia, dyslipidaemia, impaired glucose tolerance and increased insulin resistance (Punjabi 2008, Kasai, Floras et al. 2012, Phillips and O’Driscoll 2013, Cantiello, Cicione et al. 2015, Keenan, Kim et al. 2018), as well as a predisposition to obesity and cardiovascular disease (Coughlin, Mawdsley et al. 2004, Arnardottir, Bjornsdottir et al. 2016, Sutherland, Keenan et al. 2019). Various studies have suggested that OSA may contribute to and exacerbate cardiovascular disease (Marin, Carrizo et al. 2005, Peker, Carlson et al. 2006, Morsy Nesreen, Farrag Nesrine et al. 2019), with direct and deleterious effects on cardiac and vascular structure and function (Lanfranchi & Somers, 2001).

1.1.1 OSA diagnosis – Polysomnography

The gold-standard method for assessing the presence and severity of OSA is polysomnography (Chesson, Ferber et al. 1997, Boyd, Upender et al. 2016). Polysomnography monitors a set of physiological variables during an overnight sleep study, including the number of apnoeas and hypopnoeas, the percentage of arterial oxygen desaturations, heart rate, airflow, eye movements and muscle tone (Flemons, Littner et al. 2003, Pamidi, Aronsohn et al. 2010). An apnoea is defined as the complete cessation of airflow for a minimum of 10 seconds (Patil, Schneider et al. 2007, Gharibeh and Mehra 2010), whereas a hypopnoea is an airflow reduction of at least 30% below baseline for 10 seconds or more, accompanied by a drop in blood oxygen saturation (of at least 3% or 4%) and/or a measured arousal in the brain (Ruehl, Rochford et al. 2009). The respiratory events and arousals can occur over 120 times per hour (Chiang 2006). The severity of OSA is rated according to the apnoea-hypopnoea index (AHI), which is the average number of apnoeas and hypopnoeas per hour of sleep (Vasu, Grewal et al. 2012). An AHI score of < 5 is considered normal, and OSA sufferers are categorised as mild, moderate and severe with AHI
scores of 5 – 15, 15 – 30 and > 30, respectively (Badran, Ayas et al. 2014). During an apnoea or hypopnoea, OSA patients undergo varying levels of hypoxemia/hypercapnia, leading to the oxyhaemoglobin saturation dropping to 95% in mild sufferers or below 60% in very severe cases (Chiang 2006, Badran, Ayas et al. 2014). Another widely-used scale of OSA severity is the oxygen desaturation index (ODI), which measures the number of times per hour of sleep that the blood's oxygen level drops by at least 3% or 4% (Berry, Budhiraja et al. 2012).

1.1.2 OSA treatment – Continuous positive airway pressure

CPAP is the most commonly prescribed treatment for OSA (Sullivan, Berthon-Jones et al. 1981, Patel, White et al. 2003, Kushida, Littner et al. 2006, Chowdhury, Wedderburn et al. 2012). A CPAP machine consists of a facemask, a tube and a device that blows air (positive pressure) into the tube (Sahni and Wung 1998). The positive air pressure provided by the CPAP machine can prevent closure of the pharynx and larynx by distending the upper airway (Gaon, Lee et al. 1999). CPAP treatment supports an uninterrupted sleep by reducing the number of apnoeas and hypopnoeas, and normalising the sleep architecture (Sullivan, Berthon-Jones et al. 1981). CPAP treatment has been reported to reduce daytime sleepiness (Cohen-Zion, Stepnowsky et al. 2001, Marshall, Barnes et al. 2006), reduce systemic blood pressure (Bazzano, Khan et al. 2007, Marin, Agusti et al. 2012), improve daytime productivity and daily function (Weaver and Grunstein 2008), improve quality of life (Kandasamy, Almaghaslah et al. 2019), elevate mood and psychological well-being (Giles, Lasserson et al. 2006, Hobzova, Hubackova et al. 2017), and reduce the incidence of automobile accidents (Gay, Weaver et al. 2006, Weaver and Grunstein 2008). Dewan and colleagues (2015) claimed that early treatment with CPAP can maximise functional recovery and minimise residual injury (Dewan, Nieto et al. 2015). CPAP treatment is not limited to moderate and severe OSA; it has been widely used to treat mild OSA, respiratory failure in
preterm infants (Sahni and Wung 1998), upper airway resistance syndrome, and snoring (Kushida, Littner et al. 2006).

While CPAP is an effective treatment for OSA, many patients find the apparatus uncomfortable to use. Consequently, CPAP uptake is only around 50%, and of those who begin CPAP treatment, adherence is between 30% and 80% (Weaver and Grunstein 2008, Weaver and Sawyer 2010, Park, Kim et al. 2017). Even among adherent users of CPAP, it is only used for an average of 4 hours per night. A recent study showed that nearly 40% of regular CPAP users developed oral symptoms, such as dry mouth and poor oral health (Tsuda, Moritsuchi et al. 2016).

1.1.3 Risk factors for development and exacerbation of OSA

The prevalence of OSA in the overall population has been estimated to range between 2.1% and 36.5% (Table 1.2), with the wide variability being due to differences in risk between genders, ethnicity and age (Senaratna, Perret et al. 2017), as well as to differences between studies in the patient selection criteria used. Risk factors for OSA include older age, male gender, postmenopausal state, and obesity (Young, Peppard et al. 2002, Lee, Nagubadi et al. 2008, Punjabi 2008, Lam, Sharma et al. 2010, Gislason and Sunnergren 2014). The prevalence of OSA has been estimated to be 3 – 10% in adults aged less than 40 years, 10 – 20% in older adults (Punjabi 2008, Simpson, Hillman et al. 2013, Gislason and Sunnergren 2014), with this value increasing to 78% in females and 90% in males aged 60 – 85 (Senaratna, Perret et al. 2017). In a population-based study OSA prevalence was found to be nearly 50% in persons 60 – 70 years of age, while being less than 10% in those aged 30 – 39 (Durán, Esnaola et al. 2001).
Table 1.2 OSA prevalence in different genders and ethnicity.

<table>
<thead>
<tr>
<th>Country</th>
<th>Ethnicity</th>
<th>Age range</th>
<th>Prevalence of OSA (AHI ≥ 5)</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Men</td>
<td>Women</td>
</tr>
<tr>
<td>United States</td>
<td>White</td>
<td>30 – 60</td>
<td>24%</td>
<td>9%</td>
</tr>
<tr>
<td>Australia</td>
<td>White</td>
<td>40 – 65</td>
<td>126% / 3%</td>
<td>−</td>
</tr>
<tr>
<td>Spain</td>
<td>White</td>
<td>30 – 70</td>
<td>26.2%</td>
<td>28.0%</td>
</tr>
<tr>
<td>Poland</td>
<td>White</td>
<td>30 – 75</td>
<td>36.5%</td>
<td>18.5%</td>
</tr>
<tr>
<td>China</td>
<td>Chinese</td>
<td>30 – 60</td>
<td>4.1%</td>
<td>−</td>
</tr>
<tr>
<td>China</td>
<td>Chinese</td>
<td>30 – 60</td>
<td>−</td>
<td>2.1%</td>
</tr>
<tr>
<td>India</td>
<td>Indian</td>
<td>35 – 65</td>
<td>7.5%</td>
<td>−</td>
</tr>
<tr>
<td>India</td>
<td>Indian</td>
<td>30 – 65</td>
<td>13.5%</td>
<td>5.5%</td>
</tr>
<tr>
<td>Korea</td>
<td>Korean</td>
<td>40 – 69</td>
<td>4.5%</td>
<td>3.2%</td>
</tr>
</tbody>
</table>

1 RDI (respiratory disturbance index) ≥ 5 with / without excessive daytime sleepiness. Updated based on (Punjabi 2008).
Most studies have reported that OSA is more common in males, although the estimated prevalence varies greatly (Table 1.2; Figure 1.1). A study of employees 30 – 60 years old reported that 4% of men and 2% of women met the criteria of AHI ≥ 5 (Young, Palta et al. 1993); while in a review paper, OSA prevalence in the general population was estimated to be 3 – 7% in adult men and 2 – 5% in adult women (Punjabi 2008). The prevalence of OSA has also been studied in women, where it is estimated to be 0.6% in premenopausal women, 0.5% in postmenopausal women receiving hormone replacement therapy and 2.7% in postmenopausal women without hormone replacement therapy (Bixler, Vgontzas et al. 2001).

Figure 1.1 Estimated prevalence in Australia of moderate-to-severe OSA (AHI ≥ 15), stratified by age and gender.

The OSA prevalence among Australians shows a trend to increase with age, and to be more prevalent among men than women. Based on (Economics 2011).
Obesity is one of the major risk factors for OSA (Hamilton and Naughton 2013). In a worldwide study of obesity in 106 countries (approximately 88% of the world population) in 2005, the prevalence of overweight adults (BMI > 25 kg/m²) was estimated to be 23.2% (937 million), and obese adults (BMI > 30 kg/m²) to be 9.8% (396 million) (Kelly, Yang et al. 2008). The global prevalence of obesity has doubled between 1980 and 2015 in more than 70 countries, with obesity (BMI > 30 kg/m²) affecting 5.0% of children and 12.0% of adults worldwide, in a global longitudinal study over 25 years in 195 countries (Collaborators 2017). It was estimated that nearly 108 million children and more than 600 million adults were obese worldwide in 2015 (Collaborators 2017). Moreover by 2030 the number of overweight and obese adults was projected to be 1.35 billion and 570 million respectively (Kelly, Yang et al. 2008).

OSA worsens with weight gain and improves with weight loss (Peppard, Young et al. 2000). A longitudinal study investigated the independent association between weight change and change in sleep-disordered breathing severity over a 4-year period in 690 middle-aged participants (mean age 46 years; 56% male); a 10% weight gain was found to be associated with a 32% mean increase in AHI score, and a 6-fold increased risk of developing moderate to severe sleep-disordered breathing (Peppard, Young et al. 2000). Conversely, weight loss improved OSA, with a 10% weight loss being associated with a 26% mean reduction in the AHI (Peppard, Young et al. 2000).
1.1.4 Intermittent hypoxia (IH)

OSA is characterised by repeated upper airway collapse during sleep, resulting in intermittent hypoxia (IH) and sleep fragmentation (Jun and Polotsky 2007, Gharibeh and Mehra 2010, Vijayan 2012). While IH can be regarded as ‘repetitive hypoxia interspersed with episodes of normoxia’, IH elicits very different systemic and cellular responses when compared to sustained hypoxia (Neubauer 2001, Chiang 2006). The recurrent cycles of hypoxemia with reoxygenation during IH in OSA have been suggested to contribute to ischemia-reperfusion injury (Dewan, Nieto et al. 2015). An example of ischemia-reperfusion injury is when a person has a cardiac arrest and is then resuscitated; the resumption of oxidative metabolism causes an increased production of reactive oxygen species, and the consequent oxidative stress can injure the brain (Suzuki, Jain et al. 2006, Dewan, Nieto et al. 2015). IH involves innumerable episodes of ischemia-reperfusion, and the rates of reactive oxygen species production following each episode of IH are estimated to be as much as 100-fold higher than basal levels; it is this feature that differentiates between sustained hypoxia and IH (Michiels, Tellier et al. 2016).

Sustained hypoxia has been reported to be associated with the rapid stabilisation of the transcriptional hypoxia-inducible factor-1 (Ryan, Taylor et al. 2005, Chiang 2006), a sensor of systemic hypoxia, which mediates the expression of many proteins in response to hypoxic stress (Punjabi and Polotsky 2005, Lavie 2009), resulting in increased expression of erythropoietin, vascular endothelial growth factor, and inducible nitric oxide synthase, all of which contribute to an adaptive phenotype (Ryan, Taylor et al. 2005, Chiang 2006). By contrast, intermittent hypoxia does not provide the conditions needed to stabilise hypoxia-inducible factor-1, and instead nuclear factor-κB becomes activated, which leads to the activation of an inflammatory phenotype and increased production of inflammatory mediators...
(Ryan, Taylor et al. 2005, Chiang 2006). OSA patients often exhibit chronic low-grade inflammation, possibly because IH upregulates the expression of transcription factors such as nuclear factor-κB, and proinflammatory cytokines, including tumour necrosis factor-α, interleukin-6 and interleukin-8 (Calvin, Albuquerque et al. 2009). There is evidence that plasma tumour necrosis factor-α and interleukin-6 are upregulated in patients with OSA, independently of obesity (Vgontzas, Papanicolaou et al. 2000).

### 1.1.5 Oxidative stress

The fluctuation of blood oxygen saturation in OSA is similar to the conditions of hypoxia-reoxygenation and ischemia-reperfusion (Dewan, Nieto et al. 2015), which involve an initial restriction of blood supply (ischemia), resulting in limited oxygen availability (hypoxia) to an organ, that is subsequently followed by the restoration of perfusion and corresponding reoxygenation (Eltzschig and Eckle 2011). The hypoxia-reoxygenation or ischemia-reperfusion cycles initiate various processes that lead to the overproduction of reactive oxygen species (Hung, Skepper et al. 2001, Kaminski, Bonda et al. 2002, Prabhakar 2002, Xu, Chi et al. 2004), such as superoxide anion radical (O$_2^-$), hydroxyl radical (·OH) and hydrogen peroxide (H$_2$O$_2$) (Suzuki, Jain et al. 2006, Bolisetty and Jaimes 2013, Zorov, Juhaszova et al. 2014, Bhattacharya 2015). Reactive oxygen species are mainly produced by mitochondria, and their production peaks during ischemia-reperfusion events (Kalogeris, Bao et al. 2014, Lavie 2015). These chemically unstable free radicals have a dual action in cells (Kalogeris, Bao et al. 2014, Lavie 2015), on one hand they function as signal transduction mediators (Suzuki, Jain et al. 2006), while on the other hand they oxidise lipids, proteins and DNA (Suzuki, Jain et al. 2006, Birben, Sahiner et al. 2012, Finosh and Jayabalan 2013, He, He et al. 2017), leading to inflammation, cellular apoptosis and tissue damage (Uttara, Singh et al. 2009).
The cellular antioxidant system is the defence mechanism against free radicals; oxidative stress occurs when the rate of production of reactive oxygen species exceeds the capacity of the antioxidant systems to quench free radicals (Birben, Sahiner et al. 2012, Bhattcharya 2015, Liguori, Russo et al. 2018). Oxidative stress has been implicated as the cause of pathological tissue damage in cardiovascular disease, diabetes, cancer, ageing, Alzheimer’s and Parkinson’s disease (Dhalla, Temsah et al. 2000, Sayre, Smith et al. 2001, Dalle-Donne, Rossi et al. 2006, Valko, Leibfritz et al. 2007, Uttara, Singh et al. 2009, Liguori, Russo et al. 2018).

1.1.5.1 The relationship between oxidative stress and inflammation

Mitochondria are the intracellular organelles responsible for breaking down glucose in the presence of oxygen to create energy, and are key regulators of the health, integrity and function of cells (Lin and Beal 2006, Lavie 2015). Mitochondrial dysfunction and oxidative stress have been suggested to play central roles in ageing and neurodegenerative diseases (Lin and Beal 2006). Ageing is characterised by a progressive decline in the effective function of molecules, cells, tissues and organs in biological organisms over time (Campisi 2013, Liguori, Russo et al. 2018), leading to a host of pathologies including atherosclerosis, heart failure, pulmonary insufficiency, renal failure, osteoporosis and neurodegeneration (Campisi 2013). More than 300 hypotheses have been proposed to explain the ageing process (Medvedev 1990, Tosato, Zamboni et al. 2007), of interest here is the “free radical hypothesis” or the “oxidative stress hypothesis”, which posit that the accumulation of structural damage to macromolecules (lipids, DNA and proteins) caused by oxidative stress leads to functional losses (Beckman and Ames 1998).

The gradual accumulation of mutations in mitochondrial DNA (mtDNA) is thought to contribute to the ageing process by impairing the function of mitochondria and antioxidant
systems, leading to an increase in the net production of ROS (Lin and Beal 2006). Alzheimer’s disease (AD) has been reported to be associated with increased free radical damage to nDNA (nuclear DNA), and a 3-fold higher level of oxidative damage to mtDNA compared with age-matched controls (Mecocci, MacGarvey et al. 1994). AD has been linked to mitochondrial abnormalities that contribute to the production of free radicals (Christen 2000). Interestingly, mitochondrial dysfunction has also been reported in OSA/IH, where it is associated with increased oxidative stress (Douglas, Ryu et al. 2010, Lavie 2015).

Mitochondria are the primary source of cellular reactive oxygen species (Kalogeris, Bao et al. 2014) and these are rapidly detoxified through the action of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), which are supported by reserves of antioxidant molecules (e.g. glutathione (GSH), nicotinamide adenine dinucleotide phosphate (NADPH), ascorbate and alpha-tocopherol) (Finosh and Jayabalan 2013). The cellular antioxidant defense action is illustrated in Figure 1.2. Oxidative stress occurs when an oxidative burst from mitochondria temporarily exceeds the capacity of endogenous antioxidant systems (Birben, Sahiner et al. 2012, Salim 2017). In such instances, the reactive oxygen species are able to oxidise nearby proteins, lipids and DNA, particularly those within mitochondria (Birben, Sahiner et al. 2012, Finosh and Jayabalan 2013). The molecular damage sustained during oxidative stress needs to be repaired, and this need is signalled by the release of pro-inflammatory molecules from the injured cells. Neuroinflammation can be either beneficial or deleterious depending on the duration of the inflammatory response. Acute inflammation is a defensive response that leads to the repair of injured cells, whereas a chronic inflammatory response can cause neuronal degeneration, and may eventually lead to the development of neurodegenerative diseases, such as AD (Morales, Guzmán-Martínez et al. 2014).
Increased production of reactive oxygen species resulting from mitochondrial dysfunction exceeds the antioxidant capacity, leading to oxidative stress, which then can induce neuroinflammation. The chronic neuroinflammation can cause neuronal death and neurodegeneration. Modified from (Finosh and Jayabal 2013, Morales, Guzmán-Martínez et al. 2014).

**Figure 1.2 The relationship between oxidative stress and neuroinflammation.**
1.1.5.2 Oxidative stress in OSA patients and in animal models of OSA

The levels of oxidised lipids, proteins and nucleic acids that are present in tissues have been used as markers of the extent of oxidative stress recently experienced by those tissues (Thérond, Bonnefont-Rousselot et al. 2000, Griffiths, Møller et al. 2002). These markers include plasma malondialdehyde for lipid hydroperoxidation, plasma 2,4-dinitrophenylhydrazine for carbonylated proteins, urinary o,o'-dityrosine for amino acid oxidation, and urinary 8-OH-2-deoxyguanosine for oxidative DNA damage (Thérond, Bonnefont-Rousselot et al. 2000, Jordan, Cohrs et al. 2006). Since cellular antioxidant systems can be upregulated or downregulated in response to increased oxidative stress, changes in the balance of antioxidant systems can serve as indirect indicators of oxidative stress (Griffiths, Møller et al. 2002, Gozal and Kheirandish-Gozal 2008). Antioxidant enzyme activities that have been used to assess oxidative stress levels include superoxide dismutase, glutathione peroxidase and catalase (Thérond, Bonnefont-Rousselot et al. 2000, Griffiths, Møller et al. 2002, Jordan, Cohrs et al. 2006).

Increased titres of markers of oxidative stress and inflammation have been detected in the blood of OSA patients (Gozal and Kheirandish-Gozal 2008, Sales, Bruin et al. 2013, Lavie 2015, Lira and De Sousa Rodrigues 2016, Macey, Sarma et al. 2017). Urinary excretion of 8-hydroxy-2′-deoxyguanosine was tested in OSA patients as an in vivo marker of oxidative stress, and were found to correlate with several indices of OSA severity including AHI, ODI, AI (apnoea index), the duration of oxygen saturation < 90% and respiratory arousal index (Yamauchi, Nakano et al. 2005). However, when adjusted for numerous confounding factors (BMI, age, gender, smoke, diastolic blood pressure, glycosylated haemoglobin, total cholesterol), only ODI showed a significant correlation, suggesting that ODI is a robust predictor of oxidative stress in OSA (Yamauchi, Nakano et al. 2005), perhaps because ODI reflects the frequency of episodes of hypoxemia/reoxygenation. In another study,
concentrations of 8-isoprostane, an oxidative stress marker, in the morning exhaled condensate and plasma, were positively correlated with the AHI, and the levels of 8-isoprostane were reduced after two nights of CPAP therapy (Carpagnano, Kharitonov et al. 2003).

Enzyme-linked immunosorbent assays, have revealed significantly higher levels of proinflammatory cytokines (tumour necrosis factor-α and interleukin-6) and decreased levels of anti-inflammatory cytokines (interleukin-10) in OSA patients compared to controls (Alberti, Sarchielli et al. 2003). Furthermore, 8-isoprostaglandin F2α, intercellular adhesion molecule 1, tumour necrosis factor-α and interleukin-6 are all significantly elevated in OSA patients, regardless of the presence of excessive daytime sleepiness (De la Peña Bravo, Serpero et al. 2007). Antioxidant capacity has been investigated in OSA patients; while no significant difference was found between the OSA and healthy control groups, severe OSA patients (AHI > 20) had a lower antioxidant capacity that was negatively correlated with AHI, indicating that oxidative stress increases with OSA severity (Christou, Moulas et al. 2003). It has been suggested therefore, that oxidative stress is an important treatment target for OSA (Zhang and Veasey 2012).

In animal models of OSA, chronic exposure to IH during sleep is achieved by an environmental chamber which simulates the hypoxia/reoxygenation patterns of OSA. Animals subjected to these regimes of IH have consistently demonstrated evidence of oxidative stress (Row, Liu et al. 2003, Shan, Chi et al. 2007, Hung, Tipoe et al. 2008), increased lipid peroxidation and oxidative injury (Veasey, Davis et al. 2004, Zhan, Fenik et al. 2005, Sanfilippo-Cohn, Lai et al. 2006). In rats for instance, two weeks of exposure to IH resulted in an increased production of reactive oxygen species (Shan, Chi et al. 2007), and increases in brain malondialdehyde levels (Row, Liu et al. 2003). The latter study reported
that malondialdehyde production in the cerebral cortex was correlated with spatial learning deficits, and the administration of an antioxidant attenuated these deficits (Row, Liu et al. 2003). Another IH study in rats identified significantly increased levels of malondialdehyde, mRNA expression of inflammatory mediators, including tumour necrosis factor-α, inducible nitric oxide synthase, cyclooxygenase-2 and decreased levels of antioxidant enzymes, including glutathione peroxidase, superoxide dismutase and catalase in the IH group when compared with the normoxic controls (Hung, Tipoe et al. 2008). In IH rats treated with the antioxidant melatonin, serum malondialdehyde levels were reduced, the expression of inflammatory mediators decreased, and the titres of antioxidant enzymes rose (Hung, Tipoe et al. 2008).

Collectively, the findings reviewed above demonstrate that IH and OSA are associated with increased levels of oxidative stress and systemic inflammation, and that administration of antioxidants and CPAP treatment can help to attenuate this stress. However, there is not yet a clear consensus on whether OSA causes oxidative stress, since a number of recent studies have failed to find an association. For example, no significant difference in lipid-based oxidative stress markers and lipophilic antioxidant levels was found in moderate-to-severe OSA patients with 8 weeks CPAP treatment compared to a sham CPAP group (Sivam, Witting et al. 2015). Moreover, 2 weeks of CPAP withdrawal resulted in the return of OSA in regular CPAP users, yet the increased ODI and blood pressure were not associated with hypoxia-induced blood protein marker levels or endothelial-derived inflammatory substances or urinary oxidative stress markers (Turnbull, Akoumianakis et al. 2017). Furthermore, increased inflammatory markers, but not oxidative stress markers were observed in OSA patients with daytime sleepiness compared to a control group (Andaku, D'Almeida et al. 2015).
1.1.6 Cognitive impairment

Numerous studies have shown that untreated OSA patients have deficits in their cognitive functions, such as impaired judgment, slower reaction time, and impairments in visuospatial learning ability, working memory, sustained attention, vigilance and alertness, and executive functioning (Salorio, White et al. 2002, Ferini-Strambi, Baietto et al. 2003, Lim, Bardwell et al. 2007, Daulatzai 2015). It has been reported that the extent of cognitive impairments in OSA patients is related to the severity of OSA, as indicated by the AHI (Naëgelé, Thouvard et al. 1995). Although there are many confounding factors (e.g. obesity and age) that can influence cognitive outcomes in OSA studies, studies of cognitive function in healthy individuals at high altitude (sustained hypoxia) have shown similar patterns of cognitive impairment. One study reported worsened mood, attention, visual and working memory, concentration, executive functions and altered sleep patterns in healthy subjects exposed to a simulated altitude of 4,500 m for two days (Aquino Lemos, Antunes et al. 2012). A more recent study directly exposed healthy participants to mild hypoxia (10% O₂) and elicited severe neurocognitive deficits in memory, speed of processing, executive functioning, attention, cognitive flexibility and task learning (Turner, Barker-Collo et al. 2015). Since these results were obtained under conditions of sustained hypoxia, where the production of reactive oxygen species is low, they introduce the interesting possibility that oxidative stress may not necessarily be the sole cause of cognitive impairment in OSA.

In a paediatric study, children with severe OSA were found to have a significantly lower IQ and displayed significant deficits in executive functioning when compared to control children matched for age, gender and ethnicity (Halbower, Degaonkar et al. 2006). Executive functions are responsible for volition, planning, purposeful action and monitoring effective performance (Ferini-Strambi, Marelli et al. 2013), and is one of the most consistently affected
domains in OSA (Saunamäki and Jehkonen 2007). It has been reported that impaired attention, visuospatial learning, and motor performance in OSA patients returns to normal levels after 15 days of CPAP treatment, whereas executive functions and constructional abilities do not improve, indicating that some cognitive domains are refractory to CPAP treatment (Ferini-Strambi, Baietto et al. 2003). Other studies have confirmed that executive function seems not to benefit from CPAP treatment (Saunamäki and Jehkonen 2007, Lau, Eskes et al. 2010).

A recent study affirmed the association between OSA frequency and severity of cognitive impairments and affective changes, with a moderate positive correlation ($r = 0.333$, $p = 0.038$) between OSA severity (AHI) and the Epworth Sleepiness Scale (ESS). Moreover, compared to the healthy control group, OSA patients demonstrated deficiencies in several cognitive domains, including attention, memory and executive function (Bilyukov, Nikolov et al. 2018). Similarly, in aged OSA patients (55 – 89 years) with mild cognitive impairment (MCI), significantly reduced attention and global cognition were observed in patients who did not use CPAP compared to CPAP adherents, after follow-up intervals of 6 months and 1 year (Richards, Gooneratne et al. 2019). Impaired verbal episodic memory has been reported in aged OSA patients with MCI and depression, and the severity of OSA was associated with microvascular damage and lesions, implicating cerebral microvascular pathology in the cognitive decline (Kerner, Roose et al. 2017). Furthermore, the involvement of vascular factors in cognitive performance was suggested in another study, where improved OSA after CPAP treatment was associated with favourable hemodynamic changes accompanied by improved verbal memory (Buratti, Viticchi et al. 2017).

Animal IH models have also revealed cognitive deficits, including spatial learning and working memory deficits (Gozal, Daniel et al. 2001, Row, Kheirandish et al. 2002, Gozal,
Row et al. 2003, Row, Liu et al. 2003, Row, Kheirandish et al. 2007). Impaired spatial learning has been shown in rats exposed to chronic episodic hypoxia, in which O$_2$ concentrations in the chamber were cycled between 10 – 21% during daylight, and remained at normal levels (21%) during the night (Gozal, Daniel et al. 2001). Mice exposed to IH after 14 days displayed worse information retention and recognition memory, consistent with impairments in short-term memory (Kim, Martinez et al. 2015). Another study reported impaired spatial learning in rats after 7 days of IH exposure, and followed by a partial functional recovery starting at 14 days, despite continuing IH exposure (Gozal, Row et al. 2003). Rat pups exposed to IH from postnatal (PN) day 10 until PN day 30 displayed significant spatial learning impairments, and male rats but not females displayed increased locomotor activity in the open field, indicating that exposure to IH at a developmental stage equivalent to early childhood induces substantial learning impairments and gender-dependent hyperactivity (Row, Kheirandish et al. 2002). Exposure of adult rats to IH for 14 days impaired their working memory with respect to controls (Row, Kheirandish et al. 2007).

1.2 OSA effects on the brain

The human brain comprises only 2% of the total body mass, yet it consumes 20% of total body oxygen and utilises 25% of total body glucose, largely because the central nervous system (CNS) is almost entirely dependent on the aerobic metabolism of glucose for the production of energy (Bélanger, Allaman et al. 2011). Differences in the regional density of microvessels determines, to a large extent, the regional blood flow and influences the surface area available for oxygen exchange, as well as the diffusion distance required for the exchange of substrates between brain tissue and blood (Ingraham, Forbes et al. 2008). That the brain is highly dependent on its microvascular network for oxygen supply is evidenced by the fact that all cells are located within 100 µm of a capillary (Alberts, Johnson et al. 2002,
Zakrzewicz, Secomb et al. 2002), due to the passive diffusion limit for oxygen (Carmeliet and Jain 2000). The capillary bed of the brain is a dense network, with the average distances between adjacent capillaries being just 40 μm (Zlokovic 2005, Fu and Liu 2013, Volkov, Margaryants et al. 2017), and the average distance of a neuron to a capillary ranging between 8 – 23 μm (Lovick, Brown et al. 1999, Tsai, Kaufhold et al. 2009, Gould, Tsai et al. 2017). Given this dependency on blood supply, even small reductions in cerebral blood flow (CBF), as frequently occurs in OSA, can lead to transient hypoxia.

1.2.1 Hypoxic injury to the hippocampus

The hippocampus is located in the inferomedial medial part of the temporal lobe and is widely regarded as the centre of brain networks associated with memory consolidation and retrieval, long-term memory, novelty detection, pattern discrimination, spatial navigation, as well as regulation of emotion, fear, anxiety and stress (Bartsch and Wulff 2015). The hippocampus is organised into specialised subfields (Figure 1.3), including the four subfields of the cornu ammonis area (CA1 – CA4), as well as the dentate gyrus and the subiculum (Bartsch and Wulff 2015, Schmidt-Kastner 2015). The hippocampus is among the first regions of the brain to suffer from various insults, such as ischemia, ageing and AD (Robinson 2001, Bartsch and Wulff 2015).
Coronal cross-section of the hippocampus, which includes the fimbria as illustrated in yellow, dentate gyrus in pink, CA4 (dentate gyrus + hilus), CA3, CA2, CA1, and the subiculum. Note the location of alveus, prosubiculum, entorhinal cortex and collateral sulcus, which will be investigated in Chapters 3, 4, 5 and 6. Diagram is modified from (Yang, Kim et al. 2008).

The hippocampus is extremely sensitive to hypoxic injury, and hypoxia contributes to hippocampal atrophy in OSA patients (Gale and Hopkins 2004). Magnetic resonance imaging (MRI) studies have detected volumetric loss of grey matter in the hippocampus of OSA patients (Morrell, McRobbie et al. 2003, Gale and Hopkins 2004, Yaouhi, Bertran et al. 2009, Canessa, Castronovo et al. 2011, Torelli, Moscufo et al. 2011). Reduced grey matter volume has been shown in the hippocampus of OSA patients before CPAP treatment (Canessa, Castronovo et al. 2011). After 3-month CPAP treatment, grey matter volume had increased in the hippocampus (left subiculum and bilateral entorhinal cortex) and was accompanied by a significant improvement in verbal and visuospatial short-term memory (Canessa, Castronovo et al. 2011).
In a recent study of the regional volume changes in the hippocampus of OSA patients, increased volume was observed mainly in the CA1 and subiculum region, while CA3/dentate gyrus showed decreased volume; furthermore, males with OSA had similar volume variations bilaterally, whereas females only displayed significant changes on the right side; it was speculated that the sex-specific hippocampal changes may account for some sex-related OSA symptoms, such as depression (Macey, Prasad et al. 2018). In another study, there was significantly increased left hippocampus volume after CPAP treatment in male OSA patients, specifically in the anterior dentate gyrus, which may indicate neurogenesis as a result of diminished oxidative stress (Kim, Joo et al. 2016). In contrast, OSA in children had no significant effect on the volume (macrostructure) of the dentate gyrus, whereas decreased mean diffusivity (microstructure) was correlated with increased AHI (Spearman's $r = -0.50$, $p = 0.008$), arousal index ($r = -0.44$, $p = 0.017$) and decreased verbal learning capacity ($r = 0.54$, $p = 0.004$); suggesting that disrupted microstructure may be a better indicator of neurocognitive impairment than the macrostructure (Cha, Zea-Hernandez et al. 2017).

A number of reports are available regarding the regional vulnerability of the hippocampus, with the CA1 region being more commonly affected than other regions (Fung, Xi et al. 2009, Fung, Xi et al. 2012). The CA1 region has been reported to be the most vulnerable hippocampal region to hypoxia (Kreisman, Soliman et al. 2000), while CA2 and CA3 are more resistant to hypoxic injury (Hung, Tipoe et al. 2008). The same pattern of regional vulnerability in the hippocampus is seen in patients with hippocampal ischemia, neuroinflammation and other neurological conditions, including AD (Pulsinelli, Brierley et al. 1982, Bartsch, Döhring et al. 2015, Bartsch and Wulff 2015, Schmidt-Kastner 2015). In AD, the CA1 is affected before the subiculum, CA2, CA3 and CA4/dentate gyrus (West, Coleman et al. 1994, Kril, Hodges et al. 2004, Chételat, Fouquet et al. 2008).
Neural injury and impaired neural function are assumed to be the main factors that contribute to the cognitive deficits observed in animals that have been exposed to hypoxia (Goldbart, Row et al. 2003, Zhu, Fenik et al. 2007). Studies of animal models of IH have reported cognitive impairments and neuronal loss in the hippocampus (Lim and Veasey 2010), with increased excitability of CA1 neurons and apoptosis (Fung, Xi et al. 2009, Fung, Xi et al. 2012). Another hypoxia study of rats found that apoptosis within the cortex and CA1 region of the hippocampus is time-dependent, but no degenerative effect was seen in the CA3 region (Gozal, Daniel et al. 2001). Pyramidal neurons in the CA1 have been reported to be highly vulnerable to hypoxia-ischemia, whereas neurons in the CA3 region and dentate gyrus are more resistant (Kreisman, Soliman et al. 2000). Interestingly, the vulnerability of the CA1 increases with age: in old rats (20 – 22 months old) exposed to IH, CA1 neurons demonstrated greater sensitivity to hypoxia than those in young rats (3 – 4 months old), and this was accompanied by a greater decline in performance on spatial tasks (Gozal, Row et al. 2003).

A study of the proteins differentially expressed in the CA1 and CA3 provided initial insights into mechanisms underlying different susceptibility of neural tissue to hypoxia: in rats exposed for 6 h to IH, 32 proteins in the CA1 region were up-regulated in contrast to 7 proteins in the more resistant CA3 area; two pro-apoptotic proteins, α-synuclein and doublecortin-like kinase, were increased by more than 2-fold in CA1, but not in CA3; expression of heat shock protein 70 was increased 1.6-fold in the CA1 region while it was decreased (0.56-fold) in the CA3, suggesting a greater intrinsic stress response in the vulnerable CA1 region (Gozal, Gozal et al. 2002). These findings were supported by a later study in which slices of living rat brain were exposed to paraquat to induce oxidative stress; neurons in the CA1 were found to be more sensitive to oxidative stress than CA3 neurons (Wang, Zaidi et al. 2009). Comparison of neurons from these two regions in basal conditions...
revealed that CA1 neurons have a higher expression of genes associated with stress and inflammation and a lower expression of genes associated with energy generation. They concluded that the vulnerability of CA1 neurons is due to low intrinsic energy reserves coupled with high intrinsic levels of stress (Wang, Zaidi et al. 2009).

1.2.2 Cerebral blood flow (CBF) adaptation to short-term and long-term hypoxia

The entire brain is more vulnerable than other organs to hypoxia, and irreversible damage of the CNS can be caused by only 2 – 3 min of inadequate oxygen supply (Yoshihara, Takuwa et al. 2013). In acute hypoxia, the systemic blood flow is redistributed to preferentially supply the brain and heart (Faraci, Kilgore Jr et al. 1984, Boero, Ascher et al. 1999), and consequently CBF is elevated in response to hypoxia (Beck and Krieglstein 1987, Dahlgren 1990, LaManna, Vendel et al. 1992, Xu, Puchowicz et al. 2004). Rats exposed to 7% O₂ for 30 minutes displayed an increased regional CBF of 50 – 90% in the grey matter and up to 180% in the white matter (Beck and Krieglstein 1987). Similarly, rats kept in hypobaric chambers (0.5 atm) for one day had a significantly higher blood flow in all brain regions (frontal cortex 2.39-fold increase, parietal cortex 2.57-fold increase, hippocampus 2.52-fold increase, brainstem 2.50-fold increase, cerebellum 1.56-fold increase) (Xu, Puchowicz et al. 2004). The blood flow in the frontal cortex (2.23-fold) and hippocampus (1.95-fold) were still elevated on day 2 of exposure; blood flow returned to baseline in all regions by day 4 of exposure and then remained unchanged for the remainder of the hypoxic period (Xu, Puchowicz et al. 2004). By contrast, decreased rates of CBF have been recorded in OSA patients during wakefulness (Joo, Tae et al. 2007, Innes, Kelly et al. 2015), demonstrating a difference between OSA patients and animal models of IH or sustained hypoxia.

It has been proposed that there are three main adaptations of the brain in response to sustained hypoxia. Initially CBF increases by as much as two-fold, mostly due to
vasodilation (Xu and LaManna 2006). After a few days of exposure, CBF begins to fall as the oxygen-carrying capacity of the blood improves (an increase in hemocrit). After two weeks of exposure to hypoxia, the brain capillary density has increased as a result of angiogenesis, and by three weeks post-hypoxia exposure the CBF returns to the prehypoxia baseline level (Xu and LaManna 2006).

Chronic changes in blood flow are thought to alter the architecture of blood vessels (Rudic, Shesely et al. 1998). Blood flow generates mechanical forces on the vascular wall: circumferential stress is related to pressure and vessel dimensions (diameter and thickness), while longitudinal shear stress acts on the blood-endothelium interface (Lehoux and Tedgui 2003). Changes in mechanical stress caused by changes in blood flow are detected by receptors on vascular cells and sustained activation triggers signals that lead to adaptive transformations of the vessel wall (Lehoux and Tedgui 2003). Sustained increases in blood flow are associated with an increase in lumen diameter in normal conduit vessels, while decreased blood flow is associated with a decrease in lumen diameter (Rudic, Shesely et al. 1998). A 70% reduction in the rate of blood flow has been reported to lead to a 21% decrease in the diameter of the rabbit's carotid artery within 2 weeks (Langille and O'Donnell 1986).

1.3 Hypoxia and adaptations of the cerebral microvasculature

The cerebral vasculature is composed of arteries and arterioles, which deliver blood rich in oxygen and glucose to the brain; a capillary bed, consisting of capillaries and post-capillary venules that are essential for oxygen and nutrient exchange with the neuropil; and venules and veins that drain waste metabolites, including carbon dioxide from the brain (Kalaria 1996, Daneman and Prat 2015). The capillary bed or 'microvasculature' is unique as it not only supplies blood to the brain, but also directly interacts with glial cells and neurons (Del Zoppo and Milner 2006), forming a functional neurovascular unit, an integrated system that
maintains brain homeostasis by selective permeability of some molecules and regulation of the CBF (Islam and Mohamed 2015). The neurovascular unit is composed of a capillary segment with its associated endothelial cells, pericytes, astrocytes, neurons and microglial cells, as illustrated in Figure 1.4. Astrocytes act as intermediaries between neurons and the microvasculature; they control vessel diameter, and hence blood flow, in response to the metabolic requirements of neurons in their vicinity (Daneman and Prat 2015). The research in the present thesis focuses on the capillaries of the brain which comprise more than 60% of the cerebral microvasculature (Del Zoppo and Milner 2006) and have a combined length of 650 km and a total surface area of 10 – 20 m² (Cecchelli, Berezowski et al. 2007).
Figure 1.4 Schematic representation of the neurovascular unit.

The neurovascular unit permits bidirectional communication between the cerebral microvasculature and neurons via the intermediary astrocytes, with the astrocyte endfeet almost completely ensheathing the vascular tube, which is formed by a single endothelial cell folding onto itself, leaving a gap that is sealed by tight junction proteins. Pericytes are located on the abluminal surface of the endothelium between the basement membrane and astrocyte endfeet. Notice the Virchow-Robin space indicated by the black wavy lines, it is the perivascular space between the penetrating artery/venule and brain tissue that narrows and disappears as the penetrating artery branches into an arteriole and then a capillary. Redrawn and modified from (Jessen, Munk et al. 2015).
A feature of the neurovascular unit that distinguishes it from vasculature in other tissue is the presence of the blood–brain barrier (BBB) (Lawther, Kumar et al. 2011, Blanchette and Daneman 2015). The BBB is selectively permeable to the paracellular diffusion of a limited number of compounds from the blood to brain (Luissint, Artus et al. 2012). The BBB and was first identified following injection of dyes into the blood stream with the observation that these dyes would accumulate in peripheral tissues at a much greater concentration than in the CNS (Serlin, Shelef et al. 2015). The BBB forms an interface (0.3 – 0.5 μm thick) between the systemic blood circulation and the CNS (Zlokovic 2005). Its functions include protecting the CNS from an influx of blood cells, stabilizing the internal environment of the CNS by regulating the transport of nutrients and toxic substances into and out of the CNS, and establishing a consistent ionic environment that is favourable to neurotransmission (Daneman and Prat 2015).

The BBB consists of endothelial cells that form the walls of the blood vessels, adjoined by tight cell-to-cell junction proteins, basement membrane, pericytes which are embedded within the basement membrane that can often span several endothelial cells, as well as astrocyte endfeet which ensheath the vessels (Daneman and Prat 2015, Serlin, Shelef et al. 2015). The capillary is formed by a single endothelial cell that folds onto itself, forming the lumen of the vessel, which is semipermeable, allowing for regulated transport of fluids and solutes into and out of the blood (Aird 2007).

There are two main categories of transporters expressed by capillary endothelial cells, the efflux transporters at the abluminal (brain-facing) side, which transport potentially toxic molecules across the cell membrane towards the blood, and the highly specific nutrient transporters at the luminal (blood-facing) side, such as glucose transporter-1 (GluT-1), which facilitates the transfer of glucose from blood into the neuropil (Zlokovic 2005, Daneman and
Prat 2015). Tight junctions seal the adjacent endothelial cells and form a diffusion barrier, which prevents most blood-borne substances from freely entering the brain. The tight junctions contain the junction adhesion molecules claudin and occludin, as well as a number of cytoplasmic accessory proteins (Ballabh, Braun et al. 2004). The basement membrane separates the endothelium from pericytes and astrocytes, with pericytes situated on the outer side of the basement membrane (Zlokovic 2005). Pericytes maintain vascular integrity and contribute to vascular remodelling as well as tight junction formation (Balabanov and Dore-Duffy 1998). Astrocytic end-feet tightly ensheath the vessel walls and play a role in the induction and maintenance of the tight junction barriers (Ballabh, Braun et al. 2004).

The BBB is critical for maintaining brain homeostasis, assisting in proper neuronal function, and protecting neurons from agents that could be injurious (Daneman and Prat 2015). Dysfunction of the BBB can lead to altered ion regulation and signalling dyshomeostasis, and entry of plasma components, immune cells and molecules into the CNS, which increase the risk of neuronal dysfunction and degeneration (Daneman 2012, Daneman and Prat 2015). Disruption of the BBB has been identified in stroke, epilepsy, multiple sclerosis, brain trauma, tumours and AD, using both brain imaging in human patients and analysis of post-mortem brain samples (Zlokovic 2008, Daneman 2012, Blanchette and Daneman 2015, Daneman and Prat 2015). It is not known whether the BBB is disrupted in OSA.

1.3.1 Angiogenesis in response to hypoxia

'Angiogenesis' refers to a process whereby new capillaries bud from existing microvessels (Grant and Kleinman 1997, Yoo and Kwon 2013) to increase capillary density and decrease the intercapillary diffusion distances, resulting in improved tissue oxygenation (Harik, Hritz et al. 1995, LaManna, Chavez et al. 2004, Boroujerdi, Welser-Alves et al. 2015). Angiogenesis is a complex multistep process that plays a central role in various physiological
conditions, such as trauma, wound healing, tissue repair, ischemia and hypoxia (Yoo and Kwon 2013). Hypoxia is a strong trigger of angiogenesis (Carmeliet and Jain 2000, Rey and Semenza 2010, Krock, Skuli et al. 2011, Zimna and Kurpisz 2015), and promotes vessel growth by upregulating hypoxia-inducible factors (Rey and Semenza 2010, Hashimoto and Shibasaki 2015), that mediate multiple proangiogenic pathways.

Different levels of oxygen availability and blood flow combine to determine the vascular response (Table 1.3). During normoxia and normal flow, expression levels of vascular endothelial growth factor and angiopoietin-2 are low, while the constitutively expressed angiopoietin-1 activates Tie-2 (tyrosine-protein kinase receptor TIE-2), leading to vessel stabilisation as occurs in a mature, quiescent blood vessel. By contrast, in hypoxic conditions with low rates of blood flow, the expression of both vascular endothelial growth factor and angiopoietin-2 are induced, the combination of Tie-2 with angiopoietin-1 is replaced by angiopoietin-2, resulting in a diminishment of the stabilising effect of Tie-2 on the vascular wall, allowing vascular endothelial growth factor to stimulate enlargement of existing vessels and/or the formation of new vessels. Finally, under normoxic and low flow conditions, as occurs in redundant vessels that are underperfused in a well-oxygenated environment, vascular endothelial growth factor expression is suppressed but angiopoietin-2 expression is increased, the limited growth factors and destabilised vessel wall finally resulting in apoptosis and vessel regression (Zakrzewicz, Secomb et al. 2002).
Table 1.3 Vascular responses to different levels of oxygen and blood flow.

<table>
<thead>
<tr>
<th>Combined conditions</th>
<th>Oxygen level</th>
<th>Blood flow level</th>
<th>Vascular reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
<td>Normal flow</td>
</tr>
<tr>
<td></td>
<td>VGEF ↓</td>
<td>VGEF ↑</td>
<td>Ang-2 ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ang-1 −</td>
</tr>
<tr>
<td></td>
<td>VEGF-</td>
<td>VEGF-</td>
<td>Tie-2 ↑</td>
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<tr>
<td></td>
<td>R1/R2 ↓</td>
<td>R1/R2 ↑</td>
<td></td>
</tr>
<tr>
<td>Normoxia +</td>
<td>✅</td>
<td></td>
<td>Stabilisation</td>
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<tr>
<td>Normal flow</td>
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<tr>
<td>Hypoxia +</td>
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<td>✅</td>
<td>Angiogenesis</td>
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<td>Low flow</td>
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<tr>
<td>Normoxia +</td>
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<td>✅</td>
<td>Regression</td>
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<td>Low flow</td>
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</tbody>
</table>

Normoxia and normal flow leads to vessel stabilisation, not proliferation. Hypoxia and low flow lead to angiogenesis, vascular proliferation, enlargement of existing vessels and/or formation of new vessels. Normoxia and low flow leads to regression, loss of structure, destabilised vessel wall and apoptosis. Modified from (Zakrzewicz, Secomb et al. 2002). VEGF: vascular endothelial growth factor; VEGF-R1/R2: vascular endothelial growth factor receptor 1/receptor 2; Ang-: angiopoietin-; Tie-2: Tyrosine-protein kinase receptor.
Two types of angiogenesis have been identified: sprouting angiogenesis and intussusception angiogenesis (Zakrzewicz, Secomb, & Pries, 2002), as illustrated in Figure 1.5. Sprouting angiogenesis is the basic mechanism for the growth of new blood vessels, it starts with local vasodilation and increased vascular permeability (Djonov, Baum et al. 2003), leading to a localised disruption of the basement membrane. The endothelial cells are activated by vascular endothelial growth factor to become tip cells that invade the surrounding extravascular matrix; they reorganise to form a vascular lumen that fuses with another capillary and stabilises into non-leaky mature endothelium that becomes enveloped by pericytes (Djonov, Baum et al. 2003, Prior, Yang et al. 2004, De Spiegeelaere, Casteleyn et al. 2012).

Intussusception angiogenesis, also known as splitting angiogenesis, begins with endothelial cells forming an involution that extends down the centre of the vessel lumen to separate a vessel into two parallel capillaries (Zakrzewicz, Secomb et al. 2002, Mentzer and Konerding 2014). Intussusception angiogenesis is faster and metabolically cheaper than sprouting angiogenesis, as it is the reorganization of existing cells, allowing the capillary to grow ‘within itself’ without the need for endothelial migration and proliferation. Furthermore, the basement membrane may remain intact, which prevents the vessels from becoming leaky (De Spiegeelaere, Casteleyn et al. 2012). Sprouting angiogenesis precedes intussusception angiogenesis and is only responsible for the formation of new vessels, whereas intussusception angiogenesis is considered as a general mechanism for vascular remodelling and pruning (Makanya, Hlushchuk et al. 2009, De Spiegeelaere, Casteleyn et al. 2012).
Figure 1.5 Schematic representation of sprouting and intussusception angiogenesis.

Sprouting angiogenesis is localised at the abluminal aspect of vessels, endothelial cells are activated to become tip cells in response to vascular endothelial growth factor produced by hypoxic cells; a new blood vessel sprout is composed of one leading tip cell and many stalk cells, tip cells project into the degraded extracellular matrix through a disrupted BM. Intussusceptive angiogenesis is determined by the direction of pillar extension (red solid arrow head), a cylindrical microstructure that spans the capillary lumen, intussusceptive pillars may project across the vessel lumen with an intact basement membrane. i) pillar growth toward the vessel angle results in the vascular remodelling to vessel bifurcation; ii) pillar extension down the axis of the vessel results in vascular splitting to vessel duplication; and iii) asymmetric pillar growth leads to vascular pruning of a redundant or energetically inefficient vessel. Based on (Prior, Yang et al. 2004, De Spiegelaere, Casteleyn et al. 2012, Mentzer and Konerding 2014).
In the rodent brain, the process of hypoxia-induced angiogenesis takes about three weeks to reach completion (Ndubuizu, Tsipis et al. 2010). Hypoxia-inducible factor-1α rapidly accumulates during the onset of hypoxia and remains significantly elevated for at least 14 days, before returning to near baseline levels after 21 days (Chávez, Agani et al. 2000). Similarly, vascular endothelial growth factor mRNA expression increases 2-fold after 12 h exposure to hypoxia and reaches a maximum of 5-fold after 2-7 days before returning to normoxic levels after 21 days (Kuo, Benhayon et al. 1999).

Animal studies have consistently documented increased capillary density in response to exposure to chronic hypoxia (LaManna, Chavez et al. 2004). One study reported an increased capillary density of 60% in the rat cerebral cortex after 3 weeks of hypobaric hypoxia (0.5 atm, equivalent to 10% normobaric oxygen), which progressively decreased to prehypoxic levels after 3 weeks of normoxic recovery (Pichiule and LaManna 2002). In another study, mice were placed into a hypobaric chamber maintained at 0.4 atm which is equivalent to 8% normobaric oxygen; over the course of 14 days, increased capillary density was found with two different endothelial cell markers, CD31 and CD105, in both the brainstem and the cerebral cortex (Milner, Hung et al. 2008). In a chronic mild hypoxia model, mice exposed to 8% O₂ for 14 days had significantly increased total vascular area in the frontal lobe and this was followed by a vascular pruning response after two weeks of recovery in normoxic conditions (Boroujerdi, Welser-Alves et al. 2015).

There is remarkable difference between vascular responses to sustained hypoxia and intermittent hypoxia. Sustained hypoxia is associated with the rapid stabilisation of hypoxia-inducible factor-1 (Ryan, Taylor et al. 2005, Chiang 2006), resulting in increased expression of vascular endothelial growth factor (Ryan, Taylor et al. 2005, Chiang 2006). By contrast, IH cannot maintain the stabilisation of hypoxia-inducible factor-1 (Ryan, Taylor et al. 2005,
Chiang 2006), so vascular endothelial growth factor is expressed at lower levels than in sustained hypoxia and the stimulus for angiogenesis is weaker. In a study using the endothelial cell marker, GluT-1, where mice were subjected to either IH (11% O₂, 4-min cycles) or continuous hypoxia (11% O₂) for 2 weeks, the capillary density in the cerebral cortex was increased by 18% and 32%, and in the hippocampus by 20% and 38%, respectively (Kanaan, Farahani et al. 2006). However, it is not known whether the same extent of angiogenesis occurs in the hippocampus of OSA patients.

In the adult brain, mature capillaries display high electrical resistance and a tight BBB, due to tight junction proteins and the influence of astrocyte endfeet (Engelhardt 2003, Ballabh, Braun et al. 2004). These characteristics also contribute to angiogenesis in response to hypoxia. In a study by (Boroujerdi, Welser-Alves et al. 2015), mice exposed continuously to 8% O₂ for 14 days had an increased expression of the tight junction protein Claudin 5; two weeks of recovery resulted in fragmentation of Claudin 5 and the vascular pruning response. In another study, mice exposed to 0.4 atm (equivalent to 8% O₂) for 14 days showed an increased number of vessels positive for Claudin 5 by immunofluorescence, while western blotting revealed a 3.1-fold increase in the expression of Claudin 5 (Li, Welser et al. 2010).

In a technically challenging study, a closed cranial window was placed over the left parietal cortex of living mice which were then exposed to hypoxia (8 – 9% O₂) for up to 21 days. The study observed vessel sprouts after 7 – 14 days of hypoxia exposure, and after 14 – 21 days of hypoxia, the sprouting vessels had created new connections with existing capillaries to restore and increase the regional blood flow (Masamoto, Takuwa et al. 2014). In another animal study, significant BBB leakage was observed with sodium fluorescein, a fluorescent tracer that does not cross an intact BBB. After exposure to normobaric hypoxia (8% O₂) for 24 h, there was 2-fold increase in fluorescence intensity compared to normoxic controls,
suggesting that vascular permeability in the brain had increased as a result of hypoxic injury (Schoch, Fischer et al. 2002). Such BBB leakage has been suggested to alter the CNS microenvironment during IH, perturbing neuronal function and contributing to cognitive impairment (Lim and Pack 2014).

The contribution of hypoxia to angiogenesis is demonstrated in retinopathy of prematurity, a leading cause of blindness and visual impairment in premature infants (Chen and Smith 2007). Retinopathy of prematurity occurs when premature babies are given supplemental oxygen to breathe to compensate for their underdeveloped lungs; this additional oxygen retards developmental angiogenesis and may lead to a partial regression of existing vessels, resulting in the periphery of the immature retina being under-vascularised (Hellström, Smith et al. 2013). Once the baby is transferred to ambient air, the retinal tissue becomes hypoxic due to its inadequate blood supply. The hypoxic tissue then stimulates pathological angiogenesis with the newly-formed vessels being leaky and perfusing the retina poorly, leading to fibrous scar formation and retinal detachment (Hellström, Smith et al. 2013). Retinopathy of prematurity provides direct evidence that chronic hypoxia induces angiogenesis (although abnormal) in human eyes, however it is not known whether similar angiogenesis occurs in the adult human brain in response to chronic intermittent hypoxia in OSA.

1.3.2 Angioadaptation in response to hypoxia

Microvessels respond to both metabolic and hemodynamic stimuli, and hypoxia promotes vasodilation that leads to an increase of blood flow and oxygen supply (Reglin and Pries 2014). Capillary dilation is an immediate response to hypoxia; it not only helps to accommodate the increased volume of blood flow, it also reduces the resistance that occurs on the walls of vessels due to shearing stress (Boero, Ascher et al. 1999). Vessel diameter
adaptation occurs in two ways, short-term changes in smooth-muscle tone and structural, long-term changes (vascular remodelling) (Reglin and Pries 2014).

In a mouse hypobaric hypoxia model (455 Torr; equivalent to 0.6 atm; 12% normobaric oxygen), 28-days of exposure significantly increased the internal capillary diameter in several regions of the brain, including the CA1 stratum oriens (1.18-fold) in the hippocampus (Boero, Ascher et al. 1999). In another study, mice were exposed to continuous hypoxia (either 8% or 10% O2) for up to 1 month and longitudinal in vivo microvasculature within a living mouse somatosensory cortex was imaged with two-photon microscopy. Compared to the pre-hypoxic levels, mean capillary diameters were increased 1.4-fold and 1.2-fold after one week of exposure to 8% or 10% O2, respectively; after three weeks of exposure the mean capillary diameter had increased further to 1.8- and 1.4-fold, respectively (Yoshihara, Takuwa et al. 2013). With the same imaging method, another study exposed mice to three weeks of normobaric hypoxia (8 − 9% O2) and observed a 1.6-fold increase (4.7 to 7.3 µm) in parenchymal capillary diameters compared with pre-hypoxic values (Masamoto, Takuwa et al. 2014).

In chronic hypoxia, capillaries appear more elongated, dilated and have more a winding course (i.e. 'tortuous') than those in normoxic controls (Boero, Ascher et al. 1999). In an animal hypobaric hypoxia model (0.6 atm, 12% O2), capillary length per unit volume of tissue (Lv) was found to be significantly increased after 28 days of exposure throughout the brain, including in hippocampal regions: subiculum (1.58-fold), CA1 (1.33 − 1.54-fold), CA3 (1.28 − 1.47-fold) and dentate gyrus (1.23 − 1.34-fold) (Boero, Ascher et al. 1999). Moreover, significant increases in capillary surface area per unit volume of tissue were observed (Boero, Ascher et al. 1999). However, in another study, 21 days of exposure to 8%
O$_2$ did not alter the average length of the individual capillaries (< 7 μm in diameter) (Yoshihara, Takuwa et al. 2013).

In a mouse model of IH, the extent of angiogenesis was found to vary in response to the severity of hypoxia (Lim, Brady et al. 2016). The total capillary length in the hippocampus was significantly greater in mice subjected to very severe IH (SaO$_2$ nadir 37%) for 2 weeks (12 h/day) compared to those in the continuous air (SaO$_2$ 95%) and severe IH (SaO$_2$ nadir 61%), while no significant difference was found between the control and severe IH groups (Lim, Brady et al. 2016). These results indicate that under very severe conditions, IH can trigger adaptive changes in the microvasculature of the hippocampus. However, it is unclear how relevant the results from this animal study are to the IH experienced by humans with OSA. Exposure of young adult mice to 2 weeks of IH may not produce comparable outcomes to the decades of exposure to IH experienced by patients with untreated OSA. Furthermore, the severity of hypoxia used by Lim and colleagues (2016) was extreme; humans with severe OSA may experience a few instances where the nadir SaO$_2$ falls below 80% (Badran, Ayas et al. 2014), yet Lim et al induced nadirs of 61% and 37% every minute for 12 hours per day (Lim, Brady et al. 2016). Finally, there is evidence that microvessels in the brains of young adults demonstrate greater adaptability to hypoxia than those in aged brains, as discussed below.

1.3.3 Aged microvasculature in response to hypoxia

The balance between capillary density/oxygen delivery and metabolic demand decreases with age (LaManna, Chavez et al. 2004) as microvessels lose their plasticity (Casoli, Spagna et al. 1996, Riddle, Sonntag et al. 2003, Burke and Barnes 2006, Topiwala and Ebmeier 2012). The limited capacity of old capillaries to respond to increases in neural activity causes mismatches between blood flow and metabolic needs, resulting in synaptic and neurovascular
injury, which has been suggested to contribute to age-related neuropathology (Riddle, Sonntag et al. 2003, LaManna, Chavez et al. 2004, Ingraham, Forbes et al. 2008, Harb, Whiteus et al. 2013). Decreased cerebral microvascular density has been reported in aged subjects compared to young controls, and many of the remaining vessels displayed abnormal morphological alterations (Riddle, Sonntag et al. 2003, Petcu, Smith et al. 2010).

Different vessel formation events have been reported between young and old mice in response to normobaric hypoxia for 28 days (Harb, Whiteus et al. 2013). When young mice (4 – 6-week-old) were exposed to 10% O2 for 28 days, a considerable degree of angiogenesis was observed. In contrast, adult mice (3 – 4 months old) and aged mice (22 – 25 months old) exposed to 10% O2 for 28 days did not differ significantly from control mice that had been exposed to normoxia, suggesting that the cerebral microvasculature loses its plasticity by adulthood and is less responsive to metabolic perturbation (Harb, Whiteus et al. 2013). Similarly, after exposure to chronic hypoxia (11% O2) for 4 weeks, young adult rats (4-month-old) displayed a 35 – 40% increase in the number of microvessels in all subregions of the hippocampus, whereas aged rats (30-month-old) displayed more modest capillary growth: 20% increase in the dentate gyrus, 15% in the CA3, and only 6 – 7% in the CA1 region (Ingraham, Forbes et al. 2008).

Not all studies have detected age differences in respect to microvascular adaptiveness to hypoxia. Ndubuiizu and colleagues (2010) observed an increase in the density of cerebral microvessels in 24-month old rats exposed to chronic hypoxia (8% O2 for 3 weeks), that was accompanied by increased mRNA and protein levels of vascular endothelial growth factor (Ndubuiizu, Tsipis et al. 2010). Compared to normoxic controls, microvessel density increased by 48% in cerebral cortex, 53% in the corpus callosum, 35% in the striatum and 60% in the CA1 region of the hippocampus; while microvessel density in young rats (3
months old) increased by 41%, 61%, 44% and 47% respectively, demonstrating no significant difference in the angiogenic response between young and aged rats (Ndubuizu, Tsipis et al. 2010).

In a survival rate study, all young mice (4-month-old) survived throughout 3 weeks of exposure to hypoxia (8% O₂), whereas one third of the aged mice (24 months old) died between 8 – 12 days of exposure (Benderro and Lamanna 2011). It was speculated that the increased mortality was due to a slower rate of angiogenesis in the old mice, since after the first week of hypoxia capillary density in the young mice had increased by 29% compared to 13% in the aged mice (Benderro and Lamanna 2011). However, by the second week of exposure there was no significant difference between the young and aged mice (42% vs. 33%) (Benderro and Lamanna 2011).

Taken together, results from animal hypoxia models disagree as to whether the angiogenic response becomes impaired with age, or merely slowed. At present no data are available concerning adaptive changes to IH in adult human brains so it remains unclear whether results obtained from rodent models of continuous hypoxia or IH have ecological relevance to OSA in aged humans.

1.4. Neurodegeneration and microvascular disease

As noted previously, the brain is dependent on a sufficient supply of blood, and when the blood supply does not meet the metabolic demands of the tissue, neurons are unable to function normally and may degenerate (Figure 1.6). Reduced CBF, as occurs during normal ageing in association with the presence of vascular risk factors such as cardiac disease, stroke, atherosclerosis, diabetes mellitus and hypertension can result in cerebral hypoperfusion, which when it reaches a critical threshold, will promote brain capillary degeneration and energy crisis (De la Torre 1999). The damaged capillary ultrastructure
begins in specific brain regions, such as the hippocampal CA1, leading to decreased glucose transport and compromised neuronal metabolism (De la Torre 1999), which may partly explain the selective vulnerability of the CA1 region. Cerebral hypoperfusion has been regarded as an early feature of AD (De la Torre 1999, Love and Miners 2016).

Figure 1.6 Hypothesised interrelationships between degenerative changes in blood vessels and cognitive dysfunction.

Reduced CBF in the cerebral microvascular network is associated with aberrations in the capillary ultrastructure, such as local thickening of the basement membrane, partial obstruction or compression. These microvascular aberrations disturb the regular passage of blood when the usual shape of the vascular lumen becomes irregular, leading to microturbulence which compromises glucose transport across the BBB. Thus, the presence of these microvascular pathologies is indicative of suboptimal CBF and neuronal dysfunction. Based on (Farkas and Luiten 2001).
Cerebral microvascular pathology has been suggested to precede age-related neurodegeneration (Brown and Thore 2011), and the neurodegenerative processes may be aggravated by the presence of microvascular and macrovascular pathologies (Blair, Hernandez et al. 2017, Umemura, Kawamura et al. 2017, Jaswal, Swardfager et al. 2018). It has been suggested that impaired cognition during normal ageing may be partly due to the capillary changes in the hippocampus (Keuker, Luiten et al. 2000).

AD is an example of an age-related neurodegenerative disorder that is associated with microvascular dysfunction (Zlokovic 2005, Zlokovic 2011). Vascular deficiency/hypoperfusion has been associated with brain atrophy, dementia and the accumulation of Aβ in the brain (De la Torre 1999, Hagino, Kobayashi et al. 2004, Bouras, Kövari et al. 2006, Love and Miners 2016). Several ultrastructural and morphological alterations to the microvasculature have been reported in ageing and neurodegenerative disorders, including reduced packing density, increased numbers of fragmented vessels, perivascular collagen deposits, basement membrane thickening, string vessels, tortuous arterioles, twisted capillaries, marked changes in the vessel diameter and increased irregularity of capillary surfaces (Kalaria 1996, Moody, Brown et al. 1997, Farkas and Luiten 2001, Zlokovic 2008, Bell and Zlokovic 2009).

1.4.1 String vessels are indicators of degenerated capillaries

String vessels are thin tissue strands, 1 – 2 μm wide that join one capillary to another. They do not carry blood and they mark the location of capillaries that have collapsed and regressed (Challa, Thore et al. 2002), hence they are common after cerebral ischemia (Reinecke, Kuwabara et al. 1962). String vessels consist mainly of Collagen IV, one of the basement membrane connective tissue components (Brown 2010), and they are negative for CD34 (Challa, Thore et al. 2004). They can be found in human brains of all ages from preterm
babies to the very old, and they seem to eventually degrade and disappear because fewer string vessels are present in very old brains (Brown 2010). Exceptions include the grey matter of AD patients, which contains twice as many string vessels as the brains of age-matched healthy controls, and the brains of ApoE ε4 carriers, which have more string vessels than non-ε4 carriers (Hunter, Kwan et al. 2012). There were significantly more string vessels (4.0 per mm²) in the white matter of AD individuals compared to the control group (1.4 per mm²), as well as greater total string vessel length (0.29 vs. 0.10 mm/mm²) (Challa, Thore et al. 2004).

Although mostly reported in the CNS, string vessels can occur in any tissue where capillaries have died due to cessation of blood flow (Brown 2010). It is possible that string vessels develop as a result of reduced metabolism and CBF (Challa, Thore et al. 2004), while the formation of string vessels might further shut down blood flow and glucose supply to the brain, which may exacerbate neurodegeneration and the progression of AD (De la Torre 1999, Love and Miners 2016). Given that decreased regional CBF has been reported in OSA patients (Joo, Tae et al. 2007, Innes, Kelly et al. 2015), it is possible that the brains of OSA patients contain higher than normal numbers of string vessels, and that their density increases with OSA severity. However, these possibilities have not yet been investigated.

1.4.2 Thickened basement membranes in vessels from aged brains

Endothelial basement membrane is a structural component of the BBB, and creates a selectively permeable boundary between the brain parenchyma and CBF (Zarow, Barron et al. 1997). Basement membrane consists mainly Collagen IV (Farkas and Luiten 2001), that arranges into a cross-linked, three-dimensional matrix that fills the space between the vascular endothelium, and the pericytes and perivascular astrocytes (Zarow, Barron et al. 1997). The basement membrane becomes thicker with age (Serot, Foliguet et al. 2001), and
localised thickenings of the basement membrane become relatively common in old age (Farkas and Luiten 2001). For instance, a low frequency of thickenings of the basement membrane was observed in the frontoparietal motor cortex of rats aged 16, 24 and 30 months, whereas between the age of 30 − 32 months approximately 10% of all cortical microvessels exhibited basement membrane thickenings (De Jong, Traver et al. 1992). The percentage of capillaries with basement membrane thickenings in the hippocampus was low (28 ± 6%) in young monkeys (1 – 6 years), and higher (71 ± 5%) in aged rhesus monkeys (29 – 31 years) (Keuker, Luiten et al. 2000).

Variations in vascular diameter caused by constrictions and dilations directly influence blood flow by altering vascular resistance. A consistent vessel diameter is essential for the efficient flow of blood, and the existence of narrowed segments of lumen due to a thickened basement membrane will slow the CBF (Farkas and Luiten 2001). Thus, increases in basement membrane thickness are likely to contribute to age-related declines in regional CBF, and consequent reductions in cognitive functioning.

1.4.3 Tortuous vessels in aged and AD brains

During old age the course of arterioles through the deep white matter of the brain can transform from a linear one to a more winding, circuitous course that can include coils, loops and spirals (Farkas and Luiten 2001). The irregular course of the vessel implies altered haemodynamics that will inevitably impede CBF (Kalaria 1996). These 'tortuous' vessels are a result of reduced elasticity of the vessel walls combined with shrinkage of the tissue they supply. The vessels follow a recurved course in order to accommodate their excess length (Del Corso, Moruzzo et al. 1998). Tortuous vessels are rarely seen in preterm babies, children or young adults, and their number starts to increase after middle age (Thore, Anstrom et al.
Tortuous microvessels are also common in AD, presumably as a result of neurodegeneration and while matter loss (Thore, Anstrom et al. 2007).

In addition to being a marker of neurodegeneration and brain shrinkage, tortuous vessels are less efficient at delivering blood and therefore can contribute to neuronal dysfunction, thus leading to a vicious cycle of neurodegeneration and tissue shrinkage. Since areas of the human hippocampus are known to undergo shrinkage in OSA, and the extent of this shrinkage is associated with OSA severity (Owen, Benediktsdottir et al. 2019), it would be important to investigate the brains of OSA patients for the presence of tortuous vessels.

1.5 Oxidative stress and corpora amylacea (CoA)

While abnormalities in the cerebral microvasculature are an important feature of ageing and neuropathological disease, another important marker of these conditions can be found in corpora amylacea (CoA). Under the light microscope CoA appear as featureless spherical bodies with diameters ranging from 5 to 50 μm (Cavanagh 1999, Rohn 2015, Pisa, Alonso et al. 2018), although a few can be measured down to 2 μm (Cavanagh 1998) or up to 100 μm (Michaels and Levene 1957). Under the microscope the individual profiles of CoA are generally oval or elliptical, but they can have a more complex profile if they fuse into an aggregate (Cavanagh 1999). A 3-dimensional investigation claimed that 70% of CoA are interconnected into highly branched large aggregates, some of which are enclosed by the basement membranes of astrocytes (Pirici, Margaritescu et al. 2014). This claim has not yet been verified. CoA have been reported in various organs of many species (Cavanagh 1999) and consist of a polyglucosan core surrounded by an ubiquitinated outer shell (Nam, Kim et al. 2012). For this reason CoA are also referred to as 'polyglucosan bodies', as CoA are predominantly composed of glucose polymers (over 85% are hexoses), with a minor component (4 – 5%) of proteins (Abel, Hebb et al. 2010, Pisa, Alonso et al. 2018), suggesting
that a defect in glucose metabolism may cause CoA formation (Cavanagh 1999, Augé, Duran et al. 2018, Navarro, Genoud et al. 2018).

CoA were first described by Virchow in 1851 who regarded them as precipitation products of proteins (Stam and Roukema 1973). CoA are autofluorescent and can be positively stained with various histochemical stains including hematoxylin and eosin (H & E), periodic acid-schiff (PAS), luxol fast blue and cresyl violet (Cissé and Schipper 1995, Buervenich, Olson et al. 2001, Cherian, Radhakrishnan et al. 2003, Nam, Kim et al. 2012, Estupiñán-Díaz, Morales-Chacón et al. 2015, Augé, Cabezón et al. 2017); they react negatively for lipids, DNA and RNA, but positively for many proteins, such as tubulin, tau, glial fibrillary acidic protein (GFAP), neurofilament, amyloid, heat shock protein, ubiquitin and ferritin (Cavanagh 1999).

1.5.1 Association between CoA and neurodegenerative diseases

Various conditions have been suggested to favour the development of CoA including ageing, chronic hypoxia, multiple sclerosis, temporal lobe epilepsy, dementia, neuronal degeneration, AD and Parkinson’s disease (Cavanagh 1999, Estupiñán-Díaz, Morales-Chacón et al. 2015, Rohn 2015, Augé, Cabezón et al. 2017, Augé, Duran et al. 2018, Pisa, Alonso et al. 2018). Hyperglycemia in diabetes may facilitate the formation of CoA by increasing the availability of unused glucose polymers, the main component of CoA (Nam, Kim et al. 2012). In normal human brains CoA increase in number with age: CoA are rarely seen in persons younger than 40 years (Cavanagh 1999), yet their numbers climb rapidly after the fourth or fifth decade (Mizutani, Satoh et al. 1987, Mrak, Griffin et al. 1997, Cavanagh 1998, Keller 2006, Augé, Cabezón et al. 2017). Qualitative observations indicate that CoA become more numerous and larger with age (Cavanagh 1999), although no systematic quantitative data exist to support this view.
The biochemical and elemental composition of the proteins in CoA of normal ageing and neurodegenerative diseased brains have been investigated. CoA showed positive reactions with antibodies to ubiquitin, heat shock proteins (HSP) 28 and 70, which may be the result of sustained physiological stress (Cissé, Perry et al. 1993). Furthermore, 24 proteins were identified in a proteomic analysis of CoA from multiple sclerosis brain lesions that may be of neuronal origin, including proteins from the cell membrane, endoplasmic reticulum, mitochondria, cytosol and nucleus, which regulate apoptosis and senescence, as well as enzymatic pathways (Selmaj, Pawlowska et al. 2008). Another study indicated that the proteins in CoA differ between normal ageing and AD, with those from normal brains being immunoreactive for proteins associated with neuronal cytoskeleton and cell injury, whereas in AD they were derived from both neurons and glia (Singhrao, Neal et al. 1993). Indeed, a recent study argued the molecular composition of CoA was in dynamic change during the progression of mild cognitive impairment to severe AD (Bathini, Mottas et al. 2019).

1.5.2 CoA distribution patterns in ageing and neurodegeneration


With regards to the first pattern, CoA are frequently seen near blood vessels, especially the medium and large blood vessels, and are often found in the Virchow–Robin spaces, suggesting an association between CoA and the vasculature (Cavanagh 1999, Navarro,
Genoud et al. 2018). CoA are also found beneath the pial surface in areas that are close to the CSF (Meng, Zhang et al. 2009, Maurizi 2010, Nam, Kim et al. 2012), such as the pial border of the hippocampus, and in the subependymal zones of the ventricles (Cavanagh 1999, Erdamar, Zhu et al. 2000). For instance, CoA were reported in the periventricular regions of the hippocampus in three aged subjects without any neurodegenerative diseases (Augé, Bechmann et al. 2019), and within perivascular and subpial regions in four aged AD patients and one aged patient with vascular dementia (Augé, Duran et al. 2018). CoA distribution in the lateral temporal lobes was similar to the cerebral ageing pattern, with a high density of CoA in perivascular regions in temporal lobe epilepsy and focal cortical dysplasia (Estupiñán-Díaz, Morales-Chacón et al. 2015).

The second pattern of distribution is distinctive and is commonly reported in neurodegenerative diseases. Previous studies have described abundant CoA in the dentate gyrus, CA4, CA3, CA1, of the hippocampus of patients with temporal lobe epilepsy (Cherian, Radhakrishnan et al. 2003), complex partial seizures and Ammon's horn sclerosis (Erdamar, Zhu et al. 2000) and Parkinson’s disease (Navarro, Genoud et al. 2018). CoA were found to accumulate in the CA1 and CA3 regions of hippocampus in patients with temporal mesial epilepsy (Uckermann, Galli et al. 2017). CoA in the hippocampal sclerosis specimens were mainly located in the CA1 subregion and their numbers were inversely correlated with neuronal density (Van Paesschen, Revesz et al. 1997). CoA deposition has also been found in the hippocampus of patients with temporal lobe epilepsy, and mostly in the CA1 and CA2 regions (Kawamura, Morioka et al. 2002).
1.5.3 CoA may have many origins

The origin(s) of CoA remains elusive, and has been considered in many different ways over the years. At least five hypotheses have been advanced to account for their origin, none of which are mutually exclusive (Rohn 2015, Augé, Cabezón et al. 2017). The first hypothesis notes that while CoA are regarded to be a benign indicator of the ageing process, they may represent one of the earliest, though non-specific indicators of neurodegeneration (Singhrao, Neal et al. 1993, Rohn 2015). Various reports have demonstrated that the proteins in CoA originate from dying neurons, oligodendrocytes and astrocytes. The degenerated cytoskeletal elements and proteins in CoA have been identified to be of neuronal origin (Cavanagh 1999, Selmaj, Pawlowska et al. 2008), while myelin basic proteins and proteolipid proteins in CoA indicate an oligodendroglial origin (Singhrao, Neal et al. 1994, Cavanagh 1999). Cerebral CoA are found within astrocytic endfeet or as extracellular deposits (Anzil, Herrlinger et al. 1974). That dying axons are a major component of CoA is supported by the fact that large numbers of CoA were present where axons had degenerated, while lesions with preserved axons had no CoA (Selmaj, Pawlowska et al. 2008).

The second hypothesis of CoA formation stems from the discovery of proteins within CoA that originate from the blood (Meng, Zhang et al. 2009, Pisa, Alonso et al. 2016). It posits that when the BBB is compromised, serum proteins leak into the brain and are subsequently collected into CoA. It is supported by the fact that many neurodegenerative diseases that favour CoA formation, such as AD, multiple sclerosis, and temporal lobe sclerosis, are also accompanied by vascular compromise (Meng, Zhang et al. 2009). It is further supported by the observation of CoA in vascular dementia patients (Meng, Zhang et al. 2009), and by substantial numbers of CoA in the choroid plexus in conditions where the tight junctions have been destroyed (Nam, Kim et al. 2012). Finally, CoA have been immunostained with purified
IgMs, which are considered to be natural antibodies that cannot pass through the BBB into the brain parenchyma under normal physiological conditions, therefore implicating a disrupted BBB (Augé, Cabezón et al. 2017). Since OSA has been suggested to involve a disrupted BBB (Lim and Pack 2014), it might be expected that OSA will feature increased numbers of CoA near blood vessels, as a function of OSA severity.

A third hypothesis relates to CoA being indirectly caused by microbial infections, based on the observation of fungal and bacterial proteins within some CoA. For instance, CoA immunoreactive to antifungal antibodies (Candida famata, C. albicans, C. glabrata, Syncephalastrum racemosum and Phoma betae) have been observed in the entorhinal cortex and hippocampus of AD patients, whereas CoA in control subjects were almost devoid of fungal immunoreactivity (Pisa, Alonso et al. 2016). It has been suggested that CoA are not formed by infectious agents; instead the concurrent inflammation may trigger the formation of CoA, with some fungal proteins becoming incorporated in these inclusions (Pisa, Alonso et al. 2016). As cells infected by blood-borne pathogens tend to be concentrated near blood vessels, the perivascular distribution of CoA is consistent with this microbial hypothesis (Pisa, Alonso et al. 2018). Moreover, the evidence of natural IgM antibodies in CoA supports a link between the natural immune system and CoA (Augé, Cabezón et al. 2017, Augé, Pelegrí et al. 2018).

A fourth hypothesis posits that CoA are a component of the glio-pial clearance system that removes waste molecules across the glial-limiting lamina (Sbarbati, Carner et al. 1996). Thus CoA may function as containers that sequester potentially hazardous or residual cellular products, to be later eliminated via phagocytic processes or other mechanisms of the innate immune system (Augé, Cabezón et al. 2017). It is notable that CoA often aggregate in the Virchow–Robin spaces around penetrating arteries in the white matter, formed when pial
arteries penetrate the brain parenchyma (Cavanagh 1999), suggesting the involvement of the
glymphatic clearance system in eliminating CoA. This idea is supported by the close
proximity of CoA to blood vessels (37% CoA were within 5 µm of the closest blood
vessel, and 77% within 10 µm distance), and the glymphatic drainage system (92.6% of
observed CoA were located within a glymphatic area) (Navarro, Genoud et al. 2018). The
glymphatic system is a recently discovered macroscopic waste clearance system in the CNS,
that utilises the Virchow–Robin space (refer Figure 1.5) (Jessen, Munk et al. 2015). This
metabolite waste removal system is most active during sleep when neurotoxic waste products
are cleared from the brain, and it is largely deactivated upon wakefulness (Jessen, Munk et al.
2015). The glymphatic system has been shown to be important for the disposal of soluble
proteins, lipids and glucose (Thrane, Thrane et al. 2013, Lundgaard, Li et al. 2015). The
operation of the glymphatic system is affected by neurodegenerative disorders, and low
activity of glymphatic function in turn contributes to these pathologies (Jessen, Munk et al.
2015). Since OSA involves profound disruptions of sleep, it offers the opportunity to
investigate whether there are more CoA in the hippocampus of severe OSA patients,
particularly in the Virchow–Robin spaces and near the pial surface.

The fifth hypothesis links CoA formation to oxidative stress (Keller 2006). Cell culture
studies have shown that upregulated expression of the oxidative stress marker heme
oxygenase-1 promotes the transformation of normal mitochondria into CoA-like inclusions in
ageing astroglia (Cissé and Schipper 1995, Sahlas, Liberman et al. 2002). It has been
proposed that reactive oxygen species cause a cellular stress response in astrocytes that leads
to an upregulation of heme oxygenase-1, heat shock proteins (HSP 27, 72, 90) as well as
ubiquitin (Cissé and Schipper 1995), leading to the opening of the mitochondrial permeability
transition pore and mitochondrial swelling (Sahlas, Liberman et al. 2002). The abnormal
mitochondria engage in macroautophagy and incorporate lysosomes as well as peroxidative-
positive inclusions to form Gomori-positive granules (Cissé and Schipper 1995, Sahlas, Liberman et al. 2002), which are regarded to be structural precursors of CoA (Cissé and Schipper 1995). In support of this hypothesis, CoA have been reported to be more frequent in degenerative conditions that are characterised by oxidative stress and mitochondrial dysfunction, including AD, Huntington’s disease, Friedreich’s ataxia and amyotrophic lateral sclerosis (Sahlas, Liberman et al. 2002). Since OSA has been associated with increased oxidative stress (Lavie 2003, Lavie 2015, Passali, Corallo et al. 2015, Sforza and Roche 2016) and mitochondrial dysfunction (Lavie 2015), it is reasonable to suppose that increased OSA severity will be associated with an increased burden of CoA, particularly in the hippocampus which is particularly vulnerable to oxidative stress.

The most recent hypothesis relating to the formation of CoA incorporates features from several of the hypotheses mentioned above. It proposes that CoA originate in the astrocytic endfeet, and their formation are tightly linked to the neurovascular unit and the glymphatic system (Navarro, Genoud et al. 2018). It is postulated that CoA may originate from insufficient lysosomal degradation within astroglia. Small lysosomal aggregates (immature CoA, intracellular inclusions without a membrane of its own) can be cleared by the perivascular system (glymphatic system) under normal conditions. However, when CoA increase in size by incorporating more waste materials, they disrupt the cell membrane and enter the extracellular space where they are surrounded by cell debris that gradually adds to their volume. These extracellular CoA cannot be cleared and remain close to blood vessels and the glymphatic drainage system (Navarro, Genoud et al. 2018).
1.6 Summary

OSA is characterised by recurring episodes of non-breathing during sleep, resulting in chronic intermittent hypoxia, which induces several consequences such as hemodynamic, oxidative and immune-inflammatory alterations (sections 1.1.4 & 1.1.5). OSA has been linked to chronically elevated blood pressure and altered regional cerebral blood flow; both have been reported to induce functional and structural alterations of the vascular endothelium through shear stress (section 1.2.2). The cerebral vascular system plays a significant role in brain homeostasis, with the brain being highly dependent on the adequate circulation of blood for the provision of oxygen and nutrients. Consequently, the chronic intermittent hypoxia and altered cerebral blood flow in OSA can place a great burden on the normal function of hippocampus, which subserves cognition and memory (section 1.2.1).

Hypoxia is a well-known stimulus for angiogenesis (section 1.3.1) and vascular remodelling (section 1.3.2), and animal IH models have shown increased cerebral angiogenesis and vascular remodelling in various brain regions. Taking all the current reports on cerebral microvasculature into consideration, most of them were animal models (young subjects) of continuous hypoxia or intermittent hypoxia; virtually nothing is known about the capacity of the aged human vascular system to respond to the chronic IH of OSA.

Microvascular aberrations and ultrastructure capillary damage have been suggested to be detrimental to the transport of oxygen and nutrients to the neuropil (section 1.4). Among those studies that have investigated changes in microvascular morphology, most were limited to qualitative observations on the distribution of the appearance of the vasculature, rather than on quantitative measurements. The present microvascular morphology reports in humans have been concerned with the effects of ageing or AD, and nothing is known about the microvascular morphology alterations in OSA patients. It would be important to investigate
the cerebral microvascular abnormalities in the hippocampus of OSA patients, as it may indicate which subregions are most prone to vascular insufficiency, and consequent disruption of neuronal function.

There are abundant reports on the distribution of CoA in the aged human brain, and the density and size of CoA are believed to increase with ageing. CoA deposit in the periventricular, perivascular and subpial regions of CNS, demonstrating a relationship between CoA and CSF and blood vessels. The nature of this relationship may become clearer if the spatial distribution, packing density and size of CoA in different subregions of the brain were investigated in a systematic way. Based on their molecular contents, at least five different hypotheses have been formulated to explain the formation of CoA, and while each are supported by evidence, none are conclusive. Proposed causes of CoA include neurodegeneration, BBB leakage, infection, oxidative stress, and glio-pial clearance of waste metabolites. Most of these hypotheses predict that the burden of CoA should increase as a function of OSA severity, so an examination of brain tissue from OSA patients may provide insights into the origins of these enigmatic structures.

1.7 Thesis aims and hypothesis

The overall aim of the present thesis was to investigate the microvascular alterations and CoA burden in post-mortem hippocampus of OSA patients. Specifically, it investigated the effects of increased OSA severity (based on ODI) on angiogenesis, microvascular remodelling, microvascular abnormalities, as well as CoA distribution, density and size in hippocampal subregions. The effects of regular CPAP use and advancing age were also investigated. These data provided insights into the validity of the various hypotheses that have speculated about the formation of CoA.
Four specific aims were as follows (Figure 1.7):

**A1**

**Chapter 3**

- **OSA severity**
- **CPAP**
- **Age**

Figure 1.7 Schematic illustration of the research aims of the present thesis.

Each of the four data chapters (Chapters 3 – 6) are associated with a different aim (A1 – A4). The arrows indicate the main independent variables that will be considered in relation to that aim.

**A2**

**Chapter 4**

- **OSA severity**
- **CPAP**
- **Age**

- **Microvascular abnormalities**

**A3**

**Chapter 5**

- **Age**

- **CoA distribution**
- **CoA density**
- **CoA size**

**A4.1**

**Chapter 6**

- **OSA severity**
- **CPAP**

- **Microvascular abnormalities**

- **CoA distribution**
- **CoA density**
- **CoA size**

**A4.2**

**Chapter 6**

- **AB & NFT**
- **Neuropil loss**
- **Demyelination**

- **CoA distribution**
- **CoA density**
- **CoA size**
Aim 1: To investigate the effects of OSA severity, regular CPAP use and advancing age on angiogenesis and microvascular remodelling in the hippocampus (Chapter 3).

Aim 2: To investigate the effects of OSA severity, regular CPAP use and advancing age on microvascular abnormalities in the hippocampus (Chapter 4).

Aim 3: To investigate the effect of advancing age on the distribution, density and size of CoA in the hippocampus (Chapter 5).

Aim 4: To investigate whether OSA severity, AD pathology, neuropil loss or demyelination contribute to the distribution, density or size of CoA in the hippocampus, and whether regular CPAP use can ameliorate the CoA burden (Chapter 6).

The working hypothesis: There will be increased angiogenesis / microvascular remodelling / microvascular abnormalities / CoA burden in response to OSA severity. Increasing patient age will strengthen these changes, and they will be attenuated by regular CPAP use.

The results obtained through these studies are likely to provide new insights into the adaptability of the hippocampal microvasculature in OSA, as well as indicating which subregions are likely to be vulnerable to vascular insufficiency. These results will also help to elucidate the distribution, size and progression of CoA in the hippocampus, as well as providing insights into their origins.
Chapter 2 Materials and Methods

2.1 Ethics approvals

All four research projects in this thesis used *post-mortem* brain tissues kindly provided by Professors Thorarinn Gislason and Bryndis Benediktsdottir of Landspitali University Hospital, Iceland. This project was approved by the National Bioethics Committee, Iceland, reference number 09-087-CM (Appendix A) for the brain tissue samples (formalin-fixed and paraffin-embedded) and corresponding medical records to be examined at RMIT University, Melbourne, Australia. The research investigations conducted on these samples were approved by the RMIT Human Research Ethics Committee (Appendix B, reference number ASEHAPP 71-16).

2.2 Materials

Ethanol, sodium chloride, sodium dihydrogen orthophosphate, disodium hydrogen phosphate and sodium acetate were sourced from Merck Millipore. Bovine albumin serum (BSA), goat serum, diaminobenzidine-nickel sulphate (DAB-Ni), ethylenediaminetetraacetic acid (EDTA), tris hydrochloric acid (Tris-HCl), sodium dodecyl sulphate (SDS) were sourced from Sigma Aldrich Australia. Ethanolamine, hydrogen peroxide (H₂O₂) and Tween 20, were from BDH. Histolene and Depex were from Grale HSD. Triton X-100 was from APS Finechem. Reveallt antigen retrieval solution was from ImmunoSolutions. Streptavidin-biotinylated horseradish peroxidase (RPN1051) was from GE Healthcare.

2.3 OSA diagnosis

In Iceland, the diagnosis of OSA and CPAP treatment both began in 1987. The OSA diagnoses are based on whole night polysomnography. OSA severity measurement has always been determined by the number of apnoeas and hypopnoeas per hour (AHI). More
recently, the number of events involving a 4% decrease in oxygen saturation per hour (ODI) has been measured. Due to the archival nature (incomplete and older records), not all of the AHI records were fully retrieved or comparable, whereas all of the ODI records were recovered. Therefore, ODI was used as the measure of OSA severity in the present study.

Between 1987 and 2014, a total of 8,853 OSA patients (18 years and older) were registered in Iceland for the diagnosis of OSA (total population 320,000). In March 2014, 7,531 registrants were alive, 5,436 males and 2,075 females. Of those that had died, consents were received from 121 patients for donation autopsies at the Department of Pathology, Landspitali University Hospital; 61 of them included brain tissues and were sent to RMIT University for research purposes.

The cases selected for study had undergone one or more overnight sleep study using international clinical guidelines for the diagnosis of OSA. Exclusion criteria were: dementia, head trauma, stroke, multiple sclerosis, cerebral infection, pulmonary disease, as well as treatment for OSA other than CPAP. Tissues were coded to mask the patients’ identity. Among the 61 brain blocks examined at RMIT University, 35 blocks contained hippocampal brain tissues, and of these one was excluded due to a diagnosis of AD. Of the remaining 34 blocks, 3 had been fully depleted by previous research studies undertaken in our laboratory.

The hippocampal series examined in the present study consisted of autopsy tissue from 31 patients (16 males and 15 females). Seventeen patients were confirmed to have used CPAP regularly until they died, of the remainder, 3 patients were known not to have ever used CPAP, while 11 patients were not using CPAP at the time of death, although they may or may not have used CPAP at some point between OSA diagnosis and death. For the purposes of the present study, only those patients who had regularly used CPAP were considered as the CPAP group, and all other patients were considered as the non-CPAP group. While the
regular CPAP users were known to have received CPAP treatment up until their death, their nightly usage data were not available.

2.3.1 OSA severity descriptive statistics

The severity of OSA, as measured by ODI in the present study, ranged from $4.1 - 92.2$ events/h sleep. The total samples were divided into two groups at the median ODI value of 20 events/h sleep. The 15 patients with an ODI value of less than 20 events/h sleep were considered to have mild-moderate OSA, namely the 'low OSA' group, while those with an ODI value of equal to or greater than 20 events/h sleep (16 patients) were considered to have moderate-severe OSA, the 'high OSA' group. Records relating to body-mass index (BMI) were available for 27 of these 31 patients. These two groups were well matched for age at death, time lived after OSA diagnosis and BMI, as shown in Table 2.1.

Table 2.1 Descriptive statistics of sample group stratified by OSA severity.

<table>
<thead>
<tr>
<th></th>
<th>Low OSA</th>
<th>High OSA</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>15</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>M = 7, F = 8</td>
<td>M = 8, F = 8</td>
<td></td>
</tr>
<tr>
<td>CPAP users</td>
<td>Y = 6, N = 9</td>
<td>Y = 11, N = 5</td>
<td></td>
</tr>
<tr>
<td>Age at death (years)</td>
<td>66.0 ± 2.8</td>
<td>68.2 ± 2.9</td>
<td>0.589</td>
</tr>
<tr>
<td>Time lived after OSA diagnosis (years)</td>
<td>8.6 ± 1.2</td>
<td>6.8 ± 1.7</td>
<td>0.397</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.7 ± 1.0 ($n = 14$)</td>
<td>30.6 ± 2.1 ($n = 13$)</td>
<td>0.410</td>
</tr>
<tr>
<td>ODI (events/h sleep)</td>
<td>10.3 ± 1.3</td>
<td>42.4 ± 4.9</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female; Y, yes for regular CPAP use; N, No/not sure for CPAP use. Unpaired 2-tailed t-tests between low OSA (ODI < 20 events/h sleep) vs. high OSA (ODI ≥ 20 events/h sleep). Mean ± SEM.
2.3.2 CPAP use descriptive statistics

The total samples were also stratified by CPAP use; details of the descriptive statistics for these groups are provided in Table 2.2. Regular CPAP users were significantly older and had higher BMIs than non-CPAP users. No significant differences were found in terms of time lived after OSA diagnosis or OSA severity (ODI).

Table 2.2 Descriptive statistics of sample group stratified by CPAP use.

<table>
<thead>
<tr>
<th></th>
<th>Non-CPAP users</th>
<th>CPAP users</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>M = 9, F = 5</td>
<td>M = 6, F = 11</td>
<td></td>
</tr>
<tr>
<td>OSA group</td>
<td>L = 9, H = 5</td>
<td>L = 6, H = 11</td>
<td></td>
</tr>
<tr>
<td>Age at death (years)</td>
<td>63.3 ± 2.7</td>
<td>70.3 ± 2.7</td>
<td>0.085</td>
</tr>
<tr>
<td>Time lived after OSA</td>
<td>8.3 ± 5.6</td>
<td>7.4 ± 6.5</td>
<td>0.597</td>
</tr>
<tr>
<td>diagnosis (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>26.3 ± 1.2 (n=12)</td>
<td>32.3 ± 1.5 (n=14)</td>
<td>0.006</td>
</tr>
<tr>
<td>ODI (events/h sleep)</td>
<td>19.5 ± 6.2</td>
<td>32.9 ± 4.6</td>
<td>0.087</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female; L, low OSA (ODI < 20 events/h sleep); H, high OSA (ODI ≥ 20 events/h sleep). Unpaired 2-tailed t-tests between non-CPAP users vs. CPAP users. Mean ± SEM.
2.3.3 Age at death descriptive statistics

The effect of age was investigated by dividing the data into two groups based on the median age of the samples: age < 67.5 years and age > 67.5 years, the descriptive statistics are provided in Table 2.3. The older group had a significantly higher mean OSA severity than the younger group, but the groups did not differ on any of the other parameters.

**Table 2.3 Descriptive statistics of sample group stratified by age at death.**

<table>
<thead>
<tr>
<th></th>
<th>Age &lt; 67.5 years</th>
<th>Age &gt; 67.5 years</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>M = 7, F = 8</td>
<td>M = 8, F = 7</td>
<td></td>
</tr>
<tr>
<td>CPAP use</td>
<td>Y = 5, N = 10</td>
<td>Y = 4, N = 11</td>
<td></td>
</tr>
<tr>
<td>OSA group</td>
<td>L = 9, H = 6</td>
<td>L = 6, H = 9</td>
<td></td>
</tr>
<tr>
<td>Time lived after OSA diagnosis (years)</td>
<td>7.6 ± 1.4</td>
<td>7.8 ± 1.6</td>
<td>0.936</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>27.9 ± 1.2 (n=15)</td>
<td>31.8 ± 1.9 (n=12)</td>
<td>0.084</td>
</tr>
<tr>
<td>ODI (events/h sleep)</td>
<td>18.5 ± 3.7</td>
<td>35.9 ± 6.4</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female; Y, yes for regular CPAP use; N, No/not sure for CPAP use; L, low OSA group (ODI < 20 events/h sleep); H, high OSA group (ODI ≥ 20 events/h sleep). Unpaired 2-tailed t-tests between OSA patients aged < 67.5 years vs. those aged > 67.5 years. Mean ± SEM.
2.4 Brain samples

Brain tissues from dead patients were dissected by experienced pathologists in Iceland. The post-mortem interval was not recorded. Dissected brain tissues were post-fixed in 10% formalin, subsequently dehydrated in graded ethanol, embedded in paraffin wax and archived. After being shipped to RMIT University, brain blocks that contained hippocampal tissue (e.g. the wall of lateral ventricle, fimbria, parahippocampal gyrus and collateral sulcus) were selected. The investigated hippocampal regions are illustrated with diagrams in the following four chapters.

2.5 Sectioning

Formalin-fixed paraffin-embedded tissue blocks were sectioned at 20 µm with a microtome at RMIT University. Sections were then mounted onto glass microscope slides and allowed to dry in a 37°C oven overnight. Note that paraffin sections of brain are more commonly cut at 5 – 10 µm thickness; however, the present study chose the 20 µm thickness because the greater thickness affords superior visualization of capillaries and corpora amylacea (CoA).

2.6 Immunohistochemistry

The immunohistochemistry protocol was refined by a previous researcher in our laboratory (Owen, Benediktsdottir et al. 2019). Sections were deparaffinised with histolene (2 × 10 min) and graded ethanol solutions (100% 3 min, 95% 3 min, 70% 3 min, distilled water rinse 1 min), and then treated with antigen retrieval buffer for 40 min at 85°C in a water bath. Sections were then incubated with blocking solution (0.1 M phosphate buffered saline (PBS), 0.1 g BSA, 10% Triton X-100 and 1% ethanolamine) for 3 h at room temperature. Tissue sections were incubated with primary solutions of primary antibodies in a humidified chamber for 16.5 h at room temperature overnight. On the next day, sections were collected
out of the container and then incubated for 3 h with solutions of secondary antibodies diluted at 1 in 300. Between each of the above steps, sections were washed for $3 \times 10$ min in 0.1 M PBS. After tertiary incubation with a solution of streptavidin-biotinylated horseradish peroxidase (SB-HRP) diluted at 1 in 300 for 3 h, sections were washed for $2 \times 5$ min in 0.1 M PBS then for $2 \times 10$ min in 0.175 M sodium acetate buffer (NaOAc). Sections were then incubated for 10 min in a 0.05% solution of the chromogen DAB diluted in NaOAc buffer, then in 0.05% DAB in NaOAc buffer with 0.004% H$_2$O$_2$ for another 15 min to visualise the chain of antibodies bound to the tissue. Immunolabelled sections were then washed in NaOAc buffer for $2 \times 5$ min then 0.1 M PBS for $2 \times 10$ min and left overnight in 0.1M PBS at 4°C. On the following day, sections were dehydrated in graded ethanol (70% 10 min, 95% 10 min, 100% $2 \times 5$ min) and histolene ($2 \times 10$ min) before being coverslipped with Depex. Details about immunohistochemistry diluents and antibodies are provided in Tables 2.4 and 2.5.
### Table 2.4 Immunohistochemistry dilution protocol.

<table>
<thead>
<tr>
<th></th>
<th>Blocking (3 h)</th>
<th>Primary (16.5 h)</th>
<th>Secondary (4 h)</th>
<th>Tertiary (3 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% BSA</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>4% Goat serum</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>10% Triton X-100</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% Ethanolamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st antibody</td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Rabbit)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat anti-Rabbit</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>SB-HRP²</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

¹Control slides were incubated without primary antibody. ²Streptavidin-biotinylated horseradish peroxidase.
Table 2.5 Details of immunohistochemistry antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Product no.</th>
<th>Dilution</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD34</td>
<td>Abcam</td>
<td>ab81289</td>
<td>1 in 5,000</td>
<td>Cell-surface adhesion factor; expressed by vascular endothelial cells</td>
</tr>
<tr>
<td>Anti-Claudin 5</td>
<td>Abcam</td>
<td>ab131259</td>
<td>1 in 5,000</td>
<td>Tight junction protein between adjacent vascular endothelial cells</td>
</tr>
<tr>
<td>Anti-Collagen IV</td>
<td>Abcam</td>
<td>ab6586</td>
<td>1 in 20,000</td>
<td>Key component of the basal membrane of blood vessels</td>
</tr>
<tr>
<td>Anti-GluT-1</td>
<td>Abcam</td>
<td>ab15309</td>
<td>1 in 2,000</td>
<td>Primary glucose transporter; expressed by vascular endothelial cells</td>
</tr>
<tr>
<td>Anti-VWF</td>
<td>Abcam</td>
<td>ab6994</td>
<td>1 in 50,000</td>
<td>Aids clot formation; expressed by vascular endothelial cells</td>
</tr>
<tr>
<td>Anti-Rabbit IgG</td>
<td>Vector</td>
<td>BA-1000</td>
<td>1 in 300</td>
<td>Secondary</td>
</tr>
<tr>
<td>SB-HRP</td>
<td>GE Healthcare</td>
<td>RPN1051</td>
<td>1 in 300</td>
<td>Tertiary</td>
</tr>
</tbody>
</table>

VWF, von-Willebrand factor; SB-HRP, streptavidin-biotinylated horseradish peroxidase.
2.7 Image acquisition and processing

Micrographs were taken with an Olympus AX-60 microscope and Olympus DP73 camera at a final magnification of 400 × (objective lens 10 ×, ocular lens 20 ×, magnification changer 2 ×). Micrographs were taken with Olympus software CellSens using the ‘Acquisition’ feature at predetermined regions of the hippocampus with the same settings of magnification, illumination and exposure duration for all micrographs. Images were saved at a resolution of 1600 × 1200 pixels.

Scanning images of brain sections were viewed under an Olympus Virtual Microscopy Slide Scanning System (VS120) and Olympus VS-ASW-S5 software at objective magnification of 20 × (microscope: VS-BX), camera adapter of 0.5 × (camera: VC50) and camera adapter of 1 × (camera: Hamamatsu ORCA-Flash 4.0). Micrographs at investigated regions were captured with the ‘Crop’ tool in Olympus CellSens software at a resolution of 1600 × 1200 pixels.

The examiner (CX) was blinded to the OSA severity of the samples when taking the micrographs.

2.8 Image processing and analysis

As the methods of image analysis were different in each of the following four chapters, relevant methodological details are provided in each chapter.

2.9 Statistical analysis

Statistical analysis was performed using the IBM Statistical Package for the Social Sciences (SPSS version 21). A Shapiro-Wilk test was first conducted to examine whether the data were normally distributed. Normality tests of sample parameters showed that ODI had a non-normal distribution, while age, BMI and time alive between OSA diagnosis and death were
normally distributed. A significant correlation between age and ODI was found for the samples \( (r^2 = 0.175, p = 0.019) \), while no further significant correlations were found between the sample parameters.

Unpaired 2-tailed \( t \)-tests were performed to compare the differences between two groups, one way ANOVAs were performed for comparison between more than two groups, while Levene's test was included as a measure of whether the variances of the compared groups were equal; Tukey's HSD \textit{post hoc} test was used with equal variances while the Games-Howell \textit{post hoc} test was used with unequal variances. Bivariate Pearson's correlation was performed to reveal the strength of linkage of two continuous-level variables. Data and figures were present as Mean ± SEM. * \( p < 0.05 \) was considered to be significant with equal variances, alternatively, # \( p < 0.05 \) indicates significant difference with unequal variances.

\textit{Note}: Chapters 3, 4 5 and 6 have been organised and structured as draft manuscripts to be published shortly after the examination of this thesis. For this reason, some aspects of the introduction and methods of these chapters restate key concepts and details.

Throughout this thesis, ratios are calculated using the unrounded data values. Hence the derived ratios may differ slightly from ratios calculated from the rounded values given in the text.
Chapter 3 Microvascular remodelling in the hippocampus of obstructive sleep apnoea patients

3.0 Abstract

Obstructive sleep apnoea (OSA) is characterised by chronic intermittent hypoxia. The brain is a highly vascularised organ and is sensitive to minor fluctuations in the availability of oxygen. Hypoxia is a well-known stimulus for angiogenesis in the developing nervous system and is characterised by an increase in the number of blood vessels. The present study investigated whether angiogenesis occurs in the hippocampus of OSA patients. Immunohistochemistry was used to identify microvessels in the hippocampus and surrounding regions from patients with OSA. When stratified by OSA severity, the mean blood vessel count in the low OSA group (ODI < 20 events/h sleep) was not significantly different from that in the high OSA group (ODI ≥ 20 events/h sleep). There were significantly larger vessel diameters (µm) in the high OSA group in the fimbria (8.4 ± 0.2 vs. 6.8 ± 0.2, p < 0.001) and CA4 regions (7.8 ± 0.3 vs. 7.1 ± 0.2, p = 0.027). Additionally, increased vessel length (µm) was found in the fimbria region (51.1 ± 1.8 vs. 45.0 ± 1.9, p = 0.026) in the high OSA group when compared to the low OSA group. Thus, remodelled vasculature (increased vessel diameter and length), rather than angiogenesis (increase in vessel numbers), accounts for a significant increase (1.24–1.33-fold) in the area percentage occupied by blood vessels in the high OSA group in the fimbria (2.85 ± 0.26 vs. 2.14 ± 0.14, p = 0.026) and CA4 regions (6.12 ± 1.49 vs. 4.95 ± 0.82, p = 0.015). The present study found evidence of microvascular remodelling in the hippocampus of patients with moderate-severe OSA; mostly in the fimbria and CA4 regions, which could not be reversed by regular CPAP treatment. The limited capacity of microvessels to remodel in response to hypoxia in the CA1 region might underpin the selective vulnerability of this region to hypoxic injury.
3.1 Introduction

Obstructive sleep apnoea (OSA), one of the most severe types of sleep-disordered breathing, is characterised by recurrent episodes of upper airway collapse during sleep (Dempsey, Veasey et al. 2010), leading to frequent episodes of hypoxia, arousals, sleep disruption and sleep fragmentation (Malhotra and White 2002, Azagra-Calero, Espinar-Escalona et al. 2012, Kielb, Ancoli-Israel et al. 2012). OSA sufferers have reported symptoms of daytime sleepiness, morning headache, fatigue or tiredness, depression and anxiety, as well as memory loss (Kasai, Floras et al. 2012, Phillips and O’Driscoll 2013). OSA is widely recognised as a major public health problem due to its adverse impacts on health and quality of life (Leger, Bayon et al. 2012). Continuous positive airway pressure (CPAP), delivered through a facemask worn during sleep, prevents the collapse of the upper airway and supports an uninterrupted sleep (Sullivan, Berthon-Jones et al. 1981). CPAP has been proven to successfully improve daytime productivity and daily function, elevate mood and psychological well-being, and reduce daytime sleepiness in people with moderate and severe OSA (Gay, Weaver et al. 2006).

The hippocampus plays an essential role in memory consolidation, as well as contributing to other aspects of cognition (Bartsch and Wulff 2015). Additionally, the hippocampus is extremely sensitive to hypoxic injury, and hypoxia has been implicated in the hippocampal atrophy commonly observed in OSA patients with in vivo brain imaging (Morrell, McRobbie et al. 2003, Gale and Hopkins 2004, Yaouhi, Bertran et al. 2009, Canessa, Castronovo et al. 2011, Torelli, Moscufo et al. 2011). In association with this atrophy, OSA patients are frequently found to have cognitive impairment and memory deficits (Naëgelé, Thouvard et al. 1995, Salorio, White et al. 2002, Ferini-Strambi, Baietto et al. 2003, Lim, Bardwell et al. 2007). This atrophy can be partly reversed by regular CPAP use: studies have reported
significant improvements in memory and cognitive function (Canessa, Castronovo et al. 2011, Rosenzweig, Glasser et al. 2016) that paralleled increased grey matter volume in the hippocampus after three months of CPAP treatment (Canessa, Castronovo et al. 2011). Neural injury and impaired neural function have been assumed to be responsible for the cognitive deficits resulting from OSA (Goldbart, Row et al. 2003, Zhu, Fenik et al. 2007). Considering the essential role that cerebral microvasculature plays in supporting neuronal function (Ingraham, Forbes et al. 2008, Andreone, Lacoste et al. 2015), it is important to examine the adaptive response of capillaries in the hippocampus of OSA patients.

A key research study has investigated the effect of different severities of intermittent hypoxia (IH) on the dorsal hippocampal microvasculature of the mouse (Lim, Brady et al. 2016). Lim and colleagues (2016) subjected young adult mice to one of three conditions: sham (continuous air, SaO$_2$ 95%), severe IH (SaO$_2$ nadir 61%), and very severe IH (SaO$_2$ nadir 37%) for 2 weeks (12 h/day). Hippocampal capillaries were immunolabelled for CD31. It was reported that the total capillary length in the very severe group was significantly greater than those in the sham group ($p = 0.004$), and the severe group ($p = 0.05$), while no significant difference was found between the sham and severe group ($p = 0.202$) (Lim, Brady et al. 2016). mRNA expression for GluT-1 (glucose transporter type 1) on capillary walls was significantly increased in the very severe group compared with the sham group ($p = 0.014$), while no significant differences were found between the severe group vs. sham group ($p = 0.102$), or the severe group vs. very severe group ($p = 0.353$) (Lim, Brady et al. 2016). Moreover, capillaries in different hippocampal regions showed differences in GluT-1 protein expression: the very severe group had the highest GluT-1 expression among those three groups in the CA1 ($p = 0.024$), CA3 ($p = 0.002$), and the perforant pathway ($p = 0.017$), but not in the dentate gyrus ($p = 0.095$) (Lim, Brady et al. 2016). This research indicates that the
extent of angiogenesis can vary in different parts of the hippocampus in response to IH, and that the severity of IH is an important driver (Lim, Brady et al. 2016).

While (Lim, Brady et al. 2016) is an important study, it is limited in several respects. First it is an animal study that investigated the effects of IH for a relatively short duration (2 weeks) compared to decades in patients with OSA. Second, Lim and colleagues (2016) only investigated vessel length, and neglected other parameters such as vessel count, vessel diameter and area fraction. Third, the degree of hypoxia induced in this study was extreme and is not representative of human experience. In patients with severe OSA, the nadir SaO$_2$ may drop to below 80% for a few times during the night (Badran, Ayas et al. 2014), however Lim and colleagues (2016) repeatedly induced nadirs of 61% and 37% for every minute of the sleep period. In view of these limitations, it is unclear whether similar capillary remodelling would occur in the hippocampus of humans with OSA.

Both angiogenesis, the generation of new blood vessels, and vascular remodelling in terms of altered vessel diameter and length, have been reported to occur in animal models in response to hypoxia (refer section 1.3). Given the substantial IH that patients with severe OSA may experience every night, it seems likely that angiogenesis and/or vascular remodelling occurs in moderate-severe OSA. However, no data are available to confirm this likelihood. Furthermore, it is not known whether different subregions of the hippocampus undergo angiogenesis and vascular remodelling to the same extent. The aim of the present study was to investigate the effect of OSA severity on angiogenesis and microvascular remodelling in the hippocampus, and to relate these measures to CPAP use and patient age.
3.2 Materials and Method

3.2.1 Study samples

The study sample consisted of 30 of the 31 patients from the Iceland cohort described previously (section 2.3), one sample was excluded due to resistance of this tissue to immunocytochemistry, which resulted in faint staining or no staining for most blood vessel markers. The detailed descriptive statistics are provided in Tables 2.1 - 2.3. The study samples were divided into two groups, a low OSA group (ODI < 20 events/h sleep; equivalent to mild-moderate OSA) and a high OSA group (ODI ≥ 20 events/h sleep; equivalent to moderate-severe OSA). There were equal sample sizes for both groups (15 patients in each).

3.2.2 Tissue processing

Hippocampal blocks were sectioned at 20 μm on a microtome as described in section 2.5 for immunohistochemistry.

3.2.3 Immunohistochemistry

The immunohistochemistry protocol followed that previously described in section 2.6. Briefly, sections were deparaffinised prior to treatment with antigen retrieval buffer for 40 min at 85°C. After blocking, sections were incubated with primary antibodies for 16.5 h overnight. Primary antibodies were visualised by using a biotinylated secondary antibody and streptavidin-biotinylated horseradish peroxidase (SB-HRP), which reacted with DAB-Ni to demonstrate the localisation of the antigen. Following this, sections were rinsed and coverslipped.
Five capillary markers were investigated in the current study. CD34 is a cell-surface adhesion factor, expressed by vascular endothelial cells, that labels both established and newly-formed blood vessels. Von-Willebrand factor (VWF), aids clot formation following vascular injury and is synthesised by vascular endothelial cells. Claudin 5 is a tight junction protein that seals the BBB by bonding adjacent vascular endothelial cells. GluT-1 is the primary glucose transporter expressed by vascular endothelial cells, while Collagen IV is a key component of the basal membrane of blood vessels. The concentrations of the primary antibodies used in this study are detailed in Table 2.5.

### 3.2.4 Double immunofluorescence

Sections underwent the same deparaffination and antigen retrieval steps as for normal immunohistochemistry, while PBS-Tween 20 (0.05%) was used instead of PBS for the washing steps. After antigen retrieval, slides were washed in PBS-Tween 20 for 3 × 10 min, followed by 1st serum blocking (4% goat serum) for 3 h at room temperature, first primary antibody (Collagen IV; Abcam ab6586 diluted 1 in 20,000, made in rabbit) was incubated overnight for 18 h. On the second day, slides were washed with PBS-Tween 20 for 3 × 10 min, and the first secondary fluorescent antibody (goat anti-rabbit IgG H & L (Alexa Fluor® 647); Abcam ab150083, diluted 1 in 200) was incubated for 30 min at room temperature. After washing, slides were kept in PBS-Tween 20 in the dark at 4°C overnight. On the third day, slides were washed in PBS-Tween 20 for 10 min and were blocked for the second time (3 h at room temperature, 4% rabbit serum to block free binding sites of the anti-rabbit immunoglobulin), followed by the second 18 h primary antibody incubation (GluT-1; Abcam ab15309 diluted 1 in 2,000, made in rabbit). On the fourth day, slides underwent triple 10 min PBS-Tween 20 washing, and the second 30 min secondary antibody incubation (goat anti-rabbit IgG H & L (Alexa Fluor® 488); Abcam ab150077 diluted 1 in 100), after which
sections were washed with PBS-Tween 20 for $3 \times 10$ min and were kept in the dark at 4°C overnight. On the fifth day, slides were washed in PBS-Tween 20 for another 10 min, cover slipped with BrightMount and sealed with clear nail polish. Slides were kept horizontally in the dark at 4°C for long term storage.

### 3.3.5 Histological analysis of hippocampal microvasculature

Five regions of the hippocampus and overlying cortex were investigated: fimbria, Cornu Ammonis 4 (CA4 or hilus), Cornu Ammonis 1 (CA1), subiculum and collateral sulcus (near the apex), as illustrated in Figure 3.1 A. Micrographs were taken at every hippocampal region (unless a part of the tissue was missing) with an Olympus AX-60 microscope and Olympus DP73 camera at a final magnification of 400 $\times$ (objective lens 10 $\times$, ocular lens 20 $\times$, magnification changer 2 $\times$) using Olympus CellSens software at a resolution of 1600 $\times$ 1200 pixels. Regions were identified by referring to adjacent cresyl violet-stained sections that had been prepared by a previous researcher in our laboratory (Owen, Benediktsdottir et al. 2019).

Four parameters were investigated: 1) vessel count, the total number of labelled profiles in the image; 2) area fraction (%), the proportion of the image occupied by all labelled profiles; 3) vessel diameter ($\mu$m), the mean of the diameters of all selected profiles; 4) vessel length ($\mu$m), the mean of the length of all selected profiles. These parameters are illustrated in Figure 3.1 B – E.
Figure 3.1 Micrographs that illustrate the hippocampal regions investigated and parameters that were measured.

A, Micrographs were taken at five hippocampal locations as indicated by the frames in the cresyl violet-stained micrograph. B, Vessel count was defined by the enclosed outline count of one vessel segment. C, Area fraction (%) was defined as the proportion of the image area occupied by vessels. D, Vessel diameter was defined as the mean diameter of a vessel segment when measured perpendicularly to the centre line at one pixel intervals. E, Vessel length was defined as the length along the centre line from the beginning to the end.
In order to measure vessel count and area fraction, the original RGB (red-green-blue) micrographs were converted into greyscale images, auto-contrasted in Photoshop software and then analysed with Olympus CellSens software using the ‘Count and Measure’ feature. The threshold level was adjusted manually until all labelled profiles were selected, as determined by visual inspection. Profiles inside the image and those contacting a border were selected if they were greater than 100 pixels in size. Data were then exported into Excel for further analysis.

Due to a limitation of the algorithm in the CellSens software which is designed for round/oval-shaped objects such as cells, the vessel diameter and length were analysed with software that had been specifically developed for blood vessels: the Automated Retinal Image Analyzer (ARIA) v1.0 (Bankhead, Scholfield et al. 2012). ARIA automatically recognises and generates a centreline and two edge lines along each vessel segment (Figure 3.1 B). Diameters for each vessel segment were tested perpendicularly along the centreline at 1 pixel intervals (Figure 3.1 D). Vessel length was defined as the Euclidean distance along the centreline (Figure 3.1 E). Detailed data were retrieved by a separate routine, and then converted from pixels into micrometres (µm).

**3.2.6 Statistical analysis**

Statistical analysis was performed using the IBM Statistical Package for the Social Sciences (SPSS version 21). Unpaired 2-tailed *t*-tests were performed to compare the low OSA and high OSA groups, non-CPAP users to regular CPAP users, and OSA patients aged < 67.5 vs. those aged > 67.5 years. One-way ANOVAs with *post hoc* multiple comparisons were used to compare among the five blood vessel markers. *p* < 0.05 was considered significant (* with equal variances; # with unequal variances). GraphPad Prism 7 was used to generate graphs, presented as Mean ± SEM.
3.3 Results

3.3.1 Immunohistochemistry of the hippocampal microvasculature

Figure 3.2 shows the representative immunohistochemistry staining results of five blood vessel markers from the low and high OSA groups in the CA4 region: CD34 (A & B), VWF (C & D), Claudin 5 (E & F), GluT-1 (G & H), and Collagen IV (I & J). In a small number of cases the brain tissue in the investigated regions was missing, consequently not all of the 30 brain sections contained all five hippocampal regions, therefore the sample size in different hippocampal regions varies from 23 to 30 (Table 3.1).

Table 3.1 Sample sizes for each of the hippocampal regions and markers studied.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fimbria</th>
<th>CA4</th>
<th>CA1</th>
<th>Subiculum</th>
<th>Collateral sulcus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>27</td>
<td>29</td>
<td>29</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>VWF</td>
<td>28</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>Claudin 5</td>
<td>27</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>23</td>
</tr>
<tr>
<td>GluT-1</td>
<td>29</td>
<td>30</td>
<td>30</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>27</td>
<td>29</td>
<td>29</td>
<td>27</td>
<td>23</td>
</tr>
</tbody>
</table>
Figure 3.2 Representative images for five blood vessel markers in the CA4 region.

Blood vessels immunostained for CD34 (A & B), VWF (C & D), Claudin 5 (E & F), GluT-1 (G & H) and Collagen IV (I & J) in the low (A, C, E, G & I) and high (B, D, F, H & J) OSA groups.
3.3.2 The effect of OSA severity on hippocampal angiogenesis and microvascular remodelling

3.3.2.1 Increased mean blood vessel counts in the high OSA group

For each of the five blood vessel markers, the total number of blood vessels counted per image was investigated in each of the five hippocampal regions (Figure 3.3 A – E). No significant differences were found between the mean vessel counts of the low and high OSA groups in the fimbria, CA4, CA1, subiculum or collateral sulcus regions, however there were significantly more Claudin 5-positive blood vessels (1.24-fold) in the subiculum region in the high OSA group when compared to the low OSA group (143.9 ± 7.2 vs. 116.4 ± 7.2, $t(27) = 2.686, p = 0.012$; Figure 3.3 D).
Figure 3.3 Comparison of mean vessel counts between the low and high OSA groups.

Mean vessel counts in the low (ODI < 20 events/h sleep; blank bars) and high (ODI ≥ 20 events/h sleep; striped bars) OSA groups in the fimbria (A), CA4 (B), CA1 (C), subiculum (D) and collateral sulcus (E). Unpaired 2-tailed $t$-tests between low vs. high OSA groups. Mean ± SEM.
3.3.2.2 Increased mean vessel diameters in the high OSA group

The mean vessel diameter (µm) in the high OSA group was significantly larger than in the low OSA group when immunolabelled for Collagen IV in the fimbria region (1.23-fold; 8.4 ± 0.2 vs. 6.8 ± 0.2, \( t (25) = 5.370, p < 0.001 \); Figure 3.4 A) and CA4 region (1.11-fold; 7.8 ± 0.3 vs. 7.1 ± 0.2, \( t (27) = 2.336, p = 0.027 \); Figure 3.4 B). Furthermore, one-way ANOVA tests revealed that the vessels stained with Collagen IV had significantly wider diameters (1.20 – 1.46-fold) than those labelled with CD34, VWF, Claudin 5 or GluT-1 across all five hippocampal regions investigated. Details of the post hoc multiple comparison (Tukey HSD) results are shown in Table 3.2.
Figure 3.4 Comparison of mean vessel diameters between low and high OSA groups.

Mean vessel diameters (µm) in the low (ODI < 20 events/h sleep; blank bars) and high (ODI ≥ 20 events/h sleep; striped bars) OSA groups in the fimbria (A), CA4 (B), CA1 (C), subiculum (D) and collateral sulcus (E). Unpaired 2-tailed t-tests between low vs. high OSA groups. Mean ± SEM.
Table 3.2 The mean diameters of microvessels labelled with Collagen IV are larger than those labelled with the other four vessel markers in all five hippocampal regions investigated.

<table>
<thead>
<tr>
<th>Region</th>
<th>(I) Group</th>
<th>(J) Group</th>
<th>Mean Diff. (I − J) ± SEM</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fimbria</td>
<td>Collagen IV</td>
<td>CD34</td>
<td>2.39 ± 0.28</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(N = 138)</td>
<td></td>
<td>VWF</td>
<td>1.28 ± 0.28</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claudin 5</td>
<td>1.90 ± 0.28</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GluT-1</td>
<td>1.61 ± 0.27</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CA4</td>
<td>Collagen IV</td>
<td>CD34</td>
<td>2.28 ± 0.22</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(N = 147)</td>
<td></td>
<td>VWF</td>
<td>1.71 ± 0.22</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claudin 5</td>
<td>1.83 ± 0.22</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GluT-1</td>
<td>1.66 ± 0.22</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CA1</td>
<td>Collagen IV</td>
<td>CD34</td>
<td>2.08 ± 0.23</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(N = 147)</td>
<td></td>
<td>VWF</td>
<td>1.65 ± 0.23</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claudin 5</td>
<td>1.73 ± 0.23</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GluT-1</td>
<td>1.67 ± 0.23</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Subiculum</td>
<td>Collagen IV</td>
<td>CD34</td>
<td>2.07 ± 0.24</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(N = 143)</td>
<td></td>
<td>VWF</td>
<td>1.44 ± 0.23</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claudin 5</td>
<td>1.49 ± 0.24</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GluT-1</td>
<td>1.51 ± 0.24</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Collateral</td>
<td>Collagen IV</td>
<td>CD34</td>
<td>2.07 ± 0.26</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sulcus</td>
<td>(N = 117)</td>
<td>VWF</td>
<td>1.60 ± 0.26</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claudin 5</td>
<td>1.78 ± 0.26</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GluT-1</td>
<td>1.71 ± 0.26</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Mean difference in diameter is expressed in µm. One-way ANOVA with post hoc test for multiple comparisons between group I and group J, namely, Collagen IV compared to CD34, VWF, Claudin 5 and GluT-1. Tukey HSD post hoc test was used, as Levene’s test revealed equal variances.
3.3.2.3 Increased mean vessel lengths in the high OSA group

In sections immunolabelled for Collagen IV, the mean vessel length (µm) in the fimbria region was significantly greater in the high OSA group (1.14-fold) than in the low OSA group (51.1 ± 1.8 vs. 45.0 ± 1.9, t (25) = 2.372, p = 0.026; Figure 3.5 A). No significant differences between the two groups were found in any of the five regions with the other four markers.

Figure 3.5 Comparison of mean vessel lengths between the low and high OSA groups.

Mean vessel length (µm) in the low (ODI < 20 events/h sleep; blank bars) and high (ODI ≥ 20 events/h sleep; striped bars) OSA groups in the fimbria (A), CA4 (B), CA1 (C), subiculum (D) and collateral sulcus (E) region. Unpaired 2-tailed t-tests between low vs. high OSA groups. Mean ± SEM.
3.3.2.4 Increased area fraction (% vessel density) in the high OSA group

The percentage of the full image occupied by labelled blood vessels was investigated and the results are shown in Figure 3.6. In sections immunolabelled with Collagen IV, the high OSA group had a significantly greater area fraction than the low OSA group, in both the fimbria region (1.33-fold; 2.85 ± 0.26 vs. 2.14 ± 0.14, $t(19.9) = 2.401, p = 0.026^\#$, unequal variances; Figure 3.6 A) and the CA4 region (1.24-fold; 6.12 ± 0.38 vs. 4.95 ± 0.22, $t(22.0) = 2.649, p = 0.015^\#$, unequal variances; Figure 3.6 B). One-way ANOVAs corrected for post hoc multiple comparisons (Tukey's honestly significant difference (HSD) with equal variances or Games-Howell with unequal variances) showed that the area fraction of blood vessels immunostained for Collagen IV was significantly greater (1.32 − 1.99-fold) than those immunostained for CD34, VWF, Claudin 5 and GluT-1 in all 5 regions (Table 3.3).
Figure 3.6 Comparison of mean area fraction (%) between the low and high OSA groups.

Mean area fraction (%) in the low (ODI < 20 events/h sleep; blank bars) and high (ODI ≥ 20 events/h sleep; striped bars) OSA groups in the fimbria (A), CA4 (B), CA1 (C), subiculum (D) and collateral sulcus (E). Unpaired 2-tailed t-tests between low vs. high OSA groups. Mean ± SEM.
Table 3.3 The mean area fractions (%) occupied by microvessels labelled with Collagen IV are larger than those labelled with the other four vessel markers in all five hippocampal regions investigated.

<table>
<thead>
<tr>
<th>Region</th>
<th>(I) Group</th>
<th>(J) Group</th>
<th>Mean Diff. (I – J) ± SEM</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fimbria2 (N = 138)</td>
<td>Collagen IV</td>
<td>CD34</td>
<td>1.18 ± 0.18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VWF</td>
<td>1.25 ± 0.19</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claudin 5</td>
<td>0.98 ± 0.18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GluT-1</td>
<td>0.72 ± 0.19</td>
<td>0.004</td>
</tr>
<tr>
<td>CA41 (N = 147)</td>
<td>Collagen IV</td>
<td>CD34</td>
<td>2.48 ± 0.31</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VWF</td>
<td>2.58 ± 0.31</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claudin 5</td>
<td>1.72 ± 0.31</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GluT-1</td>
<td>1.67 ± 0.31</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CA12 (N = 147)</td>
<td>Collagen IV</td>
<td>CD34</td>
<td>2.51 ± 0.36</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VWF</td>
<td>2.17 ± 0.37</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claudin 5</td>
<td>1.35 ± 0.44</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GluT-1</td>
<td>1.69 ± 0.39</td>
<td>0.001</td>
</tr>
<tr>
<td>Subiculum2 (N = 143)</td>
<td>Collagen IV</td>
<td>CD34</td>
<td>2.75 ± 0.40</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VWF</td>
<td>2.29 ± 0.42</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claudin 5</td>
<td>1.96 ± 0.45</td>
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<tr>
<td></td>
<td></td>
<td>GluT-1</td>
<td>2.41 ± 0.41</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Collateral Sulcus1 (N = 117)</td>
<td>Collagen IV</td>
<td>CD34</td>
<td>3.20 ± 0.36</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VWF</td>
<td>2.65 ± 0.36</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claudin 5</td>
<td>2.88 ± 0.36</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GluT-1</td>
<td>2.72 ± 0.36</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

One-way ANOVA with post hoc tests for multiple comparisons between group I and group J, namely, collagen IV compared to CD34, VWF, Claudin 5 and GluT-1. 1 Tukey's HSD post hoc test was used when Levene's test revealed equal variances; 2 Games-Howell post hoc test was used for samples with unequal variances.
3.3.2.5 *Double immunofluorescence (Collagen IV & GluT-1)*

One-way ANOVA tests showed that both the area fraction and diameter of the capillaries immunolabelled for Collagen IV were significantly larger than those immunolabelled with the other 4 markers (Tables 3.2 and 3.3). There are two possible explanations for this phenomenon: one is that the markers label different subpopulations of blood vessels, with Collagen IV marking the largest blood vessels and the other markers preferentially labelling smaller vessels. Alternatively, the markers may bind to different parts of the same vessel, with Collagen IV marking the abluminal surface and the other markers labelling the luminal surface of vessel walls.

To distinguish between these possibilities, double immunofluorescence was performed with Collagen IV and GluT-1. It was found that most vessel segments were immunolabelled for both Collagen IV and GluT-1, as shown in Figure 3.7 A. It is evident that Collagen IV marks the abluminal surface while GluT-1 marks the luminal surface (Figure 3.7 B); in a few cases, vessels were stained only with Collagen IV, possibly indicating that the glucose transporters were absent from these vessel segments.
Figure 3.7 Double immunofluorescence micrographs of microvessels labelled for Collagen IV and GluT-1.

A selection of high magnification images from the hippocampus showing both transversely and longitudinally transected microvessels that have been immunolabelled with Collagen IV (red) and GluT-1 (green), with yellow indicating colocalisation. Note that GluT-1 is expressed at the luminal surface of the microvessels while Collagen IV is expressed at the abluminal surface. In (C) the white arrow points to a degenerating vessel that lacks GluT-1 expression. In (D) the white arrowhead indicates a 'string vessel' that represents a later stage of degeneration, as detailed in Chapter 4.
3.3.3 The effect of regular CPAP use on hippocampal angiogenesis and microvascular remodelling

Changes in area fraction (%) demonstrate whether there has been a reduction or increase in the amount of vascularisation of tissue. The effect of regular CPAP treatment on the mean area fraction of blood vessels immunostained for Collagen IV (the most consistent and strongly labelling marker) was investigated by comparing non-CPAP users to regular CPAP users in the low and high OSA groups (Figure 3.8). The mean area fraction in the subiculum region was found to be significantly lower (4.28 ± 0.30 vs. 6.50 ± 0.55, \(t\) (11) = -3.003, \(p = 0.012\); Figure 3.8 D) in regular CPAP users than in non-CPAP users in the low OSA group. However, no other significant differences were found between non-CPAP users and regular CPAP users in the low or high OSA groups in any of the five regions.
Figure 3.8 The effect of regular CPAP use on mean area fraction (vessel density).

Mean area fraction (%) stained with Collagen IV in the non-CPAP users (blank bars) and CPAP users (striped bars) in low (ODI < 20 events/h sleep; purple coloured) and high (ODI ≥ 20 events/h sleep; pink coloured) OSA groups in the fimbria (A), CA4 (B), CA1 (C), subiculum (D) and collateral sulcus (E). Unpaired 2-tailed t-tests between non-CPAP users vs. CPAP users. Mean ± SEM.
3.3.4 The effect of advanced age on hippocampal angiogenesis and microvascular remodelling

The influence of advanced age on angiogenesis and microvascular remodelling was investigated within five hippocampal regions, with the fimbria region being most sensitive to the effect of age (Figure 3.9). In the fimbria, patients older than 67.5 years had significantly fewer blood vessels immunolabelled with CD34 (68.3 ± 3.9 vs. 80.8 ± 4.3, t (25) = -2.153, p = 0.041; Figure 3.9 A), larger vessel diameters immunolabelled with Collagen IV (8.0 ± 0.3 vs. 7.2 ± 0.3, t (25) = 2.174, p = 0.039; Figure 3.9 B), and a greater area fraction when immunolabelled with VWF (1.44 ± 0.14 vs. 1.08 ± 0.09, t (26) = 2.132, p = 0.043; Figure 3.9 D), Claudin 5 (1.70 ± 0.12 vs. 1.35 ± 0.10, t (25) = 2.238, p = 0.034; Figure 3.9 D) or Collagen IV (2.84 ± 0.26 vs. 2.15 ± 0.15, t (20.5) = 2.342, p = 0.029#, unequal variances; Figure 3.9 D). There was no significant difference between the two groups in the fimbria with regards to mean vessel length (Figure 3.9 C); however, when immunolabelled with GluT-1, the mean vessel length in the subiculum region was significantly longer (45.1 ± 2.2 vs. 39.4 ± 1.4, t (27) = 2.195, p = 0.037; Figure 3.9 E).
Figure 3.9 The effect of advanced age on angiogenesis and microvascular remodelling.

Microvasculature in patients younger than 67.5 years (blank bars) and older than 67.5 years (grid bars). Graphs A-D represent the fimbria: mean vessel count (A), mean vessel diameter (B), mean vessel length (C), and mean area fraction (D). E, Mean vessel length in the subiculum region. Unpaired 2-tailed t-tests between OSA patients aged < 67.5 years vs. those aged > 67.5 years. Mean ± SEM.
3.3.5 Summary

The present study found that:

i) Microvascular remodelling (increased capillary diameter and length) occurs in the fimbria and CA4 region in response to increased severity of OSA. These changes account for an increased area fraction. There is no increase in vessel count in the fimbria or CA4 region.

ii) CPAP treatment does not reverse the microvascular remodelling in the fimbria or CA4 region.

iii) While advanced age is associated with fewer capillaries, the individual vessels are dilated and elongated, and age contributes to the OSA severity effect in the fimbria region.

iv) Apart from vessel count in the subiculum, the CA1, subiculum and collateral sulcus regions demonstrated no changes on any of the microvascular parameters measured, in response to increased severity of OSA.

These findings are summarised diagrammatically in Table 3.4
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Marker</th>
<th>Region</th>
<th>OSA severity</th>
<th>CPAP use</th>
<th>Age</th>
</tr>
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<tr>
<td>Vessel count</td>
<td>Claudin 5</td>
<td>Subiculum</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CD34</td>
<td>Fimbria</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vessel diameter</td>
<td>Collagen IV</td>
<td>Fimbria</td>
<td>↑</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Collagen IV</td>
<td>CA4</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vessel length</td>
<td>Collagen IV</td>
<td>Fimbria</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>GluT-1</td>
<td>Subiculum</td>
<td>–</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
<td>Area fraction (%)</td>
<td>Collagen IV</td>
<td>Fimbria</td>
<td>↑</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>Collagen IV</td>
<td>CA4</td>
<td>↑</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Collagen IV</td>
<td>Subiculum</td>
<td>–</td>
<td>–</td>
<td>↓</td>
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<tr>
<td></td>
<td>VWF</td>
<td>Fimbria</td>
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<td>–</td>
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</tr>
<tr>
<td></td>
<td>Claudin 5</td>
<td>Fimbria</td>
<td>–</td>
<td>–</td>
<td>↑</td>
</tr>
</tbody>
</table>

↑ = Increased; ↓ = Decreased; –, No change.
3.4 Discussion

This is the first study to examine whether angiogenesis and microvascular remodelling occur in the hippocampus of OSA patients. Our data revealed that angiogenesis does not occur in OSA patients, although there is evidence that vessels are remodelled in some subregions of the hippocampus. There were significant differences between the high OSA and low OSA groups. In the fimbria and CA4 region, the high OSA group had significantly wider vessel diameters (1.11 – 1.23-fold larger), longer vessel segments (1.14-fold longer), and a larger area fraction (1.24 – 1.33-fold greater). Regular CPAP use did not reverse the microvascular remodelling in the fimbria and CA4 that had occurred in response to OSA severity. Older age contributed to the increased microvascular remodelling resulting from OSA severity in the fimbria region. The fimbria and CA4 regions demonstrated the greatest degree of microvascular remodelling in severe OSA, suggesting that these regions are sensitive and adaptive to the IH that occurs in OSA. By contrast, the CA1, subiculum and the apex of the collateral sulcus displayed few microvascular changes in response to OSA severity, which may indicate that these regions have a limited capacity to adapt to IH.

The regional density of microvessels is a major determinant of blood flow and the consequent provision of substrates to specific regions of brain tissue (Ingraham, Forbes et al. 2008). These vascular systems are dynamic, and are able to adapt and remodel in response to changing metabolic requirements of the tissue (Secomb, Alberding et al. 2013). These changes are controlled by hemodynamic and biological signals that induce a process known as ‘angioadaptation’, which involves angiogenesis, pruning, remodelling and changes in vascular tone (Secomb, Alberding et al. 2013, Reglin and Pries 2014). Angiogenesis involves the generation of new blood vessels from existing ones by sprouting or by splitting in two (intussusception) to increase vessel density and reduce the intercapillary diffusion distance.
The opposing process is vascular pruning/regression which involves vessel narrowing and cessation of blood flow, leading to luminal occlusion and retraction, and eventually in the degeneration of redundant vessels, and a reduced regional density of microvessels (Korn and Augustin 2012). Vascular responses to changed metabolic demands can also involve changes in vessel diameter. Second-to-second changes can be accommodated by the vasoconstriction and vasodilation of the arteriolar wall due to the activity of smooth muscle cells, whereas chronic changes are effected by the expansion and proliferation of capillary endothelial cells to increase the normal diameter of the vascular lumen in a process known as 'vascular remodelling' (Reglin and Pries 2014).

The present study found little evidence of increased vessel number (angiogenesis) in the hippocampus of patients with high OSA compared to those with low OSA. Furthermore, there was no increase in the expression of the primary glucose transporter GluT-1, which plays a significant role in carrying glucose across the BBB (Brockmann 2009). These findings were unexpected, as studies of animal models of IH have reported both angiogenesis and an increased expression of GluT-1. For instance, a study that used immunocytochemistry for GluT-1 to label capillaries reported that capillary density in the hippocampus of mice exposed to 2 weeks or 4 weeks of IH increased by 1.2-fold and 1.29-fold, respectively, compared to normoxic control mice (Kanaan, Farahani et al. 2006). However, that study used neonatal mice which may have a greater capacity for angiogenesis than adult humans, since the brain in mice continues to grow for several weeks after birth. Furthermore, the mice were exposed to IH for 24 h/day throughout the experiment, whereas in humans with OSA the IH will typically last for 4 – 6 h/day. Thus it is possible that the more extreme regime of IH experienced by the mice triggered angiogenesis. In support of this possibility, another study reported that expression of the GluT-1 transporter significantly increased only in those mice that were exposed to very severe cyclical IH (SaO2 nadir of 37%), whereas there was no
significant difference between sham and severe IH (SaO₂ nadir of 61%) groups (Lim, Brady et al. 2016). As noted in the introduction, SaO₂ nadirs of 37% are far lower than the desaturation nadirs experienced by patients with severe OSA.

The present study found that the subiculum region of the high OSA group had a higher number of Claudin 5-immunoreactive vessels (1.24-fold) than the low OSA group. A possible explanation of this observation is that severe OSA selectively induced the expression of tight-junction proteins in the subiculum region. Evidence from animal models has shown that continuous hypoxia can induce Claudin 5 expression, however it does not appear to be regionally selective (Boroujerdi, Welser-Alves et al. 2015). An alternative explanation is that a limited degree of angiogenesis occurred in the subiculum region in the high OSA group. Indeed, in the subiculum, every one of the five vessel markers used in this study exhibited higher numbers of vessels for the high OSA group (Figure 3.3 D), although the differences did not reach statistical significance for the other four markers. Such systematic differences were not apparent for the other regions of the hippocampus.

In addition to angiogenesis, another adaptation to hypoxia is an increase in the diameter of blood vessels, as this increases the flow of blood, thereby improving the provision of oxygen to local tissues (Boero, Ascher et al. 1999). Increased blood flow can trigger structural and functional changes in vascular cells that are equipped with receptors to detect and respond to the mechanical forces (Lehoux & Tedgui, 2003). Vascular remodelling has been observed in various situations where the local pressure and flow are altered, such as in arterial hypertension and atherosclerosis (Lehoux & Tedgui, 2003). The present study found evidence for microvascular remodelling in the fimbria and CA4 regions. Immunolabelling for Collagen IV revealed that the mean vessel diameter and the mean vessel length in the fimbria were significantly larger (1.23-fold and 1.14-fold, respectively) in the high OSA group.
compared to the low OSA group. Similarly, in the CA4 region the mean vessel diameter was increased 1.11-fold. These changes are consistent with a previous report of a 1.6-fold increase in capillary diameters in a mouse hypoxia model (Masamoto, Takuwa et al. 2014).

While increases in vessel number indicate angiogenesis, and increases in vessel diameter or length indicate vascular remodelling, the most effective way to determine whether the tissue has acquired a more extensive blood supply is to compare the proportion of the tissue that is occupied by blood vessels (area fraction). Since area fraction indicates the total vessel area in relation to the surrounding tissue area, an increased area fraction can result from an increased vessel count or vessel size, and also from tissue shrinkage (Harik, Hritz et al. 1995). As there is a reduction of grey matter volume in some parts of the hippocampus in OSA patients (Owen, Benediktsdottir et al. 2019), there was a possibility that an increased area fraction would be found in the absence of changes in vessel size. However the results of the present study revealed that only the fimbria and CA4 regions, when labelled for Collagen IV, exhibited increases in area fraction in the high OSA group. The magnitude of these changes (1.33-fold and 1.24-fold, respectively) was comparable to those seen for vessel diameter, leading us to conclude that the increased area fraction in these two regions is likely to be due to microvascular remodelling rather than tissue shrinkage.

Regional differences in area fraction can provide an insight into regional differences in metabolic demand, since vessel density is expected to be higher in regions where the metabolic demand is higher. The present study revealed that Collagen IV consistently produced the highest area fraction for each of the five hippocampal regions, in both groups of subjects. When the area fractions of the high OSA group are compared, the highest fraction is found in the collateral sulcus region (~ 6.5%), followed by the subiculum (~ 6.0%), CA4 (~ 6.0%), CA1 (~ 5.5%) and fimbria regions (~ 2.5%). Since the neuropil in the collateral sulcus
region is thicker than in the other regions, it would be expected to have a higher metabolic requirement, and similarly, as much of the fimbria consists of axons, it might be expected to have the lowest metabolic demands.

Compared to other brain regions, the hippocampus is selectively vulnerable to ischemia (Kirino 2000, Bartsch, Döhring et al. 2015), with the CA1 region being particularly vulnerable (West, Coleman et al. 1994, Chételat, Fouquet et al. 2008). Animal hypoxia models have consistently reported neuronal apoptosis in the CA1 region (Gozal, Daniel et al. 2001, Gozal, Gozal et al. 2002, Fung, Xi et al. 2009). The results of the present study hint at why the CA1 region is vulnerable. First it has the lowest microvascular area fraction of the four grey matter regions examined, and second, unlike the CA4 and fimbria, it did not exhibit any increase in area fraction in the high OSA group. These observations raise the possibility that the CA1 region not only receives less blood flow, it is also constrained from undergoing microvascular remodelling in response to intermittent hypoxia.

One unanticipated finding of the present study was that profiles labelled with Collagen IV occupied a far greater fraction of the field of view, and individual vessels had a wider diameter in all regions examined, when compared to profiles labelled with the other four antibodies (Tables 3.2 and 3.3). This may be due to the different locations of the Collagen IV and GluT-1 proteins on vessel walls; as Collagen IV is a key component of the basal membrane (Farkas and Luiten 2001), while GluT-1 is an influx transporter, which is expressed on the luminal (blood-facing) sides of the BBB (Zlokovic 2005, Daneman and Prat 2015). This interpretation is in line with the present study’s double immunofluorescence results, with Collagen IV labelling the outside border of the vessel while GluT-1 labelled the inside border. The similar results of four blood vessel markers (CD34, VWF, Claudin 5 and
Chapter 3 Microvascular Remodelling

GluT-1) suggests that they are all located on the luminal surface of microvessels; future research is needed to confirm this novel finding.

CPAP treatment has been proven to significantly attenuate OSA-related symptoms in people with moderate and severe OSA (Giles, Lasserson et al. 2006, Hobzova, Hubackova et al. 2017). For instance, capillary density in the cerebral cortex of rats increased by 60% after 3 weeks of hypoxia exposure, and after reverting to a normoxic environment, vessel density reduced to prehypoxic levels within 3 weeks (Pichiule and LaManna 2002). However, the present study found no significant difference between non-CPAP users and regular CPAP users in the two areas that had shown an increase in area fraction in response to OSA severity: the CA4 and fimbria. A possible explanation for this lack of effect is that in elderly brains, the collagen fibrils that compose vascular walls lose their elasticity, and consequently are more resistant to remodelling. Age-related differences in vessel formation have been reported in mice exposed to 10% O2 for 28 days; young adult mice (24- to 150-days old) displayed extensive angiogenesis, whereas older mice (4- to 5-months old) displayed no microvascular changes, suggesting that aged cerebral vasculature loses its plasticity and is more resistant to remodelling (Harb, Whiteus et al. 2013).

Interestingly, in the present study, Collagen IV immunostaining in the subiculum revealed that CPAP users in the low OSA group had a significantly smaller area fraction (0.66-fold) than CPAP non-users in the low OSA group. A reduced area fraction may result from tissue expansion, and indeed, MRI studies have shown that the grey matter volume in the subiculum region of OSA patients increases after 3 months of CPAP treatment (Canessa, Castronovo et al. 2011). However, such tissue expansion would be expected to reduce the area fraction for all five vascular markers, and this was not observed. Therefore it seems more likely that this
result for Collagen IV was a spurious outcome, that arose due to the small sample size in that group \( n = 6 \).

In the present study, the fimbria of older patients (> 67.5 years) was found to have increases in vessel diameter and length, as well as an increased area fraction, but a decreased number of vessels. These results suggest that while microvascular remodelling can occur, angiogenesis in response to hypoxia is impaired, which is in agreement with the idea of age-related diminishment of the capability for angiogenesis in the brain (Rivard, Fabre et al. 1999, Ingraham, Forbes et al. 2008, Brown 2010), possibly due to the weakened hypoxia-inducible factor-1 responsiveness to hypoxia and reduced vascular endothelial growth factor expression with ageing (Frenkel-Denkberg, Gershon et al. 1999, Rivard, Berthou-Soulie et al. 2000, Chavez and LaManna 2003).

There are several unavoidable methodological limitations of the present research that will be fully discussed in Chapter 7. Nonetheless, the strikingly similar mean values and small error bars for tissue labelled with CD34, VWF, Claudin 5 and GluT-1 in the CA4, CA1 and subiculum regions supports the reliability of the methodology used in the present study, and provides confidence in the reliability of the three main conclusions of this study: i) increased OSA severity does not stimulate angiogenesis; ii) increased OSA severity stimulates microvascular remodelling in the CA4 and fimbria, but not in other regions; iii) this microvascular remodelling is not reversed by CPAP treatment.

The reduced responsiveness of the hippocampal microvessels to OSA severity contrasts with the greater microvascular plasticity observed in animal models of OSA. The diminished responsiveness of the vasculature in humans may underpin the vulnerability of the hippocampus to hypoxic injury, particularly in the most unresponsive regions: the CA1, collateral sulcus and subiculum.
Chapter 4 Microvascular abnormalities in the hippocampus of obstructive sleep apnoea patients

4.0 Abstract

Obstructive sleep apnoea (OSA) involves the repeated cessation of breathing or reductions in airflow during sleep; consequently causing intermittent episodes of tissue hypoxia, with the brain being most strongly affected due to its dependence on oxidative metabolism. The periods of reoxygenation are thought to be associated with the production of reactive oxygen species, which are believed to damage the endothelial cells on capillary walls causing microvascular abnormalities. The present study aimed to investigate whether, in the hippocampus of OSA patients, the incidence of microvascular abnormalities is associated with increased OSA severity, and if so, whether regular CPAP use is protective. 3513 vessel segments from 5 regions of the hippocampus were analysed. In four of the regions, the proportion of microvessels that had an abnormal morphology did not differ significantly between the low and high OSA groups. By contrast, there were significantly more string vessels (4.3 ± 0.9 vs. 1.7 ± 0.4, \( p = 0.014 \)), narrowing vessels (3.5 ± 1.0 vs. 0.9 ± 0.3, \( p = 0.024 \)), strictures (2.6 ± 0.4 vs. 1.1 ± 0.2, \( p = 0.002 \)) and total abnormalities (10.3 ± 1.5 vs. 3.7 ± 0.6, \( p = 0.001 \)) in the CA1 region of the high OSA group than in the low OSA group. Moreover the degree of vessel width variability in the high OSA group (1.7 ± 0.1 vs. 1.4 ± 0.1, \( p = 0.021 \)) was significantly greater in the CA1 region. No significant differences between the low and high OSA groups were found for the number of tortuous vessels in any of the five regions. There were no significant differences on any of the measures of microvascular abnormality between regular CPAP users and non-CPAP users. It is concluded that the CA1 region may be more vulnerable to oxidative stress in moderate-severe OSA than other hippocampal regions. Abnormal microvessels are permanent degenerative changes and may limit the effectiveness of CPAP to reverse memory impairments.
4.1 Introduction

OSA is characterised by chronic intermittent hypoxia (Dempsey, Veasey et al. 2010), which has been assumed to be a major contributing factor to the pathogenesis of OSA-related comorbidities, such as systemic arterial hypertension, ischemic heart disease, cardiac arrhythmias, all of which are associated with vascular dysfunction (Sforza and Roche 2016). The chronic cycles of desaturation-reoxygenation are thought to induce oxidative stress (Sforza and Roche 2016), trigger persistent systemic inflammation (Garvey, Taylor et al. 2009), and increase the activity of the sympathetic nervous system due to the stress associated with hypoxic episodes (Peng, Overholt et al. 2003). These stressors have the potential to injure microvessels and trigger pathological changes in their morphology.

The hippocampus plays a pivotal role in learning and memory (Bartsch and Wulff 2015), and is selectively vulnerable to hypoxic damage (Kirino 2000, Bartsch, Döhring et al. 2015), with the CA1 region being the most sensitive subfield (West, Coleman et al. 1994, Chételat, Fouquet et al. 2008). Recent research from our laboratory found a reduction in neuropil thickness in some parts of the hippocampus as the severity of OSA increased (Owen, Benediktsdottir et al. 2019). Significant thinning was found in the grey matter of the dentate gyrus and CA1 region as well as loss of white matter in the entorhinal cortex; these regions have been linked to memory processing (Owen, Benediktsdottir et al. 2019). Animal models of OSA have also reported neuronal loss in the CA1 region of the hippocampus (Gozal, Daniel et al. 2001, Gozal, Row et al. 2003, Fung, Xi et al. 2012).

Pathological changes to the cerebral microvessels precede and accompany impaired cognition and neurodegeneration in both normal aged brains and in Alzheimer’s disease (AD) (Brown and Thore 2011). Altered vessel morphology may hamper the supply of nutrients, leading to compromised neuronal function, disturbed synaptic activity and impaired cognition (Jong, Vos et al. 1997). In AD, several ultrastructural and morphological alterations of the cerebral
Chapter 4 Microvascular Abnormalities

Microvessels have been reported, including reduced area fraction, increased numbers of fragmented vessels, perivascular collagen deposits, basement membrane thickenings, string vessels, tortuous arterioles, twisted capillaries, changes in the vessel diameter, and increased irregularity of capillary surfaces (Farkas and Luiten 2001, Zlokovic 2008, Bell and Zlokovic 2009). Since both AD and OSA involve increased neurodegeneration and oxidative stress in the brain (Butterfield, Howard et al. 2001), it is possible that the pathological structural changes that occur in cerebral vessels in AD are also present in OSA.

Shear stress is critical for the survival of vascular endothelial cells and the patency of continued vessels (Hunter, Kwan et al. 2012). The loss of shear stress can result from both the loss of blood flow and vascular blockage (Hunter, Kwan et al. 2012). String vessels are the remnants of capillaries that have collapsed and atrophied due to the lack of shear stress, leaving behind the basement membrane connective tissue fibres, mainly composed of Collagen IV (Brown 2010). String vessels do not carry blood flow and their increased numbers in AD brains (Challa, Thore et al. 2004, Brown 2010), may be related to the decreased cerebral blood flow reported in AD (Ries, Horn et al. 1993, Matteis, Silvestrini et al. 1998, Ruitenberg, Den Heijer et al. 2005). Neuroimaging studies have reported decreased rates of blood flow in the hippocampus and parahippocampal gyrus of moderate-severe OSA patients compared to controls (Innes et al 2015; Joo et al 2007). This reduced blood flow raises the possibility that string vessels may be more common in the hippocampus in moderate-severe OSA.

In an ultrastructural study of brain tissue from AD patients, most of the terminal arterioles displayed irregular shapes and had a series of focal constrictions and dilations, resulting in altered vessel diameter along their course (Miyakawa, Uehara et al. 1988). Cerebral arterioles in white matter often become tortuous with ageing, and begin to feature coils, loops and spirals, which slow blood flow even if the lumen diameter remains constant (Farkas and
Luiten 2001). Tortuous vessels are rare in the brains of preterm babies, children and young adults, and they start to appear after middle age (Thore, Anstrom et al. 2007). The vessels become elongated due to an age-related decrease in elasticity, and since the end points of the vessels are fixed, the extra length becomes curved and twisted, forming tortuous vessels (Del Corso, Moruzzo et al. 1998). Moreover, the deposition of excessive collagen in vessel walls can occlude or narrow the lumina, further slowing blood flow (Brown and Thore 2011). Therefore, the presence of abnormal microvessels is indicative of ageing or disease, and in turn, such abnormalities contribute to a slowing of blood flow and a consequent decline in neuronal function.

Another human autopsy study of OSA patients found that the more time a patient spent with a blood oxygen saturation below 95%, the higher the risk of microinfarcts in the brain (Gelber 2015). Although microinfarcts are a consequence of cerebrovascular disease, that study did not investigate any specific characteristics of the microvasculature. An animal model utilising chronic IH demonstrated significant cerebrovascular dysfunction and oxidative stress, suggesting that these two factors could account for the increased risk of cerebrovascular disease in OSA patients (Capone, Faraco et al. 2012). While the evidence obtained from these two studies is consistent with the hypothesis that cerebrovascular changes occur in OSA, neither study reported on the structural morphology of the blood vessels.

The current study aimed to investigate whether the incidence of pathological microvascular alterations in the human hippocampus increases in association with an increased severity of OSA. Additionally, this study investigated whether the incidence of microvascular abnormalities is lower in regular CPAP users, and whether patient age is a contributing factor.
4.2 Methods

4.2.1 Study samples

The study samples consisted of 30 of the 31 patients described in section 2.3; one sample was excluded because the tissue was refractory to immunostaining for Collagen IV and GluT-1. For detailed descriptive statistics, refer to Tables 2.1 – 2.3.

4.2.2 Tissue processing

Formalin-fixed and paraffin-embedded blocks of hippocampal tissues were processed as previously described in section 2.5.

4.2.3 Double immunohistochemistry

The double-immunohistochemistry protocol was a variant of the single immunohistochemistry protocol used elsewhere. Briefly, after deparaffinisation, sections were treated with antigen retrieval buffer, followed by 3 h incubation with blocking buffer at room temperature. Sections were incubated with solutions of mixed primary antibodies at their optimal concentrations (Collagen IV at 1 in 20,000, Abcam ab6586; GluT-1 at 1 in 2,000, Abcam ab15309) for 16.5 h overnight at room temperature. Following this, sections were processed as previously described in section 2.6.

4.2.4 Image processing

The slides were scanned with the Olympus Virtual Microscopy Slide Scanning System (VS120) (microscope VS-BX; camera: VC50) and Olympus VS-ASW-S5 software at a final magnification of 10 × (objective lens 20 ×, camera adapter mag 0.5 ×). Micrographs were captured from the full view images with a ‘Crop’ tool in Olympus CellSens software at a resolution of 1600 × 1200 pixels, at the same five hippocampal regions as shown in Figure 3.1 A (fimbria, CA4, CA1, subiculum and collateral sulcus).
4.2.5 Definition of abnormal microvessels

The five types of abnormal microvessels quantified in the present study are defined and illustrated in Table 4.1. Three of these types have been described by others: string vessel, dilated/constricted vessel and tortuous vessel; whereas two types are defined for the first time in the present study: narrowing vessel and stricture. While it is not known whether these two new types of abnormal vessel are evidence of disease, their irregular profile differs from the smooth profile of healthy microvessels, and it can be anticipated that their irregular diameter and course will slow or impede blood flow.

Normal microvessels have fluent walls with a constant diameter that can range from $4 - 10 \mu m$ (data from Chapter 3), and with bifurcations at intervals to form two vessels. A 'string vessel' is a thread-like vessel segment linking two normal vessel segments/or an independent small vessel segment, with an evenly distributed narrow diameter that is less than 1.5 µm (defined in the present study, no data in previous reports). A ‘narrowing vessel’ forms when a normal vessel segment gradually tapers, the width at the narrowest point being greater than 1.5 µm but less than 2.5 µm. In a ‘striction’ the vessel segment narrows abruptly, but remains patent. A ‘dilated/constricted vessel' exhibits considerable variation in diameter along its length, and for quantitative purposes was defined as having variability in width along its length that exceeds the 2-fold standard deviation (the half-width of a 95% confidence interval) of all tested diameters. A 'tortuous vessel' is one that follows a sinuous or recurved course through the tissue. By convention, the extent of tortuosity of a vessel segment is calculated as the length of the segment divided by the distance between the two segment end points. In the present study tortuosity was automatically calculated by the algorithms in ARIA (Bankhead, Scholfield et al. 2012).
Table 4.1 Definition of the morphological features of abnormal microvessels.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Diagram</th>
<th>Micrograph</th>
<th>Annotation</th>
</tr>
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<tbody>
<tr>
<td>Normal vessel</td>
<td>Fluent vessel walls with constant widths of 4 – 10 µm.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>String vessel</td>
<td>Thread-like vessel segment linking two normal vessel segments; or an independent small vessel segment. Diameter constant, typically less than 1.5 µm.</td>
<td></td>
<td></td>
<td>Measure by count</td>
</tr>
<tr>
<td>Narrowing vessel</td>
<td>A normal vessel segment narrows gradually, the width at the narrowest point is between 1.5 – 2.5 µm.</td>
<td></td>
<td></td>
<td>Measure by count</td>
</tr>
<tr>
<td>Stricture</td>
<td>The vessel width narrows abruptly, with two vessel walls opposing at one point, distance is less than 4 µm.</td>
<td></td>
<td></td>
<td>Measure by count</td>
</tr>
</tbody>
</table>
The vessel width varies greatly due to multiple dilations and constrictions. This is tested multiple times perpendicularly to the centre line (the green line) at one pixel intervals, as illustrated with the red lines.

The degree of variability in the width of individual vessels was determined as the spread of the 95% confidence interval (2-fold standard deviation; 2SD) for all perpendicular measures along the vessel length, expressed in µm.

Width variability was determined for every vessel in a field of view. This allowed identification of the vessel with the greatest degree of variability (maximum width variability) and mean width variability of all vessels in a field of view.
The tortuosity of a vessel segment is illustrated as the Euclidean distance from A to B (the green line) divided by the Euclidean distance between point A and B (the red line), therefore a ratio equal to 1.0 indicates a straight vessel segment, and higher ratios indicate a less direct course.

The mean tortuosity of a field of view is the average tortuosity values of all selected vessel segments.

The maximum tortuosity is represented by the most tortuous vessel segment in the field of view.

While normal vessels, string vessels, dilated/constricted vessels and tortuous vessels have been reported previously, detailed definitions based on quantitative criteria are proposed here. Narrowing vessels and strictures are novel and are defined for the first time in the present study.
4.2.6 Analysis of microvascular morphology

Micrographs were visually inspected for the presence of string vessels, narrowing vessels, and strictures. Each type of vessel in a field of view was counted. Specific parameters were used to differentiate between string vessels and narrowing vessels. The width of the narrowest vessel segment was measured using Olympus CellSens software with the ‘arbitrary line’ tool. If the vessel width was ≤ 1.5 µm the vessel was categorised as a string vessel, if it was > 1.5 µm the vessel was categorised as a narrowing vessel.

The vessel width variation of dilated/constricted vessels and vessel tortuosity were analysed using ARIA: Automated Retinal Image Analyzer v1.0, a software based upon an algorithm for the automated detection and measurement of blood vessels, run from within MATLAB\textsuperscript{TM} with its associated Image Processing Toolbox (Bankhead, Scholfield et al. 2012). ARIA automatically recognises and generates a centreline and two edge lines along each vessel segment. Diameters for each vessel segment were tested perpendicularly along the centreline every 1 pixel; the standard deviation of diameter measurements from selected vessel segments was used to calculate the width variation of vessels that had noticeable dilations and constrictions. Segment length was defined as the Euclidean distance along the centreline, the tortuosity measure is the segment length divided by the Euclidean distance between the vessel segment end points. In order to allow for a minimum length of vessel segment to curve, vessels with a centreline of less than 50 pixels were excluded. Data were then exported into .csv files via a separate routine. Representative images analysed by ARIA and the output window of ARIA are shown in Figure 4.1.
Figure 4.1 Representative images of vessel profiles analysed by ARIA.

A. In this micrograph analysed with ARIA software, a total of 19 vessel segments were identified, with the 8th vessel segment being highlighted. B. ARIA main window displaying the detailed information for the 8th vessel segment. C. Analysed micrograph with the 13th vessel segment being highlighted. D. Detailed information for the 13th vessel segment. Notice the significantly greater tortuosity of the 13th vessel segment compared to the 8th vessel segment (3.13 vs. 1.02), with the tortuosity of the 8th vessel segment being close to 1.0, representing a nearly-straight vessel segment, while the 13th vessel segment is U-shaped, and three-times more tortuous.
4.2.7 Statistical analysis

Statistical analysis was performed with the IBM Statistical Package for the Social Sciences (SPSS version 21). Independent samples-\( t \) tests were performed to compare the measured parameters between the low and high OSA groups, non-CPAP users and regular CPAP users, as well as in patients grouped by age \(< 67.5\) years and age \(> 67.5\) years. \( p < 0.05 \) was considered to be significant and significant differences are indicated on graphs by an asterisk (*) with equal variances or a hash (#) with unequal variances. GraphPad Prism 7 was used to generate graphs, presented as Mean ± SEM.

4.3 Results

4.3.1 Microvascular morphology in the hippocampus

Double immunohistochemistry (Collagen IV combined with GluT-1) provided stronger and more consistent staining of vessels than that obtained with the individual antibody markers (compare Figure 3.2 G – J with Figure 4.2). Figure 4.2 shows micrographs of tissues that have been double-immunostained for Collagen IV and GluT-1 in the five hippocampal regions (fimbria, CA4, CA1, subiculum and collateral sulcus) in the low (ODI < 20 events/h sleep) and high (ODI \(\geq 20\) events/h sleep) OSA groups.
Figure 4.2 Representative images of microvascular morphology in five hippocampal regions.

Microvessels double-immunostained for Collagen IV and GluT-1 in the fimbria (A & B), CA4 (C & D), CA1 (E & F), subiculum (G & H) and collateral sulcus regions (I & J) in the low (A, C, E, G & I) and high (B, D, F, H & J) OSA groups. There is one centreline (blue line) and two edge lines (red lines) along every vessel segment, as highlighted in the inset boxes.
4.3.2 The effect of OSA severity on microvascular abnormalities

4.3.2.1 Increased incidence of microvessel abnormalities in the high OSA group

The total number of all vessel segments automatically selected by ARIA software in 5 hippocampal regions of 30 OSA patients added to 3513, of these 1658 were found in the low OSA group, and 1891 in the high OSA group. There were significantly fewer microvessels in the fimbria region than in the other four regions (Table 4.2).

Table 4.2 Numbers of vessel segments, stratified by group and hippocampal region.

<table>
<thead>
<tr>
<th>Region</th>
<th>Low OSA (n = 15)</th>
<th>High OSA (n = 15)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fimbria</td>
<td>167</td>
<td>128</td>
<td>295</td>
</tr>
<tr>
<td>CA4</td>
<td>363</td>
<td>415</td>
<td>778</td>
</tr>
<tr>
<td>CA1</td>
<td>392</td>
<td>442</td>
<td>834</td>
</tr>
<tr>
<td>Subiculum</td>
<td>392</td>
<td>485</td>
<td>877</td>
</tr>
<tr>
<td>Collateral Sulcus</td>
<td>344</td>
<td>385</td>
<td>729</td>
</tr>
<tr>
<td>Total</td>
<td>1658</td>
<td>1855</td>
<td>3513</td>
</tr>
</tbody>
</table>
The numbers of abnormal microvessels in the two groups in the five hippocampal regions are shown in Figure 4.3. In the CA1 region the high OSA group had significantly more (2.56-fold) string vessels (4.3 ± 0.9 vs. 1.7 ± 0.4, $t(19.6) = 2.703, p = 0.014^\#$, unequal variances; Figure 4.3 A) than the low OSA group. The narrowing vessel count showed a similar trend to the string vessel count, with significantly more (3.71-fold) narrowing vessels in the high OSA group (3.5 ± 1.0 vs. 0.9 ± 0.3, $t(16.3) = 2.483, p = 0.024^\#$, unequal variances; Figure 4.3 B) than in the low OSA group in the CA1 region. Moreover, there were significantly more (2.44-fold) strictures in the high OSA group in the CA1 region (2.6 ± 0.4 vs. 1.1 ± 0.2, $t(28) = 3.490, p = 0.002$; Figure 4.3 C).

As all three types of abnormal vessels were measured in the same way, and as each will limit blood flow as a result of a narrowed or constricted vessel lumen, the total number of microvascular abnormalities in a standard field of view was measured by combining the numbers of all three types of abnormal microvessels together. In the CA1 region, there were significantly more (2.82-fold) string + narrowing + strictures (10.3 ± 1.5 vs. 3.7 ± 0.6, $t(18.5) = 4.202, p = 0.001^\#$, unequal variances; Figure 4.3 D) in the high OSA group than in the low OSA group.
Figure 4.3 Number of abnormal microvessels per field of view in five hippocampal regions.

Number of string vessel (A), narrowing vessel (B), stricture (C) and total abnormal vessels (string + narrowing + strictures) (D) in the low (ODI < 20 events/h sleep; blank bars) and high OSA groups (ODI ≥ 20 events/h sleep; striped bars). Unpaired 2-tailed $t$-tests between low vs. high OSA groups. Mean ± SEM.
Of the vessels selected for analysis by ARIA, the proportion (%) of total vessels that were abnormal microvessels varied between regions, as shown in Table 4.3, with the highest proportion of abnormal morphology being observed in the fimbria region. In general, string vessels were the most common microvascular abnormality, while strictures were the least common.

Table 4.3 Proportion (%) of total vessels that had an abnormal morphology, stratified by group and hippocampal region.

<table>
<thead>
<tr>
<th></th>
<th>String vessel (low vs. high)</th>
<th>Narrowing vessel (low vs. high)</th>
<th>Stricture (low vs. high)</th>
<th>Total abnormalities (low vs. high)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fimbria</td>
<td>36.5</td>
<td>26.6</td>
<td>13.2</td>
<td>4.8</td>
</tr>
<tr>
<td>CA4</td>
<td>10.5</td>
<td>7.7</td>
<td>5.2</td>
<td>1.8</td>
</tr>
<tr>
<td>CA1</td>
<td>6.4</td>
<td>14.5</td>
<td>3.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Subiculum</td>
<td>11.7</td>
<td>9.9</td>
<td>7.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Collateral Sulcus</td>
<td>15.1</td>
<td>19.5</td>
<td>9.0</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Blue shading indicates the low OSA group, pink shading indicates the high OSA group.
4.3.2.2 *Increased vessel width variability in the high OSA group*

The vessel width variability between the low and high OSA groups was compared, as shown in Figure 4.4. The maximum width variability (max. SD; µm) in the high OSA group was larger (1.17 – 1.26-fold) than in the low OSA group, with this difference approaching the statistically significant level in the CA1 region (2.3 ± 0.1 vs. 1.9 ± 0.1, \( t(28) = 1.954, p = 0.061 \); Figure 4.4 A); moreover, a significant difference was observed in the subiculum region (2.5 ± 0.2 vs. 2.0 ± 0.1, \( t(28) = 2.334, p = 0.027 \); Figure 4.4 A). Furthermore, the mean width variability (mean 2SD; µm) in the high OSA group was significantly larger (1.18-fold) than in the low OSA group in the CA1 region (1.7 ± 0.1 vs. 1.4 ± 0.1, \( t(28) = 2.456, p = 0.021 \); Figure 4.4 B), demonstrating increased vessel width variability in response to increased OSA severity.

![Figure 4.4 Vessel width variability in five hippocampal regions.](image)

Maximum width variability (A) and mean width variability (B) in the low (ODI < 20 events/h sleep; blank bars) and high OSA (ODI ≥ 20 events/h sleep; striped bars) groups. Unpaired 2-tailed \( t \)-tests between the low vs. high OSA groups. Mean ± SEM.
4.3.2.3 *Equal tortuosity between low and high OSA groups*

The tortuosity of a vessel segment was calculated as the length along the vessel segment divided by the straight line distance between the two end points. It is therefore equal to 1.0 for a perfectly straight vessel segment, and the higher the ratio, the more tortuous the vessel. The mean and maximum tortuosity in each field of view was calculated and comparisons were made between the low and high OSA groups (Figure 4.5). No significant differences were found in the mean tortuosity of vessels between the low and high OSA groups in any of the five regions (Figure 4.5 A). Indeed, the mean tortuosity value for all regions was close to 1.0, indicating that the majority of vessel segments were straight. Although maximum tortuosity did not differ between the low and high OSA groups (Figure 4.5 B), the fimbria region had the smallest maximum tortuosity of all five regions tested, probably because the length of vessel segments in the fimbria region were relatively short.

![Graph A: Mean tortuosity comparison between low and high OSA groups.](image)

![Graph B: Mean maximum tortuosity comparison between low and high OSA groups.](image)

**Figure 4.5 Vessel tortuosity in five hippocampal regions.**

Mean tortuosity (A) and maximum tortuosity (B) of all vessel segments in the low (ODI < 20 events/h sleep; blank bars) and high OSA (ODI ≥ 20 events/h sleep; striped bars) groups. Unpaired 2-tailed *t*-tests between low vs. high OSA groups. Mean ± SEM.
4.3.3 The effect of regular CPAP use on microvascular abnormalities

The effect of regular CPAP treatment on microvascular abnormalities was investigated by comparing the non-CPAP group with the regular CPAP treatment group in the low and high OSA groups. The CA1 region had previously been shown to have the largest differences in abnormal microvessels between the low and high OSA groups. However, in the CA1 region no significant differences were found between regular CPAP users and non-CPAP users with respect to the frequency of string vessels (Figure 4.6 A), narrowing vessels (Figure 4.6 B), strictures (Figure 4.6 C), total vessel abnormalities (Figure 4.6 D), maximum vessel width variation (Figure 4.6 E) or maximum vessel tortuosity (Figure 4.6 F). This result should be viewed with caution, since the sample sizes were small.
Figure 4.6 The effect of regular CPAP use on the frequency of abnormal microvessels in the CA1 region.

Abnormal microvessel morphology in the CA1 region in the non-CPAP users (blank bars) and CPAP users (striped bars) in the low (ODI < 20 events/h sleep; purple colour) and high (ODI ≥ 20 events/h sleep; pink colour) OSA groups of string vessel (A), narrowing vessel (B), stricture (C), total abnormal vessels (D), maximum width variability (E), mean width variability (F), mean vessel tortuosity (G) and maximum vessel tortuosity (H). Unpaired 2-tailed t-tests between non-CPAP users vs. CPAP users. Mean ± SEM.
4.3.4 The effect of advanced age on microvascular abnormalities

The effect of advanced age on the frequency of abnormal microvessels was analysed by comparing tissues from patients aged < 67.5 years with those from patients aged > 67.5 years. The detailed descriptive statistics of these two groups are shown in Table 2.3. Overall, age had no significant effect on the frequency of string vessels (Figure 4.7 A), strictures (Figure 4.7 C), total abnormalities (although approaching significant level in the CA4 region, Figure 4.7 D), the mean and maximum vessel width variation (Figure 4.8 E & F), or the mean and maximum vessel tortuosity (Figure 4.7 G & H). However, there were significantly more (2.29-fold) narrowing vessels in the CA4 region in persons aged greater than 67.5 years (2.1 ± 0.5 vs. 0.9 ± 0.3, \( t(22.8) = 2.162, p = 0.041^* \), unequal variances; Figure 4.7 B). There were also twice as many narrowing vessels in the CA1 region in the older group, however this difference did not reach significance.
Figure 4.7 The effect of advanced age on abnormal microvessels in the hippocampus.

Abnormal microvessel morphology in the age < 67.5 years (blank bars) and age > 67.5 years (grid bars) of string vessel number (A), narrowing vessel number (B), stricture number (C), total abnormal vessels number (D), maximum width variability (E), mean width variability (F), mean vessel tortuosity (G) and maximum vessel tortuosity (H). Unpaired 2-tailed t-tests between OSA patients aged < 67.5 years vs. those aged > 67.5 years. Mean ± SEM.
4.3.5 Summary

The present study found that:

i) Among the five hippocampal regions examined, the fimbria region had the highest proportion of abnormal microvessels, however their number did not differ significantly between the low and high OSA groups.

ii) The CA1 had significantly more abnormal microvessels in the high OSA group than the low OSA group. This difference extended to string vessels, narrowing vessels, strictures and dilated/constricted vessels. The only other region to have more abnormal vessels in the high OSA group was the subiculum, with more dilated/constricted vessels.

iii) CPAP treatment did not reduce the incidence of microvascular abnormalities.

iv) The CA4 was only region to show an increase in microvascular abnormalities (narrowing vessels) with advancing age.

These findings are summarised diagrammatically in Table 4.4.
Table 4.4 Summary of microvascular abnormalities in the hippocampus of OSA patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Region</th>
<th>OSA severity</th>
<th>CPAP use</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>String vessel</td>
<td>CA1</td>
<td>─</td>
<td>─</td>
<td>─</td>
</tr>
<tr>
<td>Narrowing vessel</td>
<td>CA1</td>
<td>─</td>
<td>─</td>
<td>─</td>
</tr>
<tr>
<td></td>
<td>CA4</td>
<td>─</td>
<td>─</td>
<td>─</td>
</tr>
<tr>
<td>Stricture</td>
<td>CA1</td>
<td>─</td>
<td>─</td>
<td>─</td>
</tr>
<tr>
<td>String + narrowing + strictures</td>
<td>CA1</td>
<td>─</td>
<td>─</td>
<td>─</td>
</tr>
<tr>
<td>Dilated/constricted vessel</td>
<td>CA1</td>
<td>─</td>
<td>─</td>
<td>─</td>
</tr>
<tr>
<td></td>
<td>Subiculum</td>
<td>─</td>
<td>─</td>
<td>─</td>
</tr>
<tr>
<td>Tortuous vessel</td>
<td>─</td>
<td>─</td>
<td>─</td>
<td>─</td>
</tr>
</tbody>
</table>

= Increased; ─ , No change.
4.4 Discussion

This is the first study to examine the brain tissue of OSA patients for the presence of microvasculature abnormalities, and as far as we are aware, the first comprehensive quantitative study of microvascular abnormalities in the human brain. The results of the present study indicate that abnormal microvessels are surprisingly common, and that the CA1 region stands out as having more microvascular abnormalities in patients with moderate-severe OSA. By contrast, neither patient age nor CPAP use had much influence on the incidence of abnormal microvessels. Our results provide a new line of evidence relating to the selective vulnerability of the CA1 region (Kirino 1982, Pulsinelli, Brierley et al. 1982, Bartsch, Döhring et al. 2015, Bartsch and Wulff 2015, Schmidt-Kastner 2015), and they support the idea that abnormal microvessels are permanent degenerative changes (Sforza 2012). The implications of these interesting findings will be discussed below.

Among the five hippocampal regions examined, the fimbria region had the highest burden of abnormality, with approximately half of the microvessels examined exhibiting some abnormality. This is of particular concern, since the data in the previous chapter revealed that the fimbria has far fewer microvessels than the other hippocampal regions examined. Taken together, these data indicate that the fimbria region is likely to be vulnerable to perturbed regional blood flow. However, no significant differences were found between the low and high OSA groups, or between the two age groups, suggesting that factors other than hypoxia and age are responsible for the high incidence of abnormal microvessels in the fimbria.

String vessels result from the collapse of the capillary walls, caused by loss of endothelial cells (Brown 2010). Since string vessels do not carry blood, their presence indicates a reduced capacity to support blood flow to that region. It is notable that the numbers of string vessels found in AD patients are double the number found in non-demented plaque-free
controls, suggesting that AD neuropathology is associated with a reduction in cerebral blood flow (Hunter, Kwan et al. 2012). It is therefore significant that the present study found that in the CA1 region the number of string vessels in the high OSA group was 2.56-fold higher than in the low OSA group.

Narrowing vessels were defined for the first time in the present study. We speculate that narrowing vessels are a precursor of string vessels, with the narrow part of the vessel becoming increasingly narrow and extending along the length of the vessel segment to form a string vessel. Although it was not possible to confirm this speculation in the present study, the diameter of narrowing vessels is too small to permit blood flow, so they are certainly abnormal and will impede the provision of nutrients to neurons in their vicinity. The present study reported significantly more narrowing vessels (3.71-fold more) in the CA1 region of patients with high OSA, which is consistent with our observations for string vessels.

Strictures are also a novel form of abnormal microvessel, and like string vessels and narrowing vessels, they have a point of constriction where the vessel calibre is too small to permit the flow of blood. We speculate that strictures are the precursor to narrowing vessels. While it is not possible to confirm this speculation from the present data, it is supported by the fact that strictures, like string vessels and narrowing vessels, were 2.44-fold more common in the CA1 of patients in the high OSA group compared to the low OSA group.

Dilated/constricted vessels, that is, vessels with a series of focal constrictions and dilations along their course, have been reported in the cerebral cortex of AD patients (Miyakawa, Uehara et al. 1988), although they have not been quantitatively analysed before. The present study quantified the degree of variability in vessel width in various hippocampal regions of OSA patients, and found that in the CA1 region the high OSA group had 1.18-fold more vessel width variation than the low OSA group. Although this type of abnormality will not
prevent blood flow, the variation in vascular diameter will contribute to turbulence in blood flow, which in turn will slow the blood flow by increasing vascular resistance (Farkas and Luiten 2001).

Interestingly, the last type of microvascular abnormality, tortuous vessels, were equally represented in all regions examined, apart from the fimbria which had fewer. Overall, tortuous microvessels were uncommon and their frequency did not differ between the low and high OSA groups or between the two age groups. It should be noted that the present findings differ from an earlier report that the tortuosity of cerebral arterioles increases after age 50 (Thore, Anstrom et al. 2007). Since the present study focussed on capillaries, it remains possible that arterioles in the hippocampus do become more tortuous with age. Nonetheless, the fact that tortuous capillaries are not particularly common means that they are not major contributors to impaired blood supply in the hippocampus.

Combining the above findings together, the CA1 region showed significantly more microvascular abnormalities in response to increased OSA severity than any of the other subregions; the subiculum was mildly affected, whereas the fimbria, CA4 and the collateral sulcus region were unaffected. The fact that the CA1 showed a substantial increase in 4 different types of abnormal microvessel in response to increased OSA severity is particularly interesting. As mentioned in the introduction, evidence indicates that abnormalities of the cerebral microvasculature arise through injury caused to capillary endothelial cells by ROS and hypoxia (Lavie 2003). The CA1 is the hippocampal subregion most sensitive to hypoxic injury, and the present results provide a new line of evidence in support of this vulnerability. Interestingly, all of the types of microvascular pathology observed in the recent study will reduce blood flow to the CA1, leading to a vicious cycle where hypoxia causes microvascular abnormalities which in turn will worsen tissue hypoxia. The preceding chapter demonstrated
that the CA1 appears to be unable to respond adaptively to hypoxia through angiogenesis or vascular remodelling, and it is likely that this inability increases the vulnerability of the CA1 to hypoxic injury.

CPAP treatment was hypothesised to reverse the microvascular alterations and reduce abnormal microvascular morphologies that might result from the oxidative stress that accompanies OSA. However, no significant differences were found between regular CPAP users and non-CPAP users, which suggests that abnormal microvessels are permanent degenerative changes. Since the continuing presence of abnormal microvessels may limit the effectiveness of CPAP to reverse memory impairments, it may be necessary to apply CPAP treatment to diagnosed OSA patients at an early stage, in order to maximise functional recovery and minimise residual injury (Dewan, Nieto et al. 2015).

The present study compared the microvascular morphology between patients younger than 67.5 years and older than 67.5 years. Significantly more narrowing vessels were observed in the CA4 region of patients aged older than 67.5 years, whereas string vessel frequency did not increase, which is in line with a report that string vessels in healthy normal subjects do not appear to increase in prevalence with age (Challa, Thore et al. 2002). If, as suggested earlier, the narrowing vessels are a precursor of string vessels, the age-related increase in narrowing vessels in the CA4 may indicate the beginning of a trend towards the generation of string vessels in old age. This possibility will need to be explored in a future study that has a larger sample size and a broader spread of patient ages.

In conclusion, this is the first study to address the effect of OSA severity on the prevalence of abnormal microvessels in the human hippocampus and it adds to the current literature by defining two new types of abnormal vessels, as well as introducing quantitative measures for defining five types of abnormal microvessel. There were significantly more microvascular
abnormalities in the CA1 of moderate-severe OSA patients. Our finding that regular CPAP use does not reduce the incidence of microvascular abnormalities suggests that these degenerative changes are permanent, which may limit the effectiveness of CPAP to reverse memory impairments.
Chapter 5 Contribution of ageing to the distribution of corpora amylacea in the hippocampus of obstructive sleep apnoea patients

5.0 Abstract

Corpora amylacea (CoA) are spherical aggregates of glucose polymers and proteins that appear in the brain during ageing. They are primarily found within periventricular, perivascular and subpial regions of the cerebral cortex and in the hippocampal cornu ammonis (CA) subfields. The present study aimed to quantify the distribution of CoA in the hippocampus of obstructive sleep apnoea (OSA) patients, and to assess the role of ageing, while the companion study (Chapter 6) examines the contributions of OSA severity and neurodegeneration.

Using autofluorescence, CoA were observed in 29 out of 30 OSA patients (96.67%), and were most abundant in the periventricular regions (wall of lateral ventricle, alveus, fimbria, and CA4). In a few cases, sparse CoA were found in the CA3 and CA1 regions, but no CoA were detected in the CA2 or subiculum. The packing density of CoA in subpial regions was $4.36 - 18.28$-fold higher than in the corresponding deeper neuropil, and they had larger mean and maximum diameters ($1.16 - 1.26$-fold larger). On the basis of maps of CoA distribution prepared for each hippocampus, a spatio-temporal sequence was postulated, beginning in the vicinity of the fimbria and progressively spreading around the subpial layer until they extended medially around the wall of the lateral ventricle and laterally to the collateral sulcus. When brain sections were arranged in an order that reflected this sequence, it was found that the sequence was not significantly correlated with patient age. Furthermore, the packing density of CoA did not change with age. However the mean diameters of CoA were strongly correlated with age. These findings support the view that CoA gradually become larger with age, possibly due to the accretion of new material.
5.1 Introduction

Corpora amylacea (CoA) are polyglucosan bodies with a round or oval profile, that have been reported in various organs and species (Cavanagh 1999, Rohn 2015). CoA can be stained with various histochemical methods including luxol fast blue, Haematoxylin and Eosin, Periodic Acid Schiff and cresyl violet; they can also be revealed by their autofluorescence (Cissé and Schipper 1995, Buervenich, Olson et al. 2001, Cherian, Radhakrishnan et al. 2003, Nam, Kim et al. 2012, Estupiñán-Díaz, Morales-Chacón et al. 2015, Augé, Cabezón et al. 2017). Immunohistochemistry directed against tubulin, tau, GFAP, neurofilament, amyloid β has also been used to label CoA, with varying degrees of success, (Cissé and Schipper 1995, Pisa, Alonso et al. 2016, Augé, Cabezón et al. 2017).

CoA in the brain have been associated with various conditions, such as normal ageing, hippocampal sclerosis, temporal lobe epilepsy, multiple sclerosis, AD and Parkinson’s disease (Cavanagh 1999, Abel, Hebb et al. 2010, Estupiñán-Díaz, Morales-Chacón et al. 2015, Augé, Duran et al. 2018, Pisa, Alonso et al. 2018). Since 1837 when CoA were first described by J.E. Purkinje, various hypotheses have been proposed regarding the origins of CoA (Wilhelmus, Verhaar et al. 2011, Augé, Cabezón et al. 2017, Augé, Bechmann et al. 2019). Despite sophisticated analyses of the molecular and biochemical composition of CoA, no consensus has been reached.

CoA have been associated with ageing process. For instance, in patients with myopathies and polyneuropathies who underwent muscle biopsies, CoA were only detected in 1% of biopsies from patients under 20 years of age, while the proportion was 31% in patients aged 20 – 39, 49% in patients aged 40 – 59, and 48% in the 60 – 79 age group (Bernsen, Busard et al. 1989). Moreover, in another study with sural nerve biopsies, no CoA were observed under 5 years of age, while 26% of subjects aged 31 – 50 contained CoA, and the percentage reached
60% in the group aged 51 – 80 (Busard, Gabreëls-Festen et al. 1990). Three studies have related CoA count to continuous age. The first study examined CoA in the ventroposterolateral nucleus of the human thalamus: CoA were not observed in patients younger than 50, and thereafter the numbers and size of CoA appeared to increase with age, although this relationship was not analysed statistically (Mizutani, Satoh et al. 1987). The second study counted CoA in 51 non-glaucomatous human eyes (aged 2.5 – 78 years), and reported that CoA count showed a positive linear correlation with ageing ($r = 0.424, p < 0.01$) (Kubota, Holbach et al. 1993). The third was a study of the subpial white matter of human spinal cords, which detected a trend of increased CoA count with advancing age, but some aged subjects had small numbers of CoA and the correlation between number of CoA and age did not reach statistical significance (Cavanagh 1998).

In the human lung, CoA are 30 – 40 µm in diameter and can reach 100 µm (Michaels and Levene 1957), however studies of the human nervous system have consistently indicated a much smaller diameter. For instance, the diameters of CoA in the human retina and optic nerve range from 3.6 – 11.0 µm (Kubota, Holbach et al. 1993), and in the anterior horn grey matter of spinal cord, most range between 4 – 12 µm, with few exceeding 20 µm (Cavanagh 1998). While it is often claimed that CoA increase in diameter with age, this claim is based on a report that large CoA (more than 20 µm) were observed within myelinated axons after 80 years of age (Mizutani, Satoh et al. 1987). There have been no systematic studies of CoA diameter as a function of age.

Recent ultrastructure studies have distinguished immature CoA from mature CoA (Navarro, Genoud et al. 2018, Augé, Bechmann et al. 2019). Immature CoA are intracellular inclusions that are enclosed by the cytoplasmic membrane of a cell (primarily in perivascular glial cells), with diameters typically less than 10 µm (Navarro, Genoud et al. 2018), but they
do not contain a membrane of their own (Augé, Bechmann et al. 2019). By comparison, mature CoA are extracellular, are more abundant, can measure up to 30 µm in diameter, and are surrounded by myelin sheaths and cells (Navarro, Genoud et al. 2018). These recent reports focused on the composition and ultrastructure of CoA in 3 – 6 aged patients (Navarro, Genoud et al. 2018, Augé, Bechmann et al. 2019), and the sample sizes were too small to permit an assessment of whether the diameters of CoA increase over time.

In aged brains, CoA tend to aggregate in periventricular, perivascular, subpial and subependymal regions (Erdamar, Zhu et al. 2000, Nishio, Morioka et al. 2001, Meng, Zhang et al. 2009, Abel, Hebb et al. 2010, Rohn 2015), suggesting an association with cerebrospinal fluid (CSF) (Meng, Zhang et al. 2009, Maurizi 2010, Nam, Kim et al. 2012). In the hippocampus, CoA have been reported to aggregate beneath the pial border (Cavanagh 1999, Erdamar, Zhu et al. 2000). CoA were reported in the periventricular regions of the hippocampus in three aged subjects without any neurodegenerative diseases (Augé, Bechmann et al. 2019), and located within perivascular and subpial regions in four aged AD patients and one aged patient with vascular dementia (Augé, Duran et al. 2018). Other studies have described abundant CoA in the hippocampal cornu ammonis (CA) subfields of patients with neurodegenerative diseases (Erdamar, Zhu et al. 2000, Cherian, Radhakrishnan et al. 2003, Uckermann, Galli et al. 2017, Navarro, Genoud et al. 2018).

Cherian and colleagues (2003) classified the distribution of CoA in the hippocampus of patients with mesial temporal lobe epilepsy (Cherian, Radhakrishnan et al. 2003). The presence of CoA was graded by counting the number of CoA per field of view under × 400 times magnification, with an absence of CoA equating to grade 0, 1 – 5 CoA as grade 1, 6 – 10 CoA as grade 2, and more than 10 CoA as grade 3 (Cherian, Radhakrishnan et al. 2003). This semi-quantitative grading system was developed to investigate the CoA density in
specific subregions of hippocampus, and it was further applied in a study of CoA density in selected areas of neocortex (meningeal surface), white matter and perivascular region in patients with drug-resistant temporal lobe epilepsy (Estupiñán-Díaz, Morales-Chacón et al. 2015). A limitation of this grading system is that it uses high magnification fields of view, which provide unrepresentative counts when the CoA are distributed non-uniformly.

CoA have been widely reported to be located in proximity to blood vessels (Erdamar, Zhu et al. 2000, Meng, Zhang et al. 2009, Pirici, Margaritescu et al. 2014, Rohn 2015, Navarro, Genoud et al. 2018, Pisa, Alonso et al. 2018). In the only quantitative study regarding the association between CoA and blood vessels, CoA were reported to congregate in the perivascular spaces and adjacent neuropil, with 37% of CoA being located within 5 µm of a blood vessel and 77% within 10 µm (Navarro, Genoud et al. 2018). This pattern of distribution is different from that expected due to chance, and may indicate the CoA have a physiological relationship to blood vessels.

A feature lacking from all studies of CoA is that none have systematically measured the packing density of CoA or compared CoA in different subregions of the brain in a quantitative way. Thus after 180 years of knowing that CoA exist, we still do not know the density and size of CoA that are typically found in the brain tissue of patients at different ages, nor do we know whether their spatial distribution is random or conforms to a pattern. Furthermore, there are no quantitative data concerning the putative relationships between CoA and the CSF or perivascular spaces. If such information were available, it might help to inform speculation on the origins of CoA. The present study seeks to fill these major gaps in our knowledge of CoA by systematically mapping the distribution and size of CoA in hippocampal subregions of OSA patients, and relating these parameters to patient age.
5.2 Methods

5.2.1 Study sample

The study sample consisted of 30 of the 31 patients described in section 2.3. Their ages ranged from 41 – 89 years. One case was excluded due to the tissue sample containing an incomplete hippocampus.

5.2.2 Tissue processing

The brain tissues used in this study consisted of a set of control slides that had undergone immunohistochemistry without incubation with primary antibody; thus they were unlabelled, yet were part of a series that had been labelled for cresyl violet, tau, amyloid β and GFAP. The slides had been prepared by another researcher in our laboratory (Owen 2017).

5.2.3 Immunohistochemistry

The immunohistochemistry carried on control slides was similar to that previously described. Briefly, after deparaffinisation, sections were washed with 0.1 M PBS and blocked for 3 h at room temperature without antigen retrieval. Sections were washed in PBS buffer, followed by incubation in primary antibody diluent solution with no primary antibody added. Following this, sections were processed as previously described (section 2.6).

5.2.4 Image processing

An intrinsic property of CoA is that they strongly autofluoresce as green-yellow when viewed under a blue light. This property was used to map the spatial distribution of CoA in tissue sections of the hippocampal formation. The sections were viewed with an Olympus fluorescence microscope (VS-BX) and camera (Hamamatsu ORCA-Flash 4.0), using a 'Fluorescein' filter set (Emission wavelength 518 nm, filter wheel (reflected) 485, and filter
wheel (reflected) 525). Scanning images were acquired with an Olympus Virtual Microscopy Slide Scanning System (VS120) and Olympus VS-ASW-S5 software, FITC (Fluorescein isothiocyanate) channel at 500 ms exposure time, final magnification of 20 × (objective lens 20 ×, camera adapter mag 1 ×), automatic Z-stacking (20 µm range, 4 µm step size, therefore 5 planes in total) and saved as .vsi format. Images to be used for analysis were captured from the full view images with a ‘Crop’ tool in Olympus CellSens software at a resolution of 1600 × 1200 pixels (equivalent to 0.2 mm²) at pre-determined locations. The coloured images were converted into grayscale, optimised in Adobe Photoshop CS6 Extended, and then analysed with Olympus CellSens software. The minimum detection size was equivalent to 3 µm. Objects that intersected the left hand or bottom borders of the image were excluded from analysis. All brain sections were processed in an identical manner.

Seven hippocampal neuropil regions that surround the lateral ventricle were investigated in the present study, including the wall of lateral ventricle (LV), fimbria, cornu ammonis 4 (CA4), cornu ammonis 3 (CA3), cornu ammonis 1 (CA2), cornu ammonis 1 (CA1), and subiculum. In addition, the subpial regions in the following locations were investigated: alveus pial surface (APS), fimbria pial surface (FPS), prosubiculum pial surface (PPS), subiculum pial surface (SPS), medial entorhinal cortex pial surface (MPS), lateral entorhinal cortex pial surface (LPS) and collateral sulcus pial surface (CPS). These regions were chosen because they are spaced across the hippocampal formation, and they can be reliably identified in sections due to their proximity to landmarks.

5.2.5 Definition and classification of CoA stages

In previous research, the areal density of CoA within specific brain regions has been graded according to the criteria of (Cherian, Radhakrishnan et al. 2003). In the present study we observed that CoA are not randomly distributed, and hence the use of high magnification
images provides variable and unrepresentative estimates of CoA density. In order to obtain more accurate estimates, the present study examined CoA density in lower magnification images (20 ×) that encompassed a much larger area (0.2 mm²) of tissue than typically assessed. Therefore, new criteria needed to be developed to classify the stages of CoA based on the density of CoA in specific regions, as well as considering the spatial distribution of CoA across an entire section.

5.2.6 Full view diagrams of CoA distribution in the hippocampus

In order to stage individual brains, it was necessary to first ascertain the distribution and relative frequency of CoA across the hippocampus. Full view scanned images of each brain section were processed with Photoshop software to generate outline diagrams of the section. The outline was traced with the ‘Stroke’ tool; the landmarks (dentate gyrus and grey matter orientation) were traced with the ‘Pencil’ and ‘Brush’ tools into grey curves. The ‘Airbrush’ tool was used to add red dots on the diagram to diagrammatically illustrate the location and density of the CoA.

5.2.7 Quantification of CoA density in the subpial and deeper neuropil zones

On inspection of sections it was observed that the density of CoA peaks just beneath the pial border; there is a fairly distinct boundary between this high-density zone and the deeper layers of tissue that have a low density of CoA. We have termed these two regions 'subpial' and 'deeper neuropil' respectively. The subpial zone of dense CoA was enclosed with a ‘polygon’ and another line (with ‘polyline’ tool) was drawn along the centre of the enclosed area in Olympus CellSens software; the subpial zone depth was measured as the total area divided by the length of the centre line.
5.2.8 Statistical analysis

Statistical analysis was performed with IBM Statistical Package for the Social Sciences (SPSS version 21). Two-tailed student’s $t$-tests with $\alpha = 0.05$ were used to compare the sets of data between two groups. Pearson’s correlations were performed for the covariate of two continuous-level variables. GraphPad Prism 7 was used to generate scatterplots and graphs, presented as Mean ± SEM, significant differences were indicated on graphs by an asterisk (*) with equal variances or a hash (#) with unequal variances.

5.3 Results

5.3.1 CoA detection and quantification

Hippocampal sections that had been immunolabelled in our laboratory (Owen 2017) for tau, amyloid $\beta$, GFAP, or stained with cresyl violet, were examined for the presence of CoA. Comparative images from the same hippocampal region are shown in Figure 5.1. Although CoA can be easily recognised with cresyl violet (Figure 5.1 A) and tau (Figure 5.1 B), these markers produce a strong background signal in the rest of the tissue, making it difficult to be able to quantify the CoA. Immunolabelling for amyloid $\beta$ (Figure 5.1 C) and GFAP (Figure 5.1 D) show the coats of a small subpopulation of CoA but do not label their contents, leaving a 'hole' in the background labelling to signify the location of the CoA. It was found that CoA were most salient when viewed under fluorescent illumination (Figure 5.1 E); the autofluorescent signal of the CoA was easy to distinguish from background as it was much brighter and less resistant to fading than the background autofluorescence of the tissue. Hence all of the analyses of CoA in this study are based on autofluorescence images.
Figure 5.1 CoA appearance revealed by five histological staining methods at the same location of the fimbria region.

A, Cresyl violet (0.5%). B, Immunohistochemistry with anti-Tau (1:500); C, anti-Amyloid β (1:100); D, anti-GFAP (1:20,000); E, Control (without primary antibody) under fluorescent mode (FITC). Examples of individual CoA are indicated by arrows.
Of the seven hippocampal neuropil regions investigated, CoA were detected in 23 out of 24 brain samples (95.83%) that included the wall of the lateral ventricle (Table 5.1). CoA were observed in 29 fimbria regions out of 30 (96.67%), the one without any CoA throughout the hippocampus was classified into stage 0. All of the CoA found in the CA4 region (19 out of 30, 63.33%) were located close to the inferior horn of the lateral ventricle, rather than in the deeper grey matter. Sparse CoA were occasionally observed in the CA3 region (3 out of 30, 10.00%) and CA1 region (4 out of 30, 13.33%), and no CoA were observed in the grey matter of the CA2 or subiculum. Since the numbers of CoA in these regions were very low, no further analyses were performed in the CA3, CA2, CA1 and subiculum regions.

CoA were present in 58.33 – 96.67% of the seven pial surface regions, data analysis was undertaken in 10 regions in total: wall of lateral ventricle, fimbria, CA4, alveus pial surface, fimbria pial surface, prosubiculum pial surface, subiculum pial surface, medial entorhinal cortex pial surface, lateral entorhinal cortex pial surface and collateral sulcus pial surface (Figure 5.2).
Table 5.1 Hippocampal subregions examined in this study, showing the proportion of brain samples that contained CoA.

<table>
<thead>
<tr>
<th>Region</th>
<th>LV</th>
<th>Fimbria</th>
<th>CA4</th>
<th>CA3</th>
<th>CA2</th>
<th>CA1</th>
<th>Subiculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage (%)</td>
<td>95.83</td>
<td>96.67</td>
<td>63.33</td>
<td>10.00</td>
<td>0</td>
<td>13.33</td>
<td>0</td>
</tr>
</tbody>
</table>

LV: wall of lateral ventricle; APS: alveus pial surface; FPS: fimbria pial surface; PPS: prosubiculum pial surface; SPS: subiculum pial surface; MPS: medial entorhinal cortex pial surface; LPS: lateral entorhinal cortex pial surface; CPS: collateral sulcus pial surface.

Figure 5.2 Locations of the hippocampal subregions investigated in the present study.

The coloured rectangles indicate where micrographs were taken for the analysis of CoA packing density. Seven hippocampal neuropil regions that surround lateral ventricle include: LV (wall of lateral ventricle), fimbria, CA4, CA3, CA2, CA1, subiculum. Seven pial surface regions included: APS (alveus pial surface), FPS (fimbria pial surface), PPS (prosubiculum pial surface), SPS (subiculum pial surface), MPS (medial entorhinal cortex pial surface), LPS (lateral entorhinal cortex pial surface) and CPS (collateral sulcus pial surface).
Five parameters were investigated: CoA count, the total number of CoA per field of view; CoA density, calculated as the total CoA count in the region of interest (count/mm²); CoA mean diameter (µm), the average diameter of all CoA in a field of view; CoA min. diameter (µm), the mean diameter of the smallest CoA in a field of view; CoA max. diameter (µm), the mean diameter of the largest CoA in a field of view. In some cases, no CoA were detected in a field of view, in such cases a ‘0’ value was recorded for the CoA count and area, and this value was used in the analysis. However, for CoA diameter, values of 0 µm were not included in the analysis.

When diagrams of CoA burden were compared, a consistent pattern became evident: in sections with low burdens of CoA, they were concentrated in the vicinity of the fimbria, and as the burden increased, CoA extended further away from the fimbria, within the pial border, to the alveus and wall of lateral ventricle on one side, and towards the subiculum, entorhinal cortex and beyond the collateral sulcus on the other side. CoA were only observed in the deep white and grey matter in sections with the heaviest burdens. Based on these diagrams, three investigators (CX, JO and SRR) independently arranged the sections in a series, from lowest CoA burden to highest. There was strong concordance between investigators, and the minor differences in the order of the rankings were resolved by discussion. The agreed sequence was then divided into five stages, based on the spatial extent of spread of CoA and the severity of the CoA burden (Figures 5.3 and 5.4).
Figure 5.3 Representative full view hippocampus sections from four classified CoA stages.

Traced diagrams of actual hippocampal sections with the location of CoA indicated by red dots. The purpose of these diagrams is to reflect their spatial extent, so that the sections could be staged. Thus one red dot may represent a few CoA in scarce locations, and hundreds in the highly concentrated areas.
Figure 5.4 The spatial distribution of CoA in the hippocampal formation according to Stage.

These schematic diagrams are based on the outline of a hippocampus from an OSA patient. The blue colour indicates the inferior horn of the lateral ventricle, the grey lines show the landmarks of the hippocampus; the red solid circles indicate where CoA are found.
Stage 0 corresponded to no CoA being present. Stage 1 was defined as a low density of CoA (0 – 10 CoA per field of view) in the fimbria, without progression of CoA across the pial surface. Stage 2 was defined as medium density (10 – 50) CoA in the fimbria and being distributed along the pial surface of the prosubiculum and subiculum. Stage 3 was defined as medium or high density (50 – 500) CoA in the fimbria, and extending continuously across the pial surface of the prosubiculum and subiculum, as well as into the deep white matter of the wall of lateral ventricle. Stage 4 was defined as medium or high density (50 – 500 CoA); this stage resembled Stage 3, except that CoA now extended into the deep white matter of the parahippocampal gyrus and fusiform gyrus. Detailed descriptions are shown in Table 5.2 and representative micrographs of increasing CoA density in the fimbria from each stage are shown in Figure 5.5.
## Table 5.2 Definition and classification of stages of CoA extent.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No CoA observed</td>
</tr>
</tbody>
</table>
| 1     | Low density (0 – 10 count) of CoA in the fimbria, alveus, roof and walls of the inferior horn of the lateral ventricle  
Few CoA anywhere else |
| 2     | Medium density (10 – 50 count) of CoA in the fimbria, alveus, roof and walls of the inferior horn of the lateral ventricle  
CoA at the pial surface of the prosubiculum and subiculum  
CoA in depths of sulci and in cortical gyri |
| 3     | Medium or high density (50 – 500 count) of CoA in the fimbria, alveus, roof and walls of the inferior horn of the lateral ventricle  
Continuous CoA at the pial surface of the prosubiculum and subiculum  
CoA in depths of sulci and near the surface of gyri  
CoA in the deep white matter of the wall of the lateral ventricle |
| 4     | Medium or high density (50 – 500 count) of CoA in the fimbria, alveus, roof and walls of the inferior horn of the lateral ventricle  
Continuous CoA at the pial surface of the prosubiculum and subiculum  
CoA in depths of sulci and near the surface of gyri  
CoA in the deep white matter of the wall of the lateral ventricle  
CoA in the deep white matter of the parahippocampal gyrus and fusiform gyrus |
Figure 5.5 CoA micrographs and processed images from four classified stages.

CoA micrographs in the fimbria region from stages 1 – 4 (A – D) and the corresponding processed images (E – H). All micrographs are the 3rd plane of a 5-step Z-stack (Z range 20 µm, step size 4 µm), the upper and lower step images were referred to in order to generate the processed images E – H; therefore, in some cases minor mismatching occurs between the processed image and the single slice immunofluorescent micrograph.
Bivariate correlations between ranked CoA sequence and measured CoA parameters (count, area fraction, mean, minimum and maximum diameters) in seven pial surface regions were conducted to investigate the reliability of the staging criteria introduced in the present study, as shown in Table 5.3. Ranked CoA sequence was significantly correlated with increased CoA count and area fraction in five out of seven pial surface regions, demonstrating that the subjective criteria applied in the present study are supported by quantifiable parameters. Increased CoA sequence did not correlate with CoA mean diameter in any of the seven pial surface regions investigated, however, CoA sequence was negatively correlated with the CoA minimum diameter in two regions, while positively correlated with the CoA maximum diameter in two different regions. Significant correlations, highlighted in coloured shading, were displayed with scatterplots in Figure 5.6.
### Table 5.3 Pearson’s correlation of ranked CoA sequence with tested CoA parameters.

<table>
<thead>
<tr>
<th></th>
<th>APS</th>
<th>FPS</th>
<th>PPS</th>
<th>SPS</th>
<th>MPS</th>
<th>LPS</th>
<th>CPS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CoA count</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>.378*</td>
<td>.478**</td>
<td>.617**</td>
<td>.590**</td>
<td>.456*</td>
<td>.311</td>
<td>.150</td>
</tr>
<tr>
<td>$p$</td>
<td>.039</td>
<td>.008</td>
<td>.000</td>
<td>.002</td>
<td>.025</td>
<td>.139</td>
<td>.483</td>
</tr>
<tr>
<td>$n$</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td><strong>Area fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>.366*</td>
<td>.552**</td>
<td>.662**</td>
<td>.675**</td>
<td>.461*</td>
<td>.344</td>
<td>.208</td>
</tr>
<tr>
<td>$p$</td>
<td>.047</td>
<td>.002</td>
<td>.000</td>
<td>.000</td>
<td>.023</td>
<td>.100</td>
<td>.329</td>
</tr>
<tr>
<td>$n$</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
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<tr>
<td><strong>Mean diameter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>-.161</td>
<td>-.136</td>
<td>.135</td>
<td>-.125</td>
<td>.097</td>
<td>-.511</td>
<td>.259</td>
</tr>
<tr>
<td>$p$</td>
<td>.473</td>
<td>.483</td>
<td>.485</td>
<td>.570</td>
<td>.659</td>
<td>.062</td>
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<td>29</td>
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<td>23</td>
</tr>
<tr>
<td><strong>Minimum diameter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>-.232</td>
<td>-.217</td>
<td>-.180</td>
<td>-.483*</td>
<td>-.294</td>
<td>-.634*</td>
<td>-.008</td>
</tr>
<tr>
<td>$p$</td>
<td>.287</td>
<td>.259</td>
<td>.349</td>
<td>.020</td>
<td>.173</td>
<td>.015</td>
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</tr>
<tr>
<td>$n$</td>
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<td>29</td>
<td>29</td>
<td>23</td>
<td>23</td>
<td>14</td>
<td>23</td>
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<tr>
<td><strong>Maximum diameter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>.225</td>
<td>.260</td>
<td>.414*</td>
<td>.312</td>
<td>.436*</td>
<td>.314</td>
<td>.270</td>
</tr>
<tr>
<td>$p$</td>
<td>.314</td>
<td>.174</td>
<td>.026</td>
<td>.148</td>
<td>.037</td>
<td>.274</td>
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<td>$n$</td>
<td>22</td>
<td>29</td>
<td>29</td>
<td>23</td>
<td>23</td>
<td>14</td>
<td>23</td>
</tr>
</tbody>
</table>

Significant positive linear regressions were found between ranked sequence and CoA density (count and area fraction) in the alveus pial surface ($r^2 = 0.143, p = 0.039$; $r^2 = 0.134, p = 0.047$; Figure 5.6 A & B), fimbria pial surface ($r^2 = 0.229, p = 0.008$; $r^2 = 0.304, p = 0.002$; Figure 5.6 C & D), prosubiculum pial surface ($r^2 = 0.380, p < 0.001$; $r^2 = 0.438, p < 0.001$; Figure 5.6 E & F), subiculum pial surface ($r^2 = 0.348, p = 0.002$; $r^2 = 0.455, p < 0.001$; Figure 5.6 G & H) and medial entorhinal cortex pial surface ($r^2 = 0.208, p = 0.025$; $r^2 = 0.213, p = 0.023$; Figure 5.6 I & J). These correlations support the notion that the ranked CoA sequence is reflective of increasing CoA burden, and that the highest burdens are found in fimbria pial surface region.

Significant negative correlations between ranked CoA sequence and CoA minimum diameter were found in the subiculum pial surface region ($r^2 = 0.233, p = 0.020$; Figure 5.6 K) and lateral entorhinal cortex region ($r^2 = 0.402, p = 0.015$; Figure 5.6 L). Moreover, ranked CoA sequence was positively correlated with larger CoA maximum diameter in the prosubiculum pial surface region ($r^2 = 0.171, p = 0.026$; Figure 5.6 M) and medial entorhinal cortex pial surface region ($r^2 = 0.190, p = 0.037$; Figure 5.6 N). Therefore, ranked CoA sequence was negatively correlated with CoA minimum diameters while positively correlated with CoA maximum diameters.
Chapter 5 Corpora amylacea Distribution

A

$\begin{align*}
  r^2 &= 0.143, \ p=0.039, \ n=30 \\
  \\
  \text{CoA count} &\quad \text{Sequence} \\
  0 &\quad 5 \\
  10 &\quad 15 \\
  20 &\quad 25 \\
  30 &\quad \text{Alveus pial surface}
\end{align*}$

B

$\begin{align*}
  r^2 &= 0.134, \ p=0.047, \ n=30 \\
  \\
  \text{CoA area fraction (\%)} &\quad \text{Sequence} \\
  0 &\quad 5 \\
  10 &\quad 15 \\
  20 &\quad 25 \\
  30 &\quad \text{Alveus pial surface}
\end{align*}$

C

$\begin{align*}
  r^2 &= 0.229, \ p=0.008, \ n=30 \\
  \\
  \text{CoA count} &\quad \text{Sequence} \\
  0 &\quad 5 \\
  10 &\quad 15 \\
  20 &\quad 25 \\
  30 &\quad \text{Fimbria pial surface}
\end{align*}$

D

$\begin{align*}
  r^2 &= 0.304, \ p=0.002, \ n=30 \\
  \\
  \text{CoA area fraction (\%)} &\quad \text{Sequence} \\
  0 &\quad 5 \\
  10 &\quad 15 \\
  20 &\quad 25 \\
  30 &\quad \text{Fimbria pial surface}
\end{align*}$

E

$\begin{align*}
  r^2 &= 0.380, \ p<0.001, \ n=30 \\
  \\
  \text{CoA count} &\quad \text{Sequence} \\
  0 &\quad 5 \\
  10 &\quad 15 \\
  20 &\quad 25 \\
  30 &\quad \text{Prosubiculum pial surface}
\end{align*}$

F

$\begin{align*}
  r^2 &= 0.438, \ p<0.001, \ n=30 \\
  \\
  \text{CoA area fraction (\%)} &\quad \text{Sequence} \\
  0 &\quad 5 \\
  10 &\quad 15 \\
  20 &\quad 25 \\
  30 &\quad \text{Prosubiculum pial surface}
\end{align*}$

G

$\begin{align*}
  r^2 &= 0.348, \ p=0.002, \ n=24 \\
  \\
  \text{CoA count} &\quad \text{Sequence} \\
  0 &\quad 5 \\
  10 &\quad 15 \\
  20 &\quad 25 \\
  30 &\quad \text{Subiculum pial surface}
\end{align*}$

H

$\begin{align*}
  r^2 &= 0.455, \ p<0.001, \ n=24 \\
  \\
  \text{CoA area fraction (\%)} &\quad \text{Sequence} \\
  0 &\quad 5 \\
  10 &\quad 15 \\
  20 &\quad 25 \\
  30 &\quad \text{Subiculum pial surface}
\end{align*}$
Figure 5.6 CoA density in relation to classified stage and ranked sequence.

Scatterplots showing significant linear correlations between ranked CoA sequence (1 – 30) and CoA count (A, C, E, G & I), area fraction (B, D, F, H & J), minimum diameter (K & L) and maximum diameter (M & N) in the alveus pial surface region (A & B), fimbria pial surface region (C & D), prosubiculum pial surface region (E, F & M), subiculum pial surface region (G, H & K) medial entorhinal cortex surface region (I, J & N) and lateral entorhinal cortex surface region (L).
The progression of CoA along the pial border was investigated in seven pial surface regions. In stage 1, CoA were restricted to the fimbria region (Figure 5.7 A). By stage 2 CoA were concentrated in the fimbria and prosubiculum regions. In stages 3 and 4 the numbers increased further in the fimbria and prosubiculum regions, and CoA became evident in the other five pial areas, with a trend to decreasing density when moving away from the fimbria toward the collateral sulcus (Figure 5.7 B – D). The patterns of CoA distribution in the pial surface were similar in stages 3 and 4, as the main criterion distinguishing these two stages was the presence of CoA in deep white matter.
Figure 5.7 The progression of CoA packing density according to stage.

CoA progression in seven pial surface regions of stage 1 (A), stage 2 (B), stage 3 (C) and stage 4 (D). Mean ± SEM. APS: alveus pial surface, FPS: fimbria pial surface, PPS: prosubiculum pial surface, SPS: subiculum pial surface, MPS: medial entorhinal cortex pial surface, LPS: lateral entorhinal cortex pial surface and CPS: collateral sulcus pial surface.
At every stage, the pial surface of the fimbria region contained the most CoA, followed by the pial surface of the prosubiculum in second place. The CoA progression along the pial border started from the fimbria region and spread to the alveus, and the corner of lateral ventricle on one side; and towards the prosubiculum, subiculum, entorhinal cortex and collateral sulcus on the other side (Figure 5.8) with gradually reduced CoA packing density (Figure 5.7) and size, with the smallest mean and maximum diameters of CoA being found in the collateral sulcus pial surface region, as shown in Table 5.4.

**Table 5.4 CoA sizes in seven pial surface regions.**

<table>
<thead>
<tr>
<th></th>
<th>APS</th>
<th>FPS</th>
<th>PPS</th>
<th>SPS</th>
<th>MPS</th>
<th>LPS</th>
<th>CPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean diameter (µm)</td>
<td>6.07</td>
<td>6.87</td>
<td>7.08</td>
<td>7.01</td>
<td>6.76</td>
<td>6.44</td>
<td>6.28</td>
</tr>
<tr>
<td>Min. diameter (µm)</td>
<td>3.94</td>
<td>3.33</td>
<td>3.70</td>
<td>3.69</td>
<td>4.48</td>
<td>4.46</td>
<td>4.49</td>
</tr>
<tr>
<td>Max. diameter (µm)</td>
<td>7.71</td>
<td>13.47</td>
<td>9.91</td>
<td>11.19</td>
<td>9.31</td>
<td>9.55</td>
<td>7.75</td>
</tr>
</tbody>
</table>

APS: alveus pial surface, FPS: fimbria pial surface, PPS: prosubiculum pial surface, SPS: subiculum pial surface, MPS: medial entorhinal cortex pial surface, LPS: lateral entorhinal cortex pial surface and CPS: collateral sulcus pial surface. Mean diameter and maximum diameter show a trend towards a smaller size when progressing laterally from the FPS to the CPS, whereas minimum diameter trends towards a larger size.
Figure 5.8 Schematic illustration of postulated progression of CoA along the pial surface.

A, Schematic diagram of the hippocampus. B, Proposed progression of CoA along the pial surface; red curve indicates highest CoA density in the fimbria pial surface region; blue curve indicates the decreased CoA density along the pial surface when spreading bilaterally from the fimbria, as pointed by the red arrows. APS: alveus pial surface, FPS: fimbria pial surface, PPS: prosubiculum pial surface, SPS: subiculum pial surface, MPS: medial entorhinal cortex pial surface, LPS: lateral entorhinal cortex pial surface and CPS: collateral sulcus pial surface.
5.3.2 Stratification of CoA within the pial surface region: the subpial and deeper neuropil zones

Examination of micrographs of the pial region revealed that CoA were concentrated in a well-defined zone just beneath the pial border, which we have termed the 'subpial zone'. The mean thickness of the seven subpial zones was 52.4 µm at the APS (alveus), 108.4 µm at the FPS (fimbria), 72.2 µm at the PPS (prosubiculum), 43.0 µm at the SPS (subiculum), 39.8 µm at the MPS (medial entorhinal cortex), 26.6 µm at the LPS (lateral entorhinal cortex) and 32.2 µm at the CPS (collateral sulcus) (Figure 5.9 A). To simplify, for the purpose of quantitative analysis, the subpial zone in the present study was defined as extending from the pia to a depth of 100 µm at the fimbria, 70 µm at the prosubiculum and 40 µm below the pial surface at the alveus, subiculum, medial and lateral entorhinal cortex and collateral sulcus.
Figure 5.9 CoA density distribution in seven pial surface regions.

Subpial zone thickness (µm) at seven pial surface regions (A). Pial surface regions at alveus (B), fimbria (C), prosubiculum (D), subiculum (E), medial entorhinal cortex (F), lateral entorhinal cortex (G) and collateral sulcus (H). Subpial zones (top red zones) vs. deeper neuropil zones (bottom purple zones), with the width of the subpial zones being 40, 100, 70, 40, 40, 40 and 40 µm, respectively.
The deeper region with less CoA was referred to as the 'deeper neuropil zone' and for the purposes of analysis consisted of the remainder of the neuropil in each micrograph (purple enclosed zones in Figure 5.9). Comparisons between CoA packing density (count/mm²; calculated as the total CoA count in the region of interest) in the subpial zone and in the deeper neuropil zone are shown in Figure 5.10 A. CoA packing density in the subpial zones was significantly higher (4.36 – 18.28-fold) than in the deeper neuropil zones in all seven regions investigated; moreover, a similar pattern applied to the CoA area fraction comparison, with the subpial zones containing 4.44 – 20.35-fold more CoA than the corresponding deeper neuropil zones (Figure 5.10 B), detailed descriptive data for CoA packing density and area fraction comparisons are shown in Table 5.5.

The mean diameters (µm) of CoA in the seven subpial zones were similar to those in the corresponding deeper neuropil zones (Figure 5.10 C), except in the subiculum where CoA had a larger mean diameter (1.16-fold) in the subpial zone than that in the deeper neuropil zone. Interestingly, there was a trend for smaller CoA minimum diameters in the subpial zones (Figure 5.10 D), with the difference reaching significance in the prosubiculum subpial zone (0.83-fold; 4.2 ± 0.2 vs. 5.1 ± 0.3, t (41.6) = -2.702, p = 0.010 #, unequal variance). The CoA maximum diameters in the subpial zones were significantly greater than those in the deeper neuropil zones (Figure 5.10 E) in the fimbria (1.17-fold; 18.8 ± 0.6 vs. 16.1 ± 0.7, t (55) = 2.861, p = 0.006), prosubiculum (1.25-fold; 18.9 ± 0.6 vs. 15.1 ± 0.7, t (55) = 4.044, p < 0.001) and subiculum (1.26-fold; 17.1 ± 0.6 vs. 13.5 ± 0.8, t (43) = 3.645, p = 0.001).
Figure 5.10 CoA density and diameter in seven pial surface regions.

CoA in the deeper neuropil zones (blank bars) and in the subpial zones (striped bars), comparing CoA packing density (A), area fraction (B), mean diameter (C), minimum diameter (D) and maximum diameter (E). Unpaired 2-tailed t-tests. Bars show mean ± SEM.
Table 5.5 CoA packing density comparisons between seven subpial zones and corresponding deeper neuropil zones.

<table>
<thead>
<tr>
<th>Position</th>
<th>CoA density (count/mm²)</th>
<th>t value</th>
<th>p value#</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>393.0 ± 114.8 vs. 80.5 ± 29.1</td>
<td>t (32.7) = 2.640</td>
<td>0.013</td>
</tr>
<tr>
<td>FPS</td>
<td>1832.3 ± 255.3 vs. 420.0 ± 91.4</td>
<td>t (36.3) = 5.208</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PPS</td>
<td>2095.2 ± 267.4 vs. 185.5 ± 30.6</td>
<td>t (29.8) = 7.097</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>SPS</td>
<td>1464.1 ± 213.9 vs. 80.1 ± 18.1</td>
<td>t (23.3) = 6.447</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MPS</td>
<td>1135.7 ± 192.3 vs. 103.5 ± 41.5</td>
<td>t (25.1) = 5.245</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LPS</td>
<td>344.6 ± 115.9 vs. 38.1 ± 27.8</td>
<td>t (25.6) = 2.572</td>
<td>0.013</td>
</tr>
<tr>
<td>CPS</td>
<td>671.4 ± 104.8 vs. 49.2 ± 12.3</td>
<td>t (23.6) = 5.898</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Position</th>
<th>CoA area fraction (%)</th>
<th>t value</th>
<th>p value#</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>2.11 ± 0.55 vs. 0.44 ± 0.16</td>
<td>t (33.9) = 2.896</td>
<td>0.007</td>
</tr>
<tr>
<td>FPS</td>
<td>11.64 ± 1.36 vs. 2.62 ± 0.55</td>
<td>t (38.2) = 6.150</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PPS</td>
<td>15.34 ± 1.96 vs. 1.24 ± 0.22</td>
<td>t (29.7) = 7.158</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>SPS</td>
<td>11.32 ± 1.57 vs. 0.56 ± 0.15</td>
<td>t (23.4) = 6.807</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MPS</td>
<td>8.67 ± 1.59 vs. 0.81 ± 0.33</td>
<td>t (25.0) = 4.842</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>LPS</td>
<td>2.11 ± 0.70 vs. 0.19 ± 0.12</td>
<td>t (24.3) = 2.715</td>
<td>0.012</td>
</tr>
<tr>
<td>CPS</td>
<td>4.37 ± 0.78 vs. 0.29 ± 0.08</td>
<td>t (23.4) = 5.181</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

# Student’s t-tests with unequal variances. APS: alveus pial surface, FPS: fimbria pial surface, PPS: prosubiculum pial surface, SPS: subiculum pial surface, MPS: medial entorhinal cortex pial surface, LPS: lateral entorhinal cortex pial surface and CPS: collateral sulcus pial surface.
5.3.3 CoA distribution in relation to the vasculature

CoA were observed near arterioles and venules in 17 (56.7%) of the 30 OSA hippocampal tissues examined in the present study. However, it needs to be noted that in these 17 specimens, the vast majority of arterioles and venules in the deeper neuropil had no detectable CoA in their vicinity (Figure 5.11 A & B). In the rare instances where CoA were observed in association with deep blood vessels, they did not appear to be preferentially located around arterioles or venules (Figure 5.11 C & D). Although arterioles and venules located close to the pial surface were more frequently surrounded by CoA (Figure 5.11 E & F), the correspondence did not appear to be strong, and larger blood vessels such as these were infrequent. Due to the limited number of vessels that had CoA clustered around them, it was not possible to conduct a quantitative analysis of this relationship.
Figure 5.11 CoA deposition in relation to venules and arterioles in the deeper neuropil and subpial regions.

The deposition of CoA in relation to arterioles (A, C & E) and venules (B, D & F) in the grey matter (A & B), white matter (C & D) and subpial region (E & F). Examples of individual CoA are indicated by white arrows.
The distribution of CoA was compared to the distribution of microvessels, as described in Chapter 4 of this thesis. Although no quantitative comparison was made, it was evident that in all regions of the hippocampus very few microvessels are present near the pial surface, and there is a well-defined avascular zone, 80 – 200 µm wide, between the pial surface and where the first microvessels can be seen (Figure 5.12). Interestingly, CoA tend to aggregate in this avascular zone, suggesting that there is an inverse relationship between the packing densities of CoA and microvessels.

![Figure 5.12](image)

**Figure 5.12 Microvasculature and CoA density in subpial regions.**

Adjacent brain sections showing the inverse relationship between microvasculature (A & C; double-immunolabeled with Collagen IV and GluT-1) and CoA (B & D) in the pial surface regions of the alveus (A & B) and prosubiculum (C & D). There are very few microvessels within 100 µm of the pial border (avascular zone, red enclosed zone), whereas CoA reach their peak abundance in this zone (white enclosed zone). An example of an individual microvessel is indicated by the red arrow in A, and an individual CoA is indicated by the white arrow in B.
5.3.4 CoA progression in relation to natural ageing

The association of patient age with CoA stages and ranked sequence were investigated. The mean age of patients did not differ systematically as a function of CoA stage (Table 5.6). Moreover, there was no significant correlation between age and ranked CoA sequence ($r^2 = 0.080$, $p = 0.130$; Figure 5.13).

Table 5.6 Mean age of patients for each classified CoA stage.

<table>
<thead>
<tr>
<th>Stage 0</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Mean age</td>
<td>74.1</td>
<td>64.8 ± 8.0</td>
<td>68.0 ± 4.9</td>
<td>64.0 ± 2.8</td>
</tr>
</tbody>
</table>

Figure 5.13 Scatterplot showing that postulated CoA sequence is not significantly correlated with patient age.
The seven pial surface regions were examined to determine whether correlations exist between patient age and CoA number, however no significant correlations were found between age and CoA count or between age and CoA area fraction (Table 5.7). However, the sizes of CoA were found to increase with natural ageing (highlighted with coloured shading). Scatterplots of the positive linear regressions of age with CoA mean, minimum and maximum diameters are shown in Figure 5.14. Patient age was significantly correlated with larger CoA mean and maximum diameters in the fimbria pial surface ($r^2 = 0.384$, $p < 0.001$; $r^2 = 0.261$, $p = 0.005$; Figure 5.14 A & B), prosubiculum pial surface ($r^2 = 0.265$, $p = 0.004$; $r^2 = 0.196$, $p = 0.016$; Figure 5.14 C & D), subiculum pial surface ($r^2 = 0.535$, $p < 0.001$; $r^2 = 0.413$, $p < 0.001$; Figure 5.14 E & F) and medial entorhinal cortex pial surface region ($r^2 = 0.304$, $p = 0.006$; Figure 5.14 G); moreover, a significant positive linear correlation was found with CoA minimum diameter in the subiculum pial surface region ($r^2 = 0.210$, $p = 0.028$; Figure 5.14 H).
## Table 5.7 Pearson’s correlation of patient age with tested CoA parameters.

<table>
<thead>
<tr>
<th></th>
<th>APS</th>
<th>FPS</th>
<th>PPS</th>
<th>SPS</th>
<th>MPS</th>
<th>LPS</th>
<th>CPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoA count</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( r )</td>
<td>.115</td>
<td>-.216</td>
<td>-.018</td>
<td>-.105</td>
<td>.046</td>
<td>-.179</td>
<td>-.130</td>
</tr>
<tr>
<td>( p )</td>
<td>.547</td>
<td>.252</td>
<td>.926</td>
<td>.627</td>
<td>.832</td>
<td>.402</td>
<td>.545</td>
</tr>
<tr>
<td>( n )</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
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<tr>
<td>Area fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( r )</td>
<td>.115</td>
<td>.035</td>
<td>.112</td>
<td>.167</td>
<td>.202</td>
<td>-.163</td>
<td>.100</td>
</tr>
<tr>
<td>( p )</td>
<td>.546</td>
<td>.854</td>
<td>.557</td>
<td>.436</td>
<td>.344</td>
<td>.446</td>
<td>.640</td>
</tr>
<tr>
<td>( n )</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Mean diameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( r )</td>
<td>.200</td>
<td>.619**</td>
<td>.515**</td>
<td>.732**</td>
<td>.552**</td>
<td>.087</td>
<td>.334</td>
</tr>
<tr>
<td>( p )</td>
<td>.373</td>
<td>.000</td>
<td>.004</td>
<td>.000</td>
<td>.006</td>
<td>.768</td>
<td>.120</td>
</tr>
<tr>
<td>( n )</td>
<td>22</td>
<td>29</td>
<td>29</td>
<td>23</td>
<td>23</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>Minimum diameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( r )</td>
<td>.102</td>
<td>.089</td>
<td>.172</td>
<td>.458*</td>
<td>.217</td>
<td>-.288</td>
<td>.371</td>
</tr>
<tr>
<td>( p )</td>
<td>.651</td>
<td>.648</td>
<td>.373</td>
<td>.028</td>
<td>.319</td>
<td>.317</td>
<td>.081</td>
</tr>
<tr>
<td>( n )</td>
<td>22</td>
<td>29</td>
<td>29</td>
<td>23</td>
<td>23</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>Maximum diameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( r )</td>
<td>.407</td>
<td>.511**</td>
<td>.443*</td>
<td>.643**</td>
<td>.219</td>
<td>.516</td>
<td>.288</td>
</tr>
<tr>
<td>( p )</td>
<td>.060</td>
<td>.005</td>
<td>.016</td>
<td>.001</td>
<td>.316</td>
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</tr>
<tr>
<td>( n )</td>
<td>22</td>
<td>29</td>
<td>29</td>
<td>23</td>
<td>23</td>
<td>14</td>
<td>23</td>
</tr>
</tbody>
</table>

Figure 5.14 Correlations between patient age and CoA size.

Scatterplots showing significant positive linear correlations between age and CoA mean diameter (A, C, E & G), maximum diameter (B, D & F) and minimum diameter (H) in the fimbria pial surface (A & B), prosubiculum pial surface (C & D), subiculum pial surface (E, F & H) and medial entorhinal cortex pial surface region (G).
5.3.5 Summary

The major findings of the present study are as follows:

i) CoA are primarily located in the periventricular and subpial regions of the hippocampus. CoA are abundant in the wall of the lateral ventricle, alveus, fimbria, and in parts of the CA4 that abut the lateral ventricle. In rare cases CoA can be found in the CA3 and CA1, but they are not observed in the CA2 or subiculum.

ii) CoA aggregate beneath the pial border in a clearly-defined subpial zone (40 – 100 µm thick), where the CoA density is significantly greater than in the deeper tissue.

iii) It was inferred that there is a progressive spread of CoA from the fimbria region through the subpial zone towards more peripheral regions, eventually reaching the collateral sulcus.

iv) CoA do not preferentially aggregate within the perivascular spaces around arterioles or venules, and they are most abundant in the avascular subpial zone.

v) Natural ageing does not account for changes in the spatial distribution of CoA or changes in their numbers, however ageing strongly accounts for differences in the diameters of CoA, with increasing age being associated with larger CoA.
5.4 Discussion

This prospective study investigated the distribution of CoA in the hippocampus of OSA patients, and on the basis of these observations has proposed a spatiotemporal sequence to describe the spread of CoA. Importantly this sequence does not correlate with patient age, nor do the numbers of CoA in the subregions investigated, suggesting that factors other than age are more influential mediators of CoA deposition. On the other hand, patient age correlated strongly with the mean and maximum diameters of CoA, supporting the view that CoA increase in size with age. The majority (97%) of CoA in the hippocampi examined were distributed in periventricular and subpial regions, which is consistent with previous reports. Fewer CoA were observed in the CA1, CA2, CA3 and subiculum than in previous studies. In all regions where CoA were present, they were concentrated within an avascular zone that extended 40 – 100 µm below the pial surface. The implications of these interesting findings are discussed in the following sections.

CoA were detected in 97% (29 out of the 30) of the hippocampal specimens examined in the present study, which is a higher prevalence than reported by other studies: 63% (26 out of 41) of hippocampal sclerosis specimens (Van Paesschen, Revesz et al. 1997) and 57% (33 out of 58) of patients with mesial temporal lobe epilepsy (Cherian, Radhakrishnan et al. 2003). Immature CoA were found in the CA1 field of the hippocampus in 52% (24 out of 46) of patients with mesial temporal lobe epilepsy or hippocampal sclerosis (Das, Balan et al. 2011). The higher incidence of CoA observed in the present study may be due to methodological considerations, such as the greater sensitivity of autofluorescence for detecting CoA, our use of thicker brain sections, and our investigation of more extensive areas of tissue. Alternatively, the higher incidence of CoA in hippocampi from OSA patients may indicate that OSA favours the deposition of CoA. This latter possibility will be explored in Chapter 6.
In the present study, the mean diameter of CoA was found to be 9.1 ± 0.1 µm, and ranged from 6.1 – 15.3 µm; while the CoA minimum diameter varied from 3.3 – 11.5 µm, and the CoA maximum diameter ranged between 7.7 – 25.3 µm. These results are consistent with previous reports that the mean diameters of CoA in the human central nervous system range from approximately 4-12 µm and that the diameters rarely exceed 20 µm (Mizutani, Satoh et al. 1987, Kubota, Holbach et al. 1993, Cavanagh 1998). In a recent confocal microscopy ultrastructural study, immature intracellular CoA were reported to be smaller (1.87 – 11.36 µm), while mature extracellular CoA measured from 4.49 – 18.41 µm (Augé, Bechmann et al. 2019). It can therefore be concluded that the majority of CoA investigated in the present study were mature CoA. This is pertinent, since immature CoA may be disposed of and cleared by the perivascular system (glymphatic system), whereas this does not appear to be the case for mature CoA (Navarro, Genoud et al. 2018).

The present study found that CoA generally occur in proximity to the lateral ventricle and subarachnoid space. CoA were common in the walls of the lateral ventricle, fimbria and the angle of the CA4 that is closest to the lateral ventricle. This pattern, together with the CoA distribution in seven subpial regions, is consistent with reports that CoA in AD are concentrated in periventricular and subpial regions of the hippocampus (Augé, Duran et al. 2018). The present study also detected CoA in the pial surface of the medial and lateral entorhinal cortex, which is consistent with a report of CoA in the entorhinal cortex of two AD patients (Pisa, Alonso et al. 2018). In contrast, a case report observed CoA in the mediotemporal lobe and left parahippocampal white matter of a 49-year-old woman with a long history of headaches (Abel, Hebb et al. 2010), but no CoA were detected in cortical gray matter, cornu ammonis (CA) subfields, or the dentate gyrus (Abel, Hebb et al. 2010).
The present study detected CoA in the CA1 and CA3 regions of just 10 – 13% of subjects, and none were observed in the CA2 or subiculum regions. Thus, apart from the CA4 and fimbria, most of the hippocampus lacked CoA. This distribution differs substantially from the distribution of CoA in CA-subregions of the hippocampus reported by other studies. For example, in three aged healthy subjects and three aged Parkinson’s disease sufferers, CoA were observed in the CA2, as well as in the CA4 of the hippocampus (Navarro, Genoud et al. 2018). In another study, CoA were found in the fimbria, alveus, CA3, CA2, CA1 and subiculum of a 39-year-old woman with complex partial seizures (Nishio, Morioka et al. 2001). CoA were also observed in the CA4, CA3, CA2, CA1, subiculum and parahippocampal white matter of patients with complex partial seizures and Ammon's horn sclerosis (Erdamar, Zhu et al. 2000). Therefore, it is likely that the pattern of CoA deposition is linked to specific neurodegenerative conditions. This possibility will be explored in greater detail in Chapter 6.

One of the dominant hypotheses concerning the nature of CoA is that they are waste containers that are trafficked from the brain into the blood (Augé, Cabezón et al. 2017). Three lines of evidence from the present study do not support this idea. First, CoA were not commonly observed in proximity to the perivascular space around blood vessels. Only a small number of hippocampal specimens in the present study exhibited CoA clustered around the walls of any blood vessels. These sporadic vessels had large diameters and were located in the deep white matter. Second, in the rare instances where CoA were observed in the perivascular space, they were observed equally often around arterioles and venules. Since only venules, with their thinner walls and lower blood pressure convey waste from the brain, the lack of a preferential distribution around venules argues against the waste disposal hypothesis. Third, CoA are most prevalent in the subpial zone, within 100 µm or so beneath the pial surface. It is notable that adjacent brain sections that had been immunostained to
show the microvessels demonstrated a lack of microvessels in this subpial region. Taken together, these three sets of observations make it unlikely that CoA are cleared into the bloodstream, unless the clearance rate is highly efficient and results in the rapid removal of CoA from the perivascular space.

Our finding that most CoA are located within 100 µm of the lateral ventricle or subarachnoid space (adjacent to the pia) is intriguing, as in living brains both of these spaces are filled with CSF. This correspondence raises the possibility that CoA may interact with the CSF, or as several authors have speculated, CoA may originate from exudates of the CSF (Tokutake, Nagase et al. 1995, Selmaj, Pawlowska et al. 2008, Meng, Zhang et al. 2009, Maurizi 2010, Nam, Kim et al. 2012). In the context of this hypothesis, the fimbria region is unique, because it consists primarily of myelinated axons and it is bathed on three sides by the CSF. Perhaps this stronger association with the CSF is why the fimbria is the first site of CoA deposition in the hippocampus. The fact that the smallest CoA are found in the subpial layer rather than in the deeper neuropil supports the idea that newly matured CoA originate in the subpial region rather than in the deeper neuropil.

An unexpected finding to emerge from the present study was that the distribution of CoA in subpial regions of the hippocampus conformed to a pattern: when few CoA were present in a section they were invariably concentrated in and around the fimbria, and as the number of CoA increased, they systematically extended further away from the fimbria and were smaller in size. Thus the packing density and size of CoA peaked in the fimbria pial surface region, and adjacent regions displayed a progressive reduction in the density and mean diameter of CoA, specifically, alveus on one side and prosubiculum, subiculum, medial entorhinal cortex, lateral entorhinal cortex and collateral sulcus on the other side. Moreover, the CoA maximum diameter in the fimbria pial surface region was consistently greater than in the adjacent
regions, and the smallest mean and maximum CoA diameters were found in the collateral sulcus region. Since CoA size is positively correlated with age, the presence of larger CoA in the fimbria supports the idea that the fimbria region is the first site of CoA accumulation. The smaller CoA in the collateral sulcus region are consistent with the delay in CoA deposition, as the CoA gradually spread from the fimbria region along the pial border to the collateral sulcus region. If the most recently generated CoA have the smallest diameters, then the presence of the smallest minimum diameters in the fimbria region suggest that this region is the most active site of CoA generation. We speculate that the positive correlation between patient age and CoA diameter indicates that once formed, CoA remain for a long time, perhaps for several decades. During this time they gradually grow in size by fusion or by absorbing additional materials.

Despite the high concentration of CoA in tissue that is close to the CSF, we consider that it is unlikely that CoA originate from the CSF. If CoA originated from the CSF, all subpial regions might be expected to show an equal burden of CoA, whereas this is clearly not the case. The spatiotemporal sequence of CoA spread proposed in the present study implies that either CoA migrate laterally away from the fimbria, or (more likely) that the process that leads to CoA formation propagates from an affected region into adjacent tissue. This postulated spatiotemporal spread of CoA resembles the transmission of an infection, and it is notable that some authors have claimed that CoA result from microbial infection and contain DNA and proteins from bacteria and fungi (Sfanos, Wilson et al. 2009, Yanamandra, Alexeyev et al. 2009, Pisa, Alonso et al. 2015, Pisa, Alonso et al. 2016, Pisa, Alonso et al. 2016, Pisa, Alonso et al. 2018). Unfortunately it was not possible in the present study to assay our tissue for the presence of bacteria.
The postulated spatiotemporal sequence of CoA progression did not correlate with patient age, and therefore other factors must contribute to this apparent and consistent progression. Moreover, advancing age was not associated with increased CoA density (count or area fraction) in any of the regions investigated. Reports of a positive association between CoA count and patient age are limited to a single study of the retina and optic nerve (Kubota, Holbach et al. 1993), while another study was unable to find a significant correlation (Cavanagh 1998). Nonetheless, it is generally believed that there are more CoA with ageing, with a major increase after 40 years of age (Mizutani, Satoh et al. 1987, Cavanagh 1999). Since all of the brains in the present study were older than 40 years of age, our results indicate that once this age is reached, the relationship between CoA burden and patient age is weak.

In conclusion, the present study has extended our understanding of CoA in the human hippocampus. While our data are consistent with the view that the size of CoA increases with age, CoA burden has been shown not to increase with age. Furthermore, the present data strongly argue against the prevailing notion that CoA are waste containers that are disposed of via the vasculature. The present data indicate that CoA originate from the fimbria and then systematically spread through the subpial region to adjacent regions and then subsequently spread to deeper parts of the neuropil. The basis of this spread is unknown, but it is not age-related and may be related to pathological changes in the tissue. Since neurodegeneration and oxidative stress are widely regarded as causes of CoA formation, these possibilities will be explored in the following chapter.
Chapter 6 Factors that may contribute to the formation of corpora amylacea in the hippocampus of obstructive sleep apnoea patients

6.0 Abstract

A widely-subscribed view in the literature is that oxidative stress is the primary agent that causes corpora amylacea (CoA) to form. Since obstructive sleep apnoea (OSA) is widely considered to cause oxidative stress in the hippocampus, with the extent of oxidative stress increasing with OSA severity, this chapter has examined the relationship between CoA progression, numbers and size as a function of OSA severity. As treatment with continuous positive airway pressure (CPAP) is expected to reduce the severity of oxidative stress, this chapter has examined whether regular CPAP use is associated with a reduction in the CoA burden. A competing hypothesis posits that CoA are formed as a consequence of neurodegeneration; since a companion thesis (by Jessica Owen) has examined the distribution of amyloid beta (Aβ), neurofibrillary tangles (NFT), and grey and white matter loss in the hippocampus of this same hippocampal series, the present study examines the extent to which CoA burden is correlated with these markers of neurodegeneration. The results show that increased OSA severity is correlated with CoA progression along the pial surface, and that moderate-severe OSA patients have a higher CoA burden in the pial surface of the fimbria region. In contrast, CPAP use, the burden of Aβ plaques and NFTs, neuropil loss and demyelination had no appreciable effect on the distribution or numbers of CoA. Taken together, these findings imply that either OSA does not cause oxidative stress or (more likely) that oxidative stress does not influence CoA formation in the hippocampus of persons with OSA. Furthermore, the lack of correlation with markers of neuropathology strongly argues against neurodegeneration being a significant driver of CoA formation. These results provide a conceptual advance in the field, as they help to exclude influential hypotheses about CoA formation that until now have been untested.
6.1 Introduction

Obstructive sleep apnoea is a sleep disorder characterised by repeated cessations in ventilation during sleep, caused by a collapse of the muscle tone in the upper airway (Dempsey, Veasey et al. 2010). These cessations of airflow are often accompanied by drops in the blood oxygen saturation level (SaO₂), leading to chronic intermittent hypoxia (CIH) (Yadollahi, Giannouli et al. 2010). The repeated phases of tissue hypoxia followed by reoxygenation cause an overproduction of reactive oxygen species (ROS), which can result in oxidative stress if ROS production exceeds the capacity of the cellular antioxidant systems to inactivate the ROS (Eltzschig and Eckle 2011). Cellular damage caused by oxidative stress activates the inflammatory response; consistent with this, increased levels of markers of oxidative stress and inflammation have been reported in the blood of OSA patients compared to controls (Alberti, Sarchielli et al. 2003, Carpagnano, Kharitonov et al. 2003, Yamauchi, Nakano et al. 2005, De la Peña Bravo, Serpero et al. 2007, Nadeem, Molnar et al. 2013). In addition to being implicated in OSA, oxidative stress has been linked to the pathogenesis of ageing and Alzheimer’s disease (AD) (Mecocci, MacGarvey et al. 1994, Markesbery 1997).

The hippocampus plays an essential role in learning and memory (Bartsch and Wulff 2015), and is vulnerable to pathologic insults including ischemia, oxidative stress, neurodegeneration and ageing (Kirino 2000, Bartsch, Döhring et al. 2015); moreover, in AD, the hippocampus is among the first and most extensively damaged regions of the brain (Robinson 2001, Mufson, Mahady et al. 2015). Both normal ageing and AD are associated with elevations in oxidative stress and cognitive decline (Liguori, Russo et al. 2018), so it is possible that the memory loss and cognitive impairments experienced by persons with OSA (Beebe and Gozal 2002, Ferini-Strambi, Baietto et al. 2003) are associated with hypoxic injury to the hippocampus. Indirect support for this idea comes from reports that hippocampal
structural deficits and cognitive impairments in OSA patients improve after CPAP treatment (Canessa, Castronovo et al. 2011, Ferini-Strambi, Marelli et al. 2013, Rosenzweig, Glasser et al. 2016).

Memory loss and cognitive impairments correlate with reduced hippocampal volume in OSA patients (Macey, Henderson et al. 2002, Gale and Hopkins 2004, Canessa, Castronovo et al. 2011, Torelli, Moscufo et al. 2011, Wang and Wang 2019). Interestingly, hippocampal degeneration (loss of grey matter volume and reduced white matter intensity) and memory impairments are also common among AD patients (Salat, Tuch et al. 2010, Arlt, Buchert et al. 2013, Peng, Feng et al. 2015, Yi, Möller et al. 2016). These two observations may be linked, as OSA patients have increased risk of developing AD or dementia (Young, Peppard et al. 2002, Gehrman, Martin et al. 2003, Rose, Beck et al. 2011, Yaffe, Laffan et al. 2011, Chang, Liu et al. 2013). Furthermore, a researcher (Jessica Owen) in our laboratory demonstrated cortical thinning and demyelination in response to increased OSA severity (ODI: oxygen desaturation index) in the same set of brain samples as used in the present study (Owen, Benediktsdottir et al. 2019). Moreover, Owen demonstrated that two neuropathological hallmarks of AD are present in in the hippocampus of OSA patients, with the burden of extracellular amyloid beta (Aβ) plaques and intraneuronal neurofibrillary tangles (NFTs) being significantly correlated with increased OSA severity (Owen 2017).

Corpora amylacea (CoA) are spheroidal bodies consisting of glycoproteins that are found in the brains of humans and other mammals (Cavanagh 1999). The origin and function of CoA continue to be debated, with various hypotheses being proposed but not yet tested (Wilhelmus, Verhaar et al. 2011, Pisa, Alonso et al. 2016, Augé, Bechmann et al. 2019). The presence of CoA has been linked to advanced ageing and increased neurodegeneration (Cavanagh 1999, Selmaj, Pawlowska et al. 2008, Rohn 2015, Navarro, Genoud et al. 2018).
Furthermore, some CoA contain blood proteins that are normally found outside of the central nervous system, suggesting that leakage of the blood-brain barrier (BBB) is associated with the formation of CoA (Meng, Zhang et al. 2009, Navarro, Genoud et al. 2018). In this context it is noteworthy that chronic intermittent hypoxia and oxidative stress are known to impair the function of the BBB (Lim and Pack 2014, Pan and Kastin 2014). Recently, CoA have been suggested to act as ‘waste containers’ that assist in clearing or sequestering damaged cellular materials and waste products (oxidised lipids and proteins) within a matrix of polymerized glucose (Augé, Cabezón et al. 2017, Augé, Duran et al. 2018, Navarro, Genoud et al. 2018, Augé, Bechmann et al. 2019). It has been proposed that CoA are drained into Virchow-Robin spaces and then eliminated from the brain via the glymphatic system (Cavanagh 1999, Navarro, Genoud et al. 2018). Furthermore, microbial infections have been suggested to contribute to the accumulation of CoA, because some CoA have been found to contain proteins of microbial origin (Pisa, Alonso et al. 2016, Augé, Cabezón et al. 2017, Augé, Pelegrí et al. 2018, Pisa, Alonso et al. 2018).

In addition to the preceding hypotheses, the presence of CoA has been linked to cellular stress, particularly chronic hypoxia and oxidative stress (Keller 2006, Abel, Hebb et al. 2010, Pisa, Alonso et al. 2016). A recent study investigated the ultrastructure of CoA in the post-mortem hippocampus of Parkinson’s disease sufferers and aged controls, and reported that they contained lipid membrane fragments similar to the plasma membranes of cells and the membranes of organelles (e.g. mitochondria and vesicles) (Navarro, Genoud et al. 2018). The brain contains a large amount of polyunsaturated fatty acids that are highly susceptible to oxidative stress and lipid peroxidation (Chiang 2006). Since oxidative stress and lipid peroxidation are elevated in OSA patients (Barcelo, Miralles et al. 2000, Lavie, Vishnevsky et al. 2004, Gozal and Kheirandish-Gozal 2008, Lavie 2015), it is anticipated that increased numbers of CoA will be found in the brains of patients with severe OSA. CPAP treatment has
been reported to reduce systemic oxidative stress (Christou, Kostikas et al. 2009, Del Ben, Fabiani et al. 2012) and to improve the antioxidant capacity in OSA patients (Barcelo, Barbe et al. 2006); therefore, it might be expected there would be a lower CoA burden in regular CPAP users.

Perhaps the reason why there are so many competing hypotheses about the origins of CoA is that so little is known about them, and quantitative data concerning the distribution of CoA are virtually non-existent. This has made it difficult to ascertain whether the hypotheses are able to accurately predict the distribution of CoA. In order to address this issue, the present study utilises the quantitative data on CoA detailed in Chapter 5, and combines this with data previously obtained by Jessica Owen on indices of neurodegeneration (Aβ, NFT, neuropil loss and demyelination) in the same set of autopsy brain samples. This comparison has enabled an appraisal of the various hypotheses that seek to account for the formation of CoA.
6.2 Methods

The present study used the data collected from Chapter 5, so the study sample, tissue processing, immunohistochemistry protocol and image analysis process were the same as previously described (sections 5.2.1 – 5.2.4).

Five parameters of CoA were investigated: CoA count, the total number of CoA per field of view; CoA area fraction (%), the proportion of a field of view occupied by CoA; CoA mean diameter (µm), the average diameter of all CoA in a field of view; CoA min. diameter (µm), the minimum diameter of all CoA per field of view; and CoA max. diameter (µm), the maximum diameter of all CoA per field of view. The representative immunofluorescence micrographs and the corresponding processed images were shown in Figure 5.5.

The data relating to Aβ phase, NFT stage, neuropil loss and demyelination were obtained from the PhD thesis conducted on the same set of brain samples by Dr. Jessica Owen (Owen 2017).

6.2.1 Statistical analysis

Statistical analysis was performed with the IBM Statistical Package for the Social Sciences (SPSS version 21). Two-tailed student’s t-tests were used to compare the low OSA to high OSA groups, non-CPAP users to regular CPAP users; ANCOVA was performed to compare the group differences after controlling for age as a covariate factor. Pearson’s correlations were used to compare two continuous-level variables. GraphPad Prism 7 was used to generate graphs, presented as Mean ± SEM. Significant differences ($p < 0.05$) were indicated by an asterisk (*) with equal variances or a hash (#) with unequal variances.
6.3 Results

6.3.1 The effect of OSA severity on CoA burden

Hippocampal diagrams of CoA distribution were classified according to the definitions provided in section 5.3.1. There was one sample where no CoA were observable in the tissue, and this was classified as stage 0. Two samples were classified in stage 1 and were both from the low OSA group. Of the 8 samples classified as stage 2, 4 were from the low OSA group, while 4 were from the high OSA group. Stage 3 included 14 samples, 8 from the low OSA group and 6 from the high OSA group. Only 1 sample from the low OSA group was classified as stage 4, while 4 from the high OSA group were stage 4 (Figure 6.1 A). Overall, there was no significant difference between the low OSA and high OSA groups with respect to mean stage (Figure 6.1 B).

![Figure 6.1 CoA distribution and development of stages of 30 brain samples.](image)

Frequency of CoA stages in the low (ODI < 20 events/h sleep; blank bars) and high OSA (ODI ≥ 20 events/h sleep; striped bars) groups (A). Mean CoA stage in the low and high OSA groups (B). Mean ± SEM.
The association of OSA severity with ranked CoA sequence (1 – 30) was investigated; these variables were positively correlated in a log linear manner ($r^2 = 0.137, p = 0.044$), as shown in Figure 6.2 A. In contrast, CoA sequence was not significantly correlated with patient age ($r^2 = 0.080, p = 0.130$; Figure 6.2 B), BMI ($r^2 = 0.076, p = 0.173$; Figure 6.2 C), or time lived after OSA diagnosis (years) ($r^2 = 0.003, p = 0.768$; Figure 6.2 D).

Figure 6.2 Postulated CoA sequence is significantly correlated with OSA severity.

Scatterplots showing that postulated CoA sequence is significantly correlated with OSA severity (A), but not significantly correlated with age (B), BMI (C) or time lived after OSA diagnosis (D).
The relationship between OSA severity and several CoA parameters was examined in the seven pial surface regions described in section 5.3.1. The CoA count was higher (1.71-fold) in the high OSA group compared to the low OSA group in the pial surface region of the fimbria, but this difference was not statistically significant (199.6 ± 39.7 vs. 116.9 ± 18.5, \( t(19.8) = 1.889, p = 0.074 \), unequal variance; Figure 6.3 A). However, the CoA area fraction in the high OSA group was significantly larger (1.72-fold) in the fimbria pial surface region (6.28 ± 1.14 vs. 3.65 ± 0.49, \( t(19.0) = 2.110, p = 0.048 \), unequal variances; Figure 6.3 B).

The mean diameters of CoA between the low and high OSA groups were similar in six of the pial surface regions, with the exception of the lateral entorhinal cortex pial surface region, where the mean diameter of CoA was significantly smaller (0.87-fold) in the high OSA group (8.4 ± 0.4 vs. 9.7 ± 0.3, \( t(12) = -2.305, p = 0.040 \); Figure 6.3 C). Moreover, the minimum diameters of CoA in the high OSA group were also smaller (0.74 – 0.88-fold) in the fimbria pial surface region (4.3 ± 0.1 vs. 4.9 ± 0.2, \( t(27) = -2.715, p = 0.011 \); Figure 6.3 D) and lateral entorhinal cortex pial surface region (5.5 ± 0.4 vs. 7.4 ± 0.5, \( t(12) = -2.718, p = 0.019 \); Figure 6.3 D). The maximum diameter of CoA did not differ between the two groups in any pial surface region (Figure 6.3 E).
Figure 6.3 The effect of OSA severity on CoA burden.

CoA parameters in the low (ODI < 20 events/h sleep; blank bars) and high OSA (ODI ≥ 20 events/h sleep; striped bars) groups in seven pial surface regions of CoA count (A), CoA area fraction (B), CoA mean diameter (C), CoA minimum diameter (D) and CoA maximum diameter (E). Unpaired 2-tailed t-tests between low vs. high OSA groups. Mean ± SEM. APS: alveus pial surface, FPS: fimbria pial surface, PPS: prosubiculum pial surface, SPS: subiculum pial surface, MPS: medial entorhinal cortex pial surface, LPS: lateral entorhinal cortex pial surface and CPS: collateral sulcus pial surface.
Based on the results presented in section 5.3.4, it is clear that patient age strongly influences the size of CoA. Therefore a one-way ANCOVA was performed between the low and high OSA groups to remove the covariate effect of age, as shown in Table 6.1. After controlling for the age effect, the comparison of CoA count in the fimbria pial surface region between the high OSA and low OSA group reached significance \((p = 0.042)\), improving the original \(p\) value of 0.074 from the \(t\)-test. No further differences were observed between the \(t\)-tests and ANCOVA results in the fimbria pial surface or lateral entorhinal cortex pial surface regions. Moreover, patient age had no significant effect on CoA count, area fraction or minimum diameter, whereas age significantly contributed to the mean and maximum diameters, which is consistent with the results in Chapter 5.

**Table 6.1 Comparison between the low OSA and high OSA groups, showing the \(t\)-tests \(p\) values before adjustment and the ANCOVA \(p\) values after adjusting for patient age.**

<table>
<thead>
<tr>
<th>Region</th>
<th>CoA parameters</th>
<th>Before adjustment</th>
<th>Adjusted (p) value</th>
<th>Age effect Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPS</td>
<td>CoA count</td>
<td>0.074</td>
<td>0.042*</td>
<td>0.135</td>
</tr>
<tr>
<td></td>
<td>CoA area fraction</td>
<td>0.048*</td>
<td>0.049*</td>
<td>0.914</td>
</tr>
<tr>
<td></td>
<td>CoA mean diameter</td>
<td>0.748</td>
<td>0.911</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td></td>
<td>CoA min. diameter</td>
<td>0.011*</td>
<td>0.010*</td>
<td>0.391</td>
</tr>
<tr>
<td></td>
<td>CoA max. diameter</td>
<td>0.509</td>
<td>0.713</td>
<td>0.006**</td>
</tr>
<tr>
<td>FPS</td>
<td>CoA count</td>
<td>0.217</td>
<td>0.190</td>
<td>0.333</td>
</tr>
<tr>
<td></td>
<td>CoA area fraction</td>
<td>0.244</td>
<td>0.218</td>
<td>0.379</td>
</tr>
<tr>
<td>LPS</td>
<td>CoA mean diameter</td>
<td>0.040*</td>
<td>0.036*</td>
<td>0.433</td>
</tr>
<tr>
<td></td>
<td>CoA min. diameter</td>
<td>0.019*</td>
<td>0.031*</td>
<td>0.474</td>
</tr>
<tr>
<td></td>
<td>CoA max. diameter</td>
<td>0.419</td>
<td>0.602</td>
<td>0.087</td>
</tr>
</tbody>
</table>

FPS: fimbria pial surface region. LPS: lateral entorhinal cortex pial surface. The significance of the effect of patient age is shown in the last column.
6.3.2 The effect of regular CPAP use on CoA burden

In order to investigate the effect of regular CPAP treatment on CoA parameters, comparisons were performed between non-CPAP users and regular CPAP users within the low and high OSA groups. With one exception, CPAP use was found to have no significant effect on CoA count, CoA area fraction, CoA mean diameter, CoA minimum diameter and CoA maximum diameter in any of the seven pial surface regions. There was no significant difference between regular CPAP users and non-CPAP users, except for the significantly larger CoA mean diameter (1.12-fold) in regular CPAP users in the low OSA group at the fimbria pial surface (9.4 ± 0.2 vs. 8.4 ± 0.3, t (13) = 2.591, p = 0.022; Figure 6.4 C). However, after controlling for the age effect with ANCOVA, the comparison failed to reach significance (p = 0.209).
Figure 6.4 The effect of regular CPAP use on CoA burden.

CoA parameters in non-CPAP users (blank bars) and CPAP users (striped bars) in the low (ODI < 20 events/h sleep; purple colour) and high (ODI ≥ 20 events/h sleep; pink colour) OSA groups of the fimbria region (A, C, E & G) and the fimbria pial surface region (B, D, F & H) of CoA count (A & B), CoA area fraction (C & D), CoA mean diameter (E & F), CoA minimum diameter (G & H) and CoA maximum diameter (I & J). Unpaired 2-tailed t-tests between non-CPAP users vs. CPAP users. Mean ± SEM.
6.3.3 The relationship between the burdens of Aβ, NFT and CoA

The classified CoA stages were also analyzed by comparing them with their amyloid β phase (0 – 4) and neurofibrillary tangle (NFT) stage (0 – 4), as previously determined in the same set of human brain samples (Owen 2017). If the classified CoA stages are linearly correlated with increasing AD pathology, then based on the number of cases of each CoA stage, the expected relationship with Aβ phase / NFT stage is illustrated in Figure 6.5 A. In this scenario there is 1 case of CoA stage 0, 2 cases of CoA stage 1, 8 cases of CoA stage 2, 14 cases of CoA stage 3, and 5 cases of stage 4 which are distributed within 5 AD pathology stages (0 – 4).

When CoA stage is plotted against actual Aβ phase / NFT stage, the pattern does not match the expected linear relationship. As shown in Figure 6.5 B, the 17 cases in Aβ phase 0 corresponded to CoA stages 1 – 4, with most being at CoA stage 3. Moreover, the advanced Aβ phases 2-4 did not show a tendency to be associated with increasingly higher CoA stages. In contrast, the 8 cases in NFT stage 0 corresponded to CoA stages 1-3, with stage 2 being most common. For NFT stage 1, the majority of cases corresponded to CoA stage 3, and for NFT stage 2 an even higher number of cases corresponded to CoA stage 3. However, this weak linear trend did not continue for NFT stages 3-5 (Figure 6.5 C).

Figure 6.6 illustrates the pattern of spatiotemporal spread of Aβ plaques and NFTs in brain sections from the same autopsy series that were examined in the present study (Owen 2017). It is notable that Aβ and NFT first appear in the vicinity of the collateral sulcus and then progressively spread across the entorhinal cortex, subiculum and CA1 towards the CA4 region. This pattern is almost the exact opposite of the spatiotemporal spread of CoA, which begins in the fimbria and then progressively spreads towards the prosubiculum, subiculum, entorhinal cortex and finally reaches the collateral sulcus (see Figure 5.8).
Figure 6.5 The effect of AD pathology on CoA burden.

A, the expected co-distribution between CoA stage and AD pathology stage, if the severities of the two markers were linearly related. B, the actual co-distribution of classified CoA stage with Aβ phase. C, the actual co-distribution of classified CoA stage with NFT stage in the hippocampus of OSA patients.
Figure 6.6 Illustration of amyloid phases and NFT stages.

In the top four panels, the black circles indicate the presence of Aβ plaques, with larger circles indicating larger plaques. In the bottom four panels, black triangles indicate the presence of NFTs. Note that these diagrams are illustrative of the general trends and do not represent the locations of specific Aβ plaques or NFTs (reprinted from (Owen 2017)).
The CoA density (area fraction %) in seven periventricular and perivascular regions were compared as a function of each Aβ phase (0 – 4) and NFT stage (0 – 4), as shown in Figures 6.7 and 6.8. The mean CoA density in Aβ phase 0 across all ten periventricular and subpial regions was similar to those in phases 1 – 3, with an exception of the increased CoA density in Aβ phase 4 (Figure 6.7 E), which was based on a single sample. There was no significant differences in mean CoA density between the five NFT stages (0 – 4) across all ten regions investigated (Figure 6.8 A – E).
Figure 6.7 Mean CoA density in five beta amyloid phases.

Mean CoA density (area fraction %) in APS (alveus pial surface), FPS (fimbria pial surface), PPS (prosubiculum pial surface), SPS (subiculum pial surface), MPS (medial entorhinal cortex pial surface), LPS (lateral entorhinal cortex pial surface) and CPS (collateral sulcus pial surface) regions. Mean ± SEM.
Figure 6.8 Mean CoA density in five neurofibrillary tangle stages.

Mean CoA density (area fraction %) in APS (alveus pial surface), FPS (fimbria pial surface), PPS (prosubiculum pial surface), SPS (subiculum pial surface), MPS (medial entorhinal cortex pial surface), LPS (lateral entorhinal cortex pial surface) and CPS (collateral sulcus pial surface) regions. Mean ± SEM.
6.3.4 The effect of neuropil loss and demyelination on CoA burden

Hippocampal atrophy in the grey and white matter has been investigated in the same set of autopsy samples (Owen, Benediktsdottir et al. 2019). Therefore, the present study correlated CoA density with the extent of neuropil loss in the CA4 and lateral entorhinal cortex regions. Owen and colleagues (2019) reported significant negative correlations between OSA severity and CA4 length ($r^2 = 0.136, p = 0.038$; Figure 6.9 A) and layer 1 thickness of the lateral entorhinal cortex ($r^2 = 0.186, p = 0.028$; Figure 6.9 C), but not with the overall thickness of the lateral entorhinal cortex ($r^2 = 0.069, p = 0.196$; Figure 6.9 B); moreover, an analysis of unpublished data from (Owen 2017), showed that OSA severity was not correlated with the overall myelin levels in the lateral entorhinal cortex ($r^2 = 0.058, p = 0.245$; Figure 6.9 D). In the present study, the CoA density (count/mm$^2$) in the CA4 was not correlated with CA4 length (Figure 6.9 E), CoA density in the subpial zone of the lateral entorhinal cortex was not correlated with overall thickness of the lateral entorhinal cortex (Figure 6.9 F), while thickness of layer 1 of the lateral entorhinal cortex was weakly correlated (Figure 6.9 G). However, none of these associations reached significance. The only significant association found was between increased CoA density and increased myelin levels in the lateral entorhinal cortex region ($r^2 = 0.194, p = 0.031$; Figure 6.9 H).

In addition to CoA density, aspects of CoA size (mean diameter, minimum diameter and maximum diameter) also had no significant correlations with the neuropil loss/demyelination data (data not shown).
Figure 6.9 Correlations between CoA density and measures of neuropil loss or demyelination.

The association of OSA severity with neuropil loss in CA4 (A), overall lateral entorhinal cortex (B) and layer 1 of lateral entorhinal cortex (C); as well as with amount of myelin in lateral entorhinal cortex (D). The association of CoA density with length of CA4 (E), thickness of overall lateral entorhinal cortex (F), layer 1 thickness of lateral entorhinal cortex (G) and amount of myelin in lateral entorhinal cortex (H). The amount of myelin was expressed as the inverse grey scale intensity of tissue immunostained for myelin basic protein (MBP).
6.3.5 Summary

The major findings of the present chapter were:

i) Increased OSA severity is associated with a more extensive spatiotemporal spread of CoA along the pial surface.

ii) Increased OSA severity is associated with higher numbers and densities of CoA in the pial surface region of the fimbria.

iii) Increased OSA severity is associated with smaller CoA mean diameters in the pial surface region of the lateral entorhinal cortex, and with smaller minimum diameters in the pial surface regions of the fimbria and lateral entorhinal cortex.

iv) Regular CPAP treatment is not associated with any significant changes in CoA size or distribution.

v) The patterns of spatiotemporal spread of Aβ and NFT are not associated with the spatiotemporal spread of CoA, and the burdens of Aβ and NFT are not associated with the burden of CoA.

vi) The extent of neuropil loss and demyelination are not associated with the burden of CoA or their size.
6.4 Discussion

Many hypotheses have been proposed to account for the formation of CoA; however quantitative data available to support or refute these hypotheses are scarce. The research in the present chapter has investigated whether the speculated causes of CoA can account for the CoA burdens in OSA patients. It was found that ranked CoA sequence (1 − 30) positively correlates with increased OSA severity, suggesting that OSA contributes to the CoA progression along the pial surface. Furthermore, the CoA area fraction (density) in the fimbria of the high OSA group (ODI ≥ 20 events/h sleep) was 1.72-fold higher than that in the low OSA group (ODI < 20 events/h sleep). In addition, in the lateral entorhinal cortex, the high OSA group had smaller CoA mean and minimum diameters (0.74 − 0.88-fold).

In contrast to these effects, regular CPAP use had no significant influence on the distribution of CoA, suggesting that CPAP use cannot ameliorate the burden of CoA. Furthermore, the presence of CoA in hippocampi that lacked any AD pathologies, combined with the fact that the 5 Aβ / NFT stages did not differ with respect to CoA burden, suggest that no association exists between CoA and neurodegenerative changes. Moreover, there were no significant correlations between CoA density and neuropil loss / demyelination, suggesting that there is no association between CoA burden and neurodegeneration. The implications of these findings are discussed in the following pages.

As discussed in Chapter 5, the individual brain samples were ranked from 1 to 30 according to the extent of their CoA burden at the pial surface. Since the spatial sequence was found not to be significantly correlated with ageing, we speculated that additional factors must determine the CoA progression from the fimbria to the collateral sulcus. The present study investigated the effect of increased OSA severity on CoA progression, and found a significant positive correlation between ranked CoA sequence and ODI. This finding is consistent with
the possibility that OSA contributes to the distribution of CoA in the periventricular and subpial regions of the hippocampus. Interestingly, the mean and minimum diameters of CoA in the lateral entorhinal cortex decreased as OSA severity increased. This observation is consistent with the idea that newly-generated CoA are small, and they gradually increase in size over time. This interpretation is also consistent with the data presented in the previous chapter which showed that the mean size of CoA increases with age.

An influential hypothesis of CoA formation states that CoA are a marker of oxidative stress (Cissé, Lacoste-Royal et al. 1991, Schipper and Cissé 1995, Sahlas, Liberman et al. 2002). OSA is thought to be a potent source of oxidative stress, and this stress and the associated neuroinflammation are significantly attenuated after starting CPAP treatment (Carpagnano, Kharitonov et al. 2003, Alonso-Fernández, García-Río et al. 2009, Christou, Kostikas et al. 2009, Del Ben, Fabiani et al. 2012, Tichanon, Wilaiwan et al. 2016). While the present study used archived autopsy materials for which serum levels of oxidative stress markers were not available, we do have reliable data for regular CPAP use. It was expected that the regular CPAP users would experience less oxidative stress and have a correspondingly lower CoA burden than non-CPAP users with similar severities of OSA. However, the present results did not confirm this expectation, since no significant differences were found between regular CPAP users and non-CPAP users in terms of CoA count, area fraction or spatial extent. These results demonstrate that regular CPAP use is ineffective at reducing the burden of CoA, and withdraw support for oxidative stress as a primary driver of CoA formation in OSA patients.

As most patients in the present study had pre-existing moderate-severe OSA before beginning treatment with CPAP, it is possible that the full complement of CoA was deposited early in the course of OSA, and no new CoA were added, even in untreated cases of OSA. While this
possibility seems unlikely, it cannot be excluded by the present data. However, it is possible to conclude that CPAP use does not lead to a reduction in CoA burden. Thus, if CoA are only generated in early OSA, this result means that they are never cleared from the brain. Alternatively, if they continue to be generated, then CPAP use does not affect their rates of production or clearance.

If oxidative stress or neuroinflammation are not primary drivers of CoA deposition, another potential factor is sleep disruption. Sleep patterns typically get disrupted and fragmented in OSA patients (Malhotra and White 2002, Azagra-Calero, Espinar-Escalona et al. 2012, Kielb, Ancoli-Israel et al. 2012), which may compromise the capacity of the glymphatic system to clear immature CoA from the brain, resulting in an increased CoA density in the high OSA group. Such an argument has been advanced to explain the increased deposition of Aβ in OSA (Wu, Dunnett et al. 2019), and has been used to explain the proximity of CoA to blood vessels and within sites of glymphatic drainage (Navarro, Genoud et al. 2018).

Since the frequency of sleep interruptions and arousals increase with as OSA severity increases, we considered whether a compromised glymphatic clearance system could account for the higher CoA burden in severe OSA. CPAP treatment is reported to restore sleep patterns and reduce fragmented sleep (Sullivan, Berthon-Jones et al. 1981, McArdle and Douglas 2001, Gelir and Ardic 2010, Tachikawa, Minami et al. 2017); although the present study has incomplete records for compliance and duration of CPAP use, those designated as CPAP users were using CPAP until their death, so their sleep patterns should have been fairly normal, and this should have allowed their glymphatic system to function normally. In view of this, the lack of difference in CoA burden between non-CPAP users and CPAP users does not support the idea that CoA are cleared from the brain through the glymphatic system.
Since some CoA are reported to contain blood proteins (Meng, Zhang et al. 2009, Pisa, Alonso et al. 2016), a further possibility is that increased OSA severity compromises the BBB (Lim and Pack 2014), which then leads to an increased deposition of CoA. In this scenario CoA will form around those blood vessels that have a leaky BBB, and the burden of CoA would decrease with distance from these blood vessels. While such a relationship has been reported (Navarro, Genoud et al. 2018), this was based on an examination of a small number of selected vessels. The present study has examined far more brain tissue than Navarro and colleagues (2018), and has done so in a non-selective way; as noted in section 5.3.3, most CoA in the present study were located within the avascular zone beneath the pia, and only on very rare occasions were CoA clustered in the Robin-Virchow space surrounding a blood vessel. Furthermore, in a study conducted in our laboratory on this brain series in which immunohistochemistry was used to detect proteins of blood origin, very little evidence was found of BBB leakage, and the few positive cases were not associated with moderate-severe OSA (data not shown). Taken together, the present results do not support the hypothesis that a leaky BBB is the primary cause of CoA deposition in OSA.

In AD, Aβ plaques and NFTs have both been reported to begin in the vicinity of the hippocampus before spreading to other cortical regions (Braak and Braak 1991, Thal, Rüb et al. 2000, Braak, Alafuzoff et al. 2006). A study in our laboratory found that OSA severity was significantly correlated with the distribution stages of Aβ and NFTs in the hippocampus (Owen 2017). Previous studies have reported that AD is associated with an increased burden of CoA (Cavanagh 1999, Meng, Zhang et al. 2009, Abel, Hebb et al. 2010, Lee, Kim et al. 2017, Augé, Duran et al. 2018, Pisa, Alonso et al. 2018). For instance, Notter and Knuesel (2013) reported increased CoA deposition in the subiculum of AD patients compared to non-demented individuals (Notter and Knuesel 2013), while Song and colleagues (2014) reported significantly increased CoA numbers in various hippocampal regions of patients with mild
cognitive impairment, when compared to cognitively normal controls (Song, Zukor et al. 2014). Despite this apparent association between CoA and neuropathology in AD, the present study found no association between the burdens of Aβ or NFT and the burden of CoA in patients with OSA. Furthermore, the spatial distribution and spread of CoA across the subregions of the hippocampus progressed in the opposite direction to Aβ and NFT: CoA began in the fimbria and gradually spread laterally until they reached the collateral sulcus, whereas Aβ and NFT first appeared at the collateral sulcus and then gradually spread medially until they reached the CA4 and fimbria.

Two potential explanations of this discrepancy come to mind. First, since the OSA patients in the present study were known not to suffer from dementia, it follows that if AD causes CoA deposition, it may be caused by an aspect of the disease that is independent of Aβ or NFTs. A second explanation comes from the fact that OSA increases the risk of developing AD (Pan and Kastin 2014, Osorio, Gumb et al. 2015, Brzecka, Leszek et al. 2018, Sharma, Varga et al. 2018), and is commonly comorbid with AD (Cooke, Ayalon et al. 2009, Peter-Derex, Yammine et al. 2015, Emamian, Khazaie et al. 2016, Polsek, Gildeh et al. 2018). Therefore, the increased presence of CoA in AD may be due to the presence of comorbid OSA, rather than to AD itself.

It has been suggested that CoA act as ‘waste containers’ that assist in clearing or sequestering damaged cellular materials and waste products (oxidised lipids and proteins) within a matrix of polymerized glucose (Augé, Cabezón et al. 2017, Augé, Duran et al. 2018, Navarro, Genoud et al. 2018, Augé, Bechmann et al. 2019). A corollary of this hypothesis is that those areas of the hippocampus that experience the greatest levels of cell damage in OSA should have the highest burdens of CoA. In OSA, hippocampal neuropil loss and demyelination involve the length of CA4, thickness of the CA1, thickness of some layers of the entorhinal
cor cortex, and the myelin of the deep cortical white matter, and all of these losses are correlated with increases in OSA severity (Owen, Benediktsdottir et al. 2019). Therefore the present study investigated whether a correlation exists between CoA burden and thinning in the CA4 or CA1 or thinning of the lateral entorhinal cortex.

The CA1 region of the hippocampus is particularly sensitive to intermittent hypoxia and oxidative stress (Gozal, Gozal et al. 2002, Row, Liu et al. 2003, Snyder, Shell et al. 2017), and there is increased neuropil loss in the CA1 in response to increased OSA severity, as demonstrated by significant negative correlations between OSA severity and the thickness of most of the layers of the CA1 (Owen, Benediktsdottir et al. 2019). However, the present study found no association between the thickness of the CA1 and CoA burden; indeed, only 4 brains out of the 30 examined contained any CoA in the CA1 region. This absence of an association strengthens the earlier conclusion that oxidative stress is not a driver of CoA formation. Moreover, CoA density in the CA4 and subpial zone of the lateral entorhinal cortex were not significantly correlated with the length of the CA4, or the overall thickness of lateral entorhinal cortex, demonstrating that CoA burden is not associated with neuropil loss. Although CoA burden was significantly correlated with the amount of myelin in the lateral entorhinal cortex, this correlation was a positive one, so it is inconsistent with the hypothesis that CoA originate from fragments of oxidised myelin that result from demyelination (Singhrao, Neal et al. 1994, Singhrao, Morgan et al. 1995, Cavanagh 1999, Selma, Pawlowska et al. 2008). Taken together, our results are inconsistent with those hypotheses that posit that CoA are caused by neurodegeneration (Keller 2006, Wilhelms, Verhaar et al. 2011, Rohn 2015, Schipper and Song 2015, Augé, Cabezón et al. 2017).

One hypothesis of CoA formation that the present results do not rule out is the idea that CoA are formed by, or in response to, a bacterial infection of the brain. This hypothesis is
supported by evidence of bacterial and fungal proteins and neo-epitopes within CoA (Pisa, Alonso et al. 2016, Augé, Cabezón et al. 2017, Augé, Pelegrí et al. 2018, Pisa, Alonso et al. 2018). In the present study, the systematic spatiotemporal spread of CoA from the fimbria to adjacent pial surface regions may be consistent with the slow spread of an infection between adjacent regions at the pial surface. However, as the present study was unable to examine the tissue sections for the presence of bacterial markers, this will need to be a topic for a future study.

Several studies have noted that CoA contain high concentrations of metal ions. For instance Singhrao and colleagues (1993) reported that CoA in AD brains and in normal controls contain elevated levels of iron and copper, but not aluminium (Singhrao, Neal et al. 1993). However, a recent study of brain tissue from patients with multiple sclerosis has reported that extracellular deposits of aluminium, not normally found in brain, colocalise with some CoA (Mold, Chmielecka et al. 2018). Those authors postulate that as aluminium is neurotoxic, its concentration within some CoA may represent the debris of neurons that have died from neurotoxicity. This finding may be relevant to OSA, because approximately 50% of OSA patients suffer from gastroesophageal reflux disease, and as the severity of OSA increases, there is a heightened risk of gastroesophageal reflux disease (Kim, Lee et al. 2018, Wu, Yang et al. 2019). The most common treatment for gastroesophageal reflux disease is the consumption of antacid solutions or tablets that contain aluminium hydroxide (e.g. Gaviscon; Mandel, Daggy et al. 2000)). Given this link between OSA severity and the dietary consumption of aluminium, there is a need to investigate whether CoA in OSA contain elevated levels of aluminium, as this may provide insights into whether aluminium contributes to the deposition of CoA.
In conclusion, the present study has shown that oxidative stress, fragmented sleep, BBB leakage, neurodegeneration, neuropil loss and demyelination are unlikely to be significant contributors to CoA formation in OSA. Nonetheless, since CoA burden was correlated with the severity of OSA, another factor associated with OSA is likely to be a cause. Further investigations are required to determine the factors responsible for causing CoA to deposit in the hippocampus of OSA patients.
Chapter 7 General Discussion

7.0 Overview

The present thesis investigated angiogenesis and microvascular remodelling, microvascular abnormalities, and the distribution and burdens of CoA in the hippocampi of OSA patients. The research also examined whether OSA severity, regular CPAP use or advancing age influence the above-mentioned parameters. Investigations were made by comparing low vs. high OSA groups, non-CPAP users vs. CPAP users, and patients aged < 67.5 years vs. those aged > 67.5 years. Of note is the significant correlation ($r^2 = 0.175, p = 0.019$) between OSA severity (ODI) and patient age in the present study; it is likely that age contributes to some of the significant changes observed in relation to OSA severity.

Chapter 3 investigated whether OSA severity influences angiogenesis or microvascular remodelling. While no evidence of angiogenesis was found, microvascular remodelling was found to occur in response to both increased OSA severity and ageing (Figure 7.1 A).

Chapter 4 investigated the effect of OSA severity on microvascular abnormalities, which were mostly observed in the selectively vulnerable CA1 region in response to increased OSA severity and ageing (Figure 7.1 B).

Chapter 5 investigated the distribution of CoA in the hippocampus of OSA patients in relation to ageing. While the diameters of CoA were found to increase with age, no associations with increasing age were found between ranked CoA sequence or CoA density (Figure 7.1 C).

Chapter 6 investigated other factors that have been postulated to contribute to CoA formation. It was found that the ranked CoA sequence, CoA density and size were associated with OSA
severity, whereas CPAP use or markers of neurodegeneration, neuropil loss and demyelination had no association with CoA distribution, density or size (Figure 7.1 D & E).

Figure 7.1 Schematic illustration of the main findings in relation to the research aims.

The arrows indicate the main independent variables considered in relation to the research aims in the four data chapters. The ticks indicate where the variables were found to have a significant effect, while the crosses indicate where no significant effect was found. The grey colour indicates no effect of these variables or no change to the investigated parameters.
While each of these findings were discussed in the relevant chapters, the purpose of the present chapter is to review these findings together, in order to: i) gain an appreciation of how the present study has advanced our understanding of the field; ii) describe the limitations of the present findings; and iii) outline how further research can build on the current findings.

7.1 Increased microvascular remodelling in relation to advanced OSA severity and ageing

OSA is characterised by intermittent hypoxia (Kanagy 2009, Lavie 2015, Li, Ren et al. 2018), and both angiogenesis and vascular remodelling have been reported in animal IH models (Kanaan, Farahani et al. 2006, Lim, Brady et al. 2016, Suarez-Giron, Castro-Grattoni et al. 2018). Therefore it was hypothesised that angiogenesis and/or microvascular remodelling would be more common in the high OSA group when compared to the low OSA group. Indeed, the present study found that the high OSA group had significantly more microvascular remodelling (increased capillary diameter and length), however no evidence of angiogenesis was found in the hippocampi of OSA patients.

The present study found no significant difference in the number of capillaries labelled with GluT-1 between the high OSA and low OSA groups, which contrasts with a previous report of increased capillary density (numbers of vessels labelled with GluT-1) in the hippocampus of IH mice compared to normoxic control mice (Kanaan, Farahani et al. 2006). This difference may result from the fact that Kanaan and colleagues (2006) used young mice, whereas the present study examined old humans; thus there could be species differences in the brain's response to intermittent hypoxia or alternatively, the capacity for angiogenesis may decline with age. In support of this latter possibility, there were significantly fewer CD34-positive vessels in patients aged > 67.5 years compared to those aged < 67.5 years in the fimbria region (Figure 3.9 A), which is consistent with previous reports from animal
models that angiogenesis is impaired and delayed with age (Rivard, Fabre et al. 1999, Sadoun and Reed 2003, Ingraham, Forbes et al. 2008, Benderro and Lamanna 2011, Harb, Whiteus et al. 2013). Another possibility is that the degree of hypoxia experienced by mice in the IH model was more severe than the intermittent hypoxia experienced by humans with moderate-severe OSA. In support of this possibility, Lim and colleagues (2016) reported that the extent of angiogenesis in mice varies in response to the severity of IH, and was only detectable at extreme hypoxia severities that are not observed clinically in OSA patients (Lim, Brady et al. 2016).

In contrast to the lack of angiogenesis, the present study found clear evidence of microvascular remodelling (enlargement of capillary diameters and elongation of capillary lengths) in response to increased OSA severity and advanced age, in the fimbria and CA4 regions (Figures 3.4, 3.5 and 3.9). The amount of remodelling was extensive, and contributed to a 30% increase in the area of brain tissue occupied by blood vessels (Figures 3.6 and 3.9). Such an increase in area fraction will improve blood flow to the fimbria and CA4 regions, and this adaptation will almost certainly confer a degree of protection from hypoxia. Conversely, the absence of microvascular remodelling in the CA1, subiculum or collateral sulcus regions suggests that these regions are less able to adapt to hypoxia and consequently are more vulnerable to hypoxic injury. Alternatively, these regions may receive better blood supply to begin with and consequently there may be a sufficient supply of oxygen even during severe episodes of hypoxia. As discussed in the following section, the former scenario appears to be more likely.
7.2 Compromised microvasculature may explain the selective vulnerability of CA1 region

While it is widely established that the CA1 region of the human hippocampus is particularly vulnerable to hypoxia, and is one of the first regions of the brain to show neurodegenerative changes after a severe hypoxic injury (Kreisman, Soliman et al. 2000, Fung, Xi et al. 2009, Fung, Xi et al. 2012), the reasons for this vulnerability are unknown. The present study did not find significantly increased angiogenesis or microvascular remodelling in moderate-severe OSA patients in the CA1 region. By contrast, in mice exposed to hypobaric hypoxia for 4 weeks, significantly increased capillary diameters and elongated vessel length were reported in the CA1 region (Boero, Ascher et al. 1999). The conflicting results between the present study and that by Boero and colleagues (1999) are probably attributable to species differences or the fact that the mice used in Boero’s study were immature, at only 21 days old (Casoli, Spagna et al. 1996, Riddle, Sonntag et al. 2003, Burke and Barnes 2006, Topiwala and Ebmeier 2012). Regardless of the reason for the difference, the present data indicate that the CA1 region of the human hippocampus does not adapt to increased OSA severity by generating new microvessels or by remodelling existing ones. This lack of adaptation may render the CA1 more vulnerable to hypoxia than the fimbria and CA4 regions of the hippocampus that do adapt.

A further indication that the CA1 is more vulnerable to hypoxia comes from our investigations of microvessel abnormalities; such vessels are markers of physiological stress and disease. The CA1 region of the moderate-severe OSA group had significantly increased numbers of string vessels (vessel diameter < 1.5 µm), narrowing vessels (narrowest vessel diameter > 1.5 µm and < 2.5 µm), strictures (abrupt narrowing with diameter typically less than 4 µm) and total abnormal vessels (string + narrowing + strictures) (Figure 4.3). No other
regions of the hippocampus exhibited increased numbers of abnormal microvessels in moderate-severe OSA. The smaller diameters of string vessels, narrowing vessels and strictures will have the effect of limiting regional blood flow, leading to compromised metabolic support for neurons. Since vessel diameter in the CA1 appears to be unable to increase in response to worsening OSA, we speculate that the associated hypoxia damages microvessels, which subsequently develop abnormalities that further restrict blood flow, creating a vicious circle that causes the neurons in CA1 to become selectively vulnerable to hypoxia. This scenario is consistent with a hypothesis by (Riddle, Sonntag et al. 2003) that altered vessel shape and structure in old brains compromises blood flow and decreases metabolic support for neurons, while reduced microvascular plasticity in relation to ageing reduces the responsiveness of capillaries to increases in neural activity, collectively leading to cognitive disturbances. Thus the findings of the present study may have provided new insights into the basis of the CA1's vulnerability to hypoxia.

7.3 Factors that may influence CoA burden and size

Although many hypotheses have been proposed regarding the origins and functions of CoA in ageing and in neurodegenerative diseases, no study has investigated the presence of CoA in OSA. The present study investigated the distribution and progression of CoA in the hippocampus of OSA patients in relation to ageing. CoA were observed in 29 out of 30 (96.7%) OSA patients, where they were mainly located in the periventricular regions (e.g. wall of lateral ventricle, alveus, fimbria) and the subpial regions (40 – 100 µm below the pial border). The extremely high incidence of CoA observed in the present study (96.7% of brains examined) was much higher than reported by previous studies (63%, 57% and 52%) of CoA in neurodegenerative diseases (Van Paesschen, Revesz et al. 1997, Cherian, Radhakrishnan et al. 2003, Das, Balan et al. 2011), and this high incidence supports the idea that OSA is a
primary cause of CoA deposition. This idea is further supported by our findings that the ranked CoA sequence (a measure of CoA progression) is significantly and positively correlated with OSA severity (Figure 6.2 A). Furthermore, the CoA density (count and area fraction) is significantly higher in the high OSA group compared to the low OSA group in the fimbria pial surface region, after controlling for the effect of age (Figure 6.3 A & B; Table 6.1).

In addition to OSA severity, the other factor found to correlate significantly with CoA was patient age. Interestingly, age did not correlate with CoA progression or CoA density, but instead increasing age correlated linearly with CoA size (Figure 5.14). In other words, CoA tend to be larger in older brains, which is consistent with a previous report that CoA grow larger with age (Cavanagh 1999). Thus, it is possible that CoA deposition begins early in life due to OSA (or another factor) and then the material in existing CoA is gradually added to over time so that they become larger. In such a scenario, the largest CoA will be the oldest, while the smallest will be the youngest. While we were unable to determine the age of CoA in the present study, it was noted that the smallest mean and maximum diameters of CoA were observed in the vicinity of the collateral sulcus (Table 5.4), which incidentally is the last region of the hippocampal formation to display CoA. By contrast, the largest maximum diameters and highest packing density were found in the fimbria, which is the hippocampal site where CoA deposition appears to begin (Figure 5.7).

Since the findings presented in Chapters 5 and 6 of this thesis revealed that OSA severity is correlated with CoA burden and progression, the next step was to ask what features of OSA might conceivably be responsible for this association? Three features were considered: sleep fragmentation, oxidative stress and neurodegeneration. While direct data for sleep fragmentation and oxidative stress are not available for the patients included in this study, it
was reasoned that as CPAP use is known to reduce both sleep fragmentation and oxidative stress in OSA, it should be possible to detect a protective effect of CPAP in the current dataset. However, no protective effect was found for CPAP use for any of the CoA parameters measured, making it unlikely that sleep fragmentation or oxidative stress are significant factors in the deposition or growth of CoA in OSA patients. This lack of an effect was unexpected. CoA have frequently been postulated to be a marker of oxidative stress in the brain, and CoA have also been postulated to be cleared via the glymphatic drainage system (Rohn 2015, Augé, Cabezón et al. 2017, Augé, Bechmann et al. 2019) that is believed to be compromised by frequent sleep disruptions (Xie, Kang et al. 2013, Jessen, Munk et al. 2015, Ju, Finn et al. 2016, Ju, Zangrilli et al. 2019). The present data, showing the lack of a protective effect of CPAP, represent a major challenge to these two influential hypotheses of CoA deposition and clearance.

Another influential hypothesis posits that CoA represent the debris of injured and dead neurons, and CoA are formed as a consequence of neurodegeneration (Erdamar, Zhu et al. 2000, Kawamura, Morioka et al. 2002, Cherian, Radhakrishnan et al. 2003, Uckermann, Galli et al. 2017, Navarro, Genoud et al. 2018). The present study had multiple lines of direct evidence relating to this hypothesis: quantitative measurements of demyelination, neuropil thinning, Aβ plaque density and NFTs in the same tissue series as used in the present study. Remarkably, none of the measures in any of the hippocampal regions examined were significantly correlated with CoA burden, sequence or size. Indeed, the spatiotemporal spread of Aβ plaques and NFT's progressed in the opposite direction to the spatial progression of CoA. Taken together, these data rule out neurodegeneration as a causal factor in the formation of CoA.
Another hypothesis advanced to account for CoA formation is that CoA are composed of blood proteins that have leaked into the brain due to a compromised BBB barrier (Meng, Zhang et al. 2009, Pisa, Alonso et al. 2016, Navarro, Genoud et al. 2018). Since intermittent hypoxia and oxidative stress are thought to increase the leakiness of the BBB (Lim and Pack 2014, Pan and Kastin 2014), OSA should favour the formation of CoA in the vicinity of blood vessels. However, in the present study, few CoA were observed in the immediate vicinity of arterioles or venules; indeed, the vast majority of CoA were located within 100 µm of the pial border (Figures 5.9 and 5.12), which is an avascular zone. Thus the present observations are inconsistent with the hypothesis that CoA originate from a leaky BBB, at least in OSA patients.

By undertaking the first systematic, quantitative investigation of CoA distribution, density and size in the human brain, this study has provided an opportunity to test the predictions of the various hypotheses that have been advanced (but not previously tested) to explain the occurrence of CoA. While the present results do not support any of the main hypotheses of CoA formation and clearance, they do indicate a role for OSA and ageing, and by so doing they help to clarify what directions may be fruitful for future research.

7.4 Limited protective/reverse effect of regular CPAP treatment

The present study found no evidence that regular CPAP use is able to ameliorate IH induced microvascular alterations (Figure 3.8), reduce the numbers of string vessels, narrowing vessels, strictures or total vessel abnormalities in the hippocampus of OSA patients (Figure 4.6). Furthermore, CPAP use did not reduce the burden of CoA (Figure 6.4). Although there have been reports that CPAP can normalise sleep architecture, improve daytime productivity and daily function (Weaver and Grunstein 2008), and quality of life (Kandasamy, Almaghaslah et al. 2019), some aspects of impaired executive functions do not improve after
CPAP treatment, suggesting that CPAP treatment only partly reverses cognitive dysfunction in OSA patients (Ferini-Strambi, Baietto et al. 2003). It seems likely that by improving sleep quality, CPAP is able to improve those cognitive symptoms that are due to chronic tiredness, whereas neurodegenerative changes are fairly permanent and cannot be reversed by CPAP.

Hence, a recommendation originating from the present study is to screen for OSA in all at-risk individuals so that interventions may be started at an earlier stage, and the chances of minimising or reversing structural changes are maximised. If treatment is delayed until the person has had OSA for many years, the vascular changes and CoA deposition are unlikely to reverse, as the aged human brain loses its structural and functional plasticity (Casoli, Spagna et al. 1996, Riddle, Sonntag et al. 2003, Burke and Barnes 2006, Topiwala and Ebmeier 2012).

7.5 Limitations

Due to the retrospective nature of the autopsy series used, the present thesis has several limitations, specifically: i) a modest sample size (31); ii) a sample consisting entirely of Icelandic participants; iii) the absence of a healthy age-matched control group; iv) incomplete clinical records; v) the autopsy samples are limited to the hippocampus and adjacent cortex. These limitations are discussed below.

**Sample size:** Between 1987 and 2014, a total of 8,853 OSA patients (18 years and older) were registered in Iceland with a diagnosis of OSA, and by March 2014, 1322 had died. Of these, consents had been received from 121 patients (9.2% of those who had died) for donation autopsies after death. Only half of these donors (61) had autopsy material that included brain tissues, and half of these met the exclusion criteria for the present study: a diagnosis of dementia (including AD), head trauma, stroke, multiple sclerosis, cerebral infection, pulmonary disease, as well as treatment of OSA other than CPAP. Consequently,
the present study only had access to the hippocampal tissues of 31 (2.3%) of those in the program who had died.

The relatively small sample size in the present study limited the statistical power, particularly once the group had been stratified by OSA severity and CPAP use. This limited power prevented the use of multivariate statistics that can assess the relative contributions of possible co-factors, such as age, gender and BMI. In future years, as more patients enrolled in the program die, it will be possible to confirm the present findings in a larger sample, and to conduct more thorough analyses of the influence of confounding variables.

**Icelandic sample:** The samples were sourced from our collaborators (Prof. Thorarinn Gislason and Prof. Bryndis Benediktsdottir) in Iceland, therefore the OSA patients may not be representative of the global population, as Iceland is less genetically diverse (98.6% Caucasian plus 1.4 % other with no African American, Asian, or South American ethnicity) than many other countries (Keenan, Kim et al. 2018). Genetic diversity is an important consideration, as genotype is known to affect OSA risk. For instance, south-east Asians have a craniofacial structure that predisposes individuals to develop OSA at a lower BMI than in Caucasians (Keenan, Kim et al. 2018); moreover, South Americans are particularly susceptible to the effect of weight gain on OSA severity, while African Americans are least affected (Sutherland, Keenan et al. 2019). The research team of our collaborators has identified three distinct clinical phenotypes of OSA patients in Iceland, namely a disturbed sleep cluster (32.7%), minimally symptomatic cluster (24.7%), and daytime sleepiness cluster (42.6%) (Ye, Pien et al. 2014). While these three clusters can be observed in more genetically diverse cohorts, the prevalence differs (19.8%, 40.4% and 39.8%, respectively) based on a global study from six countries (Keenan, Kim et al. 2018), demonstrating ethnic differences in clinical symptoms. Therefore, while the results of the present study are relevant to the
Icelandic population, and probably to Caucasians generally, their relevance to other ethnic backgrounds may be limited.

**No control group:** The participants in the present study came from a pool of patients who were diagnosed with OSA, and who had agreed for their autopsy brain material to be used for research. Since it was not possible for a patient to join this pool if they did not have OSA, the present study lacks a control group of individuals who were diagnosed not to have OSA. Therefore, it was necessary to stratify the sample according to OSA severity, and to divide it into two groups based on the median ODI value. While some of the individuals in the low OSA group had very mild OSA, it is recognised that a future study would benefit from having a comparator group that consists entirely of individuals who had been diagnosed not to have OSA at the time of their death. Despite this limitation, the range of OSA severities encompassed by the present study (ODI range: 4.1 – 92.2 events/h sleep) was heterogeneous enough to allow assessments of whether the variables of interest were correlated with the severity of OSA.

**Incomplete clinical records:** The hippocampal tissues were sourced from patients in Iceland who had been diagnosed with OSA as early as 1987. During last century, the medical records in Iceland were paper-based and some of these records have been misplaced in the intervening period. Consequently, some of the medical records for these 31 patients lack AHI data, or data about CPAP compliance or data about the likely age of onset of OSA. Furthermore, at the time these medical records were obtained, the present study had not yet been conceived, so measures that seem obvious with the wisdom of hindsight (e.g. nadir O₂, time spent less than 90% O₂, serum markers of oxidative stress) were not recorded. Medical records collected since the turn of the century are more complete and more readily accessible, however few patients diagnosed after 2000 were able to be included in the present study.
Limited to hippocampus: The pathologist who collected the brain samples for autopsy did so before the present study was conceived. Consequently, not a great amount of care was given to obtaining identically-sized and similarly-oriented tissue blocks from each specimen. We are fortunate that the hippocampal blocks are comparable in size and orientation. However, tissue blocks from other brain regions were often unlabelled and appeared to have been collected in a less systematic manner, which prevented the present study from extending the analyses to other brain regions. In the future it will be important to systematically collect tissue from other key regions (e.g. basal ganglia, frontal cortex) in order to determine whether the findings from the present study can be generalised to other parts of the brain.

The findings regarding the hippocampal microvasculature (Chapter 3 & 4) are likely to be generalisable to other brain regions. However, the CoA distribution and progression pattern (Chapter 5) reported here is specific to the hippocampus since CoA deposition starts from the fimbria and progresses bilaterally to adjacent subpial regions. It remains to be determined whether other brain regions exhibit a similar process, whereby CoA deposit at a precise subregion and gradually spread laterally from there. When it comes to the contributing factors to CoA formation (Chapter 6), the effects of increased OSA severity, oxidative stress, sleep fragmentation, BBB leakage, ageing, neurodegeneration, neuropil loss and demyelination can all be demonstrated at other brain regions.

7.6 Directions for future research

The preceding section highlighted some of the limitations placed on the present study, due to the use of archived autopsy material that had been collected prior to the inception of the present study. It is evident that there is a need for a prospective study that is able to confirm and build on the present findings. Such a study would include a larger sample of perhaps 200 brains, plus a control group comprised of patients who had been verified not to have OSA. In
this ideal study, there would be full polysomnographic records for all participants including blood oxygen levels, continuous blood pressure measurements and CPAP compliance data. There would be detailed medical records for all participants including medication history, comorbidities, and cognitive status as confirmed by a clinical neuropsychologist. There would also be comprehensive assays of blood and urine samples for multiple markers of inflammation and oxidative stress. Furthermore, entire brains would be archived, so that identical blocks could be obtained from selected brain areas of interest. The ideal study would be a multicentre study that included a wide range of countries, so that ethnic and cultural factors could be controlled for.

Another area for investigation is related to the microvascular alterations observed in OSA. It is clear that animal models of OSA offer some advantages with respect to being able to standardise environmental and genetic variables, to minimise the post-mortem interval, and to record parameters such as oxidative stress immediately prior to death. However, current animal models of IH routinely use young adult rodents, and it seems likely from the present study that the adaptiveness of the microvasculature diminishes significantly in old age. Thus if animal models of IH are to be representative of OSA they ought to be conducted on old, overweight rodents.

The associations between microvascular abnormalities and regional CBF are worthy of future investigation. The microvessels examined in the present study demonstrated increased ultrastructural alterations in relation to OSA severity. One of the questions arising from this study is whether the microvessel abnormalities cause reduced CBF and lead to neuronal loss or whether insufficient microvascular remodelling causes stress to the tissue and leads to microvessel abnormalities. It might be possible to differentiate between these possibilities in animal models of IH.
The present study advanced our understanding of CoA distribution and progression in the hippocampus: starting from the fimbria region and spreading bilaterally to adjacent subpial regions with gradually reduced density. However, the mechanism(s) responsible for this progression cannot be determined at present. A speculation advanced here is that CoA are formed in the neuropil and then move towards the pial surface where they are cleared into the CSF. In support of this speculation, CoA were found to be more numerous and larger near the pial surface. Furthermore, individual CoA were sometimes observed on the pial surface, within the ventricular cavity (see Figure 7.2). Perhaps the rate of clearance of CoA from the ventricular surface is affected by the rate of flow of CSF through the ventricular system. Since the rates of CSF production and clearance slow down significantly with natural ageing (Masseguin, LePanse et al. 2005, Redzic, Preston et al. 2005, Kress, Iliff et al. 2014, Ma, Ineichen et al. 2017), this would reduce the rate of clearance of CoA and lead to a corresponding accumulation of CoA in the adjacent neuropil. Future studies can test this possibility by examining whether the protein components of CoA can be detected in the CSF, and if so, whether the concentration of these components decreases with age.

Figure 7.2 Immunofluorescent micrograph of CoA on the ventricular side of the pial border.

CoA were sometimes observed on the pial surface, where they appeared to be located within the ventricular cavity (arrows), rather than in the neuropil.
The present study introduced a new way of quantifying the distribution, density and size of CoA in the hippocampus. It would be important to examine whether other brain regions show similar patterns of spatiotemporal spread in response to increasing severity of OSA. The results of the present study have challenged prevailing hypotheses of CoA formation and have cast doubt on age, oxidative stress and neurodegeneration as the primary causes of CoA formation. However, as the present study was unable to investigate other potential contributors to CoA formation, such as bacterial infections or aluminium accumulation, these factors ought to be the subject of future studies.

7.7 Summary & Conclusions

The present study has investigated the microvascular alterations and CoA burden in the post-mortem hippocampi from 31 OSA patients (15 males and 16 females) from Iceland who died between 1987 and 2014, at an age that ranged between 41.7 – 89 years, with the average age at death being 67. The OSA severity (ODI) of these samples varied from very mild (4.1 events/h sleep) to very severe (92.2 events/h sleep). Note that the slightly smaller sample size (n = 30) in Chapters 3 – 6 resulted from different reasons: patient number 18 was excluded in Chapters 3 & 4 due to faint immunostaining with the blood vessel markers; while patient number 19 was excluded in Chapters 5 & 6 due to insufficient hippocampal tissue being available for analysis.

To summarise, the main conclusions are as follows:

i) Increased microvascular remodelling and microvascular abnormalities are related to elevated OSA severity and advancing age.
ii) The absence of microvascular remodelling, combined with increased numbers of abnormal microvessels may contribute to the selective vulnerability of the CA1 region to hypoxia and oxidative stress.

iii) CoA distribution and progression in the hippocampus are associated with increased OSA severity, while age is associated with an increase in the diameters of CoA. While OSA severity is likely to contribute to the formation of OSA; oxidative stress, sleep fragmentation, BBB leakage, ageing, neurodegeneration, neuropil loss and demyelination are unlikely to contribute. The actual mechanism(s) responsible for the formation of CoA is(are) yet to be elucidated.

iv) The microvascular alterations and CoA burdens that are related to OSA severity cannot be reversed by CPAP treatment. To prevent these changes from occurring it may be necessary to begin CPAP treatment at an early stage of OSA.
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Appendices

Appendix A: National Bioethics Committee of Iceland ethics approval

Efni: Regarding ref. number: 09-087 - CM
English: Brain and liver tissue changes in obstructive sleep apnea
Icelandic: Taugavefsskemdir í kæfivæfni

Enclosed is a copy of the original approval by the National Bioethics Committee, Iceland, dated 23 February 2010 (CM in English dated 16 February 2016), regarding the above mentioned research application submitted to the Principal Investigator, Þorarinn Gislason, dr. med., University Hospital Iceland.

Certain irregularities have been brought to our attention concerning the execution of the approved protocol, especially regarding the use of tissue samples abroad. We’ve investigated those irregularities and introduced amendments to the procedures of the Icelandic Biobank in order to secure that this will not happen again.

We’ve come to the conclusion that none of those incidents should have to interfere with your work or the work being conducted by your group or of your PhD students.

We therefore approve that you make use of this original Confirmation of approval to finish your work and make it possible for your PhD students to finish their thesis and the defence thereof. We do however request that no further work will be carried out on those samples, without new application/approval and that the rest of the samples will be returned to Iceland as soon as possible and no later than one year after the disputation of the latter PhD student concerned.

Sincerely Yours,

Kristján Erlendsson MD
Chairman of the National Bioethics Committee of Iceland

Copy to:
Þorarinn Gislason, Prof. University Hospital, Reykjavik, Iceland.
Dr Peter Burke, Secretary, Human Research Ethics Committee,
RMIT University, Melbourne, Australia.
Appendix B: RMIT ethics approval

RMIT UNIVERSITY
College Human Ethics Advisory Network (CHEAN)
College of Science, Engineering and Health

Email: [REDACTED]
Phone: [REDACTED]
Building: [REDACTED] Bunyip West Campus

5 May 2017

Professor Stephen Robinson
School of Health and Biomedical Sciences
RMIT University

Dear Prof Robinson,

ASEHAPP 71-16 Neurodegenerative changes in obstructive sleep apnea

Thank you for providing current institutional approval as requested and I am pleased to inform you that you have met the condition for the approval of your application. Your application is now approved for a period of 3 Years from the date of this letter to 31 December 2020 and your research may now proceed.

The CHEAN would like to remind you that:

All data should be stored on University Network systems. These systems provide high levels of manageable security and data integrity, can provide secure remote access, are backed up on a regular basis and can provide Disaster Recovery processes should a large scale incident occur. The use of portable devices such as CDs and memory sticks is valid for archiving data transport where necessary and for some works in progress. The authoritative copy of all current data should reside on appropriate network systems and the Principal Investigator is responsible for the retention and storage of the original data pertaining to the project for a minimum period of five years.

Please Note: Annual reports are due on the anniversary of the commencement date for all research projects that have been approved by the CHEAN. Ongoing approval is
conditional upon the submission of annual reports failure to provide an annual report
may result in Ethics approval being withdrawn.

Final reports are due within six months of the project expiring or as soon as possible after
your research project has concluded.

The annual/final reports forms can be found at:
www.rmit.edu.au/staff/research/human-research-ethics

Yours faithfully,

Associate Professor Barbara Polus
Chair, Science Engineering & Health
College Human Ethics Advisory Network

Cc  Student Investigator/s:  Ms Jessica Owens, School of Health & Biomedical Sciences
     Ms Caluzi Xu, School of Health & Biomedical Sciences
     Other Investigator/s:  Prof Jiming Ye, School of Health & Biomedical Sciences