FUNCTIONAL IMAGING OF THE BRAINSTEM AND CORTICAL SITES OF BLOOD PRESSURE CONTROL IN SUBJECTS WITH OBSTRUCTIVE SLEEP APNOEA

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Statement of Original Authorship

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

___________________
Rania Fatouleh
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It would not have been possible for me to write this thesis without the knowledge, enthusiasm and support of my primary supervisor Professor Vaughan Macefield, without whom this research wouldn’t have been possible. You nurtured my curiosity for clinical science and believed in me long before I was confident in my abilities.

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I cannot help but dedicate this thesis to my cherished husband George, who kept me sane throughout the stressful time. Words cannot adequately express my thanks and appreciation for your faith, support, and love all through this experience. A special thanks goes to my unborn baby who I haven’t seen yet, you have kicked and squirmed through many pages of writing this thesis, and you waited until after its conclusion for birth- I love you already.
Abstract

Obstructive sleep apnoea (OSA) is a common sleep disorder associated with repeated bouts of nocturnal hypoxaemia during collapse of the upper airways during sleep. The hypoxaemia leads to a physiologically appropriate increase in total peripheral resistance, brought about by an increase in sympathetically-mediated vasoconstriction in the muscle vascular bed. The increase in muscle sympathetic nerve activity (MSNA) persists in the awake state, leading to neurogenic hypertension and an elevated cardiovascular morbidity and mortality. However, the mechanisms underlying the elevated MSNA in OSA are poorly understood. This thesis composes a series of studies I conducted in healthy subjects and OSA patients, before and after treatment with continuous positive airway pressure (CPAP), to improve our understanding of the disturbances in autonomic control that manifests in OSA. In Studies I-IV, I recorded MSNA in healthy controls and in OSA subjects before and after treatment with CPAP. I confirmed that MSNA was significantly elevated in newly diagnosed OSA patients compared to control subjects and that there was a significant fall in MSNA after 6 months of CPAP, with no further change after 12 months. In Study I, I tested the hypothesis that respiratory-sympathetic coupling, postulated to be the underlying cause of neurogenic hypertension, is increased in OSA. In 21 OSA patients and 21 control subjects, cross-correlation analysis revealed no significant difference in the magnitude of respiratory modulation of MSNA between the OSA patients and controls, but the temporal coupling of MSNA to respiration was tighter in OSA, with more activity occurring in post-inspiration and less in inspiration and expiration. This was largely reversed following long-term treatment with CPAP. In Study II-IV, I concurrently recorded
MSNA and fMRI to identify regions within the brain that are functionally coupled to the generation of the elevated. I also measured regional grey matter volume using voxel-based morphometry. In Study II, in 17 OSA patients, the elevated MSNA drive was associated with significant changes in Blood Oxygen Level Dependent signal intensity within dorsolateral and medial prefrontal cortices (dIPFC, mPFC), dorsal precuneus, anterior cingulate (ACC) and retrosplenial cortices, caudate nucleus, as well as the right hippocampus/parahippocampus, compared to 17 healthy controls. Surprisingly, none of the regions displayed significant anatomical changes. In addition, in Study III, elevated MSNA in OSA was correlated to altered changes in signal intensity in the dorsolateral pons, rostral ventrolateral medulla (RVLM), medullary raphe and midbrain in comparison to the healthy controls. Except for the midbrain, those regions had significantly increased grey matter volumes in OSA compared with controls. Furthermore, OSA was also associated with grey matter volume increases in the region of the hypoglossal nucleus. Finally, in Study IV, I aimed to assess the changes to brain activity following 6 months of CPAP treatment in 13 OSA patients before and after 6 months of treatment with CPAP and in 15 healthy control subjects. The reduction in resting MSNA after 6 months of CPAP described earlier was coupled with significant changes in signal intensity in precuneus bilaterally, as well as in the insula, retrosplenial cortex bilaterally, right mPFC, right ACC, and right parahippocampus. In addition, CPAP treatment had no significant effect on grey matter volume in any of those brain regions. These data suggest that the elevated muscle vasoconstrictor drive that occurs in individuals with OSA may be driven by activity changes in these suprabulbar sites through influences on brainstem regulatory nuclei.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AASM</td>
<td>American Academy of Sleep Medicine</td>
</tr>
<tr>
<td>ACC</td>
<td>Anterior cingulate cortex</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AF</td>
<td>Atrial fibrillation</td>
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<tr>
<td>AHI</td>
<td>Apnoea hypopnoea index</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BOLD</td>
<td>Blood-oxygen level-dependent</td>
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<tr>
<td>BP</td>
<td>Blood pressure</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<td>CPAP</td>
<td>Continuous positive airway pressure</td>
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<tr>
<td>CVLM</td>
<td>Caudal ventrolateral medulla</td>
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<tr>
<td>dlPFC</td>
<td>Dorsolateral prefrontal cortex</td>
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<tr>
<td>dlPons</td>
<td>Dorsolateral pons</td>
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<tr>
<td>DTI</td>
<td>Diffusion tensor imaging</td>
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<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
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<tr>
<td>EDS</td>
<td>Excessive daytime sleepiness</td>
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<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
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<tr>
<td>EMG</td>
<td>Electromyogram</td>
</tr>
<tr>
<td>EOG</td>
<td>Electrooculogram</td>
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<tr>
<td>ESS</td>
<td>Epworth sleepiness scale</td>
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<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
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<tr>
<td>FWHM</td>
<td>Full-width half-maximum</td>
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<tr>
<td>GM</td>
<td>Grey matter</td>
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<td>HR</td>
<td>Heart rate</td>
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<tr>
<td>HT</td>
<td>Hypertension</td>
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<tr>
<td>IH</td>
<td>Intermittent hypoxemia</td>
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<tr>
<td>KF</td>
<td>Kölliker-Fuse nucleus</td>
</tr>
<tr>
<td>mmHG</td>
<td>Millimeters of Mercury</td>
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<tr>
<td>MNI</td>
<td>Montreal Neurological Institute</td>
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<tr>
<td>mPFC</td>
<td>Medial prefrontal cortex</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
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<tr>
<td>MSNA</td>
<td>Muscle sympathetic nerve activity</td>
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<tr>
<td>NA</td>
<td>Noradrenaline</td>
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<tr>
<td>NAA/Cho</td>
<td>N-acetylaspartate-to-choline ratio</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus tractus solitarius</td>
</tr>
<tr>
<td>OSA</td>
<td>Obstructive sleep apnoea</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal grey matter</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PSG</td>
<td>Polysomnography</td>
</tr>
<tr>
<td>REM</td>
<td>Rapid eye movements</td>
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<tr>
<td>RMS</td>
<td>Root mean square</td>
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<tr>
<td>RVLM</td>
<td>Rostral ventrolateral medulla</td>
</tr>
<tr>
<td>SaO2</td>
<td>Percentage of oxygen saturation in blood</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
</tr>
<tr>
<td>SI</td>
<td>Signal intensity</td>
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<tr>
<td>sMRI</td>
<td>Structural magnetic resonance imaging</td>
</tr>
<tr>
<td>SNA</td>
<td>Sympathetic nervous system activity</td>
</tr>
<tr>
<td>SSNA</td>
<td>Skin sympathetic nerve activity</td>
</tr>
<tr>
<td>SV</td>
<td>Stroke volume</td>
</tr>
<tr>
<td>UPPP</td>
<td>Uvulopalatopharyngoplasty</td>
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Publications, presentations and awards

The following are publications that have arisen from work conducted towards this thesis:

Publications contained in this thesis:


**Other output relating to the work contained in this thesis:**


**Conference presentations during candidature:**


**Invited talks during candidature:**


**Fatouleh R.H.** Identifying sites in the brain responsible for the increase in muscle sympathetic nerve activity in obstructive sleep apnoea. *NeuRA-Sensorimotor Control Seminar*, Sydney, July 2013.
Preface

This thesis is arranged in seven chapters. Chapter One is a general introduction to the thesis and provides an overview of the relevant literature in cardiorespiratory system and the autonomic nervous system. Then it goes in depth into the neurophysiology of obstructive sleep apnoea (OSA) and the methods of diagnosis and treatments. Chapter Two details the general methods used across the studies. Chapter Three is an investigation of the respiratory-sympathetic coupling, postulated to be the underlying cause of neurogenic hypertension, before and after long-term treatment with continuous positive airway pressure (CPAP). Chapter Four is examining the functional and structural changes in the brains of patients with OSA, who are known to develop neurogenic hypertension. Chapter Five investigates the brainstem sites responsible for this increased on-going sympathetic drive. Chapter Six investigates the brain functional and structural changes in the brain after treatment with CPAP. Finally, Chapter Seven is a general discussion, consisting of the main findings, limitations and conclusions. Appendices are attached at the end of the thesis corresponding to research that I have carried out prior to or during my candidature. Ethical approval was granted by the Human Research Ethics Committees of the University of Western Sydney (approval H8984) and the University of New South Wales (approval HREC11138) for all procedures documented herein. This project was funded by National Health & Research Council of Australia (Project Grant 1007557).
Chapter 1:

Introduction
The autonomic control of diverse organ systems provided by the sympathetic and parasympathetic divisions of the autonomic nervous system is vital for the homeostatic adjustments essential in life, and there are many pathophysiological conditions in which disorders in autonomic control have been identified - such as obstructive sleep apnoea (OSA). In patients with OSA it has been found that there is an increased level of muscle sympathetic nerve activity (MSNA) and other abnormalities in the cardiovascular regulation during normoxic daytime wakefulness. (Carlson et al., 1993, 1996; Fatouleh et al., 2014b; Hedner et al., 1995, 1988; Narkiewicz et al., 1999a; Somers et al., 1995; Elam et al., 2002; Narkiewicz & Sommers, 2003; Imadojemu et al., 2007). It is clear that there are major cardiovascular consequences of OSA: the increase in MSNA has been connected to the development of increased blood pressure (BP), heart failure, cardiac arrhythmias and increased cardiovascular morbidity and mortality (Somers et al., 1995). However, the mechanisms underlying the link between OSA and cardiovascular diseases are not completely understood. There is growing evidence showing that the chronic sympathetic activation is a key mechanism underlying this relationship between OSA and cardiovascular morbidity.

The objective of this introduction is to present a cohesive summary of the current understanding of the present literature that describes the neural regulation of the cardiovascular system, with particular reference to the sympathetic nervous control of blood pressure, and the changes that occur due to OSA. In addition, I shall consider the changes that have been reported to occur after 6 months treatment with continuous positive airway pressure (CPAP).
1.1 An overview of the cardiovascular system

The cardiovascular system is composed of the heart, blood vessels and blood. The role of this system, in partnership with the pulmonary system, is to ensure the transport of oxygen, nutrients and hormones (and the removal of metabolic wastes, including carbon dioxide) to and from all of the tissues of the body.

The bulk of the heart is composed of cardiac cells (the myocardium) that are responsible for the contraction or pumping properties of the heart. Approximately ~1% of these cells have properties that allow them to generate action potentials spontaneously; these are known as autorhythmic or pacemaker cells which are located in the sinoatrial node (Silverthorn et al, 2004), this is also known as the intrinsic regulation of the heart rate.

Extrinsic factors are those that come from both hormonal responses as well as the commands from the nervous system: the central nervous system and the autonomic nervous system. The extrinsic regulation can result in rapid changes in heart rate due to chemicals that circulate in the blood or by direct action of nerves that go to the heart. The pacemaker (autorhythmic) cells are not dependent upon neural input to generate their spontaneous activity, the rate of depolarization and firing of action potentials is regulated by neurotransmitters released by the sympathetic and parasympathetic branches of the autonomic nervous system. The rate of depolarisation in these cells determines the heart rate, with neurotransmitters either accelerating or decelerating heart rate. The sympathetic and parasympathetic nervous systems are opposing forces that affect your heart rate.

Sympathetic activation leads to an increase in heart rate via the action of noradrenaline, released by the cardiac sympathetic nerves. Conversely, parasympathetic activation slows down the pacemaker activity and hence resting
heart rate via the neurotransmitter acetylcholine (ACh), which is released from the terminals of the cardiac branches of the vagus nerve.

However, during stress or a need of increased cardiac output, the adrenal glands release the hormone adrenaline (epinephrine) into the bloodstream at the same time that the sympathetic nervous system is also triggered to increase heart rate. This hormone causes the heart to beat faster, and unlike the sympathetic nervous system that sends an instantaneous and short-lived signal, adrenalinereleased into the bloodstream increases the heart rate for several minutes or more.

1.1.1 Blood pressure

Blood pressure, the force that causes the blood to flow through the blood vessels, fluctuates significantly throughout the day, but the average BP during the 24 hour cycle is highly regulated through short-term regulation. BP is determined by the volume of blood ejected by the heart, the rate of contraction and the resistance to flow by the arterioles in the skeletal muscle and splanchnic (gut) vascular beds, the small diameter arterial vessels that are under direct sympathetic neural control. Arteriolar diameter is the primary determinant of total peripheral resistance. The volume of blood ejected from the heart’s left ventricle each minute is defined as the cardiac output (Guyenet, 2006). The amount of blood ejected during each contraction is termed the stroke volume (SV), and the heart rate (HR) is defined as the number of beats per minute. Therefore cardiac output is calculated as the product of stroke volume and heart rate [cardiac output = SV x HR]. (Guyton & Hall, 2006).

Cardiac output is dependent on three regulatory variables: the end-diastolic volume, myocardial contractility and the heart rate. The end-diastolic volume is the volume reached by the left ventricle before contraction and can be determined by the
venous pressure, which in turn, depends on the blood volume and venous vascular smooth muscle tone, which - like smooth muscle tone of the arterioles - is under sympathetic control. Myocardial contractility and heart rate are both under sympathetic and parasympathetic neural control (Guyenet, 2006).

### 1.1.2 Neural control of the cardiovascular system and blood pressure

The neural control of the cardiovascular system generally consists of two main features. The first maintains basal output via the autonomic nervous system whilst continually influencing both the heart and the vascular system. The second provides phasic modulation of this outflow, which is mediated through a large number of neural reflexes. These reflexes are composed of afferent (sensory) inputs, central nervous system regions to integrate these inputs and efferent (autonomic) neurones (Squire et al., 2003).

As noted above, neural control of the circulation depends on both parasympathetic and sympathetic innervations. Parasympathetic neurones innervate the heart via the vagus nerve, whereas sympathetic neurones innervate the heart, kidneys, adrenal medulla and blood vessels. The sympathetic nervous system innervates the vessels via barosensitive, thermosensitive and glucosensitive efferent neurones. Importantly, systemic vessels receive only sympathetic innervation, unlike the dual innervation of the heart; this sympathetic innervation of the arterioles is vasoconstrictor in function.

The autonomic nervous system controls BP in both the short- and long-term. Short-term regulation occurs at the beat-to-beat level. This enables rapid changes to occur according to the behavioural or environmental demands (i.e. feeding and exercise), the environment (i.e. cold and hot weather) and emotions. These changes
occur via rapid changes in cardiac output and arteriolar resistance. Muscle blood flow, measured using ultrasound, decreases following an individual burst of MSNA, reaching a minimum during the sixth cardiac cycle (Fairfax et al., 2013). On the other hand, long-term changes in BP may be related to pathological changes in the brain or periphery (*i.e.* stress-induced hypertension or heart failure) (Dampney et al., 2002).

The primary function of the arterial baroreceptors is to maintain homeostatic control of blood pressure on a beat-to-beat basis. Barosensitive afferents are influenced by arterial pressure (carotid and aortic baroreceptors) and lung afferents by ventilation (Guyton & Hall, 2006). Baroreceptors are stretch-sensitive mechanoreceptors located in the distensible walls of the carotid sinus, at the bifurcation of the carotid arteries, and the aortic arch to monitor the pressure of blood going towards the brain (carotid baroreceptors) and body (aortic baroreceptors) (Squire et al., 2003). Baroreceptors demonstrate ongoing tonic activity at rest but exhibit a high dynamic sensitivity, discharging in bursts during each systolic pressure pulse (Guyenet, 2006).

The thermosensitive group of sympathetic neurones consists mainly of cutaneous vasoconstrictor neurones that can be activated by changes in peripheral or core temperature, emotional stimuli and respiration. The glucosensitive group is activated by hypoglycaemia and physical exercise, and controls the release of adrenaline from the adrenal medulla. The thermosensitive and glucosensitive groups are not heavily regulated by arterial baroreceptors, but may have a secondary role in short- and long-term regulation of BP (Guyenet, 2006).

The arterial baroreflex is possibly the most important short-term regulator of arterial blood pressure (Mukkamala et al., 2003). The resistance of a vascular bed is
determined by the diameter of the arterioles, which itself is controlled by muscle sympathetic vasoconstrictor drive (Swisher et al., 2007). The release of the neurotransmitter noradrenaline by the sympathetic nerve terminals activates the adrenergic receptors on the smooth muscle to cause contraction. This results in a decrease of the vessel diameter, which increases the resistance and thereby increases arterial pressure. The primary determinant of total peripheral resistance is the degree of vasoconstriction in highly vascularised skeletal muscle. Muscle vasoconstrictor drive can be recorded directly in awake humans via a microelectrode inserted percutaneously into a peripheral nerve; this vasoconstrictor drive is referred to as MSNA, and occurs as spontaneous bursts time-locked to the cardiac cycle via the arterial baroreflex.

Core networks of neurones responsible for the regulation of BP reside in the spinal cord, medulla oblongata and hypothalamus, as well as the cerebral cortex and cerebellum. Animal studies have demonstrated that afferent fibres from the arterial baroreceptors located in the carotid sinus and aortic arch travel via the glossopharyngeal and vagus nerves respectively, to the nucleus tractus solitarius (NTS) in the medulla (Figure 1.1). When BP rises, excitatory signals from these receptors are sent to the dorsal region of the vagus motor nucleus (DMX), as well as nucleus ambiguous (NA), that supplies the heart. Excitatory neurones of the NTS also project to the caudal ventrolateral medulla (CVLM, via glutamatergic receptors) and send inhibitory signals to the rostral ventrolateral medulla (RVLM), reducing its firing via GABAergic receptors. Given that the latter neurones innervate sympathetic preganglionic neurones monosynaptically in the spinal cord (Guyenet, 2006), a reduction in RVLM activity causes a reduction in the activity of the sympathetic vasoconstrictor neurones. This reduction decreases vasoconstrictor drive to the
muscle and splanchnic vascular beds (Dampney et al., 2002; DiBona, 2004; Guyenet, 2006; Squire et al., 2003). Barosensitive sympathetic efferent neurones are primarily regulated through the RVLM. Therefore, inhibition of RVLM results in a decreased release of noradrenaline from sympathetic vasoconstrictor neurones, which allows the arteriolar smooth muscle to relax and hence dilate. Conversely, a fall in arterial blood pressure unloads baroreceptors, reducing the negative feedback provided by the baroreceptors and causing an increase MSNA that leads to an increased release of noradrenaline and ultimately arterial vasoconstriction. In addition, unloading of the baroreceptors causes the withdrawal of parasympathetic drive via the vagus nerve to the heart. This results in an increase in the rate and force of cardiac contraction (Squire et al., 2003). This accounts for the temporal relationship between MSNA and the cardiac rhythm.

In our laboratory, using functional magnetic resonance imaging (fMRI) and concurrent recording of muscle sympathetic nerve activity, we were able to confirm the operation of this same medullary circuitry in awake human subjects at rest. Associated with spontaneous bursts of MSNA, there was bilateral activation of a dorsolateral area of the medulla that appears to be the human equivalent of the RVLM. In human subjects, this nucleus is displaced dorsolaterally from its ventral position in the rat by the inferior olives. Similarly, a decrease in activity in both the dorsomedial medulla and caudal lateral medulla, equivalent to NTS and CVLM respectively, was associated with unloading of the baroreceptors (Macefield et al., 2006).
Figure 1.1: The neuronal pathway of the baroreceptor afferents and sympathetic and parasympathetic efferent fibres to control heart rate and stroke volume

Not shown are the postganglionic sympathetic axons directed to smooth muscle in the skeletal muscle vascular beds, which originate from the thoracolumbar spinal cord (Figure from Purves et al., 2001).
1.2 An overview of the respiratory system

Breathing is a vital physiological function that must be controlled continuously to maintain appropriate concentrations of oxygen, carbon dioxide and hydrogen ions in the blood. The CNS controls ventilation in all conditions and in all postures. Breathing is an automatic rhythmic behaviour which is subject to different modulatory mechanisms, such as chemoreceptor reflexes, interaction with the cardiovascular control centres, and higher brain centres that control both conscious and unconscious inputs and emotions. Automatic control of breathing is controlled by the brainstem.

1.2.1 Central neural control of breathing

Contraction of the diaphragm and the inspiratory parasternal and intercostal muscles is initiated in the pons and medulla. These neurones form a network in the CNS called the central respiratory pattern generators, clusters of neurones located bilaterally in the medulla oblongata and pons. These respiratory pattern generators are divided into three major groups: the dorsal respiratory group, which contains inspiratory motoneurones that project to the inspiratory pump muscles (the diaphragm, scalenes, parasternal and intercostal muscles), and the ventral respiratory group, which contains both inspiratory and expiratory motoneurones, are located in the medulla; the pneumotaxic center in the pons helps control the rate and pattern of respiration. In addition to motoneurones that project to the cervical and thoracic spinal cord and supply the pump muscles, motoneurones travelling in certain cranial nerves (trigeminal, facial, glossopharyngeal, vagal, and hypoglossal nerves) supply the upper airways (including the larynx) and prevent the upper airways from collapsing during inspiration.
1.2.2 Pulmonary sensory receptors

Respiratory control depends to some extent on the activity of sensory receptors in the lungs and airways. Afferent neurones from the lung are divided into three groups. The first two groups consist of large, myelinated A fibres, with receptors in the airways, and include slowly-adapting pulmonary stretch receptors and rapidly-adapting pulmonary stretch receptors. The third group consists of small unmyelinated afferents (C-fibres). These airway sensory neurones are bipolar neurones located in the nodose ganglion of the vagus nerve, one branch of the axon travelling though the vagus nerve to supply the lungs and tracheobronchial tree and the second carrying the sensory information to NTS (Hunt, 1990). Most natural stimuli activate more than one group of receptors; the CNS integrates these inputs and creates the appropriate response. The most important for cardiovascular control are the slowly adapting pulmonary stretch receptors, located in the smooth muscle of the tracheobronchial tree, and which increase their firing rate with increases in lung volume during inspiration. These afferents are responsible for the lung-inflation reflex (Hering-Breuer reflex), in which inspiration is terminated at a certain threshold; although this reflex can be demonstrated in neonates, it is not important in adult humans (Kubin et al., 2006). In addition, the slowly-adapting pulmonary stretch receptors contribute to the acceleration of heart rate during lung inflation (respiratory sinus arrhythmia), which occurs at a lower transpulmonary inflation pressure (1/6 of maximal lung expansion) than when the vagus nerves have been sectioned (Goso et al., 2001). Rapidly-adapting receptors are located in the epithelial and subepithelial layers of the airways (Squire et al., 2003), from the nasopharynx to the bronchi (Widdicombe, 2003). They have a wide range of properties according to their location. These receptors can be stimulated by rapid inflation or deflation of the lungs, and respond to inhaled irritants and CO₂ (Widdicombe, 2003; Squire et al.,
Mechanical stimulation of these receptors within the trachea, particularly at the bifurcation of the trachea (carina) evoke cough (Squire et al., 2003). Unmyelinated, slowly adapting bronchial C-fibres and pulmonary C fibres are chemosensors that respond to exogenous and endogenous substances (Yu, 2005); they can also respond to lung inflation, but only at high thresholds. These afferents are responsible for the cardiovascular suppression reflex at a higher inflation pressure from 10-30 cm H\textsubscript{2}O (Kaufman et al., 1982).

1.2.3 Oscillations in the autonomic effector organs: cardiovascular and respiratory system oscillation effects on the autonomic nervous system

Coupling between the cardiovascular and respiratory systems serves a clear purpose - to oxygenate the blood and deliver this oxygenated blood to the tissues, and remove carbon dioxide from the tissues and deliver this to the lungs for expulsion. Ventilation-perfusion is carefully matched by integrating these two systems, and ensuring that this cardiorespiratory coupling is maintained when different demands are placed on the body. Indeed, poor coupling between perfusion and respiration is a feature of many cardiovascular and respiratory diseases. During inspiration, the increase in intrathoracic volume causes a fall in intrathoracic pressure, leading to air entering the lungs. Similarly, a reduction in intrathoracic pressure leads to an increase in venous return to the heart. If more blood is returning to the heart during inspiration, it would be sensible to have a mechanism that increases cardiac output during inspiration.

There are two known mechanisms of cardiorespiratory coupling. The first comprises the central neural connections between the elements controlling the cardiovascular and respiratory systems within the brainstem. The second consists of
the common afferents that act on both control systems: the arterial and cardiopulmonary baroreceptors, peripheral and central chemoreceptors, pulmonary stretch receptors and metaboreceptors in working muscles, all of which project to the NTS in the medulla (Koepchen et al., 1981). Indeed, within NTS there is a great overlap in the terminal region of these different afferent classes (Taylor et al., 1999), which ensures that inputs from the periphery engage both cardiovascular and respiratory control mechanisms. Cardiorespiratory coupling is manifested in several ways, such as respiratory sinus arrhythmia, Traube-Hering blood pressure waves and respiratory modulation of the muscle and cutaneous vasoconstrictor neurones.

1.2.3.1 Respiratory sinus arrhythmia

Respiratory sinus arrhythmia is the normal physiological heart rate variability that occurs during respiration at rest. Heart rate increases during inspiration, decreasing during expiration. It is known that the resting heart rate is kept low by the actions of the vagus (parasympathetic) nerve (Koepchen et al., 1981; Grossman et al., 2004; Yasuma & Hayano, 2004). Sinus arrhythmia is believed to reflect cyclical fluctuations in vagal activity synchronous with respiration (Coker et al., 1984).

1.2.3.2 Traube-Hering-Mayer waves

Traube-Hering-Mayer waves, usually referred to as Traube-Hering waves, are spontaneous and rhythmic fluctuations of the blood pressure which vary on a beat-to-beat basis (Roy & Sherrington, 1890). The phenomenon was discovered by Traube in 1865, Hering in 1869 and Mayer in 1876, in separate studies. They found that rhythmic fluctuations in arterial pressure occur at the respiratory frequency, as described by Traube and Hearing, or at a lower frequency as described by Mayer.
(Koh et al., 1994). As with respiratory-sinus arrhythmia, these waves provide strong evidence of common oscillations within the cardiovascular and respiratory systems (Häbler et al., 1994).

1.2.3.3 Cardiovascular-respiratory modulation of muscle vasoconstrictor neurones

As noted above, MSNA is tightly coupled to the cardiac cycle via the arterial baroreceptors, but is also modulated by respiration. In humans, MSNA is inhibited in mid-inspiration (Hagbarth & Vallbo, 1968a; Eckberg et al., 1985, 1988; Seals et al., 1990, 1993; Macefield & Wallin, 1995a, Macefield et al., 2002), whereas in the cat vasoconstrictor outflow occurs during inspiration (Barman & Gebber, 1976; Cohen & Gootman, 1970; Bainton et al., 1985; Boczek-Funke et al., 1992b). Why muscle (or splanchnic) vasoconstrictor drive is synchronized to expiration in humans but inspiration in cats is not clear, but Boczek-Funke et al. (1992a) have shown that although sympathetic activity increases during inspiration it is initially depressed in early inspiration and reaches a nadir in post-inspiration. Experiments in anaesthetized animals are typically performed during paralysis and artificial ventilation, with the vagus nerves cut - conditions in which central (phrenic discharge) and peripheral (lung inflation) respiratory activities may become dissociated, with central inspiration occurring during peripheral expiration. Because of this entrainment of the central respiratory rhythm to the ventilator, the respiratory changes in arterial pressure also become dissociated. Boczek-Funke et al. (1992b) identified two peaks in the modulation of vasoconstrictor activity: one (direct) the result of central inspiratory drive, and the other (indirect) the result of mechanically-induced unloading of arterial baroreceptors. In animals with intact vagus nerves, however, the
inspiratory peak is replaced by an expiratory peak (as in humans), attributed to unloading of baroreceptors.

While the incidence of MSNA is inversely related to diastolic pressure (Sundløf & Wallin, 1978), a relationship that holds throughout the respiratory cycle (Eckberg et al., 1985), we now know that the respiratory modulation is independent of the changes in blood pressure: the phase relationship between MSNA burst amplitude and tidal volume is preserved when respiration is brought about either by negative-pressure or positive-pressure ventilation and the inspiratory changes in diastolic pressure are reversed (Seals et al., 1993; Macefield & Wallin, 1995a). Moreover, that for a given diastolic pressure the level of MSNA is lower during inspiration than during expiration supports the idea that the sympathetic inhibition is largely independent of the influence of the arterial baroreceptors (Seals et al., 1990, 1993). Interestingly, unlike the cat, the rat shows a pattern of respiratory modulation of sympathetic activity that is broadly similar to that of humans: inhibition of sympathetic activity commences at the onset of inspiration and reaches a minimum amplitude at mid-inspiration, maximal activity occurs in the post-inspiratory phase and, occasionally, in late expiration (Czyzyk-Krzeska & Trzebski, 1990).

1.3 An overview of the sympathetic nervous system

The sympathetic nervous system in particular can be viewed as the ultimate integrator of many physiological systems. It is almost impossible to consider systemic control of the major systems without showing the important role of the sympathetic nervous system. This system is dominant during states requiring elevated levels of energy, such as in emergencies and exercise and allows the body to be prepared to respond in the “fear, fight or flight” mode. However, it must be
emphasised that rarely does the sympathetic nervous system operate in such an all-or-nothing response; it comprises distinct modules that can be “mixed and matched” to bring about a particular outcome.

The cell bodies of these preganglionic neurons in this system arise primarily from the intermediolateral cell column in the thoracolumbar region of the spinal cord (T1-L2). Preganglionic neurones synapse onto the postganglionic neurones in the paravertebral ganglia from two chains that run parallel to the spinal cord, each chain consisting of 22 ganglia that extend into the cervical and sacral regions. Those postganglionic fibres project to their distant target tissue. The primary transmitter is noradrenaline, however, the nerve terminals also release several other co-transmitters, such as neuropeptide Y and adenosine 5'-triphosphate (ATP) (Wallin & Charkoudian, 2007).

1.3.1 Recording sympathetic nerve activity in humans.

There are two major invasive approaches to measure the activity of the sympathetic nervous system in human subjects (Figure 1.2):

(i) Using intra-arterial and intravenous lines to measure spillover of noradrenaline (NA) into blood supplying specific tissue beds or the whole body or

(ii) Using a percutaneously inserted microelectrode to record sympathetic nerve activity directly from a peripheral nerve (microneurography).
1.3.1.1 Noradrenaline spillover

As explained earlier, NA is the neurotransmitter of the sympathetic nervous system. The concentration of NA in plasma represents the transmitter released by the sympathetic nervous system from the nerve ending that has spilled over into the circulation (Esler et al., 1984 a,b). NA spillover gives the rate at which released NA enters plasma. To measure the NA spillover the technique can be further divided into measurements to estimate whole body sympathetic activity (Esler et al., 1979) and the measurement of organ-specific sympathetic activity. These techniques are simply referred to as “total” and “regional” measurements of NA spillover (Esler et al., 1984a), respectively. Both total and regional NA spillover techniques are performed by infusing radio-labelled NA and by collecting blood samples obtained from a catheter placed in an artery, and a specific artery and related vein for regional spillover.

The technique used to measure NA spillover rate, although providing a useful guide to sympathetic outflow (Esler et al., 1984a,b), does have some limitations. One of these is the dependence of plasma NA concentrations on the rate at which the neurotransmitter is cleared from the circulation, not only on the overall level of sympathetic nerve activity and hence total NA release. Secondly, the technique is invasive, in addition to the fact that infusion of radio-isotopes is prohibited in some countries (Esler, 1993). Furthermore, sympathetic nervous system activity varies in organs according to different physiological and pathophysiological states. Thus, regional sympathetic nervous activity has a greater analytical power than total sympathetic activity (Esler et al., 1984a). Moreover, very few laboratories are equipped to measure regional noradrenaline release or spillover.
1.3.1.2 Microneurography.

Microneurography, developed in Sweden between 1965-1966 by Hagbarth and Vallbo, involves inserting an insulated tungsten microelectrode through the skin and into a peripheral nerve and allows one to record action potentials from myelinated and unmyelinated axons in afferent and efferent neurones coming from, or leading to, skin, muscles or joints in awake human subjects. Microneurography is commonly used to record multi-unit activity of postganglionic sympathetic axons (Mano et al., 2006). The peroneal and tibial nerves are the most commonly used nerves in the lower limbs, while median, ulnar and radial nerves are used in the upper limbs.

While much has been learnt from direct recordings of sympathetic nerve traffic in experimental animals, the ability to record sympathetic nerve activity in awake human subjects has provided a powerful tool in understanding human physiology in health and disease. Indeed, over the last five decades microneurography has led to a better understanding of the pathological mechanisms underlying many diseases.

 Usually multi-unit bursts of sympathetic nerve traffic are recorded from fascicles supplying muscle or skin, but single-unit recordings from functionally identified postganglionic neurones have also been made. Single-unit recordings, developed by Macefield et al. (1994), allow for a better interpretation of the changes in central sympathetic drive in different disease states (Macefield et al., 2002). By recording single-unit activities, additional measurements can be obtained, such as mean firing frequency, firing probability (the percentage of cardiac intervals in which a unit fires) and the percentage of spikes a unit generates per cardiac interval, which provides additional information on how the sympathetic nervous system grades its output in health and disease.
The majority of microneurographic studies have recorded MSNA or skin sympathetic nerve activity (SSNA) due, in part, to the accessibility of peripheral nerves from which stable recordings can be obtained for over an hour. Skin sympathetic nerve activity is composed of bursts of action potentials generated by a mixture of cutaneous vasoconstrictor, vasodilator, pilomotor and sudomotor neurones, which are engaged in thermoregulation and emotional expression and occur in irregular bursts that are independent of pulsatile blood pressure (Häbler et al., 1994; Wallin & Charkoudian, 2007). On the other hand, bursts of MSNA are composed exclusively of the activity of muscle vasoconstrictor neurones, which play a major role in the control of blood pressure (Wallin, 1989). MSNA is modulated from the central nervous system and from different peripheral receptors, such as the arterial and cardiopulmonary baroreceptors (Wallin & Charkoudian, 2007), and is characterized by spontaneous activity that occurs in bursts of impulses that are time-locked to the cardiac cycle (Sverrisdóttir et al., 1998; Vallbo et al., 1979). Typically, MSNA is quantified by counting the number of bursts per minute (burst frequency) or per 100 heart beats (burst incidence).
Figure 1.2: Summary of some clinical methods for regional sympathetic nervous system activity measurement

sympathetic nervous system activity can be directly measured by microneurography, either muscle or skin sympathetic nerve activity, or indirectly by looking into the regional sympathetic activity in different organs by measuring organ-specific noradrenaline (NA) spillover to plasma (Esler & Kaye, 1998).
1.3.2 Changes in sympathetic outflow due to disease

An increase in the baseline sympathetic outflow - sympathoexcitation - is present in a number of pathophysiological conditions. Elevation of MSNA was found in patients suffering from OSA (Narkiewicz & Somers, 2003), in addition to many other disorders, such as: congestive heart failure (Macefield et al., 1999; van de Borne et al., 1999; Goso et al., 2001), renovascular hypertension (Miyajima et al., 1991; Johansson et al., 1999), hypertension associated with chronic kidney disease (Hausberg et al., 2002; Schlaich et al., 2009), essential hypertension (Grassi et al., 1998; Lambert et al., 2007; Mano et al., 2006), pregnancy-induced hypertension (Fischer et al., 2004; Schobel et al., 1996) and chronic obstructive pulmonary disease (Ashley et al., 2010; Heindl et al., 2001; Raupach et al., 2008). Moreover, microneurographic studies have shown that MSNA is increased in young hypertensive patients and populations at high risk of developing hypertension (Esler, 2000; Grassi et al., 1995). The mechanisms for the sympathoexcitation differ in each of these pathophysiological states and are generally poorly understood.

1.4 An overview of sleep-related respiratory disorders

Sleep-related respiratory disorders are those associated with regular respiratory disturbances during sleep. Over the past four decades many types of abnormal breathing during sleep have been described. The major forms of sleep-related disorders are hypopnoeas, central apnoeas, and obstructive sleep apnoeas. According to the latest review (2005) of the American Academy of Sleep Medicine (AASM) created in 1997, the definition of these major respiratory sleep related disorders is as follows:
Hypopnoea – “An episode of shallow breathing (airflow reduced by at least 50%) during sleep, lasting 10 seconds or longer, usually associated with a fall in blood oxygen saturation”, Central Apnoeas – “characterized by a cessation or decrease of ventilatory effort during sleep and is usually associated with oxygen desaturation” and Obstructive Apnoeas – “characterized by repetitive episodes of upper airway obstruction that occur during sleep, usually associated with a reduction in blood oxygen saturation”. The focus of this study will be on obstructive sleep apnoea.

1.4.1 Obstructive sleep apnoea

OSA is a breathing disorder where reversible airway obstruction routinely occurs during sleep. OSA can involve multiple systems; therefore it is associated with significant medical, cognitive and psychological coexisting confounding variables including depression, anxiety, obesity, insulin resistance, hypertension, increased risks for vascular diseases and motor vehicle accidents, poor concentration and fatigue (Somers et al., 1995). Fundamental features of OSA include excessive daytime sleepiness (EDS), snoring and intermittent hypoxemia (IH) during sleep. EDS has been suggested to result from fragmented sleep related to recurrent central nervous system arousals due to disordered breathing events (Somers et al., 1995). However, EDS seems to vary greatly between individuals who suffer from OSA (Gottlieb et al., 1999). Snoring usually is generated by the vibrations of the partially or completely collapsed and unstable pharyngeal airway walls and the soft palate (Lindberg, 1998). IH, occurring as a result of the repeated episodes of deoxygenation followed by reoxygenation, induces episodes of increased MSNA (Somers et al., 1995). Although both sleep fragmentation and IH are both believed to contribute to
the adverse effects of sleep apnoea, the exact interaction and independent
collection still more understanding is needed. However, work has been growing in
this area.

AHI (Apnoea - Hypopnoea index) has been used to characterize obstructive
sleep apnoea and measures the frequency of reductions in airflow associated with the
upper-airway collapse or narrowing that occurs during sleep. The number of apnoeas
and hypopnoeas are summed and divided by the number of hours of sleep to give the
main index of disease severity. However, this index does not quantify other causes of
the arousals during the night that could explain day-time sleepiness. Nevertheless, no
other metric has proven as good as this index in assessing the severity of OSA.

Accordingly, the severity of OSA is defined as the following: AHI of less than 5 per
hour is normal; 5-14 is mild; 15-30 is moderate, and severe is greater than 30
apnoeas per hour (Gander et al., 2005).

1.4.2 Population Prevalence

OSA is a common clinical disorder that is largely underestimated and
underdiagnosed (Kapur et al., 2002; Young et al., 1997a, 2002, 2004). Although
OSA was defined over 40 years ago, during the last two decades the awareness of the
syndrome among both the public and the medical professionals has become more
common (Chervin & Guilleminault, 1996). The landmark study investigating the
prevalence of obstructive sleep apnoea was the 1993 Wisconsin Sleep Cohort Study
(Young et al., 1993). This study reported that the prevalence of OSA was 4% in
middle-aged men and 2% in middle-aged women (aged 30-60 years). It also is
estimated that 1 in 5 Caucasian adults with an average body mass index of 25-28
Kg/m^2 has an AHI of 5 or greater and 1 of 15 has an AHI of 15 or greater. The
estimate is that up to 5% of adults in western countries properly have OSA (Young et al., 2002). Other studies reported in Australia (Bearpark et al., 1995), USA (Bixler et al., 2001; Young et al., 1993), China (Ip et al., 2001, 2004), South Korea (Kim et al., 2004) and India (Udwadia et al., 2004), showed that the community prevalence of OSA is approximately 5-10% in the middle-age population. More recent studies suggested that prevalence in high-income countries is higher than previously reported (10% in women and 20% in men) (Peppard et al., 2013; Sforza et al., 2011), which could be explained by the worsening obesity and improving technology over time (Foster et al., 2009a).

1.4.3 Risk factors for OSA

1.4.3.1 Gender

Men have a higher risk of obstructive sleep apnoea than women. OSA affects about 2% of women and 4% of men across middle-aged adults, a ratio of 2:1-3:1 men to women. Previous findings from both Busselton in rural Australia and the Wisconsin cohort indicate that about 25% of middle-aged men have an AHI >5/hr, and about 9% of middle-aged women in the Wisconsin cohort had an AHI of at least 5/hr (Bearpark et al., 1995; Young et al., 1993, 2002). It has been found that there is an association between the progression of the disorder and baseline obesity, older age, and presence of snoring (Young et al., 2002). The male dominance is apparent in both the prevalence and severity of OSA. The reason underlying this difference between women and men is not fully understood. Differences in the structural and physiological behaviour of the upper airway, craniofacial morphology, sex hormones, menopause and the pattern of fat disposition have been proposed to play a major in the higher male risk of OSA (Caples et al., 2005; Young et al., 2002). Men
tend to gain weight more centrally than women, and this pattern probably results in men having more fat stored in upper airway structures and abdomen than women (Whittle et al., 1999). Several studies suggest that the airway is longer in men than in women, independent of body height, which could explain the increased propensity for airway collapse in men (Malhotra et al., 2002). Additionally, menopause could be related to redistribution of body fat to central regions and loss of lean muscle mass (Jordan et al., 2014). Other risk factors associated with higher habitual prevalence in male compared to women could explain the higher prevalence in male to female OSA patients, such as smoking and alcohol consumption and smoking (Kauffmann et al., 1989; Resta et al., 2003).

### 1.4.3.2 Age

Risk of OSA has also been found to increase with age (Edwards et al., 2010), however daytime symptoms have been found to be less common with increasing age (Young, 1996). Older individuals might have reduced tethering of the upper airway by lung volume because of loss of elastic recoil in the lung. They might also have more easily collapsible airways caused by loss of collagen, or a reduced arousal threshold caused by poorer quality of sleep. Additionally, the efficiency of the upper airway dilator muscles might fall with age (Eikermann et al., 2007; Malhotra et al., 2006; Marcus, 2000). Ayalon and his colleagues (2010) showed that the presence of OSA and increasing age overwhelmed the brain’s capacity to respond to cognitive challenges. The brain of aged OSA patients showed compensatory cerebral activation during cognitive demand due to the neurocognitive decline, to maintain performance.
1.4.3.3 Ethnicity

Specific ethnicities may represent a risk factor for OSA. It has been reported that African-Americans showed increased risk for OSA when compared with Caucasians (Redline et al., 1997b). Additionally, Young and colleagues found that African-American and American-Indians had higher prevalence of AHI ≥ 15 (20% and 23% respectively) than that of white population (17%). Other studies suggested greater severity among Asians than Caucasians, when subjects were matched for body mass index (BMI) although the mechanism of that increase is not well understood (Li et al., 2000; Ong & Clerk, 1998). It has been suggested that Asians have craniofacial characteristics that would make the upper airway more prone to collapse (Genta et al., 2008). Also, it has been found that Asians have a higher percentage of body fat when compared to Caucasians of the same BMI and age which has been suggested as another factor.

1.4.3.4 Body Mass and Obesity

Obesity is a major risk factor for OSA (Ogden et al., 2006; Tuomilehto et al., 2008). Multiple measures of obesity have been found to predict OSA, including neck size, central obesity and general obesity (Young et al., 2002). Longitudinal increase in AHI were associated with weight gain over 4 years (Peppard et al., 2000a). A 1% increase (or decrease) in bodyweight was associated with a 3% increase (or decrease) in AHI (Young et al., 2002). Another randomised trial showed that a 10kg reduction in bodyweight caused a 5% reduction in AHI (Foster et al., 2009a,b). Therefore, losing weight through diet and exercise can be beneficial in improving the symptoms of OSA (Foster, et al., 2009a).
1.4.6 Health-related consequences of OSA

1.4.6.1 Sleepiness

Sleep fragmentation may have detrimental effects on daytime functioning as a result of excessive daytime sleepiness (EDS) (Brown, 2005). The Epworth Sleepiness Scale (ESS) is a simple and validated questionnaire for assessing subjective daytime sleepiness in the context of sleep disorders (Johns, 1991). There is a clear relationship between the measured levels of OSA and EDS. However, this relationship has been shown to be weak. Many people with EDS do not suffer from OSA and some of those who have been diagnosed with OSA do not experience EDS (Thorpy, 1992; Young et al., 1993). For example, in the Gottlieb et al. (1999) study, people with AHI >30 had a mean ESS of 9.3, whilst those with an AHI <5 had a mean Epworth score of 7.2. Twenty one percent of people with AHI <5 had Epworth scores above 10 (excessively sleepy), whilst 35% of those with AHI>30 had scores>10.

1.4.6.2 Hypertension

For years researchers have intensively investigated the relationship between OSA and daytime hypertension, and more recent evidence has given strong support to the claim that OSA causes hypertension (Stradling et al., 2001). OSA experimentally induced in four dogs over 1-3 months period was associated with a chronic increase in both night and daytime BP (Brooks et al., 1997b). Elevations of BP are known to be caused by arousals from sleep, probably via sympathetic activation (Morgan et al., 1996), but in another study, induced arousals from sleep were shown not to induce chronic rises in BP in these dogs (Brooks et al., 1997a). Additionally, IH was associated with increased BP by about 12 mmHG in rats.
(Fletcher et al., 1992). This chronic increase was not due to arousals from sleep, which are known to produce acute rises in BP (Bao et al., 1999). It seems that the effects of hypoxia, hypercapnia, increased pleural pressure and increased activation of the sympathetic nervous system - but not the sleep fragmentation associated with OSA - are the likely causes of hypertension in this condition (Leung & Bradley, 2001; Stradling et al., 2001).

The development of daytime hypertension was not seen in all genetic strains, leaving open the interesting possibility that OSA might induce hypertension only in those humans who are genetically susceptible. A linear increase in BP was observed with increasing AHI, which was independent of other factors (Lavie et al., 2000; Peppard et al., 2000b; Young et al., 1997b). However, for a given AHI, as BMI increased, the effect on BP decreased (Young et al., 1997b).

1.4.6.3 Cardiovascular diseases

The incidence of cardiovascular disease increases significantly in middle-aged OSA subjects (Peker et al., 2002). A significant linear trend exists between AHI and the risk of heart failure (Malone et al., 1991; Mansfield et al., 2004; Schwab et al., 2013; Shahar et al., 2001), coronary artery disease (Carpio et al., 2013; Mooe et al., 2001) and stroke (Dyken et al., 1996; Mohsenin, 2001; Redline et al., 2010). There are many suggested cardiovascular risk factors contributing to the increased risk of stroke in OSA. Increased thickness of the common carotid artery walls in OSA is a known risk factor for stroke (Silvestrini et al., 2002). Cardiac arrhythmias are also a leading risk factor for stroke; it has been reported that atrial fibrillation (AF) in particular seems common in people with OSA (Phillips, 2005), and CPAP has been reported to reduce AF in patients with OSA (Kanagala et al., 2003). Patients who
have had a stroke have been shown to be more likely to have OSA than matched controls (Dyken et al., 1996). More recent studies reported that OSA independently contributes to the risk of having a stroke (Capampangan et al., 2010; Yaggi et al., 2005).

1.5 An overview of the neurophysiology of OSA

1.5.1 Physiology of normal sleep

During sleep, normally there are two separate states; the Non Rapid Eye Movement (NREM) and Rapid Eye Movement (REM) states. NREM constitutes the bulk of sleep time and is subdivided into three stages through which heart rate, blood pressure and sympathetic nerve activity decreases (Somers et al., 1995). In contrast, REM sleep is characterized by an increase in sympathetic nerve activity and electrical activity in the brain, in addition to intermittent and abrupt changes in BP and HR. REM sleep occurs cyclically and more frequently during the second half of the night (Caples et al., 2005).

1.5.2 Pathophysiological mechanisms of OSA

The primary force holding the upper airway open is determined by the activity of the upper airway dilator muscles. The upper airway is a complicated structure that is usually divided into four anatomical subsegments: nasopharynx, velopharynx, oropharynx and hypopharnx. This structure forms a passage for movement of the air from the nose to the lungs, in addition to other physiological functions. If this fails during sleep, total or partial occlusion occurs. Normally during sleep various protective mechanisms maintain partial patency of the upper airway. One suggested
mechanism that might be important in producing upper airway obstruction is a
difference in timing between the hypoxic-hypercapnic drive to inspired activation
of the dilator muscles. If inspiration is initiated before the activation of the dilator
muscles, the upper airway may be at risk of closure by the negative pressure
(Chervin & Guilleminault, 1996).

The key force that promotes the closure of the upper airway is the negative
pressure generated within the thorax during inspiration, which is transmitted through
the upper airways. Any narrowing from the level of the anterior nares, nasal cavity
proper and nasopharynx will lead to greater negative pressure required to produce at
a given level of airflow into the lungs, another suggested mechanism in producing
upper airflow obstruction in OSA (Chervin & Guilleminault, 1996). The third reason
has been suggested to be the position of the hyoid bone, and abnormally large
amounts of soft tissue in the pharynx could contribute to the airway collapse (Sforza
et al., 2000). It has been suggested that the tendency for OSA to run in families may
be due to hypoxic ventilation responses (Mathur & Douglas, 1995; Redline et al.,
1997a). The pattern within families also suggests some sort of inherited susceptibility
toward airway narrowing or collapse (Redline et al., 1997a).

1.5.2.1 The pathophysiological response to OSA during sleep

Normal sleep is associated with distinct alterations in BP and heart rate. These
changes are dependent upon sleep stage and appear to mediate properly by changes
in autonomic circuitry control (Somers et al., 1993). By contrast, in OSA the
sympathetic and haemodynamic profiles are dictated primarily by the duration and
severity of apnoea rather than by the sleep stage. During the obstructive event of an
OSA patient, due to either the partial or complete collapse of the upper-airway,
which is associated with the cessation of the airflow, there is a hypercapnia and hypoxemia response during the obstructive event of an OSA patient. It has been suggested that the OSA-related hypoxemia is associated with increased sympathetic vasoconstriction and coinciding decrease in vascular protective mechanisms, which results in structural and functional changes of blood vessels (Lanfranchi & Somers, 2001). The progressive increase in sympathetic nerve activity reaches its peak at the termination of the apnoea followed by a marked decrease during recovery (Chervin & Guilleminault, 1996; Somers et al., 1995). In OSA patients, the typical progress of each apnoea event can be summarized as in Figure 1.3.

The increase in MSNA carries over to daytime wakefulness when subjects are breathing normally and have no evidence of hypoxaemia or chemoreflex activation (Somers et al., 1995). Therefore, it has been proposed that several hormonal and neural mechanisms can contribute to the maintenance of the sympathoexcitation and higher BP. That increase is evident whether or not the patients are hypertensive (Narkiewicz et al., 1999a). The main chemoreceptors that are involved in sleep apnoea are the ones located in the carotid bodies of the internal carotid arteries and the brainstem central chemoreceptors. Peripheral and central chemoreceptors are the dominant reflex control mechanisms regulating ventilatory responses to changes in arterial oxygen and carbon dioxide content. The peripheral chemoreceptors are primarily responsible for the response to low blood oxygen tension, while the brainstem central chemoreceptors are most sensitive to carbon dioxide and acid-base balance (Caples et al., 2005). If the O₂ level decreases, the carotid bodies send signals via the glossopharyngeal nerve to the medullary respiratory centres to increase ventilation.
Patients with OSA have higher peripheral chemoreceptor sensitivity compared to healthy control subjects, which results in higher ventilatory response to hypoxemia (Caples et al., 2005; Narkiewicz et al., 1999a). The increased ventilatory response is evident even during normoxia. This increase is associated with increased sympathetic outflow to the skeletal muscle vasculature during daytime in patients with OSA in response to sympathetic nervous connection with the carotid bodies, explained by the enhanced chemoreceptors sensitivity. However, in healthy individuals there is an interaction between the chemoreflex and the baroreflex responses. Baroreflex dysfunction might affect the function of the ventilatory, sympathetic and cardiac responses to peripheral chemoreflex excitation (Caples et al., 2005).

Hypoxaemia, in addition to its reflex effects via stimulation of peripheral chemoreceptors, can stimulate cardiac vagal activity directly, resulting in bradycardia (Caples et al., 2005; Harper et al., 2003). Additionally, the negative pleural pressure due to respiratory efforts against a narrowed or collapsed airway is a hallmark of OSA. This in itself reduces cardiac output and blood pressure, effects that are more profound and prolonged in those with heart failure (Caples et al., 2005).
Figure 1.3: A schematic diagram showing the physiological response to the obstructive events in obstructive sleep apnoea
1.5.2.2 Pathophysiological response to OSA during wakefulness

As noted above, it has been found that there is an increased level of muscle sympathetic nerve activity and other abnormalities in cardiovascular regulation during daytime wakefulness when patients with OSA are breathing normally and no evidence of hypoxia or chemoreflex activation is apparent (Caples et al., 2005). This is true whether these patients are newly diagnosed or never treated OSA patients or on no medications or on antihypertensive therapy (Carlson et al., 1993; Hedner et al., 1988; Narkiewicz et al., 1998; Somers et al., 1995). Several mechanisms may contribute to the maintenance of higher muscle sympathetic outflow and blood pressure. These mechanisms include chemoreflex (Hedner et al., 1992; Narkiewicz et al., 1999b) and baroreflex (Carlson et al., 1996; Narkiewicz et al., 1998) dysfunction, vasoconstrictor effects of nocturnal endothelin release (Phillips et al., 1999) and endothelial dysfunction (Kato et al., 2000).

There are major cardiovascular consequences of OSA. The daytime increase of MSNA in these patients has been connected to the development of increased BP, heart failure, cardiac arrhythmias and increased cardiovascular morbidity and mortality (Somers et al., 1995). However, the mechanisms underlying the link between OSA and cardiovascular diseases are not completely understood. There is growing evidence showing the chronic sympathetic activation is a key mechanism underlying the elevated cardiovascular morbidity in OSA.

1.5.3 Central neural manifestations in OSA

Until now much of the literature focusing on the neurophysiological changes in OSA has been limited to peripheral changes, with microneurography having provided a powerful tool in understanding the pathological mechanisms underlying
OSA. Human brain imaging technology has only recently advanced to the stage where individual nuclei in the brainstem can be structurally and functionally identified (Zimmerman & Aloia, 2006a). Neuroimaging has made great contributions to the understanding of the brain in general and the changes that happen in the brain of individuals with OSA. It can clarify abnormalities in neural control over hormonal and autonomic functions and respiratory function in OSA by studying the relationship between respiratory challenges and brain functions. It also can show damage in areas responsible for memory and cognition. Neuroimaging can also identify abnormalities in vascular function in OSA. In addition, it can be used to investigate and identify the brain’s response to treatment, which might work as potent motivator for individuals with OSA who are struggling with treatment adherence.

1.5.3.1 Neurofunctional abnormalities in brain regions due to OSA

With the increasingly sophisticated scientific approach of OSA and neuroimaging, in particular functional magnetic resonance imaging (fMRI), has come a greater understanding of the disorder. fMRI is a non-invasive technique that provides high spatial resolution. fMRI is generally acquired using the Blood-Oxygen Level-Dependent (BOLD) approach, in which signal intensity is dependent on the differences in the paramagnetic properties of oxygenated and deoxygenated blood; as such it serves as a proxy marker for changes in regional cerebral blood flow associated with an increase of synaptic activity in the brain. This technique is ideal to investigate the acute changes in the brain associated with the performance of various challenges. Current fMRI studies of OSA utilize either respiratory or cognitive challenges, designed to activate specific brain regions during brain scanning.
Respiratory challenges have employ forced alterations of breathing effort to evoke changes in the autonomic nervous system similar to those experienced by individuals with breathing disorders. Conversely, cognitive challenges represent neural mechanisms associated with performance on complex mental tasks.

1.5.3.1 Neuronal abnormalities

Functional neuroimaging studies employing cardiorespiratory challenges that alter heart rate and blood pressure, such as the Valsalva manoeuvre, cold pressor test, sustained hand grip exercise and inspiratory and expiratory loading, have shown changes in activity in the human brain, similar to those previously identified in animal studies (Cechetto, 1987), to be involved in cardiovascular control (Critchlet et al., 2000; Harper et al., 2000; King et al., 1999; Topolovec et al., 2004). Recent studies have shown widespread neural differences in motor, sensory and autonomic brain regions in OSA patients compared to controls (Harper et al., 2003, 2012; Macey et al., 2003, 2013). The Valsalva manoeuvre, in which the subject is asked to exhale vigorously against a resistance, is used clinically to assess autonomic nervous system function. Using this manoeuvre to compare regional changes between OSA and control subjects supported results of the structural changes in the grey matter loss associated with OSA in left inferior parietal lobe, left precentral gyrus, anterior superior temporal gyrus, superior frontal gyrus, posterior insula, cerebellum, anterior cingulate and hippocampus (Henderson et al., 2003; Macey et al., 2002; Woo et al., 2007). The cold pressor response, an increase in blood pressure during application of a cold stimulus to the participant’s forehead, also uncovered differences in multiple brain regions, particularly the cerebellum and limbic areas, in OSA patients (Harper et al., 2003; Woo et al., 2005).
During expiratory resistive loading, this group also found signal decreases in OSA patients in the right insula, left anterior cingulate and middle frontal gyrus. Conversely, greater signal increases occurred in the right hippocampus, ventral midbrain, left dorsal midbrain and right ventral pons in OSA patients. Variability in neural responses in patients with OSA was noted in the amygdala, cerebellum and posterior insula, regions suggested to mediate the neural response to resistive breathing challenges and to play a role in the abnormal physiology associated with OSA (Macey et al., 2003).

A more recent study examined inspiratory loading - prolonged periods of inspiration followed by brief expiration (Macey et al., 2006). This technique generates an increase in negative pressure on the upper airway, resulting in changes in BP similar to those observed in individuals with OSA. Similar to the results from the three previously mentioned respiratory challenges, altered neural signal intensities in response to inspiratory loading were observed in patients with OSA in multiple brain regions, overlapping those regions that were shown previously to be associated with grey matter volume abnormalities (Macey et al., 2002). Differences in signal intensity were mainly observed in the basal ganglia and left insula, in addition to the medial cingulate cortex, right ventral posterior thalamus, anterior thalamus, right hippocampus, left medial temporal cortex, medial midbrain and cerebellum. Changes in these motor, sensory and autonomic regions of the brain were suggested to trigger the nocturnal pathologic breathing abnormalities in patients with OSA.

1.5.3.1.2 Cognitive abnormalities

It is known that OSA is associated with various cognitive deficits, including compromised spatial and working memory. Short-term memory impairments also
appear in the majority of OSA patients, and these can persist after CPAP therapy (Ferini-Strambi et al., 2003). The structures that serve those memory roles include the hippocampus, the fornix (the principal fibre output of the mammillary bodies), the mammillary bodies and their projections to the anterior thalamus and downstream sites (Buckley et al., 2004; Lavenex et al., 2006; Ridley et al., 2004; Santín et al., 1999). It has been shown that in OSA there is a reduction in the volume of the mammillary bodies and reductions in cross-sectional area of the fornix, which conveys projections from the hippocampus to the mammillary bodies (Kumar et al., 2008, 2009). In comparison to healthy subjects, there was a reduction in the grey matter concentrations of OSA patients in the hippocampus (Joo et al., 2010; Macey et al., 2002, 2003). Other neuroimaging data have provided evidence of hippocampal atrophy in OSA patients, with a linear relationship between hippocampal volume and memory performance (Gale & Hopkins, 2004).

It has been suggested that the hippocampus also participates in several physiological functions. The hippocampus contains neurons that discharge with the respiratory and cardiac cycle, and electrical stimulation within the hippocampus evokes dramatic changes in blood pressure. A direct chemical stimulation of the hippocampus evokes marked decreases in arterial pressure and heart rate (Ruit & Neafsey, 1988). It has been suggested that this brain region may play a modulatory role in the control of blood pressure and heart rate, as hippocampal blockade in animals does not affect resting arterial pressure or heart rate (Wang & Ingenito, 1992). Interestingly, OSA and control subjects showed significant differences in heart rate responses during the cold-pressor test, which supports a reduced modulatory role of the hippocampus in OSA (Harper et al., 2003; Macey et al., 2013).
Several studies have suggested that hypoxemia in OSA increases the risk for cognitive impairment (Findley et al., 1986; Yaffe et al., 2011). An animal model of has shown that IH results in impaired executive function, excessive sleepiness and sensitivity to sleep deprivation (Sanfilippo-Cohn et al., 2006; Veasey et al., 2004; Zhan et al., 2005). This animal model has been a useful way to demonstrate cognitive impairment due to IH, and the hippocampal injury associated with significant memory and other cognitive and behavioural issues (Row et al., 2003). There is evidence of neuronal and glial injury in the hippocampus (Gozal et al., 2002; Hambrecht et al., 2007) and basal forebrain (Row et al., 2007; Veasey et al., 2004) as a result of hypoxemia.

Functional studies employing cognitive tasks reported, depending on the cognitive task, either lack of brain activation in dorsolateral prefrontal cortex (dLIPFC) or increased neural response in frontal lobe, cingulate, thalamus, cerebellum and temporoparietal junction (Ferini-Strambi et al., 2013; Zimmerman & Aloia, 2006b). Thomas and colleagues (2005) reported absence of dLIPFC activation and decreased working-memory speed in OSA patients compared to controls. However, after 8 weeks of compliant use of continuous positive airway pressure (CPAP), patients showed partial recovery of parietal activation but continued to exhibit an absence of dorsolateral prefrontal activation. dLIPFC recently has been shown to display MSNA-related activity (James et al., 2013). Therefore, the data from Thomas and colleagues (2005) support the functional autonomic dysfunction in OSA. Another study (Ayalon et al., 2006) suggested recruitment of additional neural resources consistent with an adaptive compensatory response. Inconstancies between the former two studies are likely due to differences in the cognitive-challenge paradigm and severity of OSA (Ayalon et al., 2006; Thomas et al., 2005).
1.5.3.2 Neuroanatomic abnormalities due to OSA

Structural magnetic resonance imaging (sMRI) is a very important tool that allows the examination of neuroanatomic volumetric and morphometric abnormalities that may be involved in the pathologic processes associated with OSA. There is mixed support of structural abnormalities due to OSA, but it is generally accepted that OSA is associated with several structural changes in the brain.

1.5.3.2.1 White matter changes

A few early studies showed no group differences in deep white matter and periventricular hypersensitivity between severe OSA male patients and matched healthy controls (Davies et al., 2001). In addition, the Sleep Heart Health study (Ding et al., 2004) showed no association between the frequency of apnoeas and hypopnoeas with brainstem white matter disease in adults over the age of 68 years. The authors of this study suggested that the negative findings might be related to the mild severity of sleep-disordered breathing in their population sample, survival biases or the high comorbidity of other vascular risk factors that may be present in their patient sample. In contrast, Diffusion Tensor Imaging (DTI) has revealed extensive damage to white matter tracts linking major structures within the limbic system, pons, frontal, temporal and parietal cortices, and projections to and from the cerebellum in OSA patients (Macey et al., 2008). A more recent study, utilising the same method, showed significant damage and reduced regional mean diffusivity in multiple brain sites in OSA: medulla, cerebellum, basal ganglia, the prefrontal, frontal, limbic and insular cortices, the cingulum bundle, external capsule, corpus
callosum, as well as damage to the temporal and occipital regions and the corona radiate.

1.5.3.2.2 Regional brain metabolic changes

Regional brain metabolism may provide insight into abnormalities in the neurochemical transmission that may reflect pathologic insults to the brain integrity. Changes in glucose metabolism were studied using positron emission tomography (PET) in OSA and showed impaired glucose utilisation in the frontal, temporal and/or parietal cortex (Antczak et al., 2007), despite treatment with CPAP. More recently, significant hypometabolism was observed in the bilateral prefrontal areas, left cuneus and left cingulate cortex; these persisted after CPAP (Ju et al., 2012).

Proton magnetic resonance spectroscopy (MRS) of the periventricular white matter was performed to compare cerebral metabolism in OSA and healthy controls, yet showed no anatomical abnormalities in OSA. However, OSA patients had lower N-acetylaspartate-to-choline (NAA/Cho) ratios for cerebral white matter than controls, and moderate to severe OSA patients were worse than those with mild OSA (Kamba et al., 1997). A decrease in the ratio has been utilised as an indicator of cerebral metabolic injury, such as gliosis and impairment of neuronal and axonal function (Larsson et al., 1991; Meyerhoff et al., 1994; van der Grond et al., 1995), which the authors hypothesised is due to the repetitive episodes of nocturnal hypoxia (Kamba et al., 1997). The same group studied the effect of potentially confounding comorbid conditions in OSA and found that NAA/Cho ratio decreased with age. Their findings were supportive of those previously obtained and they explained that the severity of OSA may be associated with the degree of white matter metabolic impairment that may be related, in part to comorbid cerebrovascular risk factors (Kamba et al., 2001).
The same method was used to study changes in the left hippocampus associated with OSA (Bartlett et al., 2004). They revealed a significant increase in N-Acetylaspartate-to-Creatine (NAA/Cre) ratio in the hippocampus, which was significantly correlated with arousal index but not with total respiratory disturbance index or average oxygen desaturation. Lower levels of NAA/Cre ratio in frontal regions of OSA could be related to neuronal loss (Sarchielli et al., 2008). Patients with OSA had lower levels of creatine-containing compounds related to intermittent hypoxemia associated with the disorder, which was correlated with the severity of OSA and observed neurocognitive determents. Creatine is involved in energy homeostasis and has been shown to improve performance on cognitive tests (Rae et al., 2003). Further similar examinations reflected white matter impairment in individuals with moderate to severe OSA (Alchanatis et al., 2004).

1.5.3.2.3 Greymatter changes

In patients with moderate to severe OSA, high resolution T1 weighted MRI has uncovered evidence of extensive loss of grey matter volume in areas including the raphé magnus, hypothalamus, anterior thalamus, hippocampus, caudate nuclei, cerebellum (cerebellar cortex, deep cerebellar nuclei and vermis) and various areas of cerebral cortex (temporal, parietal, prefrontal, occipital, insular, cingulate and ventral frontal cortices) (Macey et al., 2002, 2008). These areas are not only implicated in the cognitive deficits observed in OSA, but also in the fine motor regulation of the upper airway (Macey et al., 2002). These observed differences may therefore be both a cause of and/or a result of OSA (Gozal, 2002). The severity of OSA showed a positive relation to the volumetric decline (Macey et al., 2002). The same group observed that some of the differences observed are bilateral and diffuse.
in nature, as would be expected from repetitive hypoxic insults. However, some of the other changes are not the sort one might expect from the hypoxic action experienced in OSA over time. Specific sites of damage on one side of the cortex, such as in the left ventral lateral frontal cortex, are indicated as a possible cause of OSA, as this area is known to help control upper airway motor function and speech production. Damage observed in the cerebellum is also unilateral. This has been known to induce OSA, which is not surprising given its role in fine motor control during wakefulness.

Conversely, O’Donoghue and his colleagues (2005) found no evidence of grey matter changes related to OSA. A slight but significant decrease in whole brain volume, however, was observed in patients with OSA following 6 months of CPAP therapy. The positive findings of Macey and his group may have resulted from inclusion of patients with comorbid medical and psychiatric conditions. However, Macey and colleagues (2005) argue that the positive findings are explained by utilizing a statistical threshold that allowed the detection of known age-related effects of the brain, whereas the other group stated that their negative findings resulted from using an MRI scanner with a stronger field strength, homogeneous sample and statistical corrections for multiple comparison and age (O’Donoghue et al., 2005). Both groups appear to agree that the effects of OSA on brain structure are relatively small and may not be evident when highly stringent and conservative statistical methods are applied. However, there is a converging evidence suggesting that the hippocampus may be atrophic in these patients (Gale & Hopkins, 2004; Morrell et al., 2003). More recent studies showed reduction in grey matter volume in the right middle temporal gyrus and cerebellum (Morrell et al., 2010), left hippocampus, left
posterior parietal cortex and right superior frontal gyrus (Canessa et al., 2011); this was associated with cognitive dysfunction.

1.6 An overview of diagnosis of OSA.

In the view of the high prevalence of OSA, and potentially serious consequences of untreated OSA, investigations are essential to determine the most effective treatment. There are several methods that have been used to aid the clinician to determine the probability of OSA and quantify the severity of the disease. Currently, the methods used to diagnose OSA are mainly the following:

1.6.1 Morphometric examination:

Morphometric examination of the head and neck; this is very simple and time efficient (Gottlieb et al., 1999).

1.6.2 Patient questionnaires:

Patient questionnaires, (the Stanford Sleepiness Scale (SSS) or the Epworth sleepiness scale (ESS) (Hoddes et al., 1972; Johns, 1991). There are 8 questions in the ESS, which ask the patient how likely they are to doze off in certain situations. A score greater than 10 out of possible 24, indicates subjective daytime sleepiness.

1.6.3 Polysomnography:

Polysomnography is the gold standard technique for the diagnosis of OSA. This involves electroencephalography (EEG), submental electromyography (EMG), electroocculography (EOG), electrocardiography (ECG), measurement of nasal airflow and respiration from the chest and abdomen and pulse oximetry.
Generally, EEG studies use electrodes attached to the scalp overlying the frontal, central and occipital lobes of the brain, picking up electrical signals originating from neurones in the cortex. Signals of brain activity can be ‘scored’ to different stages of sleep, based essentially on the frequency content of the signals. Signals from EOG and submental EMG help to determine when sleep and rapid eye movements (REM) occur. REM is a sleep stage characterized by rapid and random movement of the eyes and low muscle tone. Additionally, signals from ECG monitors the electrical activity of the heart that can be analysed for any underlying heart pathology or abnormality.

Nasal airflow can be measured by a pressure transducer or thermocouple fitted in the nostrils. Respiration from the chest and abdomen is measured by the use of inductance or strain-guage belts around the chest and abdomen, allow respiratory rate to be measured and identifying any interruptions in breathing. Blood oxygen saturation is measured via pulse oximetry. The pulse oximeter fits around the fingertip or the ear lobe and allows a continuous monitoring of blood oxygen changes that occur with sleep apnoea and other respiratory problems. Finally, a complete polysomnongraphy test will also include a sound probe over the neck to record snoring.

### 1.7 Treatment of sleep apnoea

An effective therapeutic approach must be started as soon as the diagnosis of OSA has been established. The ultimate goal of OSA treatment is to maintain continuous sleep and to restore airway patency to improve the sleep quality and in return improve daytime functioning and quality of life. The successful treatment is reflected by a reduction, if not cessation, of the clinical symptoms that includes the
daytime sleepiness, fatigue and snoring. As well as decrease in the AHI and increase in the oxyhemoglobin saturation level (Cutler et al., 2002), it is known that extensive structural damage of the brain occurs in OSA, but it is not known whether these changes are reversible.

Although several treatments exist, they are often either poorly tolerated or only partially alleviate abnormalities. Surgical and non-surgical methods are the two major pathways used for OSA treatment.

1.7.1 Surgical treatment of OSA

Whilst surgery used to be a very popular treatment for OSA, its use now is rather more limited. Surgical treatment of OSA varies according to the cause of the obstruction, but the most common surgical procedure is the uvulopalatopharyngoplasty (UPPP) (Shepard & Olsen, 1990). This procedure involves removal of part of the soft palate, uvula and tonsils as well as part of the posterior pharyngeal wall. UPPP may improve snoring but is unlikely to cure OSA, particularly in more severe cases, and results may worsen over time (Guilleminault et al., 1983; Katsantonis et al., 1988; Simmons et al., 1983, 1984). Adenotonsillectomy is most effective when the patient has enlarged tonsils, especially in children (Bhattacharjee et al., 2010). Additionally, there are more extensive procedures available for selected cases where craniofacial abnormalities are impinging on the upper airway.

1.7.2 Non-surgical treatment of OSA

Non-surgical treatments involve the modification of behavioural factors, such as weight, smoking and consumption of alcohol, in addition to the medical use of the
oral or dental devices and nasal continuous positive airway pressure (CPAP) (Sutherland & Cistulli, 2011).

1.7.2.1 Lifestyle and behavioural changes:

Losing weight through diet and exercise can be helpful particularly amongst patients with mild OSA (Foster et al., 2009a; Sampol et al., 1998; Sánchez et al., 2009). The limitation of weight reduction as a treatment for OSA is that losing weight is challenging for many patients. Few patients achieve long-term maintenance of reduced bodyweight (Sánchez et al., 2009), but education and support is very helpful. A previous study suggested that weight increases after treatment with CPAP, therefore diet and exercise advice is necessary for those patients (Redenius et al., 2008). On the other hand, smoking was associated with increased inflammation of the upper airway, nasal stuffiness, reduced airway sensation and reduced arousal threshold or frequent arousals due to unstable sleep (Jordan et al., 2014). Alcohol can worsen OSA symptoms as it works as a depressant (Sakurai et al., 2007; Scrima et al., 1982); alcohol can relax the pharyngeal muscles allowing the pharyngeal walls to collapse more easily.

1.7.2.2 Oral devices:

Oral devices are worn during sleep and are usually individually moulded to fit the teeth. Different oral devices work in different ways but generally apply pressure to the jaw to prevent retroglossal collapse (Jordan et al., 2014) by moving the mandible forward in relation to the maxilla and hence enlarging the upper airway. The degree of advancement is individualised according to what the patient can tolerate, offset against the potential for occlusal changes (Robertson et al., 2003). Since the tongue is attached to the mandible, the tongue is held forward - preventing
it from falling backwards and causing obstruction. However the efficacy of oral devices varies and little data on the outcomes have been collected (Gotsopoulos et al., 2002; Holley et al., 2011; Itzhaki et al., 2007; Mostafiz et al., 2011). Also several visits (6-9 months) are needed for gradual titration to achieve a satisfactory outcome. Although the device and the repeated visits to the dentist can be costly, oral devices are likely to be cost effective if they are successful. Well-designed oral devices can halve the AHI in patients with moderate to severe OSA. Halving the AHI the whole night might be preferable to CPAP, eliminating the AHI for some of the night for patients unable to fully comply with CPAP therapy (Lim et al., 2004).

1.7.2.3 Continuous positive airway pressure:

Currently, CPAP is the most common and most effective treatment for OSA. The original CPAP device was first developed by Dr Colin Sullivan and his associates in 1981 in Australia (Sullivan et al., 1981). Delivered via a nasal or oronasal mask, pressurised air is used as a pneumatic stent to keep the upper airway open during sleep, preventing the collapse of these compliant airways by the negative intrathoracic pressure associated with inspiration. Randomised trials showed significant improvements in symptoms with CPAP (Gay et al., 2006; Jenkinson et al., 1999), including sleepiness (Engleman et al., 1994; Montserrat et al., 2001; Patel et al., 2003) and overall quality of life of these patients (Malhotra & White, 2002). In addition, CPAP has been shown to reduce the risk of motor vehicle accidents to control levels (Findley et al., 2000; Lindberg et al., 2001; Strohl et al., 2013).

Generally, CPAP has been shown to cause cognitive improvement (Aloia et al., 2004). More recent studies, that utilised a within-subjects design, showed after 3 and 6 month of CPAP therapy, significant improvements in memory (Joseph et al., 2009;
Zimmerman et al., 2006), attention and executive function (Aloia et al., 2003; Gale & Hopkins, 2004). Other studies that utilized case-control designs showed improvements in memory, executive attention, spatial ability and motor speed with CPAP treatment (Canessa et al., 2011; Ferini-Strambi et al., 2003; Tonon et al., 2007). Additionally, there is growing evidence that suggests improvement of brain morphology with CPAP treatment. Increased grey matter volume was reported after 3 months of CPAP treatment in hippocampal and frontal structures (Canessa et al., 2011). An important observation of the metabolic reversibility of the hippocampus was also seen after 6 months of treatment (O’Donoghue et al., 2012).

Physiologically, long-term treatment with CPAP has been found to significantly decrease MSNA (Hedner et al; 1995; Imadojemu et al., 2007; Narkiewicz et al., 1999a; Somers et al., 1995; Waradkar et al., 1996). Furthermore, some observational studies suggested that CPAP treatment reduces the incidence of fatal and nonfatal cardiovascular events in patients with moderate and severe OSA (Buchner et al., 2007; Marin et al., 2005).

Large cross-sectional and longitudinal epidemiological studies (Lavie et al., 2000; Peppard et al., 2000), randomised trials (Alajmi et al., 2007; Becker et al., 2003; Dimsdale et al., 2000; Faccenda et al., 2001; Montesi et al., 2012; Pepperell et al., 2002) and non-randomised trials showed that treatment reduced systemic BP (Hla et al., 2002). On the other hand, Engleman and his colleagues (1996) showed no effect on BP by CPAP in severe OSA patients. However, in the subgroup of patients who did not have the usual overnight dip in BP, daytime BP was reduced by CPAP. The authors interpreted these findings as indicating that CPAP would not reduce BP in a heterogeneous sample of people with OSA, but might do so in those most at risk of cardiovascular disease. Other studies (Barbé et al., 2001, 2012) showed that in
OSA patients without daytime sleepiness, CPAP had no effect on BP. Moreover, the reduction of BP seems to be small (2-3 mmHg) and less than that gained by treatment with antihypertensive drugs (Pépin et al., 2010). Nevertheless, it has been concluded that CPAP improves the symptoms of OSA in addition to the hypertension (Barbé et al., 2012).

Although CPAP has been shown to improve quality of life and survival, the use of the device is limited by poor compliance (Cutler et al., 2002). As many as 46-83% of patients fail to adhere to treatment over the long term (Weaver & Grunstein, 2008). A number of reasons limit the adherence to CPAP therapy. These include mask problems (skin irritation/allergies, claustrophobia and mask leak), pressure related problems (headaches, sinusitis, rhinitis) and equipment-related issues (noise and smell) (Gay et al., 2006). Large clinical studies have concluded that adherence was related to pre-treatment OSA severity: a higher AHI is associated with better long term adherence (Gay et al., 2006; McArdle et al., 2000). Also, mask interface type and effective humidification lead to better adherence (Massie & Hart, 2003). More recent evidence has also highlighted that in addition to the biophysical factors contributing to CPAP adherence, psychosocial attitudes and patient’s health beliefs may play a major role in determining adherence (Olsen et al., 2008; Sawyer et al., 2010).
1.8 Aims

The aims of my research are to investigate the underlying neurophysiological disturbances associated with OSA, and the effects of CPAP treatment in a longitudinal design. The research involves invasive and non-invasive studies – direct recording of muscle sympathetic nerve activity via intraneural microelectrodes, as well as functional and structural magnetic resonance imaging (MRI) of the brain. Importantly, much of the work involves concurrent recording of MSNA and MRI, an approach developed in our laboratory. My research comprises four studies:

Study I: Respiratory and cardiac modulation of MSNA in OSA

The mechanism of the sympathoexcitation and how it leads to hypertension is generally poorly understood, other than that it is related to the long-term effects of hypoxia on MSNA. Given that an increase in respiratory-sympathetic coupling has been argued as being an important contributor to the increase in BP in the spontaneously hypertensive rat, and to human hypertension (Czyzyk-Krzeska & Trzebski, 1990; Simms et al., 2009; Moraes et al., 2014), I tested the hypothesis that respiratory modulation of MSNA is increased in OSA and that this elevated modulation is reduced after CPAP therapy. Moreover, because of the higher levels of MSNA in OSA I also predicted that cardiac modulation of MSNA would be lower in OSA.
Study II: Functional and structural changes in the brain associated with the increase in MSNA in OSA

It is believed that the increase in the sympathetic drive in OSA is centrally driven through the long-lasting effects of nocturnal hypoxaemia, leading to hypertension. By recording MSNA concurrently with functional Magnetic Resonance Imaging (fMRI) I aimed to identify the central processes responsible for the sympathoexcitation. I hypothesised that the increase in MSNA in OSA is associated with increases in signal intensity in the regions that modulate MSNA, including the cingulate cortex, prefrontal cortex, insula and hypothalamus.

Furthermore, by using voxel-based morphometry (VBM) to assess regional grey matter changes, I tested the hypothesis that the increase in MSNA in OSA is associated with altered functional and structural changes in previously defined higher brain regions that modulate MSNA, resulting from hypoxic-related changes.

Study III: Functional and structural changes in the brainstem associated with the increase in MSNA in OSA

By performing high spatial-resolution imaging of the brainstem in this study I aimed to functionally identify brainstem sites, and associated structural changes, responsible for the sympathoexcitation in OSA. I tested the hypothesis that the increase in MSNA in OSA would be associated with altered functional and structural changes in brainstem regions known to modulate MSNA, such as the rostral and caudal ventrolateral medulla, nucleus tractus solitarius and dorsolateral pons, as well as the midbrain periaqueductal grey matter.
Study IV: Reversal of functional changes in the brain associated with obstructive sleep apnoea following 6 months of CPAP

CPAP is the most effective and widely used treatment for OSA. In addition to improving sleep, CPAP decreases daytime MSNA towards control levels. It remains unknown how this restoration of MSNA occurs, in particular whether CPAP treatment results in a simple readjustment in activity of those brain regions responsible for the initial increase in MSNA or whether other brain regions are recruited to over-ride aberrant brain activity. In this investigation I aimed to assess brain activity associated with individual subject’s MSNA burst patterns prior to and following 6 months of CPAP treatment. I tested the hypothesis that the activity in those brain regions associated with increased spontaneous MSNA would return to control levels as the augmented MSNA seen in OSA returned to control levels following CPAP treatment.
Chapter 2:

General Methods
2.1 Project overview

Subjects were recruited for this study if they expressed an interest in taking part and if they were either \textit{a}) well-characterized and newly diagnosed OSA patients, as assessed by overnight polysomnography, or \textit{b}) healthy controls. Potential participants were screened for their willingness to take part and for contraindications to MRI scanning, such as the presence of a pacemaker, metal shards (e.g. shrapnel) or other metal implants, or claustrophobia. The experimental session included a pre-MRI recording in the laboratory, in which participants lay on an MRI-compatible bed. This session involved insertion of a tungsten microelectrode into a muscle fascicle of the common peroneal nerve (microneurography) to record spontaneous bursts of muscle sympathetic nerve activity (MSNA), together with electrocardiography (ECG) and recording of non-invasive continuous blood pressure (BP) and respiration. Following recording of these physiological data the microelectrode was left in situ and the subjects were wheeled to the MRI facility for brain scanning. The MRI session included a structural T1-weighted image set of the entire brain and two series of concurrent recording of MSNA and fMRI - the first covering the entire brain and the second being limited to the brainstem.

Following this, OSA patients had another overnight polysomnographic study at the Prince of Wales Hospital to determine the CPAP pressure required to abolish the apnoeic events. Experimental sessions were repeated on the OSA patients after 6 months of CPAP treatment for all studies \textit{(I-IV)}. An additional microneurography session at the end of one year of CPAP treatment was performed on OSA patients in study \textit{I} to provide an insight into the physiological response to long term therapy. In addition to a face-to-face or telephone meeting to follow-up with these patients about their self-reported compliance, compliance with prescribed CPAP treatment
was based on an automated download of the CPAP device. Furthermore, each OSA subject completed the Epworth Sleepiness Scale (ESS) questionnaire to measure their daytime sleepiness before and after treatment.

### 2.2 Subject recruitment

All patients with OSA were recruited by Prof David McKenzie, Head of Respiratory Medicine, Prince of Wales Hospital. Control subjects matched for age and gender were recruited from within a healthy cohort. All subjects took part in this study during the period from May 2011 to May 2013. As each study in this thesis used a particular subject population, the specific numbers and demographics are detailed in each study’s Method’s section.

All volunteers remained on their optimal pharmacological and physiotherapeutic treatment. Informed written consent was obtained from all subjects in accordance with the Declaration of Helsinki. This study was approved by the Human Research Ethics Committee of the University of Western Sydney (approval H8984) and the University of New South Wales (approval HREC11138), with written consent signed by all participants.

### 2.3 Subject assessment

#### 2.3.1 OSA patients assessment

Prior to the experiment, all participants completed the ESS. All OSA subjects were evaluated at the sleep laboratory of Price of Whales hospital for one night and they were monitored continuously for 8 hours using 12-channel polysomnograph,
electroencephalographic (EEG) (C3/A2, C4/A1, O1/A2, O2/A1) electrooculographic (EOG) and chin electromyographic (EMG) recordings were obtained with surface electrodes according to standard methods. Nasal and oral airflow were monitored by non-invasive sensors (thermistor). Chest and abdominal movement were assessed by respiratory inductive plethysmography. Oxyhaemoglobin saturation was recorded all night by finger pulse oximetry. A microphone was placed on the lower neck to record snoring and a camera sensitive to ultraviolet light was recorded patient movements during sleep. The overnight study was analysed offline the following morning by expert sleep scientists. Apnoeas, hypopnoeas were defined according to the international classification of sleep disorders, by using both Alice software (2.8.78, Respironics 1999, 2010) and Somnologica (3.3.1.1529, 1996-2004).

After the baseline experiment the patients with OSA were willing to commence CPAP therapy. At the sleep clinic OSA subjects underwent an overnight based CPAP titration polysomnography to establish the therapeutic CPAP setting. All patients were followed up by their referring physician. In addition, all patients were fitted with a suitable mask and issued a fixed-setting CPAP machine (Series 9, ResMed, Sydney, Australia).

Patients were encouraged to use the machine as much as possible. The CPAP device (Series 9, ResMed, Sydney, Australia) used by the patients contained a memory chip for storing the duration and pressure level, as prescribed by the physician, at which the unit was in use. Additionally, the memory chip stored the AHI, compliance and any mask leak. Compliance was monitored at 1, 6 and 12 months after commencement of treatment.
2.3.2 Healthy controls assessment

All control subjects performed an overnight home apnoea screening test (via ApneaLink™, Resmed). Apnealink records the following data: respiratory nasal airflow, snoring and blood oxygen saturation. Nasal airflow is recorded via a nasal canula to a differential pressure transducer attached to the front of the patient’s chest. Flow measurements were digitalized for storage and downloaded to a computer at a later time. Analysis of flow signal was fully automated. Default settings reports AHI according to the AASM (American Academy of Sleep Medicine) definitions for scoring guidelines.

2.4 Procedures for pre-MRI session

Subjects lay supine on a MRI bed with their knees supported on a foam cushion. Multiunit MSNA, heart rate, BP and respiration from the abdomen and chest were recorded continuously for ten minutes of undisturbed resting condition, of which the final 5 minutes were used for analysis. These recordings were repeated after 6 and 12 months of treatment with CPAP.

2.4.1 Electrocardiography

Heart rate was derived from the inter-beat interval measured between consecutive R-waves of the ECG (0.3 Hz – 1.0 kHz) complexes recorded using Ag-AgCl surface electrodes placed on the chest and sampled at 2 kHz using a PowerLab 16SP hardware data acquisition system and LabChart 7 software (ADInstruments, Australia). Three ECG surface electrodes were used, one electrode was placed on the right clavicle and the other two electrodes were placed on the area around the outer margin of the left and right fifth – sixth intercostals spaces respectively.
2.4.2 Blood Pressure

Continuous non-invasive blood pressure was recorded using radial arterial tonometry at the wrist (Colin 7000 NIBP, Colin Corp., Japan). Arterial pressure was sampled at 400 Hz.

2.4.3 Respiration.

Respiration was recorded using MR-compatible piezoelectric transducers embedded in bands wrapped around the chest and abdomen (Pneumotrace II, UFI, Morro Bay CA, USA) and sampled at 100 Hz.

2.4.4 Muscle sympathetic nerve activity.

The common peroneal nerve was located at the fibular head by delivering weak electrical pulses (<5 mA, 0.2 ms, 1 Hz) from an isolated constant-current stimulator (Stimulus Isolator, ADInstruments, Sydney, Australia). An insulated tungsten microelectrode (0.2 mm diameters, ~2 mΩ impedance, Frederick Haer and Co (FHC), Bowdoinham, ME, USA) was inserted into a muscle fascicle of the common peroneal nerve as shown in Figure 2.1. An uninsulated reference microelectrode was inserted subcutaneously ~2 cm from the recording microelectrode. An Ag-AgCl ground electrode was attached to the medial aspect of the knee. Action potentials generated by axons were recorded as voltage differences between the recording microelectrode and the reference electrode. Nerve signals from the electrodes were amplified via a stainless-steel MR-compatible headstage (gain 100x, bandpass 0.1-5.0 kHz) and further amplified (total gain 2 x 10^4) and band-pass filtered (0.3 – 5.0 kHz) using an isolated amplifier (NeuroAmpEX, ADInstruments, Sydney, Australia) and stored on computer (10 kHz sampling) using a PowerLab 16SP hardware data.
acquisition system and Chart 7 software (ADInstruments, Australia). Sympathetic nerve activity was also displayed as an RMS-processed signal (root mean square, moving average time-constant 200 ms) or integrated signal (time-constant 200 ms) and sampled at 800 Hz. This is illustrated in Figure 2.1.

**Figure 2.1:** Standard multi-unit recording of muscle sympathetic nerve activity via an insulated tungsten microelectrode inserted into a muscle fascicle of the common peroneal nerve

Note that bursts of MSNA are composed of negative-going spikes that occur with a clear cardiac rhythmicity. The integrated nerve signal (or RMS-processed) signal was used to count bursts.
2.4.4.1 Confirming localization of electrode within a motor fascicle.

In order to identify a motor fascicle supplying the ankle, toe extensor and foot evertor muscles, weak electrical pulses were applied to the recording electrode to evoke muscle twitches in the leg muscles. The initial electrical search stimuli (0.1 ms pulse duration, 0.01 - 1 mA) were delivered at a frequency of 1 Hz using a programmable, optically isolated constant-current stimulator (MacLab stimulus isolator, ADInstruments, Australia). Once the electrode was advanced into the nerve muscle, twitches were observed at very low stimulation intensity (< 100 µA). Once this was achieved the stimulating lead was disconnected from the stimulator and the nerve signals amplified and routed to a loudspeaker and the data acquisition system (PowerLab 16SP; ADInstruments, Australia). Neural activity was sampled at 10 kHz.

2.4.4.2 The search for afferent nerve responses.

In order to confirm that the electrode is positioned within a muscle fascicle the nerve signal was monitored for afferent nerve activity in response to tapping and passive stretch of the muscle, as described by Vallbo and his colleagues (1979). The electrode was adjusted until a clear increase of muscle afferent activity was established; there was no response to stroking of the skin, indicating that the microelectrode was not located in a cutaneous fascicle.

2.4.4.3 The search for spontaneous sympathetic nerve activity

Characteristic differences have been found in the spontaneous sympathetic bursts recorded in fascicles supplying muscle or skin. For the purpose of this project, only MSNA was studied. Therefore, a functional test of the autonomic discharge was applied to confirm that the sympathetic outflow recorded is from a muscle nerve.
fascicle and not skin. These functional tests involve first asking the subjects to relax with their eyes closed, when the researcher made sudden stimuli such as a clap or a loud nose. Muscle sympathetic nerve activity was stable and was not influenced by minor disturbances or short arousal stimuli (Hagbarth & Vallbo, 1968a; Nyström & Hagbarth, 1981; Vallbo et al., 2004). Additional tests involve asking the patient to take a deep breath (inspiratory capacity apnoea) and to hold it for 40 s, a manœuvre known to cause a sustained increase in MSNA (Macefield & Wallin, 1995b).

2.5 Procedures for during-MRI session

Once the spontaneous nerve activity was recorded for ten minutes at rest at the laboratory, the ECG electrodes and BP monitor were removed and the subject was carefully transported to the 3T whole body MRI scanner (Intera, Philips Medical System) with the microelectrodes and respiratory bands in situ. An MR compatible piezoelectric transducer was attached to a finger pad to monitor the pulse and calculated heart rate. The subject’s head was enclosed in a 32-channel SENSE head coil and stabilised with foam pads to minimise head movement. Headphones were provided to minimise noise experienced from the magnet. Subjects could communicate via both a microphone and a pneumatic buzzer.

2.5.1 Wholebrain fMRI and MSNA acquisition

Muscle sympathetic nerve signals continued to be recorded as mentioned earlier (section 2.4.4). Signal intensity changes within the brain were measured using echo echo-planar images, sensitive to blood oxygen level dependent (BOLD) contrast. 200 volumes (46 axial slices, TR=8s, TE=40ms, flip angle=90˚, field of view:240 mm and raw voxel size =1.5 mm³) were collected in the first 4s of each 8s
TR. For each subject two series of 200 volumes was recorded. The first 200 series covered the entire brain; the second was limited to the brainstem. Each series took around 27 minutes.

We used a 4s ON and 4s OFF protocol, recording from MSNA during the 4s- OFF period and scanning during the 4s ON period. This interval was chosen carefully due to the temporal delays inherited in BOLD imaging. It is known that the neurovascular lag by some~5s (Logothetis et al., 2001) and that we need to allow ~1s for muscle vasoconstrictor volley to travel from the brain to the peripheral recording site at the knee (Fagius & Wallin, 1980). Accordingly, we reasoned that the changes in BOLD signal intensity would reflect changes in neural activity associated with emission of sympathetic volleys recorded in the previous 4s epoch. Axial slices have been collected sequentially from caudal to rostral during the 4s; extending from the rostral midbrain to the vertex of the cerebral cortex.

2.5.2 Brainstem fMRI and MSNA acquisition

To focus on the brainstem with high spatial resolution, the scan was limited to region extending from the top of the thalamus to the top of the cervical spinal cord. As previously mentioned, axial slices were collected sequentially from caudal to rostral during the 4s(46 axial slices, TR=8s, TE=40ms, flip angle=90º, raw voxel size=1.5mm³). Therefore, for the brainstem, the first parts of the scan were limited to the medulla whereas the later parts of the scan included the pons and thalamus.
2.5.3 sMRI, VBM of T1-images acquisition.

In each subject, a high-resolution 3D average T1-weighted anatomical image set covering the entire brain was collected (Turbo field echo; TE=2.5ms, TR=5600ms, flip angle=8°, Voxel size: 0.8 mm$^3$).

2.6 Analysis.

2.6.1 Pre-MRI physiology analysis

For the physiology measures made prior to entering the MRI scanner mean BP, systolic BP, diastolic BP, HR and respiratory rate were determined. All MSNA signals were RMS-processed (root mean square, moving average, time constant 200 ms). MSNA during the pre-MRI recording period was quantified according to standard time-domain analysis of the RMS-processed signal as burst frequency (bursts min$^{-1}$) and burst incidence (bursts per 100 heart beats). Analysis of variance, coupled with Tukey’s multiple comparisons test, was used to assess statistical significance across each group (Prism 6.0, GraphPad Software, USA). All values are expressed as means and standard errors, and p<0.05 was considered statistically significant.

2.6.2 MSNA analysis during MRI

For MSNA recording made in the MRI scanner and during fMRI collection, a high-pass digital filter at 300 Hz was applied to the recorded signal to remove artefacts picked up by the cable from the headstage to the amplifier. MSNA bursts were manually measured from the root mean square of the filtered nerve signal during the 4 s inter-scan OFF period. This period was divided into 4 x 1 s intervals.
and the total numbers of MSNA bursts during each of the 4 seconds fMRI collection periods determined.

### 2.6.2.1 Whole brain fMRI processing

Using SPM8 (Friston et al., 1995), fMRI images were realigned, spatially normalized to the Montreal Neurological Institute (MNI) template and intensity normalized to eliminate any slow drift in signal intensity. Scans were then smoothed by a 6mm full-width half-maximum (FWHM) Gaussian filter. For the whole brain, ifocused the investigation on brain regions rostral to the midbrain (seconds 2, 3 and 4), given that I performed a brainstem specific analysis separately. Therefore in each individual subject, the number of MSNA bursts during each of these 1s periods was determined and a 200 time point model derived for each individual subject for the 2nd, 3rd and 4th second time periods. That is, for each brain volume, a value of the total number of MSNA burst that occurred during the 2nd second of the 4 seconds TR was entered into a search model. The same analysis was then performed for the 3rd and 4th second periods.

Signal intensity changes that matched each individual subject’s MSNA burst model were then identified. Second level analyses were performed to determine in which brain regions signal intensity increased or decreased during each MSNA burst in both control and OSA subjects (random effects, p<0.005, uncorrected minimum cluster size 20 voxels). In addition, regions in which changes in signal intensity were significantly different between control and OSA subjects were determined (random effects, p<0.005, uncorrected, minimum cluster size 20 voxels).

Since I was essentially correlating on-going signal intensity with spontaneous MSNA bursts, and that MSNA bursts were significantly more frequent in OSA
subjects than controls, it is possible that differences in contrast values between OSA and controls may have been partially due to differences in search models. To ensure that this was not the case, for each significant cluster I extracted the raw signal intensity changes and compared signal intensity during MSNA bursts to signal intensity during periods where there were no bursts. Significant differences in signal intensity between controls and OSA subjects were then determined (p<0.05, two sample t-test).

2.6.3.2 Brainstem fMRI processing

Using SPM8 (Friston et al., 1995), the functional image sets for each individual subject were preprocessed and realigned and co-registered to their T1-weighted image set and global signal intensity drifts removed using a linear de-trending method. Manual correction of the images was performed to create an accurate match between the functional and anatomical image sets. Using the SUIT toolbox (Diedrichsen, 2006), the brainstem and cerebellum were isolated and the images spatially normalized into Montreal Neurological Institute space using a spatially unbiased atlas template of the cerebellum and brainstem.

To assess brainstem regions involved in the generation of MSNA, we analysed the 1st, 2nd and 3rd second periods of the 4 second TR separately. These three 1 s periods encompassed the medulla, pons and midbrain regions respectively. In each individual subject, the number of MSNA bursts during each of these 1 s periods was determined and a 200 time point model derived for each individual subject for the 1st, 2nd and 3rd second time periods. That is, for each brain volume, a value of the total number of MSNA bursts that occurred during the 1st second of the 4 s TR was
entered into a search model. The same analysis was then performed for the 2\textsuperscript{nd} and 3\textsuperscript{rd} second periods’ as well.

Signal intensity changes, which were significantly related to MSNA, were then determined using this model including the 6 parameter movement parameters as nuisance variables. The resulting contrast images were then smoothed using a 3mm FWHM Gaussian filter to account for small anatomical variations. Brain regions that displayed significant increases or decreases were then assessed in the control and OSA groups using a random effects procedure (one-sample t-tests; p<0.05, small volume corrected, minimum cluster size 5 voxels). In addition, differences in the signal intensity changes between controls and OSA subjects (two-sample t-tests; p<0.05, small volume corrected, minimum cluster size 3 voxels). I also compared signal intensity during MSNA burst periods to that during no MSNA burst periods (two sample t-test, p<0.05).

2.6.3.3 After CPAP treatment processing

Changes in signal intensity that matched each individual subject’s MSNA burst model were determined as described in section 2.6.3.1. Second level, random effects analyses were then performed to compare signal intensity changes during each MSNA burst in OSA subjects prior to and following 6 months CPAP treatment (random effects, p<0.005, uncorrected, minimum cluster size 20 voxels). In addition, regions in which changes in signal intensity were significantly different between control and OSA subjects were determined (random effects, p<0.005, uncorrected, minimum cluster size 20 voxels). Given that the bursts of MSNA were significantly more frequent in OSA subjects prior to CPAP treatment than after treatment and in controls, it is possible that differences in contrast values between OSA before and
after CPAP, and compared with controls, may have been partially due to differences in search models. To ensure that this was not the case, for each significant cluster we extracted the raw signal intensity changes and compared signal intensity during bursts of MSNA to signal intensity during periods where there were no bursts. Significant differences in signal intensity between controls and OSA subjects prior to CPAP treatment were determined (p<0.05, two sample t-test), and also between OSA subjects prior to and following CPAP treatment (p<0.05, paired t-test).

2.6.3 T1-images analysis

2.6.3.1 Whole brain T1-image analysis

T1-weighted images from each subject were bias corrected using SPM8 unified segmentation (Ashburner & Friston, 2005). And then segmented and spatially normalized using a second pass of the unified segmentation algorithm. The result of the segmentation and spatial normalization were wholebrain “maps” of grey matter probabilities, spatially normalized into the MNI template space, and “modulated” by the volume changes due to the normalization. The normalized, modulated grey matter images were then smoothed using a 6mm FWHM Gaussian filter, and analysed for group differences.

Significant differences in grey matter between OSA and control subjects were determined using random effects analysis with age, sex and total brain volume as nuisance variables (p<0.0005, false discovery rate corrected for multiple comparisons, minimum cluster size 20 voxels). The statistical threshold (p<0.005, uncorrected, minimum cluster size 20 voxels) was also lowered to explore less significant grey matter volume differences. Significant grey matter volume differences were then overlaid onto an individual’s T1-weighted image.
To determine if there were any brain regions in which OSA subjects displayed both significant differences in MSNA-related fMRI signal intensity and grey matter volume, I created binary images of the statistic maps derived from these two analyses. The intersection of these two statistic maps was then determined.

### 2.6.3.2 Brainstem T1-image analysis

The T1-weighted image from each subject was segmented and spatially normalized with a dedicated symmetrical brainstem template. In brief, the image was cropped and the brainstem masked before normalisation such that no supratentorial grey matter can bias the results using the SUIT toolbox. The subsequent normalisation and re-slicing process produces brainstem "maps" of grey matter probabilities, spatially normalised into the brainstem template space, and modulated by the volume changes due to the normalisation. Finally, the images were re-sliced into the new atlas space and smoothed (FWHM 3mm). Significant differences in grey matter between OSA subjects and controls were determined using a voxel-by-voxel analysis (two sample t-tests; p<0.05, small volume corrected, minimum cluster size 3 voxels). Significant volume differences were then overlaid onto a T1-weighted template for visualization. To explore the direction and overall grey matter volume differences, individual grey matter volumes (probability*volume) were extracted from clusters of difference and the means compared between groups (p<0.05, two tailed two sample t-test).

Brainstem regions in which there was a significant difference in both the fMRI response and grey matter volume were also determined. Individual grey matter volumes in OSA subjects were extracted from the resulting clusters and grey matter volumes were then plotted against the number of bursts per minute during the fMRI...
collection period. The significance of these correlations were determined (two tailed Spearman r, p<0.05).

2.6.3.3 After CPAP treatment T1-image analysis

T1-weighted images from each subject were bias corrected using SPM8 unified segmentation (Ashburner & Friston, 2005). And then segmented and spatially normalized using a second pass of the unified segmentation algorithm. The result of the segmentation and spatial normalization were whole brain “maps” of grey matter probabilities, spatially normalized into the MNI template space, and “modulated” by the volume changes due to the normalization. The normalized, modulated grey matter images were then smoothed using a 6mm FWHM Gaussian filter.

We then determined if in those regions which displayed MSNA-related functional changes associated with CPAP treatment, grey matter volumes also changed as a consequence of CPAP treatment. Grey matter volumes were extracted in control, OSA pre-CPAP and OSA post-CPAP subjects. Significant differences in grey matter volumes between controls and OSA subjects pre-CPAP treatment and between controls and OSA subjects post-CPAP were determined (two-tailed, two sample t-test, p<0.05), and also between OSA subjects prior to and following CPAP treatment (two-sample, paired t-test, p<0.05).
Chapter 3: Study I

Respiratory modulation of muscle sympathetic nerve activity in obstructive sleep apnoea

This chapter has been edited from the original paper that was accepted in the journal *Experimental Physiology* on the 22nd of May 2014. It was authored by Rania H Fatouleh, David K McKenzie, & Vaughan G Macefield (PMID: 24887112; in press)
3.1 Abstract

Obstructive sleep apnoea (OSA) is associated with elevated muscle sympathetic nerve activity (MSNA) during normoxic daytime wakefulness, leading to hypertension. In this study, I tested the hypothesis that respiratory-sympathetic coupling, postulated to be the underlying cause of neurogenic hypertension, is increased in OSA. MSNA, blood pressure, ECG and respiration were recorded in 21 normotensive control subjects and 21 newly diagnosed patients with OSA before and after 6 and 12 months of treatment with continuous positive airway pressure (CPAP). MSNA was significantly elevated in newly diagnosed OSA patients compared to control subjects (53 ± 2 vs 28 ± 2 bursts/min). There was a significant fall in MSNA after 6 months of CPAP (37 ± 2bursts/min) with no further change after 12 months (37 ± 2bursts/min). There were no significant differences in the magnitude of respiratory modulation of MSNA between the OSA patients and controls (40 ± 3.1% vs 39 ± 3.4%), but the temporal coupling of MSNA to respiration was tighter in OSA. Cardiac modulation was significantly lower in the patients (77 ± 4% vs 87 ± 3.5%), which fits with the lower baroreflex sensitivity in OSA. Respiratory and cardiac modulation did not change significantly with CPAP. Therefore, I conclude that, despite a difference in the temporal profile of respiratory modulation of MSNA, an increase in respiratory-sympathetic coupling is not responsible for the neurogenic hypertension associated with OSA.
3.2 Introduction

As described previously (Chapter 1), obstructive sleep apnoea (OSA) is a common disorder, characterized by repeated episodes of nocturnal hypoxaemia that causes enhanced muscle sympathetic nerve activity (MSNA), leading to neurogenic hypertension (Hedner et al., 1988, 1995; Carlson et al., 1993, 1996; Somers et al., 1995; Narkiewicz et al., 1999a; Elam et al., 2002; Narkiewicz & Sommers, 2003; Imadojemu et al., 2007) and a high mortality rate (He et al., 1988; Partinen et al., 1988). The increase in MSNA carries over into daytime wakefulness when subjects are breathing normally and have no evidence of hypoxaemia (Somers et al., 1995). However, the mechanisms underlying sympathoexcitation in this syndrome remain unclear. OSA has been associated with. Continuous positive airway pressure (CPAP) is an effective and widely used method for treatment of OSA (Sullivan et al., 1981). Long-term treatment with CPAP decreases MSNA in OSA (Hedner et al., 1995; Waradekar et al., 1996; Narkiewicz & Somers, 2003; Imadojemu et al., 2007), but this is not reflected in a fall in blood pressure (Hedner et al., 1995).

Muscle sympathetic nerve activity is vasoconstrictor in function and hence plays a major role in the control of arterial pressure. MSNA is tightly coupled to the cardiac cycle via the arterial baroreceptors, but is also modulated by respiration. In humans, MSNA is maximal during expiration and is inhibited in mid-inspiration (Eckberg et al., 1985; Hagbarth & Vallbo, 1968b; Macefield et al., 2002; Macefield & Wallin, 1990, 1995a,b; Seals et al., 1993). It has been argued from work in the spontaneous hypertensive rat (SHR) that an increase in respiratory modulation of sympathetic vasoconstrictor drive may contribute to the development of hypertension, and that this augmented respiratory-sympathetic coupling is responsible for the generation of hypertension in humans (Czyzyk-Krzeska &
Trzebski, 1990; Simms et al., 2009). I recently compared respiratory and cardiac modulation of MSNA in patients with essential hypertension, and showed that there was no increase in respiratory modulation (Fatouleh & Macefield, 2011; as in Appendix A). Nevertheless, it may be that an exaggerated respiratory modulation of MSNA may appear in other cases of neurogenic hypertension, such as OSA. In the current study, I tested the hypothesis that respiratory modulation of MSNA is increased in OSA compared to healthy-age matched control subjects, and that this elevated modulation is reduced following treatment with CPAP. Because of the higher levels of MSNA in OSA, I also predicted that cardiac modulation of MSNA would be lower in OSA.

3.3 Methodology

3.3.1 Participants

Data were obtained from 21 patients with obstructive sleep apnoea (18 males and 3 females, mean ±SEM 55 ±2, range 35–69 years). Five patients were lost to follow up at 6 months and one more patient was lost at 12 months. A Total of 16 of the initial OSA patients came back after 6 months of treatment (13 males and 3 females), and 15 came back after 12 months of treatment with CPAP (13 males and 2 females). Age and gender matched 21 controls (17 males and 4 females, Ages 35–68; 52 ± 2) were recruited from a healthy cohort.

3.3.2 Procedures and analysis

For the purpose of this study, patients underwent procedures explained in section 2.3 and 2.4 entirely (Chapter 2; General Methods). All patients with OSA
underwent a pre-MRI laboratory session, which included microneurography, electrocardiography, blood pressure and respiration. This was repeated after 6 and 12 months of CPAP treatment.

In addition to the standard analyses of MSNA reported in General Methods, the analysis of MSNA in this study was conducted on the raw, negative-going, sympathetic spikes to avoid any contamination from spikes generated by positive-going myelinated axons (such as spontaneously active muscle spindles) or motor units associated with spontaneous EMG activity. This approach has been described previously, and provides a more sensitive means of analysing sympathetic outflow than the standard method of simply counting the number of sympathetic bursts. (Fatouleh & Macefield, 2011, 2013, *Appendices A&B*). Briefly, negative-going spikes in the neurogram (half-width 0.2–0.5 ms) were detected using window discriminator software (Spike Histogram for Macintosh v2.2; ADInstruments), while the times of occurrence of the R-waves of the ECG and of the inspiratory peaks of the respiratory signal were computed using Peak Analysis software (ADInstruments).

Auto-correlation histograms for the cardiac and respiratory signals, and cross-correlation histograms between the MSNA and ECG or respiration, were generated by the Spike Histogram software (50 ms bins). Discriminator levels of the neural activity were adjusted so that negative-going (C-fibre) spikes exhibited a robust cardiac modulation, as revealed by cross-correlation between the neural activity and the ECG. These same discriminator settings were used for construction of cross-correlograms between the MSNA and the inspiratory peaks of the respiratory signal (either the chest or abdomen, depending on which signal was cleanest).

Smoothed polynomial curves were fitted to the histogram data using a graphical analysis program; zero-order polynomials were used to fit curves to the
slower respiratory cross-correlograms while second-order polynomials were required to fit curves to the cardiac cross-correlograms (Prism 5.0, GraphPad Software, La Jolla, Ca, USA). Quantification of the modulation of MSNA was performed by measuring the difference in the number of spikes at the peak of the modulation and the number at the trough for each subject, as computed from the smoothed polynomial:

\[ \text{Modulation index} = \frac{\text{peak} - \text{trough}}{\text{peak}} \times 100. \]

For calculating the modulation index the peak–trough difference extended over the same interval as the respiratory or cardiac periods. In addition, to illustrate the average time profile of respiratory modulation we computed a normalized cross-correlation histogram from the smoothed data across subjects. Data were normalized by defining the peak event count in an individual subject’s cross-correlogram and auto-correlogram as 100%.
3.4 Results

Based on overnight polysomnography, all OSA subjects were diagnosed with moderate to severe OSA. Based on apnoea-hypopnoea indices, three with moderate and 18 with severe OSA (AHI 44±5; range 18–106). In OSA subjects, the minimum SaO₂ on the night of polysomnography was 80±3% (range 48–93%), baseline SaO₂ during wakefulness was 95±1% (range 91–99%) and the baseline Epworth Sleep Scale score was 9±1 (range 3–19). The mean AHI for the control subjects following an in-home overnight assessment of sleep patterns was 4±1. Overnight monitoring of sleep was made at variable times after the scanning had been conducted, and revealed that, while the majority had an AHI of 1–3, two of the control subjects had an AHI of 8 one has an AHI of 10. Nevertheless, these subjects were otherwise healthy and did not identify as snorers or report being tired during the day; one subject reported drinking on the night of the test, which may well have affected his sleep patterns. Given the absent clinical history of sleep disorders, it was not deemed necessary to undertake a full polysomnographic assessment in these two individuals because they were symptom-free and normotensive.

Cardiorespiratory details of the healthy age-matched control subjects and the patients with obstructive sleep apnoea at the time of diagnosis and after 6 and 12 months of treatment with continuous positive airway pressure (CPAP) are provided in Table 1.1. It can be seen that there were no significant differences in heart rate or respiratory period across the groups. However, compared to the controls, OSA patients had significantly elevated systolic and diastolic pressures (P<0.01) and muscle sympathetic nerve activity (MSNA, P<0.001). There was a significant reduction in MSNA after 6 months (P < 0.005) of CPAP, with no further change after
12 months of compliant use, but there were no significant falls in blood pressure with treatment.

Experimental records from a female patient with OSA are shown in Figure 3.1. Negative-going spikes (MSNA) in the neurogram have been discriminated and presented as standard spikes, used to generate cross-correlation histograms between the inspiratory peaks of the respiratory record and the R-waves of the ECG. As illustrated in Figure 3.2A, cross-correlation histograms between the times of occurrence of the sympathetic spikes and of the ECG show a very tight coupling of MSNA to the cardiac cycle, while the respiratory rhythmicity is weaker (Figure 3.2B). Cardiac and respiratory modulation of MSNA was calculated from the smoothed polynomials fitted to the cross-correlation histograms.
Table 3.1: Cardiorespiratory parameters of the healthy control subjects, OSA patients prior, and after 6 months and 12 months of treatment with CPAP

<table>
<thead>
<tr>
<th></th>
<th>CON (n = 21)</th>
<th>OSA-0M (n = 21)</th>
<th>OSA-6M (n = 16)</th>
<th>OSA-12M (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F/M</td>
<td>4/17</td>
<td>3/18</td>
<td>3/13</td>
<td>2/13</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>120 ± 3</td>
<td>139 ± 4</td>
<td>129 ± 6</td>
<td>133 ± 4</td>
</tr>
<tr>
<td></td>
<td>**</td>
<td></td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>68 ± 3</td>
<td>80 ± 2</td>
<td>75 ± 4</td>
<td>75 ± 2</td>
</tr>
<tr>
<td></td>
<td>**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>66 ± 3</td>
<td>70 ± 3</td>
<td>68 ± 2</td>
<td>69 ± 2</td>
</tr>
<tr>
<td>Respiratory period (s)</td>
<td>4.0 ± 0.2</td>
<td>4.0 ± 0.3</td>
<td>4.0 ± 0.3</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>Burst incidence (bursts/100beats)</td>
<td>45 ± 3</td>
<td>76 ± 4</td>
<td>56 ± 3</td>
<td>53 ± 3</td>
</tr>
<tr>
<td></td>
<td>****</td>
<td>**</td>
<td>* ++</td>
<td>+++</td>
</tr>
<tr>
<td>Burst frequency (burst/min)</td>
<td>28 ± 2</td>
<td>53 ± 2</td>
<td>37 ± 2</td>
<td>37 ± 2</td>
</tr>
<tr>
<td></td>
<td>****</td>
<td>** +++</td>
<td>** +++</td>
<td>** ++++</td>
</tr>
</tbody>
</table>

Cardiorespiratory parameters of the healthy control subjects (CON), obstructive sleep apnoea patients prior (OSA-0M), and after 6 months (OSA-6M) and 12 months (OSA-12M) of treatment with Continuous Positive Airway Pressure (CPAP). Values presented are mean ± SEM. Statistical comparisons (ANOVA) are shown relative to data obtained from the Control (CON) group (*=P<0.05, **=P<0.01, ****=P<0.0001) and, for the OSA subjects, relative to data obtained at 0 months of CPAP (+=P<0.05, +=P<0.01, +++=P<0.001, ++++=P<0.0001).
Figure 3.1: Multiunit recording of muscle sympathetic nerve activity from a 34-year-old female patient with OSA

The mean-voltage neurogram is shown in the RMS-nerve trace; this was used to quantify the number of sympathetic bursts. Note that bursts of MSNA occurred in many, but not all, cardiac intervals in this subject. Discriminated spikes extracted from the nerve recording are illustrated as standard pulse (MSNA spikes). The times of occurrence of each heart beat (R-waves) and peaks of each breath (inspiratory peaks) are also shown as standard pulses. These timing events were used to generate the cross-correlation and auto-correlation histograms shown in Figure 3.2. MSNA: muscle sympathetic nerve activity; OSA: obstructive sleep apnoea
Figure 3.2: Cross-correlation histograms auto-correlation histograms between sympathetic spikes and respiration and ECG

Cross-correlation histograms (upper traces in each panel) and auto-correlation histograms (lower traces in each panel) between sympathetic spikes and respiration (A) and ECG (C). Auto-correlation histograms for respiration and ECG are shown in B and D, respectively. Data obtained from the same subject illustrated in Figure. 3.1. Smoothed polynomials (thick lines) have been fitted to the histograms. The numbers on the y-axis refer to the numbers of spikes per 50 ms bin. Time zero - corresponding to the triggering event in the cross- or auto-correlograms - is indicated by the vertical broken lines. ECG: Electrocardiogram; MSNA: muscle sympathetic nerve activity, Resp: respiration.
Because of the higher levels of MSNA in OSA I predicted that cardiac modulation would be lower in the untreated OSA patients (77.3 ± 4.0%) than the controls (87.3 ± 3.5%), and this was borne out (one-tailed t-test, P=0.0337). However, there was no significant difference in respiratory modulation in the OSA patients (39.8±3.1%) before treatment and the controls (39.1±3.4%). Mean data are shown graphically in Figure3.3. As expected from my earlier work (Fatouleh & Macefield, 2011, 2013), respiratory modulation of MSNA was significantly lower than the cardiac modulation in both groups (P<0.0001). Repeated-measures analysis of variance revealed no significant change in cardiac modulation [F(1.783, 16.04)=0.2629; P=0.7474] at 0 (baseline), 6 and 12 months of compliant CPAP use. Even though respiratory modulation appeared greater than baseline after 6 months of CPAP, RMANOVA uncovered no significant differences over the three time points [F(1.937, 21.30)=1.845; P=0.1831].

It should be emphasised that the respiratory modulation indices presented in Figure 3.3 were calculated from the peak-trough difference in the cross-correlograms for each subject, regardless of where in time the peak and trough occurred. To illustrate the temporal profile of the respiratory modulation I constructed normalised cross-correlation and auto-correlation histograms from all subjects and pooled the data to assess whether there were any differences in the mean times at which the peak and trough occurred with respect to time zero (i.e. the peak of inspiration). These are illustrated in Figure 3.4. It is apparent that the mean normalised respiratory modulation of MSNA is higher in the OSA patients, at baseline (0 months) and following 6 and 12 months of CPAP. This can also be seen in the higher normalised peak-trough differences in the OSA patients prior to CPAP, as shown in Table 3.2, though this was not significantly different.
Figure 3.3: Mean cardiac and respiratory modulation indices across the four groups studied

CON, age-matched controls (n=21); OSA 0 m CPAP, patients with Obstructive Sleep Apnoea before treatment (n=17); OSA 6 m OSA CPAP, patients after 6 months of CPAP treatment (n=16); OSA 12m CPAP, patients after 12 months of treatment (n=15). Cardiac and respiratory modulation could not be calculated for all subjects because of technical issues with the ECG or respiratory recordings. Cardiac modulation was significantly lower than control in the 0 m OSA group, but there were no significant differences in respiratory modulation between groups. Con: controls; CPAP: continuous positive airway pressure; ECG: Electrocardiogram; OSA: obstructive sleep apnoea.
Figure 3.4: Cross-correlation histograms and auto-correlation histograms between sympathetic spikes and respiration

Cross-correlation histograms (upper traces in each panel) and auto-correlation histograms (lower traces in each panel) between sympathetic spikes and respiration. Normalised data (mean ± SE) for control subjects (A), and patients with OSA at 0 months (B), 6 months (C) and 12 months (D) of treatment with CPAP. Data were normalized by defining the peak event count in an individual subject’s cross-correlogram and auto-correlogram as 100%. Time zero - corresponding to the peak of inspiration in the cross- and auto-correlograms - is indicated by the vertical broken lines. Statistical significance was assessed using a t-test. Because the maximal value (100%) occurs at different times during the respiratory cycle for each subject this dispersion means that the graphs do not show a peak of 100%. CPAP: continuous positive airway pressure; OSA: obstructive sleep apnoea.
Table 3.2: Normalised peaks and troughs computed from the averaged cross-correlation histograms

<table>
<thead>
<tr>
<th></th>
<th>normalised peak</th>
<th>normalised trough</th>
<th>normalized peak-trough</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>78.68 ± 3.81</td>
<td>65.22 ± 3.51</td>
<td>13.45 ± 5.18</td>
</tr>
<tr>
<td>OSA 0 m</td>
<td>81.47 ± 5.27</td>
<td>57.33 ± 4.81</td>
<td>24.14 ± 7.14</td>
</tr>
<tr>
<td>OSA 6 m CPAP</td>
<td>80.57 ± 4.43</td>
<td>60.64 ± 4.60</td>
<td>19.93 ± 6.40</td>
</tr>
<tr>
<td>OSA 12 m CPAP</td>
<td>83.60 ± 2.93</td>
<td>66.48 ± 4.73</td>
<td>17.12 ± 5.56</td>
</tr>
</tbody>
</table>

As illustrated in Figure 3.4. Normalised peaks and troughs (mean ± SE of highest and lowest values, expressed as a percentage) computed from the averaged cross-correlation histograms, illustrated in Figure 3.4, for the control (n=21) and OSA subjects at 0 months (n=18), 6 months (n=16) and 12 months (n=15) of CPAP. Compared to the control subjects, there were no significant differences in the amplitude of the mean peak or trough of the normalized respiratory modulation. CPAP: continuous positive airway pressure; OSA: obstructive sleep apnoea.
Despite the lack of significant difference when considering only the peak-trough differences, which are point measures, it is clear from Figure 3.4 that the peak modulation of MSNA occurred before the peak of inspiration for the control subjects but after the peak, i.e. in the post-inspiratory phase, for the OSA patients prior to CPAP. As shown in Figure 3.5, analysis of the normalized MSNA within the post-inspiratory period (measured over 0.05-0.50 s from the peak of inspiration) revealed a significant difference between the two groups, activity being significantly higher in the OSA patients (P<0.0001). Conversely, MSNA was significantly lower in the OSA patients during both inspiration (-1.5 to 0 s) and expiration (-0.55 to 2.00 s). Numerical data are presented in Table 3.3. It can also be seen that 6 months of CPAP resulted in a normalization of the amount of activity in inspiration and post-inspiration, but not in expiration – which remained significantly lower than in the control subjects. Indeed, it was not until after 12 months of CPAP that MSNA levels in expiration returned, and significantly exceeded, baseline levels; MSNA during inspiration and post-inspiration were also higher than in the controls, presumably reflecting the fact that while CPAP reduced the overall levels of MSNA (as measured by burst incidence and burst frequency, Table 3.1), it did not reduce MSNA completely to control levels. It would appear that, prior to CPAP, OSA is associated with a greater temporal coupling of MSNA to respiration than seen in controls. Accordingly, although the magnitude of the modulation in individual subjects is not significantly different between control subjects and subjects with OSA, I suggest that the temporal jitter in respiratory modulation in the control subjects result in a modulation profile that is less pronounced than that of the OSA patients.
Figure 3.5: Cross-correlation histograms and auto-correlation histograms between sympathetic spikes and respiration

Cross-correlation histograms (upper traces in each panel) and auto-correlation histograms (lower traces in each panel) between sympathetic spikes and respiration. Normalised data (mean ± SE) for control subjects (open symbols), and patients with OSA at 0 months (closed symbols). Data were calculated by counting the percentage of spikes in each bin over specific time periods: inspiration (-1.5-0 s), post-inspiration (0.05-0.50 s) and expiration (0.55-2.00 s). Time zero - corresponding to the peak of inspiration in the cross- and auto-correlograms - is indicated by the vertical broken lines. I= inspiration, P-I = post-inspiration, E=expiration. Statistical significance was assessed using a t-test. OSA: obstructive sleep apnoea.
Table 3.3: Normalised MSNA, computed from the averaged normalized cross-correlation histograms for the control and OSA subjects

<table>
<thead>
<tr>
<th></th>
<th>inspiration</th>
<th>post-inspiration</th>
<th>expiration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>control</strong></td>
<td>76.97 ± 0.23</td>
<td>74.40 ± 0.31</td>
<td>69.23 ± 0.52</td>
</tr>
<tr>
<td><strong>OSA 0 m</strong></td>
<td>74.38 ± 0.58</td>
<td><strong>79.41 ± 0.82</strong></td>
<td>62.14 ± 0.75</td>
</tr>
<tr>
<td></td>
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<td>****</td>
</tr>
<tr>
<td><strong>OSA 6 m CPAP</strong></td>
<td>76.62 ± 0.67</td>
<td>74.46 ± 0.77</td>
<td>63.89 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>****</td>
<td>****</td>
<td>****</td>
</tr>
<tr>
<td><strong>OSA 12 m CPAP</strong></td>
<td>81.07 ± 0.24</td>
<td>80.67 ± 0.44</td>
<td>69.94 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>****</td>
<td>****</td>
<td>*</td>
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</table>

As shown in Figure 3.5. Mean values (mean ± SE) were calculated over the bins extending from -1.5 to 0 s (inspiration), 0.05 to 0.5 s (post-inspiration) and 0.55 to 2.0 s (expiration). Statistical differences (repeated-measures one-way ANOVA) are shown relative to the OSA baseline (0 months) data: *=p<0.05, **=p<0.01, ****=p<0.0001. CPAP: continuous positive airway pressure; OSA: obstructive sleep apnoea.
3.5 Discussion

I have compared the magnitude of the respiratory modulation of MSNA in OSA patients with that of healthy controls, and found no significant difference, despite the greatly augmented levels of muscle sympathetic nerve activity and blood pressure in obstructive sleep apnoea measured across individual subjects. Moreover, a reduction in MSNA after continuous positive airway pressure (CPAP) was not associated with changes in the magnitude of the respiratory modulation of MSNA. However, when considering the normalised temporal profile there were changes in the respiratory patterning of MSNA in OSA, with more activity occurring in post-inspiration and less in inspiration and expiration. This was largely reversed following long-term continuous positive airway pressure, although blood pressure remains elevated because total MSNA remains higher than in the control subjects. This will be expanded on in General Discussion.
Chapter 4: Study II

Functional and structural changes in the brain associated with the increase in muscle sympathetic nerve activity in obstrusive sleep apnoea

This chapter has been edited from the original paper that was accepted in the journal *NeuroImage Clinical* on the 26th of August 2014. It was authored by Rania H Fatouleh, Elie Hammam, Linda C Lundblad, Paul M Macey, David K McKenzie, Luke A Henderson and Vaughan G Macefield. (in press)
4.1 Abstract

By recording MSNA concurrently with functional Magnetic Resonance Imaging (fMRI) I aimed to identify the central processes responsible for the sympathoexcitation. Spontaneous fluctuations in MSNA were recorded in 17 OSA patients and 15 healthy controls while lying in a 3T MRI scanner. Fluctuations in Blood Oxygen Level Dependent (BOLD) signal intensity covaried with the intensity of the concurrently recorded bursts of MSNA. In both groups there was a positive correlation between MSNA and signal intensity in the left and right insula, dorsolateral prefrontal cortex (dIPFC), dorsal precuneus, sensorimotor cortex and posterior temporal cortex, and the right mid-cingulate cortex and hypothalamus. In OSA left and right dIPFC, medial PFC (mPFC), dorsal precuneus, anterior cingulate cortex, retrosplenial cortex and caudate nucleus showed augmented signal changes compared with controls. While the right hippocampus/parahippocampus signal intensity decreased in controls but did not change in the OSA subjects. In addition, there were significant increases in grey matter volume in the left mid-insula, the right insula, left and right primary motor cortex, left premotor cortex, left hippocampus and within the brainstem and cerebellum, and significant decreases in the mPFC, occipital lobe, right posterior cingulate cortex, left cerebellar cortex and the left and right amygdala in OSA, but there was no overlap between these structural changes and the functional changes in OSA. These data suggest that the elevated muscle vasoconstrictor drive in OSA may result from functional changes within these brain regions, which are known to be directly or indirectly involved in the modulation of sympathetic outflow via the brainstem. That there was no overlap in the structural and functional changes suggests that asphyxic damage due to repeated episodes of nocturnal obstructive apnoea is not the main cause of the sympathoexcitation.
4.2 Introduction

Over the past decade, a number of investigations in humans have begun to describe both anatomical and functional brain changes associated with OSA. These studies have found that OSA is associated with significant functional and grey matter changes in a number of regions, including those that can modulate MSNA (Canessa et al., 2011; Harper et al., 2003, 2012; Joo et al., 2010, 2013; Macey et al., 2002, 2003; Morrell et al., 2010). Although these studies investigated neural substrates responsible for evoked changes in sympathetic drive, none have explored brain function in structures responsible for the increased MSNA at rest and the hypertension associated with OSA.

The aim of the current investigation was to identify brain sites potentially responsible for the increased MSNA associated with OSA. I used concurrent recordings of MSNA and functional magnetic resonance imaging (fMRI) to assess brain activity associated with the pattern of an individual subject’s MSNA burst activity, as described previously (James et al., 2013; Macefield and Henderson, 2010). Furthermore, I assessed regional grey matter changes using voxel-based morphometry (VBM) (Ashburner & Friston, 2000). I tested the hypothesis that the increase in MSNA in OSA would be associated with altered function and anatomy in higher brain regions that modulate MSNA, including the cingulate cortex, prefrontal cortex, insula and hypothalamus.
4.3 Methodology

4.3.1 Participants
Seventeen subjects with obstructive sleep apnoea (15 males, mean±SEM age 55±3, range 35–69 years) and 15 healthy controls (12 males, age 53±3, 35–68 years) were recruited for this study.

4.3.2 Procedures and analysis
As explained in Chapter 2 (General Methods), all subjects underwent a pre-MRI laboratory session, which included microneurography, electrocardiography, blood pressure and respiration. Followed by an MRI session and collected a concurrent recording of MSNA and wholebrain fMRI as well as a 3D T-weighted image.

4.4 Results
Based on apnoea-hypopnoea indices (AHI: mild 5–15 events per hour, moderate 15–30, severe>30), two subjects were diagnosed with mild, two with moderate and 13 with severe OSA (AHI 36±4; range 7–62). In OSA subjects, the minimum SaO2 on the night of polysomnography was 83±2% (range 67–93%), baseline SaO2 during wakefulness was 95±1% (range 91–99%), and the baseline Epworth Sleep Scale score was 9±1 (range 3–19). The mean AHI for the control subjects following an in-home overnight assessment of sleep patterns was 3.3±1. Overnight monitoring of sleep was made at variable times after the scanning had been conducted, and revealed that, while the majority had an AHI of 1–3, two of the control subjects had an AHI of 8 and 10. Nevertheless, these subjects were otherwise
healthy and did not identify as snorers or report being tired during the day; one subject reported drinking on the night of the test, which may well have affected his sleep patterns. Given the absent clinical history of sleep disorders, it was not deemed necessary to undertake a full polysomnographic assessment in these two individuals because they were symptom-free and normotensive. Although there was no significant difference in age between OSA and control subject groups (two sample t-test; p>0.05), as expected there was a significant difference in body mass index (BMI: OSA 31±2, controls 25±1, p=0.001).

4.4.1 Physiology

Resting mean BP was significantly higher in OSA subjects (OSA 99±3mmHg, controls 86±4mmHg; p=0.008), but there were no significant differences in resting heart rate (OSA 69±3 beats/minute, controls 66±3 beats/minute, p=0.5). As expected OSA subjects had significantly elevated MSNA burst incident (OSA 78±5 bursts/100 heart beats, controls 40±2 bursts/100 heart beats; p<0.0001) and burst frequency (OSA 53±3 bursts/minute, controls 26±2 bursts/minute, p<0.0001). Figure 4.1A shows a recording of MSNA, together with heart rate and respiration, in a patient with severe OSA. Raw data from a healthy control subject are shown in Figure4.1B; MSNA was clearly elevated in the subject with OSA.
Figure 4.1: Multiunit recording of muscle sympathetic nerve activity from a 50-year-old male patient with OSA and a 42 year-old male healthy control subject.

The mean-voltage neurogram is shown in the nerve RMS (root mean square) trace; this was used to quantify the number of sympathetic bursts. Four consecutive sets of the 4s-ON and 4s-OFF scanning sequences are shown; the black areas represent the scanning artifacts. MSNA burst amplitudes were measured during the OFF periods. Heart rate was calculated from a piezoelectric pulse transducer on the fingerpad; respiration was monitored via a piezoelectric transducer around the abdomen. Note the higher level of MSNA in the OSA patient; the control subject was a fit individual with low resting heart and respiratory rates. MSNA: muscle sympathetic nerve activity; OSA: obstructive sleep apnoea.
4.4.2 Changes in fMRI signal intensity:

Analysis of MSNA-related changes in BOLD signal intensity revealed that in control, significant increases occurred in those regions which we have previously identified as being associated with spontaneous bursts of MSNA: the left and right insula, dorsolateral prefrontal cortex (dlPFC), dorsal precuneus, sensorimotor cortex and posterior temporal cortex, and the right mid-cingulate cortex (MCC) and hypothalamus (Figure 4.2, Table 4.1), areas we had previously shown were temporally coupled to bursts of MSNA in young control subjects (James et al. 2013).

Analysis of MSNA-related changes in signal intensity in OSA compared with control subjects revealed a number of brain regions in which signal intensity changes were significantly greater in OSA subjects. When bursts of spontaneous MSNA were present, signal intensity in the left and right dlPFC, medial PFC (mPFC), dorsal precuneus, anterior cingulate cortex (ACC), retrosplenial cortex and caudate nucleus increased significantly in OSA subjects, but either did not increase or decreased in controls (Figure 4.3, Table 4.2). The mean (±SEM) percentage change in signal intensity when MSNA was present compared to when MSNA was absent were as follows: signal intensity was higher in OSA than controls in the dlPFC (controls vs OSA: left: -0.02±0.03 vs 0.10±0.04, p=0.01; right: 0.01±0.03 vs 0.140±0.034, p=0.005); mPFC (left: -0.10±0.04 vs 0.11±0.04, p=0.0004; right: -0.12±0.04 vs 0.10±0.04, p=0.0001); ACC (-0.05±0.04 vs 0.11±0.03, p=0.0005); retrosplenial cortex (left: -0.07±0.05 vs 0.14±0.04, p=0.0004; right: -0.04±0.04 vs 0.15±0.04, p=0.002) and in the dorsal precuneus (left: -0.08±0.07 vs 0.30±0.08, p=0.001; right: -0.02±0.04 vs 0.22±0.04, p=0.0001). As in Figure 4.4, within the right hippocampus/parahippocampus, signal intensity decreased significantly in controls but did not change in the OSA subjects (-0.12±0.03 vs 0.04±0.03, p=0.0005).
brain region was fMRI signal intensity greater in controls compared with OSA subjects.

4.4.3 Changes in grey matter volume

Comparison of grey matter volumes revealed that OSA is associated with significant regional changes in grey matter volume (Figure 4.5, Table 4.3). At a p<0.05 corrected threshold, increases in grey matter volume occurred in a cluster encompassing the medulla/pons and cerebellum in OSA vs controls (probability*volume: 0.35±0.006 vs 0.31±0.006, p=0.00005), as well as in the left mid-insula (0.51±0.01 vs 0.48±0.01, p=0.008), right insula (0.37±0.01 vs 0.35±0.01, p=0.015), right primary motor cortex (0.43±0.01 vs 0.36±0.01, p=0.003), left primary motor cortex (0.43±0.01 vs 0.36±0.01, p=0.00006), left premotor cortex (0.43±0.01 vs 0.36±0.01, p=0.0003) and left hippocampus (0.58±0.02 vs 0.53±0.01, p=0.019). At a corrected statistical threshold, none of these regions displayed significantly lower grey matter volume in OSA subjects compared with controls. However, lowering the statistical threshold to an uncorrected level revealed a number of regions in which grey matter volume was lower in OSA subjects. These included the mPFC, occipital lobe, and the left and right amygdala, right posterior cingulate and left cerebellar cortices. Surprisingly, even using this lower statistical threshold, no brain region displayed both a significant difference in grey matter volume and a significant difference in MSNA-related fMRI signal intensity change in OSA compared with control subjects.
Figure 4.2: Brain regions in which signal intensity changes were significantly correlated to MSNA pattern at rest

Hot colour scale represents regions in which signal intensity increased during MSNA bursts. Significant clusters are overlaid onto a mean T1-weighted anatomical template image. The dark shading indicates brain regions not included in the analysis. Slice location in Montreal Neurological Institute space are indicated at the lower right of each image. dIPFC: dorsolateral prefrontal cortex; MCC: midcingulate cortex; MSNA: muscle sympathetic nerve activity; Signal intensity (SI).
Table 4.1: Location, T-score and cluster size for regions showing significant signal intensity changes that were coupled to spontaneous MSNA in control and subjects with OSA

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
<th>T-score</th>
<th>Cluster size</th>
</tr>
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<td>Signal intensity increases during spontaneous MSNA bursts</td>
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Cluster locations are given in Montreal Neurological Institute (MNI) space. MSNA: muscle sympathetic nerve activity; OSA: obstructive sleep apnoea.
Figure 4.3: Brain regions in which fMRI signal intensity changes correlated to MSNA were significantly different in controls compared with subjects with OSA.

Hot colour scale indicates regions in which signal intensity changes were greater in OSA subjects compared with controls. Significant clusters are overlaid onto a T1-weighted anatomical template image. The dark shading indicates brain regions not included in the analysis. Slice location in Montreal Neurological Institute space are indicated at the lower right of each image. ACC: anterior cingulate cortex, dPFC: dorsolateral prefrontal cortex; mPFC: medial prefrontal cortex; MSNA: muscle sympathetic nerve activity; OSA: obstructive sleep apnoea; RSC: retrosplenial cortex.
Table 4.2: Location, T-score and cluster size for regions showing significant differences in the signal intensity changes that were coupled to spontaneous MSNA in control compared with OSA subjects

<table>
<thead>
<tr>
<th>Brain regions</th>
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<th>Z</th>
<th>T-score</th>
<th>Cluster size</th>
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Cluster locations are given in Montreal Neurological Institute (MNI) space. MSNA: muscle sympathetic nerve activity; OSA: obstructive sleep apnoea.
Figure 4.4: Plots of percentage signal intensity change differences during MSNA bursts compared with periods of no MSNA activity in brain regions identified as significantly different in subjects with OSA compared with controls

Note that in all regions except for the hippocampus, signal intensity increased in OSA subjects (grey bars) and did not change or decreased modestly in controls (white bars). In the hippocampus, signal intensity decreased dramatically in controls and did not change in OSA subjects. ACC: anterior cingulate cortex, dlPFC: dorsolateral prefrontal cortex, mPFC: medial prefrontal cortex; MSNA: muscle sympathetic nerve activity; OSA: obstructive sleep apnoea.
Figure 4.5: Brain regions in which GM was significantly different in subjects with OSA compared with controls

Hot colour scale indicates increases in grey matter volume in OSA subjects compared with controls at a corrected statistical threshold (p<0.05). Cool colour scale indicates decreases in grey matter volume in OSA subjects compared with controls at a more liberal uncorrected statistical threshold (p<0.001). Significant clusters are overlaid onto a T1-weighted anatomical template image. Slice location in Montreal Neurological Institute space are indicated at the lower right of each image. GM: grey matter volume; M1: primary motor cortex; mPFC: medial prefrontal cortex; PCC: posterior cingulate cortex; OSA: obstructive sleep apnoea.
Table 4.3: Location, T-score and cluster size for regions showing significant grey matter volume differences in OSA subject compared with controls

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<th>Z</th>
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<th>Cluster size</th>
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</tbody>
</table>

Cluster locations are given in Montreal Neurological Institute space. OSA: obstructive sleep apnoea.
4.4.4 Functional and structural overlap

To confirm that those regions displaying significant functional changes in OSA did not display structural changes, I compared grey matter volumes in these regions in OSA and control subjects. Confirming the voxel-by-voxel analysis, I found no significant differences in grey matter volumes between controls and OSA subjects in any brain region: the ACC (0.49±0.080 vs 0.52±0.096, p=0.08), the dIPFC (0.50±0.081 vs 0.51±0.098, p=0.40), the left insula (0.53±0.069 vs 0.54±0.063, p=0.23), right hippocampus (0.51±0.057 vs 0.50±0.073, p=0.29) and right precuneus (0.47±0.060 vs 0.48±0.055, p=0.35).

4.5 Discussion

Consistent with previous studies, I found that OSA is associated with significant increases in muscle sympathetic nerve activity (MSNA) (Carlson et al., 1993, 1996; Hedner et al., 1988, 1995; Narkiewicz et al., 1999; Somers et al., 1995; Fatouleh et al., 2014b) and elevated blood pressure (Pepperell et al., 2002). This elevated muscle vasoconstrictor drive was associated with significant changes in signal intensity in a number of brain regions during each MSNA burst in OSA subjects but not healthy controls. Within the left and right dorsolateral and medial prefrontal cortices (mPFC, dIPFC), dorsal precuneus, anterior cingulate, retrosplenial cortices and caudate nucleus, signal intensity increased significantly during each MSNA burst in OSA subjects but not in controls. In contrast, within the right hippocampus and parahippocampus, signal intensity decreased significantly during each MSNA burst in healthy controls, but did not change in OSA subjects. Thus, the MSNA-related neural activity in higher brain regions differs in OSA patients relative to controls. Surprisingly, none of the regions displayed significant anatomical
changes. These data suggest that the elevated muscle vasoconstrictor drive that occurs in individuals with OSA may be driven by activity changes in higher cortical regions, possibly through influences on brainstem regulatory nuclei. In addition, the cortical changes in signal intensity associated with increased MSNA do not result from localized structural changes that may occur as a result of the repeated hypoxic events that occur in individuals with OSA.

In both controls and OSA patients, spontaneous bursts of MSNA associated with functional changes within the insula, dorsal precuneus, right hypothalamus, dorsolateral prefrontal, posterior temporal, mid-cingulate and sensorimotor cortices. Furthermore, OSA is associated with significant changes in grey matter volume in autonomic control brain regions, which in theory would be expected to impact the normal functioning of those structures. The OSA subjects displayed increases in grey matter volume in medulla/pons and cerebellum, left mid-insula, right insula, bilateral primary motor cortex, left premotor cortex and left hippocampus and decreases in the mPFC, occipital lobe, and the left and right amygdala.
Brainstem changes associated with increased muscle sympathetic drive in obstructive sleep apnoea

This chapter has been edited from the original paper that was submitted to the journal *NeuroImage*. It was authored by Linda C Lundblad, Rania H Fatouleh, Elie Hammam, David K McKenzie, Vaughan G Macefield and Luke A Henderson. (Currently in review after resubmission)
5.1 Abstract

In this study I aimed to investigate brainstem sites that contribute to the increased on-going sympathetic drive in obstructive sleep apnoea patients (OSA). I measured regional grey matter volume in 20 subjects with OSA and 19 healthy age-matched controls. I also performed concurrent recordings of MSNA and brain signal intensity of the brainstem, in 15 subjects with OSA and 15 controls. OSA subjects had significantly elevated MSNA, which was correlated to altered signal intensity changes in the dorsolateral pons, rostroventrolateral medulla, medullary raphe and midbrain. The medullary raphe, rostroventrolateral medulla and dorsolateral pons also had significantly increased grey matter volumes in subjects with OSA compared with controls. Furthermore, I also found that OSA was associated with increases in grey matter volume in the region of the hypoglossal nucleus. These data suggest that the elevated muscle vasoconstrictor drive in obstructive sleep apnoea may result from functional and anatomical changes within the dorsolateral pons, rostroventrolateral medulla and medullary raphe. These brainstem regions are known to modulate sympathetic output either directly or indirectly via sympathetic preganglionic neurons within the spinal cord. In addition, the known increase in genioglossus muscle activity in OSA may reflect the increase in grey matter volume of the hypoglossal nucleus.
5.2 Introduction

As explained earlier, a number of investigations in humans have begun to describe both anatomical and functional brain changes associated with OSA. However, no human study has focused its attention on the role of brainstem nuclei in these changes. This is surprising, given that experimental animal studies have revealed that brainstem nuclei are critical for the generation and modulation of resting sympathetic outflow and that in an experimental animal model of OSA, neural activation occurs in these cardiovascular regulating brainstem regions (Greenberg et al., 1999; Sica et al., 2000). It is therefore likely that altered brainstem function underlies the expression of increased MSNA in humans with OSA.

The aim of this investigation was to determine brainstem sites responsible for the increased MSNA associated with OSA. I used concurrent recording of MSNA and functional magnetic resonance imaging (fMRI) to assess regional brainstem activity associated with individual subject’s MSNA burst patterns (James et al., 2013; Macefield and Henderson, 2010). Furthermore, we assessed regional grey matter changes using voxel based morphometry (VBM) of the brainstem. I hypothesized that the increase in MSNA in OSA would be associated with altered function and anatomy in brainstem regions known to modulate MSNA, such as the rostral and caudal ventrolateral medulla, nucleus tractus solitarius, the dorsolateral pons as well as the midbrain periaqueductal grey matter.
5.3 Methodology

5.3.1 Participants

Twenty subjects with obstructive sleep apnoea (OSA) and 19 healthy controls were recruited for the study. Fifteen of the 20 OSA subjects and 15 of the 19 control subjects completed the fMRI portion of the experimental paradigm.

5.3.2 Procedures and analysis

All subjects underwent a pre-MRI laboratory session, which included microneurography, electrocardiography, blood pressure and respiration. Followed by an MRI session and collected a concurrent recording of MSNA and brainstem fMRI as well as a 3D T-weighted image.

5.4 Results

Of the 20 OSA subjects, 1 subject had mild OSA, 3 subjects had moderate OSA and 16 had severe OSA (mean±SEM AHI 38 ± 3.4; range 7–62). In OSA subjects, the minimum SaO2 was 77±2.8% (range 43–93%), baseline SaO2 during wakefulness was 95±0.6% (range 91–99%) and Baseline Epworth Sleep Scale score was 9±1.1 (range 2–19). The controls subjects had a mean (±SEM) AHI of 3.5±0.9, two of which had AHI values in the mild OSA range (8 and 10). There was no significant difference in age between the subjects with OSA (54±2.1, 35–69 years) and the controls (50±2.4, 35–68 years; two sample t-test; p>0.05) or gender composition (OSA: 3 females, controls: 4 females; chi-squaredtest, p>0.05). There was, however, a significant difference in body mass index (BMI) between OSA and control subjects (OSA 31.7±1.3, controls 26.0±1.2, p=0.003).
5.4.1 Physiology

During the recording period prior to entering the MRI scanner, comparison of the 15 OSA and 15 control subjects that completed the fMRI study revealed that the OSA subjects had significantly elevated systolic (OSA vs controls; 138±5 vs 121±4 mmHg, p=0.03) and diastolic pressures (80±2 vs 68±4 mmHg, p=0.004) and significantly elevated MSNA (77±6 vs 38±3 bursts/100 heart beats, p=0.0001). In contrast, there were no differences in heart rate (70±3 vs 67±4 beats/min, p=0.50), or respiratory rate (3.9±0.3 vs 3.8±0.4 breaths/min, p=0.80) between the subjects with OSA and the controls. Figure 5.1 shows a recording of MSNA, together with heart rate and respiration, in a 50 year-old male subject with OSA, immediately prior to brainstem fMRI and during the scanning sequences.

5.4.2 Changes in fMRI signal intensity:

In control and OSA groups, fMRI signal intensity matched the spontaneous pattern of MSNA bursts in a number of brainstem regions (Figure 5.2, Table 5.1). These included the region of the rostral ventrolateral medulla (RVLM) and the dorsolateral pons. In controls a significant correlation between fMRI signal and MSNA also occurred in the midbrain in the region of the periaqueductal grey matter (PAG). Assessment of pooled data from control and OSA subjects revealed changes in signal intensity within other regions involved in the baroreflex, including the region of the dorsal medulla and caudal ventrolateral medulla (Figure 5.3). Extraction of signal intensity changes within these regions revealed that signal intensity increased bilaterally within the regions of the RVLM in both controls and OSA subjects (OSA vs controls: left RVLM; 0.07±0.05 vs 0.15±0.05, p=0.28; right RVLM; 0.05±0.02 vs 0.16±0.05, p=0.06) and dorsal medulla (0.14±0.07 vs
0.24±0.09, p=0.37) and decreased within the region of the caudal ventrolateral medulla (left CVLM; -0.28±0.13 vs -0.30±0.16, p=0.96; right CVLM; -0.18 ± 0.08 vs -0.32±0.14, p=0.38).

Comparison of signal intensity changes in OSA subjects and controls revealed significant differences in a number of brainstem regions (Figure 5.4, Table 5.2). Control subjects displayed significantly greater signal intensity compared with OSA subjects in the region of the medullary raphe nuclei, left RVLM, right and left dorsolateral pons and right midbrain in the region of the PAG. Extraction of signal intensity changes revealed that in all of these regional differences, control subjects displayed increased and OSA subjects decreased signal intensity or no change during MSNA bursts (OSA vs controls: medullary raphe; -0.21±0.08 vs 0.15±0.06, p=0.0008; left RVLM; -0.24±0.16 vs 0.32±0.012, p=0.007; right dorsolateral pons; 0.02±0.07 vs 0.32±0.11, p=0.02; midbrain; -0.08±0.07 vs 0.42±0.18, p=0.02).
Figure 5.1: Multiunit recording of MSNA from a 50-year-old male patient with OSA

The mean-voltage neurogram is shown in the nerve RMS (root mean square) trace; this was used to quantify the number of sympathetic bursts. The left panel shows the recording while the patient was laying in the scanner bore but prior to the onset of scanning to illustrate the high level of spontaneous MSNA in this patient. The right panel shows four consecutive sets of the 4s-ON and 4s-OFF scanning sequences; the black areas represent the scanning artifacts. MSNA burst amplitudes were measured during the OFF periods. Heart rate was calculated from a piezoelectric pulse transducer on the fingerpad; respiration was monitored via a piezoelectric transducer around the abdomen. MSNA: muscle sympathetic nerve activity; OSA: obstructive sleep apnoea.
**Figure 5.2: Brainstem regions in which signal intensity changes were significantly correlated to MSNA pattern at rest**

Hot colour scale represents regions in which signal intensity increased during MSNA bursts, whereas the cool colour scale indicates signal intensity decreases during MSNA bursts. Significant clusters are overlaid onto a mean T1-weighted anatomical template image. The cerebellum is shaded since it was not included in the analysis. Slice location in Montreal Neurological Institute space is indicated at the top right of each image in the top row. dlPons: dorsolateral pons; MSNA: muscle sympathetic nerve activity; RVLM: rostral ventrolateral medulla; SI: signal intensity.
Table 5.1: Location, T-score and cluster size for regions showing significant signal intensity changes that were coupled to spontaneous MSNA in control and subjects with OSA

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Cluster locations are given in Montreal Neurological Institute space. RVLM: rostral ventrolateral medulla. MSNA: muscle sympathetic nerve activity; OSA: obstructive sleep apnoea.
Figure 5.3: Baroreflex related medullary regions in which fMRI signal intensity changes correlated to MSNA were significantly increased or decreased in both controls and subjects with OSA

The cerebellum is shaded since it was not included in the analysis. Slice location in Montreal Neurological Institute space are indicated at the top right of each image. Below are plots of percentage changes in signal intensity during MSNA bursts relative to periods of no MSNA bursts. Note that signal intensity increased in controls (white) and OSA (grey) subjects in the region of the rostroventrolateral medulla (RVLM) and dorsal medulla and decreased within the region of the caudal ventrolateral medulla (CVLM). MSNA: muscle sympathetic nerve activity; OSA: obstructive sleep apnoea.
Figure 5.4: Brainstem regions in which fMRI signal intensity changes correlated to MSNA were significantly different in controls compared with subjects with OSA

Hot colour scale indicates regions in which signal intensity changes were greater in control subjects compared with obstructive sleep apnoea (OSA) subjects. Significant clusters are overlaid onto a T1-weighted anatomical template image. The cerebellum is shaded since it was not included in the analysis. Slice location in Montreal Neurological Institute space are indicated at the top right of each image. Below are plots of percentage changes in signal intensity during muscle sympathetic nerve activity (MSNA) bursts relative to periods of no MSNA bursts. Note that signal intensity increased in controls (white) but decreased in subjects with OSA (red). dlPons: dorsolateral pons; RVLM: rostral ventrolateral medulla.
Table 5.2: Location, T-score and cluster size for regions showing significant differences in signal intensity changes coupled to spontaneous MSNA in control compared with OSA

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Cluster locations are given in Montreal Neurological Institute space. RVLM: rostral ventrolateral medulla. MSNA: muscle sympathetic nerve activity; OSA: obstructive sleep apnoea.
5.4.3 Changes in grey matter volume

Comparison of grey matter volumes in controls compared with OSA subjects revealed that OSA is associated with significant regional changes in grey matter volume (Figure 5.5, Table 5.3). Significant increases occurred in the region of the medullary raphe, extending dorsally to include the region of the hypoglossal nucleus and the RVLM (probability*volume±SEM: OSA vs controls 0.33±0.03 vs 0.20±0.02, p=0.0002) and the region of the dorsolateral pons bilaterally (left dorsolateral pons: 0.39±0.02 vs 0.28±0.02, p=0.0006; right dorsolateral pons: 0.29±0.02 vs 0.20±0.02, p=0.0003).

5.4.4 Functional and structural overlap

In the region of the medullary raphe, left RVLM and right dorsolateral pons, OSA subjects displayed both significant differences in MSNA related changes in fMRI signal intensity as well as significantly increased grey matter volume (Figure 5.6). Within all three of these brainstem regions, although MSNA burst/min was not significantly correlated with fMRI signal intensity (medullary raphe: r=0.08, p=0.78; RVLM: r=0.25, p=0.36; dorsolateral pons: r=0.14, p=0.62), it was inversely correlated with grey matter volume (medullary raphe: r=0.08, p=0.78; RVLM: r=0.25, p=0.36; dorsolateral pons: r=0.14, p=0.62). The region of both fMRI and grey matter volume change within the dorsolateral pons was located immediately lateral to and including the region of the parabrachial and Kölliker-Fuse nuclei.
Figure 5.5: Brainstem regions in which GM was significantly greater in subjects with OSA compared with controls

Hot colour scale indicates increases in grey matter volume in OSA subjects compared with controls. Significant clusters are overlaid onto a T1-weighted anatomical template image. The cerebellum is shaded since it was not included in the analysis. Slice location in Montreal Neurological Institute space are indicated at the top right of each image. dlPons: dorsolateral pons; RVLM: rostral ventrolateral medulla. To the lower right are plots of grey matter volume (GM) in controls (white) and Obstructive sleep apnoea (OSA) (red) subjects. dlPons: dorsolateral pons.
Table 5.3: Location, T-score and cluster size for regions showing significant grey matter volume differences in control compared with OSA

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Cluster locations are given in Montreal Neurological Institute space. OSA: obstructive sleep apnoea.
Figure 5.6: Brainstem regions in which signal intensity changes correlated to MSNA and grey matter volume were both significantly different in subjects with OSA

Significant clusters are overlaid onto a T1-weighted anatomical template image. Slice location in Montreal Neurological Institute space are indicated at the top right of each image. Plots of grey matter volume (GM) against muscle sympathetic activity (MSNA) bursts/minute are shown for the medullary raphe, left rostral ventrolateral medulla (RVLM) and right dorsolateral pons (dIPons). Note that GM in all three regions is significantly correlated to MSNA bursts/min. The location of change within the dIPons is shown overlaid onto a line-drawing modified from (Duvernoy, 1995). Note that the area of change overlaps with and extends ventrally to the parabrachial (PB) and Kölliker-Fuse nuclei (KF). LC: locus coeruleus; OSA: obstructive sleep apnoea; SI: signal intensity.
5.5 Discussion

In addition to the well-documented increase in MSNA and high blood pressure in OSA is a dampened baroreflex (Carlson et al., 1996; Narkiewicz et al., 1998), which likely results from changes in the function of central neural structures involved in the baroreflex. The results of this study show that OSA is associated with altered MSNA related activity changes and significant increase in grey matter volume in the region of the medullary raphe, RVLM and dorsolateral pons. As expected, we found that both subjects with OSA and controls displayed changes in signal intensity associated with MSNA in a number of brainstem regions, including those shown to mediate the baroreflex (Dampney, 1994). This confirms a previous study, in which we showed MSNA-related activity changes within the RVLM (Macefield and Henderson, 2010). In the current study I found that signal intensity changes within the RVLM, were significantly different between controls and OSA subjects. Interestingly, control subjects displayed significantly greater signal intensity than OSA subjects within the left RVLM, although we are at a loss to explain this. Surprisingly, despite elevated MSNA, OSA subjects displayed decreases in MSNA-related signal intensity within the region of the RVLM. It has been proposed that BOLD signal intensity reflects synaptic activity rather than electrical output (Logothetis et al., 2001), so our counterintuitive finding of a decrease in signal intensity within RVLM in OSA may be a reflection of a reduction in active inhibition of RVLM in OSA.

These data suggest that the elevated sympathetic drive in obstructive sleep apnoea may result from functional and anatomical changes within discrete regions of the brainstem, including the dorsolateral pons, RVLM and medullary raphe. These brainstem regions are known to modulate sympathetic output via either direct or
indirect inputs to the sympathetic preganglionic neurons in the spinal cord. It remains unknown if these brainstem changes can be reversed by treatments such as continuous positive airway pressure, which are known to result in a reduction in resting MSNA to control levels. If not, then these changes may be more permanent and may significantly influence development of treatment regimens aimed at reducing the OSA-related hypertension.
Chapter 6: Study IV

Reversal of functional changes in the brain associated with obstructive sleep apnoea following 6 months of CPAP
6.1 Abstract

As Continuous positive airway pressure (CPAP) is the most effective and widely used treatment for preventing collapse of the upper airways in obstructive sleep apnoea (OSA). In addition to improving sleep, CPAP decreases daytime muscle sympathetic nerve activity (MSNA) towards control levels. It remains unknown how this restoration of MSNA occurs, in particular whether CPAP treatment results in a simple readjustment in activity of those brain regions responsible for the initial increase in MSNA or whether other brain regions are recruited to over-ride aberrant brain activity. By recording MSNA concurrently with functional Magnetic Resonance Imaging (fMRI) I aimed to assess brain activity associated with each individual subject’s pattern of MSNA prior to and following 6 months of CPAP treatment. Spontaneous fluctuations in MSNA was recorded in 13 newly diagnosed patients with OSA before and after 6 months of treatment with CPAP and in 15 healthy control subjects. MSNA was significantly elevated in newly diagnosed OSA patients compared to control subjects (55±4 vs 26±2 bursts/min), and there was a significant fall after 6 months of CPAP (39±2 bursts/min). The reduction in resting MSNA was coupled with significant changes in signal intensity in precuneus bilaterally, the left and right insula, right medial prefrontal cortex, right anterior cingulate cortex, right parahippocampus and the left and right retrosplenial cortices. CPAP treatment had no significant effect on grey matter volume in any of these brain regions. These data support our contention that functional rather than structural changes in these suprabulbar sites are, via projections to the brainstem, driving the augmented sympathetic outflow to the muscle vascular bed in OSA.
6.2 Introduction

It has been shown by a number of investigators that treatment of OSA with continuous positive airway pressure (CPAP) results in a decrease in daytime MSNA towards control levels (Hedner et al., 1995; Imadojemu et al., 2007; Fatouleh et al., 2014b; Stenlöf et al., 1996; Waradekar et al., 1996). It remains unknown how this reduction in MSNA occurs, in particular whether CPAP treatment results in a simple readjustment in activity of brain regions responsible for the initial increase in MSNA as explained in study II&III, or whether other brain regions are recruited to over-ride the aberrant brain activity. Elucidating the underlying mechanisms is important as it may provide evidence that changes in brain activity in individuals with conditions such as OSA are reversible by effective treatment regimens. The aim of this investigation is to determine the effects of CPAP on resting MSNA and associated brain activity patterns in individuals with OSA, by using concurrent recording of MSNA and functional Magnetic Resonance Imaging (fMRI) to assess brain activity associated with each individual subject’s sympathetic burst patterns prior to and following 6 months of CPAP treatment. I hypothesized that activity in those brain regions associated with increased spontaneous MSNA, such as the prefrontal, cingulate, and parahippocampus, would return to control levels as the augmented MSNA seen in OSA returned to control levels following CPAP treatment. Furthermore, I assessed regional grey matter changes using voxel-based morphometry (VBM) (Ashburner & Friston, 2000); I hypothesized that in those regions the reversibility in the activity in those brain regions would be associated with altered grey matter volume.
6.3 Methodology

6.3.1 Participants

Data were obtained from 13 well-characterised and newly diagnosed patients with OSA (11 males, mean±SEM age 53±3, range 35–67 years) and 15 healthy controls (12 males, age 53±3, 35–68 years).

6.3.2 Procedures and analysis

All subjects underwent a pre-MRI laboratory session, which included microneurography, electrocardiography, blood pressure and respiration. Followed by an MRI session and collected a concurrent recording of MSNA and brainstem fMRI as well as a 3D T-weighted image. This was repeated after 6 months of CPAP treatment.

6.4 Results

Two of the OSA subjects were diagnosed with mild, one with moderate and 10 with severe OSA (AHI range 7–62; mean±SEM AHI 38±5). In OSA subjects, the minimum SaO\textsubscript{2} on the night of polysomnography was 83±2% (range 67–93%), baseline SaO\textsubscript{2} during wakefulness was 95±1% (range 91–99%), and the baseline Epworth Sleep Scale score was 9±1 (range 3–19). I monitored compliance during the 6 months CPAP treatment period and found that OSA patients used CPAP for an average of 6±0.4 h/night, as reported by the ResScan software. Also as reported by the software, there was a significant reduction in AHI after 6 months of compliant treatment (AHI range 1–21; mean±SEM AHI 4±2). Furthermore, during the CPAP treatment period, there was no significant change in BMI in OSA subjects (mean±SEM BMI: pre-CPAP 31±2, post-CPAP 33±2; p= 0.7).
The mean AHI for the control subjects following an in-home overnight assessment of sleep patterns was 3±1. Overnight monitoring of sleep was made at variable times after the scanning had been conducted, and revealed that, while the majority had an AHI of 1–3, 3 of the control subjects had an AHI of 8,9 and 10. Although there was no significant difference in age between OSA and control subject groups (two sample t-test; p>0.05), as expected there was a significant difference in body mass index (BMI: pre-CPAP 31±2, controls 24±1, p=0.0005).

6.4.1 Physiology

During the physiology recording session, compared to controls, OSA subjects before pre-CPAP had significantly elevated systolic blood pressure (pre-CPAP vs controls; 141±5 vs 118±4mmHg; p=0.002), diastolic blood pressure (82±2 vs 67±3mmHg, p=0.0003) and MSNA burst frequency (55±4 vs 26±2 bursts/min; p<0.0001) and MSNA burst incident (80±6 vs 40±2 bursts/100 heart beats; p<0.0001). In contrast, there was no significant difference in heart rate (72±3 vs 66±3beats/min; p=0.22).

Six months of CPAP treatment resulted in a significant reduction in resting MSNA burst frequency (pre-CPAP vs post-CPAP; 55±4 vs 39±2 bursts/min; p=0.0005), and burst incident (80±6 vs 56±4 bursts/100 heart beats; p=0.004) in all 13 OSA subjects. Nevertheless, despite a significant reduction in MSNA, CPAP treatment did not have a significant effect on systolic blood pressure (141±5 vs 136±5mmHg, p=0.3), diastolic blood pressure (82±2 vs 76±3mmHg, p=0.08), or heart rate (72±3 vs 70±2beats/min, p=0.6). Figure 6.1 shows a recording of MSNA during fMRI in one subject with OSA prior to and following 6 months of CPAP; it can be seen that there are dramatically fewer bursts of MSNA after CPAP treatment.
Figure 6.1: Multiunit recording of MSNA from a 50-year-old male patient with OSA during scanning of the brain, obtained prior to (A) and following (B) 6 months of CPAP treatment.

The mean-voltage neurogram is shown in the nerve RMS (root mean square) trace; this was used to quantify the number of sympathetic bursts. The black areas represent the scanning artifacts. Muscle sympathetic nerve activity (MSNA) burst amplitudes were measured during the OFF periods. Resting levels of MSNA were greatly reduced following CPAP.
6.4.2 Changes in BOLD signal intensity:

Voxel-by-voxel comparison of MSNA-related BOLD signal intensity changes pre- and post-CPAP treatment revealed that the reduction in resting MSNA was coupled with significant changes in signal intensity in a number of brain regions (Figure 6.2, Table 6.1). Significantly reduced signal-intensity changes post-CPAP compared with pre-CPAP occurred in the region of the precuneus bilaterally, the left and right insula, right medial prefrontal cortex (mPFC), right anterior cingulate cortex (ACC), right parahippocampus and the left and right retrosplenial cortices. Direct comparison of percentage changes in signal intensity during MSNA bursts compared with periods of no bursts showed that CPAP significantly reduced the signal intensity changes within all of these brain regions (mean±SEM signal intensity OSA-0M vs OSA-6M: precuneus: 0.22±0.05 vs -0.06±0.05, p=0.0001; left insula: 0.18±0.03 vs -0.09±0.06, p=0.001; right insula: 0.18±0.04 vs -0.05±0.03, p=0.0005; right mPFC: 0.11±0.05 vs -0.03±0.06, p=0.02; right ACC: 0.07±0.04 vs -0.06±0.05, p=0.01; right parahippocampus: 0.08±0.04 vs -0.10±0.06, p=0.008; left retrosplenial cortex: 0.18±0.04 vs -0.04±0.03, p=0.0006; right retrosplenial cortex: 0.16±0.03 vs -0.12±0.05, p=0.0003).

In all of these brain regions, with the exception of the left insula and right parahippocampus, signal intensity was significantly increased in OSA subjects OSA-0M compared with controls (controls vs OSA pre-CPAP: precuneus: -0.04±0.07 vs 0.22±0.05, p=0.004; left insula: 0.09±0.08 vs 0.18±0.03, p=0.19; right insula: 0.01±0.07 vs 0.18±0.04, p=0.03; right mPFC: -0.02±0.05 vs 0.11±0.05, p=0.04; right ACC: -0.07±0.06 vs 0.07±0.04, p=0.03; right parahippocampus: 0.01±0.05 vs 0.08±0.04, p=0.14; left retrosplenial cortex: -0.10±0.07 vs 0.18±0.04, p=0.002; right retrosplenial cortex: -0.09±0.07 vs 0.16±0.03, p=0.006). Furthermore, within all of these brain regions apart from the left insula, signal intensity returned to
control levels post-CPAP (controls vs OSA post-CPAP: precuneus: p=0.45; left insula: p=0.04; right insula: p=0.22; right mPFC: p=0.38; right ACC: p=0.44; right parahippocampus: p=0.29; left retrosplenial cortex: p=0.24; right retrosplenial cortex: p=0.37).

6.4.3 Changes in grey matter volume

Interestingly in those brain regions in which CPAP treatment was associated with significant changes in MSNA-related BOLD signal intensity. As in Figure 6.3, CPAP treatment had no significant effect on grey matter volume (grey matter volume OSA-0M vs OSA-6M: precuneus: 0.52±0.01 vs 0.53±0.02, p=0.10; left insula: 0.33±0.01 vs 0.34±0.01, p=0.08; right insula: 0.49±0.01 vs 0.50±0.02, p=0.61; right mPFC: 0.42±0.02 vs 0.42±0.02, p=0.55; right ACC: 0.57±0.03 vs 0.57±0.03, p=0.64; right parahippocampus: 0.59±0.01 vs 0.59±0.01, p=0.62; left retrosplenial cortex: 0.45±0.02 vs 0.45±0.02, p=0.36; right retrosplenial cortex: 0.48±0.02 vs 0.48±0.01, p=0.70). In all of these brain regions, grey matter volumes were also not significantly different in OSA subjects pre-CPAP compared with controls (controls vs OSA OSA-0M: precuneus: 0.55±0.02 vs 0.52±0.01, p=0.26; left insula: 0.34±0.01 vs 0.33±0.01, p=0.42; right insula: 0.48±0.01 vs 0.49±0.01, p=0.37; right mPFC: 0.44±0.02 vs 0.42±0.02, p=0.46; right ACC: 0.57±0.03 vs 0.57±0.03, p=0.94; right parahippocampus: 0.60±0.02 vs 0.59±0.01, p=0.85; left retrosplenial cortex: 0.47±0.02 vs 0.45±0.02, p=0.33; right retrosplenial cortex: 0.50±0.02 vs 0.48±0.01, p=0.27). Furthermore, within all of these brain regions, grey matter volume was not different between controls and OSA post-CPAP subjects (controls vs OSA OSA-6M: precuneus: p=0.28; left insula: p=0.95; right insula: p=0.30; right mPFC: p=0.52; right ACC: p=0.97; right parahippocampus: p=0.97; left retrosplenial cortex: p=0.26; right retrosplenial cortex: p=0.21).
Figure 6.2: Brain regions in which signal intensity changes correlated to resting MSNA were significantly different in subjects with OSA prior to CPAP, compared with following 6 months of CPAP treatment.

Cool colour scale represents regions in which signal intensity changes during each muscle sympathetic nerve activity (MSNA) burst were significantly reduced following continuous positive airway pressure (CPAP) treatment. Significant clusters are overlaid onto a mean T1-weighted anatomical template image. Slice location in Montreal Neurological Institute space are indicated at the lower right of each image. ACC: anterior cingulate cortex, mPFC: medial prefrontal cortex; OSA: obstructive sleep apnoea.
Table 6.1: Location, T-score and cluster size for regions showing significant signal intensity differences that were coupled to spontaneous MSNA in subjects with OSA prior to, compared with after 6 months of continuous positive airway pressure treatment.

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<td>right parahippocampus</td>
<td>24</td>
<td>-42</td>
<td>-11</td>
<td>3.64</td>
<td>26</td>
</tr>
<tr>
<td>right medial prefrontal cortex</td>
<td>8</td>
<td>60</td>
<td>-2</td>
<td>3.78</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>53</td>
<td>19</td>
<td>4.16</td>
<td>20</td>
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<tr>
<td>retrosplenial cortex</td>
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<tr>
<td>right</td>
<td>9</td>
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<td>13</td>
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</tr>
<tr>
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<td>30</td>
<td>5.22</td>
<td>923</td>
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</tbody>
</table>

Cluster locations are given in Montreal Neurological Institute (MNI) space. MSNA: muscle sympathetic nerve activity; OSA: obstructive sleep apnoea.
Figure 6.3: Plots of percentage signal intensity changes in regions in which signal intensity changes correlated to MSNA were significantly different in subjects with OSA prior to, compared with following 6 months CPAP treatment.

Graphs show mean (±SEM) signal intensity (SI) changes during resting muscle sympathetic nerve activity (MSNA) bursts compared with periods of no bursts in controls (black), obstructive sleep apnoea (OSA) pre-CPAP (white) and OSA post-CPAP (grey). In addition, plots of grey matter volumes (GM) for each cluster are shown. Note that, continuous positive airway pressure (CPAP) treatment results in a significant reduction in signal intensity during each MSNA burst in all seven brain regions. Furthermore, these signal reductions return to control levels in all regions except for the left insula. Note also that the fMRI signal intensity changes are not accompanied by changes in grey matter volume. * indicates p<0.05.
6.5 Discussion

In this study, I have shown similar to *study I*, that CPAP caused a significant fall in MSNA. This reduction in MSNA was not associated with a significant fall in blood pressure (BP), which contradicts with some previous findings that CPAP does reduce BP (Becker et al., 2003; Narkiewicz et al., 1999). However, it is worth pointing out that, while MSNA fell significantly following 6 months of CPAP, it did not return to resting levels in control subjects. Importantly, the fall in resting MSNA following CPAP was associated with significant changes in BOLD signal intensity within a number of brain regions, including precuneus bilaterally, the left and right insula, right medial prefrontal cortex (mPFC), right anterior cingulate cortex (ACC), right parahippocampus and the left and right retrosplenial cortices, despite the fact that blood pressure remained elevated. Those brain regions have shown altered in signal intensity in OSA patients prior to treatment, *StudyII*. That could mean that the functional changes in those brain regions are responsible for the changes in muscle sympathetic outflow.

I found no effect of CPAP treatment on grey matter volume in any of these brain regions that showed altered MSNA-related signal intensity. Additionally, none of those regions were different between healthy controls and OSA. Therefore, we suggest that altered in signal intensity associated with increased MSNA do not result from localized structural changes that may occur as a result of the repeated hypoxic events that occur in individuals with OSA, and CPAP reduces the activity in those cortical and subcortical regions, without any change to the grey matter volume.
Chapter 7:

General Discussion
The chapters of this thesis cover the research I undertook to investigate the neurophysiological disturbances associated with obstructive sleep apnoea (OSA), and the effects of continuous positive airway pressure (CPAP) treatment, in a longitudinal design. While the main findings of each study have been summarised within their respective chapters, the following section will discuss, in more detail, the findings in this thesis.

7.1 Physiological changes due to OSA before and after treatment

It is well documented, and further confirmed by my research, that OSA is associated with a significant increase in muscle sympathetic nerve activity (MSNA) (Study I – IV), leading to neurogenic hypertension both during sleep and waking periods (Carlson et al., 1993, 1996; Fatouleh et al., 2014b; Hedner et al., 1995, 1988; Narkiewicz et al., 1999; Somers et al., 1995; Elam et al., 2002; Narkiewicz & Sommers, 2003; Imadojemu et al., 2007) and a high mortality rate (He et al., 1988; Partinen et al., 1988). Furthermore, we have previously argued, on the basis of single-unit recordings obtained in patients with OSA and in otherwise healthy obese individuals, that this elevated MSNA in OSA is largely independent of any elevated body mass index (Macefield, 2012). As reported in Studies I & IV, I have shown that long-term compliant CPAP treatment caused a significant fall in MSNA after 6 months of CPAP with no further change after 12 months (Hedner et al., 1995; Narkiewicz et al., 1999a; Stenlöf et al., 1996; Waradekar et al., 1996; Fatouleh et al., 2014b). However, as demonstrated previously, the reduction in MSNA was not associated with a significant fall in blood pressure (Hedner et al., 1995), though MSNA remained higher than in the controls, which contradicts with some previous findings -that CPAP does reduce blood pressure (Becker et al., 2003; Narkiewicz et al., 1999).
The mechanisms underlying the sympathoexcitation and how it leads to hypertension are generally poorly understood, other than that it is related to the long-term effects of hypoxia on MSNA (Lanfranchi & Somers, 2001; Nieto et al., 2000; Peppard et al., 2000b). An increase in respiratory-sympathetic coupling has been argued as being an important contributor to the increase in blood pressure in the spontaneously hypertensive rat (SHR), and to human hypertension (Czyzyk-Krzeska & Trzebski, 1990; Simms et al., 2009; Moraes et al., 2014). However, in Study I (included as Appendix C), I have shown, for the first time, that the magnitude of the respiratory modulation of MSNA in OSA patients was not significantly different to that of healthy controls. Moreover, a reduction in MSNA after CPAP was not associated with changes in the magnitude of the respiratory modulation of MSNA.

MSNA is controlled on a beat-to-beat basis by the arterial baroreceptors, such that MSNA shows a very tight coupling to the cardiac cycle and an inverse relationship to diastolic pressure. In addition, previous studies showed that there is also a close relationship between MSNA and respiration, bursts of MSNA occurring preferentially in expiration and being inhibited in mid-inspiration (Hagbarth & Vallbo, 1968b; Eckberg et al., 1985; Macefield & Wallin, 1995a,b; Macefield et al. 1999; Seals et al., 1990, 1993). Respiratory modulation of MSNA is independent of the changes in BP, so it cannot be explained by indirect effects via the baroreceptors (Seals et al., 1993; Macefield & Wallin, 1995a). Like MSNA, skin sympathetic nerve activity (SSNA) also exhibits respiratory modulation, the magnitude of which is statistically identical to that of MSNA (Fatouleh & Macefield, 2013 - Appendix B).

As noted above, studies in the neonatal and juvenile SHR showed an increased respiratory modulation of vasoconstrictor drive in the thoracic (Simms et al., 2009) and the cervical and lumbar (Moraes et al., 2014) sympathetic outflows. It was
hypothesized that amplification of this modulation leads to hypertension, not just in the rat but in human hypertension (Simms et al., 2009; Moraes et al., 2014). Unlike humans, in the SHR the lowest sympathetic activity occurs during the post-inspiratory period and the highest activity occurs in mid-inspiration and mid-expiration; given that the rats had been sinoartically denervated and were vagotomised, the respiratory modulation must be independent of the arterial baroreceptors, peripheral chemoreceptors and inputs from the lungs (Czyzyk-Krzeska & Trzebski, 1990). Interestingly, recent studies by Moraes and colleagues (2014) found that, in the juvenile SHR pre-inspiratory and post-inspiratory medullary respiratory neurones show an increased excitability compared to the normotensive Wistar-Kyoto rat; importantly, this was reflected in an increase in post-inspiratory activity in rostral ventrolateral medulla (RVLM) neurones.

Recently, as part of my Bachelor of Medical Science Honours year, I explored the idea that essential hypertension in humans is due to an increase in respiratory-sympathetic coupling, by quantifying respiratory modulation of MSNA in a group of patients with essential hypertension and increased levels of MSNA at rest (Fatouleh & Macefield, 2011; see Appendix A). Compared to a group of normotensive age-matched control subjects, there was no increase in respiratory modulation in the hypertensive group. In addition, I also found no increase in respiratory modulation in patients with chronic obstructive pulmonary disease (COPD). Given that the latter patients are chronically asphyxic, are in a state of elevated respiratory drive (Gandevia et al., 1996), and have very high levels of MSNA (Heindl et al., 2001; Raupach et al., 2008; Ashley et al., 2010), I would have thought that respiratory modulation would have been higher in this group than in the essential hypertension group, but it was actually lower (Fatouleh & Macefield, 2011). Interestingly,
respiratory modulation in COPD (37.5±6.3%) was statistically identical to that seen in the patients with OSA (39.8±3.1%). Accordingly, my research does not support the idea that an increase in respiratory modulation of MSNA can explain the sympathoexcitation and, for essential hypertension and obstructive sleep apnoea, the high blood pressure. However, there were significant changes in the temporal profile of the respiratory modulation of MSNA in the OSA patients; when examining the temporal profile of respiratory modulation, measured from the normalised MSNA during the respiratory cycle, it was clear that the temporal coupling of MSNA to respiration was stronger in the OSA patients than in the control subjects. In this study (Study I), I have shown that prior to the initiation of CPAP, MSNA was higher in the post-inspiratory phase than in inspiration and expiration; these changes were largely reversed following long-term CPAP. I should point out that all analyses were conducted after all data collection had been completed; I was unaware that there was no significant difference in respiratory modulation of MSNA in the OSA patients prior to undertaking the analysis of the data following CPAP.

It has been suggested that the sympathoexcitation and increased blood pressure in OSA patients is likely a result of changes in the function of central neural structures involved in the baroreflex. It is known that the sensitivity of the baroreflex is reduced in OSA (Carlson et al., 1996; Ryan et al., 2007), as well as in COPD (Raupach et al., 2008), but the blunted baroreflex sensitivity in OSA has been shown to be unrelated to the overall increase in muscle sympathetic outflow (Carlson et al., 1993). While I did not specifically measure baroreflex sensitivity in my study, cardiac modulation of MSNA clearly does reflect the efficacy of the baroreflex; the finding that cardiac modulation was significantly lower in the patients with OSA than in a group of age-matched control subjects further confirms that the sympathetic
baroreflex is indeed blunted in OSA. However, there was no significant change in this measure following 6 or 12 months of CPAP, although an improvement in baroreflex sensitivity, as measured from spontaneous fluctuations in MSNA, has been reported (Ryan et al., 2007).

7.2 Neurophysiological changes due to OSA before and after treatment

A number of investigations have explored the neural basis of evoked changes in blood pressure and heart rate in both controls and OSA subjects during cardiorespiratory challenges such as the Valsalva manoeuvre, cold pressor test, sustained hand grip and inspiratory and expiratory loading (Harper et al., 2003, 2012; Macey et al., 2003, 2013). In general, these studies have shown that OSA subjects display aberrant cardiovascular responses which are associated with altered activity in regions such as the hypothalamus, amygdala, hippocampus, and insular, cingulate and prefrontal cortices (Harper et al., 2003, 2012; Henderson et al., 2003; Macey et al., 2003, 2013; Woo et al., 2005, 2007). Furthermore, many of these areas of altered function overlapped with regions of grey matter loss and altered water diffusion parameters (Kumar et al., 2012; Macey et al., 2002, 2003, 2006), indicative of cellular and axonal damage. Although these previous studies are valuable, the brain activation patterns evoked during such challenges are complicated by behavioural changes, including alterations in motor drive, volition, sensory input and cognition. In contrast, in my studies, I determined the activation patterns associated with spontaneous bursts of MSNA while the subject was relaxed and at rest, i.e. in the absence of any manoeuvre (Studies II-IV).

Using concurrent microneurography and fMRI, I have shown, for the first time, that the elevated levels of spontaneous MSNA seen in subjects with OSA are related to functional changes in the activity of several discrete cortical and subcortical
regions. Surprisingly, none of the regions displayed significant anatomical changes (Study II). These data suggest that the elevated muscle vasoconstrictor drive that occurs in individuals with OSA may be driven by changes in activity in higher cortical regions, possibly through influences on brainstem regulatory nuclei, independently of the loss of grey matter associated with OSA. Therefore, by looking in depth into the functional and structural changes to the brainstem due to OSA (Study III), I found that the elevated sympathetic drive in OSA may result from functional and anatomical changes within discrete regions of the brainstem known to modulate sympathetic output via either direct or indirect inputs to the sympathetic preganglionic neurones in the spinal cord. Interestingly, I have shown that the functional changes in the brain in patients with OSA return essentially to control levels after 6 months of compliant CPAP treatment (Study IV). Importantly, that the changes in signal intensity following CPAP essentially paralleled the changes in MSNA, despite the fact that blood pressure remained elevated, means that the functional changes in the brain are responsible for the changes in muscle sympathetic outflow.

7.2.1 Changes in the brain due to OSA before and after treatment

Our laboratory previously used concurrent recording of MSNA and fMRI to determine brainstem and cortical sites underlying spontaneous changes in MSNA in healthy controls (James et al., 2013). The results from my study (Study II) in controls and OSA patients show similar patterns, with spontaneous bursts of MSNA associated with functional changes within the insula, dorsal precuneus, right hypothalamus, dorsolateral prefrontal, posterior temporal, mid-cingulate and sensorimotor cortices. However, in addition, a number of brain regions displayed significant increases or decreases in signal intensity during each MSNA burst in
OSA subjects but not healthy controls. Within the left and right medial prefrontal cortex (mPFC), dorsolateral prefrontal cortex (dLPFC), dorsal precuneus, anterior cingulate cortex (ACC), retrosplenial cortex and caudate nucleus, signal intensity increased significantly during each MSNA burst in OSA subjects but not in controls. In contrast, within the right hippocampus and parahippocampus, signal intensity decreased significantly during each MSNA burst in healthy controls, but did not change in OSA subjects. Thus, the MSNA-related neural activity in higher brain regions differs in OSA patients relative to controls. Interestingly, as I have shown in Study IV, in the precuneus bilaterally, the left and right insula, right mPFC, right ACC, right parahippocampus and the left and right retrosplenial cortices, signal intensity was altered and returned to control levels after 6 months of CPAP treatment.

In OSA subjects, I found an increase in signal intensity in mPFC, which covaried with MSNA. The mPFC is known to send projections to specific nuclei in the brainstem related to control of muscle sympathetic outflow, such as the nucleus tractus solitarius (NTS) and rostral ventrolateral medulla (RVLM) (Sica et al., 2000a; Weisz et al., 2001). Although mPFC lesions do not affect resting mean blood pressure and heart rate, they do reduce baroreceptor reflex gain (Verberne et al., 1987). As I mentioned earlier, baroreceptor sensitivity is reduced in OSA (Carlson et al., 1996; Ryan et al., 2007), but this reduction is unrelated to the overall increase in muscle sympathetic outflow (Carlson et al., 1993). Furthermore, electrical stimulation of the mPFC reduces the discharge of sympathoexcitatory neurones in RVLM, and microinjection of glutamate into the mPFC reduces sympathetic vasoconstrictor drive and blood pressure (Verberne, 1996). Moreover, in an animal model of OSA, the chronic intermittent hypoxia (CIH) model, there is a sustained
increase in sympathetic vasoconstrictor drive and increased c-fos expression in mPFC, a cellular marker of neural activity, in the NTS and RVLM, implicating this structure in the sympathoexcitation (Sica et al., 2000b). It is likely that any changes in mPFC operate via changes in RVLM, which in turn result in increased spontaneous MSNA. After 6 months of CPAP treatment, there was a reduction in signal intensity in mPFC associated with the reduction of MSNA in those patients.

In addition to the functional changes in the mPFC, OSA subjects displayed MSNA-related changes in other cortical areas which differed from healthy controls, including the precuneus, retrosplenial cortex, dIPFC and sensorimotor cortex. Although cardiovascular-related function of the precuneus has received little attention, our laboratory has previously shown that this region, along with the dIPFC, displays MSNA-related activity (James et al., 2013). Furthermore, precuneus activity declines during deep sleep (Maquet, 2000), when MSNA and blood pressure also fall, suggesting that this region may provide a resting drive to MSNA during wakefulness. The precuneus and dIPFC are functionally coupled to the RVLM at rest in healthy controls, which suggests that the activity within these cortical regions can influence activity within the RVLM (James et al., 2013). Furthermore, treatment with CPAP reduces the MSNA-related activity of precuneus - signal intensity in this region was reduced after treatment. However, we currently do not know the significance of any coupling between the sensorimotor cortex and MSNA. Such coupling may reflect projections from the insula, precuneus or PFC to the sensorimotor cortex, all of which, as mentioned earlier, are coupled to spontaneous bursts of MSNA (James et al., 2013).
Unlike the precuneus, there is considerable interest in the role of the ACC in sympathetic control (Beissner et al., 2013; Critchley et al., 2003; Kimmerly et al., 2013). Pyramidal neurones within the ACC project directly and indirectly to subcortical brain regions associated with homeostasis and autonomic control, including the hypothalamus (Ongür et al., 1998). Electrical stimulation of the ACC in experimental animals evokes autonomic responses affecting heart rate and blood pressure (Ward, 1948) and in humans, ACC stimulation causes prompt and usually complete arrest of respiration, bradycardia and a gradual increase in blood pressure (Pool & Ransohoff, 1949). In addition, in humans, ACC lesions are associated with disrupted sympathetic cardiovascular regulation and impaired generation of cardiovascular arousal during cognitive efforts (Critchley et al., 2003). Furthermore, it has been shown during manoeuvres, such as the Valsalva manoeuvre and the cold pressor test, which evoke significant increases in blood pressure and heart rate, that OSA subjects display aberrant cardiovascular responses that are associated with altered changes inactivity in brain regions including the ACC and retrosplenial cortex (Harper et al., 2003; Macey et al., 2013), and in experimental animals, 30 days of CIH exposure evokes c-fos expression in the cingulate cortex (Sica et al., 2000b). Consistent with my findings, orthostatic stressors, such as lower-body negative pressure and inspiratory-capacity apnoea, which unload baroreceptors and cause a sustained increase in MSNA, are associated with increased BOLD signal intensity in ACC (Goswami et al., 2012; Macefield et al., 2006). In addition, heart rate variability is associated with activity changes within both the ACC and retrosplenial cortex (Critchley et al., 2003). The combined evidence demonstrates a key role for the ACC and retrosplenial cortex in the autonomic circuitry, and hence the present findings of altered ACC activity in OSA raise the possibility that dysfunction in this brain region
contributes to the sympathoexcitation in the condition. The normalisation of ACC activity following CPAP treatment further demonstrates a key role for the ACC in contributing to the sympathoexcitation in OSA.

In contrast to the regional differences described above, within the hippocampus/parahippocampus, subjects with OSA displayed no signal intensity changes that covaried with MSNA, whereas controls displayed decreases in signal intensity. Interestingly, CPAP essentially caused a normalisation of this activity towards control levels. This region contain neurones that discharge with the respiratory and cardiac cycles, and electrical stimulation evokes dramatic changes in blood pressure (Harper et al., 2013). A direct chemical stimulation of these regions in an animal model evoked marked decrease in arterial pressure and bradycardia (Ruit & Neafsey, 1988; Wall & Davis, 1951). Additionally, other animal experiments showed the presence of sympathetic-related neurones in these cortical regions (Westerhaus & Loewy, 2001). Blockade of hippocampal activity in experimental animals does not affect resting arterial pressure and heart rate (Wang & Ingenito, 1992), which suggest that these regions form part of the higher-brain central autonomic network. In human studies, using static and grip exercise and the cold-pressor test, OSA and control subjects showed significant differences in heart rate and respiratory responses, which supports their reduced modulatory role in OSA (Harper et al., 2003; Macey et al., 2013). Therefore, the functional changes within this brain region in OSA might be a factor contributing to the altered sympathetic outflow. That BOLD signal intensity is back to control levels after CPAP treatment provides further evidence that this region is functionally related to the elevated muscle sympathetic nerve activity in OSA.

In contrast to regional differences described above, although signal intensity decreased significantly following CPAP treatment in the left insula and right
parahippocampus, it was not different to controls prior to treatment. Somewhat surprisingly, signal intensity declined after CPAP treatment in both these regions to control levels in the right parahippocampus, and even significantly more than control in the left insula. The insular cortex has been considered to play an important role in autonomic control in humans (Oppenheimer et al., 1990), as well as in animals; Indeed, we know that baroreceptors project to the insular cortex in the rat and monkey (Zhang & Oppenheimer, 1997, 2000a,b), and that electrical stimulation of the anterior insula can elicit changes in arterial pressure, heart rate and sympathetic activity (Butcher & Cechetto, 1995). The insular cortex has also been implicated in the cardiovascular regulation (Harper et al., 2003; Oppenheimer et al., 1996; Woo et al., 2005, 2007). Furthermore, cardiovascular challenges evoked increases in signal intensity in the anterior insula (Harper et al., 2000, 2003; Henderson et al., 2002, 2004; Macey et al., 2002, 2003; Sander et al., 2010; Wong, 2007a, b; Woo et al., 2007). The precise route via which the insula alters sympathetic outflow remains unknown. Although the insula does not project directly to RVLM (Saper, 1982), electrical stimulation of the insula excites some RVLM sympathoexcitatory neurones (Sun, 1992). Therefore, it must achieve this via a polysynaptic pathway.

Strikingly, as shown in Study II, I found no voxels displaying both an MSNA-related difference in signal intensity in OSA and a significant change in grey matter volume. This finding was unexpected, since numerous investigations, including this study, have shown that OSA is associated with significant changes in grey matter volume in autonomic control brain regions, which in theory would be expected to impact the normal functioning of those structures. Our OSA subjects displayed increases in grey matter volume in medulla/pons and cerebellum, left mid-insula, right insula, bilateral primary motor cortex, left premotor cortex and left
hippocampus and decreases in the mPFC, occipital lobe, and the left and right amygdala. Previous studies have revealed changes in grey matter volume in areas such as the hippocampus, parahippocampal gyrus, fronto-parietal cortices, temporal lobe, anterior cingulate and cerebellum (Joo et al., 2010; Kumar et al., 2014; Macey et al., 2008; Torelli et al., 2011; Zimmerman & Aloia, 2006b). Given the presumed detrimental effects of hypoxia on brain structure, I hypothesised that changes in MSNA-related brain activity in OSA would be associated with changes in grey matter volume resulting from hypoxic-related changes. This data strongly suggests that cortical changes in BOLD signal intensity associated with increased MSNA do not result from localized structural changes that may occur as a result of the repeated hypoxic events that occur in individuals with OSA. The changes in grey matter volume I showed may not be solely due to changes in neuronal density, but could be due to changes in glia.

### 7.2.2 Brainstem changes due to OSA

In *Study III* I performed high-resolution imaging of the brainstem to assess whether OSA is associated with functional and structural changes. Despite numerous studies reporting altered cortical structure and function in OSA subjects, until now no investigation has focussed on changes within the brainstem. Given that resting MSNA, as well as the primary characteristic of OSA - a lack of upper airway tone - are functions driven by structures within the brainstem, it is likely that altered function of brainstem nuclei is responsible for the autonomic and respiratory changes associated with OSA. Indeed, my data show that OSA is associated with changes in MSNA-related activity and grey matter volume in the region of the medullary raphe, RVLM and dorsolateral pons (dlPons). As expected, I found that both subjects with
OSA and controls displayed changes in signal intensity associated with MSNA in a number of brainstem regions, including those shown to mediate the baroreflex (Dampney, 1994). This confirms a previous study from our laboratory, in which MSNA-related activity changes within the RVLM were first documented in awake humans (Macefield and Henderson, 2010). In Study III, I found that changes in BOLD signal intensity within the RVLM, were significantly different between controls and OSA subjects. Interestingly, control subjects displayed significantly greater signal intensity than OSA subjects within the left RVLM, although I am at a loss to explain this. Surprisingly, despite the elevated MSNA, OSA subjects displayed decreases in MSNA-related signal intensity within the region of the RVLM. It has been proposed that BOLD signal intensity reflects synaptic activity rather than electrical output (Logothetis et al., 2001), so a decrease in signal intensity within RVLM in OSA may be a reflection of a reduction in active inhibition of RVLM in OSA.

It is well established that the RVLM contains premotor neurones which project directly to the sympathetic preganglionic neurones in the intermediolateral cell column of the spinal cord (Dampney, 1994). In addition to its role in mediating the baroreflex, the RVLM provides the major output nucleus from which almost all brain regions, including the cerebral cortex, control arterial pressure. For example, experimental animal investigations have shown that during physical and psychological stressors, higher brain regions such as the cingulate, insular, and prefrontal cortices project to the RVLM to alter arterial pressure and sympathetic drive (Dampney et al., 2002; Gabbott et al., 2005). In addition, other brainstem sites such as the midbrain periaqueductal grey matter (PAG) and dlpons, regions which also displayed significantly MSNA-related signal intensity changes in OSA subjects,
project directly to the RVLM and can mediate changes in sympathetic outflow (Carrive et al., 1988; Lovick, 1992; Saper and Loewy, 1980). Recent human imaging studies support the role of the RVLM in mediating evoked changes in sympathetic drive. For example, increases in MSNA evoked by maximal inspiratory capacity apnoeas and sustained handgrip are associated with increases in BOLD signal intensity within the region of the RVLM (Macefield et al., 2006; Sander et al., 2010).

My data reveals that resting MSNA-related signal intensity changes within the midbrain, dlpons, RVLM and medullary raphe are significantly altered in subjects with OSA. Consistent with these results, it has been previously shown that OSA is associated with altered cardiovascular responses and changes in brainstem signal intensity during a number of cardiovascular manoeuvres. For example, during the cold presser challenge, OSA subjects display smaller heart rate decreases and significantly reduced signal intensity changes in the dorsal midbrain, dorsal and ventral medulla (Harper et al., 2003). Similarly, during loaded breathing, OSA subjects display reduced heart rate responses and altered signal intensity changes in the dorsal midbrain (Macey et al., 2003). Although this study provides the first evidence of altered brainstem function associated with increased MSNA in OSA subjects, a number of studies have investigated brainstem function in an experimental animal model of OSA, the chronic intermittent hypoxia (CIH) model. CIH results in chronic elevated sympathetic activity and increased arterial pressure (Bao et al., 1997; Fletcher et al., 1992) and is associated with increased c-fos expression, a neural activity marker, in the NTS and RVLM (Greenberg et al., 1999; Knight et al., 2011). Although CIH did not result in baseline activation within the dlpons, acute c-fos induction was enhanced in the dlpons, specifically within the
noradrenergic neurones in the locus coeruleus during an acute stressor following CIH exposure (Ma et al., 2008).

In addition to altered MSNA-related activity changes, the dlPons, RVLM and medullary raphe also displayed significantly increased grey matter volumes in OSA subjects compared with controls. This dlPons region overlapped with, and extended ventrally, to the region of the Kölliker-Fuse nucleus (KF). Although inactivation of the dlPons, including the KF nucleus does not alter resting arterial pressure and heart rate (Shafei et al., 2011), chemical stimulation of the same region can evoke increases or decreases in arterial pressure and sympathetic nerve activity (Dampney, 1994; Hade et al., 1988; Miyawaki et al., 1991). Furthermore, neurones within the dlPons display resting sympathetic discharge patterns corresponding to both 10 Hz and cardiac activity (Barman et al., 1999), and chemical inactivation blocks this 10 Hz sympathetic-related rhythm (Barman et al., 1997). These dlPons neurones do not appear to project directly to spinal preganglionic sympathetic neurones (Barman et al., 1999), although they do project directly to the RVLM (Dampney, 1994; Saper and Loewy, 1980). The caudal medullary raphe also contains neurones which display a 10 Hz sympathetic-related rhythm (Barman et al., 1995), and it is thought that this rhythm involves activity in serotonergic neurones (Orer et al., 1996). It appears that both the dlPons and medullary raphe contain neurones which can modulate sympathetic activity and whose activity corresponds to a 10 Hz resting sympathetic activity discharge. Altered activity within these two brainstem regions would almost certainly affect resting sympathetic outflow and potentially underlie the increases in MSNA that occur in OSA.

My data cannot determine the underlying cellular changes that lead to increases in grey matter volume within the medullary raphe, RVLM and dlPons in OSA.
subjects. Numerous investigations have explored changes in grey matter volume associated with OSA and found decreases in several regions - including the hippocampus, cingulate, cerebellar and prefrontal cortices (Canessa et al., 2011; Macey et al., 2002; Morrell et al., 2003). As explained earlier, these decreases in grey matter volume may result from the repeated hypoxic damage that occurs due to years of night-time apnoeas and consequent periods of episodic hypoxaemia. In contrast, I found that within the lower brainstem, OSA subjects had increased grey matter volumes in the medullary raphe, RVLM and dlpons. Previous studies have shown that increased use can lead to an increase in grey matter volume within a relevant cortical region (Amunts et al., 1997; Maguire et al., 2000; Sluming et al., 2002), although it is not known if increased activity within brainstem sites also results in increased volume. And, while I cannot determine the mechanisms responsible for increased grey matter volume, the finding that the medullary raphe and dlpons display both functional and anatomical changes strongly suggests that these changes are coupled.

In addition to volumetric changes in cardiorespiratory-related brainstem regions, I also found increased grey matter volume in OSA subjects in the region encompassing the hypoglossal nucleus. Multiunit electromyography of the genioglossus is increased during wakefulness in OSA subjects (Fogel et al., 2001; Mezzanotte et al., 1992) and changes in the timing and level of firing of groups of inspiratory motor units within the genioglossus have been suggested to indicate changes in the output of the hypoglossal nucleus (Saboisky et al., 2007). It is likely that the increase in hypoglossal nucleus volume seen in OSA subjects is associated with altered neural processing and ultimately changes in the activity of the...
genioglossus muscle. Whether these changes seen in OSA are reversible following long periods of CPAP (6 & 12 months) remains to be determined.

It is well known that MSNA is modulated by respiration (Eckberg et al., 1985; Fatouleh & Macefield, 2011, 2013; see Appendices A & B; Hagbarth & Vallbo, 1968; Seals et al., 1990). Much of the coupling between respiratory rhythm and sympathetic outflow may result from “cross-talk” between brainstem circuits, and since pontomedullary transection significantly attenuates respiratory modulation of sympathetic outflow, it has been proposed that this modulation occurs within the pontine region (Baekey et al., 2008). It is possible that altered input to the dlpons, either from other brainstem regions or higher cortical regions that also display altered structure and function in OSA subjects, results in altered neural numbers and/or complexity, which in turn results in increased grey matter volume and activity and, ultimately, an increase in resting MSNA.
7.3 Limitations

While the methodological approach I have described provides a higher temporal resolution between direct measure of sympathetic outflow and fMRI than is afforded by indirect indicators of sympathetic activity, the general applicability of concurrent microneurography and fMRI is rather limited. Microneurography is a very skilled procedure and requires that the subject remain very still, such that the recording site remains stable; slight movement of the subject could dislodge the recording site, especially while transporting the subject to the MRI room with the microelectrode in situ.

OSA is a heterogeneous condition, with several confounding factors known to influence the central nervous system in general and autonomic outflow in particular. Obesity is one such confounder, although elevated MSNA in OSA is largely independent of any elevated BMI (Macefield, 2012). The effect of BMI on the observed results was not controlled in this study by matching for BMI, so we could not perform a comprehensive assessment of the influences of BMI on the functional and structural changes in the brain seen in OSA. To some extent, this limits interpretation regarding the interaction of OSA and obesity - which is a great risk factor in OSA. A further limitation of our study is that healthy control subjects were not re-evaluated after 6 and 12 months, a restriction caused by practical considerations of study cost and participant availability for repeated scanning.
7.4 Conclusions

I have shown that, despite the greatly augmented levels of muscle sympathetic nerve activity and blood pressure in OSA, the magnitude of respiratory modulation of MSNA is not increased in OSA when measured across individual subjects. However, when considering the normalised temporal profile there were changes in the respiratory patterning of MSNA in OSA, with more activity occurring in post-inspiration and less in inspiration and expiration. This was largely reversed following long-term continuous positive airway pressure, although blood pressure remained elevated because total MSNA remains higher than in the control subjects.

Furthermore, utilizing microneugraphy and fMRI, I have shown, for the first time, that the elevated levels of spontaneous muscle sympathetic nerve activity seen in subjects with OSA are related to functional changes in the activity of several discrete brainstem, cortical and subcortical regions. These cortical and subcortical regions are known to modulate sympathetic output either directly or indirectly. Moreover, that the functional changes within suprabulbar regions of the brain did not match the structural changes indicates that the increase in MSNA in OSA may well be independent of the loss of grey matter associated with OSA. Interestingly, the functional changes revert back to normal in OSA patients after 6 months of CPAP treatment, which is known to result in a reduction in resting MSNA to control levels. On the other hand, within the brainstem regions, including the dorsolateral pons, RVLM and medullary raphe there were both functional and anatomical changes. It remains unknown if these brainstem changes seen in OSA are reversible following long-term CPAP treatment.

These findings are bringing us closer to a clearer understanding of how increases in MSNA are brought about in OSA, and the changes that happen to the
brain following treatment. But we still need to know how the nocturnal episodes of hypoxemia translate into changes we observe and to understand how those changes occur in the brain following treatment. Nevertheless I am confident that the provision of this mechanistic data may lead to the development of novel treatment strategies to improve the health of patients with OSA. Additionally, these results, in particular the reversibility of physiological changes and the brain changes associated with OSA after long-term treatment with CPAP, could serve as a motivator for patients to adhere to treatment.
Bibliography


Appendices
Appendix A

Respiratory modulation of muscle sympathetic nerve activity in obstructive sleep apnoea

Respiratory modulation of muscle sympathetic nerve activity is not increased in essential hypertension or chronic obstructive pulmonary disease

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**Non-technical summary.** High blood pressure is known to be caused by an increase in activity of the sympathetic nerves that constrict blood vessels in skeletal muscle and the gut. Experiments in the spontaneous hypertensive rat (SHR) suggest that the hypertension is brought about by an increase in coupling between respiration and sympathetic outflow. We tested whether this mechanism occurs in two models of elevated muscle sympathetic nerve activity (MSNA) in human subjects: essential hypertension (HT) and chronic obstructive pulmonary disease (COPD). Unlike the SHR model, respiratory modulation of MSNA was not increased in either HT or COPD. These results help us understand how the cardiovascular and respiratory systems interact in health and disease.

**Abstract.** We examined cardiac and respiratory modulation of muscle sympathetic nerve activity (MSNA) in 13 patients with essential hypertension (HT) and 15 with chronic obstructive pulmonary disease (COPD), and compared these with a group of young healthy controls (YHC) and older healthy controls (OHC). There were no significant differences in age of the OHC and HT subjects. MSNA was recorded via a tungsten microelectrode inserted percutaneously into the common peroneal nerve. Respiration was recorded by a strain-gauge transducer around the chest and ECG recorded by surface electrodes. Cardiac and respiratory modulation of MSNA was quantified by fitting polynomials to the cross-correlation histograms constructed between the sympathetic spikes and ECG or respiration. Cardiac modulation was high across all groups, but was significantly lower in COPD (75.9 ± 4.4%) than in the HT (92.4 ± 3.0%), OHC (93.7 ± 1.3%) or YHC (89.1 ± 1.6%) groups. Across all groups, respiratory modulation was significantly lower than cardiac modulation. Respiratory modulation in HT (45.2 ± 5.7%) and COPD (37.5 ± 6.3%) was not higher than in the OHC (47.2 ± 5.4%) or YHC (49.5 ± 6.0%) groups. We have shown that respiratory modulation of MSNA is present in all groups, is consistently lower than the magnitude of cardiac modulation, and is not increased in HT or COPD, arguing against an amplified respiratory–sympathetic coupling in hypertension. Moreover, given that patients with COPD are chronically asphyxic, these data indicate that an increased chemical drive does not increase respiratory modulation of MSNA.

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**Abbreviations.** COPD, chronic obstructive pulmonary disease; HT, essential hypertension; MSNA, muscle sympathetic nerve activity; OHC, older healthy controls; OSAS, obstructive sleep apnoea syndrome; SHR, spontaneous hypertensive rat; YHC, young healthy controls.
Introduction

Muscle sympathetic nerve activity (MSNA), as recorded via microelectrodes from peripheral nerves in awake human subjects (microneurography), is entrained to the cardiac cycle by the arterial baroreceptors. In addition, MSNA is modulated by respiration, the pulse-synchronous vasoconstrictor discharges tending to occur in expiration and decreasing in intensity during mid-inspiration (Hagbarth & Vallbo, 1968; Eckberg et al., 1985, 1988; Seals et al., 1990, 1993; Macfarlane & Wallin, 1995; Macfarlane et al., 2002). Elevated levels of muscle sympathetic nerve activity feature in many cardiovascular and cardiorespiratory diseases. MSNA is high in patients with congestive heart failure (Leimbach et al., 1986; Grassi et al., 1995; Macfarlane et al., 1999), essential hypertension (Grassi et al., 1998; Schlaich et al., 2004; Lambert et al., 2007), pregnancy-induced hypertension (Schoel et al., 1996; Fischer et al., 2004), renovascular hypertension (Johansson et al., 1999; Miiptima et al., 2001) and the hypertension associated with chronic kidney disease (Hausberg et al., 2002; Schlaich et al., 2009). MSNA is also greatly elevated in the obstructive sleep apnoea syndrome (Hedner et al., 1988; Carlsson et al., 1993; Somers et al., 1995; Narkiewicz et al., 1999; Elam et al., 2002) and in chronic obstructive pulmonary disease (Hindli et al., 2001; Raupach et al., 2008; Ashley et al., 2010). The mechanisms for the sympathetic overactivation differ in each of these pathophysiological states and are generally poorly understood. Yet, given the importance of the physiological coupling between the cardiovascular and respiratory systems, it is surprising that little research has assessed how strong this coupling is in different disease states.

Based on recent findings in the spontaneously hypertensive rat, in which respiratory modulation of sympathetic vasoconstrictor drive was shown to be increased and a theory proposed that ascribes the hypertension to this increase in respiratory modulation (Czyzyk-Krzeska & Trzebski, 1990; Simmons et al., 2009), we tested the hypothesis that the magnitude of respiratory modulation of sympathetic vasoconstrictor drive is likewise increased in essential hypertension in human subjects. We also tested the hypothesis that respiratory modulation of MSNA is also increased in other states of elevated MSNA without hypertension. Specifically, we assessed the magnitude of respiratory modulation in patients with chronic obstructive pulmonary disease (COPD); these patients suffer from marked airflow limitation, primarily in expiration, and a compromised gas exchange (Hindli et al., 2001; Raupach et al., 2008; Ashley et al., 2010). Interestingly, although the levels of MSNA seen in COPD are similar to those seen in the obstructive sleep apnoea syndrome (OSAS) – in which repeated episodes of nocturnal hypoxaemia cause an increase in MSNA and neurogenic hypertension (Hedner et al., 1988; Carlsson et al., 1993; Narkiewicz et al., 1999; Elam et al., 2002) – in COPD there is no hypertension (Hindli et al., 2001; Raupach et al., 2008; Ashley et al., 2010). This then provides a nice means of factoring out the increase in blood pressure per se from the increase in MSNA observed in both essential hypertension and COPD. Furthermore, in COPD the chronic hypoxaemia and hypercapnia lead to an increase in inspiratory drive; mean firing rates of motor units recorded from the diaphragm, scalene and parasternal inspiratory muscles are all increased in COPD (Gandevia et al., 1996; De Troyer et al., 1997). So it is not unreasonable to posit that respiratory modulation of MSNA will also be augmented. Indeed, an elevated resting level of MSNA and an elevated respiratory drive may well be expected to increase, by central neuronal means, the coupling between the cardiovascular and respiratory systems, which will be evidenced by a magnitude of respiratory modulation of MSNA which is higher than what we expect to see in essential hypertension.

Methods

Participants

Data were obtained from 22 healthy participants, subdivided into a younger group (n=12; 8 male, 4 female) with a mean age (±SEM) of 29±1.1 years and an older group (n=10; 5 male, 5 female) with a mean age of 50±3.6 years, and 10 male and 3 female patients with long-standing essential hypertension (age 58±2 years). A retrospective analysis was performed on data obtained from 8 male and 7 female patients with chronic obstructive pulmonary disease (COPD) with a mean age 71±2 years, a subset of data previously reported from our laboratory (Ashley et al., 2010). Both the hypertensive and COPD patient groups remained on their scheduled pharmaceutical and physiotherapeutic treatment during the recordings. The hypertensive patients were on combinations of Ca2+ channel blockers (n=5), angiotensin II receptor antagonists (n=5), β-adrenergic blockers (n=4), angiotensin-converting enzyme (ACE) inhibitors (n=2) and statins (n=5). One COPD patient was being treated with an angiotensin II receptor antagonist, one with thyrroxine and another was on supplemental oxygen (discontinued 1 h before the recording); inhaled respiratory medications included salbutamol, ipratropium, fluticasone propionate, salmeterol and beclomethasone dipropionate. Each participant provided informed written consent to the procedures, which conformed to the standards set by the Declaration of Helsinki and which were conducted under the approval of the Human Research Ethics Committees of the University of Western Sydney and the University of New South Wales.
Appendix A

General procedures
Participants lay semi-recumbent in a chair with their backs at 45 deg and their legs supported horizontally. Spontaneous MSNA was recorded from fascicles of the common peroneal nerve supplying the ankle and toe extensor and foot evertor muscles via tungsten microelectrodes (Frederick Haer Co., Bowdoinham, ME, USA) inserted percutaneously at the level of the fibular head; a nearby subdermal electrode with a larger uninsulated tip served as the reference electrode. The impedances of the microelectrodes (~100–150 kΩ), favoured oligo-unitary and multi-unit, rather than unitary, recordings. Neural activity was amplified (gain 2 × 10^5, bandpass 0.3–5.0 kHz), using an isolated amplifier and headstage (NeuroAmpXR, ADInstruments, Sydney, Australia) and stored on computer (10 kHz sampling) using a computer-based data acquisition and analysis system (PowerLab 16SP hardware and LabChart 7 software, ADInstruments). ECG (0.3 Hz to 1.0 kHz) was recorded with Ag–AgCl surface electrodes on the chest and sampled at 2 kHz. Respiration (DC to 100 Hz) was recorded using a strain-gauge transducer (Pneumotrace, UPI, Morro Bay, CA, USA) wrapped around the chest and sampled at 100 Hz. Continuous non-invasive blood pressure was recorded using radial arterial tonometry (Colin 7000 NIBP, Colin Corp., Japan) or digital arterial plethysmography (Finometer, Finapres Medical Systems, The Netherlands), sampled at 400 Hz. Spontaneous MSNA, ECG, blood pressure and respiration were recorded for at least 30 min and periods of stable activity (>10 min) were sampled for analysis.

Analysis
MSNA was displayed as an RMS-processed (root mean square, moving average, time constant 200 ms) signal but the primary analysis was conducted on the raw, negative-going, sympathetic spikes to avoid any contamination from spikes generated by positive-going myelinated axons (such as spontaneously active muscle spindles) or motor units associated with spontaneous EMG activity. This approach has been described previously, and provides a more sensitive means of analysing sympathetic outflow than the standard method of simply counting the number of sympathetic bursts (Bent et al. 2006). Briefly, negative-going spikes in the neurogram (with a half-width of 0.2–0.5 ms) were detected using window discriminator software (Spike Histogram for Macintosh v2.2, ADInstruments), while the times of occurrence of the R-waves of the ECG and of the inspiratory peaks of the respiratory signal were computed using Peak Analysis software (ADInstruments). Autocorrelation histograms for the respiratory and cardiac signals, and cross-correlation histograms between the MSNA and ECG or respiration, were generated by the Spike Histogram software (50 ms bins). Discriminator levels of the neural activity were adjusted so that negative-going (C-fibre) spikes exhibited a robust cardiac modulation, as revealed by cross-correlation between the neural activity and the ECG. These same discriminator settings were used for construction of cross-correlograms between the MSNA and the positive peaks of the respiratory signal. Smoothed polynomial curves were fitted to the histogram data using a graphical analysis program; lower-order polynomials were used to fit curves to the slower respiratory cross-correlograms while higher-order polynomials were required to fit curves to the cardiac cross-correlograms (Prism 5.0, GraphPad Software, USA). Quantification of the modulation of MSNA was performed by measuring the difference in the number of spikes at the peak of the modulation and the number at the trough, expressed as a percentage: Modulation Index = (Peak – Trough)/Peak × 100. For calculating the modulation index the peak–trough difference extended over the same interval as the respiratory or cardiac periods. In addition, in each subject we calculated the number of sympathetic spikes in different phases of the respiratory cycle, as defined by the peak of inspiration: expiratory activity was calculated from −3.0 to −1.5 s of the inspiratory peak (time 0), inspiratory activity was calculated from −1.5 to 0 s and post-inspiratory activity from 0 to 1.5 s. For this analysis the neural activity was displaced back in time 1.2–1.3 s in each subject to account for the baroreflex delay. The total number of sympathetic spikes was divided by the number of minutes in the recording and presented as spikes per minute. Mean blood pressure was also calculated in each of these intervals to assess respiratory fluctuations in blood pressure. MSNA was also quantified according to standard time-domain analysis of the RMS-processed signal as burst frequency (bursts min^-1) and burst incidence (bursts 100 heart beats^-1). Analysis of variance, coupled with Tukey’s multiple comparisons test, was used to assess statistical significance across each group (Prism 5.0, GraphPad Software, USA). All values are expressed as means and standard errors of the mean, and P < 0.05 was considered statistically significant.

Results
Cardiovascular and respiratory details of the younger and older healthy controls (YHC and OHC) and the patients with essential hypertension (HT) and chronic obstructive pulmonary disease (COPD) are provided in Table 1. The mean age of the hypertensive patients was not significantly different from that of the older healthy control subjects, but was significantly higher than that of the younger healthy controls and significantly lower than that of the
Table 1. Cardiorespiratory parameters of the young healthy controls (YHC), older healthy controls (OHC), patients with hypertension (HT) or obstructive pulmonary disease (COPD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>YHC (n = 12)</th>
<th>OHC (n = 10)</th>
<th>HT (n = 13)</th>
<th>COPD (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>126 ± 4**</td>
<td>130 ± 6</td>
<td>152 ± 4*</td>
<td>133 ± 5</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>70 ± 4</td>
<td>75 ± 4</td>
<td>88 ± 2</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>Mean BP (mmHg)</td>
<td>95 ± 4</td>
<td>95 ± 6</td>
<td>110 ± 2**</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>Heart rate (beats min⁻¹)</td>
<td>74 ± 5</td>
<td>60 ± 3</td>
<td>63 ± 5</td>
<td>70 ± 3</td>
</tr>
<tr>
<td>Resp. period (s)</td>
<td>3.9 ± 0.2</td>
<td>4.0 ± 0.2</td>
<td>3.9 ± 0.3</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>Burst freq. (bursts 100 hbs⁻¹)</td>
<td>22 ± 2</td>
<td>29 ± 3</td>
<td>51 ± 3**</td>
<td>62 ± 2***</td>
</tr>
<tr>
<td>Burst incidence (bursts (100 hbs⁻¹)</td>
<td>33 ± 3</td>
<td>49 ± 6</td>
<td>80 ± 4**</td>
<td>85 ± 2***</td>
</tr>
</tbody>
</table>

Values presented are mean ± SEM. For clarity, only statistical comparisons are shown relative to data obtained in the OHC group, for which there was no significant age difference from the HT group: *P < 0.05, **P < 0.01, ***P < 0.001. See text for further comparisons. BP: blood pressure; hbs, heart beats.

COPD patients. For convenience we have made statistical comparisons with the older healthy controls. It can be seen that there were no significant differences in heart rate or respiratory period across groups but, as expected, systolic and diastolic pressures (and hence mean arterial pressure) were significantly higher in the HT group. There were no differences in any of the blood pressure parameters across the YHC, OHC or COPD groups. As previously described, MSNA was significantly elevated in HT and COPD, with burst frequency (but not burst incidence) being significantly higher in COPD than in HT (P < 0.01). Burst incidence was significantly lower in the YHC group than in the OHC (P < 0.05), HT (P < 0.005) or COPD (P < 0.005) groups. Blood gas analysis in the COPD patients revealed significant hypoxaemia and hypercapnia, as reported in the original study from which the current data were obtained (Ashley et al. 2010).

Experimental records from a normotensive healthy female control subject (OHC) are shown in Fig. 1; records from a female subject with essential hypertension are shown in Fig. 2. In both figures event markers are shown for the sympathetic spikes, the ECG and the peaks of inspiration. These event markers were used to construct the autocorrelation and cross-correlation histograms shown in Fig. 3. As illustrated in Fig. 3B, cross-correlation histograms between the times of occurrence of the sympathetic spikes and of the ECG show a very tight coupling of MSNA to the R-wave of the ECG, while the respiratory rhythmicity is weaker (Fig. 3A). Cardiac and respiratory modulation of MSNA was calculated from the smoothed polynomials fitted to the cross-correlation histograms.

Mean data are shown graphically in Fig. 4. Cardiac modulation was high across all groups, but was significantly lower in COPD (75.9 ± 4.4%; P < 0.01) than in the HT (92.4 ± 3.6%, OHC (93.7 ± 1.3%) or YHC (89.1 ± 1.6%) groups. Across groups there was no difference in respiratory period, although respiratory rate tended to be higher (and respiratory period lower) in the COPD patients (presumably because of the elevated chemical drive to breathe). Respiratory modulation of MSNA was significantly lower than cardiac modulation across all groups. Interestingly, while we had predicted that respiratory modulation would be higher in HT, there was no significant difference between this group (45.2 ± 5.7%) and the YHC (49.3 ± 6.0%) and OHC (47.2 ± 5.4%) groups. Moreover, given the chronic asphyxia, we had predicted that respiratory modulation in COPD would be higher than in HT, but in fact it was lower (37.5 ± 6.3%) than in the HT, YHC and OHC groups, though this difference failed to reach statistical significance. The phase shift between the peak of inspiration and the peak of the respiratory modulation of MSNA was not significantly different across groups: 3.40 ± 0.3 s (YHC group), 3.48 ± 0.3 s (OHC), 3.53 ± 0.4 s (HT) and 2.50 ± 0.4 s (COPD). There were also no significant differences in the phase lag between the R-wave and the peak of cardiac modulation for the YHC (0.69 ± 0.1 s), OHC (0.54 ± 0.1 s), HT (0.55 ± 0.1 s) and COPD (0.52 ± 0.1) groups.

Table 2 shows the total MSNA, calculated as the number of sympathetic spikes in a one-minute period across the different phases of the respiratory cycle (see Methods). Although MSNA tended to be higher during inspiration in the YHC, OHC and HT groups, but higher in expiration in the COPD group, these differences failed to reach statistical significance. There were also no significant respiratory variations in mean blood pressure within each group (Table 2).

Discussion

This is the first study to compare the magnitude of respiratory modulation in two pathophysiological states of increased muscle sympathetic nerve activity – essential
hypertension and chronic obstructive pulmonary disease—
with that of healthy subjects. Contrary to our expectations,
compared to age-matched healthy subjects, respiratory
modulation was not increased in the hypertensive group,
despite the clear evidence of a significantly elevated
blood pressure. This would argue against an amplified
respiratory-sympathetic coupling as being a causative
factor in the generation of hypertension. Moreover, given
that patients with chronic obstructive pulmonary disease
are chronically asphyctic, our observations of a slightly
lower (or at least statistically unchanged) respiratory
modulation in COPD indicates that an increased chemical
drive does not increase respiratory modulation of MSNA.
That cardiac rhythmicty of MSNA was significantly lower
in COPD suggests perhaps that the sympathetic bursts
are not completely inhibited by the arterial baroreceptors
in COPD; indeed, we know that the sensitivity of the
sympathetic baroreflex is reduced in COPD (Raupach et al.
2008).

**Respiratory modulation of MSNA**

In humans, MSNA is inhibited in mid-inspiration
(Hagbarth & Vallbo, 1968; Eckberg et al. 1985, 1988; Seals
et al. 1990, 1993; Maclefield & Wallin, 1995; Maclefield
et al. 2002), whereas in the cat vasoconstrictor outflow
occurs during inspiration (Bainton et al. 1985; Cohen
& Gootman, 1970; Koizumi et al. 1971; Barman &
(or splanchnic) vasoconstrictor drive is synchronized
to expiration in humans but inspiration in cats is not
clear, but Bocek-Funkke et al. (1992) have shown that
although sympathetic activity increases during inspiration
it is initially depressed in early inspiration and reaches a
nadir in post-inspiration. Experiments in anaesthetized
animals are typically performed during neuromuscular
blockade and artificial ventilation, conditions in which
central (phrenic discharge) and peripheral (lung inflation)
respiratory activities may become dissociated, with
central inspiration occurring during peripheral expiration. Because of this entrainment of the central respiratory rhythm to the ventilator, the respiratory changes in arterial pressure also become dissociated. Indeed, Boczek-Funke et al. (1992) identified two peaks in the modulation of vasoconstrictor activity: one (direct) the result of central inspiratory drive, and the other (indirect) the result of mechanically induced unloading of arterial baroreceptors. In animals with intact vagus nerves, however, the inspiratory peak is replaced by an expiratory peak (as in humans), attributed to unloading of baroreceptors. While the incidence of MSNA is inversely related to diastolic pressure in humans (Sandlöf & Wallin, 1978), a relationship that holds throughout the respiratory cycle (Eckberg et al. 1985), we now know that the respiratory modulation is independent of the changes in blood pressure: the phase relationship between MSNA burst amplitude and tidal volume is preserved when respiration is brought about by negative-pressure or positive-pressure ventilation and the inspiratory changes in diastolic pressure reversed (Seals et al. 1993; MacEachfield & Wallin, 1995). Moreover, the finding that for a given diastolic pressure the level of MSNA is lower during inspiration than during expiration supports the idea that the sympathetic inhibition is largely independent of the influence of the arterial baroreceptors (Seals et al. 1990, 1993). In the current study, we found no significant respiratory fluctuations in blood pressure in any group. Interestingly, unlike the cat, the rat shows a pattern of respiratory modulation of sympathetic activity that is broadly similar to that of humans: inhibition of sympathetic activity commences at the onset of inspiration, reaches a minimum at mid-inspiration, and maximal activity occurs in the post-inspiratory phase and, occasionally, in late expiration (Gryzyl-Krzeska & Trugelski, 1990).

**Sympathoexcitation in HT and COPD**

In our recent paper, in which we compared the firing properties of single muscle vasoconstrictor neurons in COPD (Ashley et al. 2010) with those obtained in the obstructive sleep apnoea syndrome (Elam et al. 2002), we pointed out that it is highly likely that the ongoing hypoxaemia and hypercapnia associated with severe COPD (but not OSAS) are responsible for the augmented MSNA. We believe the peripheral
Chemoreceptors contribute to the elevated MSNA in COPD because oxygen administration has been shown to lower MSNA (Heindl et al. 2001) and slow, deep breathing has also been shown to reduce MSNA in COPD, presumably by improving gas exchange (Rausch et al. 2008). Conversely, the aetiology of the elevated MSNA in essential hypertension is (by definition) unknown, but it is generally accepted that the hypertension is neurogenic (Grassi et al. 1998; Schlaich et al. 2004; Lambert et al. 2007). A provocative hypothesis has been proposed to explain the hypertension: an increase in respiratory coupling of sympathetically mediated vasoconstriction (Czaryk-Krzeska & Trzebski, 1990; Simms et al. 2009). In the normotensive Wistar-Kyoto rat (WKY), the lowest level of cervical and renal sympathetic activity occurs in mid-inspiration and the highest in the post-inspiratory phase; in the spontaneously hypertensive rat (SHR), on the other hand, the lowest sympathetic activity occurs during the post-inspiratory period and the highest activity occurs in mid-inspiration and mid-expiration (Czaryk-Krzeska & Trzebski, 1990). Using the working heart-brainstem preparation (in which the animal is decerebrated, its lungs removed and the heart and brainstem supported by retrograde artificial perfusion via the descending aorta; Paton, 1996) Simms et al. (2009) showed that perfusion pressure (and hence vascular resistance) and thoracic sympathetic nerve activity were elevated in the neonatal and young (3–5 weeks) SHR but not in the normotensive WKY rat (Simms et al. 2009). Moreover, phrenic-nerve-triggered averaging revealed a specific increase in respiratory-related activity of the sympathetic vasoconstrictor drive, with sympathetic outflow occurring in the post-inspiratory phase in the WKY rat but with the peak occurring progressively earlier in inspiration in...
the 3-week- and 5-week-old SHR rats. Given that this survived denervation of the arterial baroreceptors (and peripheral chemoreceptors), and is independent of inputs from the lungs (which had been removed), the authors conclude that this reflects an increase in coupling between the inspiratory and vasoconstrictor drives, and that this contributes to the generation of hypertension.

So, why do we not see an increase in respiratory rhythmicity of MSNA in human essential hypertension, or indeed in patients with COPD? Obviously, the awake human subject is not the same as the anaesthetized rat, and especially the highly reduced working heart–brainstem preparation, but it is not unreasonable to think that respiratory coupling of MSNA would be increased in human hypertension and especially in COPD. However, we do know that another state of sympathetic excitation – congestive heart failure (CHF) – is associated with a rightward shift in the distribution of burst amplitudes (Sverrisdottir et al. 1998, 2000); in other words there are more large bursts and fewer small bursts. Given that respiratory modulation of MSNA is reflected in respiratory-related changes in burst amplitude (and the temporal distribution of sympathetic firing when expressed as a cyclic histogram) it would appear that the reduced variance in burst amplitudes of itself suggests a lower modulation. It may be that in states of elevated MSNA (such as CHF, HT and COPD) the overall increase in vasoconstrictor drive limits the expression of any increase in respiratory-related vasoconstrictor drive.

Alternatively, given that respiratory modulation of MSNA in human subjects appears to be largely independent of central respiratory drive at rest (it is observed even in artificially ventilated subjects in whom central inspiratory drive has been suppressed; Macfarlane & Wallin, 1995), it is quite possible that the mechanism proposed for the sympathetic excitation in the spontaneously hypertensive rat differs from the sympathetic excitation observed in either human essential hypertension or COPD.

**Methodological considerations**

As noted in our previous studies of patients with CHF, OSAS or COPD (Macfarlane et al. 1999; Elam et al. 2002; Ashley et al. 2010), we did not withdraw pharmacological or physiotherapeutic treatment; despite this, MSNA remained very high, so it is unlikely that the ongoing treatment affects our conclusions. Nevertheless, we should acknowledge that 5 of our 13 HT patients were on angiotensin II receptor antagonists and two were on ACE inhibitors. Given that the renin–angiotensin system has been shown to contribute to respiratory–sympathetic coupling in the hypertensive rat (Toney et al. 2010), it is possible that in these seven patients pharmacological blockade may have attenuated the respiratory modulation. Of course, this would not affect respiratory modulation of MSNA in COPD, which, because of the elevated chemical drive to breathe, would be expected to be high. There were differences in age between those patients with essential hypertension and those with COPD, but at least those with HT were matched in age to the older healthy controls. We know that MSNA increases with age (Sverrisdottr et al. 2000; Hart et al. 2009); we observed this difference between the younger and older healthy controls in our study. Nevertheless, we do not believe age is an important parameter in this situation: what is important is that we have examined four groups of subjects with differing levels of MSNA, with or without hypertension.

It is worth noting that, unlike some studies that have addressed respiratory rhythmicity in human subjects, we specifically did not constrain the respiratory pattern by asking subjects to breathe to a metronome, subjects breathed spontaneously and there was no significant difference in respiratory period across each of the groups. However, the respiratory excursions we recorded were not calibrated with respect to volume, so we do not know whether the depth of respiration differed across groups. The times of occurrence of the inspiratory peaks were used to generate cross-correlation histograms.
Respiratory-sympathetic coupling in humans

between the times of occurrence of sympathetic spikes and respiration; the times of occurrence of the R-waves were used to generate cross-correlation histograms between MSNA and ECG. By using the raw nerve data, rather than the RMS-processed signal, we believe we have used a more sensitive measure of calculating respiratory rhythmicity, particularly as we generated the cross-correlation and autocorrelation histograms with a bin width of 30 ms. Moreover, by fitting low- or high-order smoothed polynomials to the histograms we could differentiate between the slower respiratory-related modulation and the faster cardiac-related modulation of MSNA, and calculate modulation from the peak-to-trough difference in the numbers of spikes. However, it should be pointed out that our method of analysis differed from that used by Simms et al. (2009) in the rat: their analysis was based on phrenic-nerve-triggered averaging of the integrated bursts recorded from the thoracic sympathetic chain, so the means of assessing respiratory modulation differed in the two studies. Nevertheless, despite these differences, both studies compared respiratory modulation across groups; accordingly, the conclusions reached in both studies stand on their own.

Conclusions

In conclusion, we have shown that respiratory modulation of muscle sympathetic nerve activity in human essential hypertension is not increased, as suggested by the increased occurrence observed in the neonatal and adult spontaneously hypertensive rat. Moreover, respiratory modulation of MSNA in COPD is also not increased, despite the presence of a high chemoreflex drive to respiration. These observations argue against the utility of an increase in respiratory-related MSNA as being causal for the increase in muscle vasconstrictor drive seen in essential hypertension.

References


Author contributions

V.G.M. was responsible for the conception and design of the study. R.E. and V.G.M. were each involved in data acquisition. R.E. was responsible for the analysis and interpretation of the data. V.G.M. drafted the article and both authors approved the final submission. The study was conducted at the School of Medicine, University of Western Sydney.
Appendix B

Cardiorespiratory coupling of sympathetic outflow in humans: a comparison of respiratory and cardiac modulation of sympathetic nerve activity to skin and muscle.

Cardiorespiratory coupling of sympathetic outflow in humans: a comparison of respiratory and cardiac modulation of sympathetic nerve activity to skin and muscle

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New Findings

- What is the central question of this study?
  Muscle sympathetic nerve activity (MSNA) is well known to be modulated by the arterial baroreceptors and respiration, but what are the magnitudes of cardiac and respiratory modulation of skin sympathetic nerve activity (SSNA), which primarily subserves thermoregulation?

- What is the main finding and what is its importance?
  Using direct microelectrode recordings of MSNA and SSNA in awake humans, we show that the magnitude of respiratory modulation of SSNA is identical to that of MSNA, the primary difference between the two sources of sympathetic outflow being the greater cardiac modulation of MSNA. This emphasizes the role of the baroreceptors in entraining sympathetic outflow to muscle.

It is well known that microelectrode recordings of skin sympathetic nerve activity (SSNA) in awake human subjects reveal spontaneous bursts of activity with no overt modulation by changes in blood pressure or respiration, in contrast to the clear cardiac and respiratory modulation of muscle sympathetic nerve activity (MSNA). However, cross-correlation analysis has revealed that, like individual muscle vasconstrictor neurones, the firing of individual cutaneous vasconstrictor neurones is temporally coupled to both the cardiac and respiratory rhythms during cold-induced cutaneous vasconstriction, and the same is true of single sudomotor neurones during heat-induced sweating. Here, we used cross-correlation analysis to determine whether SSNA exhibits cardiac and respiratory modulation in thermoneutral conditions and to compare respiratory and cardiac modulation of SSNA with that of MSNA. Oligounitary recordings of spontaneous SSNA (n = 20) and MSNA (n = 18) were obtained during quiet, unrestrained breathing. Respiration was recorded by a strain-gauge transducer around the chest and ECG recorded by surface electrodes. Respiratory and cardiac modulation of SSNA and MSNA were quantified by fitting polynomial equations to the cross-correlation histograms constructed between the sympathetic spikes and respiration or ECG. The amplitude of the respiratory modulation (32.5 ± 3.4%) of SSNA was not significantly different from the amplitude of the cardiac modulation (46.6 ± 3.2%). Both were comparable to the respiratory modulation of MSNA (47.7 ± 4.2%), while cardiac modulation of MSNA was significantly higher (89.8 ± 1.9%). We conclude that SSNA and MSNA share similar levels of respiratory modulation,
the primary difference between the two sources of sympathetic outflow being the marked cardiac modulation of MSNA provided by the baroreceptors.

Introduction
For over 40 years of microelectrode recordings from muscle or cutaneous fascicles of human peripheral nerves (microneurography), it has been recognized that the spontaneous discharge of postganglionic sympathetic axons occurs as bursts of varying amplitude and duration (Hagbarth & Vallbo, 1968; Delius et al. 1972a,b). It is also well known that the patterns of sympathetic outflow to muscle and skin differ, which fits with their fundamentally different physiological roles.

Muscle sympathetic nerve activity (MSNA), which supplies vasconstrictor neurones to the skeletal muscle vascular beds, plays a primary role in the control of arterial pressure and, as such, is strongly influenced by the arterial and cardiopulmonary baroreceptors. Accordingly, MSNA shows a very tight cardiac rhythmicity, increasing during spontaneous or evoked falls in arterial pressure and decreasing when blood pressure (BP) increases. Specifically, it is the cardiac baroreceptors that seem to provide the dominant temporal coupling of MSNA to the cardiac cycle (Wallin et al. 1975; Walin & Eckberg, 1982). In addition, MSNA is modulated by respiration; bursts of MSNA appear in expiration and decrease in intensity during mid-inspiration (Hagbarth & Vallbo, 1968; Eckberg et al. 1985; Seals et al. 1990, 1993; MacEiﬁeld & Wallin, 1995; Fatouleh & MacEiﬁeld, 2011). In humans, single postganglionic muscle vasconstrictor neurones ﬁre with a respiratory rhythm, in addition to the dominant cardiac rhythm (MacEiﬁeld et al. 1994). Furthermore, the respiratory periodicity is independent of the associated changes in blood pressure, so it cannot be explained by baroreceptor inﬂuences (MacEiﬁeld & Wallin, 1995).

Skin sympathetic nerve activity (SSNA) primarily subserves thermoregulation, through its actions on blood vessels in the skin, sweat glands and (phylogenetically) the hair; the sympathetic innervation of the skin has also been commandeered as a means of emotional expression, with SSNA increasing during states of heightened arousal or emotional engagement (Delius et al. 1972b; Hagbarth et al. 1972; Brown et al. 2012). Unlike the clear cardiac modulation of MSNA, SSNA appears not to be inﬂuenced by beat-to-beat fluctuations in blood pressure. When baroreceptor afferents are blocked, by bilateral anaesthesia of the glossopharyngeal and vagus nerves, muscle sympathetic nerve activity loses its normal entrainment to the cardiac rhythm (Fagius et al. 1985). Interestingly, in addition to MSNA being greatly augmented by loss of the negative feedback provided by the baroreceptors, the sympathetic outflow takes on much of the character of SSNA, including activation by arousal stimuli that would normally activate only SSNA (Fagius et al. 1985), a pattern also seen during the prolonged bursts produced during syncope (Ivase et al. 2002) and in Guillain–Barre syndrome (Fagius et al. 1985). This suggests that the two sympathetic outflows share common sources of modulation.

One source of modulation that is common to both MSNA and SSNA is the baroreceptors themselves, although this is certainly stronger for MSNA. Indeed, SSNA is little inﬂuenced by changes in arterial pressure and hence activation of arterial baroreceptors (Hagbarth et al. 1972; Delius et al. 1972b; Bini et al. 1980a, 1981; Jungen et al. 1983; Drot et al. 1995). Multitubular bursts of SSNA often contain both vasconstrictor and sudomotor (and in some instances vasodilator or pilomotor) impulses, and it is known that exposing subjects to a cold or a warm environment can bias the sympathetic outflow towards essentially ‘pure’ vasconstrictor or sudomotor neural trafﬁc, respectively (Bini et al. 1980a,b; MacEiﬁeld & Wallin, 1996, 1999). Although baroreflex modulation of SSNA is known to be weak (Bini et al. 1981; Drot et al. 1995), at least when measured from multitubular recordings, an overt increase in cardiac modulation has been observed when subjects are heated and sudomotor activity is increased (Bini et al. 1981). It has been suggested that the low-pressure (cardiopulmonary) baroreceptors, which are known to display cardiac rhythmicity (Thorén, 1979), are responsible for this weak coupling to the cardiac cycle (Drot et al. 1995). These same afferents may also be responsible for the cardiac modulation of the discharge of single cutaneous vasconstrictor as well as single sudomotor neurones (MacEiﬁeld & Wallin, 1996, 1999). Another source of input shared by MSNA and SSNA is respiration. We know that a burst of SSNA can be evoked by asking a subject to take a brisk sniff, but unlike MSNA it is often reported that the breath-to-breath modulation of SSNA during tidal breathing is weak (Delius et al. 1972a, b; Hagbarth et al. 1972; Bini et al. 1980a), but it certainly has not been quantiﬁed to the extent that respiratory modulation of SSNA has been examined.

The purpose of the present study was to compare the magnitude of respiratory and cardiac modulation of SSNA and MSNA, to test the hypothesis that respiratory
modulation of SSNA is of comparable magnitude to that of MSNA.

Methods

Data were obtained from 39 healthy participants (25 male and 14 female) with a mean (±SD) age of 24 ± 6 years (range 18–36 years). The study was conducted with the approval of the Human Research Ethics Committee, University of Western Sydney, and satisfied the Declaration of Helsinki. Subjects were seated in a semi-reclined posture in a comfortable chair, with the legs supported in the extended position. The laboratory was a temperature-controlled room maintained at 22°C. Skin sympathetic nerve activity was recorded from cutaneous fascicles of the left common peroneal nerve in 21 subjects (12 male and nine female), via tungsten microelectrodes (THC, Bowdoinham, ME, USA) inserted percutaneously at the level of the fibular head. In 18 subjects (12 male and six female), MSNA was recorded from muscle fascicles of the left common peroneal nerve supplying the ankle and toe extensor and foot eveter muscles. The impedances of the microelectrodes (~100–150 kΩ) favoured oligounitary and multunit, rather than unitary, recordings. Neural activity was amplified (gain 20,000, bandwidth 0.3–5.0 kHz) using an isolated amplifier (NeuroAmp EX; ADInstruments, Sydney, NSW, Australia) and stored on computer (10 kHz sampling) using a computer-based data acquisition and analysis system (PowerLab 16SP hardware and LabChart 7 software; ADInstruments, Sydney, NSW, Australia). A cutaneous fascicle was identified by the following criteria: (i) electrical stimulation through the microelectrode induced radiating paraesthesia in the fascicular innervation territory without muscle twitches; (ii) light stroking of the skin evoked nerve impulses in tactile afferents; and (iii) tapping or passive stretching of muscles supplied by the common peroneal nerve did not cause afferent mechanoreceptor impulses. The microelectrode was manually advanced until spontaneous bursts of SSNA were encountered, identified by the following features: (i) a burst could be evoked by a brisk sniff and, with the subject’s eyes closed, an arousal burst could be evoked by an unexpected tap on the nose or a loud shout (Delius et al. 1972b); (ii) the bursts were typically longer than those comprising muscle sympathetic nerve activity; and (iii) unlike MSNA, there was no sustained increase in burst amplitude and frequency during an inspiratory-capacity apnoea, in which the subject takes a maximal inhalation and holds it against a closed glottis for 40 s (Macefield & Wallin, 1993; Macefield, 1998). A muscle fascicle was identified by the following criteria: (i) electrical stimulation through the microelectrode induced twitches in the muscle supplied by that fascicle without radiating paraesthesia; (ii) light stroking of the skin did not evoke any afferent activity; and (iii) activity in muscle spindle afferents could be evoked by percussion over the tendon or muscle belly or passive stretching of the muscle supplied by that fascicle. The microelectrode was manually advanced until spontaneous bursts of MSNA were encountered, identified by the following features: (i) bursts occurred spontaneously with a clear cardiac rhythmicity; (ii) a burst could not be evoked by a brisk sniff or arousal stimulus; (ii) the bursts were typically shorter than those comprising skin sympathetic nerve activity; and (iii) unlike SSNA, there was a sustained increase in burst amplitude and frequency during an inspiratory-capacity apnoea.

Spontaneous sympathetic nerve activity, ECG, BP and respiration were recorded for at least 30 min, and periods of stable activity (>5 min) were sampled for analysis. Blood pressure was recorded continuously using finger-plethysmography (Finometer; Finapres Medical Systems, Amsterdam, The Netherlands) and sampled at 400 Hz. The ECG (0.3–10 kHz) was recorded with Ag–AgCl surface electrodes on either side of the chest over the fifth or sixth intercostal space (earth on right clavicle), and sampled at 2 kHz. Respiration (DC–100 Hz) was recorded using a strain-gauge transducer (Pneumotrace; UFI, Morro Bay, CA, USA) wrapped around the chest, sampled at 100 Hz. Subjects were not informed that we were interested in respiratory rhythmicity and were given no instructions on how to breathe; subjects were simply asked to relax and not to talk.

Analysis

Sympathetic nerve activity was displayed as an RMS-processed root mean square, moving average, time constant 200 ms) signal, but the primary analysis was conducted on the raw, negative-going sympathetic spikes to avoid any contamination from spikes generated by positive-going myelinated axons (such as spontaneously active muscle spindles) or motor units associated with spontaneous EMG activity. This approach has been described previously, and provides a more sensitive means of analysing sympathetic outflow than the standard method of simply counting the number of sympathetic bursts (Bent et al. 2006; Fatouleh & Macefield, 2011). Briefly, negative-going spikes in the neurogram (with a half-width of 0.2–0.5 ms) were detected using window discriminator software (Spike Histogram for Macintosh v2.2; ADInstruments), while the times of occurrence of the R waves of the ECG and of the inspiratory peaks of the respiratory signal were computed using Peak Analysis software (ADInstruments). Autocorrelation histograms for the respiratory and cardiac signals, and cross-correlation histograms between the MSNA and ECG or respiration, were generated by the Spike Histogram software (50 ms bins). Discriminator levels of the neural activity were adjusted so that negative-going
(C-fibre) spikes exhibited a robust cardiac modulation, as revealed by cross-correlation between the neural activity and the ECG. These same discriminator settings were used for construction of cross-correlograms between the sympathetic nerve activity and the positive peaks of the respiratory signal. Smoothed polynomial curves were fitted to the histogram data using a graphical analysis program; second-order polynomials were used to fit curves to the cardiac cross-correlograms, while zero-order polynomials were required to fit curves to the slower respiratory cross-correlograms (Prism 5.0; GraphPad Software, La Jolla, CA, USA). Quantification of the modulation of MSNA or SSNA was performed by measuring the difference in the number of spikes at the peak of the modulation and the number at the trough, expressed as a percentage, as follows: modulation index = [(peak − trough)/peak] × 100. For calculating the modulation index the peak−trough difference extended over the same interval as the respiratory or cardiac periods. Analysis of variance, coupled with Tukey’s multiple comparison test, was used to assess statistical significance of the modulation indices across each group (Prism 5.0). Student’s unpaired t-tests were used to compare blood pressure, heart rate and respiratory period between groups of subjects in whom MSNA or SSNA were recorded. All values are expressed as means and standard errors, and P < 0.05 was considered statistically significant.

Results
Experimental records of spontaneous SSNA and MSNA are shown in Figs 1 and 2, respectively. In both figures, event markers are shown for the negatively-going sympathetic spikes, the ECG and the peaks of inspiration. These event markers were used to construct the autocorrelation and cross-correlation histograms shown in Figs 3 and 4 for SSNA and MSNA, respectively. It can be seen that SSNA exhibits both respiratory (Fig. 3A) and cardiac modulation (Fig. 3B). The same is true for MSNA, the difference being the much higher modulation of MSNA by the cardiac cycle (Fig. 4B). Moreover, the peak of the respiratory modulation occurred close to the peak

![Figure 1. Multisunit recording of skin sympathetic nerve activity (SSNA) from a 21-year-old female subject](image)

Discriminated negative-going (sympathetic) spikes extracted from the nerve recording are illustrated as standard pulses (spikes) below the root mean square (RMS)-processed nerve signal. The times of occurrence of each heart beat (R waves) and peaks of each breath (imp. peaks) are also shown as standard pulses. These timing events were used to generate the cross-correlation and autocorrelation histograms shown in Figs 3 and 4. Note that spontaneous SSNA occurs as broad bursts that span several cardiac intervals. The three large spikes towards the centre of the recording are EMG artifacts that did not contribute to the sympathetic spike events. Abbreviation: BP, blood pressure.
(C-fibre) spikes exhibited a robust cardiac modulation, as revealed by cross-correlation between the neural activity and the ECG. These same discriminator settings were used for construction of cross-correlograms between the sympathetic nerve activity and the positive peaks of the respiratory signal. Smoothed polynomial curves were fitted to the histogram data using a graphical analysis program; second-order polynomials were used to fit curves to the cardiac cross-correlograms, while zero-order polynomials were required to fit curves to the slower respiratory cross-correlograms (Prism 5.0; GraphPad Software, La Jolla, CA, USA). Quantification of the modulation of MSNA or SSNA was performed by measuring the difference in the number of spikes at the peak of the modulation and the number at the trough, expressed as a percentage, as follows: modulation index = [(peak – trough) / peak] × 100. For calculating the modulation index the peak-trough difference extended over the same intervals as the respiratory or cardiac periods. Analysis of variance, coupled with Tukey’s multiple comparison test, was used to assess statistical significance of the modulation indices across each group (Prism 5.0). Student’s unpaired t-tests were used to compare blood pressure, heart rate and respiratory period between groups of subjects in whom MSNA or SSNA were recorded. All values are expressed as means and standard errors, and P < 0.05 was considered statistically significant.

Results

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of inspiration for SSNA (Fig. 3A), but in late expiration for MSNA (Fig. 4A), yet the magnitude of the respiratory modulation was similar. Between the groups of subjects in whom MSNA or SSNA were recorded, there were no significant differences in systolic pressure (125.9 ± 3.1 versus 131.0 ± 3.6 mmHg), diastolic pressure (67.3 ± 3.1 versus 68.6 ± 3.0 mmHg), heart rate (72.5 ± 3.7 versus 75.5 ± 3.8 beats min⁻¹) or respiratory period (3.83 ± 0.81 versus 4.09 ± 0.18 s).

Cardiac and respiratory modulation of MSNA was calculated from the smoothed polynomials fitted to the cross-correlation histograms. Mean data are shown graphically in Fig. 5. All parameters showed significant modulation, significantly different from zero. It can also be seen that the amplitude of the respiratory modulation of SSNA (52.6 ± 3.0%) was not significantly different from the amplitude of the cardiac modulation (46.6 ± 3.2%), and both were of similar magnitude to the respiratory modulation of MSNA (47.7 ± 4.2%, P > 0.5), but cardiac modulation of MSNA was significantly higher (89.8 ± 1.5%, P < 0.001). There were no significant gender differences in either respiratory or cardiac modulation of MSNA or SSNA.

Discussion

This study has shown that, in awake human subjects, sympathetic outflow to muscle and skin share certain similarities, in that both are modulated by respiration and the cardiac cycle, but there are also differences, in that the magnitude of the cardiac modulation of MSNA is much greater than that of SSNA, but the magnitude of respiratory modulation is identical for both MSNA and SSNA. This is contrary to the accepted dogma that SSNA is little influenced by respiration, other than the characteristic burst of impulses when subjects perform a brisk sniff. Here, we have shown that spontaneous SSNA, recorded in thermoneutral conditions and with no external influences, is modulated on a breath-to-breath basis during tidal breathing. Furthermore, the magnitude of this modulation, approximately 50%, is similar to the magnitude of the cardiac modulation of SSNA, which is known to be much weaker than that of MSNA. Indeed, it would appear that a major difference in the bursting pattern between MSNA and SSNA is the marked entrainment of MSNA to the cardiac rhythm, which is close to 100% for MSNA but about 50% for SSNA. The cardiac modulation of MSNA is known to be

![Image: Multifunit recording of muscle sympathetic nerve activity (MSNA) from a 34-year-old female subject. Discriminated spikes extracted from the nerve recording are illustrated as standard pulses (spikes) below the RMS-processed nerve signal. The times of occurrence of each heart beat (R waves) and peaks of each breath (imp. peaks) are also shown as standard pulses. Note that bursts of MSNA are much briefer than those of SSNA and are tightly locked to the cardiac rhythm.]

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evoked primarily from the carotid arterial baroreceptors (Wallin et al. 1975; Wallin & Eckberg, 1982), and it is this entrainment by the baroreceptors that constrains the duration of a burst of MSNA to being less than that of a cardiac interval; conversely, it is the weaker entrainment of skin sympathetic outflow to the arterial baroreceptors (Wallin et al. 1975) that allows bursts of SNA to extend across cardiac intervals, resulting in much broader bursts of impulses than one sees with MSNA.

While cardiac rhythmicity has been seen in SNA during whole-body heating, in which skin sympathetic outflow is dominated by sudomotor activity (Bini et al. 1981), overt cardiac rhythmicity has not previously been detected in multunit recordings of SNA dominated by cutaneous vasoconstrictor activity (Hagbarth et al. 1972; Wallin et al. 1975; Bini et al. 1980a, 1981; Vissing et al. 1994). However, only the study by Bini and colleagues (1981) used ECG-triggered averaging to assess cardiac rhythmicity in the cold state, and it should be noted that recordings were made from the median nerve, which supplies the glabrous skin of the hand. Given that glabrous skin is not believed to be influenced by the arterial baroreceptors (Eckberg & Sleight, 1992), this may explain why cardiac rhythmicity was not detected in the median nerve. Conversely, the other studies were conducted in the peroneal nerve, as in the present study, but did not use a quantitative method to assess cardiac rhythmicity. Indeed, we believe that cross-correlation analysis is the most sensitive means of detecting an underlying source of rhythmic modulation, one that has previously allowed us to quantify cardiac modulation of SNA in thermoneutral conditions (James et al. 2010).

Given that SNA is not greatly influenced by the arterial baroreceptors (Delius et al. 1972a; Hagbarth et al. 1972; Wallin et al. 1975; Bini et al. 1980a, 1981), the low-pressure (cardiopulmonary) baroreceptors may be seen as a potential source of cardiac rhythmicity. Lower-body negative pressure or head-up tilt maneuvers that cause unloading of the cardiopulmonary baroreceptors, decrease sudomotor traffic in sweating subjects (Dodd et al. 1995). Conversely, lower-body negative pressure has no effect on SNA or skin blood flow in thermoneutral conditions (Vissing et al. 1994, 1997), arguing against

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Figure 3. Cross-correlation (A and B) and autocorrelation histograms (C and D) between sympathetic spikes and respiration (A and C) or ECG (B and D) for a recording of SNA. Smoothed polynomials (thick lines) have been fitted to the histograms. The numbers on the y-axes refer to the numbers of counts per 50 ms bin. Time zero, corresponding to the triggering event in the cross- or autocorrelograms, is indicated by the vertical dotted lines. Numbers of events comprising each average are shown in the top right corner of each panel.
a role of the low-pressure baroreceptors in the cardiac modulation of cutaneous vasoconstrictor activity.

The finding that cardiac modulation of SSNA is weaker than that of MSNA may mean that the central coupling of baroreceptor inputs to SSNA is weaker and/or that the modulation is expressed by only a subpopulation of cutaneous sympathetic neurones. Single-unit recordings in human subjects certainly support both options. Cross-correlation analysis revealed that a subpopulation of sudomotor neurones, activated by whole-body heating, express cardiac rhythmicity (Macefield & Wallin, 1996), although the modulation is certainly weaker than that expressed by individual muscle vasoconstrictor neurones in the awake human (Macefield et al. 1994) or anaesthetised cat (Janig, 1985). Cross-correlation analysis also identified cardiac modulation of individual cutaneous vasoconstrictor neurones during whole-body cooling (Macefield & Wallin, 1999). Again, this was weaker than that of muscle vasoconstrictor neurones and also weaker than that of sudomotor neurones; unitary recordings in the cat also revealed a subpopulation of cutaneous vasoconstrictor neurones that exhibit weak cardiac rhythmicity (Janig, 1985).

Respiratory modulation of SSNA is believed to be weak, but until now the magnitude of this modulation has never been quantified. It is known that a single burst of SSNA can be evoked during a brisk sniff, and that, unlike MSNA, there is no sustained discharge during a maximal inspiratory apnoea (Macefield, 1998). This suggests that skin sympathetic outflow is less influenced by static modulatory inputs, and more so by dynamic inputs. Cross-correlation analysis of unitary recordings of sudomotor and cutaneous vasoconstrictor neurones have shown that both classes of neurone also exhibit respiratory modulation (Macefield & Wallin, 1999). Such modulation can be difficult to discern by simply looking at the pattern of multimodal sympathetic bursts; as noted above, cross-correlation analysis is an intrinsically more sensitive means of identifying modulation of sympathetic outflow. Indeed, we recently used this approach to compare the magnitude of respiratory modulation of MSNA in healthy subjects and patients with essential hypertension or chronic obstructive pulmonary disease (Fatouleh & Macefield, 2011).

Respiratory modulation in the discharge of postganglionic sympathetic neurones has been well...
documented in anaesthetized animals. In the cat, muscle vasoconstrictor neurones fire during inspiration, with a depression in activity during the postinspiratory phase, while sudomotor neurones fire primarily in postinspiration and early expiration and the majority of cutaneous vasoconstrictor neurones exhibit no respiratory modulation or, for those supplying glabrous skin, display a narrow peak during inspiration (Boczek-Funkke et al. 1992a,b). Conversely, in the rat both muscle and cutaneous vasoconstrictor neurones discharge only during postinspiration and early expiration (Häßler et al. 1993, 1994, 1996; Johnson & Gilbey, 1996). While the discharge of some cutaneous vasoconstrictor neurones exhibited respiratory rhythmicity, the discharge of others was independent of the central or peripheral respiratory rhythm; for all cutaneous vasoconstrictor neurones, there was no evidence of cardiac rhythmicity (Johnson & Gilbey, 1996), which differs from observations in human subjects (and in cats).

Another source of modulation of both muscle and skin sympathetic outflow is provided by the vestibular system. We have previously shown that sinusoidal galvanic vestibular stimulation, a means of selectively modulating vestibular inputs, induces a potent modulation of both MSNA (Bent et al. 2006; Grewal et al. 2009; James & Maciefield, 2010; Hammam et al. 2011; El Sayed et al. 2012) and SSNA (James et al. 2010; Hammam et al. 2012; El Sayed et al. 2012). While the vestibular modulation of MSNA is much weaker than that provided by the baroreceptors, vestibular modulation of SSNA is significantly greater than the cardiac modulation. This emphasizes the differential control of sympathetic outflow, but also illustrates the fact that different types of sympathetic outflows share common sources of modulation.

Conclusions

The present study has shown that respiratory modulation of SSNA, recorded in thermoneutral conditions, is not statistically different to that of MSNA recorded at rest in young healthy subjects. Moreover, the magnitude of the respiratory modulation of SSNA is statistically identical to the magnitude of the cardiac modulation of SSNA, indicating that skin sympathetic outflow shares two common sources of modulation with muscle sympathetic outflow, or three common sources if we include the vestibular system. What appears primarily to differentiate SSNA and MSNA is the very tight coupling of MSNA to the cardiac rhythm via the arterial baroreceptors; loss of this primary source of modulation causes MSNA to revert to a pattern that closely resembles that of SSNA. Why there should be coupling between skin sympathetic outflow and the cardiac and respiratory rhythms we do not know, but given the need for coupling between the control of blood flow in skin and in muscle, and between sweat release and blood volume, it makes physiological sense that, like MSNA, SSNA also exhibits cardiac and respiratory modulation.

References

Appendix B


**Additional information**

**Competing interests**

None declared.

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None declared.
Appendix C

Respiratory modulation of muscle sympathetic nerve activity in obstructive sleep apnoea

Research Paper

Respiratory modulation of muscle sympathetic nerve activity in obstructive sleep apnoea

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New Findings

- What is the central question of this study?
  Muscle sympathetic nerve activity (MSNA) is increased in obstructive sleep apnoea (OSA), leading to hypertension. Is this due to an increase in respiratory-sympathetic coupling, as has been demonstrated in the spontaneously hypertensive rat?
- What is the main finding and its importance?
  Using direct microelectrode recordings of MSNA in hypertensive OSA patients and normotensive control subjects, we show that the magnitude of respiratory modulation is not increased in OSA, arguing against an amplified respiratory-sympathetic coupling as the underlying cause of the neurogenic hypertension, although the temporal coupling of MSNA to respiration was stronger in OSA.

Obstructive sleep apnoea (OSA) is associated with elevated muscle sympathetic nerve activity (MSNA) during normoxic daytime wakefulness, leading to hypertension. We tested the hypothesis that respiratory-sympathetic coupling, postulated to be the underlying cause of neurogenic hypertension, is increased in OSA. Muscle sympathetic nerve activity, blood pressure, ECG and respiration were recorded in 21 normotensive control subjects and 21 newly diagnosed patients with OSA before and after 6 and 12 months of treatment with continuous positive airway pressure. Muscle sympathetic nerve activity was recorded via tungsten microelectrodes inserted percutaneously into the peroneal nerve. Cardiac and respiratory modulation of MSNA was quantified from the cross-correlation histograms constructed between the sympathetic spikes and either ECG or respiration. Muscle sympathetic nerve activity was significantly elevated in newly diagnosed OSA patients compared with control subjects (55 ± 2 versus 28 ± 2 bursts min⁻¹). There was a significant fall in MSNA after 6 months of continuous positive airway pressure (37 ± 2 bursts min⁻¹), with no further change after 12 months (37 ± 2 bursts min⁻¹). There were no significant differences in the magnitude of respiratory modulation of MSNA between the OSA patients and control subjects (40 ± 3.1 versus 39 ± 3.4%). However, when considering the normalized temporal profile there were changes in the respiratory patterning of MSNA in OSA, with more activity occurring in postinspiration and less in inspiration and expiration. This was largely reversed following long-term continuous positive airway pressure.

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Appendix C
Appendix C

Introduction
Obstructive sleep apnoea (OSA) is a common disorder, characterized by repeated episodes of nocturnal hypoaxemia that cause an enhanced muscle sympathetic nerve activity (MSNA), leading to neurogenic hypertension (Hedner et al. 1988, 1995; Carlson et al. 1993, 1996; Somers et al. 1995; Narkiewicz et al. 1999; Elam et al. 2002; Narkiewicz & Somers, 2003; Imadojemu et al. 2007) and a high mortality rate (He et al. 1988; Partinen et al. 1988). The increase in MSNA carries over into daytime wakefulness, when subjects are breathing normally and have no evidence of hypoaxemia (Somers et al. 1995); however, the mechanisms underlying the sympathoexcitation in this syndrome remain unclear. Continuous positive airway pressure (CPAP) is an effective and widely used method for treatment of OSA (Sullivan et al. 1981). Long-term treatment with CPAP decreases MSNA in OSA (Hedner et al. 1995; Wardakar et al. 1996; Narkiewicz & Somers 2003; Imadojemu et al. 2007), but this is not reflected in a fall in blood pressure (Hedner et al. 1995).

Muscle sympathetic nerve activity is vasoconstrictor in function; hence, it plays a major role in the control of arterial pressure. Muscle sympathetic nerve activity is tightly coupled to the cardiac cycle via the arterial baroreceptors, but is also modulated by respiration. In humans, MSNA is maximal during expiration and is inhibited in mid-inspiration (Hagbarth & Vallbo, 1968; Eckberg et al. 1985; Macefield & Wallin, 1995a,b; Macefield et al. 2002; Seals et al. 1993). It has been argued from work in the spontaneously hypertensive rat that an increase in respiratory modulation of sympathetic vasoconstrictor drive may contribute to the development of hypertension, and that this augmented respiratory–sympathetic coupling is responsible for the generation of hypertension in humans (Gayeck-Krzeska & Trebzi, 1990; Simms et al. 2009). We recently compared respiratory and cardiac modulation of MSNA in patients with essential hypertension and showed that there was no increase in respiratory modulation (Fatouleh & Macefield, 2011). Nevertheless, it may be that an exaggerated respiratory modulation of MSNA may appear in other cases of neurogenic hypertension, such as OSA. In the present study, we tested the hypothesis that respiratory modulation of MSNA is increased in OSA patients compared with healthy, age-matched control subjects and that this elevated modulation is reduced following treatment with CPAP. Owing to the higher levels of MSNA in OSA, we also predicted that cardiac modulation of MSNA would be lower in OSA.

Methods
Participants
Data were obtained from 21 (18 males and three females, aged 35–69 years, mean ± SEM 55 ± 2 years) well-characterized and newly diagnosed patients with moderate to severe OSA (apnoea–hypopnoea index 44 ± 5). Five patients were lost to follow-up at 6 months and one more patient was lost at 12 months; a total of 16 of the initial OSA patients came back after 6 months of treatment, and 15 came back after 12 months of treatment with CPAP. Age- and sex-matched 21 control subjects (17 males and four females, aged 35–68 years, 52 ± 2 years) were recruited from a healthy cohort. Each participant provided informed written consent to the procedures, which were approved by the Human Research Ethics Committees of the University of Western Sydney and the University of New South Wales and satisfied the requirements set out in the Declaration of Helsinki.

Polysonmography
All OSA subjects were evaluated at the sleep laboratory of Prince of Wales Hospital for one night. Patients were monitored continuously for 8 h using 12-channel polysomnography, Electroencephalographic, electro-oculographic and chin electromyographic recordings were obtained with surface electrodes according to the standard methods. Nasal and oral airflow were monitored by thermistor. Chest and abdominal movements were assessed by respiratory inductive plethysmography. Oxyhaemoglobin saturation was recorded all night by finger pulse oximetry. A microphone was placed on the lower neck to record snoring, and a camera sensitive to ultraviolet light recorded the movements of the subject during sleep. The overnight study was analysed offline, and apnoeas and hypopnoeas were defined according to the international classification of sleep disorders, by using both (Phillips Medical Systems, The Netherlands) and Somnomed (Medcare Flaga, Reykjavik, Iceland) software. Therapeutic CPAP was determined during a full night with respiratory monitoring. The treating specialist determined the CPAP pressure that resulted in the cessation of the apnoeic events. Subsequently, subjects were treated at home with a CPAP machine individually calibrated for their optimal pressure (Series 9, ResMed, Sydney, NSW, Australia). Compliance with prescribed CPAP therapy was based on an automated download of the CPAP machine at 1, 6 and 12 months.
General procedures

Subjects lay supine on a bed with their knees supported on a foam cushion. Multicunit muscle sympathetic nerve activity was recorded by a tungsten microelectrode that was inserted percutaneously into a muscle fascicle of the common peroneal nerve at the level of the fibular head (Frederick Haer and Co., Bowdoinham, ME, USA). A second microelectrode was inserted nearby, subdermally (1–2 cm), to act as reference electrode. Neural activity was amplified (gain 2 × 10^4, bandwidth 0.3–5.0 kHz) using an isolated headstage (NeuroAmp Ex; ADInstruments, Sydney, NSW, Australia). Data were stored (10 kHz sampling) using a computer-based data acquisition and analysis system (PowerLab 16 SP hardware and LabChart 7 software; ADInstruments, Sydney, NSW, Australia). Electrocardiographic activity (0.3–1.0 kHz) was recorded with Ag-AgCl surface electrodes on the chest and sampled at 2 kHz. Respiration (DC to 100 Hz) was recorded using strain-gauge transducers (Pneumotrace; UFI, Morro Bay, CA, USA) wrapped around the chest and abdomen and sampled at 100 Hz. Continuous non-invasive blood pressure was recorded using radial arterial tonometry (Colin 7000 NIBP; Colin Corp., Aichi, Japan), sampled at 400 Hz. Subjects were asked to relax and were given no instructions on breathing. Spontaneous MSNA, heart rate, BP, and respiration from the abdomen and chest were recorded continuously for 10 min of undisturbed rest, of which the final 5 min were used for analysis.

Analysis

Muscle sympathetic nerve activity was displayed as a root mean square (RMS)-processed (root mean square, moving average, time constant 200 ms) signal, but the primary analysis was conducted on the raw, negative-going, sympathetic spikes to avoid any contamination from spikes generated by positive-going myelinated axons (such as spontaneously active muscle spindles) or motor units associated with spontaneous EMG activity. This approach has been described previously, and provides a more sensitive means of analysing sympathetic outflow than the standard method of simply counting the number of sympathetic bursts (Fatouleh & MacIntosh, 2011, 2013). Briefly, negative-going spikes in the neurogram (half-width 0.2–0.3 ms) were detected using window discriminator software (Spire Histogram for Macintosh v2.2; ADInstruments, Sydney, Australia), while the times of occurrence of the R waves of the ECG and of the inspiratory peaks of the respiratory signal were computed using Emc Analysis software (ADInstruments, Sydney, Australia). Autocorrelation histograms for the cardiac and respiratory signals, and cross-correlation histograms between the MSNA and ECG or respiration, were generated by the Spire Histogram software (50 ms bins). Discriminator levels of the neural activity were adjusted so that negative-going (C fibre) spikes exhibited a robust cardiac modulation, as revealed by cross-correlation between the neural activity and the ECG. These same discriminator settings were used for construction of cross-correlograms between the MSNA and the inspiratory peaks of the respiratory signal (either the chest or the abdomen, depending on which signal was closest). Smoothed polynomial curves were fitted to the histogram data using a graphical analysis program; zero-order polynomials were used to fit curves to the slower respiratory cross-correlograms, while second-order polynomials were required to fit curves to the cardiac cross-correlograms (Prism 5.0; GraphPad Software, La Jolla, CA, USA). Quantification of the modulation of MSNA was performed by measuring the difference in the number of spikes at the peak of the modulation and the number at the trough for each subject, computed from the smoothed polynomial as follows: modulation index = [(peak – trough)/peak] × 100. For calculating the modulation index, the peak–trough difference extended over the same interval as the respiratory or cardiac periods. In addition, to illustrate the average time profile of respiratory modulation we computed a normalized cross-correlation histogram from the smoothed data across subjects. Data were normalized by defining the peak event count in an individual subject’s cross-correlogram and autocorrelogram as 100%. Muscle sympathetic nerve activity was also quantified according to standard time-domain analysis of the RMS-processed signal as burst frequency (in bursts per minute) and burst incidence (in bursts per 100 heart beats). Analysis of variance, coupled with Tukey’s multiple comparisons test, was used to assess statistical significance across each group (Prism 6.0, GraphPad Software). All values are expressed as means and SEMs, and P < 0.05 was considered statistically significant.

Results

Cardiorespiratory details of the healthy age-matched control subjects and the patients with OSA at the time of diagnosis and after 6 and 12 months of treatment with CPAP are provided in Table 1. It can be seen that there were no significant differences in heart rate or respiratory period across the groups. However, compared with the control subjects, OSA patients had significantly elevated systolic and diastolic blood pressures (P < 0.01) and MSNA (P < 0.001). There was a significant reduction in MSNA after 6 months (P < 0.005) of CPAP, with no further change after 12 months of compliant use, but there were no significant falls in blood pressure with treatment. Experimental records from a female patient with OSA are shown in Fig. 1. Negative-going spikes (MSNA) in the neurogram have been discriminated and presented as standard spikes, used to generate cross-correlation histograms between the inspiratory
Table 1. Cardiorespiratory parameters of the healthy control subjects and of obstructive sleep apnoea (OSA) patients prior to (0 months) and after 6 and 12 months of treatment with continuous positive airway pressure (CPAP)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 21)</th>
<th>OSA, 0 m CPAP (n = 21)</th>
<th>OSA, 6 m CPAP (n = 16)</th>
<th>OSA, 12 m CPAP (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/male (n)</td>
<td>4/17</td>
<td>3/18</td>
<td>3/13</td>
<td>2/13</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>120 ± 3</td>
<td>139 ± 4**</td>
<td>129 ± 6*</td>
<td>133 ± 4*</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>68 ± 3</td>
<td>80 ± 2**</td>
<td>75 ± 4</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>Heart rate (beats min⁻¹)</td>
<td>66 ± 3</td>
<td>70 ± 3</td>
<td>68 ± 2</td>
<td>69 ± 2</td>
</tr>
<tr>
<td>Respiratory period (s)</td>
<td>4.0 ± 0.2</td>
<td>4.0 ± 0.3</td>
<td>4.0 ± 0.3</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>Burst incidence (bursts 100 beats⁻¹)</td>
<td>45 ± 3</td>
<td>76 ± 4****</td>
<td>56 ± 3***</td>
<td>53 ± 3**</td>
</tr>
<tr>
<td>Burst frequency (bursts min⁻¹)</td>
<td>28 ± 2</td>
<td>53 ± 2****</td>
<td>37 ± 2***</td>
<td>37 ± 2**</td>
</tr>
</tbody>
</table>

Values presented are means ± SEM. Statistical comparisons (ANOVA) are shown relative to data obtained from the control group (*P < 0.05, **P < 0.01 and ***P < 0.001) and, for the OSA patients, relative to data obtained at 0 months of CPAP (†P < 0.01, ††P < 0.001 and †††P < 0.0001).

Figure 1. Multunit recording of muscle sympathetic nerve activity from a 34-year-old female patient with obstructive sleep apnoea (OSA)

The mean voltage neurogram is shown in the root mean square (RMS) nerve trace; this was used to quantify the number of sympathetic bursts. Note that bursts of MSNA occurred in many, but not all, cardiac intervals in this subject. Discriminated spikes extracted from the nerve recording are illustrated as standard pulse (MSNA spikes). The times of occurrence of each heart beat (R waves) and peaks of each breath (respiratory peaks) are also shown as standard pulses. These timing events were used to generate the cross-correlation and autocorrelation histograms shown in Fig. 2.
peaks of the respiratory record and the R waves of the ECG. As illustrated in Fig. 2A, cross-correlation histograms between the times of occurrence of the sympathetic spikes and of the ECG show a very tight coupling of MSNA to the cardiac cycle, while the respiratory rhythmicity is weaker (Fig. 2B). Cardiac and respiratory modulation of MSNA was calculated from the smoothed polynomials fitted to the cross-correlation histograms.

Owing to the higher levels of MSNA in OSA, we predicted that cardiac modulation would be lower in the untreated OSA patients (77.3 ± 4.0%) than in the control subjects (87.3 ± 3.5%), and this was borne out (Student’s unpaired t test, P = 0.0337). However, there was no significant difference in respiratory modulation between the OSA patients before treatment (39.6±3.1%) and the control subjects (39.1 ± 3.4%). Mean data are shown graphically in Fig. 3. As expected from our earlier work, respiratory modulation of MSNA was significantly lower than the cardiac modulation in both groups (P < 0.0001). Repeated-measures ANOVA revealed no significant change in cardiac modulation [F(1.783, 16.04) = 0.2629; P = 0.7474] at 0 (baseline), 6 and 12 months of compliant CPAP use. Even though respiratory modulation appeared greater than baseline after 6 months of CPAP, repeated-measures ANOVA uncovered no significant differences over the three time points [F(1.957, 21.30) = 1.845; P = 0.1831].

It should be emphasized that the respiratory modulation indices presented in Fig. 3 were calculated from the peak–trough difference in the cross-correlograms for each subject, regardless of when in time the peak and trough occurred. To illustrate the temporal profile of the respiratory modulation, we constructed normalized cross-correlation and autocorrelation histograms from all

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**Figure 2. Correlations between MSNA and ECG and respiration**

Cross-correlation histograms (upper traces in each panel) and autocorrelation histograms (lower traces in each panel) between sympathetic spikes and inspiration (A) and ECG (C). Autocorrelation histograms for respiration and ECG are shown in B and D, respectively. Data were obtained from the same subject illustrated in Fig. 1. Smoothed polynomials (thick lines) have been fitted to the histograms. The numbers on the y-axes refer to the numbers of spikes per 50 ms bin. Time zero, corresponding to the triggering event in the cross- or autocorrelograms, is indicated by the vertical dotted lines.

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subjects and pooled the data to assess whether there were any differences in the mean times at which the peak and trough occurred with respect to time zero (i.e. the peak of inspiration). These are illustrated in Fig. 4. It is apparent that the mean normalized respiratory modulation of MSNA is higher in the OSA patients, at baseline (0 months) and following 6 and 12 months of CPAP. This can also be seen in the higher normalized peak–trough differences in the OSA patients prior to CPAP, as shown in Table 2, though this was not significantly different.

Despite the lack of significant difference when considering only the peak–trough differences, which are point measures, it is clear from Fig. 4 that the peak modulation of MSNA occurred before the peak of inspiration for the control subjects but after the peak, i.e. in the postinspiratory phase, for the OSA patients prior to CPAP. As shown in Fig. 5, analysis of the normalized MSNA within the postinspiratory period (measured over 0.05–0.50 s from the peak of inspiration) revealed a significant difference between the two groups, with activity being significantly higher in the OSA patients ($P < 0.0001$). Conversely, MSNA was significantly lower in the OSA patients during both inspiration (from $-1.5$ to 0 s) and expiration (from $-0.55$ to $-2.00$ s). Numerical data are presented in Table 3. It can also be seen that 6 months of CPAP resulted in a normalization of the amount of activity in inspiration and postinspiration, but not in expiration, which remained significantly lower than in the control subjects. Indeed, it was not until after 12 months of CPAP that MSNA levels in expiration returned to and significantly exceeded baseline levels. MSNA during inspiration and postinspiration were also higher than in the control subjects, presumably reflecting the fact that while CPAP reduced the overall levels of MSNA (as measured by burst incidence and burst frequency; Table 1), it did not reduce MSNA completely to control levels. It would appear that, prior to CPAP, OSA is associated with a greater temporal coupling of MSNA to respiration than seen in control subjects. Accordingly, although the magnitude of the modulation in individual subjects is not significantly different between control subjects and patients with OSA, we posit that the temporal jitter in respiratory modulation in the control subjects results in a modulation profile that is less pronounced than that of the OSA patients.

![Figure 3. Mean cardiac and respiratory modulation indices across the four groups studied. Abbreviations: CON, age-matched control subjects ($n = 21$); OSA 0m CPAP, OSA patients before treatment with continuous positive airway pressure ($n = 17$); OSA 6m CPAP, OSA patients after 6 months of CPAP treatment ($n = 18$); and OSA 12m CPAP, OSA patients after 12 months of treatment ($n = 15$). Cardiac and respiratory modulation could not be calculated for all subjects because of technical issues with the ECG or respiratory recordings. Cardiac modulation was significantly lower than control in the OSA 0m CPAP group, but there were no significant differences in respiratory modulation between groups.](image)

**Discussion**

It is well established that MSNA is greatly elevated in OSA, during both sleep and waking periods (Hedner et al. 1988, 1995; Carlson et al. 1993, 1996; Somers et al. 1995; Narkiewicz et al. 1999; Elam et al. 2002; Narkiewicz & Somers, 2003; Imadogbe et al. 2007). The mechanism of the sympathoexcitatory and how it leads to hypertension is, in general, poorly understood, other than that it is related to the long-term effects of hypoxia on MSNA. Given that an increase in respiratory–sympathetic coupling has been argued to be an important contributor to the increase in blood pressure in the spontaneously hypertensive rat, as well as to human hypertension (Czyzyn-Krzeska & Trzebski, 1990; Simms et al. 2009; Moraes et al. 2014), we tested the hypothesis that respiratory modulation of MSNA is increased in OSA. We compared the magnitude of the respiratory modulation of MSNA in OSA patients with that of healthy control subjects, but found no significant difference. Moreover, a reduction in MSNA after CPAP was not associated with changes in the magnitude of the respiratory modulation of MSNA. As described previously, the reduction in MSNA after 6 or 12 months of CPAP was not associated with a significant fall in blood pressure (Hedner et al. 1995), although MSNA remained higher than in the control subjects.
Figure 4. Cross-correlation histograms (upper traces in each panel) and autocorrelation histograms (lower traces in each panel) between sympathetic spikes and respiration. Normalized data (means ± SEM) for control subjects (A) and for patients with OSA at 0 (B), 6 (C) and 12 months (D) of treatment with CPAP. Data were normalized by defining the peak event count in an individual subject’s cross-correlogram and autocorrelogram as 100%. Time zero, corresponding to the peak of inspiration in the cross- and autocorrelograms, is indicated by the vertical dotted lines. Statistical significance was assessed using an unpaired Student’s t-test. Given that the maximal value (100%) occurs at different times during the respiratory cycle for each subject, this dispersion means that the graphs do not show a peak of 100%.
Muscle sympathetic nerve activity is controlled on a beat-to-beat basis by the arterial baroreceptors, such that MSNA shows a very tight coupling to the cardiac cycle and an inverse relationship to diastolic pressure. In addition, previous studies showed that there is also a close relationship between MSNA and respiration, with bursts of MSNA occurring preferentially in expiration and being inhibited in mid-inspiration (Hagbarth & Vallbo, 1968; Edberg et al. 1985; Seals et al. 1990, 1995; Macefield & Wallin, 1995a,b; Macefield et al. 1999). Respiratory modulation of MSNA is independent of the changes in blood pressure, so it cannot be explained by indirect effects via the baroreceptors (Seals et al. 1993; Macefield & Wallin, 1995a). Like MSNA, skin sympathetic nerve activity also exhibits respiratory modulation, the magnitude of which is statistically identical to that of MSNA (Fatouleh & Macefield, 2013).

As noted in the Introduction, studies in the neonatal and juvenile spontaneously hypertensive rat showed an increased respiratory modulation of vasomotor drive in the thoracic (Simms et al. 2009) and the cervical and lumbar sympathetic outflows (Moraes et al. 2014). It was hypothesized that amplification of this modulation leads to hypertension, not only in the rat, but also in human hypertension (Simms et al. 2009; Moraes et al. 2014). Unlike humans, in the spontaneously hypertensive rat the lowest sympathetic activity occurs during the postinspiratory period and the highest activity occurs in mid-inspiration and mid-expiration; given that the rats had been sinoaortically denervated and were vagotomized, the respiratory modulation must be independent of the arterial baroreceptors, peripheral chemoreceptors and inputs from the lungs (Czyzyk-Krzeska & Trzebski, 1990). Interestingly, recent studies by Moraes and colleagues (2014) found that, in the juvenile spontaneously hypertensive rat pre-inspiratory and post-inspiratory medullary respiratory neurones show an increased excitability compared with the normotensive Wistar-Kyoto rat; importantly, this was reflected in an increase in postinspiratory activity in the rostral ventrolateral medulla (RVLM).

Recently, we explored the idea that essential hypertension in humans is due to an increase in respiratory-sympathetic coupling, by quantifying respiratory modulation of MSNA in a group of patients with essential hypertension and increased levels of MSNA at rest (Fatouleh & Macefield, 2011). In comparison to
Table 3. Normalized muscle sympathetic nerve activity, computed from the averaged normalized cross-correlation histograms (as shown in Fig. 5) for the control subjects and OSA patients

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Inspiration</th>
<th>Postinspiration</th>
<th>Expiration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>76.97 ± 0.23</td>
<td>74.40 ± 0.31</td>
<td>69.23 ± 0.52</td>
</tr>
<tr>
<td>OSA, 0 months</td>
<td>74.38 ± 0.58**</td>
<td>79.41 ± 0.82****</td>
<td>62.14 ± 0.75****</td>
</tr>
<tr>
<td>OSA, 6 months CPAP</td>
<td>76.62 ± 0.67</td>
<td>74.46 ± 0.77</td>
<td>63.99 ± 0.44****</td>
</tr>
<tr>
<td>OSA, 12 months CPAP</td>
<td>81.07 ± 0.24****</td>
<td>80.67 ± 0.44****</td>
<td>69.94 ± 0.59**</td>
</tr>
</tbody>
</table>

Mean values ± SEM were calculated over the 21 bins extending from −1.5 to 0 s (Inspiration), the 10 bins extending from 0.05 to 0.5 s (Postinspiration) and the 30 bins extending from 0.55 to 2.0 s (Expiration). Statistical differences (repeated-measures one-way ANOVA) are shown relative to the OSA baseline (0 months) data. *P < 0.05, **P < 0.01 and ****P < 0.0001. Note that statistical power is greater for this analysis than that of Table 2 because differences are computed over multiple bins.

a group of normotensive age-matched control subjects, there was no increase in respiratory modulation in the hypertensive group. In addition, we also found no increase in respiratory modulation in patients with chronic obstructive pulmonary disease. Given that the latter patients are chronically asphyxiated, are in a state of elevated respiratory drive (Gandevia et al., 1996), and have very high levels of MSNA (Heindl et al., 2001; Raupach et al., 2008; Ashley et al., 2010), we would have thought that respiratory modulation would have been higher in this group than in the essential hypertension group, but it was, in fact, lower (Fatouleh & Macefield, 2011). Interestingly, respiratory modulation in chronic obstructive pulmonary disease (37.5 ± 6.3%) was statistically identical to that seen in the patients with OSA (39.8 ± 3.1%). Accordingly, the present data, together with our previously published data (Fatouleh & Macefield, 2011), do not support the idea that an increase in respiratory modulation of MSNA can explain the sympathoexcitation and, for essential hypertension and OSA, the high blood pressure. However, there were significant changes in the temporal profile of the respiratory modulation of MSNA in the OSA patients; when examining the temporal profile of respiratory modulation, measured from the normalized MSNA during the respiratory cycle, it was clear that the temporal coupling of MSNA to respiration was stronger in the OSA patients than in the control subjects. Prior to the initiation of CPAP, MSNA was higher in the postinspiratory phase than in inspiration and expiration; these changes were largely reversed following long-term CPAP. We should point out that all analyses were conducted after all data collection had been completed; we were unaware that there was no significant difference in respiratory modulation of MSNA in the OSA patients prior to undertaking the analysis of the data following CPAP.

Finally, it is known that the sensitivity of the baroreflex is reduced in OSA (Carlson et al., 1996; Ryan et al., 2007), as well as in chronic obstructive pulmonary disease (Raupach et al., 2008), but the blunted baroreflex sensitivity in OSA has been shown to be unrelated to the overall increase in muscle sympathetic outflow (Carlson et al., 1993). While we did not specifically measure baroreflex sensitivity in the present study, cardiac modulation of MSNA clearly does reflect the efficacy of the baroreflex; the finding that cardiac modulation was significantly lower in the patients with OSA than in a group of age-matched control subjects provides further evidence that the sympathetic baroreflex is indeed blunted in OSA. However, there was no significant change in this measure following 6 or 12 months of CPAP, although an improvement in baroreflex sensitivity, as measured from spontaneous fluctuations in MSNA, has been reported (Ryan et al., 2007).

Conclusions

We have shown that, despite the greatly augmented levels of MSNA and blood pressure in OSA, the magnitude of respiratory modulation of MSNA is not increased in OSA when measured across individual subjects. However, when considering the normalized temporal profile there were changes in the respiratory patterning of MSNA in OSA, with more activity occurring in postinspiration and less in inspiration and expiration. This was largely reversed following long-term CPAP, although blood pressure remains elevated because total MSNA remains higher than in the control subjects.

References

data acquisition, analysis and writing. All authors approved of
the final manuscript.

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