NEURAL PLASTICITY IN OBSTRUCTIVE SLEEP APNOEA AND IN MEMORY FORMATION

By

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Abstract

Neural plasticity is the brain's ability to build new neural connection throughout life to adapt for several factors. The work in this thesis is built on two kinds of neural plasticity, respiratory long-term facilitation (rLTF) and memory formation.

Respiratory LTF: Obstructive sleep apnoea (OSA) is a chronic disorder which is characterised by repeated partial or complete airway obstruction during sleep, which leads to intermittent hypoxia (IH). Chronic IH is strongly linked to the increased blood pressure, pulmonary artery pressure, and oxidative stress associated with OSA. Ironically, many studies found that IH can induce rLTF which enhances ventilation and maintains airway patency, but the utilisation of this intervention is limited due to the complications of IH.

Respiratory LTF has several therapeutic potentials as will be seen later in this thesis and applying intermittent negative airway pressure (INAP) has shown to induce rLTF in couple of studies. The use of INAP for LTF is a new approach and little is known about its effectiveness and adverse effects. Two studies were conducted in our laboratory to investigate the effectiveness of INAP to induce LTF of ventilation (abstract in the following link: http://erj.ersjournals.com/content/50/suppl_61/PA2189) and of upper airway dilator reflex response to negative pressure (Chapter 3). INAP did not seem to

induce LTF of the upper airway dilators as will be seen in Chapter 3, but it did cause LTF of ventilation.

Until now, nothing is known about the safety of INAP as it is newly ventured area. In Chapter 4 looked at the effects of prolonged exposure to the INAP protocol on the blood pressure, pulmonary artery pressure, and oxidative stress on healthy subjects. The results show that INAP is a potential safe option for rLTF.

Memory formation: Memory is formed and strengthened by enhancing the connection between some neurons (synaptic plasticity) and by the creation of new neurons in the hippocampus (neurogenesis). Both mechanisms can be modified by external stimuli. In Chapter 5 looked at the effect of experimental sleep fragmentation on the consolidation of episodic memory. Episodic memory is composed of an item and associative elements to that item. The study found that the fragmentation maintains the item memory and negatively affects the associative memory.

Chapter 6 looked at the effect of targeted memory reactivation (TMR) paradigm during wakefulness or during nap on memory consolidation. The study found that TMR successfully enhanced memory consolidation, providing it is delivered during the nap. Also, the effect found to be not immediate, as it was only revealed after a night of sleep.

TO MY MOTHER (SANA)

AND

MY HUSBAND (MANSOUR)

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As a respiratory therapist, the brain was a new area for me to venture; starting from the EEG analysis software until reaching the complex TMR

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ABBREVIATIONS

AHI	Apnoea hypopnoea index
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
BP	Blood pressure
CB	Carotid body
CO2	Carbon dioxide
CPAP	Continuous positive airway pressure
CWD	Continuous wave Doppler
DBP	Diastolic blood pressure
ECG	Electrocardiography
EEG	Electroencephalogram
fMRI	Functional magnetic resonance imaging
FRAP	Ferric reducing ability of plasma
HR	Heart rate
HPV	Hypoxic pulmonary vasoconstriction
HRV	Heart rate variability
IH	Intermittent hypoxia
INAP	Intermittent negative airway pressure
LTD	Long term depression
LTF	Long term facilitation

LTP	Long term potentiation
MAP	Mean arterial pressure
MDA	Malondialdehyde
NREM	Non-rapid eye movement
O2	Oxygen
OSA	Obstructive sleep apnoea
PET	Positron emission tomography
PH	Pulmonary Hypertension
PSG	Polysomnography
RAS	Renin-angiotensin system
REM	Rapid eye movement
rLTF	Respiratory long-term facilitation
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Standard deviation
SE	Sleep efficiency
SEM	Standard error of the mean
SPAP	Systolic pulmonary artery pressure
TBARS	Thiobarbituric acid reactive substances
TMR	Targeted memory reactivation
TST	Total sleep time
vLTF	Ventilatory long term facilitation

1. GENERAL INTRODUCTION

1.1 OBSTRUCTIVE SLEEP APNOEA (OSA)

1.1.1 Overview

OSA is a chronic sleep disorder that is caused by changes to the anatomy and physiology of the airway. This results in upper airway collapse during sleep causing recurrent partial or complete airway obstruction leading to hypopnea and/or apnoea. Each OSA event is associated with hypoxia, which is corrected after the reestablishment of patent airway, exposing the patient to episodes of intermittent hypoxia (IH). This increases the risk of oxidative stress as will be discussed in Chapter 4 of this thesis and will also leads to hypertension and increase the pulmonary artery pressure. OSA is estimated to affect 9% of adult men and 4% of adult women in the general population (Neelapu et al., 2017), and yet it is still underdiagnosed and its prevalence is growing due to increased obesity and aging in the population (Kezirian et al., 2014). The risk of OSA increase with obesity, which is a fact well established from wealth of observational studies done in several countries (Barvaux et al., 2000, Young et al., 2005, Rejón-Parrilla et al., 2014). One of the effects of obesity is the increased fat accumulation around the pharynx which leads to narrower airways predisposing it for collapse. The increased fat deposition around the pharynx is indicated by increased neck circumference (Barvaux et al., 2000, Horner et al., 1989, Shelton et al., 1993, Fogel et al., 2004). Therefore, neck circumference more than 40.6 cm in women or more than 43.2 cm in men and BMI

more than 30 kg/m^2 are used as prognostic indicators for OSA in clinical practice (Shah and Tayade, 2018, NHS, 2016).

OSA is diagnosed with overnight sleep studies which are usually performed after noticing the key signs of OSA as loud snoring, daytime sleepiness and the cessation of breathing during sleep. This leads to frequent arousals from sleep to overcome the obstruction, which results in sleep fragmentation and negatively impact the cognitive functions (Jackson et al., 2011). Early diagnosis and treatment of OSA is vital because if not treated, it can cause several health complications and increase the mortality and morbidity rates for patients (Punjabi, 2008). Continuous Positive Airway Pressure (CPAP) is the standard treatment for OSA, which works by pneumatically splinting the airways during sleep to prevent it from collapse. CPAP is very effective if used per clinical recommendations, but the treatment itself has its own limitations. CPAP therapy requires wearing a mask that causes discomfort, as it constantly leaks and irritate the eyes, leads to nasal dryness, and is associated with increased prevalence of accumulation of abdominal gas (Broström et al., 2010, NHS, 2016). These limitations lead to poor tolerance and low compliance of the treatment, and that limits the benefit of the therapy as will be expanded on in Chapter 3 of the thesis (Phillips et al., 2013, Dempsey et al., 2014). This urges the need of alternative treatments for OSA, and LTF is a considered a potential option.

1.1.2 Pathophysiology of OSA

Breathing with both of its components (inspiration and expiration) eliminates carbon dioxide and provides the body with oxygen. For inspiration to occur, the diaphragm and external intercostal muscles contract to increase the volume of the intrathoracic cavity leading to lung expansion. This creates a negative intrathoracic pressure, which then causes air to move from the atmosphere into the lungs. The negative pressure during inspiration creates a suction effect that predisposes the upper airway to collapse, but in the same time it activates the peripheral mechanoreceptors located in the airway. The activation of the mechanoreceptors triggers a neural response that causes the upper airway dilator muscles to react. When those muscles are activated, they contract to maintain the patency of the airway (Horner et al., 1991, Fogel et al., 2004, Edwards and White, 2011).

The genioglossus (GG) muscle is the largest and the most studied upper airway dilator as will be expanded on in Chapter 3, which focus on the reflex response of the GG muscle to negative pressure. The activity of this muscle is affected by several factors as oxygen and carbon dioxide levels, the sleep/wake status, and the exposure to negative pressure (Bradford et al., 2005, Jordan and White, 2008, Edwards and White, 2011). Application of external intermittent negative airway pressure (INAP) has successfully triggered the GG reflex response in several studies (Horner et al., 1991, Horner et al., 1994). Furthermore, one study found that the reflex response to negative pressure was more significant when the negative pressure is applied at end expiration when compared to the beginning of expiration

(Tantucci et al., 1998). LTF aims to enhance this reflex as will be discussed later in this chapter.

Sleep is found to decrease the activity of the upper airway dilators, including the activity of GG muscle. Furthermore, it decreases the muscles' reflex response to the negative pressure by reducing its magnitude and delaying its onset. This increases the risk of upper airway collapse during sleep (Horner et al., 1994, Fogel et al., 2003, Fogel et al., 2004, Eckert et al., 2007). The reduction of negative pressure reflex response is found to progress as the sleep move to deeper stages as sleep is divided to stage I, II and III Non Rapid Eye Movement (NREM) sleep and REM sleep predisposing the airway to collapse as we move to deeper sleep stages (Shea et al., 1999).

In addition to the effect of sleep on the upper airway dilators which affects even healthy people, OSA patients have several factors that increase their risk of upper airway collapse during sleep. They have thick pharyngeal wall, large soft palate, inferior hyoid bone, smaller mandible and larger more posteriorly placed tongue (Schwab et al., 2003, Fogel et al., 2004, Dempsey et al., 2014, Neelapu et al., 2017). To compensate for that, the GG muscle of OSA patients shows greater baseline activity even during wakefulness when compared to age and weight matched controls (Mezzanotte et al., 1992, Fogel et al., 2001, Fogel et al., 2004, Mateika and Syed, 2013, Mateika and Sandhu, 2011). OSA patients succeed in maintaining and/or re-establishing the patency of upper airway during sleep some of the time with the help of the peripheral mechanoreceptors and chemoreceptors.

Those receptors respond to the hypoxia and hypercapnia and to the increased negative pressure that happens with each OSA event leading to increased ventilation and to airway maintenance, but in many occasions, an arousal must occur to re-establish a stable airway. This results in a recurrent sleep fragmentation and reduced sleep quality (Edwards and White, 2011).

1.1.3 Neural pathway that controls breathing

Ventilation and upper airway dilator muscle activity are controlled by several peripheral receptors which respond to multiple factors as the changes in oxygen and carbon dioxide partial pressures, sleep–wake state and changes in the upper airway pressure (Malhotra et al., 2000). Central chemoreceptors also contribute to the control of ventilation, and evidence strongly suggests that serotonergic neurons are part of the central chemosensory system responsible for ventilation and airway maintenance (Corcoran et al., 2009). The serotonergic neurons are stimulated by hypercapnia, and they have an excitatory effect on the nerves that control respiration as the phrenic and the hypoglossal motor neurons so when they are activated they will increase ventilation and lead to upper airway dilation (Lipford et al., 2016). The activity of these neurons is reduced as sleep progress to deeper stages, and that might explain why the OSA events are worsening overnight. Furthermore, the activation of these neurons has been linked to the arousals that occur after the apnoea episodes implying for their essential role in OSA (Lipford et al., 2016). The importance of the serotonin receptors for LTF will be discussed in Chapter 3.

1.1.4 Morbidity and mortality

Patients with untreated OSA are at high risk of endocrine disturbances, daytime sleepiness, decreased cognitive function, decreased quality of life and increased road accidents (Kezirian et al., 2014). OSA also leads to hypertension and other cardiovascular complications; a risk that increases proportionately with the severity of OSA (Peppard et al., 2000, Phillips, 2005). The OSA severity is mainly defined by the apnoea hypopnoea index (AHI) which is the number of apnoea and hypopneas/total sleeping time, where 5-14 is considered mild, 15-30 is moderate and more than 30 is severe OSA (NHS, 2016).

Apnoea is defined as a cessation of breathing for more than 10 s, and hypopnea is defined as reduction of flow from baseline that last for 10 s or reduced oxygen saturation S_aO_2 more than 4% (NHS, 2016). Furthermore, OSA is associated with pulmonary hypertension and increased oxidative stress (Golbin et al., 2008). Oxidative stress results from an imbalance between the production and the elimination of the reactive oxygen species (ROS), which are reactive molecules with free electron. The oxidative stress occur when the increase of ROS is not compensated for by increasing the body's antioxidant capacity (Lavie, 2003, Pialoux et al., 2009).

Repeated collapse of the airway and the resulting IH is the major cause for the oxidative stress found in patients with OSA as will be discussed in Chapter 4. Furthermore, the oxidative stress is found to be associated with increased production of some vasoconstrictors as endothelin-1 and angiotensin II which

contribute to the hypertension that occur due to OSA (Brindeiro et al., 2007, Lavie, 2003, Serebrovskaya et al., 2008). In addition to that, oxidative stress leads to sympathetic excitation by enhancing the chemoreflex and suppressing the baroreflex which regulates blood pressure (BP) and that also contribute to the OSA associated hypertension (Prabhakar and Kumar, 2010, Tamisier et al., 2010, Freet et al., 2013).

Baroreflex is an essential BP regulator where stretch receptors in the aortic arch and carotid sinus sense the change in blood pressure and send signals to the brain stem. When the blood pressure increases, the brain increases the parasympathetic output and decreases the sympathetic output which leads to decrease in HR and stroke volume and to vasodilation. Together these factors work to bring the BP back to normal level.

Chemoreceptors on the other hand are located in the carotid and the aortic body and they monitor the levels of oxygen, carbon dioxide and pH. When they sense hypoxia, hypercapnia and/or acidosis they send signals to the brain which decrease the parasympathetic and increase the sympathetic output leading to increased HR, increased stroke volume and vasoconstriction; resulting in increased BP (Freet et al., 2013).

1.1.5 Treatment for OSA

CPAP is the current treatment for moderate and severe OSA, and it works by pneumatically splinting the airway and preventing it from collapse. Although CPAP is efficient treatment for OSA patients compliance with it is low as will be discussed in chapter 3 (Cortés-Reyes et al., 2017). The search for alternative treatment is still ongoing, but nothing is achieving the same outcome of CPAP yet. Lifestyle changes can help in the OSA treatment process (NHS, 2016).

Weight loss either with diet or weight reduction surgeries found to reduce the severity of OSA, but it doesn't eliminate the need for CPAP (Barvaux et al., 2000). Smoke cessation is highly recommended for OSA patients, as smoking induces upper airway inflammation. Furthermore, nicotine acts as a stimulus to the upper airway muscles, and the withdrawal during night can lead to rebound (Lin et al., 2012). Reducing alcohol consumption is also highly recommended as mean blood alcohol concentration of 0.07 g x dL(-1), caused significant increase in the OSA events, raising the AHI to 9.7 ± 2.1 compared to 7.1 ± 1.9 without alcohol consumption events x h(-1) (mean+/-SEM, p=0.017) (Scanlan et al., 2000). Finally, changing the sleep position from supine to lateral is found to decrease the airway collapsibility reflected by significantly reduced pharyngeal critical pressure (Penzel et al., 2001).

Other therapeutic modalities are also used for the treatment of OSA. For instance, mandibular advancement device (MAD) is an oral appliance that protrudes the jaw during sleep increasing the size of the upper airway. Yet, patients with severe

OSA or high BMI will not benefit from MAD (Cortés-Reyes et al., 2017). Moreover, long term use of MAD can result is dental malformation which may result in pain in the tooth and the face (Doff et al., 2013, Cortés-Reyes et al., 2017). Surgeries may also be used as last option to correct for anatomical causes of OSA and increase the pharyngeal size, but this is not routinely recommended due to the lack of evidence for the surgeries' effectiveness (NHS, 2016).

This lack of ideal treatment for OSA triggered the ongoing search for a way to address the neural deficit that leads to the obstructive events. The aim is to find a way to permanently reverse the disease process. The scope of interest of the current research is finding a way to enhance the neural feedback system in response to one or more of the events that occur with each apnoea episode; hypoxia, hypercapnia and increased negative pressure aiming to produce an amplified response to the event which should avoid or overcome the obstructions the occur during sleep.

1.1.6 Respiratory Long-term facilitation (rLTF)

Respiratory LTF is a form of neural plasticity induced after exposure to a stimulus, characterised by augmented respiratory motor output that lasts for 90 minutes after the elimination of the stimulus (Mitchell and Johnson, 2003, Mateika and Sandhu, 2011). The most well-known trigger for rLTF is intermittent hypoxia (IH), but rLTF has also been demonstrated following electrical stimulation of the phrenic nerve (Zhang et al., 2003) and by application of intermittent negative airway pressure (INAP), *the second study published as an abstract* (Ryan and Nolan, 2009b, Griffin et al., 2017). Both phrenic and hypoglossal nerves can be influenced by LTF (Mahamed

and Mitchell, 2007). Stimulation of the phrenic nerve leads to increase in ventilation, while stimulation of the hypoglossal nerve maintains airway patency (Baker-Herman and Strey, 2011). For LTF to present itself in the hypoglossal nerve (hLTF) and phrenic nerve (pLTF), the stimulus that triggers the LTF should be intermittent rather than continuous. Moreover, LTF for both nerves require the facilitation of serotonin receptors. The difference between the two is the location of the receptors; where it is proved that pLTF requires the activation of the spinal serotonin receptors, it is still not clear which serotonin receptors are activated for hLTF although some evidence suggest the hypoglossal motor neurons receptors as mediators for the hLTF. In addition, hLTF might be achievable by activation of other receptors as α1-adrenergic receptors, but more work has to be done in order to find out the mechanism of action for those receptors (Baker-Herman and Strey, 2011).

LTF BY IH

IH induced LTF of the hypoglossal nerve in rats (Fuller, 2005, Ryan and Nolan, 2009a), cats (Mateika and Fregosi, 1997) and in sleeping humans (Chowdhuri et al., 2008). On the other hand, several studies failed to achieve LTF through IH in awake humans (Jordan et al., 2002, Mateika et al., 2004, Khodadadeh et al., 2006, Morelli et al., 2004). The evidence of LTF in animals implied that it is possible in humans, so researchers kept trying until they found out that elevating the CO_2 above its baseline level unmasked the expression of rLTF in awake humans (Mateika and Sandhu, 2011, Harris et al., 2006, Griffin et al., 2012).

This is because the control of ventilation during sleep is solely led by chemoreceptor feedback which allows the expression of LTF. During wakefulness on the other hand, ventilation is controlled by arousal and/or behaviour inputs, so the hypoxia protocol leads to hyperventilation and hypocapnia. Sequentially, the hypocapnia inhibits the chemoreceptors and mask the expression of LTF during wakefulness. Once the carbon dioxide is elevated, the control of ventilation is led by the chemoreceptors again, allowing the expression of LTF (Harris et al., 2006, Gerst et al., 2011). Although LTF is achievable by IH, the use of this method is still limited, not only by the complexity of delivering IH to individuals, but also due to the negative side effects that are associated with IH which will be expanded on in Chapter 4.

LTF BY INAP

The stimulation of negative pressure receptors in the airway leads to activation of a neural reflex that maintains airway patency (Horner et al., 1991). As mentioned in section 1.1.2, application of external INAP successfully activated this reflex. It was hypothesised that repetitive stimulation of these receptors will elicit rLTF. This hypothesis was confirmed by achieving rLTF in anaesthetised rats after exposure to INAP, which was the first study that did not include IH for rLTF (Ryan and Nolan, 2009b). The shared physiological characteristics between humans and rats highly suggest the possibility for initiation of rLTF in humans in response to INAP.

In fact, a study which is published as an abstract successfully achieved ventilatory LTF in awake healthy human who were kept under elevated levels of CO_2

during the protocol.13 healthy male volunteers participated in this crossover study, and each participant went through a control and an INAP trial. LTF was manifested as increased minute ventilation compared to baseline after one hour of INAP, an increase that was significantly more than observed after one hour of control (Griffin et al., 2017).

1.2 SLEEP AND COGNITIVE FUNCTIONS

1.2.1 Memory systems

Human memory can be divided into two categories: short-term and long-term memory, and the long-term memory can be split into further classifications as shown in Figure 1.1 (Rauchs et al., 2005, Tulving, 1985).

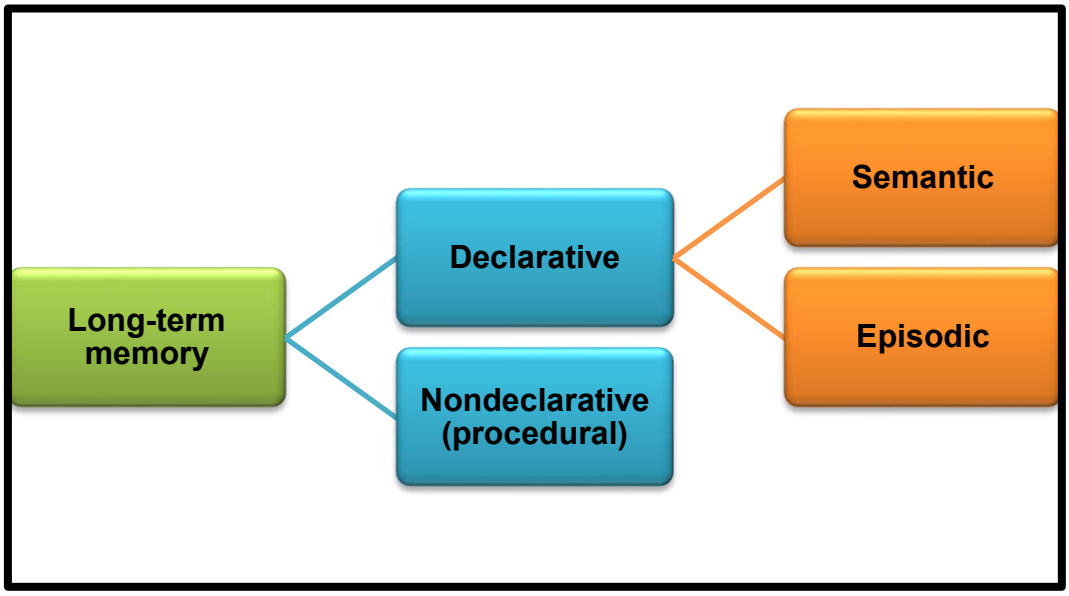

Figure 1-1. Long-term memory classification.

Declarative memory is the memory accessible for conscious recollection and it includes two memory systems; the *semantic memory system*, which deals with facts, and the *episodic memory system* which deals with memories of events (Diekelmann et al., 2009, Diekelmann and Born, 2010, Born and Wilhelm, 2012, Tulving and Markowitsch, 1998). This thesis will focus on the *episodic memory*, which is

described as a memory with a context. This type of memory can be mentally relived with all its details (Tulving, 1995).

1.2.2 Memory processing

Memory is processed through three stages: encoding, consolidation and retrieval. Learning initiates the encoding process, which creates a memory trace for the encountered stimulus. Consolidation happens during the offline period after learning during both sleep and wakefulness. During this time, memory traces are stabilised and enhanced to protect them from decay. Retrieval allows the utilisation of the stored memory exposing it to destabilisation, which triggers reconsolidation, (Walker and Stickgold, 2006, Diekelmann and Born, 2010, Rasch and Born, 2013, Born and Wilhelm, 2012), Figure 1.2.

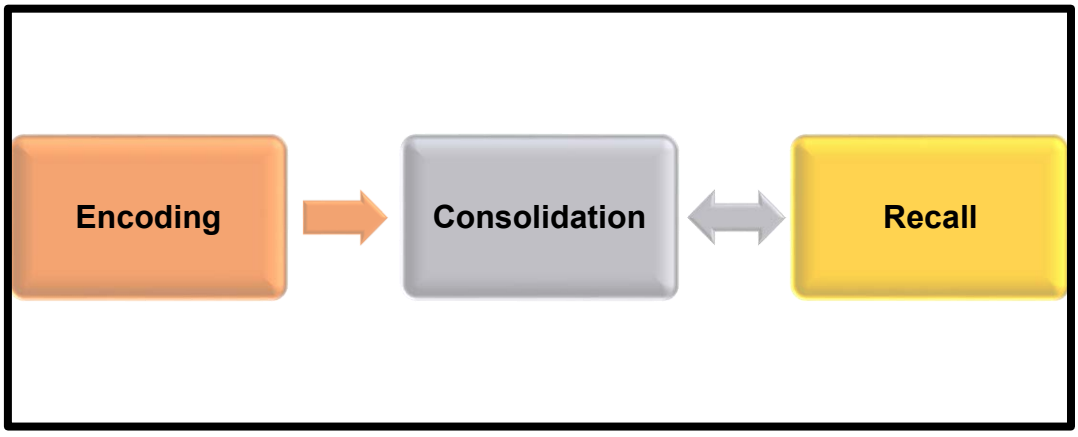

Figure 1-2. A schematic diagram of memory processing,

The way we understand the consolidation process is evolving with time and it depends on the type of memory being stored. *The two-stage memory system* theory proposes that the declarative memory trace is created by *synaptic consolidation*, which is a form of neural plasticity. This trace is saved temporarily in an unstable format in the hippocampus. Many factors can affect what happens to the memory next, as not all memories are stored. Nonetheless, if the memory was meant to be remembered, it is transferred during an offline period, which could be during sleep or quiet wakefulness, to a more stable format in the brain's long-term storage space, which is suggested to be the neocortex. This chain of events happens through the *system consolidation process* (Rasch and Born, 2013, Born and Wilhelm, 2012, Diekelmann et al., 2011).

As mentioned earlier, evidence shows that certain factors can affect which memories will go through the system consolidation process, especially during sleep. For instance, consolidation of memories with strong emotional impact is selectively enhanced during sleep (Hu et al., 2006). Furthermore, a study showed that when the participants knew about or expected that there will be a retrieval task, the consolidation was also enhanced during sleep (Wilhelm et al., 2011). It was also shown that reward anticipation could also affect the consolidation during sleep (Fischer and Born, 2009).

Moreover, the type of learning also affects the consolidation process as learning can be explicit (intentional and with awareness) or implicit (which happens without awareness of learning). Explicit learning is known to activate the

hippocampus system during the encoding, and that is assumed to make the memory trace more liable for sleep dependant memory consolidation (Diekelmann et al., 2009).

1.2.3 Sleep architecture

Human sleep goes through several stages that occur in repeated 90-minute cycles. Each cycle is typically composed of stage I, II and III non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. Each stage has its unique characteristics, which are summarised in Table 1.1 (Iber et al., 2007). Studies comparing the sleep cycle between a night's first and second half consistently showed that stage II sleep was similar in both, while the first half was rich with SWS and the second half had more REM sleep (Walker and Stickgold, 2004, Stickgold, 2005).

	Stage	Characteristics
NREM	I	- Light sleep - Slow rolling eye movement - Loss of alpha activity
	II	- K complex (large electrical sharp wave) - Sleep spindles (short synchronised oscillations)
	III (SWS)	- Deep sleep - Some spindles - Slow wave sleep (SWS), 0.5-4 Hz frequency
REM		- Desynchronised cortical oscillation, 30-80 Hz frequency - Brain activity like wake - Episodes of rapid eye movement - Low muscle tone compared to wake and NREM

Table 1-1. Characteristics of the sleep stages.

1.2.4 The function of sleep

Although the consolidation of declarative memory occurs with the passage of time during both wakefulness and sleep, sleep is found to promote brain plasticity, which enhances memory consolidation (Maquet, 2001, Gais and Born, 2004). A wealth of evidence show that sleep serves as an optimal condition for consolidation of episodic memory when compared to wakefulness as the communication between the hippocampus and the neocortex is facilitated by sleep spindles and the slow oscillations (Ellenbogen et al., 2006, Gais et al., 2006, Diekelmann and Born, 2010, Mölle et al., 2011, Diekelmann et al., 2012).

The mechanism of consolidation has received major attention from researchers. Functional Magnetic Resonance Imaging (fMRI) scans of the brain showed a similar activity during encoding and during sleep, suggesting that the aforementioned system consolidation process happens through the replay of the memory traces during sleep (Rasch and Born, 2007, Born and Wilhelm, 2012, Cairney et al., 2016). This replay was also seen during wakefulness, but in reversed order (Foster and Wilson, 2006).

In a study that compared the performance of a declarative memory task in participants who slept for a certain period of time to those who stayed awake for the same length of time, the researchers observed a superior performance in participants who tested under the sleep condition, which was noticed in the 48-hour retrieval and in the 6-month retest but to a lesser extent. Participants in that study performed the retrieval in an fMRI scanner, which showed that sleep increased the connection between the medial prefrontal cortex and the hippocampus when compared to wakefulness (Gais et al., 2007). Furthermore, several studies showed a consistent improvement in the performance of declarative tasks after a night's sleep and even after half a night's sleep when compared to wakefulness (Rasch and Born, 2013). Moreover, even short naps had a positive effect on performance when compared to the same duration of wakefulness (Diekelmann et al., 2009).

1.2.5 Sleep stages and memory type

As stated earlier, memory consolidation is the process that converts a non-stable memory trace into a permanent form by establishing connections between the hippocampus in the medial temporal lobes and the neocortical areas (Rauchs et al., 2005). After confirming the superior impact of sleep on memory consolidation, several studies tried to find which memory type was affected by each sleep stage, and that resulted in several hypotheses. The following sections will discuss the dual process hypothesis, the sequential hypothesis and the active system consolidation hypothesis.

The dual process hypothesis assumes that different sleep stages are responsible for the consolidation of specific types of memory (Diekelmann et al., 2009). Several testing paradigms supported this theory and showed evidence that nondeclarative memory is consolidated during REM sleep while declarative memory is consolidated during NREM, particularly SWS (Yaroush et al., 1971, Smith, 2001, Gais and Born, 2004, Rauchs et al., 2005). An example is the “night half paradigm”, where participants do the encoding in the evening and woken for retrieval after three to four hours or sleep for three to four hours before woken to do the encoding task then sleep for rest to the night and do the recall in the morning (Rasch and Born, 2013). The limitation of this theory lay behind some studies’ findings that some elements of the declarative memory benefit from REM consolidation while some nondeclarative memories benefit from NREM sleep (Rauchs et al., 2005) making this classification not practical, especially with the limitations of the paradigms used to support this theory.

In contrast, the sequential hypothesis suggests that both REM and NREM components are essential for the consolidation of both memory types and propose that SWS and REM balance and complement each other in a certain way (Diekelmann and Born, 2010, Rasch and Born, 2013). This theory is supported by studies of human naps, which found that 90-minute naps that contain NREM and REM sleep can improve the performance of the declarative memory tasks. By contrast, 60-minute naps that did not contain REM failed to show the same effect. This finding contradicts the assumption that REM is not considered an ideal brain condition for the consolidation of declarative memory, instead it supports that REM role might compliment the SWS role in the consolidation process (Mednick et al., 2003).

Another approach to support this theory consisted of disturbing the NREM-REM cycle throughout the night and comparing the performance to a night with a non-disturbed sleep cycle. This result was achieved by the experimental sleep fragmentation protocol, in which the results showed a decline in performance after the disturbed sleep cycle (Ficca et al., 2000, Tucker et al., 2006, Rauchs et al., 2005, Lau et al., 2010).

The research then took another approach to understand the effect of sleep on memory consolidation. Evidence showed that sleep spindles, occurring in stage II and III, were found to contribute to the neural plasticity and aid with the information transfer from the hippocampus to other brain regions (Schabus et al., 2004, Van Der Helm et al., 2011, Cairney et al., 2018, Gais et al., 2002). For instance, a study found

that the spindles' density increased during the first 90 minutes of a night's sleep in participants who had previously trained for a declarative task when compared to non-learning controls. This increased density was correlated to the improvement in performance (Gais et al., 2002).

This discovery moved the scope of research towards the importance of elements of NREM sleep, including sleep spindles in stage II and III, as well as SWS for consolidation of declarative memory (Gais et al., 2002). As a result, the focus of the research started to shift away from the SWS/REM thinking towards concentrating on more specific characteristics of sleep like sleep spindles and slow oscillations as mediators for memory consolidation (Diekelmann et al., 2009).

Thus, the active system consolidation hypothesis emerged. This hypothesis integrates components of both the dual process and the sequential hypotheses, and it proposes that consolidation occurs with repeated activation of the memory trace during SWS. This process is proposed to shift the trace from the short-term to the long-term memory store, which is facilitated by the sharp wave-ripples and sleep spindles. This step is followed by a stabilisation process that, according to this hypothesis, happens during REM (Rasch and Born, 2013).

This theory led to the development of the targeted memory reactivation technique, in which the encoding is associated with a cue that is used to reactivate the memory trace during the offline period. TMR successfully improved the retrieval

of the cued memory, indicating that it facilitated the consolidation process (Cairney et al., 2016). TMR paradigms and their outcomes will be discussed in Chapter 6.

1.2.6 Sleep and neural plasticity

Neural plasticity of the brain is manifested by forming new synapses between the brain neurons, enhancing current synaptic connections, generation of new neurons and/or modification of specific neurons. This leads to improved communication between the neurons and results in Long-term potentiation LTP (Timofeev and Chauvette, 2017). In contrast, neural plasticity of the brain can be displayed by a complete opposite way, which is by the weakening of the unnecessary synaptic connections to maintain the brain efficiency and avoid signal saturation by a process known as long term depression LTD (Timofeev and Chauvette, 2017).

Although there is agreement in the research about the importance of SWS for declarative memory consolidation, the mechanism is a source of debate. *The synaptic homeostasis hypothesis* on one hand suggests that it works by downscaling all synaptic connections during sleep, which eliminates all traces with weak synaptic connections and maintains the traces that formed a strong connection during encoding. This process is assumed to reduce the noise to signal ratio of encoded information (Tononi and Cirelli, 2003, Tononi and Cirelli, 2006, Diekelmann and Born, 2010). *The active system consolidation* on the other hand suggests that the encoded memory traces first go through synaptic consolidation then they are reactivated offline, and this reactivation transfer the fragile memory trace from the hippocampus to more stable format which is stored in the neocortex and integrated to the long-term

memory network in a process known as system consolidation (Takashima et al., 2009, Diekelmann and Born, 2010, Born and Wilhelm, 2012). This is achieved by synchronized brain activities which shows a pattern of memory trace replay during SWS and quite wakefulness, known as memory reactivation (Sutherland and McNaughton, 2000, Rasch and Born, 2013). The reactivation hypothesis was supported by several animal studies. For example, one study recorded the brain activity of rats while they navigate through a maze, and reported the same pattern of activation during SWS (Wilson and McNaughton, 1994).This reactivation was also reported in humans using positron emission tomography (PET), functional magnetic resonance imaging (fMRI), and EEG; and it was noticed during the SWS rather than REM (Rasch and Born, 2013).

The synaptic homeostasis hypothesis received many critiques and it is not supported by the findings of behaviour data (Diekelmann and Born, 2010, Rasch and Born, 2013). For instance, it can't explain the memory improvement after the evident reactivation of memory during sleep, which is seen to occur both spontaneously and with external stimuli; a finding that favour the active system consolidation hypothesis (Rasch et al., 2007, Rudoy et al., 2009). Yet, despite the difference between those theories, there is possibility that both mechanisms are complementing each other (Diekelmann and Born, 2010).

Several pathways were proposed to cause LTF or LTD; as it could be due to changes in neural transmitters or changes in gene expression, but the pathway more correlating to the research in this thesis is through the changes of the brain's

electrical activities, which is correlated with sleep events like sleep spindles, ripples and slow oscillations (Timofeev and Chauvette, 2017). The following sections will briefly define each brain activity and their proposed contribution to memory formation.

Slow oscillations

These are the main elements in the SWS, which are mainly generated in the neocortical network and are defined by frequency of 0.5 to 4 Hz; ~0.75 Hz on average (Born and Wilhelm, 2012). The membrane of the neurons alternates between depolarisation “up-states” and hyperpolarisation “down-states” in a synchronised way during the slow oscillation (Rasch and Born, 2013).

The role of slow oscillations in memory consolidation is established by noticing an increased oscillations activity during SWS after encoding of new declarative memory. The increase is seen in the up-states amplitude and the oscillations frequency of the slow wave activity. Furthermore, an increase in the slope between the up and down states is also noted (Mölle et al., 2004, Mölle et al., 2009). This increase in oscillations activity is directly related to the amount of encoded information during wakefulness (Born and Wilhelm, 2012).

SLEEP SPINDLES

Spindles are EEG activity originating from the thalamo-cortical region, with a frequency of 10 to 15 Hz which can be seen in stage II and III NREM sleep, being more prominent in stage II. Spindles and slow wave activity have a reciprocal

relationship as when slow wave activities decrease as the night progress, spindle activity increases. Also, it is noted that the recovery sleep after a period of sleep deprivation has increased slow wave activity and decreased spindles activity.

Humans has two types of spindles; fast 13-15 Hz and slow 10-12 Hz. The slow spindles are mainly seen during SWS more than stage II. Researchers showed association between the fast spindles and the consolidation of hippocampus dependent memories (Diekelmann and Born, 2010, Rasch and Born, 2013).

Encoding of declarative memories increased the number of fast spindles especially during the first half of the night (Gais et al., 2002, Schabus et al., 2004). Furthermore, the correlation between spindles and overnight enhancement of declarative memory is well established (Schabus et al., 2004, Cairney et al., 2018). Not only that, but evidence indicated that spindles helps in the integration of the new memory to existing information (Takashima et al., 2009, Tamminen et al., 2010). This improvement was also noted during nap, not only during overnight sleep (Van Der Helm et al., 2011).

These findings together with the noticed reactivation of the newly encoded memory trace in both hippocampal and neocortex regions in synchrony with spindle activity in NREM sleep (Bergmann et al., 2012) indicates the important role of sleep spindles in the communication between the hippocampus and the neocortex and in memory consolidation during sleep (Rasch and Born, 2013).

SHARP WAVE-RIPPLES (SW-R)

SW-R are oscillation with high frequency (100-300 Hz) which usually noticed during SWS and quite wakefulness and are linked to memory reactivation, especially the hippocampus dependent memories (Rasch and Born, 2013). Elective post learning ripples disruption by electrical stimulation led to impaired consolidation process, indicating the importance of the ripples for the process. It is highly suggested that sharp wave-ripples could lead to synaptic potentiation (Diekelmann and Born, 2010).

HOW SLOW OSCILLATIONS, SPINDLES AND RIPPLES INTERACT

The active system consolidation hypothesis propose that memory consolidation occur through reactivation of the memory trace via synchronised activation of the neocortical slow oscillations, hippocampal sharp-wave ripple and thalamocortical sleep spindles during the depolarising upstate of SWS creating a dialog between the hippocampus and neocortex, and that in turn strengthen the memory trace (Tamminen et al., 2010, Born, 2010, Diekelmann and Born, 2010, Born and Wilhelm, 2012, Cairney et al., 2014), Figure 1.3.

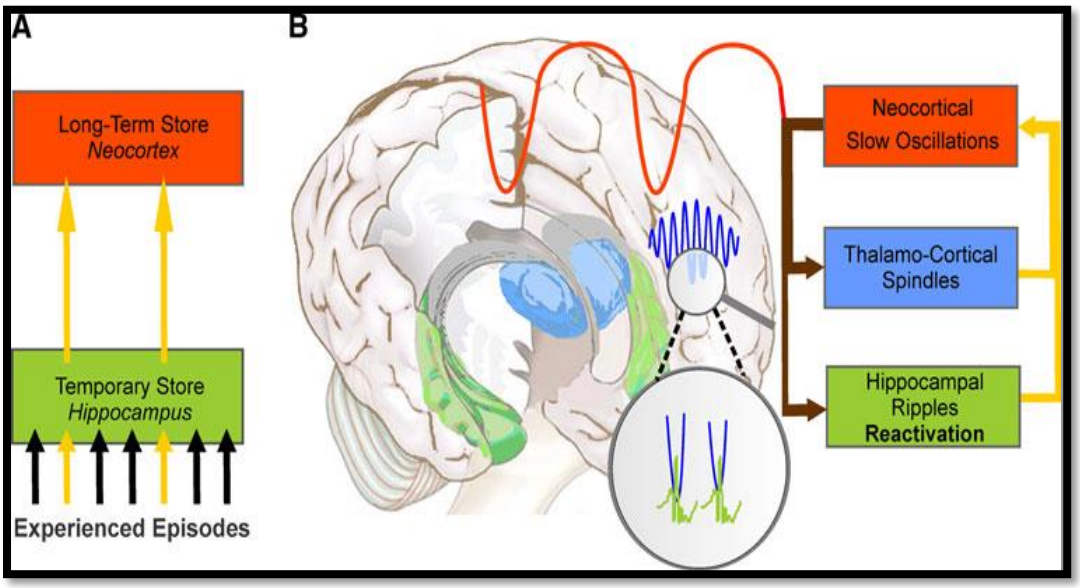

Figure 1-3 Active System Consolidation. A: Memory consolidation during SWS, moving the trace from hippocampus to neocortex. B: Synchronized activation of ripples, spindles and slow oscillations to transfer the memory trace. Image has an open access and was taken from (Born and Wilhelm, 2012)

1.3 QUESTIONS AND HYPOTHESES

This thesis includes two parallel lines; in one side there are studies about the possibility of LTF by INAP which had two questions; first would INAP result on hLTF? And if so, would it be a safe alternative to IH? These questions will be addressed in Chapter 3 and Chapter 4 of this thesis. The hypothesis was that INAP will be an alternative to IH for rLTF with less side effects.

The other line is about sleep and memory. Chapter 5 investigates the effects of sleep fragmentation on the consolidation of the episodic memory. The hypothesis

was that the fragmentation would negatively affect the consolidation, which would be reflected as reduced retrieval performance.

Chapter 6 examines the effect of TMR on the consolidation of episodic memory during sleep and wakefulness. The hypothesis was that TMR would improve the retrieval of the cued memory, especially when applied during sleep.

2. GENERAL METHODS

2.1 THE NEGATIVE PRESSURE SYSTEM

We used the negative pressure system, which was built in our laboratory in two experiments with variation in the protocols. This section will describe the system while the protocols will be detailed in Chapter 3 and Chapter 4.

In both experiments, participants were breathing through a comfortable nasal mask (a modified TrueBlue, Philips Respironics, USA) with tight seal during the experiment. The mask was connected to a 50-litre negative pressure reservoir, which was maintained at -45 cmH_2O by using a vacuum pump. A continuous flow of room air was provided via a CPAP machine (REMstar Pro M series CPAP, Respironics, USA). Exhalation was directed away from the breathing circuit via a one-way valve to prevent rebreathing (5800 Giant, Hands Rudolph Inc, USA).

The negative pressure was initiated when a solenoid valve was used to activate a piston which rapidly switched between breathing room air at ambient pressure or at negative pressure. This resulted in instant evacuation of air from the breathing circuit (0 to -15 cmH_2O in less than 10 ms). The pressure was maintained at -15 cmH_2O by a spring loaded reversed positive end-expiratory pressure (PEEP) valve placed near the mask. Upper airway pressure was monitored by a pressure transducer placed close to the nasal mask throughout the experiment (40PC,

Honeywell, USA) and the flow was measured by a pneumotachograph (4813, Hands Rudolph, USA), Figure 2.1.

Spike2 software was used to automatically activate the solenoid valves and switch between breathing room air at ambient at negative pressures. Negative pressure breathing was introduced at the beginning of inspiration and was maintained for 30 seconds, while ambient pressure was introduced at the beginning of expiration and was maintained for 1 minute, Figure 2.2.

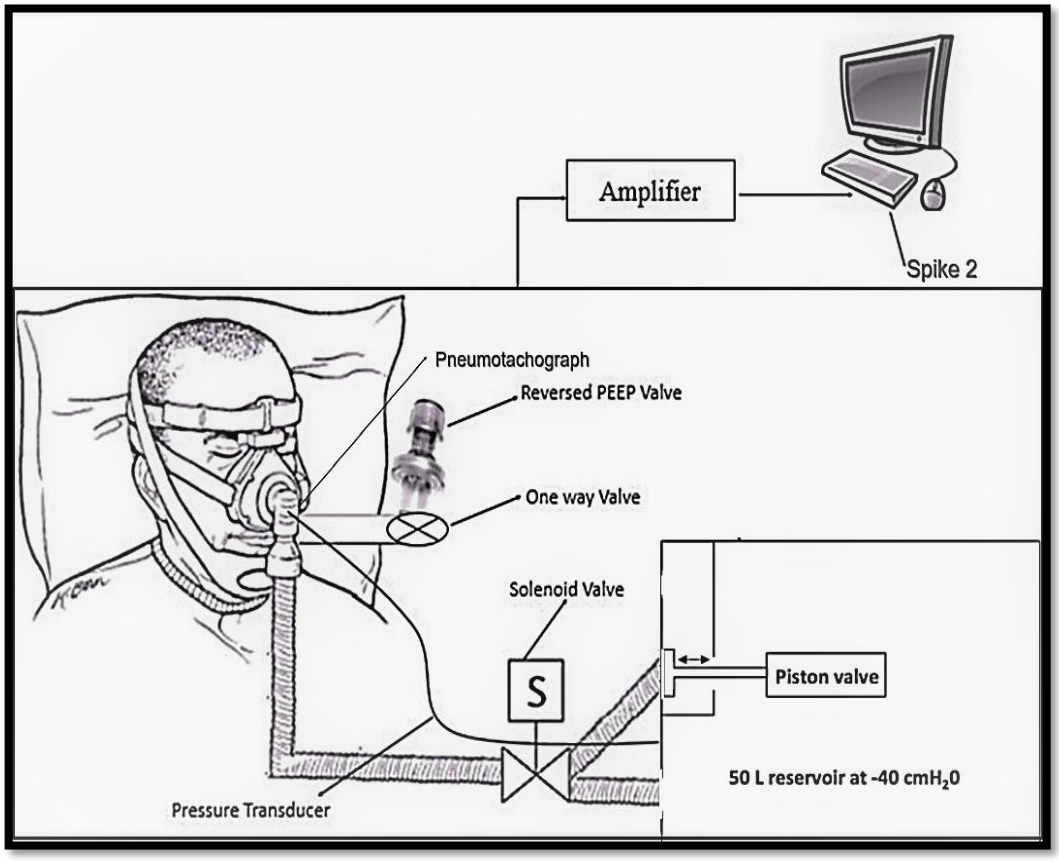

Figure 2-1. A Schematic diagram of the INAP experiment setup.

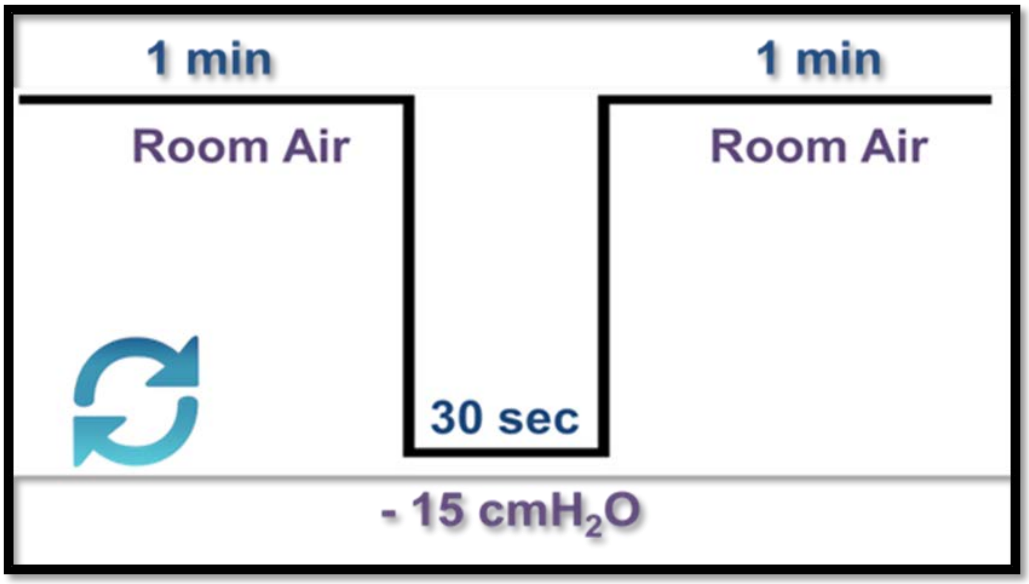

Figure 2-2. The negative pressure exposure protocol.

2.2 BLOOD PRESSURE MEASUREMENTS

Blood pressure was measured using an automated blood pressure monitor (OMRON 705IT) while subjects were positioned in a 45 angle from the supine position (semi fowler). The blood pressure value was recorded as a mean of three consistent readings.

2.3 VENEPUNCTURE

A trained phlebotomist collected 6 ml blood sample in an anticoagulant-treated tube by venepuncture from a vein in the arm. Blood was centrifuged for 10 minutes at 1700xg and 4 degrees Celsius temperature. Plasma was transferred to multiple 0.5 ml aliquots by Pasteur pipette, and stored at (-70˚C) until later oxidative stress analysis.

Blood Analysis

Plasma was separated by centrifuge at speed of 1700 RPM for 10 min at a temperature of 4ºC. Plasma was stored at -80 degrees Celsius in several aliquots for later analysis of oxidative stress parameters. At the end of the experiment, after collecting all the samples from the participants FRAP protocol was used (appendix 8.1) to measure the total antioxidant capacity and the MAD protocol (appendix 8.2) to measure the lipid oxidation at 3 time points; beginning of day 1, beginning of day 3 and end of day 3 to establish the baseline value, see the effect mid intervention and see the affect after the intervention.

2.4 CARDIAC ULTRASOUND

Ultrasound was used for the non-invasive measurement of pulmonary arterial pressure (PAP) and cardiac output which was required for Chapter 4. The measurement required the utilisation of the two-dimensional image and the Doppler technology. The following sections will describe each feature, then explain how it was used to obtain the required measurements.

2.4.1 Two-dimensional image

The ultrasound transducer contains crystals which vibrate when exposed to electrical current. This vibration creates pressure waves/sound waves which can pass through the soft tissues. The crystals then receive the reflected sound waves and convert them to voltage which is presented as two-dimensional image by a

computer. The denser the tissue the brighter the presented image, as more sound waves would be reflected. Two-dimensional ultrasound can give a clear image of the heart valves and chambers despite the small size of the transducer. This is because the sound waves spread in a fan shape which produces larger image compared to the transducer size, Figure 2.3.

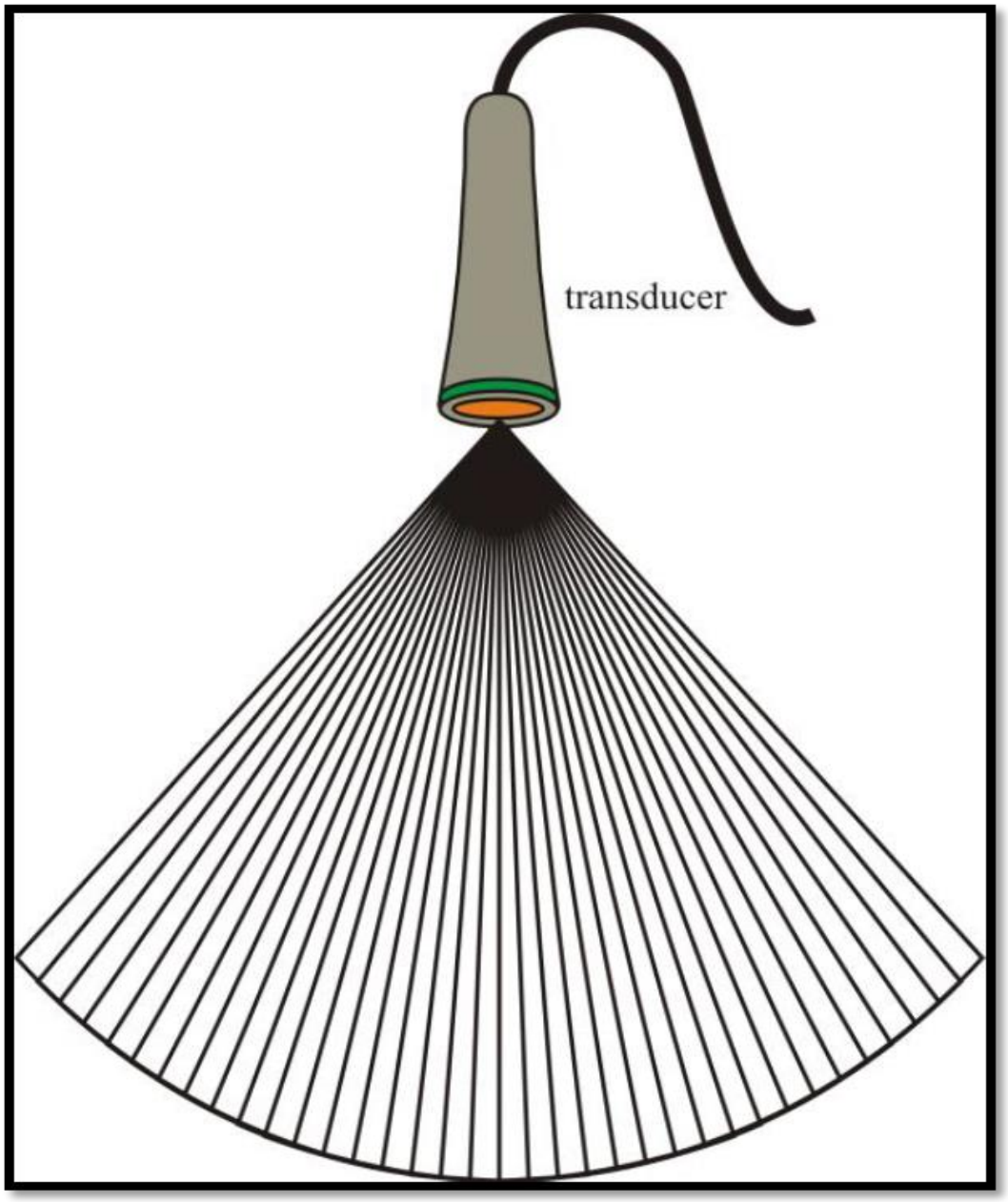

Figure 2-3. Scan lines from the ultrasound transducer.

2.4.2 Using doppler effect to measure blood flow

The Doppler effect was first introduced in 1842 by the Austrian physicist Christian Doppler. Doppler linked the direction of stars movement to their colour as he noticed that the stars moving away from the earth appeared red, while the stars moving toward the earth appeared blue.

The same concept is now used in Doppler ultrasound to measure the blood flow through the heart valves, where a pulse with known frequency is sent from the transducer and the reflected waves are analysed to determine the velocity and direction of flow. Higher reflected frequency means the blood is flowing toward the transducer (red) and lower reflected frequency means the blood is flowing away from the transducer (blue), Figure 2.4.

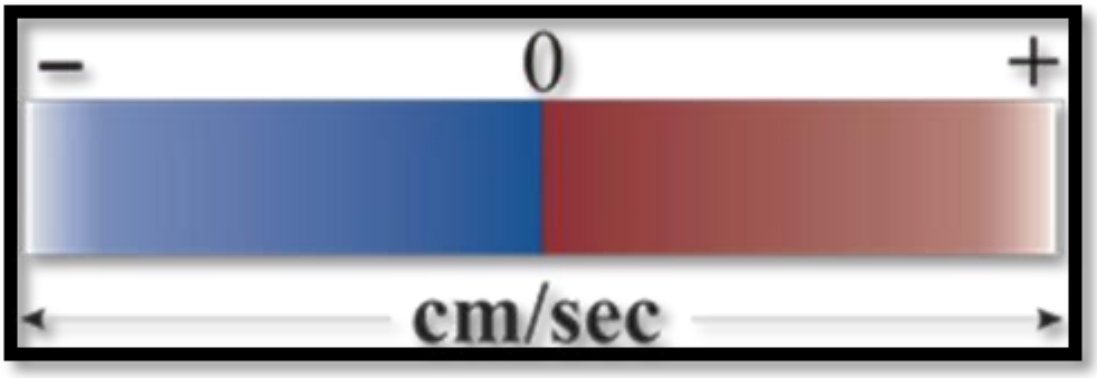

Figure 2-4. Two colour doppler ultrasound.

Blood velocity can be calculated from the following formula:

$$v = \frac{\Delta f \mathrm{C}}{2f \cos\theta}$$

Where v is the blood velocity, Δf is a frequency shift measured by the ultrasound machine, C is equal to (1540 m/sec) which is the velocity of sound in tissues, f is the transmitted frequency and θ is the angle between the direction of the flow and the sound wave (assumed to be 0º).

2.4.3 Doppler modes

Doppler ultrasound could be delivered as pulsed wave (PW) or continuous wave (CW). PW measures low flow at specific location while the CW measures fast flow. In PW doppler the transducer sends a pulse then waits for the sound reflection before sending another pulse. In CW doppler there are two transducers on the ultrasound probe, one to send the pulses and one to receive them with no ‘’listening’’ pause.

The waves returning from both Doppler modalities are separated to waves with positive shift (moving toward the transducer) and waves with negative shift (moving away from the transducer), Figure 2.5.

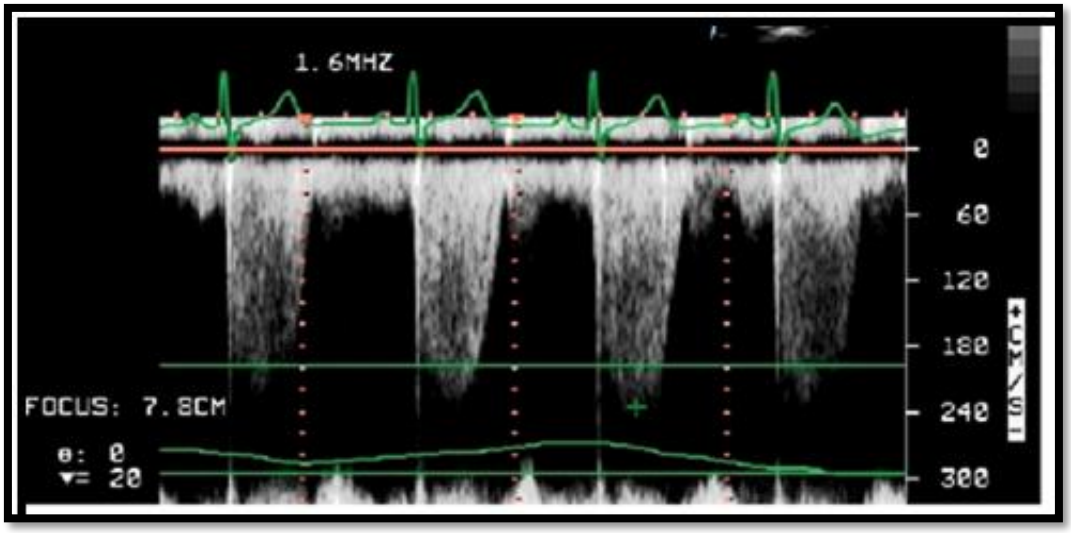

Figure 2-5. Spectral waveform of tricuspid regurgitation measured in continuous wave Doppler mode (CWD). Upper: electrocardiogram; Middle: Doppler waveform (frequency shift converted to positive and negative velocity); Lower: respiratory cycle. Green cross indicates peak velocity of tricuspid regurgitation.

2.4.4 Measurement

CARDIAC VIEWS

Three different cardiac views were used to assess pulmonary artery pressure (PAP) and cardiac output for the purpose of this thesis; four-chamber apical view, five-chamber apical view and parasternal long axis view.

The four-chamber apical view was used to measure the blood flow velocity across the tricuspid valve while the five-chamber apical view was used to measure the blood flow velocity across the aortic valve, Figure 2.6. The parasternal long axis view was used to measure the diameter of the aortic valve, Figure 2.7.

The apical view was obtained laterally from the 4th or 5th intercostal space with the participant being on the left side leaning slightly forward. The parasternal view was obtained from the 2nd or 3rd intercostal space lateral to the sternum with the participant being in the supine position.

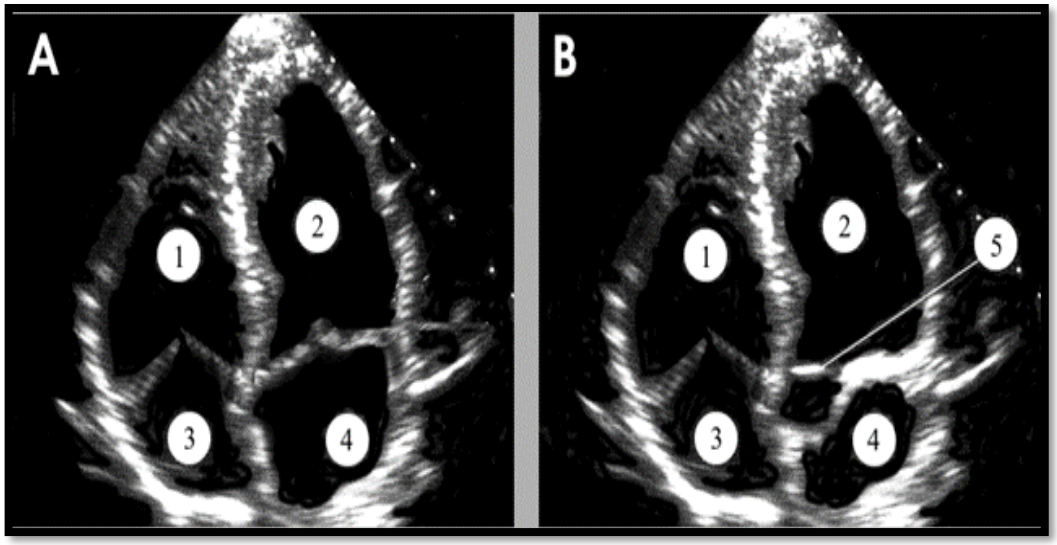

Figure 2-6. Apical 4-chamber (A) and 5-chamber (B) 2-D ultrasound images. Right ventricle (1), left ventricle (2), right atrium (3), left atrium (4), aortic valve (5).

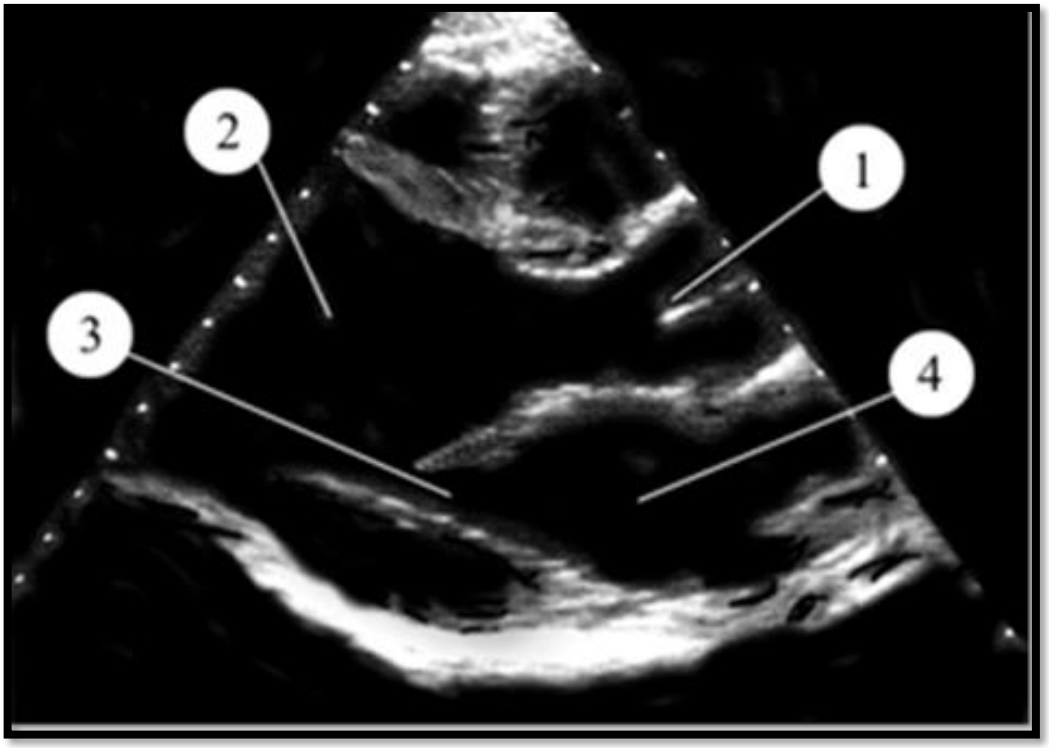

Figure 2-7. Parasternal long-axis view. Aortic valve (1), left ventricle (2), mitral valve (3), left atrium (4).

ESTIMATION OF PULMONARY PRESSURE FROM TRICUSPID REGURGITATION

Tricuspid regurgitation is a fast-flowing jet flowing from the right ventricle to the right atrium during systole with a velocity up to 2m/sec, and it is measured by CW doppler in the 4-chamber view, Figure 2.8.

Physiological tricuspid regurgitation is presented in about 75% of the population and it can be used to estimate pulmonary pressures in a non-invasive way. The peak velocity of the jet is measured at the most distal part of the waveform, Figure 2.5., then it is converted to peak pressure difference ($\Delta \mathrm{Pmax}$) between the

right ventricle and right atrium pressures (RAP) using the following modified Bernoulli's equation ($\Delta Pmax = 4v^2$) where $\Delta\mathrm{Pmax}$ is the pressure drop across the valve in mmHg and v is velocity.

Because right ventricular pressure during systole is equivalent to systolic pulmonary artery pressure (SPAP) the relationship can be shown as: $\Delta\mathrm{Pmax}$ = SPAP-RAP. As RAP is relatively constant at a low value of ~5 mmHg, SPAP is assumed to be $\Delta\mathrm{Pmax}$ +5 mmHg.

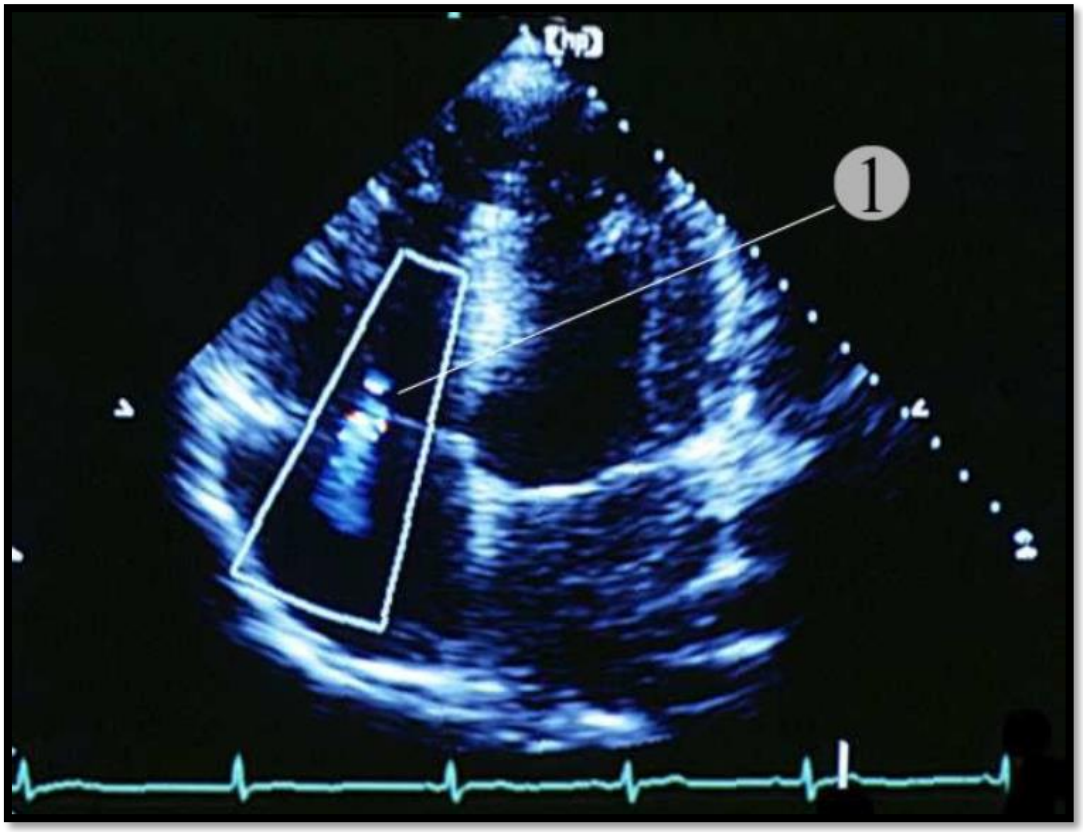

Figure 2-8. Tricuspid regurgitation (1) visualised in colour Doppler mode from an apical 4-chamber view.

CARDIAC OUTPUT

Two echocardiography measurements are required to calculate cardiac output using Doppler ultrasound. First, the velocity of blood flow through the aortic valve which is obtained from the 5-chamber view is needed, Figure 2.9.

This is used to calculate the velocity time integral (VTI), which is the distance that blood travels in the aorta with each ventricular contraction. To do that, PW Doppler is used with the sampling gate positioned at the aortic orifice and the beam aligned with the flow. The trace is then saved for offline analysis, Figure 2.10.

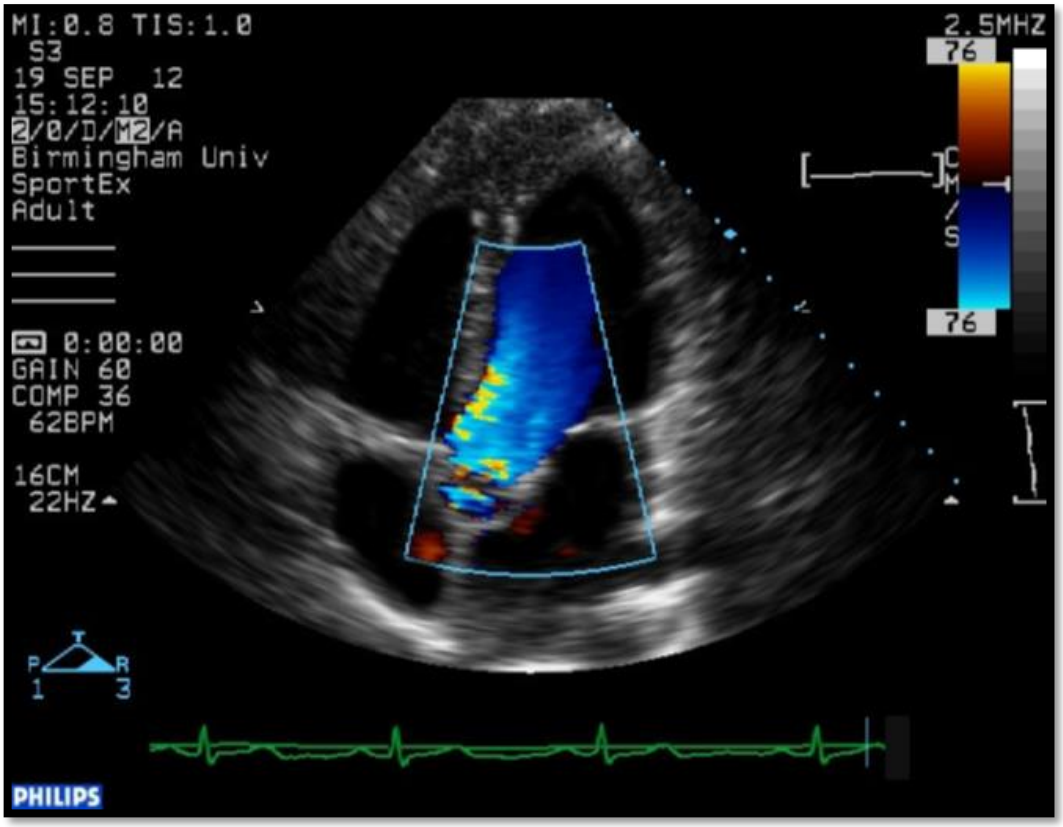

Figure 2-9. The flow of blood exiting the heart through the aortic valve during systole, visualised in colour Doppler mode from an apical 5-chamber view

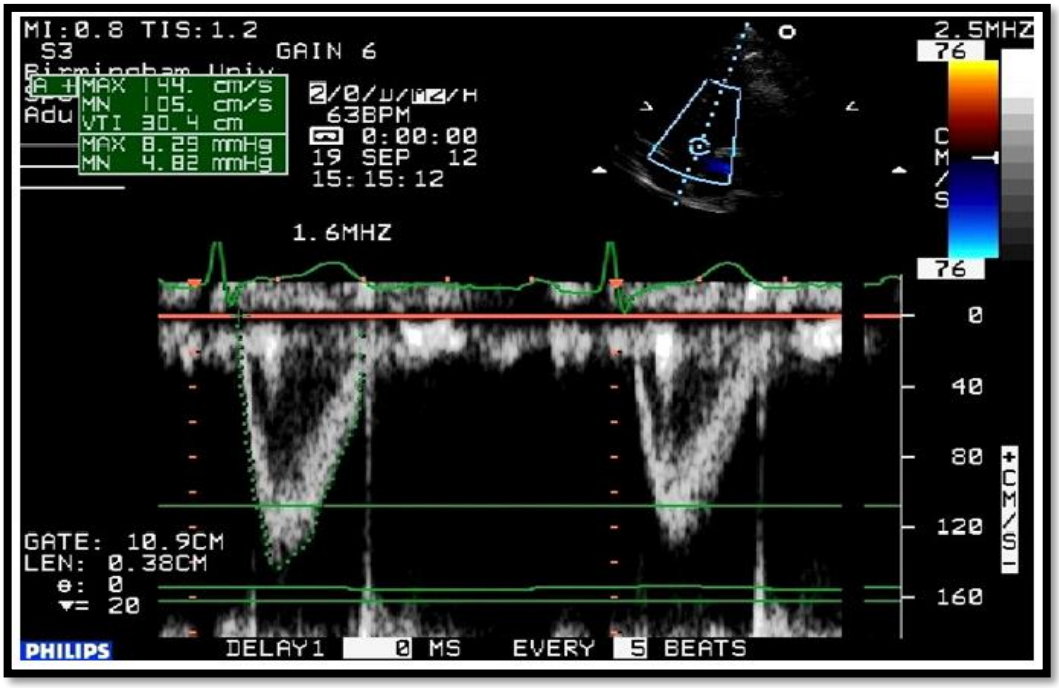

Figure 2-10. A PWD spectral trace of flow through the aortic valve. The mean velocity (VTI) is measured using the green trace.

Second, the diameter of the aortic valve orifice needs to be obtained from the parasternal view. Assuming the aortic valve orifice is a circle, the area of the valve (A) can be calculated using the following formula: πr^2, Figure 2.11.

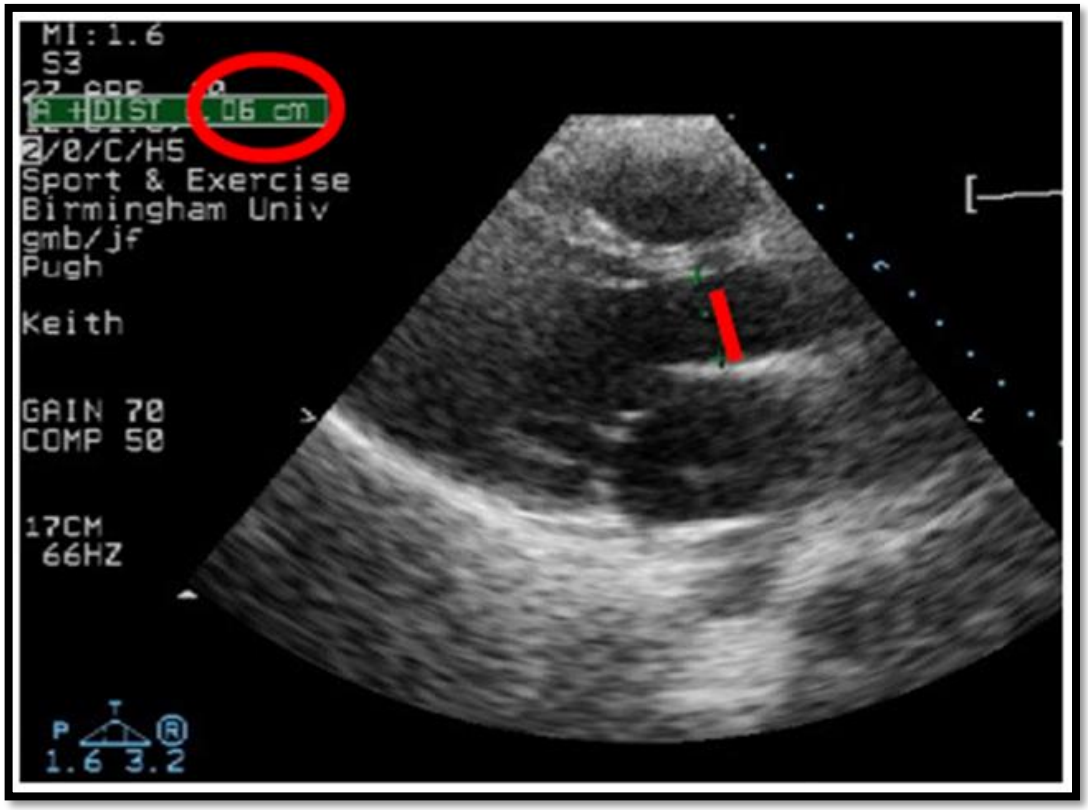

Figure 2-11. The aortic diameter from the parasternal view and the calculated area.

Cardiac output is then calculated with the following equation:

$$\dot{Cardiac\ output} = VTI \times A \times HR$$

2.5 POLYSOMNOGRAPHY

Polysomnography was used in two studies of this thesis. Both studies were nap studies which were performed in a specially designed sound proofed sleep laboratory with well-maintained temperature and light in the school of sport and exercise science.

2.5.1 Electrode Setup

In Chapter 5 the EEG was recorded using the 10-20 system while in Chapter 6 the easy cap was used. For both studies the brain activity was monitored through electroencephalograph (EEG), muscle tone through submental electromyography (EMG) and eye movement through electrooculography (EOG). The study protocol will be explained in each chapter along with the cognitive functions performed for each study.

The signals were amplified, filtered and montaged according to the American Academy of Sleep Medicine (AASM) manual for the scoring of sleep and associated events guidelines (Iber et al., 2007).

2.5.2 Sleep Scoring

Sleep was staged in 30 sec. epochs according to the AASM guidelines which classify sleep according to the criteria explained in the following sections.

STAGE WAKE (W)

Stage W ranges from full alertness to early stages of drowsiness. The epoch is scored as W when it has 50% or more alpha rhythm, which is a train of sinusoidal activity (8-13 Hz frequency) recorded over the occipital brain region when the eyes are closed. Eye movement tends to slow down as person progress to drowsiness and the chin EMG will show high amplitude.

STAGE ONE (N1)

This is the first stage of sleep, which is presented by a decreased alpha activity and increased low amplitude mixed frequency activity which consist more than 50% of the epoch. Other features of N1 include Vertex sharp waves less than 0.5 sec. over the central brain region, slow rolling eye movement and lower chin EMG activity compared to stage W, Figure 2.12.

STAGE TWO (N2)

N2 is characterised by the presence of K complex and sleep spindles. K complex is a sharp negative wave followed by a positive component which is equal to or more than 0.5 sec. in duration over the frontal brain region. Sleep spindles are train of waves with frequency of 11-16 Hz lasting for equal to or more than 0.5 sec. in duration over the central brain region. N2 has minimal and slow to none eye movement with low chin EMG activity, Figure 2.13.

STAGE THREE (N3)

N3 is the deep sleep which is characterised by slow brain frequency 0.5 to 2 Hz with high amplitude equal to or more than 75 μV over the frontal brain region which consist 20% or more of the epoch. N3 has no eye movement with very low to none chin EMG, Figure 2.14.

RAPID EYE MOVEMENT STAGE (REM)

REM is presented by saw tooth low amplitude mixed frequency EEG (2-6 Hz) over the central head region with low chin EMG and rapid eye movement, Figure 2.15. Table 2.1 will summarise the features of each sleep stage.

Stage		Criteria
Wake		More than 50% of the epoch is Alpha EEG wave (train of sinusoidal 8-13 Hz waves in the occipital trace appears when the eyes are closed). Alpha was noticeable in all participants
NREM	I	- Slow rolling eye movement - Low amplitude, mixed frequency EEG waves (4-7 Hz) - V waves (vertex sharp wave for less than 0.5 seconds on the central trace)
	II	- K complex (more than 0.5 seconds duration present in the frontal trace) - Sleep spindles (more than 0.5 seconds duration, 11-16 Hz, seen on the central trace)
	III (SWS)	Slow wave activity, 0.5-2 Hz frequency, with an amplitude more than 75μV seen in the frontal trace, seen in more than 20% of the epoch
REM		- Rapid eye movement - Low EMG tone - Sawtooth brain wave - Transient muscle activity

Table 2-1. Sleep Staging Criteria, based on the recommendation of AASM Manual for the Scoring of Sleep and Associated Events

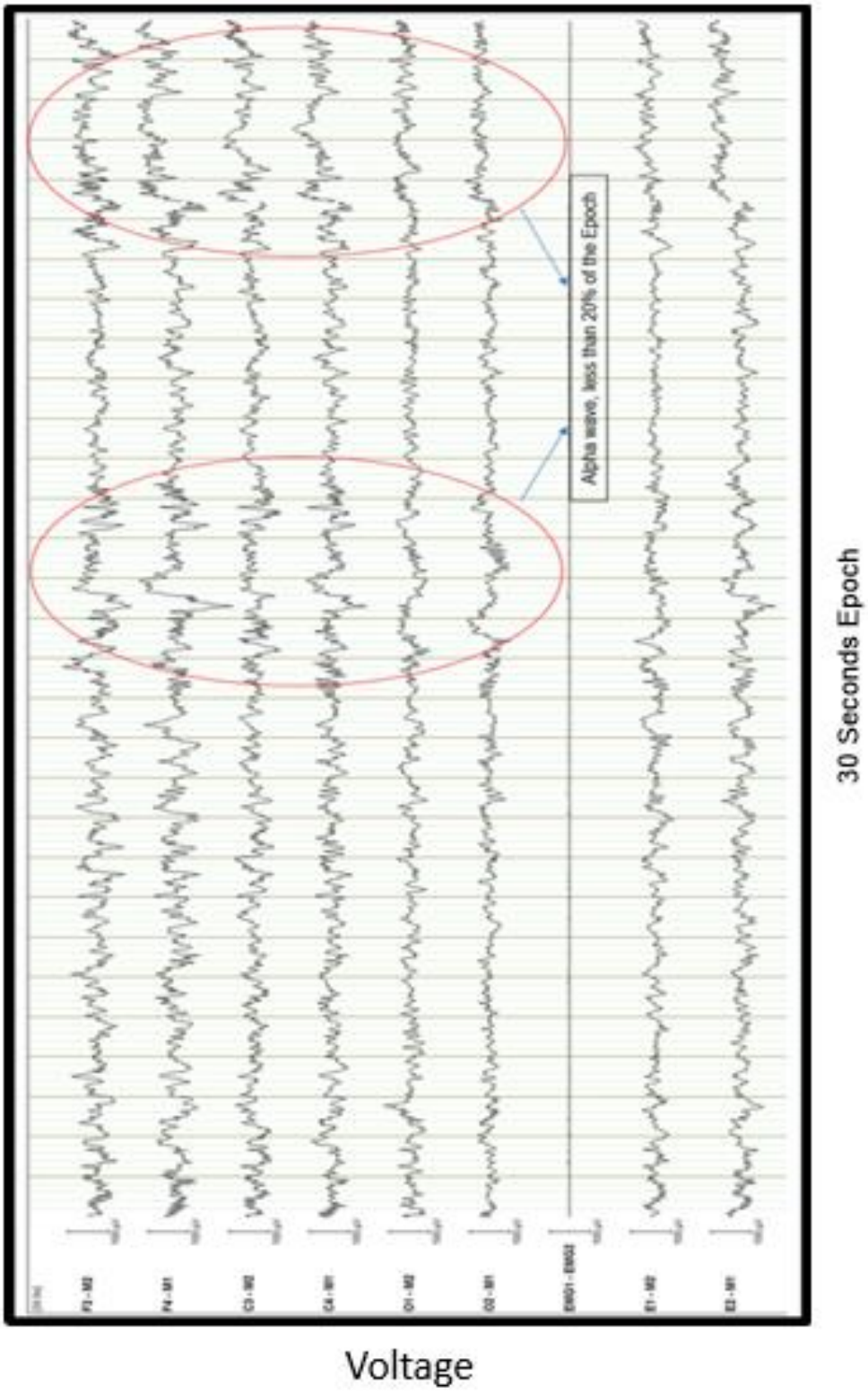

Figure 2-12. Sleep stage I showing attenuated Alpha activity and slow eye movement, from a participant in the experiment.

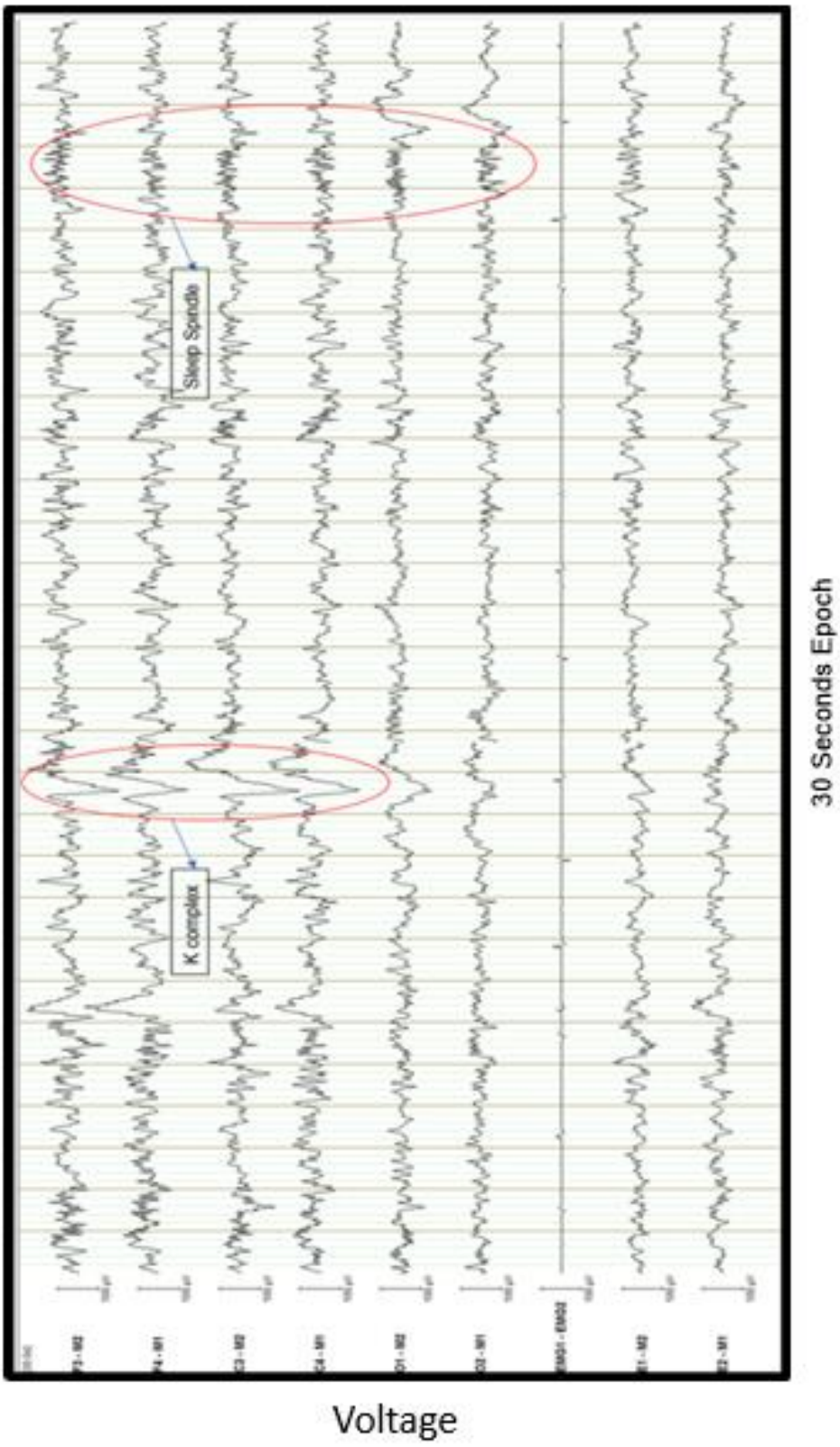

Figure 2-13. Sleep Stage II , with an example of a K complex and a Sleep Spindle, obtained from a participant in the experiment.

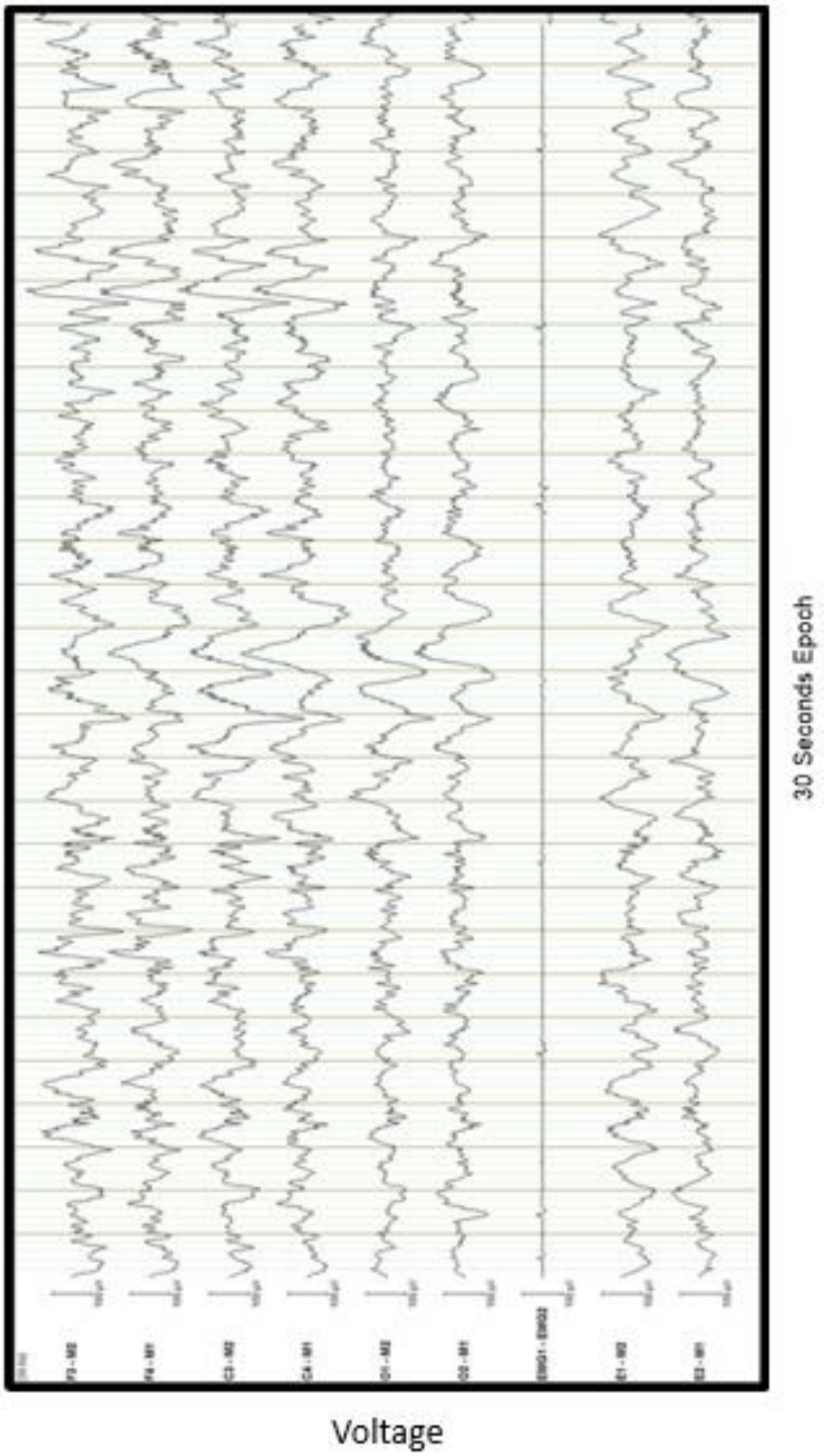

Figure 2-14. Sleep Stage III , showing the SWS pattern, obtained from a participant in the experiment.

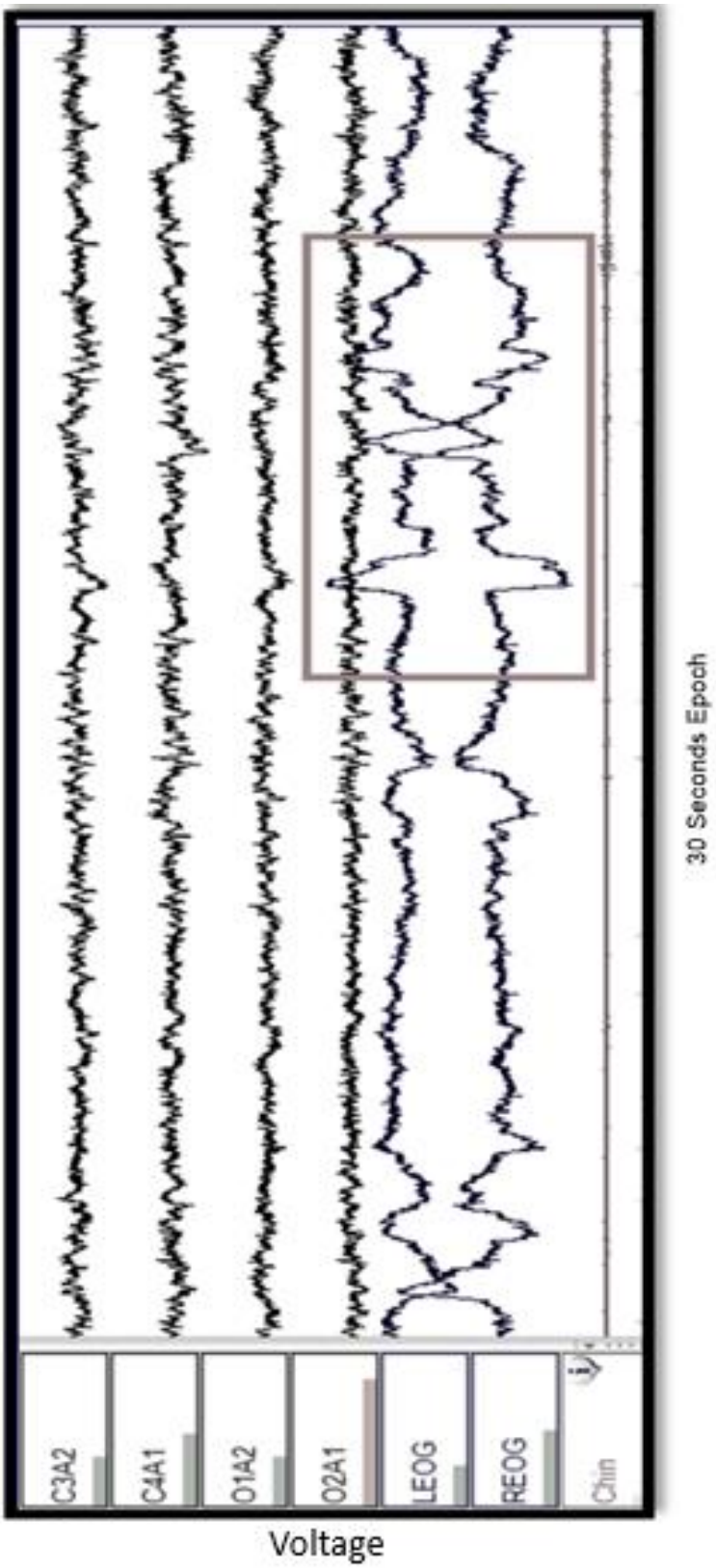

Figure 2-15. REM sleep , showing the rapid eye movement and the increased brain activity. A picture with open access.

2.5.3 Microarousals

Microarousals are defined as abrupt shift of EEG frequency to 16 Hz or more lasting for 3 seconds or more, which occur after stable sleep that lasted for at least 10 sec. This frequency shift is usually noticed over the central and occipital brain regions. In REM stage, this EEG frequency shift has to be accompanied by increase in chin EMG activity that last at least 1 sec. Microarousals don't cause the person to fully wake up, Figure 2.16.

Chapter 5 and 6 both utilised the microarousals, but each experiment used it in a different way and for a different reason. More details will be provided in each chapter.

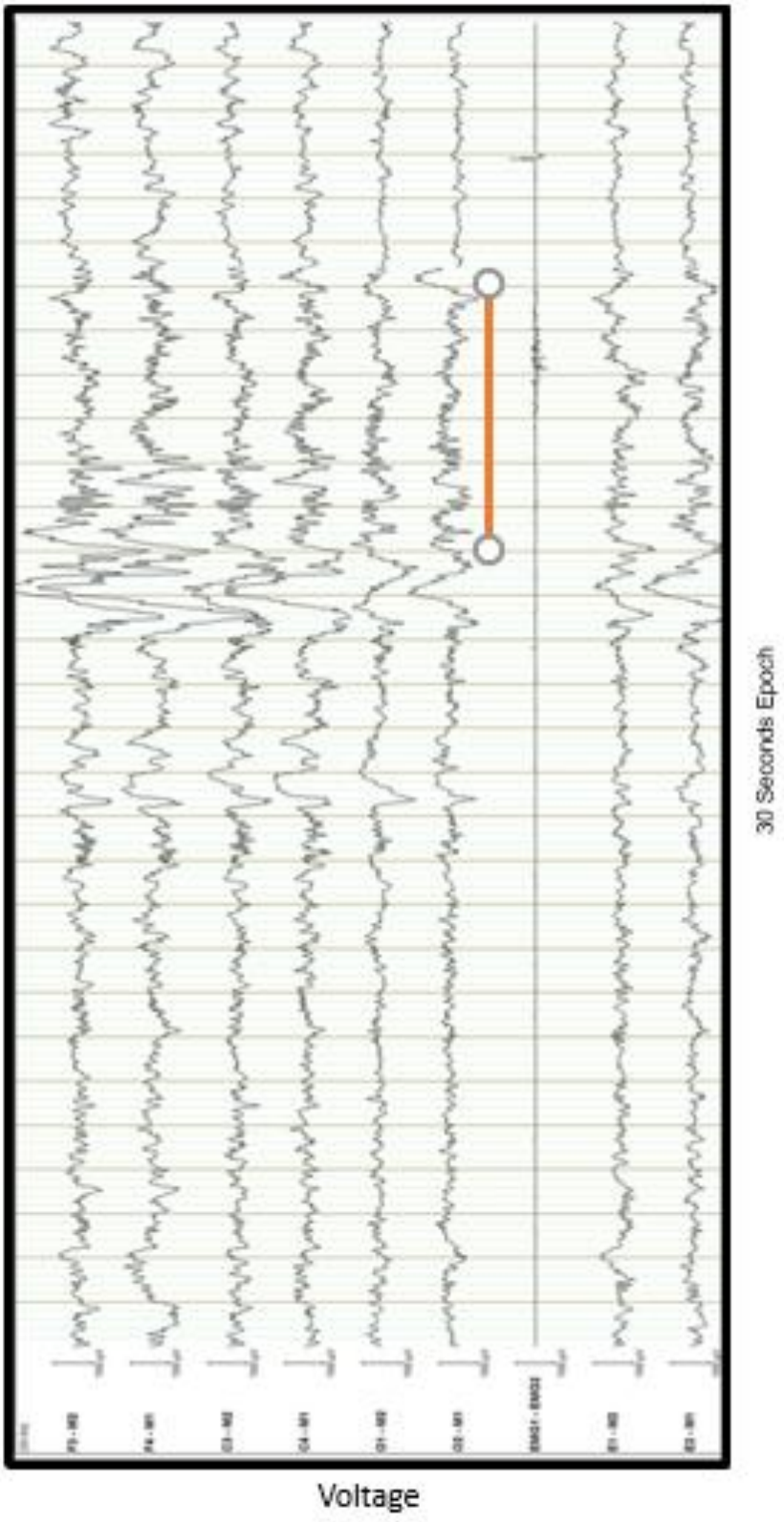

Figure 2-16. An example of microarousal. Obtained from a participant in the experiment.

3. LONG TERM FACILITATION OF THE GENIOGLOSUS REFLEX TO INTERMITTENT NEGATIVE AIRWAY PRESSURE

3.1 INTRODUCTION

3.1.1 Current treatment of OSA

As can be seen from the general introduction, the pathophysiology of OSA is a combination of anatomical and physiological abnormalities which exist with variable degrees from one patient to the other, so not all OSA patients have identical characteristics. Therefore, various treatment strategies are used to prevent the airway obstruction during sleep as weight loss and modification of sleeping position. Some pharyngeal exercises are also suggested as a supportive treatment.

Furthermore, several treatment modalities are used to alleviate the symptoms of OSA such as CPAP, mandibular advancement, and surgical treatment; yet none of these modalities is considered as an optimal strategy.

CPAP, which is the standard therapy for OSA is poorly tolerated by patients as reports show that 21-46% of CPAP users use it less than the recommended 4 h/night, 70% of the nights. Worse than that, 8-15% of patients refuse to start the treatment (Engleman and Wild, 2003, Weaver and Grunstein, 2008, Chai-Coetzer et

al., 2013). Several factors can affect the adherence to CPAP, with some of them showing a measurable correlation with adherence while others remain subjective.

For instance, patients with increased level of daytime sleepiness show better adherence to the therapy as they notice the improvement in their symptoms. Also patients who have improvement in the snoring level show more long term adherence to therapy (Weaver and Grunstein, 2008, Chai-Coetzer et al., 2013)

On the other hand, patients with small nasal airway are less likely to adhere to the therapy as the low diameter results in increased resistance to the flow of air. Furthermore, patient personality and their perception of the device affect their level of adherence. Claustrophobia for example reduces the adherence to treatment, which can be addressed by positive reinforcement and motivation. Moreover, the side effects from CPAP can result in less adherence. For example, the leak from the mask, the pressure sores, the noise and bulkiness of the mask and the device can all affect the adherence the therapy (Weaver and Grunstein, 2008, Broström et al., 2010).

3.1.2 Respiratory LTF

Although CPAP is an effective therapy for OSA, the benefits are associated with the use of the device. Any withdrawal of treatment, even for a short time show a rebound of the OSA complications (Young et al., 2013). The lack of treatment that can reverse the OSA process urges the search for other alternative way which could support or even replace the current treatments, for at least some of the patients.

Respiratory LTF is proposed as a possible pathway for the treatment of OSA. IH was used in several studies to trigger rLTF, but it is not widely used and not strongly supported due to the side effects of IH. Just recently, INAP was considered as a stimulus for rLTF, but it is not examined enough.

3.1.3 Reflex response to negative pressure

This section is an overview of the concept behind using INAP as a trigger for rLTF. The upper airway is subjected to negative pressure with each inspiration. This negative pressure activates the mechanoreceptors located on the walls of the upper airways (Ryan et al., 2001, Berry et al., 2003), which triggers a reflex response that increases the upper airway dilator muscle activity. This is a response which is mediated by several nerves, especially the supra laryngeal nerve (SLN) (Ryan et al., 2001, Eckert et al., 2010). This increase in the muscle activity during inspiration is found to maintain the upper airway patency in rats (Ryan et al., 2001) and humans (Horner et al., 1991, Mezzanotte et al., 1992, Tantucci et al., 1998, Fogel et al., 2001).

The genioglossus muscle (GG) is the most studied upper airway dilator muscle due to its substantial functional value in keeping the upper airway open and due to its anatomical advantages as it is large muscle which is easily accessible for reliable instrumentation and easy recording of the muscle activity (Mezzanotte et al., 1992, Jordan and White, 2008, Eckert et al., 2010). The GG muscle is a fan-shaped extrinsic tongue muscle, which is innervated by the hypoglossal nerve (cranial nerve XII). When activated, it depresses and protrudes the tongue, Figure 3.1.

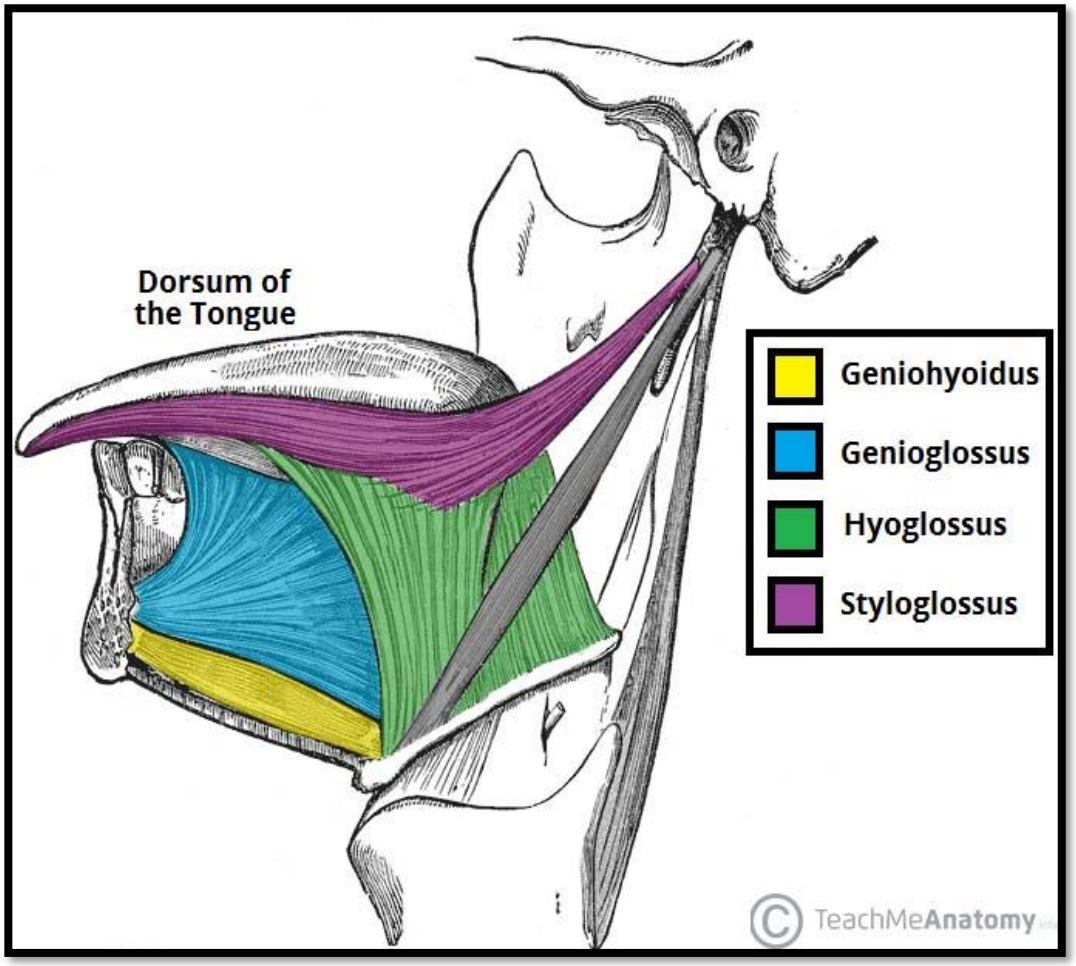

Figure 3-1. Anatomical illustration of the genioglossus muscle. Picture taken from https://teachmeanatomy.info/head/muscles/tongue/

Patients with OSA have the disadvantage of a narrow and compliant airway, which increases the risk of airway collapse during inspiration (Fogel et al., 2001, Dempsey et al., 2014). To maintain the airway patency, OSA patients have higher GG muscle activity compared to normal people. This is evident by higher GG EMG during wakefulness when OSA patients are compared to age and weight matched controls (Mezzanotte et al., 1992, Fogel et al., 2001, Berry et al., 2003). This increased activity while awake puts the muscle at risk of fatigue, which might have a

role in the pathophysiology of OSA and may explain the progressive increase in the apnoea/hypopnoea severity during the course of night (McSharry et al., 2012).

Another significant aspect in the pathophysiology of OSA is the well documented loss of respiratory neuromuscular responses to various stimuli at sleep onset, including the reduction of the upper airway reflex response to the negative upper airway pressure. This predisposes the airway to collapse, especially for OSA patients who rely on this reflex more than individuals with a normal airway (Mezzanotte et al., 1992, Horner et al., 1994, Fogel et al., 2001, Berry et al., 2003).

Despite this, patients with OSA are not exposed to airway collapse with each breath, indicating the presence of some protective mechanism. Long-term facilitation (LTF) of hypoglossal nerve activity has been considered as one of the naturally occurring protective mechanisms through the course of OSA (Fogel et al., 2001, Mateika and Syed, 2013). LTF of the hypoglossal nerve is a form of neural plasticity manifested as progressive and sustained increase in hypoglossal nerve discharge after exposure to an intermittent stimulus, an increase that is maintained after the stimulus is removed (Mateika and Sandhu, 2011).

This increase in the motor output of the hypoglossal nerve is linked to the activation of the muscles that protrude and retract the tongue, which aids in the establishment of airway patency (Fuller, 2005). LTF of GG activity through the increase in hypoglossal nerve activity has been observed in rats (Ryan et al., 2001, Fuller, 2005, Ryan and Nolan, 2009a), cats (Mateika and Fregosi, 1997), awake

humans (Harris et al., 2006, Griffin et al., 2012) and in sleeping humans (Chowdhuri et al., 2008).

3.1.4 Respiratory LTF by IH

Ironically, intermittent hypoxia (IH), which is part of the pathophysiology of OSA proved to trigger LTF and it is proposed to stabilise the OSA condition and to achieve respiratory homeostasis (Mateika and Komnenov, 2017). Several studies tried to induce rLTF through IH, but the effectiveness of the intervention was highly dependent on the selected protocol. Finding the right hypoxia severity, duration, and frequency of exposure which result in rLTF without causing side effects was challenging as the area between the benefits and adverse effects of IH is not well defined leading to a narrow therapeutic window (Navarrete-Opazo and Mitchell, 2014, Mateika et al., 2014).

On one hand IH can result in LTF with the right protocol, and on the other hand it is highly associated with increased blood pressure, increased pulmonary artery pressure, increased oxidative stress and impairment of cognitive function (Harris et al., 2006, Foster et al., 2007, Gerst et al., 2011, Mateika and Sandhu, 2011, Griffin et al., 2012).

3.1.5 Respiratory LTF by INAP

Each apnoea/hypopnea exposure is caused by complete or partial collapse of the airway. These events cause the repeated exposure to hypoxia, and at the same time they are associated with increased negative pressure below the site of airway collapse. This increase in the negative pressure enhances the reflex response of the upper airway dilators (Ryan and Nolan, 2009b).

This led to the assumption that exposing the airway to intermittent negative airway pressure (INAP) can result in LTF of the upper airway dilators in a similar way that IH does. Indeed, INAP resulted in LTF of the GG muscle response to negative pressure in spontaneously breathing anaesthetised rats (Ryan and Nolan, 2009a). This novel finding in rats which have genetic, biological and behavioural characteristics which closely resemble those of humans, raises the question whether humans may exhibit the same response to INAP. As mentioned previously, a study INAP has shown the ability to enhance the ventilatory response in healthy subjects (Griffin et al., 2017). To our knowledge, the effect of INAP on the hypoglossal nerve activity in humans was not investigated before.

3.2 AIM OF THE STUDY

The aim of this study was to determine if INAP can induce LTF of the GG muscle activity in healthy awake adults. The hypothesis was that exposing the upper airways to repeated bouts of INAP will augment the GG reflex response to negative pressure which will be reflected by an increase in EMG activity after the INAP

exposure when compared to control trial. A secondary aim is to evaluate the effect of INAP on HR and BP and compare it to the effect of IH.

3.3 METHODS

The study was approved by the local ethics committee (University of Birmingham Ethical Review Committee – ERN_14-0685). Subjects were recruited via emails, posters and word of mouth. After receiving detailed information about the experiment and the potential adverse effects of the study, an informed written consent was obtained from all participants.

3.3.1 Participants

Nine participants successfully completed the experiment protocol, seven males and two females. Their mean age was 26 years ± 8, mean BMI was 23 kg/m² ± 2, and mean collar size was 36 cm ± 2.

All participants were healthy, non-smokers, and had no documented history of respiratory, cardiac, sleep or upper airway diseases. Participants were excluded if they had false teeth or had significant facial injuries in the past. They were all instructed to abstain from moderate and vigorous exercise and from alcohol intake for 24 h, and from caffeine intake for 12 h prior to the experiment. They were also asked to have a light meal ~3 hours prior to the start of the study.

3.3.2 Study Design

This was a crossover study, so each participant visited the laboratory three times. The first visit was a familiarisation visit and the following two were the experimental sessions (control and intervention) which were conducted in random order with one-week gap.

During the familiarisation session, participants were oriented to the study instrumentation and the negative pressure protocol. Participants were asked to lie on a bed and breathe freely through a comfortable nasal mask. They were then exposed to intermittent negative airway pressure via the negative pressure system described in the General Methods Chapter for 15 minutes.

Participants who felt comfortable with the study design and decided to proceed signed an informed consent, filled a general health questionnaire and completed a dental referral form. They were then given an appointment with the dentist who took an individualised dental impression of the lower jaw for the participants. Each participant had a custom-made gum shield from that impression, which was used as the electrode carrier as will be described later in this chapter. These electrodes were used to measure the activity of the GG muscle.

During the intervention (INAP visit), each participant tried the electrode carrier and it was adjusted for their comfort. The participant was then positioned on a bed wearing a nasal mask which was described in the General Methods Chapter. Participants were breathing at ambient pressure for the first 10 minutes and a

baseline blood pressure was recorded at the end of this period. Following this, the electrode carrier was fitted, and participants were exposed to 15 minutes of intermittent negative pressure (-15 cmH_2O) through the system described earlier in the General Methods Chapter.

Each negative pressure exposure was started at the beginning of inspiration and lasted for two full breaths. The exposures were separated by breathing ambient pressure for 1 minute, and they were used to establish the baseline EMG response to negative pressure. Once the baseline response of the GG to the negative pressure was established, the electrode carrier was removed, and participants were exposed to 1 h of intermittent negative airway pressure, breathing at repeated cycles of -15 cmH_2O pressure for 30 seconds and ambient pressure for 1 minute.

Following the last exposure to negative pressure, participants went through 1 hour of recovery during which the electrode carrier was placed back in the mouth and the GG response to negative pressure was measured immediately after the intervention, at 30 minutes of recovery and at 60 minutes of recovery. Negative pressure was delivered in an identical fashion to that used during the baseline exposures, and the blood pressure was measured before each negative pressure exposure at the three recovery points.

The electrode carrier was removed between exposures for comfort. Participants were watching TV with noise isolating headphones during the experiment. The protocol is illustrated in Figure 3.2.

The Control visit was identical to the intervention visit, except that the one-hour of negative pressure was replaced by one hour of breathing at ambient pressure through the nasal mask. The vacuum pump was turned on during the control visit without delivering the INAP to establish the same testing environment.

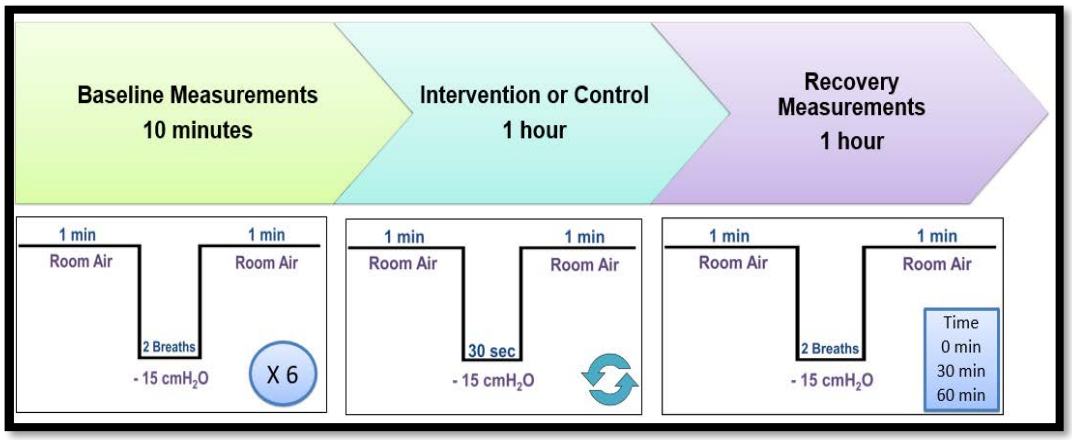

Figure 3-2. A schematic illustration of experiment protocol.

3.3.3 GG EMG activity

A pair of silver electrodes were used to measure the activity of the GG muscle utilizing the design from (O'Connor et al., 2007) which will be elaborated on further in this section. A custom-made gum shield was used as the electrodes carrier which held the surface EMG electrodes in contact with the floor of the mouth mucosa during measurements. The electrodes were sewed to the base of the gum shield and it had an exposed uninsulated part which was resting on the mouth mucosa, Figure 3.3.

Six participants were tested while the electrodes were unilaterally aligned, based on the improved surface EMG electrode design for measuring genioglossus muscle activity described by (O'Connor et al., 2007). In this design, two 5 mm long silver electrodes were placed unilaterally and were separated by 5 mm. The electrodes were positioned perpendicular to the GG muscle fibres, Figure 3.4 b.

Three other participants were tested using a bilateral arrangement of the electrodes which is an older design described by (Doble et al., 1985). This was the most used design for the non-invasive measurement of GG EMG activity, but it was critiqued by (O'Connor et al., 2007). In the bilateral design, two 10 mm long electrodes were placed, each in one side of the electrode carrier and were positioned parallel to the GG muscle fibres, Figure 3.4 a. In addition to the electrodes position, the older design used of stainless-steel electrodes, but the design was used with silver electrodes. The electrodes were placed 20 mm away from the lower incisors in both designs.

A reference electrode was placed on the participant's forehead, and the EMG signal was amplified to 10,000x. The signals were recorded by Spike2 for offline analysis.

Figure 3-3. The costume made gum shield with the unilateral electrode's alignment.

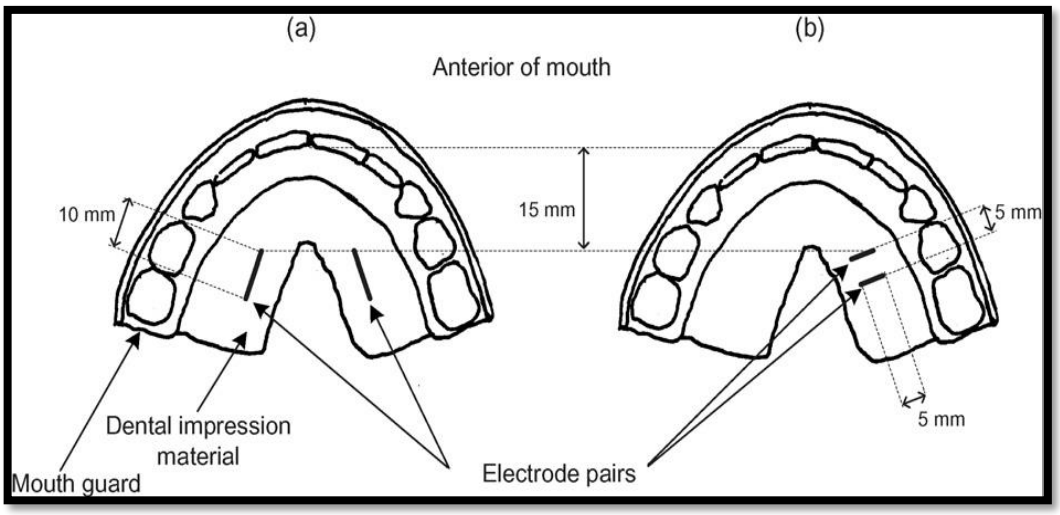

Figure 3-4. Schematic diagram of the two electrode configurations for GG EMG. (a) Bilateral electrode (b) Unilateral electrode configuration. Illustration taken from (O'Connor et al., 2007) with permission.

3.3.4 Maximum Voluntary Contraction (MVC) of the Tongue

MVC of the tongue was measured before and after both the INAP and control period and the difference were then calculated offline to examine the effect of each trial on the tongue force to see if the INAP leads to muscle fatigue.

The measurement was conducted by a force transducer apparatus which was fixed to a bench and aligned with the participant mouth, while setting upright and facing the unit. Each participant was positioned comfortably with their mouth around a soft bite pad and was asked to push the transducer button with their tongue to record the MVC. The resultant force was displayed live on a computer monitor placed in front of the participants to provide visual feedback, Figure 3.5. The measurement was repeated with one-minute rest after each trial until 3 readings within 5% of each other were achieved, and the highest value was recorded as the tongue MVC.

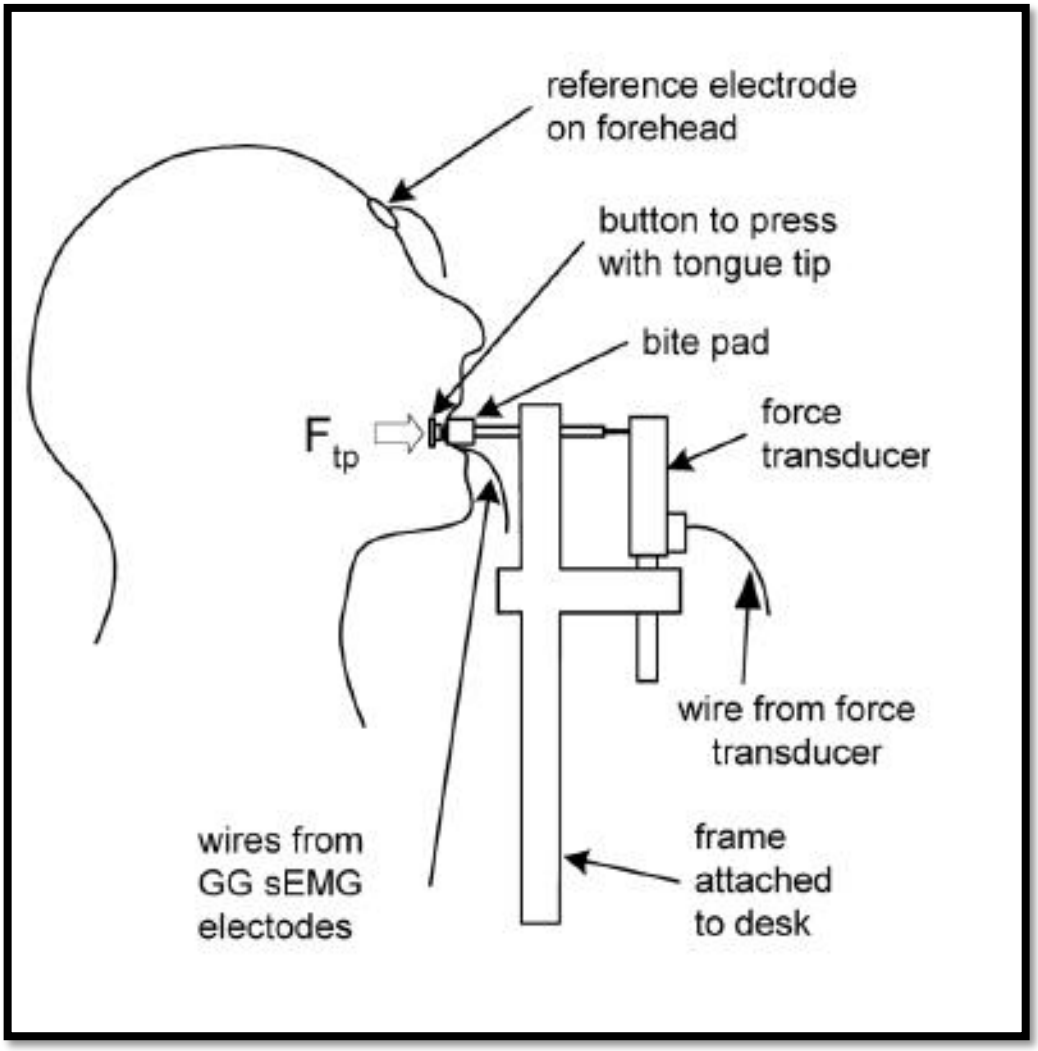

Figure 3-5. MVC Measurement Apparatus. Illustration taken from (O'Connor et al., 2007) with permission.

3.3.5 Data Processing

The genioglossus surface EMG signal was then amplified and filtered (amplification: 10,000, filtered: 1-450 Hz) using a pre-amplifier (Bagnoli-2, Delysys, USA). Signals were recorded at 4000 Hz using the computer-based signal acquisition system Spike2 software (CED, Cambridge, England) for offline analysis.

To find out the magnitude of the GG EMG response, the EMG signal was rectified, and the *baseline GG activity* was measured (which is the activity during expiration taken 80 ms before INAP initiation), and the *peak GG activity* (taken within 80 ms of the negative pressure application to avoid any voluntary activity). This is because the voluntary GG activity typically takes 150-230 ms post stimulus to be initiated. This will typically result in readings that reflect the GG reflex response to INAP (Horner et al., 1991, Horner et al., 1994). The magnitude of the response was calculated as the % change of peak activity from baseline activity.

3.3.6 Statistical Analysis

A two-way analysis of variance (ANOVA) with repeated measures was used to assess the effect of INAP on EMG activity, HR and BP when compared to control at different time points. A post hoc Bonferroni adjustment was used if required. A non-parametric Wilcoxon signed-rank test was used to assess the effect of INAP on the MVC of the tongue. Differences were considered significant if $P \leq 0.05$, and data were expressed as mean ± SD. Statistical analysis was performed using SPSS version 23. Although not ideal way to check the statistical power, a post hoc power

analysis was carried using G*Power version 3.1.9.2 because it can guide the data generalisation process as will be elaborated on during the discussion of the results (Faul et al., 2007).

3.4 RESULTS

3.4.1 Effect of INAP on Baseline and Magnitude of Change in EMG Activity

A two-way mixed design ANOVA was used to determine the effect of protocol on the EMG (the dependent variable), with the time being the repeated measure within subjects' factor (baseline, immediate recovery, 30 min. recovery and 60 min. recovery) and the intervention was the between subjects' factor (INAP/Control). Data showed normality as assessed by the Shapiro-Wilk test of normality, had no outliers as assessed by no studentized residuals greater than ± 3 standard deviations and there was sphericity for the interaction term as assessed by Mauchly's test of sphericity ($p > .05$).

Opposing to the initial hypothesis, there was no statistically significant two-way interaction between the protocol and time, $F(3, 24) = 0.258$, $p = 0.85$. Mean change in GG EMG activity was nearly identical across the time points in the control and the INAP trials Figure 3.6 and Table 3.1.

	% Change in EMG Activity in INAP	% Change in EMG Activity in Control
Baseline	72.65 ± 14.89	74.56 ± 11.00
Immediate Recovery	74.94 ± 17.65	73.14 ± 18.07
30 m Recovery	75.57 ± 16.77	76.48 ± 12.41
60 m Recovery	77.16 ± 16.73	76.81 ± 16.40

Table 3-1. The mean values of the change in GG EMG activity ± SD during baseline and 3 recovery time points in both INAP and control.

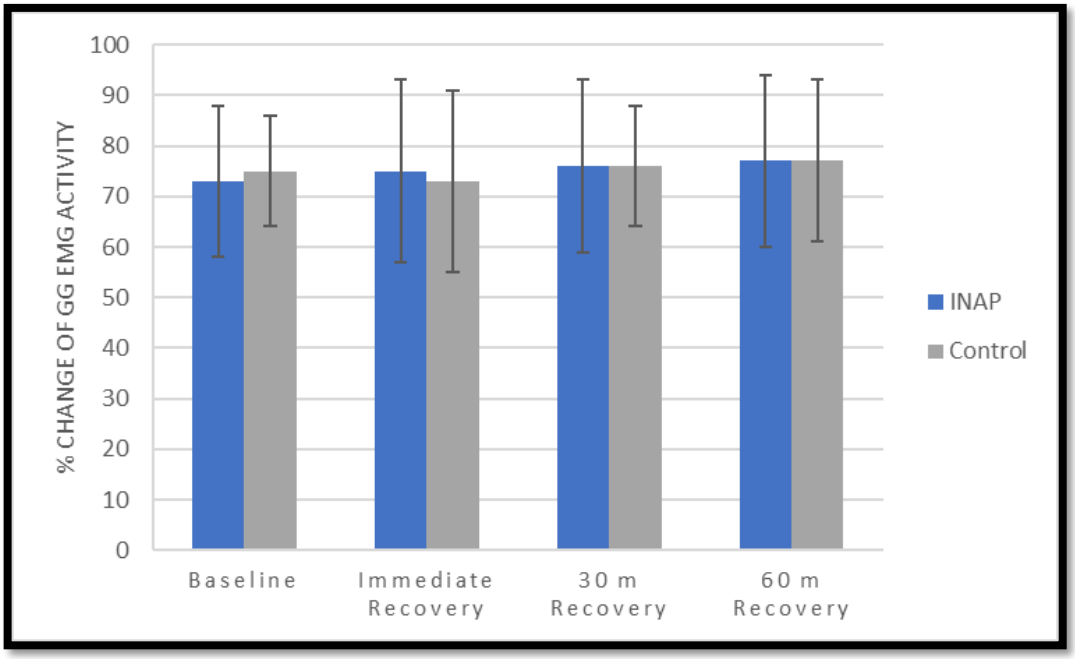

Figure 3-6. Magnitude of Change of GG EMG activity at baseline, Immediate Recovery, 30 m Recovery and 60 m Recovery.

This finding was also true for the tonic GG EMG activity, as there was no significant difference in the tonic GG EMG value between the protocols at all time points; $F(3, 24) = 0.48$, $p = 0.69$. Figure 3.7 and Table 3.2.

	Tonic GG EMG Value during INAP (μV)	Tonic GG EMG Value during Control (μV)
Baseline	0.07 ± 0.03	0.07 ± 0.03
Immediate Recovery	0.06 ± 0.03	0.07 ± 0.03
30 m Recovery	0.06 ± 0.02	0.06 ± 0.02
60 m Recovery	0.06 ± 0.02	0.07 ± 0.03

Table 3-2. The mean values of the tonic GG EMG activity ± SD during baseline and 3 recovery time points in both INAP and control.

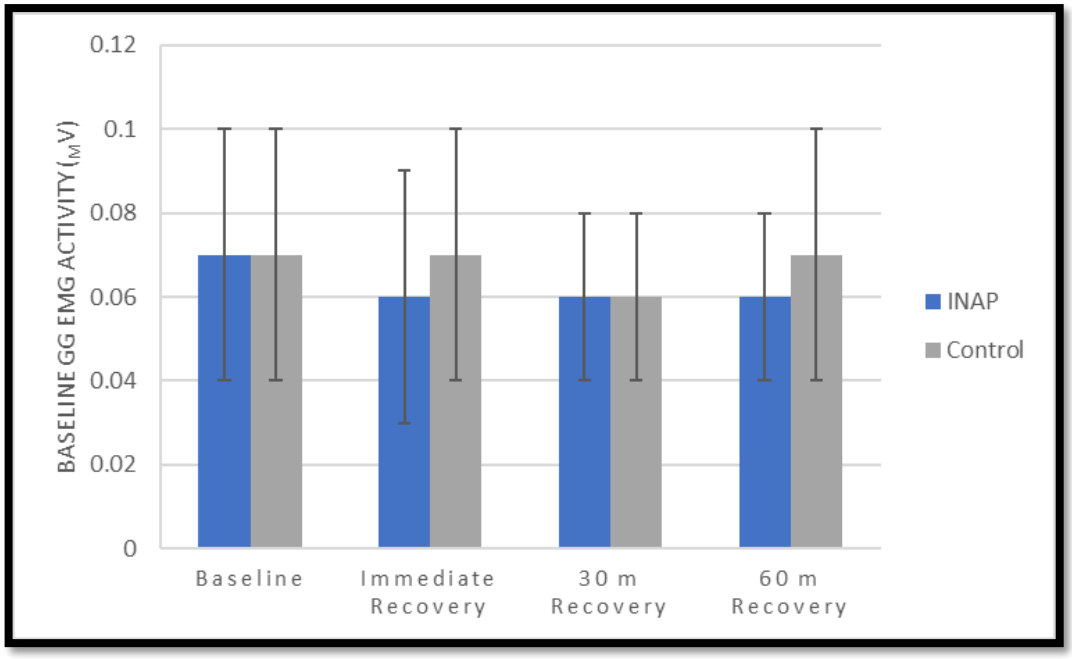

Figure 3-7. Tonic GG EMG activity at baseline, Immediate Recovery, 30 m Recovery and 60 m Recovery.

3.4.2 Effect of INAP on the MVC of the Tongue

A non-parametric Wilcoxon signed-rank test was performed to examine the effect of INAP on the of MVC activity during baseline and recovery when compared to control protocol. This test was used as the data was not normally distributed and it had outliers.

The results indicate that INAP caused a median increase in MVC activity (Mdn = 0.56 newton), which is more than the increase caused by control protocol (Mdn = 0.19 newton); a median difference of 0.23 newton; which is a not statistically significant change p = 0.76. Figure 3.8. shows the mean force before and after the protocol for both control and INAP.

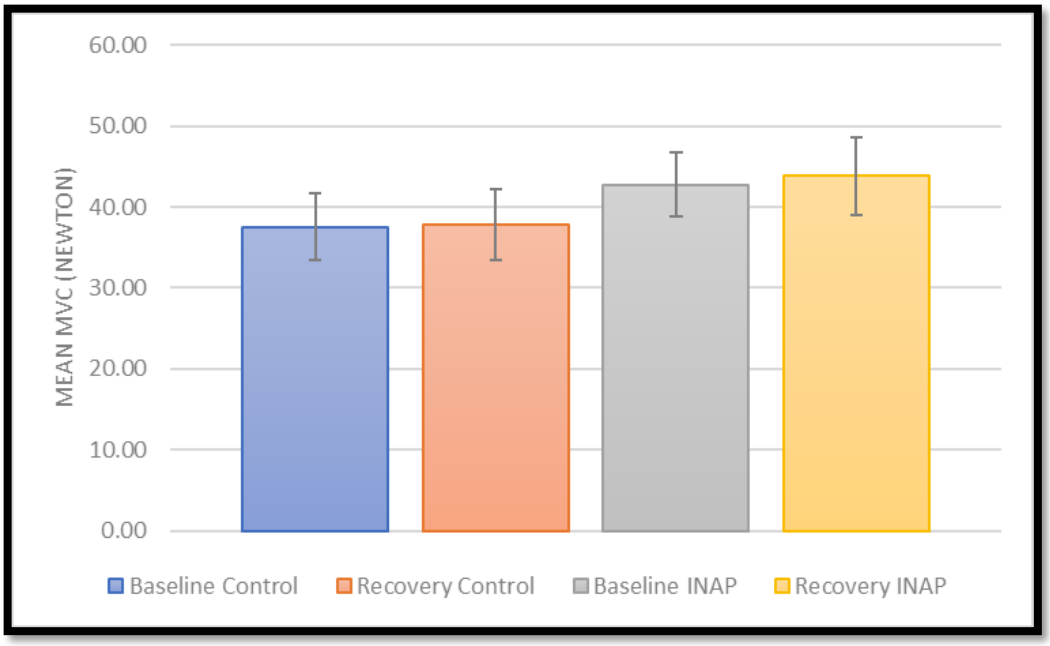

Figure 3-8. Mean MCV values at baseline and recovery in INAP and control.

3.4.3 Effect of INAP on Heart Rate (HR)

A two-way mixed design (ANOVA) with repeated measures was used to show the effect of INAP compared to control on HR (dependent variable). Time was the repeated measure within subjects' factor (baseline, immediate recovery, 30 min. recovery and 60 min. recovery) and the intervention was the between subjects' factor (INAP/Control). There was no statistically significant two-way interaction between the protocols at the different time points on HR, $F(3, 24) = 1.156$, $p = 0.347$. The results are presented in Figure 3.9 and Table 3.3.

	INAP beat/min	**Control beat/min**
Baseline	64.22 ± 12.22	61.78 ± 13.80
Immediate Recovery	58.89 ± 8.13	55.22 ± 9.63
30 m Recovery	59.33 ± 11.93	57.56 ± 12.90
60 m Recovery	61.56 ± 12.32	62.89 ± 15.46

Table 3-3. Mean HR beat/min ± SD in both INAP and control across the different time points.

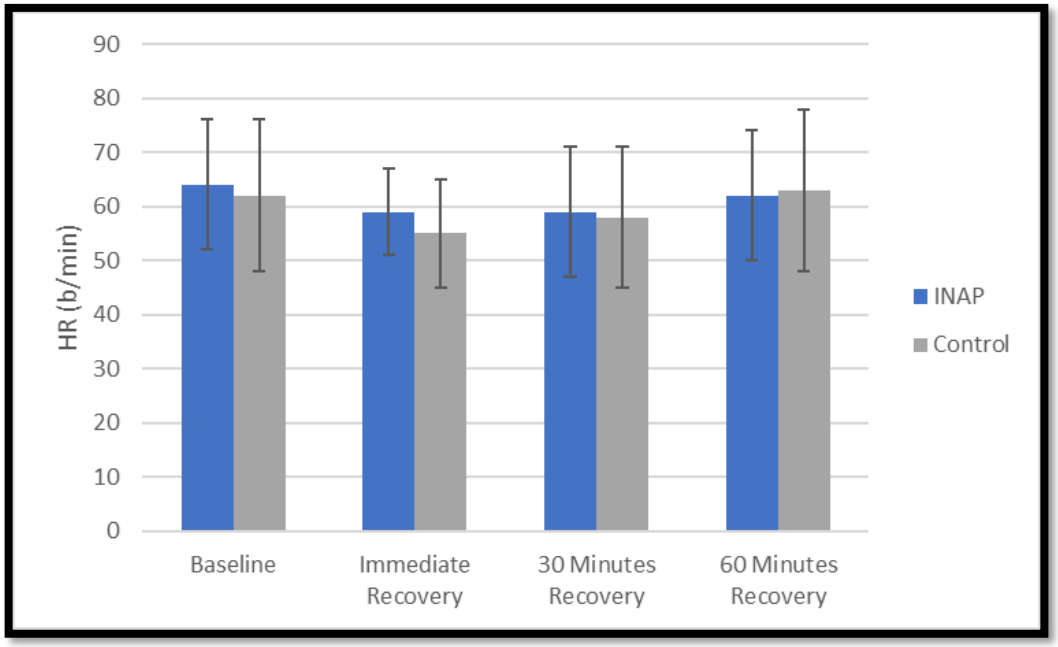

Figure 3-9. Mean HR beat/min in both INAP and control across different time points.

There was no difference in the simple main effect between the INAP and control trials on HR, $p = 0.4$. On the other hand, there was a statistically significant effect of time on HR, $F(3, 24) = 3.574$, $p = 0.029$.

Post hoc tests using the Bonferroni correction revealed a significant decrease in HR in the immediate recovery point in both groups $p = 0.04$, a finding which was not presented in the other time points as HR went to values similar to baseline on the 30 minutes and 60 minutes recovery.

3.4.4 Effect of INAP on Mean Arterial Blood Pressure (MABP)

The same mixed design ANOVA was used to determine the effect of protocol on the MABP (the dependent variable), with the time being the repeated measure within subjects' factor (baseline, immediate recovery, 30 min. recovery and 60 min. recovery) and the intervention is between subjects' factor (INAP/Control). There was no statistically significant two-way interaction between intervention and time on MABP, $F(3, 24) = 0.211$, $p = 0.888$. Furthermore, there was no simple main effect on BP neither for time $F(3, 24) = 1.294$, $p = 0.299$ nor for trial $F(1, 8) = 3.055$, $p = 0.119$. The effect of INAP compared to control on MABP is presented in Figure 3.10 and Table 3.4.

	INAP mmHg	**Control mmHg**
Baseline	87.44 ± 3.28	85.89 ± 6.17
Immediate Recovery	87.78 ± 7.12	86.44 ± 7.80
30 m Recovery	88.67 ± 4.41	86.11 ± 6.81
60 m Recovery	90.22 ± 6.11	87.89 ± 7.69

Table 3-4. MABP mmHg ± SD in both INAP and control across different time points.

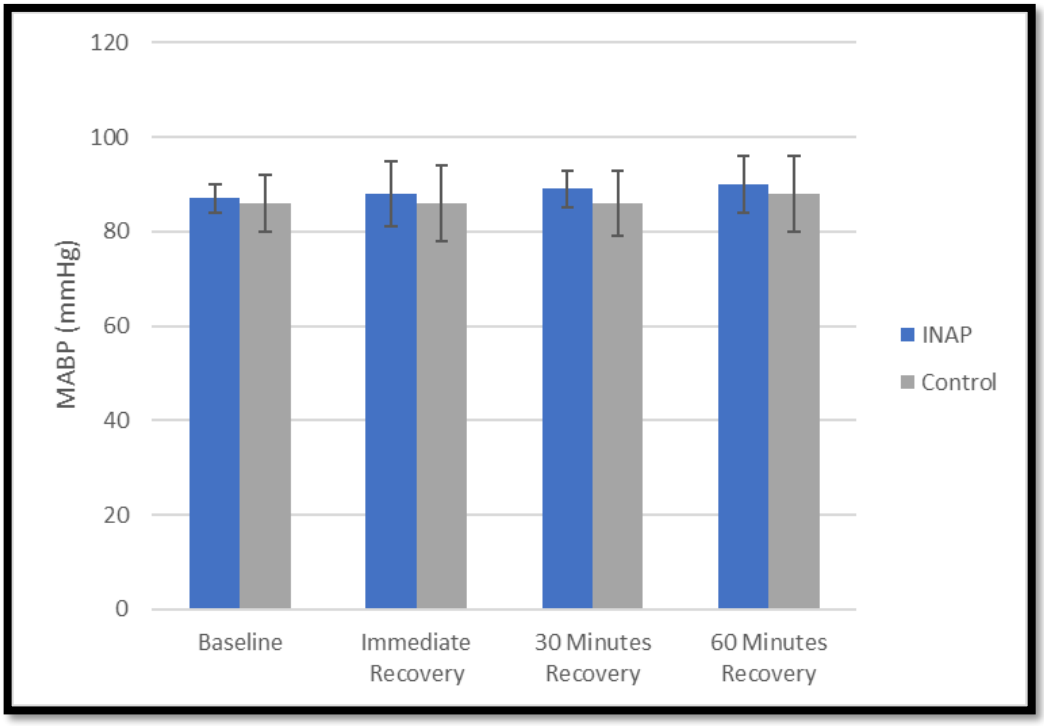

Figure 3-10. MABP mmHg in both INAP and control across different time points.

3.5 DISCUSSION:

The main aim of this study was to find out if INAP can lead to LTF of GG muscle activity in awake healthy participants. Contrary to the initial hypothesis, one hour of INAP did not result in LTF of the GG muscle activity when compared to one hour of control. The GG muscle response to the negative pressure was almost identical across all time points in both conditions. Furthermore, the tonic muscle activity stayed constant in both trials across all time points. The secondary aim was to see the effect of INAP protocol on HR and BP. The study found that INAP did not have an effect on HR or BP when compared to control.

The use of INAP for LTF is still in its early stages as it is supported by two studies so far, and one of them is published as an abstract (Ryan and Nolan, 2009b, Griffin et al., 2017). Although the data of the current study did not show rLTF after one hour of INAP, the effect might still exist but needs some changes to the study protocol to be revealed as will be discussed later in the discussion. This believe is emphasised by a post hoc statistical power analysis which was conducted with G*Power program. The analysis showed that the current study is underpowered, with a power estimate of 0.67 due to small sample size and effect size. This increased the chance of type II error during the hypothesis testing, which is defined as ‘’failure to reject a false null hypothesis’’. For the same reason, the findings of the INAP effect on the BP and HR should be considered with caution until it is confirmed with a larger scale studies.

In contrast to INAP, intermittent hypoxia (IH) is a well-established trigger for LTF, which have resulted in increased respiratory motor output that can last for up to 90 minutes after the restoration of normal oxygenation (Mateika and Narwani, 2009). The use of IH for LTF to increase the ventilatory output and enhance the activity of the upper airway dilator muscles has been studied for decades until a fair understanding of its mechanism was established and the right protocol was proposed, and yet the use of IH for rLTF is not optimal (Baker and Mitchell, 2000, Aboubakr et al., 2001, Mitchell et al., 2001, Jordan et al., 2002, Babcock et al., 2003, Fuller, 2005, Harris et al., 2006, Ryan and Nolan, 2009a, Xing et al., 2013, Mateika

and Syed, 2013, Navarrete-Opazo and Mitchell, 2014, Lipford et al., 2016, Zou et al., 2018).

The following sections will discuss the proposed mechanism of action for IH and INAP induced rLTF, tracking the similarities and the differences. It will also discuss the possible limitations of the methods used in this experiment which could have affected the results. Furthermore, it will propose modifications to the protocol used in this study to guide the future research about INAP induced rLTF.

3.5.1 Serotonin Dependent Mechanism of LTF

OVERVIEW

Serotonin (5-HT) is a neurotransmitter and neuromodulator that has a major role in the initiation of LTF. The serotonergic neurons (mainly found in the medullary raphe and ventrolateral medulla) release 5-HT, which has an excitatory effect on respiration (Sood et al., 2003, Lipford et al., 2016). Pre-treatment with serotonin receptor antagonist (*methysergide)* led to lack of LTF(Ling et al., 2001), proving the importance of these receptors. IH has been shown to increase the release of 5-HT, which in turn leads to the excitatory effect of the phrenic motor neurons (Baker and Mitchell, 2000, Xing et al., 2013) and hypoglossal nerve (Xing et al., 2013). The mechanism will be discussed in the following section.

PHRENIC MOTOR NEURONS LTF

Figure 3.11. shows the proposed mechanism of phrenic long-term facilitation (pLTF) where IH triggers the release of serotonin in the spinal cord, which then activates 5-HT_2 receptors on the phrenic motor neurons. This triggers a signalling cascade that stimulates synthesis of new proteins, such as the Brain-derived neurotrophic factor BDNF protein. The new proteins are proposed to increase the phrenic motor neurons synaptic efficacy and enhance their motor output (Baker-Herman and Mitchell, 2002, Baker-Herman et al., 2004).

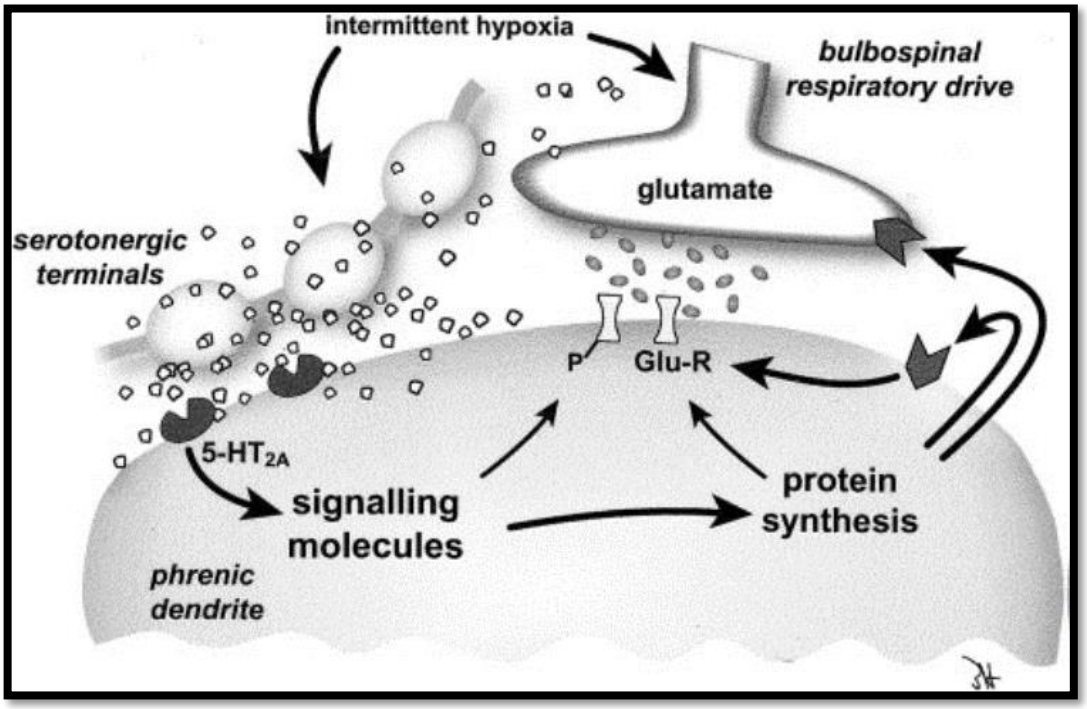

Figure 3-11. Proposed mechanism of phrenic long-term facilitation. Taken from (Baker-Herman and Mitchell, 2002) with permission.

HYPOGLOSSAL NERVE LTF

The hypoglossal nerve receives its signal from raphe neurons located in the medial medulla, a signal which is 5-HT mediated and which is affected by arousal status, being high during wakefulness and reduced with sleep (Sood et al., 2003, Lipford et al., 2016). IH successfully triggered hypoglossal LTF and it is highly speculated that it achieves that through the same serotonin mediated mechanism that result in pLTF (Fuller, 2005, Wilkerson et al., 2017).

3.5.2 Conditions for rLTF in Previous Studies

The rLTF triggered by IH was not achieved unless the participant was sleeping (Babcock et al., 2003) or was awake with controlled levels of CO_2 as will be discussed later in this chapter (Jordan et al., 2002, Chowdhuri et al., 2008). This raises the possibility that sleep can function as the ideal media for serotonin dependent LTF. This possibility is supported by the evidence which show a clear reduction of the 5-HT production during sleep. This reduction will free the 5-HT receptors allowing the IH mediated neural plasticity to function (Chowdhuri et al., 2008).

Based on that, despite that INAP did not induce GG LTF in this study, applying the protocol during sleep might show different results as participants were awake and that might have occupied the 5-HT receptors blocking the rLTF channels. Nonetheless, sleeping might not be easily achieved with the experiment setup.

CO_2 AND LTF

Hypercapnia and/or hypoxia are potent triggers for the peripheral chemoreceptors and they lead to increase in ventilation and in the motor drive to the upper airway dilators (Horner, 2011). It is evident that there is a required level of $PaCO_2$ to activate the peripheral chemoreceptors. This means that the chemoreceptors will not be triggered when the $PaCO_2$ is below that level. This level is called the Apnoeic Threshold, and once it met it will result in activation of the tongue muscle and the intercostal muscles (Janssen and Fregosi, 2000).

The excitation of the chemoreceptors is essential for the manifestation of LTF by IH (Mateika et al., 2017), while IH leads to hyperventilation and result in CO_2 levels which are below the apnoeic threshold which restrict the expression of LTF. Consequently, LTF was not achieved by IH in awake humans unless CO_2 levels were maintained above the apnoeic threshold. As a result, raising the CO_2 levels above the baseline and maintaining that elevation successfully expressed the LTF in awake humans (Harris et al., 2006, Griffin et al., 2012).

This could be a potential limitation to this study, as the levels of CO_2 for the awake participants were not controlled. In fact, one study showed evidence of ventilatory LTF vLTF after one hour of INAP when the CO_2 was elevated above baseline (Griffin et al., 2017).

3.5.3 INAP Protocol

In this study, a pressure of -15 cmH_2O was selected for the protocol as the GG response to negative pressure was shown to saturate at pressure more than -20 cmH_2O (Ryan and Nolan, 2009b). This level might have been higher than the optimal pressure, which could have resulted in the lack of change in the GG EMG activity in this study as excessive stimulation was found to suppress the GG activity (Sood et al., 2003). This possibility is supported by the success of vLTF by using a pressure of -10 cmH_2O (Griffin et al., 2017).

Another possible reason for the finding is that GG LTF needed more time to manifest itself, as studies in awake cats showed an increased corticomotor activity within a week of IH exposure, but GG activity in that study did not change until the fourth week of exposure (Zou et al., 2018). The duration and frequency of exposure in this experiment were chosen as a starting point placing feasibility in mind, so there is room to explore alternative protocols.

3.5.4 Surface Electrodes

Surface EMG electrodes are considered a good option for the measurement of GG activity. They are not invasive; they guarantee constant placement of electrodes across several trials and they cover a larger area of the GG muscle compared to intramuscular electrodes.

The GG EMG reflex response to negative pressure was measured using the improved design for surface GG EMG electrode (O'Connor et al., 2007). This design was introduced as a superior alternative to the older design by (Doble et al., 1985), which is the most frequently used design to non-invasively measure GG EMG activity. The modified electrode setup changed both the setup of the electrodes in the electrode carrier and the electrode material. The major characteristics of each design are presented in Table 3.5 which also show the difference between the designs.

	Doble design	**O'Connor design**
Electrode Material	Stainless-steel (SS)	Silver–silver chloride (Ag–AgCl)
Electrode Alignment	One on either side of the mid-line of the mouth such that each electrode lies next to one of the two GG muscles (bilateral configuration)	Both electrodes are placed on the same side of the mouth (unilateral configuration)
Orientation	The bar electrodes are orientated with their long axis parallel to the fibres of the GG muscles.	The electrode bars are orientated perpendicular to the muscle fibres.

Table 3-5. Main differences between the unilateral and bilateral electrodes' configuration

This design resulted in a non-consistent signal which was extremely variable not only between but also within participants and that was against the purpose of surface GG EMG. Although surface EMG is known to be variable between subjects, it

is supposed to be fairly consistent within subjects (Shin et al., 2016), but that was not the case for this electrode design, as can be seen in the example provided in Figure 3.12.

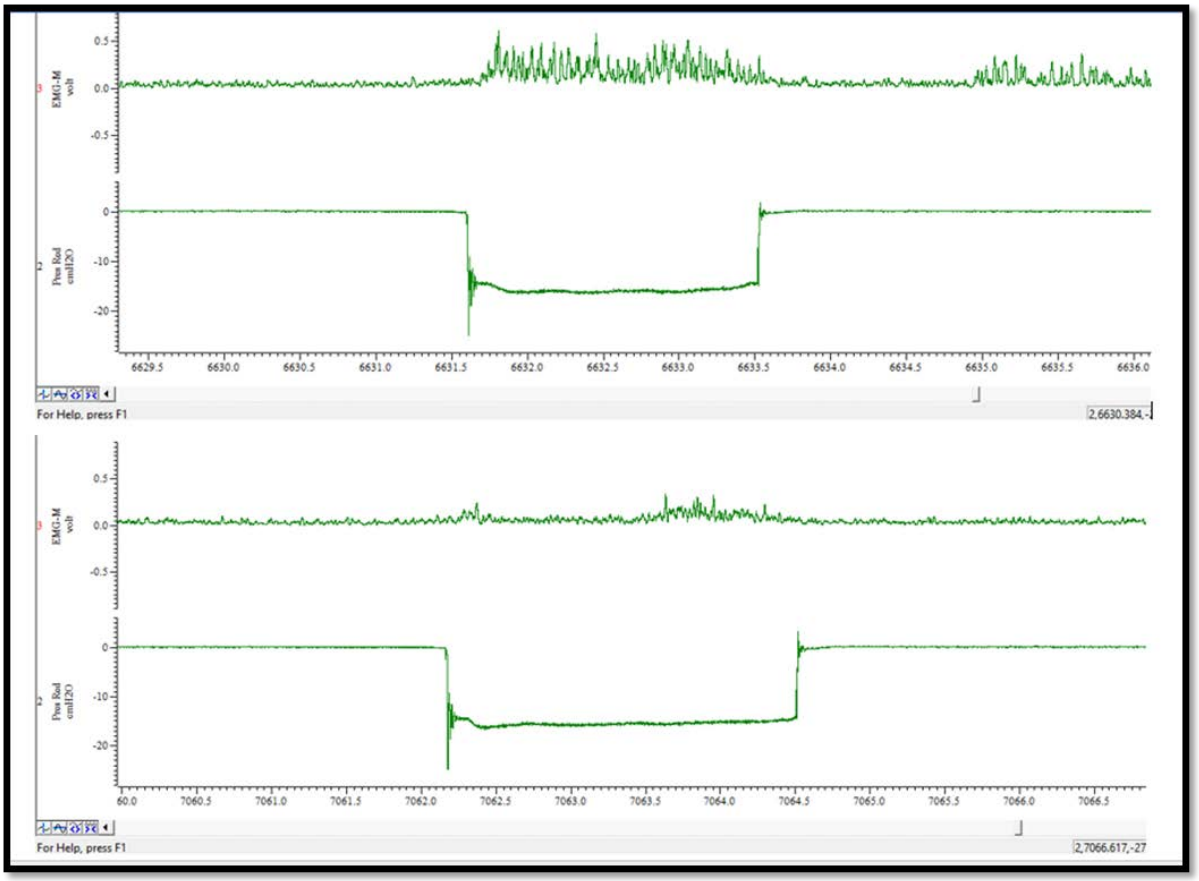

Figure 3-12. Two rectified trace of genioglossus EMG activity from the same participant which are short time apart during the exposure to -15 cmH2O, presenting the EMG signal variability.

This observed variability of the signal could be due to several limitations of the design. First, using a unilateral alignment of the electrodes could have led to connection between the two electrodes with saliva, which can alter the signal quality. This is still a possibility although the design was tested in O'Connor's laboratory, as it

was not used in an actual experiment with extended time of use. This could initially mask the limitation of the design and affect its validity as noticed in this study. Second, the distance between the electrodes (5 mm) was chosen by the researchers as a starting point as they mentioned, so it is not known whether this distance is optimal for experimenting or not, and the findings suggest that it might not be ideal.

After the confirmation of the signal variability with the unilateral design, the same silver electrode was used as it is introduced as superior to stainless steel electrodes for this kind of measurements (O'Connor et al., 2007) and integrated it in the bilateral electrode setup, but this did not change the magnitude of the signal variability obtained.

Another major limitation for the use of surface EMG for measurement of GG activity is the lack of standard protocol for data analysis. Each laboratory performs their analysis in a different way, which increases the risk for subjectivity and interpretation bias, affecting the reliability of the reported results. Establishing an evidence-based guideline for data collection, processing and analysis for surface GG EMG with clear protocol will lead to more precise data that could be compared between laboratories.

Nonetheless, although the variability in the signal was a major limitation of the study, it is highly unlikely to be the sole cause of the finding of this study, as the other factors mentioned earlier have a greater applicability as this current study was performed on awake participants without controlling the $PaCO_2$.

3.5.5 Why Should We Consider INAP for LTF?

Although IH has shown to be a potent stimulant for LTF, there is no optimal protocol for using it yet. The line between its advantageous and its adverse effects is a very fine one. Strong evidence linked IH to increased HR, BP, pulmonary artery pressure and oxidative stress. Although the effect of INAP on those variables is not known, the data show that it did not affect neither BP nor HR. If INAP had the same effect (or lack of it) on the other physiological parameters, it would be a better way to establish LTF compared to IH especially that it has already shown the potential to induce vLTF (Griffin et al., 2017). The effect of INAP on those parameters will be expanded on in chapter 4.

3.5.6 Conclusion

Despite the absence of evidence for GG LTF in this study, its likelihood cannot be disregarded, specially that the study is underpowered. The possibility is supported by strong theory and the data suggests that it is evident in anaesthetised rats (Ryan and Nolan, 2009b). INAP could be a good alternative to IH for stimulation of LTF, but more studies are required to find the optimal way to achieve it. Future research on healthy subjects during sleep or during wakefulness with CO_2 levels elevated above baseline might show better results. Furthermore, the unilateral electrode setup should be improved and validated before being used again. Until then, the use of invasive electrodes seems to be a better option. The bilateral non-invasive design with stainless-steel electrodes could also be explored as a way to measure GG EMG.

4. THE PHYSIOLOGICAL EFFECTS OF INAP

4.1 INTRODUCTION

4.1.1 Overview

The respiratory system has the ability to adapt for changing conditions throughout the life, and when these changes are persistent as during pregnancy or when living at high altitude it is known to trigger a form of neural plasticity. This neural plasticity can be manifested as enhanced efficiency of the synaptic connections, increased release of the neuromodulators as the serotonin, change in the anatomical or physiological neural structure and/or by the growth of new synaptic connections between existing neurons. Furthermore, neural plasticity of the respiratory system can be manifested as enhanced synchronisation between the brain stem respiratory neurons (Mitchell and Johnson, 2003).

Respiratory long-term facilitation is a form of neural plasticity, which is characterised by enhanced respiratory motor output in response to a stimulus; a response that lasts even after the removal of the stimulus (Mitchell and Johnson, 2003). IH is the most studied stimulus for rLTF in animals and humans (Mateika and Sandhu, 2011). Many IH protocols and different approaches have been used in the past to cause rLTF, varying from acute to chronic exposure and from mild to severe hypoxia trying to find the right protocol to induce rLTF without causing the side effects associated with exposure to hypoxia.

As mentioned in the general introduction, rLTF includes both phrenic LTF (pLTF) and hypoglossal LTF (hLTF) which enhance the ventilation and maintain the airway patency respectively. The strongest evidence has shown that rLTF of both ventilation and upper airway dilator muscle activity is more likely to be unmasked with moderate acute IH with the CO_2 maintained above the baseline levels (Harris et al., 2006, Navarrete-Opazo and Mitchell, 2014).

In addition to rLTF, neural plasticity has been shown to have a beneficial effect for other conditions such as spinal injuries, where IH successfully improved respiratory function on rats after the injury (Golder and Mitchell, 2005). Furthermore, IH induced neural plasticity has shown to improve the motor functions for patients with incomplete spinal cord injury (Trumbower et al., 2012, Lovett-Barr et al., 2012) and improved the walking ability of persons with chronic incomplete spinal cord injury (Hayes et al., 2014).

Although IH proved to be an effective stimulus for neural plasticity if the right protocol is used, it has some serious side effects. Hypoxia is a strong stimulus for the peripheral chemoreceptors, especially the carotid body (Iturriaga, 2018b, Prabhakar et al., 2018). Sustained stimulation of the carotid body leads to sympathetic overactivation (Iturriaga, 2018a), which leads to hypertension and alters the heart rate variability (HRV) (Río Troncoso, 2010).

HRV is used as an indicator of the interaction between the sympathetic and the parasympathetic systems, which are branches of the autonomic nervous system.

Power spectral density analysis of HRV show high frequency HF which reflect the parasympathetic effect on the heart, and low frequency LF which reflects the sympathetic regulation of the heart. The LF/HF ratio reflects the balance between both systems, as when it increases it reflects the dominance of the sympathetic system and vice versa. Sympathetic dominance is a known result of hypoxia (Nesterov, 2005, Iturriaga et al., 2009). It is suggested that the change in HRV balance indicated by increased ratio can be used as an early alarming sign for hypertension (Iturriaga et al., 2009).

Furthermore, IH results in oxidative stress, which will be discussed in the following section. The oxidative stress also plays an important role in the activation of the carotid body, adding to the mechanism in which IH lead to its associated side effects (Iturriaga et al., 2009, Río Troncoso, 2010).

4.1.2 IH and Oxidative stress

Oxidative stress results from imbalance between the production and elimination of the reactive oxygen species (ROS). ROS are unstable atoms or molecules with free electrons in their outer orbit (Sies, 1997). Patients with OSA suffer from recurrent airway obstruction during sleep which leads repeated cycles of hypoxia and reoxygenation which could be comparable to recurrent episodes of ischemia- reperfusion injury, a condition that is associated which excess release of ROS (Yamauchi et al., 2005). In fact, the evidence show that exposure to 6 hours of IH per day for 4 days leads to an increase in ROS production without causing a change in the antioxidant capacity. Thus, the way IH cause oxidative stress is

probably linked to ROS production without having effect on the antioxidant capacity (Pialoux et al., 2009). Lipid peroxidation is identified as one of the major oxidative stress pathways (Barcelo et al., 2000, Lavie et al., 2004).

4.1.3 IH and Autonomic Dysfunction

Figure 4.1. shows the organisation of the nervous system networks, as it is divided to central and peripheral nervous systems. The central nervous system is composed of the brain and the spinal cord while the peripheral nervous system has the sensory neurons and the ganglia.

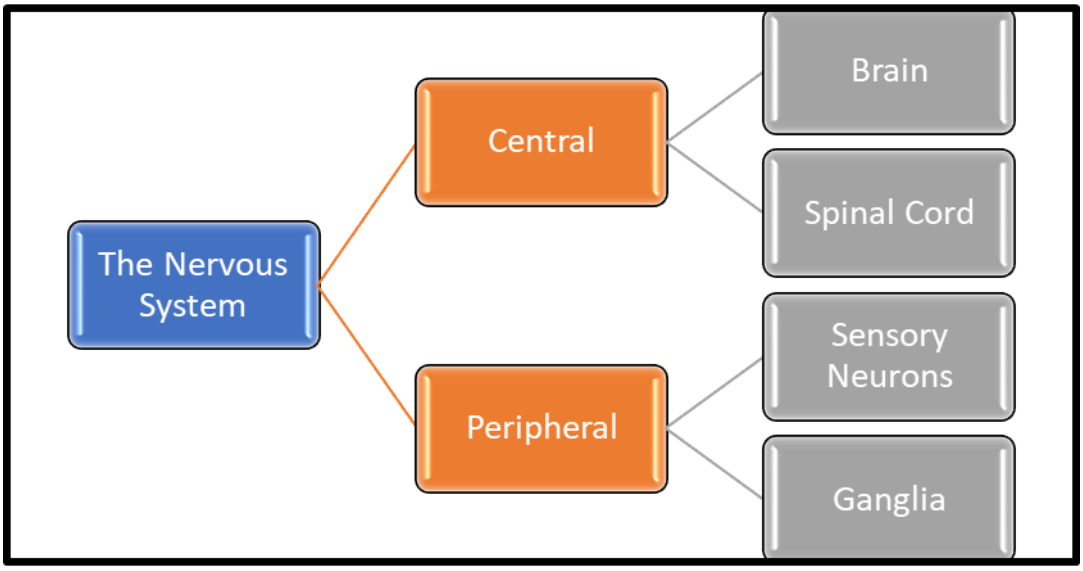

Figure 4-1. Basic structure of the Nervous System.

Functionally, the nervous system is classified to voluntary (somatic) and involuntary (autonomic), with the autonomic system further divided to the sympathetic

and parasympathetic branches. Increased sympathetic activity increase the HR and BP while increased parasympathetic activity has the opposite effect.

As mentioned earlier, IH results in increased sympathetic activity and decreased parasympathetic activity (Wadhwa et al., 2008, Tamisier et al., 2010). The effect of IH on mean arterial pressure (MAP) has been consistently demonstrated using a variety of protocols. For example, exposing healthy humans to 4 days of IH for 6 h/day has increased MAP by ~4 mmHg (Foster et al., 2009), 14 days of IH exposure for 8-9 h/night increased MAP by ~5 mmHg (Tamisier et al., 2009), and a single 6-hour bout of IH increased MAP by ~7 mmHg (Foster et al., 2010). The findings of these experiments and others show the direct link between IH and hypertension.

4.1.4 IH and Pulmonary Hypertension

Hypoxia leads to pulmonary vasoconstriction (hypoxic pulmonary vasoconstriction, HPV). HPV has clear physiological advantages as it helps to maintain a good ventilation/perfusion ratio when hypoxia is localised. This is achieved by diverting blood away from the alveoli which are not well ventilated through the vasoconstriction of those areas. However, hypoxia that affects the entire pulmonary system creates a restriction of blood flow at the pulmonary circulation and because this circulation accepts the entire cardiac output, global HPV leads to increased pulmonary artery pressure (PAP) (Zieliński, 2005, Ward and McMurtry, 2009)

Persistent HPV as it happens in sustained exposures to continuous hypoxia (for example in prolonged exposure to high altitude), or as a result of repeated cycles of IH (as it happens every night in OSA patients and chronic IH protocols) may cause the pulmonary arteries to undergo a remodelling process of their walls that can eventually results in the development of pulmonary hypertension (PH) (Zieliński, 2005, Ward and McMurtry, 2009).

4.1.5 Alternative Triggers for Neuroplasticity

IH is the most studied mechanism to induce neuroplasticity which includes rLTF. Neuroplasticity is a possible treatment modality with good potential, but it has limited applicability although it has been studied for decades. On one hand neural plasticity has several therapeutic implications for rLTF and for patients with incomplete spinal injury, and there is a wealth of evidence to support that it can be achieved by IH. And on the other hand, the correlation between IH with hypertension, pulmonary hypertension and oxidative stress is also supported by strong evidence. This led to the search for alternative triggers for neuroplasticity, including rLTF.

Intermittent negative airway pressure (INAP) was recently studied as a method for stimulation of rLTF and was effective in both rats (Ryan and Nolan, 2009b) and humans (Griffin et al., 2017), but as it is a newly studied intervention, little is known about its physiological effects. With the lack of IH in this method, there is a good possibility that it will be a safer alternative to induce rLTF and maybe other types of neural plasticity as well.

4.2 AIM OF THE STUDY

The aim of this study was to investigate the effect of repeated INAP exposures on the main variables that are negatively affected by IH (BP, SPAP and oxidative stress). The hypothesis was that INAP will be safer than IH and that it will not cause a significant increase in any of the variables mentioned here.

4.3 METHODS

The study was approved by the local ethics committee (University of Birmingham Ethical Review Committee – ERN_17-1048). Subjects were recruited via emails, posters and word of mouth. An informed written consent was obtained from all participants after receiving detailed information on the procedures and risks of the study.

4.3.1 Participants

Ten participants (5 female, 5 male) were recruited for this study from the general population of the University of Birmingham students. Participant characteristics are shown in Table 4.1. All participants were healthy, non-smokers, had a BMI of less than 25, and they had no documented history of respiratory, cardiac, sleep or upper airway disorders. Participants abstained from moderate or vigorous exercise and alcohol intake for 24 hours and caffeine intake for 12 hours prior to experimental visits to the laboratory. All participants were asked to keep their

meals consistent for the protocol duration and they recorded their meals in a food diary which was handed to us at the end of the experiment.

	Age (years)	BMI (kg/m^2)
1	22	21.63
2	22	15.57
3	24	23.42
4	29	24.84
5	27	23.04
6	24	22.48
7	25	23.31
8	20	19.88
9	30	23.71
10	27	23.94

Table 4-1. Physiological effects of INAP Participant characteristics.

4.3.2 Study Protocol

Each participant visited the laboratory on four occasions, once for orientation and then on three consecutive days for the study protocol.

THE ORIENTATION VISIT

The purpose of this visit was to familiarise the participants with the laboratory environment and with the experimental procedures which they were going to undertake in the experimental visits. After asking any questions in their mind and discussing the protocol, participants were exposed to short bouts of INAP to ensure that they can tolerate this intervention for the duration of the experimental visits. During this visit the ability to detect a tricuspid valve regurgitation using ultrasound in these participants was also established.

INAP VISITS

Day one: Participants arrived at the laboratory in the morning and they were given 15 minutes to settle. The experimental protocol started with EEG electrodes attachment and cardiac ultrasound scanning in order to measure SPAP and cardiac output as described in the general methods chapter (Chapter 2). This was followed by a blood sample obtained via venepuncture in order to allow measurement of baseline values of the antioxidant capacity and the markers of oxidative stress. After that, participants were instrumented for the INAP protocol using the system described in Chapter 2 but continued to breathe room air at ambient pressure to allow for a baseline reading of BP and ECG to be obtained.

In order to achieve reliable baseline readings, participants were left to relax and breathe normally for 15 minutes before BP measurements were made; meanwhile a continuous reading of ECG was stored for offline analysis. The INAP

protocol was started after the 15 minutes baseline and it was continued for the next three and a half hours with repeated cycles of 30 s breathing at -15 cmH_2O negative pressure and 1 min breathing at atmospheric pressure, Figure 4.2.

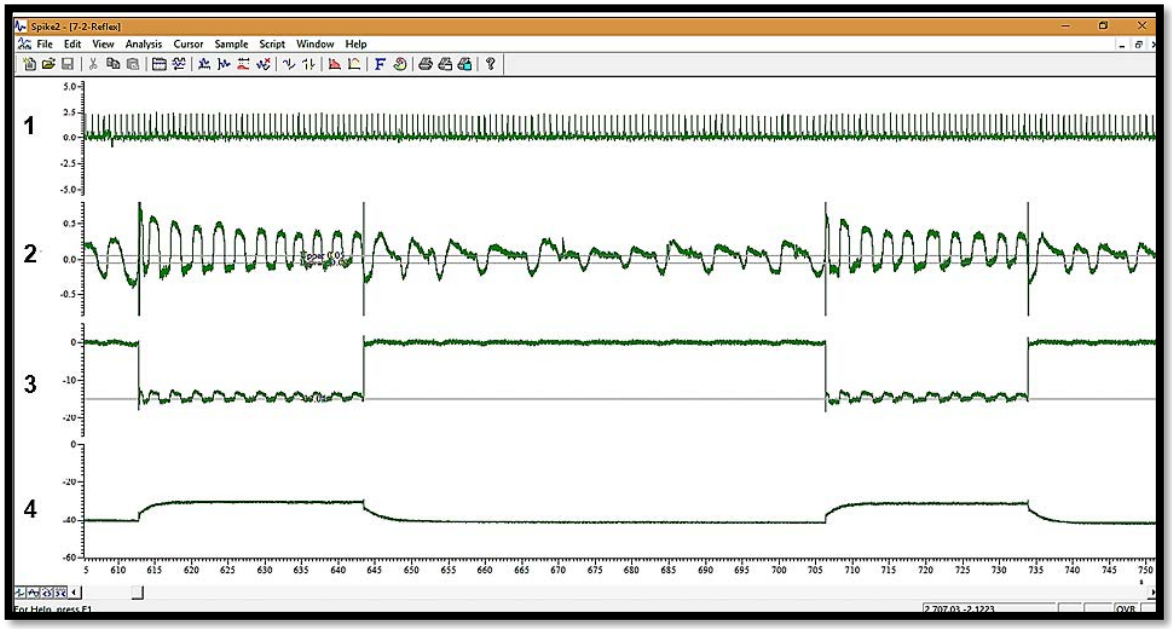

Figure 4-2. Example of the INAP protocol. The traces show the ECG (1), flow by pneumotachometer reading (2), pressure at the mouth (3) and pressure at the box. The traces show the transition from atmospheric pressure to the negative pressure and the duration of exposure.

During that time, participants were lying in a supine position with their backs raised slightly on a comfortable bed watching movies of their choice while wearing noise isolating headphones. BP was measured every 30 minutes, and an ECG was recorded continuously for later offline analysis. At the end of this period a further cardiac ultrasound scan was performed in a similar way to the baseline measurement. A summary of the protocol is illustrated in Figure 4.3.

Day two: The second day was identical to the first day with the exception that no blood sample was obtained in the day.

Day three: The third day was identical to the first day with the exception that we took two blood samples, one in the beginning of the day and one at the end of the protocol.

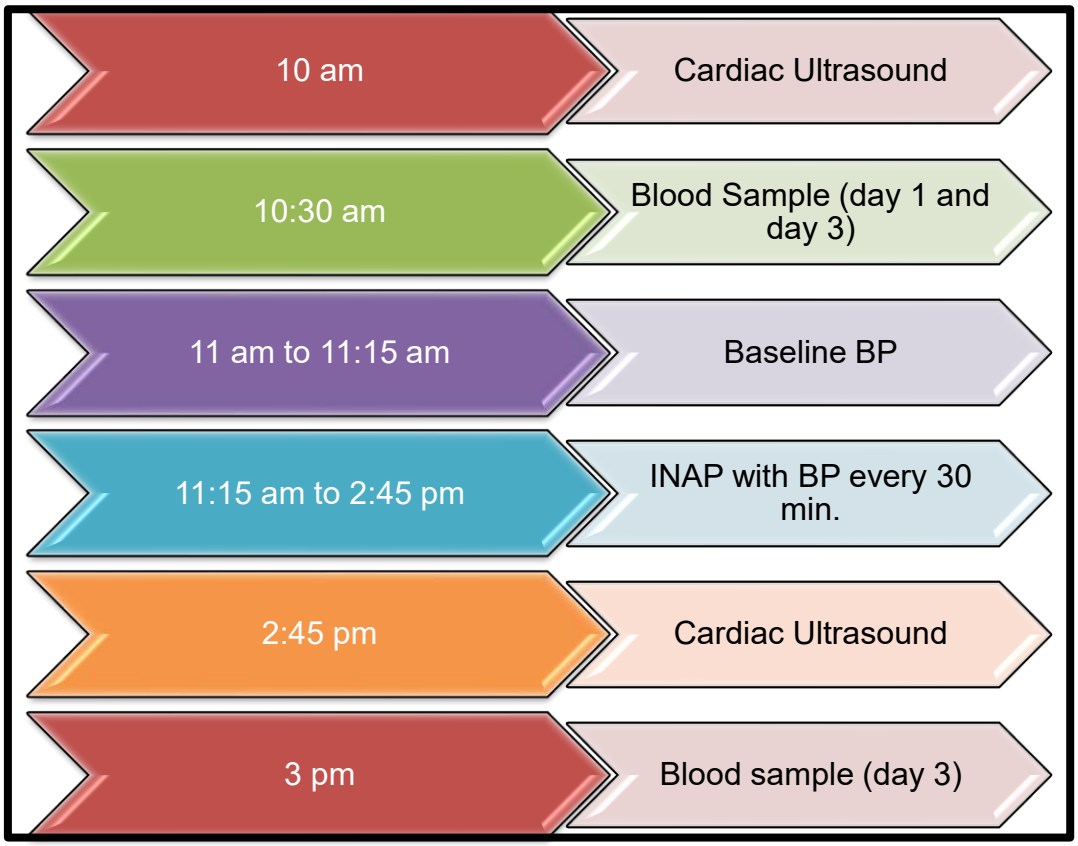

Figure 4-3. The physiological effects of INAP study protocol.

4.3.3 Heart rate variability (HRV)

The heart beat is composed of several waves which are PQRST, and the RR interval is used to monitor the variation between the length of the cardiac cycles. Spike2 software was used (CED, Cambridge, England) to transform the RR intervals to bands with various spectral frequencies then transform them to hertz (Hz). The script identified the low frequency (LF) and high frequency (HF) bands and calculated the LF/HF ratio. LF/HF ratio was measured at baseline and every 30 minutes of the INAP for each day to see the effect of the INAP on the HRV.

4.3.4 Doppler ultrasound assessment of cardiac output and SPAP

Philips Sonos 7500 ultrasound machine with an S3 two-dimensional transducer (1-3 MHz) was used to measure the cardiac output and SPAP as described in Chapter 2. In summary, the four-chamber apical view was used to measure the velocity of the tricuspid regurgitation jet by using continuous wave Doppler, and SPAP was estimated from the peak velocity of the jet using the modified Bernoulli equation. To measure cardiac output the five-chamber view was used to measure the velocity of the blood flow at the aortic orifice using the pulsed wave Doppler, and this value was used to derive the velocity time integral (VTI). The diameter of the aortic orifice was also measured using the parasternal long axis view, and this was used to calculate the area of the aortic orifice (A) using the formula $A = \pi r^2$, where r is the radius. Heart rate was obtained from the ECG trace. Finally, we calculated cardiac output from the following equation (cardiac output = VTI x A x HR).

4.4 RESULTS

4.4.1 Blood Pressure

A one-way repeated measures ANOVA was used to determine whether there was a statistically significant difference in BP over the course of 3 days of INAP. BP was the dependent variable and time was the within subject factor which had twelve levels; baseline, after one hour, after two hours and after three hours recorded for three consecutive days. There were no outliers and the data were normally distributed at each time point, as assessed by boxplot and Shapiro-Wilk test ($p > .05$), respectively. The assumption of sphericity was not met, as assessed by Mauchly's test of sphericity, so the results were calculated according to Greenhouse & Geisser correction. INAP did not elicit statistically significant changes in MAP over time, $F(4.01, 36.12) = 1.437$, $p = 0.242$. Table 4.2, Figure 4.4.

The same one way repeated measure ANOVA design used for the MAP showed that the change in systolic blood pressure was also not statistically significant as the mean SBP was 112 mmHg ± 6 SD in the beginning of the protocol and 113 mmHg ± 8 SD at the end of the protocol, $F(4.58, 41.22) = 2.477$, $p = 0.052$. Furthermore, the change in diastolic blood pressure was not statistically significant either as the mean DBP was 66 mmHg ± 7 SD in the beginning of the protocol and 69 mmHg ± 9 SD at the end, $F(3.94, 35.48) = 1.099$, $p = 0.372$.

Time		Mean ± SD mmHg
Day 1	Baseline	81.3 ± 5.57
	One Hour	81.2 ± 5.22
	Two Hours	81.0 ± 5.04
	Three Hours	83.1 ± 7.0
Day 2	Baseline	82.1 ± 5.76
	One Hour	81.0 ± 5.40
	Two Hours	82.7 ± 4.27
	Three Hours	81.3 ± 7.0
Day 3	Baseline	81.2 ± 6.42
	One Hour	81.0 ± 1.64
	Two Hours	83.1 ± 6.23
	Three Hours	82.1 ± 7.11

Table 4-2. Change in Mean BP over 3 days of INAP.

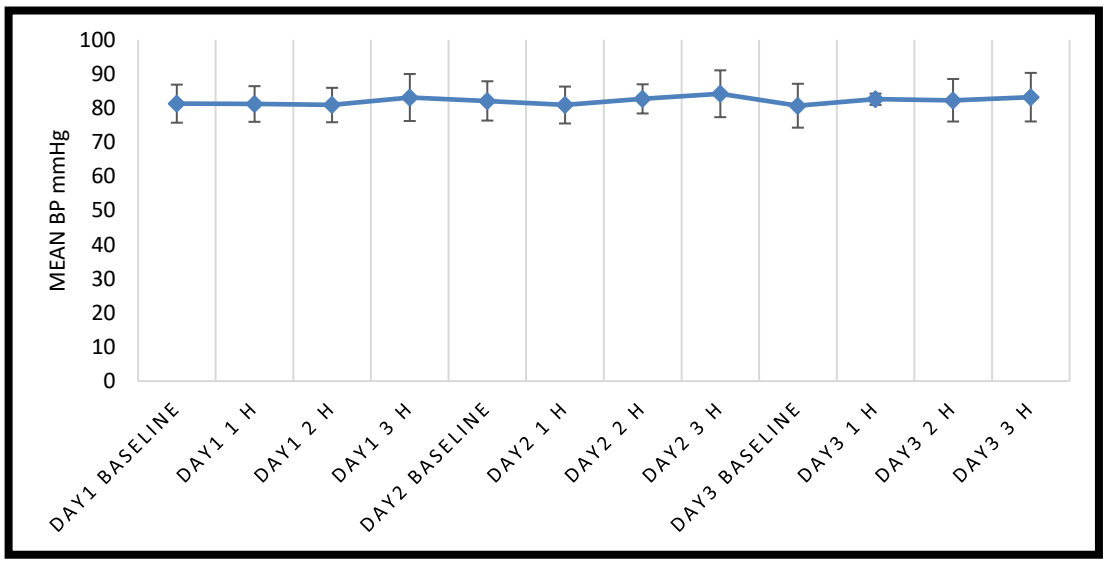

Figure 4-4. Change in mean BP over 3 days of INAP exposure. Error bars represent SD.

4.4.2 Heart Rate Variability

The heart rate variability data were not normally distributed, so a Friedman analysis of variance on ranks was used to determine if there was change in the LF/HF ratio during the course of INAP. The results are presented in Table 4.3 and Figure 4.5, which show a non-statistically significant median change in the ratio over time during the INAP, $\chi 2(2) = 28.028$, $p = 0.215$.

Time		Median LF/HF ratio
Day 1	**Baseline**	0.63
	One Hour	1.05
	Two Hours	0.81
	Three Hours	0.80
Day 2	**Baseline**	0.62
	One Hour	0.70
	Two Hours	0.63
	Three Hours	0.65
Day 3	**Baseline**	0.70
	One Hour	1.0
	Two Hours	1.0
	Three Hours	0.74

Table 4-3. Median change in the LF/HF ratio over the 3 days of INAP.

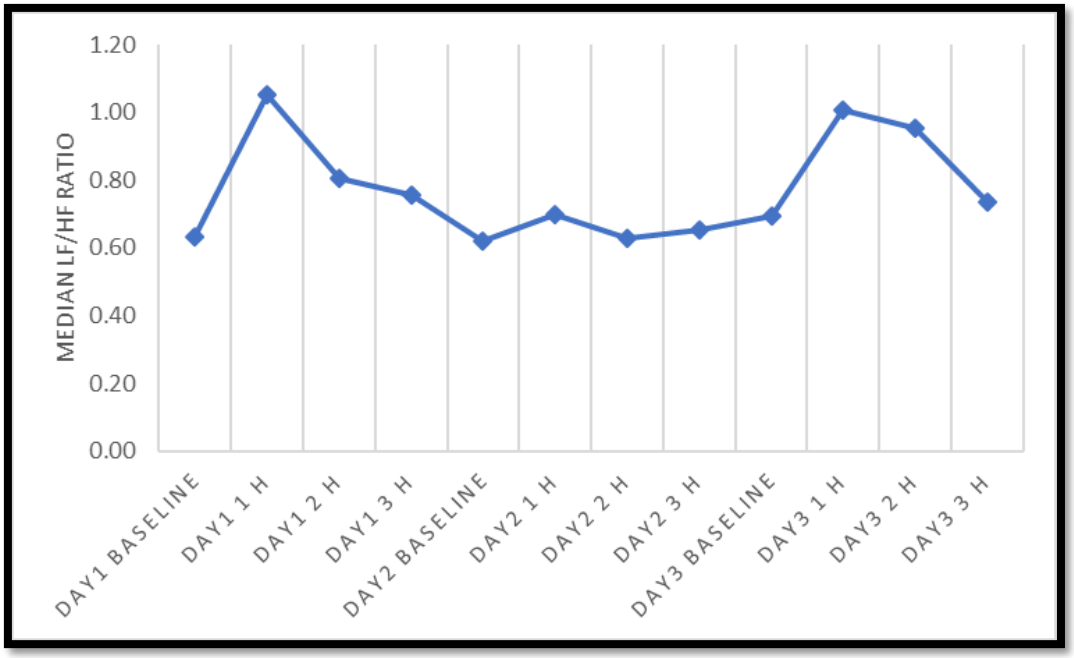

Figure 4-5. Change in median LF/HF ratio over 3 days of INAP exposure.

4.4.3 Systolic Pulmonary Artery Pressure (SPAP)

A one-way repeated measures ANOVA was conducted to determine whether there was a statistically significant difference in SPAP over the course the INAP intervention. SPAP was the dependent variable and time was the within subject factor which had twelve levels; baseline, after one hour, after two hours and after three hours recorded for three consecutive days. There were no outliers and the data were normally distributed at each time point. One participant was excluded because the measurements were not possible. The assumption of sphericity was not met, as assessed by Mauchly's test of sphericity so the Greenhouse-Geisser correction was used. The results show that INAP did not elicit statistically significant changes in SPAP over time, $F(3.8, 1.78) = 2.13$, $p = 0.142$, Figure 4.6.

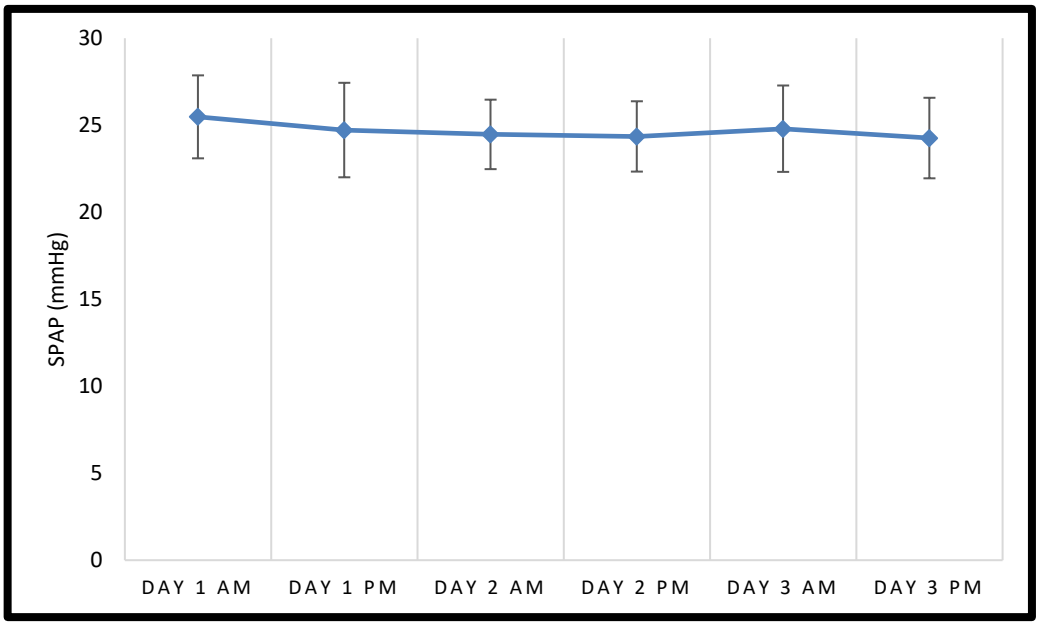

Figure 4-6. Mean change in SPAP over 3 days of INAP exposure. Error bars represent SD.

4.4.4 Cardiac Output

The one-way repeated measures ANOVA was used for the analysis. Cardiac output was the dependent variable and time was the within subject factor which had twelve levels; baseline, after one hour, after two hours and after three hours recorded for three consecutive days. The result showed no statistically significant change in the cardiac output over the course of INAP, $F(5, 40) = 1.98$, $p = 0.102$, Figure 4.7.

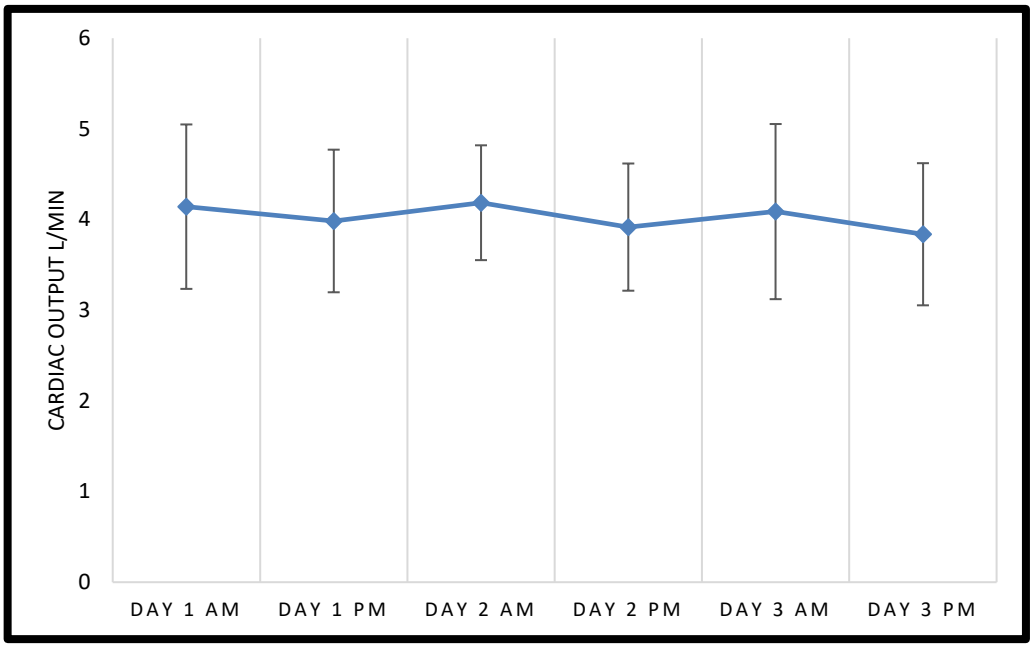

Figure 4-7. Mean change in cardiac output over 3 days of INAP exposure. Error bars represent SD.

4.4.5 Antioxidant capacity

The results of the FRAP assay showed that the total plasma antioxidant capacity was not affected by the INAP protocol. The data is presented in Table 4.4 and Figure 4.8 and it shows a slight increase mid intervention and a slight decrease at the end, a change which was not statistically significant. $F(2, 16) = 4.71$, $p = 0.633$.

Data expressed as μM of antioxidant power relative to ascorbic acid as determined from a seven-point linear standard curve. One participant was excluded from the analysis due to missing data.

	Mean ± SD µM
Baseline	228.04 ± 58.0
Mid Protocol	232.0 ± 70.0
Post INAP	219.9 ± 73.43

Table 4-4. Antioxidant capacity over 3 days of INAP.

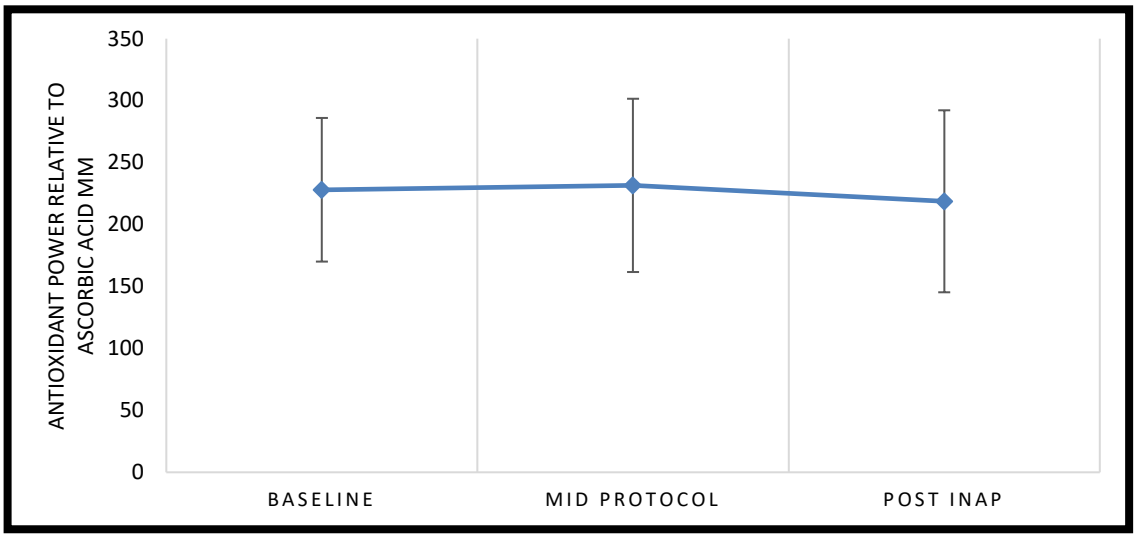

Figure 4-8. Antioxidant capacity over 3 days of INAP. Error bars represent SD.

4.4.6 Lipid Oxidation

Lipid peroxidation product in the plasma was measured with the TBARS assay using the MDA standards as reference. The results of the TBARS assay showed that the lipid oxidation was not affected by the INAP protocol. The data is presented in Table 4.5 and Figure 4.9, which show a slight increase at the end of the protocol which was not statistically significant. $F(2, 18) = 0.635$, $p = 0.542$.

	Mean ± SD µM
Baseline	7.60 ± 1.06
Mid Protocol	7.60 ± 1.60
Post INAP	8.11 ± 1.60

Table 4-5. Plasma MDA over 3 days of INAP.

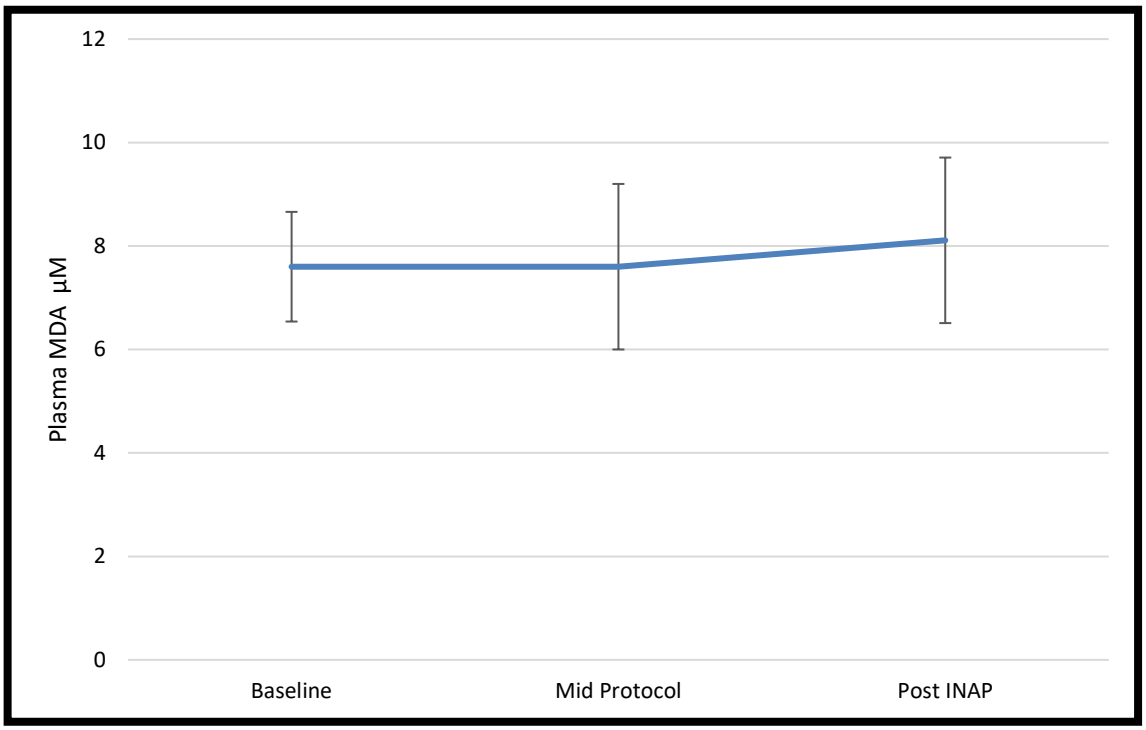

Figure 4-9. Plasma MDA over 3 days of INAP. Error bars represent SD.

4.5 DISCUSSION

4.5.1 Overview

The results of this experiment indicate that INAP does not affect BP, cardiac output, SPAP, or oxidative stress in the three-day protocol used in the study. Although these are promising results, a post hoc power analysis with G*Power version 3.1.9.2 showed that the current study is underpowered with a power estimate of 0.72 due to small sample and effect size. As in the previous study, the study might have failed to reject the null hypothesis although it is false due to type II error because of the small sample size of the study.

Considering the positive findings of the studies that showed potential rLTF in response to INAP as discussed previously in this thesis (Ryan and Nolan, 2009b, Griffin et al., 2017), the results of the present study are promising and the data suggest that INAP might be a safer potential trigger for rLTF compared to IH. This finding should be confirmed by a larger scale studies before taken as granted.

By comparing INAP to IH, which is the most studied method for rLTF, we can see how several IH protocols have led to an increase in oxidative stress (Barcelo et al., 2000, Lavie et al., 2004, Pialoux et al., 2009), and increased BP (Tamisier et al., 2010, Foster et al., 2009, Foster et al., 2010). This was not the case with INAP which show some promise but needs to be explored by future research.

4.5.2 Oxidative Stress

IH is associated with increased ROS production in the body which result in protein oxidation, lipid peroxidation, and DNA damage (Iturriaga et al., 2009). Peroxidation of lipid produces several by-products, with Malondialdehyde (MDA) being the major one and one that is studied the most. MDA reacts with other molecules, such as DNA and proteins, altering their physiological mechanisms (Del Rio et al., 2005). Chronic exposure to IH has shown to double the plasma MDA in rats (Savransky et al., 2007). In addition to the oxidative stress analysis, the body antioxidant capacity is usually measured by the FRAP assay to assess the body's capability to fight against oxidative stress. IH protocols mostly resulted in increased MDA without change in the total antioxidant capacity, leading to oxidative stress. That's why these values were selected for the experiment.

The findings of this study show that three days of INAP exposure, for three and a half hours per day did not affect the plasma MDA which highly suggests that INAP does not cause oxidative stress, at least in protocol used in this study. This finding is reasonable as although breathing against resistance like what happens during negative pressure could alter the oxygenation and ventilation the protocol did not incorporate any change to the blood gases. Even if it caused a change, it is so mild and did not have any major effects on the oxygenation and as a result did not cause any sign of oxidative stress.

4.5.3 Autonomic Dysfunction

Carotid bodies, which are the main peripheral chemoreceptors, are very sensitive to the level of oxygen in the blood. The Dejours test is usually used to assess the sensitivity of the carotid body to oxygen, and it works by exposing participants to 100% O_2 and examining the carotid body response to that. The test ideally shows a decreased sensory activity leading to hypoventilation. OSA patients who are exposed to recurrent IH show a stronger hypoventilation during the Dejours test compared to controls, reflecting an enhanced carotid body response (Prabhakar et al., 2005).

Moreover, IH results in increased sympathetic activity and reduced parasympathetic activity. A series of experiments has shown that the effect of IH on the autonomic nervous system is mediated by the carotid body. For instance, bilateral carotid body denervation prevented the rats in one experiment from developing hypertension in response to IH (Iturriaga et al., 2009). Furthermore, it was found that OSA patients with resected carotid bodies did not develop hypertension compared to the OSA patients with intact carotid bodies. These findings highly suggest that IH cause an increase in BP by stimulating the peripheral chemoreceptors, and that in turn increase the sympathetic outflow (Prabhakar et al., 2005, Iturriaga et al., 2009).

The mechanism of the carotid body enhanced response to hypoxia which is stimulated by IH is not well understood yet, but it is linked to the increase ROS in the body, associating the mechanism with the oxidative stress. Providing rats with

compounds that increased their antioxidant capacity for 10 days then exposing them to IH prevented the enhanced carotid body response to hypoxia and prevented hypertension, and that supports the link between oxidative stress and hypertension (Iturriaga et al., 2009).

This complicated cascade does not seem to be triggered by INAP as the protocol did not affect blood pressure during the three-day intervention we used because as mentioned in the previous section, it does not provide a means by which blood gases would be altered. Moreover, the protocol did not reveal an effect on the LF/HF ratio which serves as an early indicator for any sympathetic activation or parasympathetic depression (Nesterov, 2005, Iturriaga et al., 2009). This shows that the INAP protocol did not even show the tendency to affect the blood pressure.

4.5.4 Pulmonary Hypertension

Opposite to the systemic circulation, hypoxia causes pulmonary vasoconstriction with variable degrees of intensity between individuals, leading to an increase in mean pulmonary artery pressure SPAP between 2-15 mmHg with each hypoxia exposure (Zieliński, 2005). Prolonged exposure to hypoxia eventually results in pulmonary arteries remodelling, causing thicker walls and narrower lumens which increase the pulmonary vascular resistance and the right ventricle afterload. This leads to pulmonary hypertension and right ventricle hypertrophy (Zieliński, 2005). Patients with severe OSA show an increase in SPAP with each apnoeic episode, which is highly suggested to be triggered by the repetitive hypoxia exposure and in

the long run it could lead to the same remodelling process caused by sustained hypoxia.

IH protocols that result in rLTF can lead to pulmonary hypertension (Zieliński, 2005, Golbin et al., 2008), which is a major complication that limits the utilisation of IH for rLTF. This is not the case with the INAP, as the protocol did not appear to induce a change in the pulmonary blood pressure at any point during the protocol giving another indicator for the safety of the intervention.

4.5.5 INAP in comparison to IH

IH is a well-established trigger for neuroplasticity which has several clinical advantages but is associated with multiple adverse effects which are all linked to the hypoxic exposure. INAP on the other hand is a newly explored trigger for neuroplasticity with limited information about it but with a promising potential. Although there is a good possibility that INAP shares the same mechanism of serotonin activation with IH (Ryan and Nolan, 2009a, Ryan and Nolan, 2009b, Griffin et al., 2017), it does not seem to cause the same adverse effects of IH.

4.5.6 Conclusion

Exposing participants to three days of INAP in this experiment did not affect BP, SPAP, cardiac output or oxidative stress. This is because INAP does not induce any

changes to blood gases, which is the main reason for all the advert effects of IH. There is a lot to be explored about INAP, and more research is required to understand its mechanism of action and explore its therapeutic potentials. More research about INAP as a trigger for neuroplasticity should be motivated by the signs of its potential benefits and the current evidence that suggests it does not cause the same advert effects as IH. It has to be noted, although the results of the current study are promising, they are limited by the small sample size. A larger scale studies are recommended to confirm the findings of this study.

5. THE IMPACT OF SLEEP FRAGMENTATION ON EPISODIC MEMORY CONSOLIDATION

5.1 INTRODUCTION

5.1.1 Episodic memory

An episodic memory is a memory that can be mentally recalled and relived. At least two parallel memory systems function in synchrony to form the episodic memory trace: the item memory system (which codes the item) and a supportive associative system or systems to code the other elements of the memory as *when* (temporal-order memory system), *where* (spatial memory system) or *how* (source memory system) (Davachi, 2006, Van Der Helm et al., 2011). For example, an episodic memory might consist of remembering a meal (item memory) by such details as the venue, the atmosphere, the smell and taste of the food (associative memory). The methods section will explain the paradigm used in this study to test the episodic memory.

5.1.2 Effects of Sleep Fragmentation

Sleep fragmentation is defined as recurrent interruption of sleep with microarousals lasting between three to ten seconds, which usually happen without the awareness of the sleeping subject because it usually doesn't cause the person to wake up. Sleep fragmentation is common but poorly diagnosed and it happens with several conditions such as OSA, periodic leg movement, post-traumatic stress

disorder, narcolepsy, and depression (Tartar et al., 2006, McCoy and Strecker, 2011, Short and Banks, 2014). Sleep fragmentation can lead to many adverse effects on several physiological and psychological parameters, one of which is cognition. Frequent arousals are highly correlated with excessive daytime sleepiness, altered mood, and increased feeling of worsened memory. Overall, sleep fragmentation imposes a greatly negative effect on health and safety (Cote et al., 2003, Tartar et al., 2006).

Obstructive sleep apnoea patients are often selected to study the effect of sleep fragmentation on memory due to the correlation between the conditions. Patients with untreated obstructive sleep apnoea (OSA) are chronically exposed to sleep fragmentation. The affected population complain of difficulty concentrating, forgetfulness and difficulty in taking decisions, all of which affect their careers adversely. They are also at higher risk for road traffic accidents because of daytime sleepiness (Short and Banks, 2014). Those findings are supported by the research that found correlation between the sleep fragmentation and the adverse effects on several memory systems, including the episodic memory system (Daurat et al., 2008, Andreou et al., 2014). The findings that support the adverse effects of sleep fragmentation on memory are contradicted by the findings of other line of research that does not correlate the sleep fragmentation with the impaired cognitive functions as reviewed by (Beebe et al., 2003). This disagreement in the research could be due the lack of consistency and clear memory classification in the paradigms used to assess the effect of fragmentation on memory.

Adding to the complication of OSA, although patients have memory complaint, the objective memory measures are not conclusive (Beebe et al., 2003, Wallace and Bucks, 2013, Vaessen et al., 2015) and there appear to be a dissociation between their perception of their memory performance and the results of the objective memory assessment (Daurat et al., 2010). This leads to wrong assessment of their condition which might lead to wrong treatment plans. Therefore it is important to understand the effects of OSA's on cognition as this will aid in developing the right assessment tool and lead to the right treatment strategy for this increasingly prevalent high-impact syndrome (Jackson et al., 2011).

Another point of conflict on the research about the effect of sleep fragmentation on memory rise from using patients with OSA for this kind of investigation. The confusion is because it is not clear whether the cognitive impact of OSA is due to the sleep fragmentation or to the intermittent hypoxia (IH) that occurs with OSA (Daurat et al., 2008, Daurat et al., 2010, Jackson et al., 2011). Several studies found a correlation between IH and the patients' cognitive deficits, but because IH occurs with sleep fragmentation, it could be the main reason for the cognitive impact of OSA (McCoy and Strecker, 2011). This possibility is supported by a finding of one study which was done on rats, which proved that exposing rats to 24 and 72 hours of sleep fragmentation without hypoxia altered the hippocampal function leading to impaired processing of the declarative memory (Tartar et al., 2006). Another point of debate about the effect of sleep fragmentation on memory is the mechanism of this effect, and whether it is mediated by the alteration of the sleep architecture or by the lack of sleep continuity as it is difficult to isolate the effect of

one factor in patients with OSA (Daurat et al., 2008). In one hand, it is evident that OSA reduces the time spent in SWS (Andreou et al., 2014, Short and Banks, 2014) which is an essential stage for the consolidation of the declarative memory (Gais and Born, 2004, Rauchs et al., 2005). On the other hand, lack of sleep continuity is OSA is a fact and the association of this factor with cognitive impact is a fact too (McCoy and Strecker, 2011).

It is suggested that both IH and sleep fragmentation affect the hippocampus in the same way which leads to the memory impairment (Daurat et al., 2010). To isolate the effects of sleep fragmentation from IH, experimental sleep fragmentation with normal oxygenation of healthy subjects emerged as the experiment protocol of choice instead of studying the effect on OSA patients. The most frequently used method of fragmentation is delivering audio stimulation during sleep to induce microarousals without waking the subject. Sleep fragmentation studies proved the negative impact of sleep fragmentation on attention and daytime sleepiness, yet still revealed conflicted results about the effects on episodic memory (Cote et al., 2003, Short and Banks, 2014).

5.1.3 Nap Studies

Sleep fragmentation in nap is a more feasible way to address the effect of sleep fragmentation in memory compared to an overnight study. Before considering this protocol, it was important to know if the nap was adequate timeframe for offline memory consolidation. To answer this question a study comparing the benefits of a 60-minute nap to a 90-minute nap on memory consolidation established that the 90-

minute nap, which included both REM and NREM sleep and was rich in SWS, succeeded in improving the declarative memory task performance when compared to the awake period and to the 60-minute nap that did not contain REM sleep (Mednick et al., 2003). The daytime nap's benefits on memory consolidation were confirmed by other studies (Tucker et al., 2006, Lau et al., 2010, Diekelmann and Born, 2010).

To our knowledge, the effect of sleep fragmentation on memory during naps has not been explored yet. Nap fragmentation could be very a useful paradigm in simplifying experiments with sleep fragmentation and facilitating the exploration of its effect on memory.

5.2 AIM OF THIS STUDY

This study examined the effect of nap fragmentation on sleep architecture and on episodic memory consolidation in normal subjects without hypoxia. The hypothesis was that nap fragmentation will reduce the consolidation of both episodic memory components (item and source) compared to non-fragmented naps and that will be reflected by worsening performance after the fragmented nap compared to the non-fragmented nap. The other hypothesis was that sleep fragmentation will reduce the time spent in SWS.

5.3 METHOD

The study was approved by the local ethics committee (University of Birmingham Ethical Review Committee – ERN_15-0034B). Subjects were recruited

via emails, posters and word of mouth. After receiving detailed information about the study, informed written consent was obtained from all participants.

5.3.1 Participants

21 individuals successfully completed the experiment protocol. All participants were healthy non-smokers and had no documented history of respiratory, cardiac, sleep or upper airway diseases. All participants were native English speakers and they were used to taking mid-day naps. Participants were excluded if they were shift workers or if they were taking medications that affected their sleep. Participants were asked to abstain from moderate or vigorous exercise and alcohol intake for the duration of the study and caffeine intake for eight hours prior to the experiment. More details about the participants' demographics are provided in the results section.

5.3.2 Study Design

This was a crossover study in which each participant came to the sleep laboratory for three sessions: an orientation visit, and two testing sessions in consecutive days, once for control and once for intervention. The study was not randomised as the control visit served as the familiarisation visit to overcome the ''first night effect'' on sleep, but participants were blinded about which day the fragmentation would occur. The study was conducted in in a purpose-built sleep laboratory in the School of Sport and Exercise Sciences which was sound- and light-proofed, Figure 5.1.

Figure 5-1. The sleep laboratory in the School of Sport and Exercise Sciences.

5.3.3 Study protocol

ORIENTATION DAY

In the orientation, participants met the researcher to discuss the study and to confirm their eligibility to participate. They were then given an activity watch that would monitor their sleep pattern during the study nights.

EXPERIMENT DAYS

On each day, participants were asked to wake up at 7 am, and they arrived at the laboratory at 12 pm. Once they were comfortable and felt ready, they performed the encoding task, which is described in section 5.3.4. Afterward, several electrodes were attached for polysomnography recording according to the 10-20 system described in section 5.3.7, a procedure that took around one hour allowing for an offline consolidation widow. Once the electrodes were attached, and when the participants were ready, they did the first retrieval task, which is described in section 5.3.5.

Once that was done, the participants had a light snack and were given the opportunity to go to the toilet. The lights were dimmed to 10 lumens at 2 pm and the participants were left in bed. The researcher was monitoring the electrodes output from a separate control room. After the nap, a second retrieval was done. The schematic of the study protocol is presented in Figure 5.2.

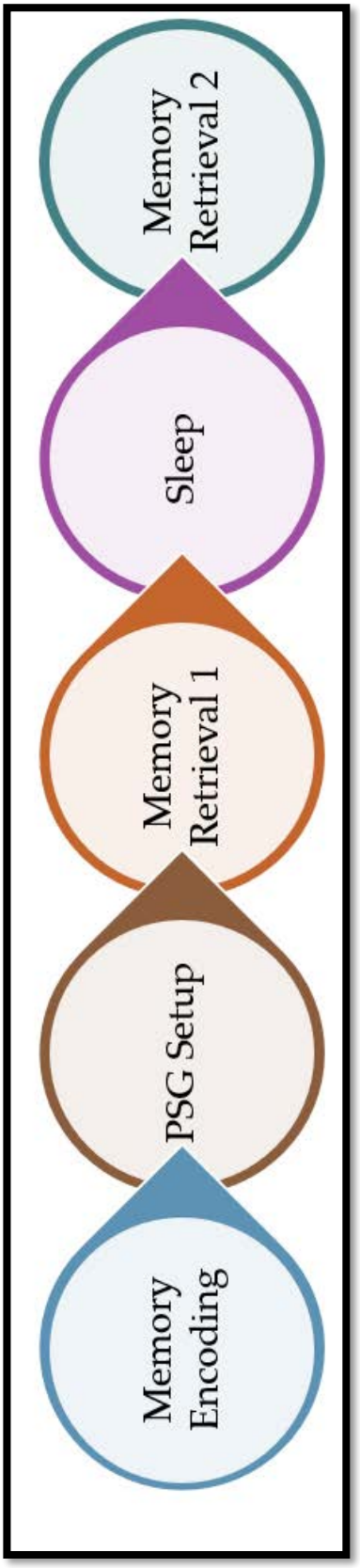

Figure 5-2. A schematic diagram of the study protocol.

Control day: No intervention was done in the control visit and participants were left to sleep for two hours. A few audio stimuli were played at the last few minutes of the nap before participants were woken up, with the intent of blinding the participants to the protocol and to establish their arousal threshold to aid in the fragmentation on the following day.

Fragmentation day: Fragmentation was initiated after two minutes of stable stage II sleep was reached. The target was to induce a microarousal every two minutes to achieve a fragmentation level similar to the moderate OSA. This was achieved with variable tone durations (two seconds to ten seconds), frequencies (1000 Hz to 2000 Hz) and volume (65 dB to 100 dB). The implemented fragmentation protocol is described in Table 5.1 and the sound files used for the fragmentation are described in Figure 5.3.

Sound Delivery	Sound isolating earbuds
When to start	After two minutes of stable stage II sleep
The Protocol	- 1000 Hz sine-wave auditory tone of two seconds duration and 65 dB was played every two minutes. The frequency was changed every 20 minutes to avoid adaptation. - If microarousal was not elicited with the sound stimulus, subsequent stimuli were tried while increasing the volume in 10 dB increments, up to a maximum of 100 dB. - If that did not cause an arousal, the three seconds 65 dB audio was used, and the volume was increased in 10 dB until 100 dB was reached. - If that did not cause arousal the five-second file was used in a similar manner. - At occasions, especially during REM, the ten-second files with their variable frequencies were used. -Once an arousal was noted, the next stimulation was chosen based on the sleep stage that appears on the EEG after arousal.
When the participant woke up	The sound was stopped until stable stage II sleep was achieved, and fragmentation was initiated with the lowest settings.

Table 5-1. The fragmentation protocol used in the experiment.

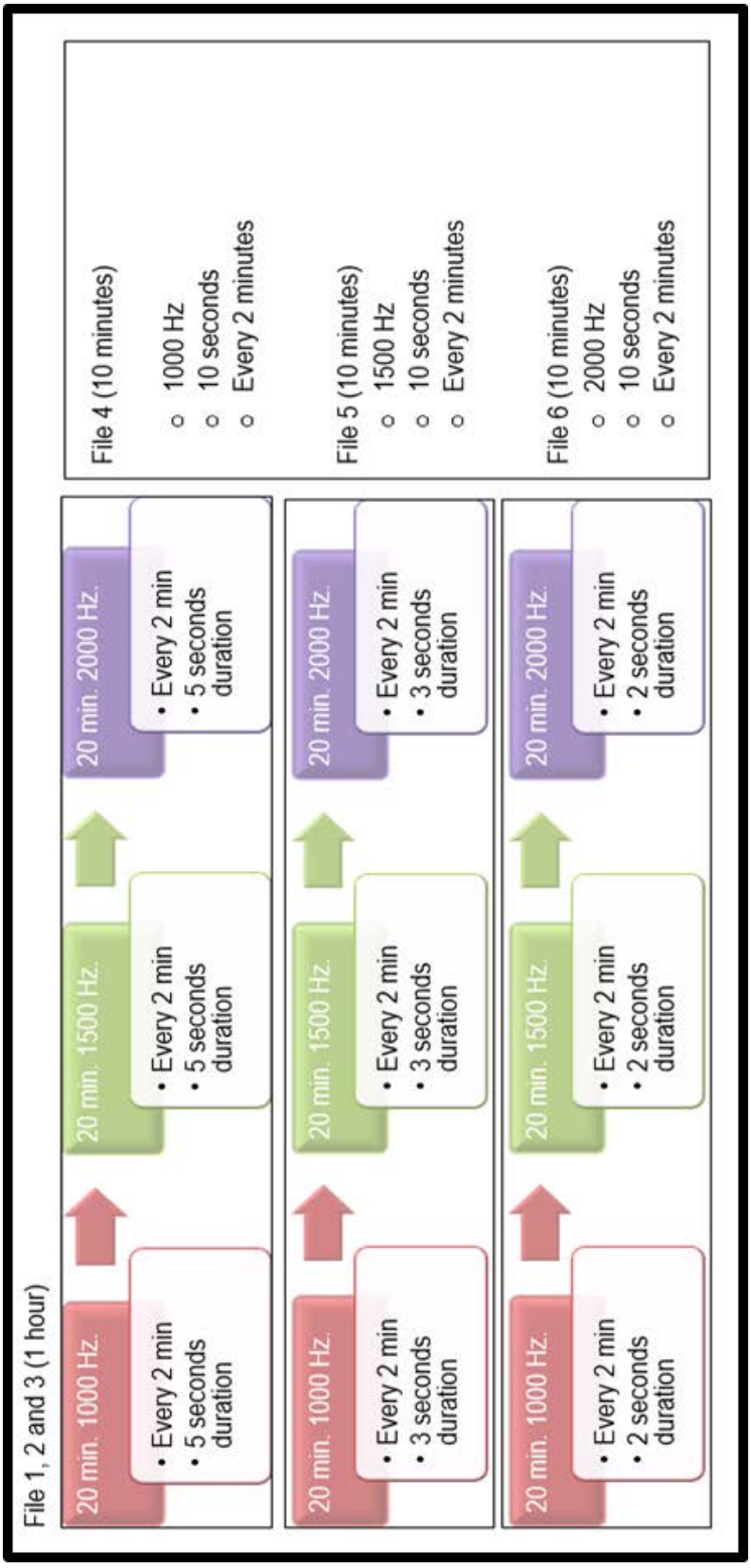

Figure 5-3. Description of the audio files used for the fragmentation.

5.3.4 Encoding task

Participants were presented with 60 adjectives from a pool of 270 adjectives together with one of four possible pictures; two of these pictures were objects (from a pool of 60) and two were scenes (from a pool of 60). This arrangement guaranteed a unique list for each participant that was not repeated between the control and the fragmentation. The participant's task was to indicate how well the adjective described the picture; the left arrow key was for 'yes, adjective and picture DO match' and the right arrow key was for 'no, adjective and picture DO NOT match'. Each adjective/picture combination remained on the screen for four seconds, and the participants were supposed to give their answer within this time for the response to be valid. Stimuli were created and displayed using MATLAB (MathWorks, Sherborn, MA) in combination with Psychophysics Toolbox extensions (Brainard, 1997), Figure 5.4.

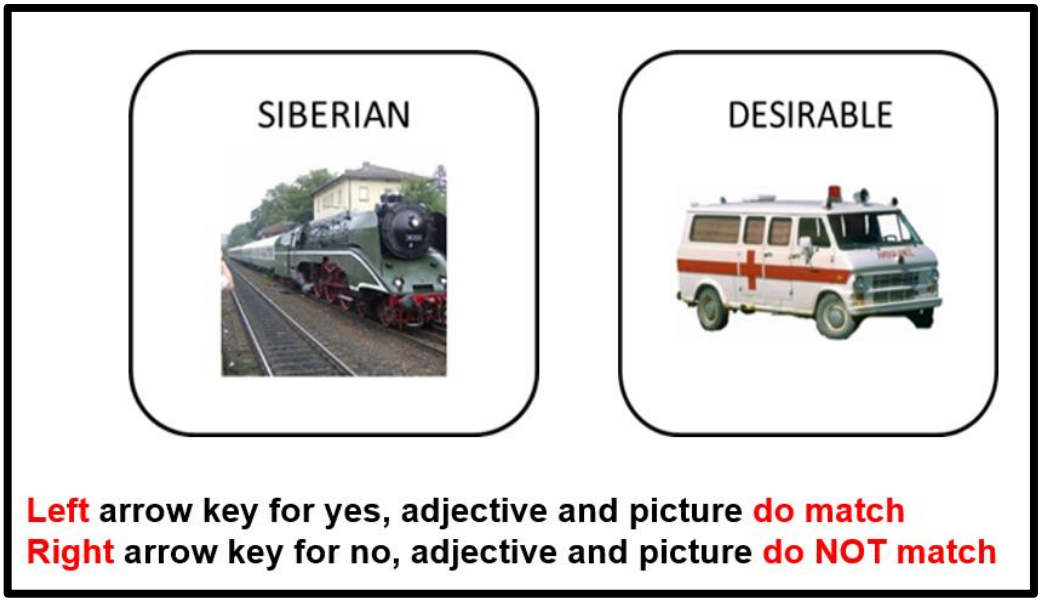

Figure 5-4. The Encoding Task for the fragmentation study.

5.3.5 Retrieval task

During retrieval, participants saw the adjective on the screen for two seconds and were then presented with the response scheme in Figure 5.5.

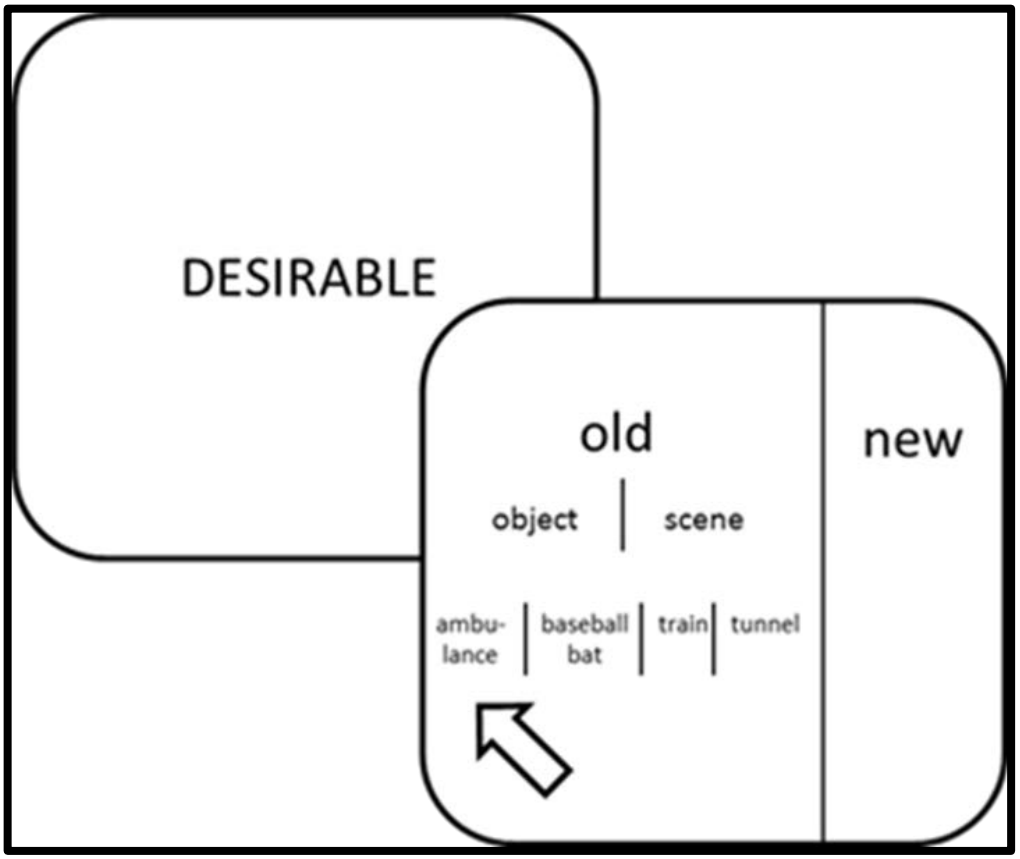

Figure 5-5. Response scheme of the retrieval task for the fragmentation study.

In that scheme shown above, participants had to indicate whether or not they remembered the adjective, and if so, to what detail. Participants were encouraged not to guess; instead, they were supposed to answer to the best of their memory. They either remembered only whether the adjective was old or new with no

accompanying details (item memory), the classification alone (object/scene) or the exact picture that came with the adjective (source memory).

5.3.6 Data Processing

The answers given by participants during the retrieval fell under one of four categories derived from the signal detection classification (Van Der Helm et al., 2011), which is described in Table 5.2.

	Adjective was presented during encoding (old)	**Adjective was not presented during encoding (new)**
Participants indicate that they have seen adjective before (old)	Hit	False Alarm (FA)
Participants indicate that they have not seen the adjective before (new)	Miss	Correct Rejection (CR)

Table 5-2. The signal detection classification.

The change in HIT response before and after the nap was calculated in both control day and fragmentation day to find the effect of fragmentation on memory performance.

Encoding and retrieval tasks were both discussed in detail with the participants prior to the task and a practice session was conducted before the actual task. The practice session was standard, and it had 30 adjectives, two scenes and two objects that were not included in the testing sessions. This process insured the activation of the explicit learning system, which is activated when learning happens with conscious intention for later retrieval. Explicit learning is linked with the activation of the sleep-dependent consolidation (Diekelmann et al., 2009).

5.3.7 Electrodes attachment

Brain activity was measured using electroencephalography (EEG) attached using the simplified 10-20 system as described in Table 5.3 and illustrated in Figure 5.6. Eye movement was measured by electrooculography (EOG), muscle activity was recorded by chin electromyography (EMG), and oxygen saturation and heart rate were measures by a pulse oximeter. Gold-plated electrodes were used for EEG, EOG and EMG. The skin was prepared with alcohol and Nuprep scrub before the electrodes were attached with the Ten20 conductive paste, then secured with either tape or gauze with extra paste.

1	Measure over the centre line of the scalp, from the Nasion (bridge of the nose) to the Inion (occipital bulge)	
2	Measure 50% of the distance	1st step to locate Cz
3	Measure 10% of the distance from Nasion toward the centre of the head	Initial Fpz
4	Measure 10% of the distance from Inion toward the centre of the head	Initial Oz
5	Measure from the right preauricular point to the left preauricular point	
6	Measure 50% of the distance	2nd step to locate Cz
7	Measure 10% of the distance from the left preauricular point toward the centre of the head	T3
8	Measure 10% of the distance from the right preauricular point toward the centre of the head	T4
9	Measure 50% of the distance between T3 and Cz	C3
10	Measure 50% of the distance between T4 and Cz	C4
11	Make a line across the nose to cross with Fpz line	True Fpz
12	Measure head circumference through Fpz, T3, T4 and Oz	
13	Measure 50% of head circumference	True Oz
14	Measure 5% of head circumference to the left of Oz	O1
15	Measure 5% of head circumference to the right of Oz	O2
16	Measure 5% of head circumference to the left of Fpz	Fp1
17	Measure 5% of head circumference to the right of Fpz	Fp2

Table 5-3. Steps to locate Fp1&2, C1&2, O1&2 using the simplified 10-20 system.

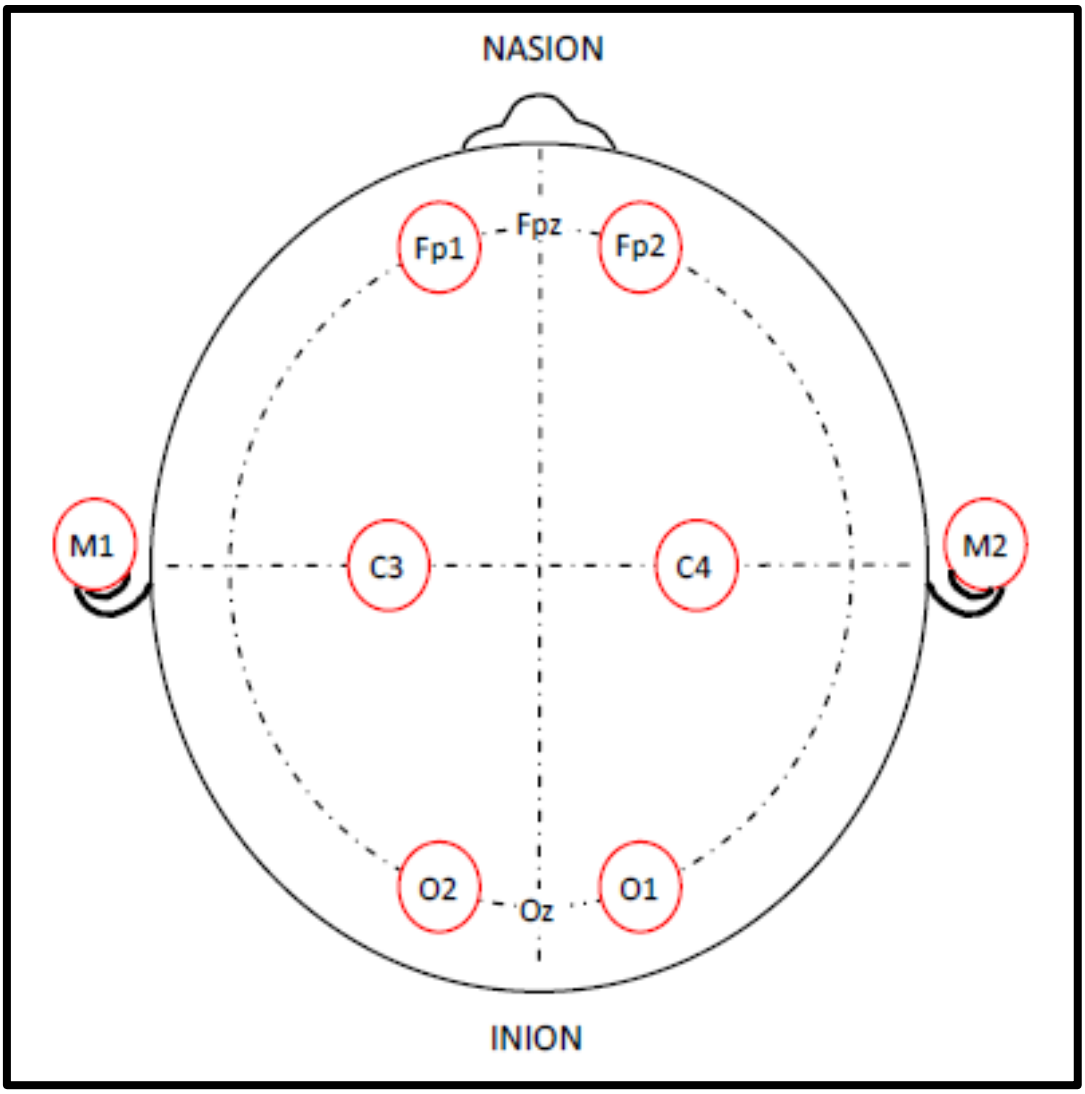

Figure 5-6. EEG electrodes placement using the simplified 10-20 system A picture with open access.

EOG was recorded for right and left eyes; both electrodes were located 1 cm away from the corner of the eye. The left EOG electrode was placed 1 cm down from that point, and the right EOG electrode was placed 1 cm up. EMG was recorded from 2 chin electrodes.

Two electrodes were placed on the mastoid bone on both sides and were used for offline montage. Cz was used as reference and Fpz was used as a ground. All signals were processed through an Embla titanium system and analysed by the remlogic software.

5.3.8 Sleep scoring

Sleep was staged in 30-second epochs according to the AASM criteria as described in the general methods chapter.

5.3.9 Microarousals

Microarousals were identified based on the AASM criteria and were defined as shifts in EEG frequency that produced alpha, theta and/or any frequencies greater than 16 Hz that lasted for at least three seconds in NREM sleep. In REM sleep, arousals were identified by an increased EMG tone that lasted for one second minimum (Iber et al., 2007).

5.3.10 Statistical Analysis

A paired sample t-test and two-way repeated measures ANOVA were used to analyse the data. Results are presented as mean ± SD, unless mentioned otherwise. In the t-test, there were no outliers and the data were normally distributed unless mentioned otherwise. For the ANOVA, there were no outliers, and data were normally distributed as assessed by Shapiro-Wilk's test of residuals ≥ 0.05 unless mentioned otherwise. When Mauchly's test of sphericity indicated that the

assumption of sphericity for the two-way interaction had been violated, the data were interpreted using the Greenhouse-Geisser adjustment.

5.4 RESULTS

5.4.1 Participants

21 participants took part in this experiment, three of whom were males. The average BMI was 23 kg/m² ± 1.71; the average age was 20 years ± 1.48. One participant was excluded from analysis as she neither slept during the control day nor returned for the fragmentation day. Another participant generated the behaviour data without the EEG data because of bad EEG signal quality.

5.4.2 Actiwatch results

Because of the nature of the data, a non-parametric exact sign test was used to compare the median TST and SE before the control night and the fragmentation night. Table 5.5 shows the median total sleeping time (TST) in hours and sleep efficiency (SE) % ± SD on the night before the control and the night before the fragmentation visit. On average, participants had the same sleeping duration before testing with a slightly worse sleep efficiency before the fragmentation. The data show a median increase in TST before the control night, which is a non-significant difference ($p = 0.648$), and a median increase in SE before the fragmentation night, which was also not significant ($p = 0.815$).

	Control	Fragmentation
Total sleep time (hours)	5.45	5.40
Sleep efficiency (%)	79.50	80.31

Table 5-4. Total sleep time and sleep efficiency (median) in the night prior to control and fragmentation.

5.4.3 Sleep stages

There was one outlier, who had a studentized residual value of 3.46, which was excluded from the analysis. The data were not normally distributed as some values of the Shapiro-Wilk's test of residuals were less than 0.05. Mauchly's test of sphericity indicated that the assumption of sphericity had been violated for the two-way interaction, $\chi2(2) = 34.02$, $p \leq 0.001$, so the interpretation was done using the Greenhouse-Geisser adjustment.

Table 5.6 presents the sleep architecture during both the control and fragmentation naps. Fragmentation led to increased time awake and stage I sleep. On the other hand, it led to decreased stage III and REM sleep but did not affect stage II. A two-way repeated measures ANOVA with ten variables was used for the analysis; intervention was the first within subjects' factor and it had two levels (control and fragmentation) and the sleep stages was the other within subjects' factor and had five levels (wake, stage I, II, III and REM). The difference in the sleep stages did not reach a statistical significance in the two-way repeated measures ANOVA; $F(2.34, 39.79) = 1.288$, $p = 0.29$.

	Control ± SD	Fragmentation ± SD
Awake %	25.82 ± 13.22	28.41 ± 15.0
Stage I %	13.70 ± 6.15	18.50 ± 5.51
Stage II %	37.53 ± 11.51	37.46 ± 12.71
Stage III %	13.30 ± 8.11	8.45 ± 9.12
REM %	9.70 ± 7.34	7.16 ± 7.65
Total Sleep Time in hours	1.80 ± 0.46	1.76 ± 0.34
Number of Arousals	31.05 ± 17.29	57.11 ± 12.32

Table 5-5. sleep architecture in both control and fragmentation naps.

In contrast, a paired sample t-test showed that the fragmentation caused a statistically significant increase in the number of arousals compared to the control nap, with a mean difference of 25.7 arousals (SE =3.8), $t(18) = 6.6$, $p < .001$.

5.4.4 Memory performance

CHANGE IN HIT (ITEM MEMORY)

A paired t-test showed that the fragmented nap did not affect the HIT response, showing a mean increase of 1% ± 0.1 SD reflecting data noise, while the non-fragmented nap decreased the HIT response by 4.7% ± 0.08 SD. This mean difference of 5.7 % (SE 0.028) approached but did not reach a statistical significance; $t(19) = 2.025$, $p = 0.057$, Figure 5.7.

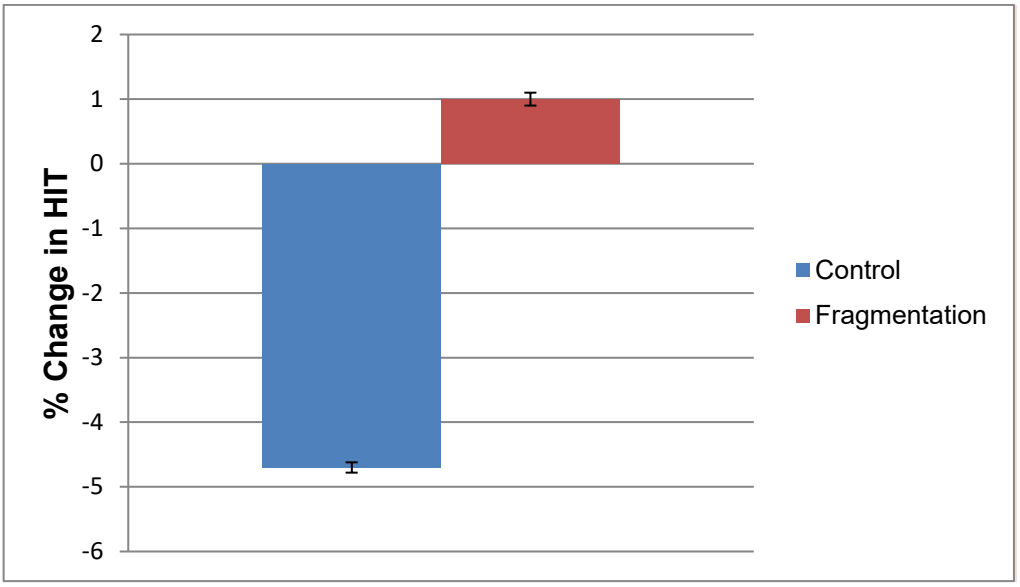

Figure 5-7. The effect of fragmentation on item memory.

CHANGE IN HIT (SOURCE MEMORY)

A paired t-test showed that fragmented nap decreased the HIT response by mean of 2.2% ± 0.089 SD while the non-fragmented nap increased the HIT response by 1.8% ± 0.084 SD. This mean difference of 4 % (SE 0.0197) approached but did not reach a statistical significance t (20) = -2, p = 0.059, Figure 5.8.

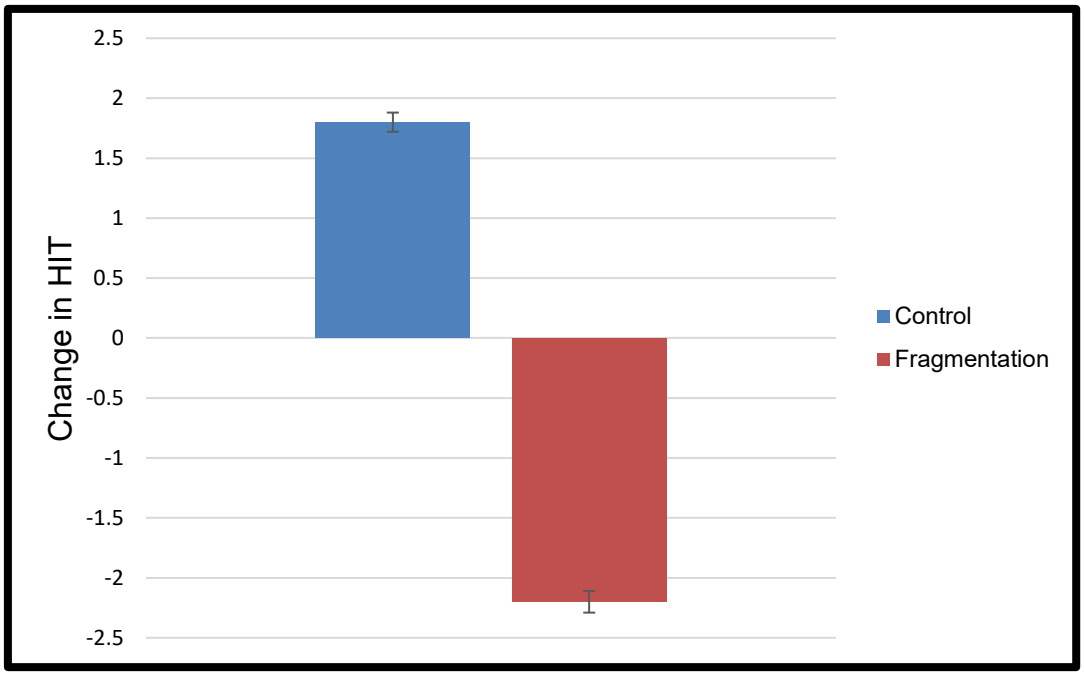

Figure 5-8. The effect of fragmentation on source memory.

MEMORY DISSOCIATION

Noticing fragmentation's opposite effects on the item and source memory, a 2x2 repeated-measures ANOVA was used to examine fragmentation's effect on the two levels of memory: condition (control/fragmentation) and memory level (item/source).

The repeated measure ANOVA revealed a statistically significant two-way interaction between the condition and the memory level: $F(1, 19) = 4.77$, $p = 0.042$. The main effect of the memory level was statistically significant ($f[1,19] = 5.11$, $p = 0.036$), while the main effect of the condition approached but did not reach a statistical significance ($F[1, 19] = 4.257$, $p = 0.053$), Figure 5.9.

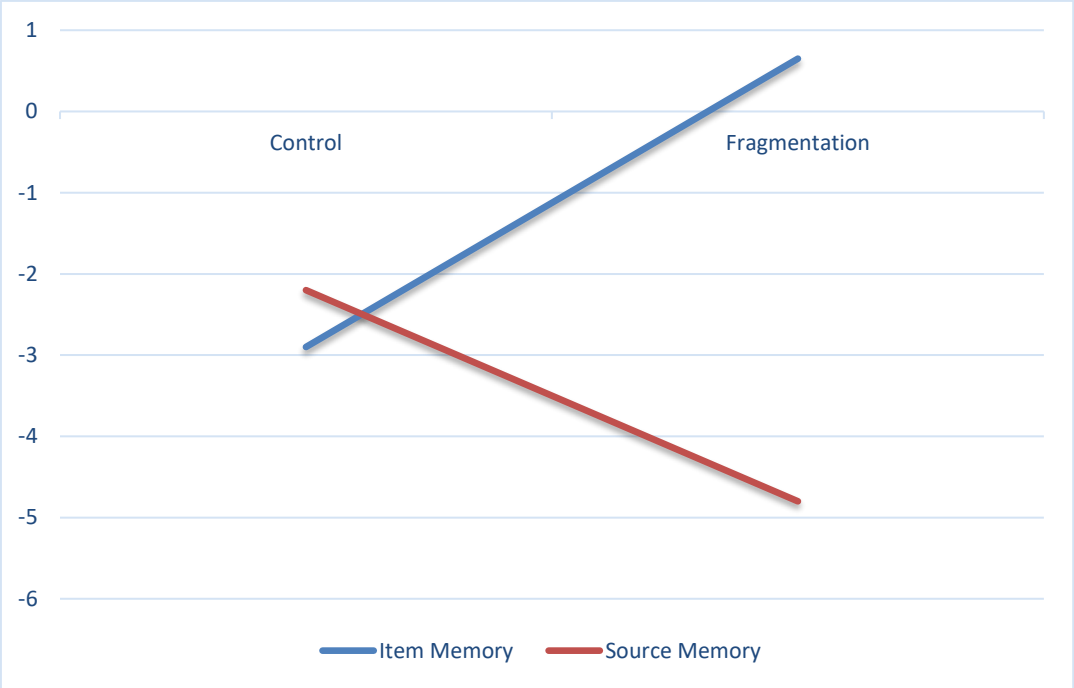

Figure 5-9. The two-way interaction between the experimental condition and memory level.

MAIN EFFECT OF MEMORY LEVEL

The difference between the performance in the item memory compared to the source memory in the control was 0.7% ± 1.3 SE which was not statistically significant p = 0.588. Fragmentation on the other hand caused a difference of 5.5% ± 2.4 SE, which was a statistically significant effect p = 0.036.

MAIN EFFECT OF CONDITION

The change in the HIT for item memory between the control and the fragmentation was 3.6% ± 1.72 SE. A change that approached but did not reach statistical significance p = 0.053. In contrast, the change in the HIT for source memory between the control and the fragmentation was 2.6% ± 2.0 SE. A change that was not statistically significant p = 0.203.

5.4.5 Correlation between sleep stages and memory performance

A Spearman rank correlation coefficient correlation was used to test for the correlation between the % of sleep spent at each sleep stage and memory performance. This test was selected due to the non-linearity of the data, and the results are summarised in Table 5.6.

	Item Memory	**Source Memory**
Stage I	0.17	- 0.17
Stage II	- 0.08	0.1
SWS	0.002	0.22
REM	0.21	- 0.006

Table 5-6. The correlation between the sleep stages and the memory performance

The results show that there were no observed statistically significant sleep-stage and memory performance correlations (all $p > 0.05$) although a post hoc power analysis showed that this study had high statistical power, with a power estimate of 0.93. Yet, although not statically significant, a positive correlation can be observed between SWS and the performance in the source memory.

5.5 DISCUSSION

This experiment investigated the effect of sleep fragmentation on sleep architecture and examined fragmentation's effect on episodic memory. This study led to two main findings: First, fragmenting the nap with audio stimulation did not affect the sleep architecture, but it significantly increased arousals. Second, this increase in arousals affected episodic memory in two different ways, maintaining the performance of item memory and negatively affecting the performance of source memory.

5.5.1 Episodic memory

As mentioned in the introduction, the episodic memory is a memory within a context which has two components: item and source memory. The findings of the study showed that fragmentation maintained the performance in the item memory but negatively affected the source memory performance.

The mechanism by which fragmentation affects memory is open for exploration. The current evidence suggests that item memory is processed via the

perirhinal and Para-hippocampal cortices. The associative elements, by contrast, appear to be bonded to the item by the hippocampus. Finding that the source memory was negatively affected by fragmentation support the notion that the fragmentation affects the hippocampus itself and disturbs its function (Tartar et al., 2006). This study's findings are in line with the other studies which indicated that the consolidation of the hippocampus-dependent associative memory depends on sleep more than the consolidation of the item memory, and that justifies the impact of disturbed sleep on the source memory, which is a type of associative memory (Mayes et al., 2007, Staresina and Davachi, 2008, Lau et al., 2010, Van Der Helm et al., 2011). Furthermore, the positive correlation found between the SWS and the performance of the source memory is in line with other research that emphasises on the essential role of SWS in the consolidation of hippocampus-dependent memory consolidation (Diekelmann and Born, 2010). As this is a nap study, the time spent in SWS is relatively short compared to an overnight sleep. In addition to that, some participants did not reach the SWS at all. This can explain the lack of statistical significance in the correlation found in this study.

The dissociation between the item memory and the source memory found in this study can explain the mismatch between the subjective feeling of bad memory repeatedly reported by OSA patients (cognitive complaint) and the results of the objective assessment that show no significant impairment (Daurat et al., 2010, Vaessen et al., 2015). The current assessment tools for memory may only be capable to assess the item memory, while the fragmentation affects the source memory. Furthermore, this study shows that sleep fragmentation *per se*, rather than

the IH, is responsible for OSA's negative impact on memory. Alternatively, they might have a combined effect, which will further compromise the memory.

5.5.2 Proposed effect of fragmentation

There is an ongoing debate about fragmentation's effects on memory (Cote et al., 2003). Some evidence suggests that the distribution of the sleep cycle is the main influence on memory disturbance. These studies link the SWS characteristics with the processing of the declarative memory through the hippocampus and argue that the memory is affected by the reduction of spindles and ripples (Rasch and Born, 2013). This argument does not align with the findings of the current study as the performance was affected although the sleep cycle was maintained.

The other set of evidence holds the lack of sleep continuity and the increase in alpha EEG responsible for the memory disturbance (Cote et al., 2003, Daurat et al., 2008, Andreou et al., 2014). The results of this study support the second notion, as the fragmentation protocol did not alter the sleeping cycle, yet by causing 22-27 arousals/hour the effect of the fragmentation protocol was reflected on memory performance.

The way in which fragmentation maintained the item memory compared to the non-fragmented nap is not clear. A study that used pink noise for audio stimulation to trigger slow oscillation and spindle activity in the brain successfully enhanced the performance in a paired-associates learning task (Ngo et al., 2013). The EEG data

from the fragmentation protocol cannot be analysed in the same way as that experiment, so the possibility that the audio stimulation caused the same effect can be only left for speculation.

5.5.3 Conclusion

This chapter showed a dissociation between the effects of sleep fragmentation on the item memory versus those on the source memory. Furthermore, it showed that these effects were caused by lack of sleep continuity even though the sleep cycle was maintained. Finally, the results of this study show the positive correlation between the time spent in SWS and the source memory performance.

As already established, memory formation is a complex process affected by several factors; the type of memory and the way it is encoded and consolidated as well as the sleep stages and their characteristics contribute to memory formation. Therefor the paradigms used for memory testing are complex and need to be designed carefully as every factor can influence the results. The current evidence suggests that the ongoing debate about which stage of sleep affects which memory may be better directed to how each stage affects the memory as all the sleep stages seem to contribute to memory formation.

Choosing a purely episodic task causes some challenges as some of the used paradigms test more than one memory type at once and that leads to conflicted findings. The paradigm used in this study might have succeeded in measuring the

episodic memory and the dissociation found can help in the memory assessment for patients with OSA and the other conditions that lead to sleep fragmentation. The findings of this study could be confirmed by establishing a standard way to evaluate the source memory and the item memory and evaluate the performance difference for people with sleep fragmentation in future research.

5.5.4 Study limitations

This study have several results which approached but did not reach statistical significance and this could be due to the sample size of the study. Having more participants could have led to more conclusive findings. However, a post hoc power analysis suggests that the study have a good power. Nonetheless, this kind of power analysis should be taken with caution as post hoc power analysis are strongly critiqued.

Moreover, not being able to discuss how sleep fragmentation maintained the item memory could have been avoided by having an awake arm in this experiment. Comparing the effect of wakefulness vs. fragmented nap in addition to the control nap could have gave a clearer picture about this finding, and that could be a good idea for future research. Also, not randomizing the fragmentation and control protocols can be considered a limitation for this study but this arrangement was chosen so participants can get used to the laboratory environment and setup during the control day to minimize the spontaneous arousals during the fragmentation day.

A quote by Tulving may be the best end to this chapter: ‘We have succeeded in adding more to what there is to know than what we do know’ (Tulving, 1995).

6. EFFECTS OF TARGETED MEMORY REACTIVATION ON THE ASSOCIATIVE MEMORY

6.1 INTRODUCTION

6.1.1 Overview

As already established in the general introduction, there is a strong evidence that sleep plays an essential role in the consolidation of memory (Ellenbogen et al., 2006, Gais et al., 2006, Takashima et al., 2006, Rasch and Born, 2007, Rasch et al., 2007, Wilhelm et al., 2011). Slow wave sleep (SWS) in particular, which includes slow oscillations and sleep spindles, is a vital component of the memory consolidation process, especially for emotionally neutral declarative memories (Mölle et al., 2011, Moelle and Born, 2011, Staresina et al., 2015).

The benefit of SWS on memory consolidation was established based on several observations: the positive correlation between SWS duration and memory performance (Marshall et al., 2006, Diekelmann et al., 2012), the increased spindle density after extensive learning (Gais et al., 2002) and the observed neural replay during SWS in which the hippocampus and the neocortex show the same patterns seen during encoding (Peigneux et al., 2004).

According to the active system consolidation hypothesis mentioned in the general introduction, the newly encoded memory traces are reactivated and spontaneously replayed during offline consolidation. This replay triggers a form of neural plasticity, which results in transferring the memory trace from the temporary hippocampus store to more stable stores in the neocortex, where it is associated with existing knowledge (Rasch and Born, 2007, Born and Wilhelm, 2012, Rasch and Born, 2013). In fact, strong evidence suggests that consolidation during sleep facilitates the generalisation and the integration of knowledge (Landmann et al., 2014), a finding that is less evident during wakefulness (Tamminen et al., 2010).

The current evidence show that this replay can be triggered by external factors, and that led to the development of the targeted memory activation paradigms (TMR) (Rasch et al., 2007, Rudoy et al., 2009, Diekelmann et al., 2011, Oudiette et al., 2013, Cairney et al., 2014, Schreiner and Rasch, 2014, Groch et al., 2016, Cairney et al., 2017). TMR was successfully used to facilitate the memory consolidation process for several memory types. The focus of this chapter will be about the effect of TMR on the consolidation of the declarative memory. As will be discussed below, the TMR paradigms work by linking memories to cues (as odour or sound) and playing half of the cues or playing the cues for half of the participants during SWS or wakefulness, then comparing the recall of cued memories to that of non-cued memories.

The leading TMR study exposed participants to a rose scent while they learned object/location association. The experiments then presented the cue during

three different conditions; wakefulness, SWS and REM stage and then tested the recall accuracy after the cueing. The study found that participants who were cued during SWS gave more accurate answers in the retrieval, which was not the case when the cue was delivered during REM or wakefulness. The lack of effect was also shown when the cue was presented during SWS but was not previously associated with learning (Rasch et al., 2007).

Furthermore, another study using the object/location association paradigm linked the encoded memory with sound cues that represented each object. The study then tested for retrieval accuracy after using the associated sound to reactivate the memory. Cueing was done either during wakefulness or SWS , and the study found that the retrieval of the cued objects did improve compared to the non-cued objects in the napping group, but not the waking group (Rudoy et al., 2009).

The lack of positive effects of cueing during wakefulness was noticed in another object/location study, which, in addition to the behavioural outcome, showed activation of different brain regions in response to TMR during wakefulness than appeared during sleep. An fMRI scan showed that TMR in wakefulness activates the prefrontal cortex while in sleep it activates the hippocampal and posterior areas of the brain. This finding can explain the different effects of cueing during wakefulness as opposed to cueing during sleep. Furthermore, the study suggested that TMR during wakefulness destabilises the memory trace and due to the active brain status during the wakefulness predispose it to interface, therefore leading to worse performance in the retrieval (Diekelmann et al., 2011).

However, another study showed the possibility of improving performance on object/location task with TMR during wakefulness regardless of the previous findings (Oudiette et al., 2013). This difference in the findings could be due to the nature of the encoding process, as the second experiment linked the performance with a reward promise, which could lead to an enhanced consolidation process (Fischer and Born, 2009).

Moreover, quiet wakefulness after encoding showed an improvement in performance when compared to equal time spent playing a game, implying a positive effect on consolidation (Dewar et al., 2012), a finding that was confirmed by another study (Dewar et al., 2014). These findings could be explained by the opportunistic consolidation theory, which proposes that the hippocampus utilises any offline time to consolidate newly encoded memories (Mednick et al., 2011). Additionally, it is proposed that wakeful consolidation is optimised when the brain is in idle condition such as daydreaming or involvement in a non-demanding activity (Brokaw et al., 2016).

The evidence of memory consolidation during wakefulness, together with the conflicting results about TMR during wakefulness, opens this topic for more research about the possibility of TMR benefits during wakefulness and the mechanism of action if it was achievable.

Several studies have established that TMR has a positive effect on memory consolidation if applied during sleep, but the mechanism of action and the extent of

the benefits are still being explored. The positive correlation between the benefits of TMR and the duration of SWS, which includes both low oscillations and spindle activity suggests a causal effect (Cairney et al., 2014, Farthouat et al., 2016, Cairney et al., 2018). Furthermore, it is also suggested that the TMR results are affected by the strength of encoding, as neither weak memories nor very strong memories benefited from TMR (Creery et al., 2015).

In terms of cueing, comparing verbal cues to non-verbal cues during SWS found that both acoustic stimuli resulted in the same positive effects during TMR, although there were initial thoughts that verbal cueing needed a more complicated cognitive process and might not have the same positive effect during TMR (Cairney et al., 2017).

Nowadays, it is established by a wealth of evidence that when a sound is linked to a memory during encoding, then played during SWS, performance will improve for the cued memory (direct cue-memory association) as shown in the previous sections. The focus of the research is now expanded from the direct cue-memory association to linking the cued memory to additional memories, and seeing if TMR can enhance the consolidation of the linked memory in addition to the directly linked memory (Cairney et al., 2016). For instance, a nap study has linked pictures' locations to words, then pictures' locations to sounds, then played the sounds during the TMR protocol. The study then tested the recall accuracy after the nap of both pictures' location and words. The study found benefits in the directly cued association (pictures' locations) but not in the linked memory (words). Although the study did not

find benefits in the linked memory, it opened the door to more research in this area by implying the importance of REM and longer sleeping cycles in the consolidation of linked memories (Cairney et al., 2016).

Continuing this research, another study examined the cueing effect on linked memories directly after an offline period of either wakefulness or napping, then when followed by an overnight sleep. Like the previous study, this experiment found no difference in the retrieval of the linked memory when the recall was performed directly after the offline period. Interestingly, the study showed that the TMR had a clear effect on the linked memory which appeared only after a night of sleep (Cairney et al., 2018).

This overnight improvement is supported by the findings of another study on the effects of offline consolidation during wakefulness and sleep on establishing the relational memory, which is an essential ability of human memory that links information. The study compared performances at three time points after learning: 20 minutes, 12 hours (waking or overnight sleep) and 24 hours. The study showed a superior performance after the overnight sleep and the 24-hour period compared to the post 20-minute test (Ellenbogen et al., 2007). Although the nap effect was not tested in this experiment, the study showed that longer SWS and REM sleep is essential to facilitate the establishment of the relational memory and provide more plasticity than can possibly be achieved with short naps. This suggestion is also supported by the findings of other studies (Cairney et al., 2016, Batterink et al., 2017, Tamminen et al., 2017, Cairney et al., 2018).

6.2 OVERVIEW OF THE STUDY

This study investigated the effects of using non-verbal cueing to trigger memory reactivation on linked memories. Each participant learned object-scene-face associations, with each object being paired with a specific sound which is related to the object. Participants who met a predefined encoding requirement, which will be discussed later in this chapter, proceeded to the next stage of the experiment, in which the sounds were used to cue the memory during either a nap or wakefulness. Memory performance was tested before cueing, after 30 minutes of cueing and after an overnight sleep.

6.3 HYPOTHESIS

We hypothesised that memory performance would be better for the sequences that were cued compared to the sequences that were not cued, particularly in the napping group. We also hypothesised that the performance of the cued memory would further improve after a night of sleep in both groups.

6.4 MATERIALS AND METHODS

The study was approved by the local ethics committee (University of Birmingham Ethical Review Committee ERN-14-1379). Subjects were recruited via emails and word of mouth. After receiving detailed information about the study, informed written consent was obtained from all participants.

6.4.1 Participants

58 participants (41 female), mean age ± SD 21 years ± 2.5, were recruited for the study. Participants had no history of neurological or psychological illness or sleep problems; they also had normal to corrected hearing and eyesight, and they agreed to abstain from caffeine (if they were in the napping group) and alcohol for 24 hours before the experiment. 19 participants were excluded from the study because they did not meet the encoding requirement, were not able to complete the experiment, kept waking up during the cueing in the napping group, or because of technical complications. The final sample size was 39 participants, 18 in the waking group (5 male, mean ± SD age, 22 ± 3.7 years) and 21 in the napping group (7 male, mean ± SD age, 20 ± 1 years). Each participant filled the Pittsburgh Sleep Quality Index (PSQI) to assess the quality and pattern of their sleep at the beginning of the experiment.

6.4.2 Stimuli

90 pictures of objects were selected from a prior study (Brady et al., 2008). Each object was paired with a representative sound that was 500 ms in duration and was selected from http://www.audiomicro.com. Each object was then linked to a face (Barack Obama or Angelina Jolie) and a scene (Macchu Picchu or Westminster Abbey), creating 90 sequences for each participant.

6.4.3 Procedure

The study began at 10 am in the sleep laboratory at the School of Sport and Exercise Sciences in the University of Birmingham. The study started with EEG cap setup then proceeded to the protocol illustrated in Figure 6.1, which will be explained in detail in the following sections.

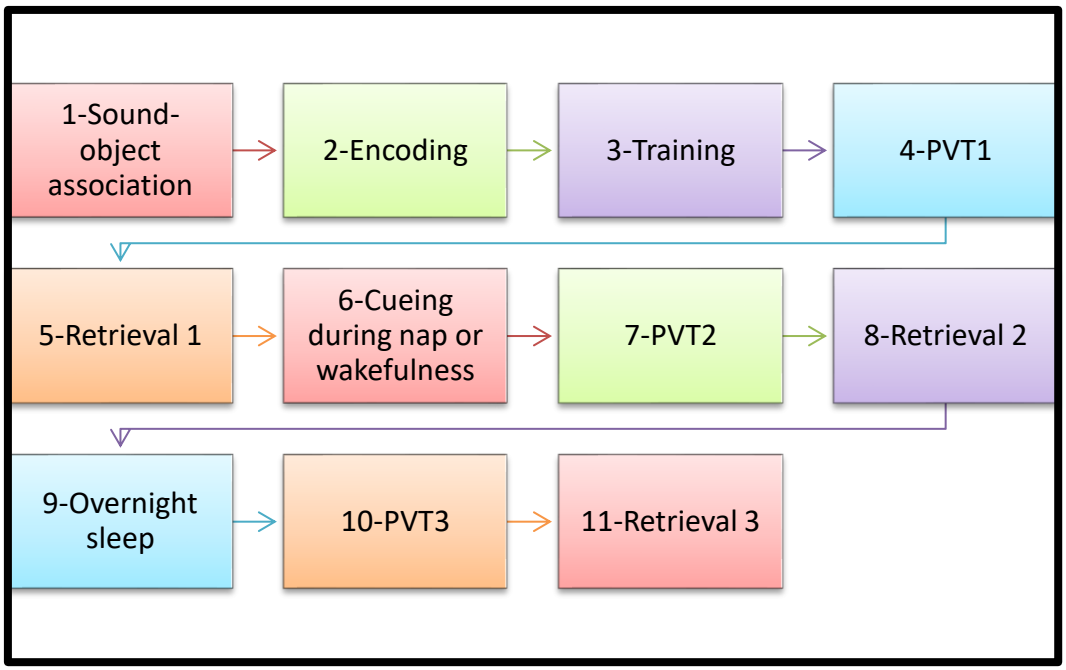

Figure 6-1. The TMR study protocol.

Before proceeding to the protocol details, it is worth mentioning here that each participant practiced the complete protocol with a short version of each task to gain a full understanding of the experiment before starting it, but they were unaware that targeted memory reactivation (TMR) would take place.

6.4.4 Sound-Object association

In this part of the experiment, each participant saw 90 objects and heard an associated sound for each object. All participants were informed that the association they formed would be tested later in the experiment, and they had the chance to repeat the sound as many times as they required to link it with the object, Figure 6.2.

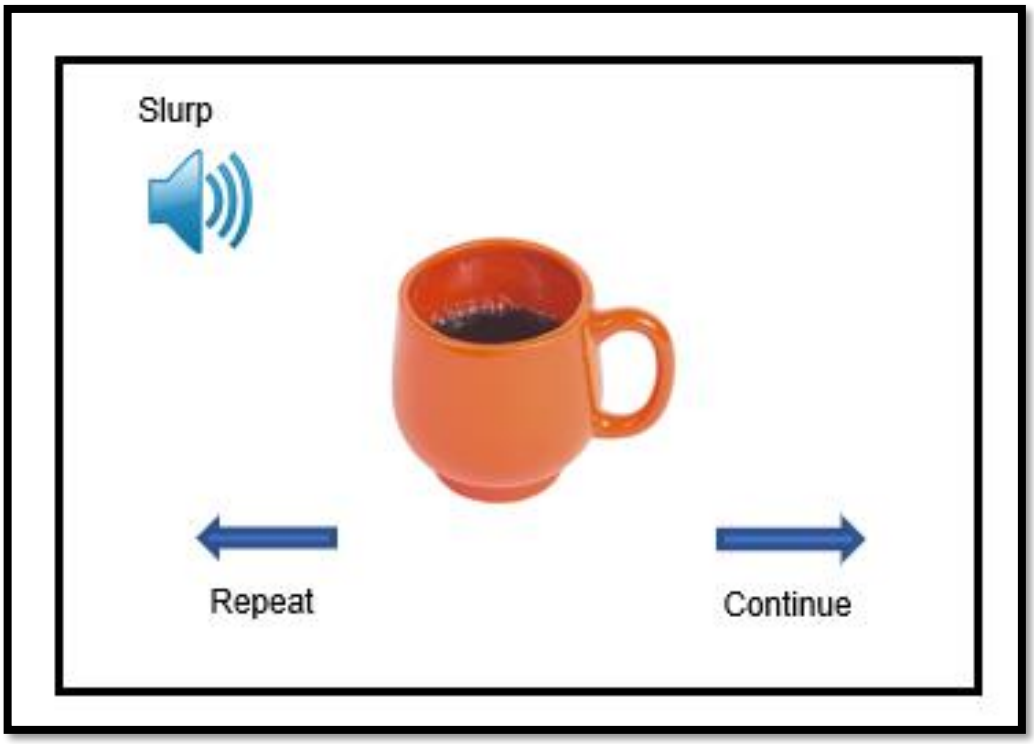

Figure 6-2. The Sound-Object Association Task for the TMR.

6.4.5 *Encoding*

Once the participants formed a link between the object and its sound in the previous task they moved to this task. Each object was presented again with the associated sound in the beginning of this part of the experiment, then followed by a face (Barack Obama or Angelina Jolie), then a scene (Macchu Picchu or Westminster Abbey). The participants then took all the time they needed to create a vivid story that mentally linked the three elements together for 90 sequences, Figure 6.3.

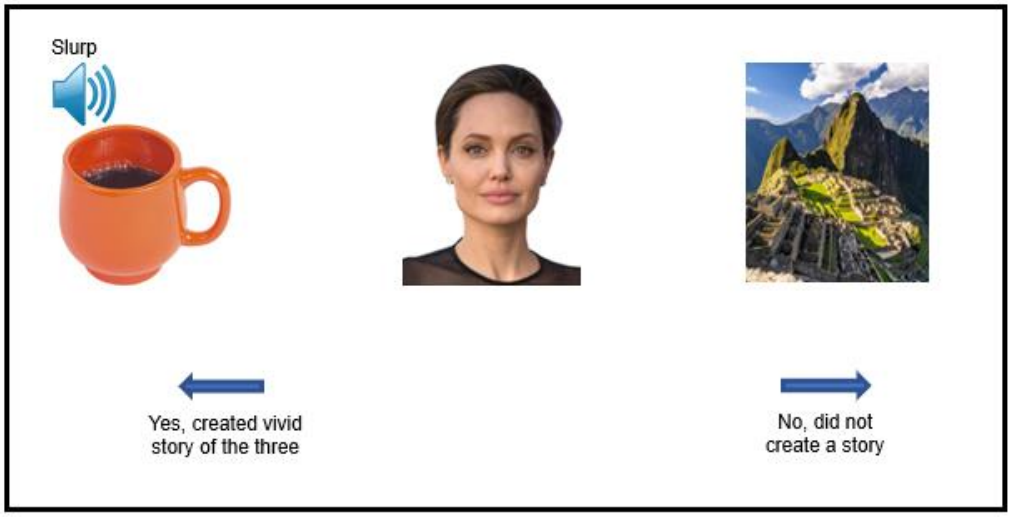

Figure 6-3. The Encoding Task for the TMR.

6.4.6 *Training*

After the encoding of the 90 sequences, participants were presented with the object/sound, followed by 5 s pause giving them a chance to mentally recollect the associated face and scene. They were then presented with the pictures of Barack

Obama (associated with the right arrow on the keyboard) and Angelina Jolie (associated with the left arrow on the keyboard), and they had to indicate either which face was presented with which object/sound or that they did not remember (down arrow on the keyboard). Afterwards, they were presented with the pictures of Macchu Picchu (right arrow on the keyboard) and Westminster Abbey (left arrow on the keyboard), and they again had to indicate either which scene was presented with which object/sound or that they did not remember (down arrow on the keyboard).

When the participant remembered the right sequence, it was not presented again in this part of the experiment, but if they answered wrong for one of the element's or indicated that they did not remember, the sequence kept appearing until they answered correctly. This part lasted until each participant remembered 75 sequences out of the 90.

6.4.7 Psychomotor Vigilance Test (PVT)

This test was done before each retrieval to measure the participant's attention level. The 10-min version of the PVT was used; in which participants were required to press the space key as soon as a fixation cross disappeared from the screen. Their reaction time (RT) was recorded several times, then the average RT for each participant at each PVT time point (1, 2, and 3) was calculated.

6.4.8 First Retrieval (R1)

In this part of the experiment, participants were presented with the sound alone without seeing the object, then they had a 5 s. pause to mentally recollect the sequence. After that, they had to indicate which face and which scene were associated with the sound, and they were also able to indicate if they could not remember the association. Responses were given with the keyboard, as in the training task. No feedback was provided for the answers in this task, and only answers with both the right face and the right scene were considered correct.

After the retrieval, participant took 30 minutes lunch break before proceeding to the TMR part of the experiment, either during a nap or wakefulness. Only participants who remembered at least 40 sequences correctly proceeded to the next part of the experiment.

6.4.9 Cueing during nap

Participants were left to sleep in a sound- and light-isolated room, with speakers located on both sides of the bed, about 1 meter away from the head. Once the participant entered the SWS, cueing was started and continuously played every 5 s ± 200 ms. through MATLAB 2016 (MathWorks Inc., Natick, MA, USA) unless a sign of arousal was noticed. When an arousal occurred, the cueing stopped and then continued when the participant went back to SWS. Only half of the sounds from the correctly retrieved sequences were cued. After two hours, participants were woken

up only when they were in stage 1 or 2, and they were given 30 minutes break before proceeding with the experiment.

6.4.10 Cueing during wakefulness

This part of the experiment started with the participants *playing Bubble Shooter* for 30 minutes then cueing was started afterwards. Participants were involved in a change detection task during cueing, in which they had to count how many times a fixation cross went from light grey to dark grey and type the answer every minute to receive feedback about their response. This task aimed to take their direct attention away from the cueing. Each awake participant was matched to a napping participant and received the same cueing, then spent whatever time remaining from the two hours playing *Bubble Shooter*.

6.4.11 Second Retrieval (R2) and Third Retrieval (R3)

These two parts were similar to R1, while R2 was conducted 30 minutes after the TMR and R3 was conducted the next morning after a night of sleep.

6.4.12 Equipment

Sounds were heard through Advent speakers (model ASP20BK15). A 24″ flat screen monitor (resolution = 1920 x 1080 pixels) was positioned at eye level (~0.5 m from participants). Words and sounds were presented during the learning and recall sessions, and during sleep or wakefulness TMR using the Psychtoolbox-3 software (Brainard, 1997) implemented in MATLAB 2016 (MathWorks Inc., Natick, MA, USA).

EEG was monitored with Brain Vision recorder version 1.21.0004 (Brin Products GmbH).

6.4.13 Statistical analysis

An independent samples t-test was used for the PSQI score and R1, while two-way mixed ANOVA was used for PVT, R2 and R3. The statistical significance threshold was set at $p < .05$ and data were analysed with SPSS statistics 23.

6.5 RESULTS

The data had four outliers, defined by SPSS by 1.5 box length away from the edge of their box using the boxplot method. Two were in the napping group and two were in the waking group, so they were excluded from the analysis. The data were normally distributed, as assessed by Shapiro-Wilk's test of normality ($p > .05$). There was homogeneity of variances ($p > .05$) and covariances ($p > .05$), as assessed by Levene's test of homogeneity of variances and Box's M test, respectively. When Mauchly's test of sphericity indicated that the assumption of sphericity was not met, the Greenhouse-Geisser correction was used for analysis.

6.5.1 PSQI

The wake group had a slightly lower PSQI score ($M = 5.38$, $SD = 2.44$) than the nap group ($M = 5.42$, $SD = 2.36$). An independent samples t-test showed that the difference was not statistically significant ($M = 0.46$, 95% CI [-1.70, 1.61]), ($t(33) = -0.057$, $p = 0.95$).

6.5.2 PVT

A two-way mixed ANOVA was used to test how is the dependent variable (reaction time) is affected by the between subject factor (nap/wake) and the within subject factor (time point 1, 2 and 3). The results showed that there was no statistically significant interaction between the PVT time points (1, 2 or 3) and the experimental conditions (napping/waking) on reaction time, $F(2, 66) = 1.06$, $p = 0.353$, Data is presented in Table 6.1 and Figure 6.4.

PVT	Condition	Mean Reaction time in s. ± SD
1	Wake	0.35 ± 0.04
	Nap	0.36 ± 0.05
2	Wake	0.37 ± 0.05
	Nap	0.36 ± 0.04
3	Wake	0.33 ± 0.04
	Nap	0.32 ± 0. 04

Table 6-1. Mean reaction time in the PVT test in seconds ± SD.

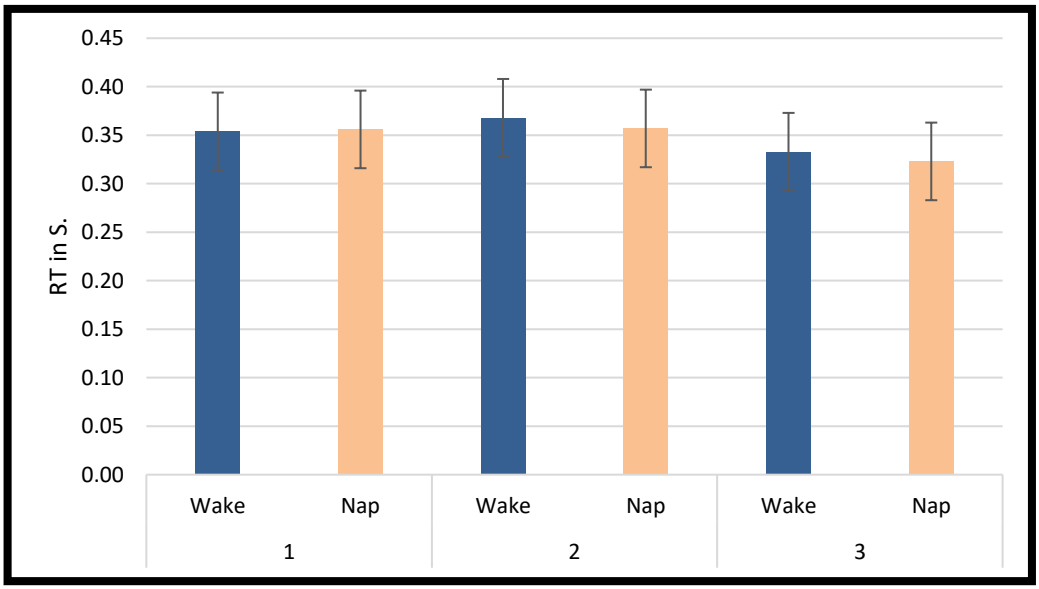

Figure 6-4. Reaction Time in s. for PVT 1, 2 and 3. Error bars represent SD

The main effect of the PVT time, however, showed a statistically significant difference in mean reaction time (RT) at the different time points: $F(2, 66) = 19.41$, $p < 0.001$, Table 6.2.

PVT	RT s. ± SE
1	0.354 ± 0.011
2	0.361 ± 0.007
3	0.330 ± 0.006

Table 6-2. Reaction time is seconds ± SE in PVT 1, 2, and 3.

By contrast, the main effect of the experimental conditions did not show a statistically significant difference in the mean reaction time between the groups: $F(1, 33) = 1.265$, $p = 0.269$.

6.5.3 *First retrieval (R1)*

R1 was calculated as the percentage of remembered sequences from the learned 90 sequences. Participants in the wake group remembered more sequences (M = 73.63%, SD = 12.2%) than the participants in the nap group (M = 64.44%, SD = 13.18%), an independent samples t-test showed that the difference was statistically significant: t(33) = 2.12, p = .041, Figure 6.5.

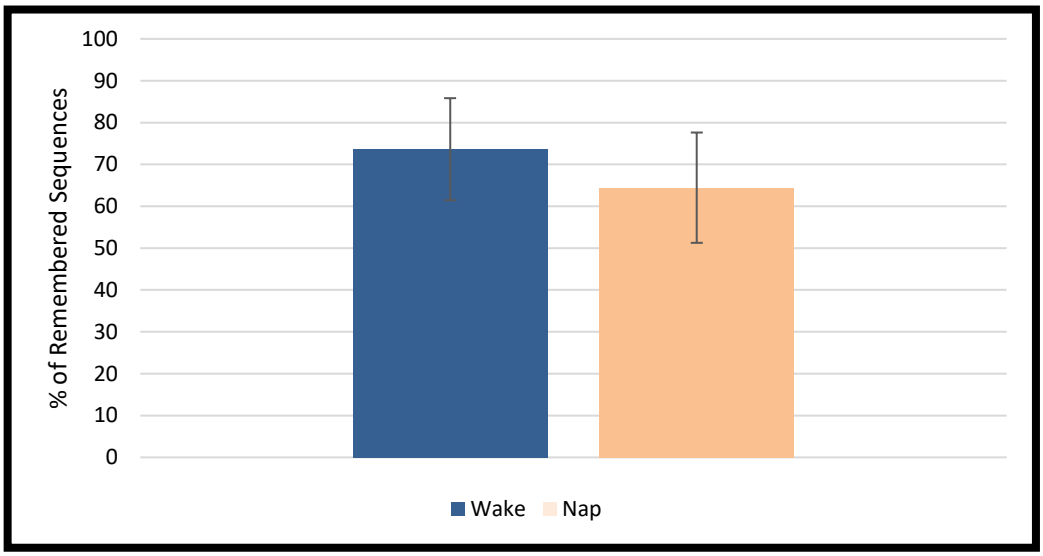

Figure 6-5. The percentage of remembered sequences from the 90 sequences in the first retrieval. Error bars represent SD.

6.5.4 *Effect of TMR on relational memory, second retrieval (R2)*

R2 was calculated as the % of remembered sequences from R1. Data was analysed with a 2 (TMR: cued/not cued) × 2 (condition: wake/nap) repeated-measures analysis of variance (ANOVA). The results showed that there was no statistically significant interaction between the experimental conditions (wake/nap)

TMR (cued/non-cued) in the percentage of remembered sequences from R1 after the offline period: $F(1, 33) = 0.707$, $p = 0.406$. Table 6.3, Figure 6.6.

	Condition	% Remembered from the 1st Retrieval ± SD
Cued	Wake	87.0 ± 10.0
	Nap	86.0 ± 5.0
Non-Cued	Wake	89.0 ± 7.0
	Nap	86.2 ± 8.0

Table 6-3. The percentage of remembered sequences from the first retrieval after the offline period.

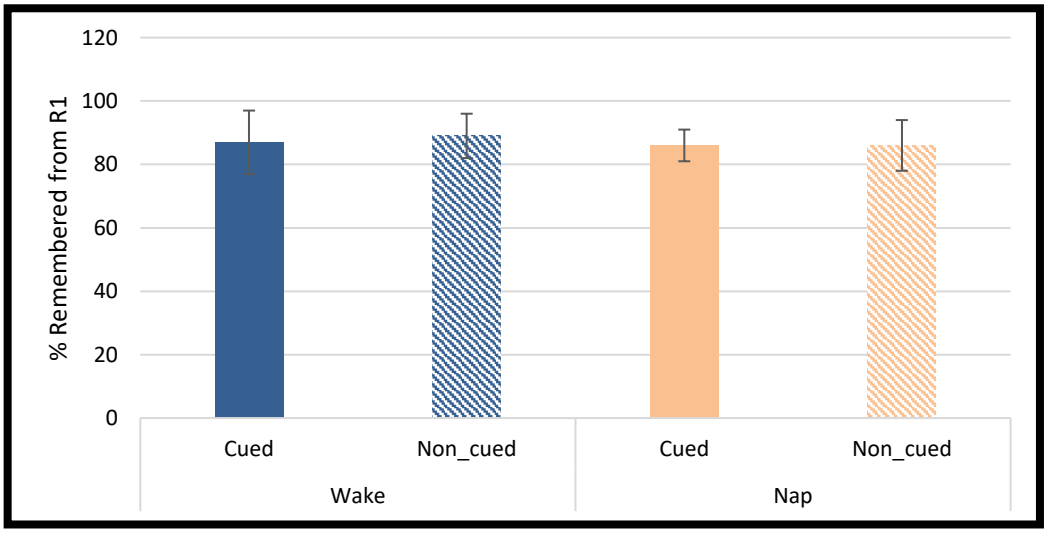

Figure 6-6. The percentage of remembered sequences from the first retrieval after the offline period.

Furthermore, the main effect of TMR did not show a statistically significant difference in the mean percentage of remembered sequences between the cued and

non-cued sequences: F(1, 33) = 1.08, p = 0.306. In addition, the main effect of condition did not show a statistically significant difference in the mean percentage of remembered sequences between intervention groups: F(1, 33) = 0.601, p = 0.444.

6.5.5 Effect of TMR on relational memory, third retrieval (R3)

R3 was calculated as the % of remembered sequences from R2. Data was analysed with a 2 (TMR: cued/not cued) × 2 (condition: wake/nap) repeated measures analysis of variance (ANOVA). There was no statistically significant interaction between the experimental conditions and the TMR in the percentage of remembered sequences from R2 after a night of sleep: F(1, 33) = 0.343, p = 0.562, Table 6.4, Figure 6.7.

	Condition	**% Remembered from the 2nd Retrieval ± SE**
Cued	Wake	104 ± 2.1
	Nap	104 ± 2.0
Non-Cued	Wake	101 ± 2.0
	Nap	99 ± 2.0

Table 6-4. The percentage of remembered sequences from the second retrieval after a night of sleep.

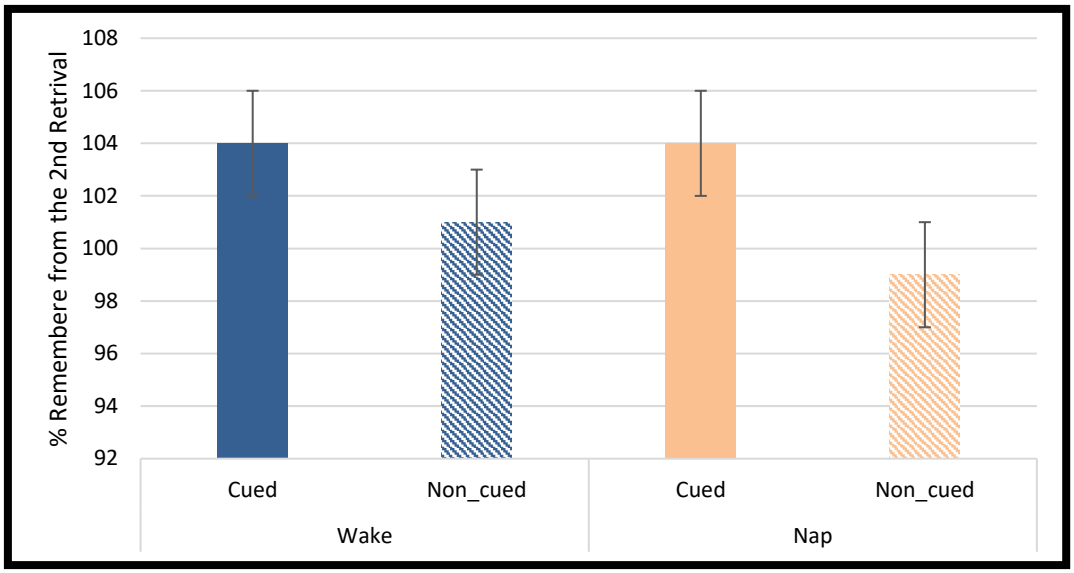

Figure 6-7. The percentage of remembered sequences from the second retrieval after a night of sleep.

The main effect of TMR, however, showed a statistically significant difference in the percentage of content remembered between the cued and non-cued sequences: $F(1, 33) = 5.73$, $p = 0.023$.

A post-hoc t-test showed a 5.4%, 95% CI [1.52, 9.22] increase in the remembered sequences that were cued during naps compared to the non-cued sequences. An increase which was statistically significant: $t(18) = 2.93$, $p = 0.009$, $d = 0.70$. This finding was not true of the wake group, as there was no statistically significant effect of cueing on remembered sequences: $t(15) = 0.99$, $p = 0.336$.

6.5.6 Correlation between sleep stages and memory performance

A Spearman rank correlation coefficient was used to test for the correlation between the time spent at each sleep stage and memory performance. This test was selected due to the non-linearity of the data, and the results are summarised in Table 6.5.

	Time in minutes*	Correlation with 3rd Retrieval
Stage I	15.58 ± 9.25	- 0.07
Stage II	47.10 ± 12.70	0.08
SWS	13.23 ± 10.82	0.1
REM	17.68 ± 12.20	- 0.13

Table 6-5. The correlation between the sleep stages and memory performance. Time is presented as mean ± SD. *Sleep staging data is shared with another PhD project.

There were no observed statistically significant sleep-stage correlations (all $p > 0.05$) although a post hoc power analysis showed that the study had good statistical power, with a power estimate of 0.96. Yet, although not statically significant, a positive correlation can be observed between both stage II and SWS with the performance in the third retrieval.

6.6 DISCUSSION

6.6.1 Overview

We carried out this experiment to investigate the effects of TMR during wakefulness and sleep on the non-directly cued associative memory using environmental sounds cues. The retrieval accuracy was assessed directly after TMR and again after a night of sleep. The data showed improvement of the recall accuracy for the non-directly cued associations only in the third retrieval, which was specific to the napping group. This finding was consistent with another TMR study (Cairney et al., 2018). We also found positive correlation between time spent in stage II and in SWS with memory performance in the 3rd retrieval, which although did not reach statistical significant, it is in line with the findings of other researches that link the time in SWS to improvement in memory performance (Diekelmann et al., 2012).

6.6.2 PSQI and PVT

We used the PSQI to evaluate the quality of sleep prior to the study, which was similar in both groups. PVT was also used to assess the vigilance and attention before each retrieval, which showed that the reaction time of the participants was consistent and less than 500 ms, indicating good levels of attention and vigilance (Short and Banks, 2014). Although the reaction time was the shortest at PVT3, indicating better vigilance, this finding was similar for both groups making the results comparable. Together, these two measures suggest the same cognitive baseline levels between the groups at baseline and with each retrieval.

6.6.3 TMR during wakefulness vs. sleep

We believe that by matching the participants in the wake group with participants in the nap group, we established a foundation for comparing the groups' performances. The lack of TMR benefits in the wake group was against the initial hypothesis, and that could be due to several factors. First, TMR during wakefulness is proposed to destabilise the memory trace, while the active brain status prevents the immediate reconsolidation that occurs during sleep (Rasch and Born, 2007, Born and Wilhelm, 2012). Second, the performance in R1 was better in the waking group than in the napping group. The relatively weak association in the napping group may have opened a better opportunity for the TMR benefits, as weaker memories have better opportunity to be enhanced by TMR, providing that the memory trace is not too weak as weak memory traces do not benefit from the TMR either (Diekelmann et al., 2009, Born and Wilhelm, 2012, Creery et al., 2015, Cairney et al., 2016).

6.6.4 Second vs. third retrieval

As did the previous TMR studies, this study found that the accuracy of retrieval for the linked memory did not improve in the second retrieval in both groups (Cairney et al., 2016, Cairney et al., 2018). This finding is in line with the hypothesis that indirect cue-memory association requires more sleep cycles with more SWS and adequate REM periods, which are not possible during a nap, especially with the complicated paradigm used in this study (Cairney et al., 2016). Theoretically, this finding is supported by the active system consolidation theory (Rasch and Born, 2013).

One of the other TMR studies which used similar paradigm to ours utilised sounds that were not representative of the object, so the author proposed that the lack of TMR effects in their study could be avoided by using sounds with clear semantic links to the objects (Cairney et al., 2016). The current study showed the lack of TMR effects in the second retrieval, although there was a link between each sound and the associated object. This finding ruled out the impact of the sound used for cueing and showed it is not the reason for the evident lack of effects in the second retrieval. This has shifted the focus of interpretation to other reasons; mainly the importance of the sleep cycle efficiency during the overnight sleep, which is supported by the literature (Oudiette and Paller, 2013).

Nonetheless, similarly to a recent study, the results showed the benefit of TMR for the recall accuracy in the nap group after a night of sleep (Cairney et al., 2018). To explain this result, it is suggested that TMR during the nap tagged which sequences would benefit from the enhanced overnight memory consolidation. Furthermore, it is suggested that the second retrieval might have triggered a reconsolidation process that enhanced the benefits of the TMR which was clear after the overnight sleep (Cairney et al., 2018).

The lack of statistical significance in the correlation found between the time spent in SWS and performance in the third retrieval could be due to the short time participants spent in SWS, and the fact that it is a nap study, which can affect the circadian rhythm and the way information are processed. The findings of this study

are similar to another study that found a significant correlation between memory performance and SWS when the time spent on that stage was about 40 minutes, but not when it was about 17 minutes (Diekelmann et al., 2012), and the participants in this study only spent about 13 minutes in SWS. This is supported by other nap studies which showed the correlation existed but was not significant either (Cairney et al., 2016, Ashton et al., 2018).

From another perspective, studies that found statistical correlation between memory performance and SWS looked at more than the duration of SWS. TMR studies in particular looked at more specific components that reflects the effect of reactivation as the sleep spindle power and density, timing of spindles activation, changes in the slow oscillation slope (Creery et al., 2015, Schouten et al., 2017, Cairney et al., 2018). This study has the required data, but analysis is beyond the scope of this thesis as it is part of another PhD student thesis.

6.6.5 Conclusion

This experiment showed that TMR can enhance the consolidation of cued associations when applied during sleep rather than during wakefulness. Furthermore, the benefits found to appear after a night of sleep rather than immediately after the cueing. TMR is a newly explored way to enhance memory consolidation, so there is much to be found about its underlying mechanism and its possible applications.

7. CONCLUSION

This thesis looked at the neural plasticity from two angles; respiratory long-term facilitation and memory formation.

7.1 RESPIRATORY LONG-TERM FACILITATION

7.1.1 Overview

In Chapter 3, a custom-built model was used to expose the upper airways of healthy humans to bouts of INAP aiming to induce long term facilitation of the hypoglossal nerve which would be indicated by a change in the EMG signal of the GG muscle. The protocol did not result in measurable change of the GG EMG, which was against the initial hypothesis and in contradiction of other studies which showed that INAP is capable to induce rLTF in rats (Ryan and Nolan, 2009b) and in healthy humans (Griffin et al., 2017). A difference that needs further research to understand and to be clarified.

As little is known about the physiological effects of INAP, the second study (Chapter 4) was conducted to show the effect of extended exposure to INAP on the systemic and pulmonary blood pressure and on the oxidative stress. Aligned with the initial hypothesis, INAP did not affect any of these parameters. This makes INAP a good candidate for rLTF compared to IH, which is the most used trigger in the rLTF research. Although promising, this finding should be taken with cautious until confirmed by a larger scale studies.

IH has several adverse effects, which limited its practical use for rLTF and urges the search for an alternative method that could result in rLTF as rLTF has shown to be a possible pathway for the treatment of OSA. Furthermore, neural plasticity, which includes more than rLTF was achieved by IH and has shown potentials in the motor rehabilitation for patients with incomplete spinal cord injury. It is not known if INAP is capable to cause the same effect, but it certainly worth exploring.

7.1.2 Limitations

As shown in Chapter 3, there was no change in GG EMG after exposing the participants to one hour of INAP (-15 cmH_2O) compared to the control day. This finding could be the result of some limitations to the study protocol.

First, it was clear that using the unilateral electrodes' alignment for the non-invasive measurement of the GG EMG signal was not reliable in this experiment. Also using the bilateral alignment with the silver electrodes did not improve the reliability of the signal. The signal variability was a major limitation of the study.

Second, conducting the study on healthy awake subjects without controlling the CO_2 levels is another limitation of the study. Other studies on rLTF which utilised IH were either conducted during sleep or while subjects were awake but with CO2 levels maintained above the apnoeic threshold. As this was the first study to investigate the possibility of hypoglossal LTF in humans by INAP the protocol was

designed to start with basic setup then advance with other steps as needed which will be expanded on in the next section.

Finally, the INAP protocol used in this thesis should be considered as a starting point and there is a possibility that the negative pressure level was higher than the optimal level as pLTF was achieved in another study by -10 cmH_2O (Griffin et al., 2017) compared to the chosen setting of -15cmH2O in the study.

7.1.3 Future research

The use of INAP for has potential for rLTF and the possibility is supported by a solid theory. Future research can use the same protocol applied here but accompanied by maintaining the CO_2 level 5 mmHg above the subjects' baseline level. To overcome the limitation of the EMG measurement, each participant could have two electrode carriers, one with (O'Connor et al., 2007) design and one with (Doble et al., 1985) with the measurement taken with both carriers at each time point. If that did not show results, the same protocol could be repeated with a pressure of -10 cmH_2O. The second step is to apply the protocol on patients with OSA instead of healthy subjects and see the effect of INAP on the AHI.

7.2 MEMORY FORMATION

7.2.1 Overview

This part of the thesis focused on the brain's neural plasticity in memory formation. In the first experiment which was about the effect of sleep fragmentation on episodic memory consolidation, fragmentation seems to maintain the item memory but negatively affected the associative memory. This finding is in line with the evidence which show that associative memory is consolidated through the hippocampus, which is very sensitive to sleep.

Clinically, the findings of the study can explain the discrepancy between the cognitive complaints of patients with OSA who chronically suffer from sleep fragmentation and the objective assessment of their memory which fails to show the impairment they feel. It is possible that the current assessment tools might not be designed to assess the associative memory. This inconsistency has a major impact on patients as it can affect their diagnosis and treatment.

The second experiment showed the effect of TMR during wakefulness and sleep on the consolidation of non-directly cued associative memory. Our findings were consistent with the current evidence about TMR. We have shown that cueing has enhanced the consolidation only when applied during SWS compared to wakefulness. Furthermore, we showed that the TMR effect did not show until a night of sleep. This finding could indicate that the nap we used in the sleep group was not

sufficient for the consolidation process for the memory sequence we selected in our paradigm.

7.2.2 Brain plasticity and memory consolidation

Several neuroimaging studies showed that memory formation depends on brain plasticity which is manifested as permanent change in the brain's neurons structure or function; especially during sleep (Walker and Stickgold, 2006, McCoy and Strecker, 2011). For example, one study showed a hippocampal activation during learning a declarative task, and then showed a replay of this activity during SWS. This replay was correlated with performance improvement post sleep (Peigneux et al., 2004). Hippocampal reactivation is suggested to strengthen the brain's synaptic connection (Walker and Stickgold, 2006, McCoy and Strecker, 2011). This reactivation is mostly evident during sleep, but can be also observed during quite wakefulness, as it is suggested that the hippocampus can use any opportunity of idle brain status as a chance for consolidation according to the opportunistic consolidation hypothesis (Mednick et al., 2011).

There are several hypotheses to interpret the interaction between the sleep stages and the memory type during consolidation. The dual process theory which is supported by the night half paradigm propose that the declarative memory consolidation depends on SWS while the consolidation of non-declarative memory depends on REM sleep. This theory has many limitations as ignoring the role of stage II sleep and the contradicting findings of beneficial effects of REM to declarative memory and NREM to non-declarative memory.

The sequential hypothesis on the other hand suggests that both REM and NREM sleep are important for the memory consolidation regardless what type of memory is being processed, and it emphasise the importance of sleep continuity for efficient memory consolidation (Ambrosini and Giuditta, 2001, Rasch and Born, 2013). The theory suggests that NREM sleep is essential for the early consolidation process while REM sleep is important for the final consolidation stages (McCoy and Strecker, 2011). This theory is supported by sleep fragmentation studies which showed that the lack of sleep continuity negatively affected memory consolidation, and by the half night paradigm as well (Rasch and Born, 2013).

As both dual process and sequential theories are supported by evidence, a third theory has emerged which integrate components from both theories. The active system consolidation theory links memory consolidation to the replay of the memory trace during sleep. The theory suggests that the replay help to transfer the memory from their short-term storage, which is the hippocampus for the declarative memory, to the long-term storage where it is integrated to a bigger network in a process called system consolidation, which occurs during SWS. This is proposed to be followed by synaptic consolidation process which is suggested to occur during REM to stabilize the memory trace (Rasch and Born, 2013).

7.2.3 TMR

Evidence show that hippocampal replay of the memory trace is essential for memory consolidation (Mölle et al., 2011), and it has been shown that this replay can

be enhanced by external stimuli as in the TMR paradigms. It was also shown that TMR can be used to facilitate memory consolidation during SWS, which improves the performance (Ngo et al., 2013, Cairney et al., 2014, Groch et al., 2016, Cairney et al., 2017). It should be noted that the strength of the memory during encoding can affect the TMR outcome, as neither very weak nor very strong traces can benefit from the external reactivation (Creery et al., 2015, Schapiro et al., 2018).

The effect of TMR during wakefulness is not well established yet, as fMRI scans show that it activates the prefrontal cortex instead of the hippocampus (Diekelmann et al., 2011). Furthermore, the memory replay during wakefulness is noticed to be in reverse (Foster and Wilson, 2006), how does that affect the TMR is still not known, so there is still a lot to be found about TMR, specially during wakefulness.

The TMR paradigm we implemented showed the possibility to enhance the consolidation of non-directly cued memory during a 90 minutes nap, but the effect will not be evident unless proceeded by an overnight sleep. We also showed that cueing during wakefulness did not cause the same effect. Both findings were in line with the current evidence about TMR.

7.2.4 Sleep fragmentation

Sleep fragmentation has shown to impair the hippocampal synaptic efficiency and therefore negatively affect the memory performance (Tartar et al., 2006). It is also evident that hippocampal damage can negatively affect the associative memory

selectively without affecting the item memory (Mayes et al., 2002, Holdstock et al., 2005). The results of the sleep fragmentation protocol in the study showed that the sleep fragmentation has negatively affected the consolidation of the associative memory, which is an element of the episodic memory. The study also showed that the fragmentation maintained the item memory, but this finding can't be explained with the data gathered from the current experiment.

7.2.5 Summary

This section showed that sleep can be used as a window to manipulate the memory; either by strengthening or weakening the consolidation efficiency. We enhanced the consolidation of episodic memory using audio cue which was played during SWS. In the same time, we negatively affected the consolidation of the associative element of the episodic memory using audio stimulation. Both studies established a positive correlation between SWS and memory consolidation.

Not following the fragmentation protocol with an assessment after an overnight sleep limited our ability to compare the results. The factor that seems to influence how does consolidation affected by the audio stimulation is linking the sound to the memory during the encoding process. There might be other factors which was not highlighted by the data, and that would be a fruitful area for future research.

On another note, the dissociation between the item memory and the source memory seen in the fragmentation study has important clinical manifestation. Future work can focus on establishing assessment tools for the associative element of the

episodic memory for patients with chronic sleep fragmentation and that could reduce the gap between the subjective feeling of bad memory for patients and the lack of evidence to support the complaint in the current objective assessment tools.

8. APPENDICES

8.1 FRAP ASSAY METHODOLOGY

Sodium acetate ($C_2H_3NaO_2$) = **82.03379 g/mol**

2, 4, 6-tris (2-pyridyl)-S-triazine ($C_{18}H_{12}N_6$) = **312.33 g/mol**

Ferric Chloride ($FeCl_3$) = **166.2 g/mol**

8.1.1 SODIUM ACETATE

1. 82.03379 g/L = 1 mol
2. We want 300mM
3. 24.610137 g/L = 300mM @ pH=3.6 (Benzie, 1996 – low pH)
4. 3.1 g sodium acetate into 16 ml of neat glacial acetic acid per litre of buffer solution.

8.1.2 2, 4, 6-TRIS (2-PYRIDYL)-S-TRIAZINE [TPTZ w/40mM HCl)

1. 312.33 g/L = 1 mol
2. We want 10mM
3. 3.1233 g/L = 10mM
4. HCL (40mM)
5. 12M conc. diluted 300 fold
6. 2.5mls acid in 750mls dH_2O

7. 3.1233g TPTZ in 1L 40mM HCL
8. Make up 160mM solution: 0.05g/ml – put 0.1g TPTZ in 2ml methanol – add 2ml into 30mls of 40mM HCl = 10mM TPTZ (10.6mM).

8.1.3 FERRIC CHLORIDE (in doubly distilled deionized water)

1. 166.2 g/L = 1 mol
2. We want 20mM
3. 3.324 g/L = 20mM
4. 0.332g in 100mls dH_2O.

8.1.4 Methodology (for 1 plate)

1. Get out plasma samples and thaw slowly on ice
2. Get everything out that you need for the day (plates, pipettes, chemicals, bottles etc)
3. Dilute samples 1:1
   - To make the assay high throughput (if you are doing a whole plate or more, this is good) conduct the dilutions in a spare 96 well plate
   - Add 20ul plasma from eppendorf to 20ul dH20 in the spare 96 well plate... mix with multichannel pipette
   - Transfer 10 ul of diluted samples to the plate you want to do the FRAP assay in using a multichannel pipette
4. Dilute standards and load onto the plate you want to do the FRAP assay in
5. Store plates in fridge

6. Make up all of the separate FRAP ingredients
7. 30ml acetate buffer
8. 3ml TPTZ solution
9. 3ml $FeCl_3.6H_2O$ solution
10. Add 300µl FRAP reagent to 96 well plate
11. Plates left for 8 minutes at room temperature
12. Read at 650nm on a plate reader
13. Values were determined by linear regression from a 7-point standard curve and expressed as µM of antioxidant power relative to ascorbic acid

8.1.5 Standards

Ascorbic acid (MW = 176.13) – standards concentration range 0 - 1000µM

1M = 176.13g/L

Make10mM stock solution, then 1mM (1000µM) solution:

Solution 1: 10mM = 1.7613g/L

STOCK 10mM = 0.17613g/100mls dH_2O

Solution 2: 1000µM = 100µl solution 1 with 900µl dH_2O

1 ML

µl of Ascorbic Acid (1000µM)	µl of Water	Concentration (µM)
0	100	0
12.5	187.5	62.5
25	175	125
50	150	250
100	100	500
150	50	750
200	0	1000

8.2 TBARS ASSAY

Colorimetric Standard Preparation (Abs: 532nm)			
Tube	125uM 1,1,3,3-Tetramethoxypropane Stock (ul)	ddH_2O Water (ul)	1,1,3,3-Tetramethoxypropane Concentration (uM)
A	0	1000	0
B	5	995	0.625
C	10	990	1.25
D	20	980	2.5
E	40	960	5
F	80	920	10
G	200	800	25
H	400	600	50

8.2.1 Reagents ***(volumes in brackets are amounts needed for one plate)***

1. 1.23M TCA (trichloroacetic acid) (Sigma, T0699 – 15mls): (in fridge)

   3.014g in 15mls water

2. 0.05M H_2SO_4 (30mls):

   1.5mls in 30 mls water

3. TBA (thiobarbituric acid) Solution (30mls needed per plate):

   0.67g/100ml ddH_2O and dilute 1:1 w/ glacial acetic acid – make fresh daily.

4. 200uM Butylated hydroxytoluene (BHT):

   Make 2mM stock: 44.07 mg/100mls ethanol (100%).

   Dilute on day 1:10 in ethanol (100%) to make 200uM stock

5. Tetramethoxypropane standard (125uM) (Sigma, 108343):

   Molecular weight: 1M = 164.2 ml/L

   Molarity = (wt% × density (g/ml) × 10) / MW

   Molarity= (99 * 0.997 * 10)/ 164.2 = 6.01M

   48090-Fold Dilution needed

   10ul Tetramethoxypropane (6.01M) + 48.08mls ddH_2O then dilute 1/10 dilution for 125uM TMP stock solution.

8.2.2 Solutions ***(per plate)***

1. TCA solution (0.75ml per sample: 0.25ml of TCA (1.23M) and 0.5ml of H_2SO_4 (0.05M) per sample: for one plate mix <u>15ml TCA (1.23M) with 30ml H_2SO_4 (0.05M)</u>
2. Colour Reagent (2.51ml: 0.5ml TBA Solution, 2ml ddH_2O and 10ul BHT (20uM). Make colour reagent for 1 plate: <u>30mls TBA, 120mls ddH_2O and 500ul BHT</u>. Make fresh daily.

8.2.3 Protocol

1. Add 100ul of sample or standard to eppendorf tubes.
2. Add 100ul of TCA solution to tube and swirl to mix.
3. Add 800ul of the colour reagent to each tube and vortex.
4. Place tubes to vigorously boiling water- 100 degree celcius. Boil for one hour. Caps may pop up during boiling, close cap immediately.
5. After one hour, immediately remove the tubes and place in ice bath to stop reaction. Incubate in ice for 10 minutes.
6. After 10 minutes, centrifuge the vials for 10 minutes at 1,600xg at 4 degree celcius.
7. Tubes are stable at RT for 30 mins.
8. Carefully remove 200ul from each tube without disturbing pellet and transfer to wells.
9. Read absorbance at 540nm.

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