Sleeping Soundly: Effects of Auditory Closed-Loop Stimulation on Sleep and Memory

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Contents

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List of Figures

List of Tables

Abbreviations

AASM	**A**merican **A**cademy of **S**leep **M**edicine
AD	**A**lzheimer's **D**isease
BBSRC	**B**iotechnology and **B**iological **S**ciences **R**esearch **C**ouncil
BF	**B**ayes **F**actor
EEG	**E**lectro**e**ncephalo**g**raphy
EMG	**E**lectro**m**yo**g**raphy
FDR	**F**alse **d**iscovery **r**ate
FFT	**F**ast **f**ourier **t**ransform
FT	**F**inger **t**apping
ISI	**I**nter-**s**pindle **i**nterval
KC	**K**-**c**omplex
MCI	**M**ild **c**ognitive **i**mpairment
MRI	**M**agnetic **r**esonance **i**maging
N1/NREM1	**N**on-**REM** sleep stage 1 (AASM)
N2/NREM2	**N**on-**REM** sleep stage 2 (AASM)
N3/NREM3	**N**on-**REM** sleep stage 3, also termed SWS (AASM)
NREM	**N**on-**r**apid **e**ye **m**ovement (sleep)
PAL	**P**aired **a**ssociate **l**earning
PANAS	**P**ositive **a**nd **n**egative **a**ffect **s**cale
PASA	**P**osterior to **a**nterior **s**hift in **a**geing
PE	**P**icture **e**ncoding
PSG	**P**oly**s**omno**g**raphy
PVT	**P**sycho**m**otor **v**igilance **t**ask
REM	**R**apid **e**ye **m**ovement (sleep)
RM ANOVA	**R**epeated-**m**easure **an**alysis **o**f **va**riance
RMS	**R**oot **m**ean **s**quare
S1	Non-REM sleep **s**tage 1 (Rechtschaffen & Kales)
S2	Non-REM sleep **s**tage 2 (Rechtschaffen & Kales)
S3/S4	Non-REM sleep **s**tage 3, also termed SWS (Rechtschaffen & Kales)

SD	**S**tandard **d**eviation
SEM	**S**tandard **e**rror of the **m**ean
Sham	Non-stimulation condition
SHY	**S**ynaptic **h**omeostasis h**y**pothesis
SO	**S**low **o**scillation
SQ	**S**leep **q**uality
SSS	**S**tanford **s**leepiness **s**cale
Stim	**Stim**ulation condition
SWA	**S**low **w**ave **a**ctivity
SWS	**S**low **w**ave **s**leep
tACS	**t**ranscranial **A**lternating **C**urrent **S**timulation
tDCS	**t**ranscranial **D**irect **C**urrent **S**timulation
TMR	**T**argeted **m**emory **r**eactivation
TMS	**T**ranscranial **m**agnetic **s**timulation
TST	**T**otal **s**leep **t**ime
WP	**W**ord **p**airs
η_p^2	Partial eta squared

Abstract

Sleeping Soundly:

Effects of Auditory Closed-Loop Stimulation on Sleep and Memory

Julia Schneider, The University of Manchester

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Sleep is a unique behavioural state, whose exact purpose remains an unsolved mystery. A wealth of evidence posits that sleep facilitates the consolidation of memories. In particular slow oscillations (SOs) during deep slow wave sleep are thought to play a vital functional role in driving this consolidation process by orchestrating phase-coupled thalamo-cortical sleep spindles. To elucidate functional mechanisms of these oscillations, a new experimental technique called auditory closed-loop stimulation has been trialled, which applies brief sound stimuli in phase with on-going endogenous oscillations, and thereby enhances both SO and spindle activity, as well as overnight memory consolidation. The aim of the work presented in this thesis was to use the technique's potential in combination with behavioural measures of declarative and procedural memory and overnight polysomnography (PSG) to investigate oscillatory dynamics and their functional purpose during sleep.

In **Chapter 2**, we examined the applicability of auditory closed-loop stimulation in a cohort of healthy, late middle-aged adults, as SO and spindle activity naturally decline during the lifespan and have been linked to impaired memory. We further compared stimulation outcome in this group to an existing young adult cohort. While the ageing brain responded to the stimulation, its susceptibility was markedly decreased with age, with no favourable impact on overnight memory consolidation observed. Our results demonstrate the need for stimulation optimisation to translate functional protocols to different age groups prior to clinical application. In **Chapter 3**, we investigated whether slow and fast sleep spindle types could be differentially modulated by applying variations of auditory closed-loop stimulation protocols, as particularly the respective functional contribution of slow spindles in the consolidation process remains unclear. A multi-stimulus protocol was found to enhance slow spindles to a greater extent than fast spindles, and revealed further temporal differences which suggest slow and fast spindles may underlie different neural dynamics in their generation. Auditory closed-loop stimulation constitutes a useful non-invasive tool to probe their potential functional differences in future investigations. Finally in **Chapter 4**, we considered the psychological impact associated with receiving auditory closed-loop stimulation during sleep and asked whether merely anticipating specific effects in a placebo protocol would have an impact on sleep and cognition. We discovered placebo and nocebo effects in the form of transiently altered slow spindle activity throughout the night, and suggest psychological aspects of applying stimulation could present a crucial factor and require due consideration in future research designs.

In conclusion, our results shed new insights on the potential of using customised auditory closed-loop stimulation protocols as a suitable technique in the investigation of sleep oscillation dynamics and their functional implications. However, we also highlight limitations of susceptibility in an older population, and provide evidence of psychological aspects presenting a potentially confounding variable in stimulation outcome.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Rationale for submitting in alternative format

The work presented in this thesis comprises of experimental Chapters 2-4 which were written in the style of scientific papers, and are at various stages of being prepared and submitted for publication. While they each include a brief review of the literature relevant to the respective experiment, Chapter 1 provides a broader introduction to the literature upon which the work undertaken in this thesis is based and outlines key research questions and aims.

The author was the primary investigator of all work presented in this thesis and performed the majority of experimental conceptualisation, design, recruitment, data collection, analysis, interpretation, and manuscript preparation. Professors Penelope Lewis, Wael El-Deredy, and Dr Alexander Casson supervised the development of experimental designs, technical setup, analyses, and provided feedback. Further individual contributions are detailed in the acknowledgements sections at the end of each experimental chapter.

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This thesis is dedicated to my family - biological and other.

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The Author

Julia Schneider completed an undergraduate degree with joint honours in Geography and Psychology at the University of Strathclyde, with a semester spent at the City University of Hong Kong. She then obtained a Masters in Global Mental Health from the University of Glasgow, followed by a 6-month internship in the Laboratory of Neuroscience and Psychology of Sleep at the University of Manchester, where she subsequently commenced her PhD funded by the Biotechnology and Biological Sciences Research Council and the University of Manchester. She later followed her supervisor and laboratory to the Cardiff University Brain Research Imaging Centre. Her previous research experience covers sleep and overnight memory consolidation, cross-cultural assessment of post-traumatic stress disorder, subjective evaluations of happiness, biases in emotion perception, psychopathy, as well as urban flooding, and environmental security.

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"I love sleep. My life has the tendency to fall apart when I'm awake, you know?"

Ernest Hemingway

Chapter 1

General Introduction

1.1 Preface

Something truly curious and remarkable happens to us with every rotation of the earth around its own axis. Each night, we lose conscious control and command over our mental and physical manifestations of self, and sever our connection to the outside world. When morning comes, we regain consciousness and resume our lives. Yet we are no exception in displaying this behaviour; in fact, the vast majority of living organisms on earth have evolved to follow this alternating rhythm of diurnal activity and nocturnal rest (Siegel, 2008). Considering that any unresponsive creature renders itself immensely vulnerable to passing predators, choosing to adopt this behaviour over staying alert and maximising chances of survival must therefore be based on a strong incentive in the form of functional advantages for the committing organism (Allada & Siegel, 2008, Eban-Rothschild *et al.*, 2017). The unsolved question is, what are they?

Scientific and technological advances, such as the first recording of human brain activity with an electroencephalogram (EEG) in 1924, have enabled an increasing number of in-depth investigations into the nature of sleep over the past century (Dement, 1998). Contrary to previously assumed inactivity, the sleeping brain was discovered to be anything but idle during its regular downtime intervals. Instead, sleep was found to be a non-homogenous state consisting of different sleep stages, oscillatory patterns, and a range of associated functional purposes have since been proposed. Suggested benefits include energy conservation, restorative cellular and systems-level mechanisms, as well as the facilitation of sleep-dependent cognitive functions, such as overnight memory consolidation. However, many details of these mechanisms remain a mystery (Mignot, 2008). In an attempt to uncover these, modern sleep research has begun to specifically target processes and oscillations by applying different forms of stimulation to the sleeping brain (Cellini & Mednick, 2019, Diekelmann, 2014). Beyond the aim of advancing scientific knowledge, another objective of such experimentation is the development of interventions for individuals who show sleep difficulties or altered oscillatory patterns, as is the case in healthy and pathological ageing for example. The work presented in this thesis aims to add to our current understanding of sleep oscillations and their relationship to sleep-dependent memory processes by using a relatively novel manipulation technique called

auditory closed-loop stimulation. The relevant literature supporting and inspiring this experimental approach is introduced in this chapter. I will begin by summarising our contemporary understanding of sleep architecture, stages, and brain oscillations, and review brain stimulation techniques aimed at enhancing these, with a focus on auditory closed-loop stimulation together with the physiological background of sound processing during sleep. Following on, I will present a selective summary on physiological and functional changes in late adulthood to provide context for the idea of using auditory closed-loop stimulation to combat age-related deterioration of sleep and cognition, before concluding with an outline of the research objectives which stimulated the work conducted for this thesis.

1.2 Sleep physiology and oscillatory features

As humans, we spend roughly one third of our lives asleep. Across a full night's sleep, an individual passes through a number of sleep cycles, each encompassing various stages of sleep. The latter are broadly divided into rapid eye movement (REM) sleep and non-REM (NREM) sleep, which in itself consists of the lighter sleep stages 1 (N1/S1) and 2 (N2/S2), as well as the deeper sleep stages 3 and 4 (Iber *et al.*, 2007, Rechtschaffen & Kales, 1968). The latter two have been combined in newer sleep scoring frameworks and are now jointly referred to as slow wave sleep (SWS). Healthy young sleepers pass through these stages in a sequential order within repeating cycles of 60 to 90 minutes on average, beginning with the smallest numbered NREM stage and ending in REM sleep (see hypnogram in Figure 1.1). The proportional amount of time spent in each stage per cycle varies throughout the night. The length of SWS periods decreases as REM and N2 duration increases towards morning due to a gradually dissipating sleep pressure. In the theoretical model on sleep and wake regulation, Borbély and colleagues (2009, 2016) posit that homeostatic sleep pressure (Process S) interacts with a circadian pacemaker, i.e. an endogenous body clock synced to an approximately 24-hour rhythm (Process C). With increasing time spent awake, sleep pressure rises and is reflected in the amount of time spent in SWS during the subsequent sleeping period. Throughout sleep, the pressure dissipates, hence the proportionally higher amount of SWS briefly after sleep onset while sleep pressure is still at its highest (Van Dongen & Dinges, 2003).

Sleep architecture can be recorded using polysomnography (PSG), a term which denotes the combined use of electroencephalographic (EEG) recordings of neuronal activity occurring in neocortical layers of the brain. Electrooculography (EOG) and electromyography (EMG), which measure eye movements and muscle activity respectively, are added to facilitate the discrimination between different sleep stages. Distinct patterns of brain oscillations hallmark each sleep stage as outlined below and shown in Figure 1.2 (Lee-Chiong, 2005). It should be emphasised that normative percentages of time per sleep stages provided in this section are based on data from healthy, young adult sleepers. Sleep undergoes considerable changes throughout the early years, as well as adulthood (Bathory & Tomopoulos, 2017, Scullin & Bliwise, 2015), as explored further in section 1.5.2.

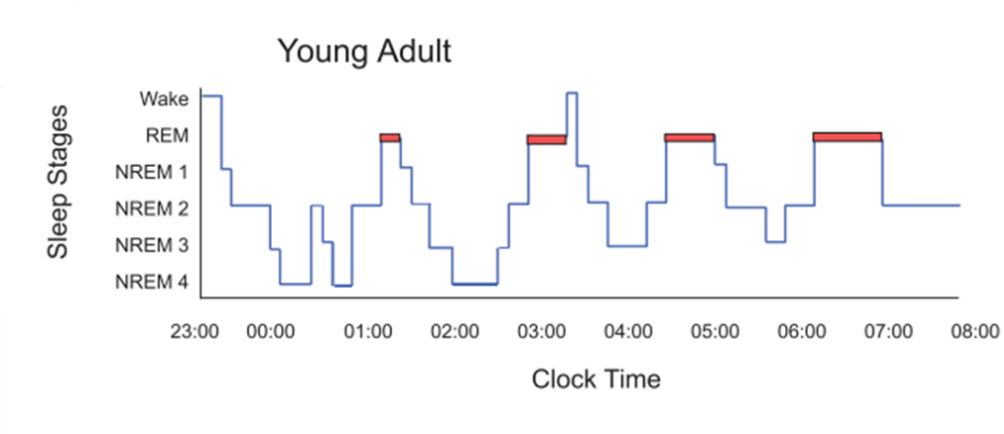

Figure 1.1: Hypnogram showing sleep stage distribution across a night of sleep from sleep onset to morning. Most sleep cycles generally begin with a transition from wake to shallow stage NREM1 sleep, followed by NREM2 and eventually deep SWS (NREM stages 3 & 4) before ending with REM (red). Throughout the night, the proportion of time spent in each stage per cycle changes due to dissipating sleep pressure, with deeper NREM sleep dominating the first half of the night, and NREM2 and REM the latter half. *Source: adapted from Mander* et al. *(2017)*

Upon wake to sleep transition, we briefly experience N1 which only comprises 3-8% of total sleep time (TST). N1 shows a lower amplitude, mixed frequency theta (4-8 Hz) pattern. It is initially interspersed with bouts of alpha activity (8-13 Hz) indicating wakefulness and consciousness, as well as rolling eye movements in the EOG, both of which continuously cease along with muscle tone observed in the EMG as sleep gradually deepens.

Following on from N1 is N2 sleep, which accounts for 45-55% of TST and is hallmarked by sleep spindles (9-15 Hz bursts for at least 0.5-3 s) and K-complexes (KCs) (Lee-Chiong, 2005), see Figure 1.2. The latter are waveforms made up of high amplitude, sharp negative deflections, closely followed by a positive component with a total minimum duration of 0.5 s. The KC poses the largest known event in the human EEG with maximal amplitude observed over frontal EEG derivations, however, KCs occur over wide cortical areas (Bellesi *et al.*, 2014, Forget *et al.*, 2011). The name of the KC was derived from the word 'knock', based on the observation that this waveform can be evoked by arousing stimuli in the sleeper's environment, such as a knock on the door, but also occur spontaneously without external interference (Halász *et al.*, 2014, Zygierewicz *et al.*, 2009). No significant differences were found between evoked and spontaneous KCs in terms of peak amplitudes and power (Cash *et al.*, 2009). Auditorily evoking KCs resulted in an acute increase in delta power (Forget *et al.*, 2011). The KC commences with a brief depolarisation, followed by a consecutive sharp negative deflection which is created by groups of neurons engaging in synchronous hyperpolarisation (also termed down-state). After a moment of neuronal silence, neurons then enter a depolarisation phase (up-state). Colrain (2005) posits that these opposite components of the KC are likely to serve different functional roles: part miniature arousal to process the stimulus and scan the environment for threats during the initial smaller depolarisation and part sleep-promoting arousal inhibition during the silent down-state in case no reason to wake up was identified, before internal processing resumes with the up-state (Jahnke *et al.*, 2012).

Sleep spindles are bursts of oscillatory brain activity characterised by a waxing and waning pattern, and are often divided into slower (9-12 Hz) and faster (12-15 Hz) subgroups (Mölle *et al.*, 2011). Fast spindles have been shown to preferentially occur during the up-states of slow waves (defined below) and in the company of sharp-wave ripples, and are thought to prime the cortex for plasticity, while slow spindles predominantly occur in the up-to-down transition (Mölle *et al.*, 2011, Timofeev & Chauvette, 2017). These oscillatory rhythms, their interactions, and suggested functions will be explored in more detail in sections 1.2.2 & 1.3.

As sleep continually deepens, sleepers transition from N2 to SWS, which makes up 15-20% of TST and constitutes the deepest sleep stage. SWS is dominated by delta activity (1-4 Hz) and slow oscillations (SOs, 0.5-1 Hz) (combined denomination: slow wave activity (SWA), 0.5-4 Hz), see Figure 1.2. These slow waves are thought to be paramount for the restorative effect of sleep and the cognitive benefit attributed to SWS (Mölle & Born, 2011,

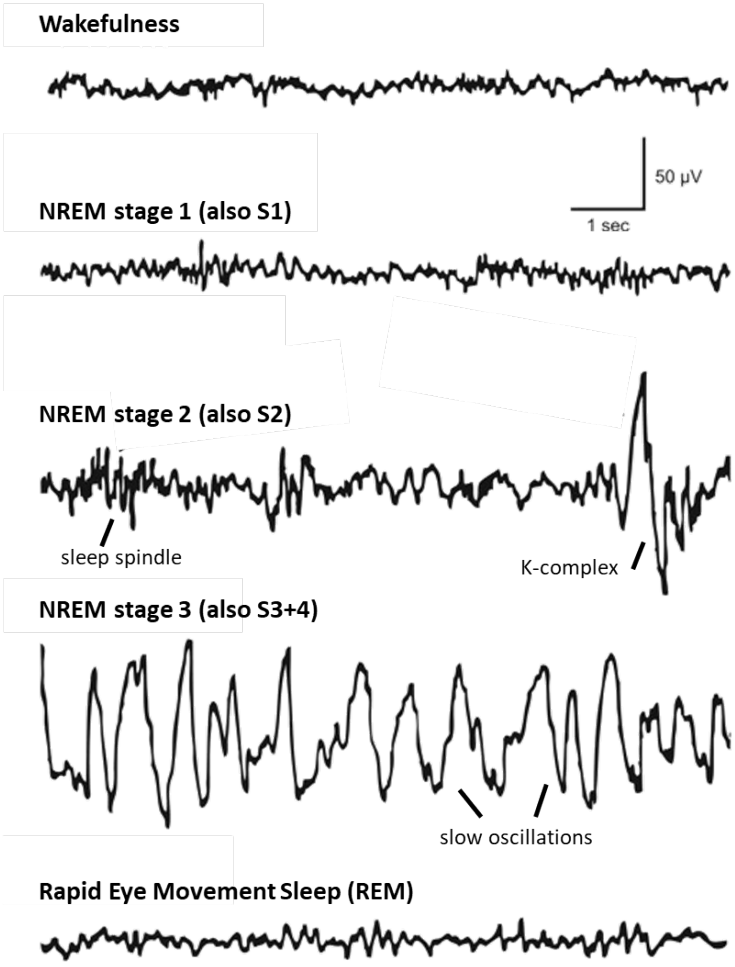

Figure 1.2: Characteristic oscillatory EEG patterns and features of sleep stages. High frequency, low amplitude activity prevails in relaxed wakefulness. Frequency decreases while amplitude increases from shallow N1 to deep SWS (3/4). Stage N2 shows characteristic sleep spindle and K-complex, whereas slow waves (delta and slow oscillations (SOs)) are depicted in SWS. REM sleep activity resembles low amplitude wake EEG. *Source: adapted from Brown* et al. *(2012)*

Tononi & Cirelli, 2006). Their proposed dynamics and functions will thus be considered in greater detail in sections 1.2.1 & 1.3.

Finally, REM sleep, in which we typically spend 20-25% of TST, is characterised by low voltage and mixed frequency EEG activity, including beta range (>12 Hz) and sawtooth waves (2-6 Hz), muscle atonia in the EMG, and rapid eye movements during phasic REM periods in the EOG (Lee-Chiong, 2005). With its mixed high frequency and low amplitude profile in the EEG, it most closely resembles the EEG activity seen in wake and is therefore often referred to as paradoxical sleep. REM sleep is associated with vivid dream imagery, and a certain degree of consciousness due to its subjective phenomenological element (Siclari *et al.*, 2017). Unlike previously held, new research suggests NREM sleep is not devoid of dreams, but NREM dreams appear to be of more analytical content (Siclari *et al.*, 2017).

Importantly, while sleep has previously been considered an all-encompassing global brain state, recent research has provided evidence for the concept of local sleep specific to selective brain regions only (Krueger *et al.*, 2019, Siclari & Tononi, 2017, Tamaki *et al.*, 2016). In addition, multiple sleep stages have been found to prevail simultaneously in the brain during sleep stage transitions, and with sleep onset at intracranial sites preceding cortical areas (Emrick *et al.*, 2016, Sarasso *et al.*, 2014).

1.2.1 Sleep slow waves

The rhythmic waveforms of slow oscillations and delta waves have frequencies between 0.5-4 Hz, peak-to-peak amplitudes of at least 75 μV over frontal regions and an average peak-to-peak duration of 1 s (Iber *et al.*, 2007). They arise from large populations of cortical neurons engaging in synchronous activity patterns of membrane de- and hyperpolarisation, owing to an underlying neuronal bistability in deep SWS (Berry *et al.*, 2017, Steriade *et al.*, 1993). The initiating cellular mechanisms behind the summation of exhibitory and inhibitory post-synaptic potentials causing the observed large-scale polarisations are not yet fully understood. For instance, it is unclear how local excitation is initiated following a down-state with vast neuronal silence (Vyazovskiy & Harris, 2013).

Topographically, the majority of slow oscillations are thought to be generated in frontal networks such as the prefrontal-orbitofrontal region prior to their anterior-to-posterior propagation and towards the medial temporal lobes and hippocampus (exemplary typical propagation topography across the scalp in Figure 1.3) at speeds of 1.2-7.0 m/s (Massimini *et al.*, 2004, Murphy *et al.*, 2009, Nir *et al.*, 2011). Further research found SO amplitude to be strongly indicative of spatial occurrence, with larger SOs (>140 μV) associated with activation in the para-hippocampal gyrus, cerebellum and brainstem, compared to smaller SOs in frontal areas (Rasch & Born, 2013). Previous studies also suggest a possible involvement of other subcortical structures, such as the thalamus (Lemieux *et al.*, 2014). Moreover, although SOs occur as global cortical events, they have also been observed to

occur locally within relatively confined cortical space (Siclari *et al.*, 2018, Nir *et al.*, 2011). While causal, mechanistic and functional distinctions behind this variance in origin, travelling path, and distance are unclear, correlates with time of night and sleep stages have been found (Nir *et al.*, 2011).

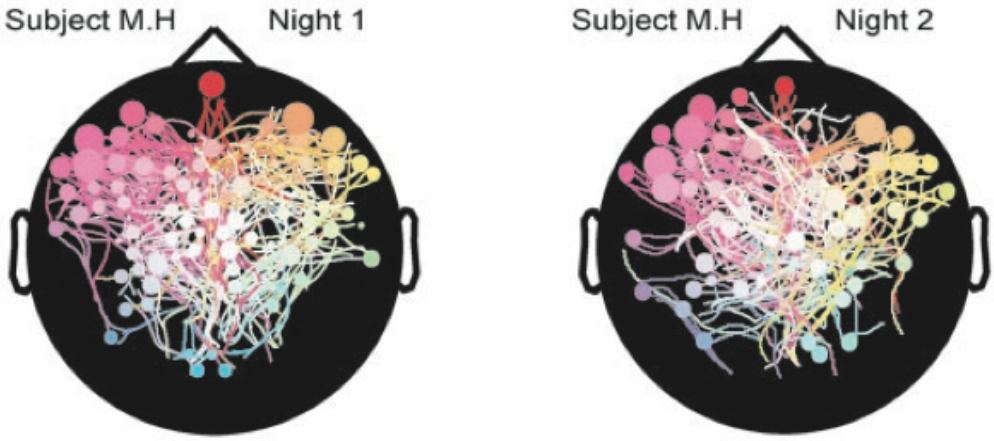

Figure 1.3: SO origin and propagation in the same subject across two nights. The size of dots indicates the number of SO cycles originating from each location, and lines represent travel direction. Colour for differentiation purposes only. The topoplot illustrates that the majority of SOs are generated frontally and travel in anteroposterior direction. *Source: Massimini* et al. *(2004)*

It should be noted that the terminology surrounding slow oscillations, delta waves, slow waves, and KCs is confounded, with these terms often used inconsistently and interchangeably across the literature, rendering inter-study comparisons difficult. There further is an ongoing debate whether slow waves and KCs in their core represent distinct oscillations as initially thought or constitute one and the same oscillatory feature in different variations (Genzel *et al.*, 2014). This debate is mirrored in a recently added note in the sleep scoring manual update by the American Academy of Sleep Medicine (AASM) which states that *"K-complexes would be considered slow waves if they meet the definition of slow wave activity"* (Berry *et al.*, 2017, p.24). Recent studies have attempted to identify sub-types of slow waves and posit that larger waves dominate during the falling asleep period, whereas smaller ones occur later in sleep. They also appear distinguishable by factors such as time of night, density, amplitude, slope, duration, and origin (Bernardi *et al.*, 2018, Siclari *et al.*, 2014), but have yet to be linked to differential functions to make this distinction meaningful. It was proposed to change the terminology to slow waves type I and II to highlight similarities in both waveforms, possibly initiating mechanisms, and intended function (Siclari *et al.*, 2014). Similarly, Halász (2016, p.1) has previously labelled the KC a *"special reactive sleep slow wave"*, stressing similarities while also acknowledging differences.

Frequency, density, amplitude, and slope steepness of slow waves and KCs decrease from sleep onset to waking (Nicholas *et al.*, 2002, Halász *et al.*, 2014). During the earlier night, SOs appear more frequently with shorter depolarisation periods and longer hyperpolarised

components. This relationship is inverted towards morning when longer up-states are interrupted by briefer down-states (Vyazovskiy *et al.*, 2009). These time-of-night effects were confirmed by Nir *et al.* (2011), who also observed that more locally limited SOs seemed to occur in the latter half of the night, with less than 30% of recorded brain areas being involved in an SO event.

SOs and SWS percentage are subject to inter-individual differences and factors such as age, sex, body weight, and ethnicity among others. Age-related changes in SWS and SOs will be discussed in more detail in sections 1.5.2 & 1.5.3. With regard to sex differences, men show less SWS and SWA compared to women, i.e. lower SO density, smaller amplitudes, and flatter slopes. While one study failed to find any statistically significant sex difference in SWS obtained in a sample of young adults (possibly due to its relatively small sample size, as a numerical difference did exist), it observed higher power densities in young women across different frequency ranges and sleep stages, including SWS (Dijk *et al.*, 1989). This observed sex difference persists across different age groups: in a study with more than 2500 37- to 92-year-olds, Redline *et al.* (2004) found that women had on average 106% more SWS than men.

Another influential factor is body weight. Increases in body mass index negatively correlate with the amount of SWS obtained, even after statistical adjustment for the severity of obstructive sleep apnoea (breathing difficulty commonly found in overweight individuals, particularly with increasing age) (Mokhlesi *et al.*, 2012). While SWS differences have also been reported for different ethnic groups, e.g. Caucasian Americans obtain about 48% more than African Americans (Mokhlesi *et al.*, 2012), and despite a chance of genetic influences, such results need to be interpreted with caution as they may be prejudiced by socioeconomic circumstances or cultural customs of different ethnic groups.

Interestingly, the distinct origin, propagation path, and waveform characteristics of SOs have been found to be reproducible within individuals across different nights as depicted in Figure 1.3, essentially rendering them a blueprint and reliable measure of individual cortical connectivity and cortical excitability (Massimini *et al.*, 2004). Such a measure could potentially be valuable in monitoring changes over time, such as age-related SWS and SO deterioration.

Lastly, while SO occurrence has historically been almost exclusively linked to sleep in the literature, recent research has challenged this view by providing evidence for local slow waves in the brains of awake rats and humans (Hung *et al.*, 2013, Vyazovskiy *et al.*, 2011), as well as in anaesthetised humans, and comatose patients (Amzica & Kroeger, 2011, Ní Mhuircheartaigh *et al.*, 2013). Such findings point towards pivotal functional implications of SOs, now thought to extend beyond the realm of sleep. We will explore suggested functions of slow waves in section 1.3.

1.2.2 Sleep spindles

Sleep spindles are waxing and waning bursts of oscillatory brain activity. By definition, they last between 0.5 to 3.0 s, and occupy the sigma frequency spectrum of 9-15 Hz in the human brain (Gennaro & Ferrara, 2003, Lüthi, 2014). Spindles occur during N2 and SWS. Slow and fast spindle types have previously been identified (Anderer *et al.*, 2001, Diekelmann, 2014), but it is presently unclear whether they share the same generation and regulation mechanisms, or functional purpose (Piantoni *et al.*, 2016, Schabus *et al.*, 2007, Timofeev & Chauvette, 2013).

Spindles are thought to originate in the thalamic reticular nucleus through a reciprocal interaction between excitatory relay neurons and the inhibitory reticular thalamus (Barthó *et al.*, 2014, Clawson *et al.*, 2016, Murata & Colonnese, 2019). Neuronal fast spindle-generating networks appear to underlie refractory durations between 3–6 s (Antony *et al.*, 2018, Ngo *et al.*, 2015). No such suppression period has previously been reported for slow spindles. Subsequently propagated to various other regions, both types of this waveform share a common activation pattern in thalami, paralimbic areas, and superior temporal gyri. However, while slow spindles additionally engage the superior frontal gyrus, fast spindles occur in sensorimotor cortices, the mesial frontal cortex, and the hippocampus (Schabus *et al.*, 2007). Related observations have been made in scalp EEG recordings, which locate slow spindles primarily over (pre-)frontal sites and fast spindles over centro-parietal topographies (Gennaro & Ferrara, 2003). Notably, spindle oscillations have been shown to occur in the hippocampus prior to neocortical sleep onset (Sarasso *et al.*, 2014), thus rendering scalp measures of spindles only an approximation of the actual activity.

Temporally, about 60% of fast and 70% of slow spindles co-occur within 3 s around negative peaks of cortical SOs (Mölle *et al.*, 2011). The phase-coupling between SOs and spindle oscillations occurs in a systematic manner: fast spindles ride on the positive-going slope near the up-state, whereas slow spindles dominate on the negative-going slope towards the SO down-state (Klinzing *et al.*, 2016a, Mölle *et al.*, 2011, Timofeev & Chauvette, 2013). New research suggests many spindle oscillations occur in a rotating wave commonly travelling in temporal-parietal-frontal direction at propagation speeds of 2-5 m/s (Muller *et al.*, 2016).

With regard to oscillatory frequency, a number of differently defined frequency ranges have been used across previous studies. In addition, various optimal cut-off frequencies of e.g. 12 Hz (Mölle *et al.*, 2011), 13 Hz (Schabus *et al.*, 2007), and 13.5 Hz (Lustenberger *et al.*, 2015) have been proposed to distinguish slow and fast spindles, making the comparison of results across studies difficult. Defining slow and fast spindle frequency peaks individually has been suggested and attempted to circumvent this issue. However, a large-scale analysis comprising of 11,000+ recordings noted how defining this cut-off in such a manner could prove difficult in some individuals who lacked a clear distinction between slow and fast spindle frequency peaks (Purcell *et al.*, 2017). A study examining this

frequency division under consideration of sleep stage and topographical differences came to the same conclusion (Cox *et al.*, 2017). Additionally, the mean frequency of visually identified spindles has been found to vary intra-individually within and across sleep bouts and cycles, with faster frequencies observed at the beginning and end of each sleep bout, and with a trend of mean frequencies increasing across cycles (Himanen *et al.*, 2002). Differences in brain anatomy as well as variable hyperpolarisation levels could explain these variations, linking spindle activity to the degree of sleep pressure and depth (Andrillon *et al.*, 2011, Himanen *et al.*, 2002). Sleep stages per se also appear to influence spindling frequency. For example, when comparing N2 and SWS, fast spindle activity was found to be comparable between these sleep stages (N2: 13.46 Hz, SWS: 13.40 Hz), but slow spindles demonstrated more variability with mean frequencies of 11.44 Hz in N2 and 10.23 Hz in SWS (Mölle *et al.*, 2011).

Additional variables mediating general spindle activity have been identified. For instance, reduced spindle density and power have been demonstrated in males compared to females, or with increasing age in adulthood (Clawson *et al.*, 2016, Nicolas *et al.*, 2001, Purple *et al.*, 2017). Moreover, recurring variation has been observed across the menstrual cycle, and throughout the circadian cycle linked to melatonin levels. Spindles are thought to underlie genetic influences, with fast spindles showing greater heritability than slow spindles (Adamczyk *et al.*, 2015). Lastly, Lustenberger *et al.* (2015) stress the importance of differentiating between state- and trait-like aspects of spindles. They define trait-like characteristics, which are relatively consistent over time, as a 'biological fingerprint'. Meanwhile, state-like aspects are thought to be dependent on situational circumstances, e.g. increased spindle activity after intense learning episodes. Changes, such as those induced by learning, point towards particular functions of these waveforms. As part of the proposed functions of sleep and its oscillations, these will be explored in the following section.

1.3 Proposed functions of sleep and its oscillations

Despite efforts to unravel the reasons behind sleep, a definite and comprehensive explanation remains elusive (Allada & Siegel, 2008). In the words of Allan Rechtschaffen, an early pioneer in sleep research, *"if sleep does not serve an absolutely vital function, then it is the biggest mistake the evolutionary process has ever made"* (in Walker, 2009, p.168). An organism is unlikely to choose to spend regular time intervals immobile and with considerably diminished fight or flight response, essentially rendering itself easy prey for any passing predator, if it did not profit from entering such a state in return (Mignot, 2008). At present, researchers are still divided over whether sleep evolved as a direct evolutionary advantage or as a by-product of another developmental process (Allada & Siegel, 2008). Existing theories in the field can be broadly categorised into three overarching ideas which respectively reason for either energy conservation, cellular and metabolic restoration, or cognitive processes of learning and memory.

Firstly, the idea of sleep being the body's way of reducing its energy consumption has been common discourse for centuries and was fostered by the notion of the idle sleeping brain. Given that the human brain shows an above-average energy expenditure-by-size ratio when compared to other organs, the presumed decrease in activity during sleep would be an economic solution for counterbalancing its 'running' costs (Mignot, 2008). In addition, body temperature has been observed to drop slightly during sleep, hinting at metabolic alterations (Krueger & Takahashi, 1997). This course of reasoning was supported by correlations between sleep amount and brain-relative-to-body size (Siegel, 2005), but unfortunately cannot be supported by data from all sleeping species, including our own. While lower-than-wake energy demands have been measured during NREM sleep in humans, the opposite was found for REM sleep, thus diminishing any overall energy saving during sleep (Zhang *et al.*, 2007).

Another proposed reason for sleep is cellular recovery. All living cells consume energy and produce waste. While the brain was just recently discovered to contain lymphatic vessels, it is unclear whether these suffice for disposing of all its metabolic waste (Louveau *et al.*, 2015, Sun *et al.*, 2018). Recent research has implied sleep as a potential driving agent for waste clearance (Xie *et al.*, 2013). During sleep, brain volume decreases slightly, allowing the circumfluent cerebrospinal fluid to flush through the widened cavities more effectively and take with it any waste products such as tau or β-amyloids, for example. Both are proteins which increase across periods of wake, and can clutter and eventually incapacitate nerve cells when deposited in a confined space long-term (Holth *et al.*, 2019, Iliff *et al.*, 2012). During repeated de- and hyperpolarisation cycles of SOs in SWS, cells in neuronal populations engage in a pattern of shrinking and expanding synchronously. As such, SOs are thought to enhance the flushing of extracellular spaces by acting as an initiating or co-facilitating force in this waste-clearance pumping mechanism.

A similar idea has been that of sleep enabling individual cell rest, thereby allowing recovery and renewal. Vyazovskiy & Harris (2013) propose that the primary role of NREM sleep lies in permitting individual neurons time for cellular maintenance after highly active periods of synaptic activation during wake. They suggest this is done in a prophylactic and preventative manner in order to avoid a build-up of cellular damage and consequently and possibly permanent adverse functional repercussions. As neurons are highly interconnected, such periodical rest from demanding metabolic activity can be more efficiently entered collectively during slow wave down-states. Local off periods during wake prove to be qualitatively inferior in restorative power due to the increased possibility of rest interruption by neighbouring cells firing (Piantoni *et al.*, 2013). While providing a plausible explanation for NREM sleep on a microcellular level, this idea fails to account for the existence of highly active REM sleep.

Moreover, neuroendocrine processes, such as the regulation of hormone secretion, are linked to sleep through the interplay of Processes C and S. For instance, cellular stress ex-

pression is neutralised through biosynthesis during sleep (Born & Fehm, 2000). Similarly, changes in gene expression are evident between sleeping and waking states (Mignot, 2008), and increases of SWA have been linked to heightened activity of the immune response system (Besedovsky *et al.*, 2012, Majde & Krueger, 2005). Furthermore, observations in the relationship of metabolism and sleep include associations between altered SWA and decreased glucose tolerance or heightened insulin sensitivity (Armitage *et al.*, 2013, Herzog *et al.*, 2013). However, most of these observations have been made in NREM sleep (and in animal experiments), ignoring the role of REM sleep in these processes, as well as the sheer complexity and likely interactions of these systems.

Finally, a wealth of studies testify to the beneficial influence of sleep on cognitive processes such as learning and memory (Diekelmann *et al.*, 2009, Rasch & Born, 2013, Sara, 2017, Straube, 2012). Different hypotheses have been proposed on the interaction of sleep and learning. In the following section, the most prominent of these are introduced with regard to their relevance to SWS, SOs, and healthy cognitive functioning for memory.

1.3.1 The memory functions of sleep

The ability to form long-term memories is crucial to our survival. Memories are commonly divided into different types; in the context of the research conducted for this thesis, the focus will be on declarative and procedural memory. Declarative memories can be consciously and explicitly recalled, e.g. the contents of last night's dinner, or the street you grew up on. Meanwhile, procedural memories consist of implicit knowledge, e.g., the ability to ride a bike or swim. The memory process is comprised of three distinct but complimentary stages of encoding, consolidation, and retrieval. During encoding, novel declarative information is saved as short-term memories in the hippocampus temporarily due to the latter's limited capacity. To be available for long-term recall, the pieces of information considered worthy of preservation subsequently undergo consolidation (McGaugh, 2000). This process not only occurs during wake, but has also been repeatedly demonstrated to operate more efficiently in the sleeping brain, presumably due to less external interference (Ellenbogen *et al.*, 2006). By transferring recently encoded neuronal representations to neocortical long-term storage, these hitherto labile memory traces are reorganised, stabilised and strengthened, rendering them less vulnerable to later decay and forgetting (Dudai *et al.*, 2015, Rasch & Born, 2013). Meanwhile, novel procedural memories are thought to be initially stored in the motor cortex and later transferred to the striatum for long-term retrieval (Vahdat *et al.*, 2017).

Applying the idea of sleep as maintenance for networks on a neuronal level, the synaptic homeostasis hypothesis (SHY) proposed by Tononi & Cirelli (2003, 2014) focuses on the equilibrium of connections between neuronal assemblies (see Figure 1.4). In short, SHY argues that synaptic potentiation occurs for the connections between activated nerve cells during encoding in the hours spent awake and results in a net increase of synaptic weights. Synaptic down-regulation processes become necessary to avoid oversaturation

and to maintain a meaningful signal-to-noise ratio (i.e. more efficient storage of information), and occur during subsequent SWS (Cirelli, 2017). Synapses of memories tagged as important become strengthened overnight (this refers to relative strengthening, i.e. they are less weakened). The memories considered disposable are weakened in turn, thereby restoring a synaptic and energetically sustainable equilibrium, and freeing up new capacity for cortical excitability and new synaptic potentiation (i.e. learning) in the following wake episode (Huber *et al.*, 2007). It is thought that this regular depotentiation is accomplished by SWA. Such synaptic up- and down-scaling has recently been demonstrated in a mouse model (de Vivo *et al.*, 2017). In this view, sleep is considered to be the price paid for brain plasticity (Tononi & Cirelli, 2006), enabling the preservation of important newly learnt information while discarding irrelevancies and ensuring regular return to a homeostatic baseline, which is evidenced in improved post- compared to pre-sleep encoding (Mander *et al.*, 2011). While convincing in its overall logic, SHY has been criticised for falling short of explaining the exact act of synaptic tagging (Rasch & Born, 2013).

The relevance of SHY for SWS and SOs can be explained in relation to the observed principles of wake- and use-dependencies. The longer an organism remains awake, the more its sleep or homeostatic pressure increases, a direct measure of which has been found in the duration of SWS and number of SOs occurring during subsequent sleep (Bellesi *et al.*, 2014). Based on the principles put forth by SHY, more potentiation during the preceding wake period results in more time needed for down-regulation, manifested in longer SWS. Similarly, the use-dependency concept denotes the idea that cortical areas involved in learning throughout wake show more SWA in the following sleep period (Lesku *et al.*, 2011). Local brain processes re-establish synaptic homeostasis locally in cortical areas where potentiation had occurred in prior wake (Borbély & Tobler, 2011).

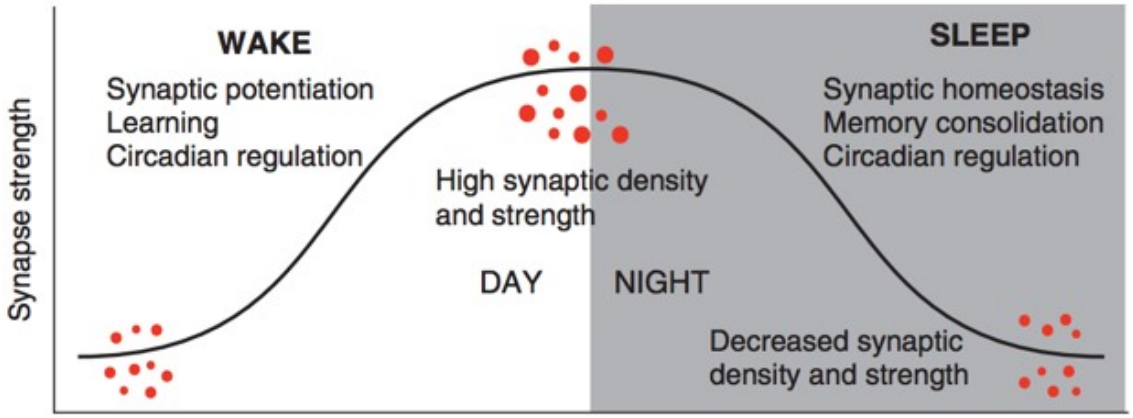

Figure 1.4: Synaptic homeostasis hypothesis. Synapses involved in learning processes throughout the day become potentiated, resulting in a net increase of synaptic strength. In order to prevent oversaturation, synaptic down-regulation occurs during sleep, restoring a synaptically sustainable equilibrium. *Source: Wang* et al. *(2011)*

In contrast to SHY, the dual process hypothesis holds that sleep benefits memory consolidation processes insofar as different types of memories are consolidated by particular sleep stages (Plihal & Born, 1997). Declarative memories benefit primarily from SWS. REM sleep on the other hand helps the consolidation of non-declarative memories, such as implicit, procedural or emotional ones (Gais & Born, 2004). The evidence supporting this hypothesis is usually collected in night-half paradigm experiments, in which participants either encode before going to sleep and are tested for recall in the middle of the night, or encode after sleeping for half a night and undergo recall tests in the morning. This methodology makes use of the observation that the first half of night is considerably richer in SWS compared to the latter REM- and N2-dominated half. Potential confounds owing to sleep deprivation are avoided this way, yet instead this method introduces bias through dissimilar encoding and recall time points and associated levels of wakefulness (Wagner *et al.*, 2004). Also, neither night half exclusively contains one stage of sleep only. The idea that procedural memory consolidation solely relies on REM sleep for example is contradicted by the fact that previous research has shown the process to depend on spindle activity (Fogel *et al.*, 2015, 2007).

In an attempt to add to the previous idea of dual processes, the sequential process hypothesis emphasises the cyclical nature of sleep stage progression as key to successful overnight memory consolidation. Similarly to SHY, the sequential process hypothesis considers SWS as the sleep stage for synaptic depotentiation, while REM sleep is implicated in the strengthening of synaptic links (Ambrosini *et al.*, 1995, Giuditta, 2014). Mednick *et al.* (2003) showed that even a 60 to 90 minute nap containing both SWS and REM proved sufficient for sleep-dependent consolidation of a texture discrimination task. Post-sleep recall on a declarative word-pair test was impaired in a study using experimental manipulation to induce sleep cycle fragmentation (Ficca *et al.*, 2000). Unfortunately, little investigation has been carried out to unravel specific details about the stage-proportionality within each cycle required to achieve this effect.

The active system consolidation hypothesis assumes that observed overnight consolidation effects of newly encoded memories are based on their reactivation, followed by reorganisation and storage during sleep (Diekelmann & Born, 2010) (see Figure 1.5). Freshly acquired declarative memories are held in the hippocampus, a subcortical formation which acts as a temporary store. During SWS, temporal coupling of SOs in the cortex, fast spindles in thalamo-cortical regions, and sharp-wave ripples in the hippocampus facilitates the gradual incorporation of these new memories into the cortex (Clemens *et al.*, 2007, Staresina *et al.*, 2015). Here they become potentiated and integrated in qualitatively organised schema networks of long-term memories to enable efficient later recall (Albouy *et al.*, 2013, Landmann *et al.*, 2014, Lewis & Durrant, 2011, Oyanedel *et al.*, 2014). In this process, SOs are considered to be the orchestrating force (Rasch & Born, 2013). A vast number of studies have lent support to this hypothesis by demonstrating SO and fast spindle enhancements after learning, and revealing associations between spindle measures

and post-sleep behavioural performance (Cairney *et al.*, 2018, Gais *et al.*, 2002, Schabus *et al.*, 2004, Rasch & Born, 2013). Following the relocation of a memory trace, REM sleep is thought to be the stabilising agent for assisting the synaptic consolidation in long-term storage.

Over recent years, the functional significance behind single graphoelements, such as spindles in particular, has attracted considerable research interest. In general, sleep spindles have been linked to intellectual and mnemonic abilities in information processing, and are seen as primers for cortical plasticity and synaptic strengthening, as well as information carriers (Fogel & Smith, 2011, Holz *et al.*, 2012, Lüthi, 2014, Maier *et al.*, 2018, Ujma *et al.*, 2015, Ulrich, 2016). Fast spindles were found to support emotional memory consolidation (Cairney *et al.*, 2014), the integration of new information into existing knowledge (Tamminen *et al.*, 2010), related to schema maintenance (Hennies *et al.*, 2016), and have also repeatedly been linked to overnight enhancements in procedural memory (Barakat *et al.*, 2013, Fogel *et al.*, 2015).

Meanwhile, the purpose of slow spindles is more obscure, and at present only few experiments have shed light on potential functions. For example, decreases in slow spindles were indicative of procedural memory impairments in major depression (Nishida *et al.*, 2016), while experimentally boosting these waveforms in healthy subjects led to improved declarative overnight memory retention (Marshall *et al.*, 2006). Based on their temporal occurrence with regard to the SO phase, fast spindles were postulated to account for thalamo-cortical network coupling to transfer recently encoded memories, while slow spindles could reflect cortico-cortical interactions (Mölle *et al.*, 2011). In this picture, slow spindles may play a role in cross-linking information for optimal long-term integration (Astori *et al.*, 2013).

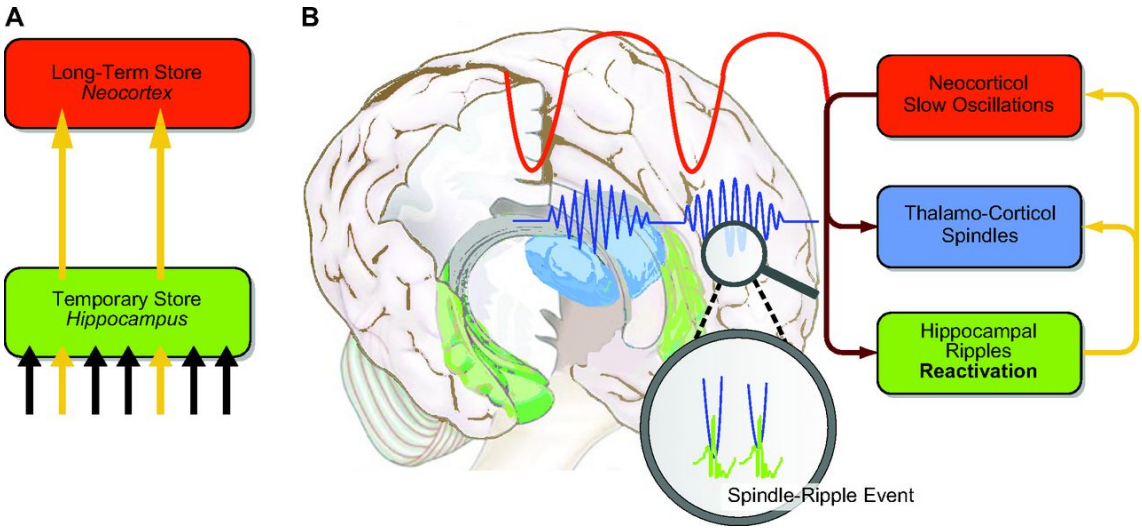

Figure 1.5: Active system consolidation hypothesis. **(A)** Newly acquired memories are temporarily stored in the hippocampal formation and are incorporated into long-term storage in the neocortex during SWS. **(B)** Neocortical SOs orchestrate lower-level oscillatory activity involved in the transfer process, such as thalamo-cortical spindles and hippocampal ripples. *Source: Rasch & Born (2013)*

1.4 Stimulating the sleeping brain to enhance sleep and cognition

Given the assumed functional implications of SWS, SOs, and spindles, experimental sleep research has attempted to manipulate these sleep features with a range of different methods to date. From a neurological point of view, an early idea was to alter the chemical properties which have been identified to determine processes of sleep and wakefulness, such as neurotransmitters and hormones (Walsh, 2009, Marrosu *et al.*, 1995). Particular combinations of these compounds have been observed to support hippocampal-neocortical memory incorporation in NREM sleep (Born & Fehm, 2000). However, longitudinal neurochemical manipulation to enhance sleep and memory raises the problem of tampering with a highly complex and inherently fine-tuned system. Such interference could thus result in a number of repercussions, i.e. unwanted side effects in overall cognitive functioning or lead to substance dependencies (Diekelmann, 2014, Feld *et al.*, 2013). Particularly in a neurochemical system, which is already undergoing changes throughout the natural ageing process, puzzling out the perfect combination of chemical agents for achieving such an enhancing yet harmless effect will require substantial future research efforts.

Research has also sought to assess the possibility of enhancing SWA by applying magnetic or electrical stimulation on the scalp. Using transcranial magnetic stimulation (TMS) at just below 1 Hz succeeded in evoking slow waves at the site of stimulation which then propagated over the cortex in a manner similar to spontaneous SOs (Massimini *et al.*, 2007). This method successfully led to an increase in SWS and SWA. However, further investigation with TMS found that due to the highly variable decline in brain plasticity with age, the success of this application varies greatly between age groups, and associated effects of concurrent cognitive boosts have been inconsistent (Pascual-Leone *et al.*, 2011).

Other studies have applied transcranial alternating or direct current stimulation (tACS/ tDCS). Marshall *et al.* (2006) used tDCS at 0.75 Hz and observed an acute enhancement of SWS, including SOs and spindles in the frontal cortex. This enrichment of SWS was mirrored in enhanced memory consolidation for declarative word-pairs, and numerically on a procedural finger-tapping task. In a recent study, phase- and frequency-matched tACS between 0.5–1.2 Hz resulted in temporary SWA increases during post-stimulation intervals, though these effects were in turn followed by slow wave power decreases (Ketz *et al.*, 2018). Nonetheless, the paradigm improved overnight recognition memory. Reversely, using tACS to disrupt SWA led to performance impairments in declarative memory (Garside *et al.*, 2015). A meta-analysis on electrical stimulation protocols concluded that while there was clear evidence for tACS/tDCS stimulation enhancing declarative memory consolidation processes, procedural memory appeared unaffected (Barham *et al.*, 2016). However, procedural (but not declarative) memory was enhanced across a sleep period by a tACS protocol applying a 12 Hz frequency which enhanced spindles (Lustenberger *et al.*, 2016).

The methods for sleep enhancement detailed so far share a number of disadvantages. They require high-tech apparatuses, incur considerable expenses, and have yet to be tested for safe, longitudinal application. Transcranial stimulation further presents the problem that its exact direction of flow through neuronal tissue cannot be very well controlled, thus limiting any definite conclusions being drawn on affected and implicated brain networks. Additionally, conscious perception of stimulation cannot be avoided, as participants usually report a tingling sensation on their skin during application, although this may be reduced during sleep. Researchers have hence turned to experimenting with lower-tech interventions with easy setup, lower cost, and a possibility for larger-scale use in home environments. For instance, Bayer *et al.* (2011), Perrault *et al.* (2019) used vestibular stimulation by asking participants to sleep in a bed rocking at a frequency of 0.25 Hz, which shortened sleep onset, increased the time spent in SWS, SO and spindle activity, subsequently leading to improved declarative memory recall post-sleep. A similar experiment stimulated the vestibular system electrically, but was less successful (Krystal *et al.*, 2010). With regard to psychological interventions, hypnotic suggestion has been successfully applied to increase SWS and SWA while reducing the time spent awake during a 90 minute nap opportunity in healthy young and older adults (Cordi *et al.*, 2014, 2015). Yet this approach was only successful in individuals high in suggestibility, and no effects on cognitive performance were investigated. Similarly, participants instructed overtly to either enhance or worsen certain aspects of their sleep were only able to conform to the latter command (Combertaldi & Rasch, 2017). Lastly, (mostly pharmacological) placebos have been successfully used to improve sleep parameters, but to date this has only been attempted in insomniacs (Rogev & Pillar, 2013, Winkler *et al.*, 2015). Whether sleep and associated cognitive processes can be enhanced by placebos in healthy sleepers is currently unknown.

Further systematic investigation of the effects of stimulation on sleep has considered various types of sensory modalities. While somatosensory stimulation effects were limited, auditory closed-loop stimulation on the other hand has proven very successful and popular over recent years (Bellesi *et al.*, 2014, Laurino *et al.*, 2014, Tononi *et al.*, 2010), and will be explored in greater detail in Section 1.4.2 after a brief summary of auditory processing during sleep. For completeness, the extensive body of research using auditory and semantically meaningful stimuli in targeted memory reactivation (TMR) paradigms to reactivate previously learnt materials (mostly irrespective of the phase of endogenous rhythms) needs to be mentioned at this point. However, a summary on the wealth of TMR studies is beyond the scope of this thesis, whose focus lies on the enhancement of specific sleep graphoelements and associated cognitive processes in a closed-loop manner, but can be found elsewhere (Oudiette & Paller, 2013).

1.4.1 Auditory processing during NREM sleep

Physiologically, the auditory system can be divided into three structures of outer, middle, and inner ear. Sounds in the form of pressure waves with varying frequency and amplitude

characteristics enter the outer ear through the pinna and travel along the ear canal to the eardrum, which marks the beginning of the middle ear (Velluti, 2008). In the middle ear, pressure waves are converted into mechanical vibrations with the help of the three ossicles (hammer, anvil, stirrup), thereby amplifying these waves. Subsequently, in the inner ear apparatus, these waves are translated into an electrical signal by the cochlea and adjacent structures. The new signal travels along the auditory nerve to the brain stem, and onto the thalamus. Each structure is involved in decoding a particular type of information in the signal. When it eventually reaches the auditory cortex, where it can be detected as auditory evoked potential in scalp EEG recordings, integration to a complex stimulus occurs (Atienza *et al.*, 2001).

Differences in auditory processing have been described across vigilance states. Auditory stimuli during stable NREM sleep failed to produce the same degree of activation during wake in left parietal, bilateral prefrontal and cingulate cortices, as well as the thalamus, whereas activity in the left amygdala and left prefrontal cortex were increased (Portas *et al.*, 2000). These differences likely reflect added layers of processing during consciousness. Despite the lack of consciousness in sleep, sleepers appear able to discriminate between stimuli of different salience levels (Legendre *et al.*, 2019). Furthermore, replaying semantically meaningful information to the sleeping brain as part of a TMR procedure benefits the consolidation of memories previously associated with these sounds (Schreiner & Rasch, 2014).

The success of auditory stimulation during sleep is thought to be grounded in the thalamocortical system, which appears susceptible to auditory input in this state (Bellesi *et al.*, 2014). However, during SWS, the degree to which auditory stimuli elicit evoked auditory responses largely depends on ongoing oscillatory activity. SO up-states appear to be brief time windows during which such processing is preferentially supported (Batterink *et al.*, 2016, Cox *et al.*, 2014, Dang-Vu *et al.*, 2010b, Schabus *et al.*, 2012). Exposed to a non-threatening and non-salient auditory stimulus at this point in time, and eager to maintain sleep, the brain typically responds by inducing an immediate neuronal down-state to prevent arousal. The non-lemniscal auditory processing pathway is thought to be responsible for the production of such large-scale neuronal depolarisations following sound input, which then spread across large areas of the cortex in the form of a KC or SO (Bellesi *et al.*, 2014, Cash *et al.*, 2009, Dang-vu *et al.*, 2011, Hu, 2003). Due to this inherent mechanism, auditory stimuli timed in synchrony with ongoing oscillatory activity have been employed as an effective and yet non-invasive stimulation method to elicit responses resembling endogenous slow waves.

1.4.2 Auditory closed-loop stimulation

The previously described, inherent mechanism of the sleeping brain to prevent arousal when faced with a non-threatening and non-salient auditory stimulus can be utilised to purposefully and systematically manipulate oscillatory features in SWS. In a seminal study,

Ngo *et al.* (2013) demonstrated how to apply closed-loop auditory stimulation timed precisely to co-occur with the SO up-state. In essence, an automated algorithm is used to monitor spontaneous SO activity and detect any signal indicating a down-state below a pre-defined amplitude threshold in real-time EEG (see Figure 1.6). Once detected, auditory stimuli are then administered to the sleeper in-phase with the SO up-state, which can be anticipated from prior assessed (e.g. fixed delay) or real-time adjusted (e.g. phase-locked) individual SO parameters. By playing brief 50ms pink noise bursts to their participants during two consecutively targeted SO up-states, Ngo *et al.* (2013) increased SO power, and phase-coupled fast spindles following the first stimulus during the stimulation period. Importantly, spontaneous and stimuli-evoked SOs did not differ in terms of topographical origin, propagation, or slope steepness, with the most pronounced SOs observed on fronto-central EEG derivations. Moreover, the stimulation enhanced overnight consolidation of declarative memory in their healthy, young adult cohort.

Following this finding, a growing number of studies employing an identical or similar closed-loop stimulation protocol to probe the relationship between SOs, spindles, and various proposed functions, as well as to elucidate on optimal stimulation settings, have since been conducted. The summarised studies presented in Table 1.1 constitute the current literature on auditory closed-loop experiments in human participants published to date (as of February 2019). Considered in sum, these studies corroborate the view that auditory closed-loop stimulation presents an effective experimental method to enhance immediate SO activity, phase-locked but temporally limited fast spindle activity, and on a behavioural level facilitates post-sleep benefits to declarative, procedural, spatial, recognition, and working memory (Antony *et al.*, 2018, Choi *et al.*, 2018, Diep *et al.*, 2018, Göldi *et al.*, 2017, Leminen *et al.*, 2017, Ngo *et al.*, 2013, Ong *et al.*, 2016, Shimizu *et al.*, 2018). However, behavioural benefits were not found in all studies (Leminen *et al.*, 2017,

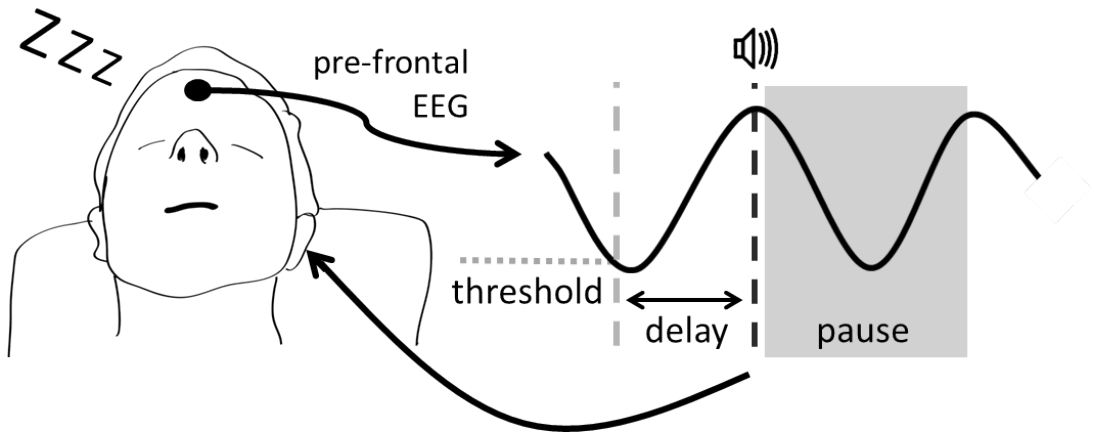

Figure 1.6: Example of an auditory closed-loop stimulation protocol. The real-time signal is monitored by an automated algorithm to detect values below a pre-defined threshold. An amplitude drop below this threshold in the pre-frontal EEG indicates an endogenous SO down-state and imminent up-state. An auditory stimulus is then delivered after a short delay to co-occur with the SO up-state. Following stimulation, the algorithm may pause for a while before resuming SO down-state detection. *Source: Own work. Drawing: 'Sleeper' by Gregory Matesich.*

Ong *et al.*, 2018), which may have been due to stimulation or task parameters, task load, or further unidentified factors. Interestingly also, 'more' stimulation does not necessarily appear to be better. Ngo *et al.* (2015) demonstrated that administering sounds to four consecutive SOs did not exceed the behavioural or fast spindle effects of a 2-click protocol.

Auditory closed-loop stimulation further supported immune functions of SWS, and had a positive effect on autonomic nervous system activity (Grimaldi *et al.*, 2019), but did not impact on metabolic processes (Besedovsky *et al.*, 2017, Santiago *et al.*, 2019). One study applied the stimulation to down-states to selectively perturb local slow waves, which impaired post-sleep encoding of a novel sequence on a procedural finger-tapping task, but interestingly did not affect consolidation of another sequence encoded prior to sleep (Fattinger *et al.*, 2017). Meanwhile, Cox *et al.* (2014) report that despite timing semantically meaningful stimuli to the optimal processing window of SO up-states, learning new information during SWS was not possible. Attempts to utilise this stimulation technique to manipulate fast spindles reveal that such activity can be enhanced only in the imminent and temporally limited aftermath of application of the first stimulus per trial, but not be entrained acutely during endogenous spindle activity (Antony *et al.*, 2018, Ngo *et al.*, 2018). Additionally, a refractory period in fast spindle-generating networks imposes further limitations. No detailed effects of this stimulation paradigm on slow spindle activity have been examined to date. It is furthermore unclear to what degree slow spindles are implicated in the reported 'fast' spindle results in some of the listed studies due to some supposedly fast spindle frequency ranges being defined from 11 Hz onward. Lastly, two studies have so far applied the closed-loop method in older adult cohorts, with results showing significant SO enhancement (Debellemaniere *et al.*, 2018, Papalambros *et al.*, 2017). However, it is unclear to what extent these oscillatory improvements mirror those found in younger adults. It is likely that any impact could be reduced due to general changes the human brain undergoes in late adulthood. As part of the work presented in this thesis sought to address this question of susceptibility to auditory closed-loop stimulation in relation to age, a brief summary of the vast literature on human brain ageing is provided in the following section in order to provide context for this work in terms of potentially challenging and limiting factors when applying the technique in older adults.

In summary, auditory closed-loop stimulation presents a non-invasive, easily administrable, and promising experimental approach to improve specific aspects of SWS and explore their functional implications. Previous studies illustrate that, while generally beneficial to different types of overnight memory consolidation, not all variations of this technique have resulted in beneficial behavioural outcomes. Furthermore, any psychological aspects of expecting or experiencing auditory closed-loop stimulation during sleep, and their possible impact on stimulation outcome are equally unexplored. In this light, the exact underlying oscillatory mechanisms, optimal technical settings and parameters required for obtaining favourable behavioural effects through auditory closed-loop stimulation as well as associated psychological impact demand further investigation.

Table 1.1: Summary of auditory closed-loop studies

Reference	Population	Design	Stimulation parameters	Behaviour	Results
Antony *et al.* (2018)	20 healthy, young adults (Exp. 3).	Within-subject conditions of early and late post-spindle TMR in N2 and SWS during one 90min afternoon nap (Exp. 3).	Stimulation: fixed delay after spindle detected in 11-16 Hz RMS. Early condition: 0.25s delay, late condition: 2.5s. 40dB white background noise. Stimuli: semantically meaningful sounds <500ms.	Item-spatial location associative learning.	Late cueing enhanced post-cue fast spindle density and memory recall. Cues in both conditions enhanced immediate SO density.
Besedovsky *et al.* (2017)	14 healthy, young men.	Counter-balanced, within-subject Stim and Sham overnights. Stimulation for 3hrs in stable N2 and SWS.	Stimulation: fixed delay to target two consecutive SO peaks after threshold -80μV crossed (updated over 5s intervals), last stimulus followed by 2.5s pause. Stimuli: 50ms pink noise at individually adjusted volumes.	None.	Stim enhanced SO power during stimulation period, reduced T and B cell counts, cortisol, and increased aldosterone levels, but did not affect growth hormone.
Choi *et al.* (2018)	4 healthy, young men.	Counter-balanced, within-subject Stim and Sham conditions. Stimulation in stable N2 and SWS during 90min afternoon naps.	Stimulation: immediate stimulus onset after spindle detected in 11-16 Hz RMS. No pause applied. Stimuli: 50ms pink noise at 62dB.	Procedural finger-tapping.	Stim increased delta, theta, and alpha activity in immediate aftermath, and enhanced post-sleep performance in all participants.
Cox *et al.* (2014)	12 healthy, young adults.	Within-subject conditions of SO peak-TMR, trough-TMR, and no TMR (each a third of stimuli). Stimulation in SWS during 2-2.5hrs evening nap. No pre-sleep exposure to sounds.	Stimulation: middle of stimulus duration phase-locked to target SO trough or peak. Stimuli: 60 semantically meaningful sounds <500ms, embedded in background white noise, at 35-45dB.	Sound familiarity task.	Conditions had no impact on post-sleep sound recognition. Stimuli elicited response regardless of targeted SO phase, but differed in their timing.

Reference	Population	Design	Stimulation parameters	Behaviour	Results
Debellemaniere *et al.* (2018)	Exp. 1: 20 healthy, young adults. Exp. 2: 90 healthy, middle-aged adults (mean age 40.8ys).	Counter-balanced, within-subject Stim and Sham conditions with ambulatory device at home. Exp. 1: conditions in separate nights. Exp. 2: 10 nights, each with both conditions selected randomly throughout night.	Stimulation: phase-locked to target two consecutive SOs followed by 9s pause in SWS. Stimuli: 40dB pink noise, duration N/A.	None.	Exp. 1: Sleep stage detection and reasonable phase accuracy of protocol confirmed. Exp. 2: Increased delta power following both stimuli. No difference in magnitude of stimulation effects between first and tenth night.
Diep *et al.* (2018)	24 healthy, middle-aged men (35-48ys, mean N/A).	Counter-balanced, within-subject Stim and Sham condition overnights. Further details N/A.	N/A	Tasks on letter fluency, category fluency, working memory.	Stim increased delta power throughout night. Post-sleep performance enhanced on all tasks. Improvement on tasks correlated with SWA increase in Stim.
Fattinger *et al.* (2017)	15 healthy, young adults.	Counter-balanced, within-subject Stim and Sham conditions in overnights. Stimulation throughout night in stable NREM sleep.	Stimulation: fixed timing upon 0.5-2 Hz filtered signal crossing threshold of -30μV indicating slow wave down-states to disturb ongoing activity. Stimulation applied to approx. 50% of all slow waves during stable NREM sleep. EMG monitored to stop stimulation upon detection of arousals. Signal derived from electrode closest to primary motor cortex per participant. Stimuli: 50ms pink noise at approx. 50dB.	Procedural finger-tapping.	Stim reduced 1-2 Hz SWA locally near detection electrode, acutely increased slope steepness, and reducing duration of targeted slow wave. No difference in sleep architecture or global SWA between conditions. Stim impaired learning of new motor sequence post-sleep, but did not impact consolidation of pre-sleep learnt sequence.

Reference	Population	Design	Stimulation parameters	Behaviour	Results
Garcia-Molina *et al.* (2018)	28 healthy, middle-aged adults (mean age 36.9ys).	Counter-balanced, within-subject Stim and Sham conditions. 5 nights Stim/Sham each, with one night per condition spent in the lab and remainder at home. Median split across age range performed to create two age groups (median = 40ys).	Stimulation: fixed delay to target consecutive SO up-states after endogenous SO crossed threshold -40μV in SWS & when slow wave detected. No pause. Stimuli applied at 1 Hz rhythm. Stimuli: 50ms tones at automatically adjusted volume based on sleep-depth (20-65dB).	None.	Stim enhanced SWA in younger age group, but not in older (who received only about half the number of stimuli compared to younger). Slow waves align to stimulus rhythm.
Göldi *et al.* (2017)	22 healthy, young adults.	Within-subject conditions of down- to up-state TMR, up- to down-state TMR, and no TMR in one overnight.	Stimulation: fixed delay to target SO up- to down-state or down- to up-state transition after endogenous SO crossing -75μV threshold in stable N2 and SWS, followed by 8s pause. Stimuli: 2/3 of previously recalled and unrecalled Dutch-German word pairs, respectively, sounds 300-500ms at 55dB.	Declarative memory task with 120 Dutch-German word pairs.	Cues presented during SO up-state improved post-sleep recall. Successful TMR was associated with elicited theta and 11-15 Hz spindle increases.
Grimaldi *et al.* (2019)	20 healthy, young adults	Within-subject Stim and Sham conditions in overnights during stable NREM sleep.	Stimulation: phase-locked to predict SO up-state. Five stimuli applied to five consecutive SOs, or five Sham markers placed, each followed by a pause lasting for the next five SOs. Stimuli: 50ms pink noise at individual adjusted volume.	None.	Stim increased acute slow wave and spindle (10-15 Hz) activity compared to Sham. Stim positively affected autonomic nervous system activity post-sleep. Sleep cycles 2-3 showed increase in parasympathetic activity.

Reference	Population	Design	Stimulation parameters	Behaviour	Results
Leminen *et al.* (2017)	15 healthy, young adults	Counter-balanced, within-subject Stim and Sham conditions in overnights during SWS.	Stimulation: fixed delay to target one SO peak after threshold -50μV crossing, followed by 2s pause. Stimuli: 50ms noise at -5 to 15dB around individual hearing threshold.	Tasks of declarative memory with 240 word pairs, procedural finger-tapping, picture recognition (119 encoded, 119x2 tested), 30 face-name associations.	Stim increased slow wave, spindle activity and post-sleep declarative memory recall. No benefits found on other tasks.
Ngo *et al.* (2015)	Exp. 1: 18 and Exp. 2: 16 healthy, young adults.	Counter-balanced, within-subject. Stimulation for 3hrs in SWS for 210min after sleep onset. Exp. 1: Driving Stim & Sham, Exp. 2: Driving & 2-click.	Stimulation: fixed delay to target two (in 2-click Stim) or up to four (in Driving Stim) consecutive SO peaks after endogenous SO crossed threshold -80μV, followed by 2.5s pause. Driving Stim used repeated threshold updating to determine whether consecutive SO would receive stimulus. Stimuli: 50ms pink noise at 55dB.	Declarative memory task with 120 word pairs (cued recall).	Driving Stim prolonged SO trains, enhanced SO and spindle power, and post-sleep recall compared to Sham. Driving Stim compared to 2-click Stim enhanced SO amplitudes but not fast spindle activity, and had no differential effect on memory recall.
Ngo *et al.* (2013)	11 healthy, young adults.	Counter-balanced, within-subject Stim and Sham overnights. Stimulation for 3hrs in N2 and SWS after stable SWS sleep onset.	Stimulation: fixed delay to target two consecutive SO peaks after threshold -80μV crossed, followed by 2.5s pause. Stimuli: 50ms pink noise at 55dB.	Declarative memory task with 120 word pairs (cued recall).	Stim enhanced SO amplitude, fast spindle power on second targeted SO, slow spindle power after first targeted SO peak, and post-sleep recall.

Reference	Population	Design	Stimulation parameters	Behaviour	Results
Ngo *et al.* (2018)	Exp. 1 and 2: each 12 healthy, young adults.	Counter-balanced, within-subject conditions. Exp. 1: Spindle Stim and Arrhythmic Stim. Exp. 2: Spindle Stim and Sham. For 3hrs in N2 and SWS after stable N2 sleep onset.	Stimulation: fixed delay after SO trough -80μV detection and stimulus. Stimuli: Spindle Stim: Seven clicks of 25ms pink noise at individual's fast spindle peak frequency, Arrhythmic Stim: as Spindle Stim but in irregular frequency with added temporal jitter (5-138ms) between within-trial clicks. Volume individually adjusted.	Declarative memory task with 120 word pairs (cued recall).	Exp. 1: No difference in evoked ERP or fast spindle power between Spindle Stim and Arrhythmic. Exp 2.: Enhanced slow wave amplitude and fast spindle power following stimulus offset, but no immediate enhancement during acute stimulation. No memory benefit identified between any conditions.
Ong *et al.* (2016)	16 healthy, young adults, partially sleep-deprived.	Counter-balanced, within-subject Stim and Sham conditions during stable NREM in 90min afternoon naps.	Stimulation: phase-locked to stimulate just before slow wave up-state. Five consecutive SOs Stim, following five Sham. Stimuli: 50ms pink noise at adaptable volume.	Declarative memory task with 40 word pairs (cued recall).	Stim increased slow wave, theta, and fast spindle activity during trial, and enhanced post-sleep recall.
Ong *et al.* (2018)	37 healthy, young adults, partially sleep-deprived.	Counter-balanced within-subject Stim and Sham conditions in N2 and SWS during 90min afternoon naps.	Stimulation: phase-locked to target SO up-state. Stimuli: 50ms pink noise at 16dB in N2 and SWS. Two consecutive SOs stimulated, two paused.	Picture recognition task (80 encoded, 160 tested)	Stim enhances SOs. No condition effect on recognition memory post-sleep. Degree of SO enhancement correlated with recall performance.

Reference	Population	Design	Stimulation parameters	Behaviour	Results
Papalambros *et al.* (2017)	13 healthy, older adults aged 60 - 84ys (mean 75.2ys).	Counter-balanced, within-subject Stim and Sham conditions in overnights throughout night.	Stimulation: phase-locked to predict SO up-state -40μV. Five stimuli applied to five consecutive SOs, or five Sham markers placed, each followed by a pause lasting for the next five SOs. Stimuli: 50ms pink noise, 30-50dB.	Declarative memory task with 88 word pairs (cued recall).	Total night SWA unchanged between Stim and Sham, but spindle density and amplitude increased. SWA increased during acute Stim intervals compared to Sham. Stim improved post-sleep recall.
(Santiago et al., 2019)	22 healthy, young men.	Counter-balanced, within-subject Stim and Sham overnights. Stimulation for 3hrs in N2 and SWS after stable SWS onset.	Stimulation: fixed delay to target two consecutive SO peaks after threshold -80μV crossing, followed by 2.5s pause. Stimuli: 50ms pink noise at 55dB.	None.	Stim enhanced SWA throughout night in SWS but did not differentially affect measures on glucose homeostasis, consecutive food intake, or energy expenditure.
Santostasi *et al.* (2015)	5 healthy, young adults.	Counter-balanced, within-subject Stim and Sham in SWS during afternoon naps.	Stimulation: phase-locked to target SO up-state after delta power and spindle presence criteria met. Stimuli: Five consecutive 50ms sine-Gaussian pulses at 500 Hz between 30-45dB.	None.	Phase-locked loop well-suited for stimulating SOs. Stim increased SWA and fast spindle (14-16 Hz) power during acute stimulation.
Shimizu *et al.* (2018)	19 healthy, young adults.	Between-subject Stim and Sham conditions in N2 and SWS during 90min afternoon naps (multiple-day protocol).	Stimulation: fixed delay to target the down- to up-state transition of SOs after endogenous SO below threshold -80μV detected. Stimuli: 18 700ms TMR sound cues at individually adjusted volume.	Spatial navigation task, with assessment on cued recall and recognition memory.	Stim did not affect sleep architecture, but increased fast spindle power (12-15 Hz), and improved post-sleep navigation efficiency on day 2.

1.5 The ageing brain

We are growing older. Owing to scientific and medical advances as well as changes in societal lifestyle, the increasing life expectancy of humans in Western societies is not only a test of our welfare and healthcare systems, but also from a more existential viewpoint, of the inherently limited, biological durability of the human body (Bloom *et al.*, 2015, Kirkwood, 2011). At present, the complex mechanisms behind ageing are far from comprehensively understood, but theories on ageing agree that this biological process is the accumulative result of molecular changes and damage to cells throughout the lifetime (Morgan & Kunkel, 2011). Numerous structural and functional changes are known to occur in healthy and pathological brain ageing, and evidence linking their extent to levels of diminished cognitive functioning is mounting (Grady, 2012). A healthy diet, regular physical exercise, and social interaction can delay the onset or lessen the impact of age-related cognitive decline (Kim *et al.*, 2018, Södergren, 2013). Beyond these suggestions, a growing body of evidence posits that sleep may have an equally protective effect (Scullin & Bliwise, 2015). Both sleep architecture and oscillations undergo considerable changes during the middle and later years in life, which have been linked to sleep's diminished restorative quality for brain health and consequent cognitive decline (Lo *et al.*, 2014a, Pace-Schott & Spencer, 2011). In the following section, the nature of physiological changes in the ageing brain will be briefly explored. Alterations observed in sleep structure, slow wave and spindle oscillations, as well as implications thereof on altered function, particularly in terms of memory abilities, will be summarised. Finally, attempts of experimental sleep research to either delay or undo such changes, and the potential of auditory closed-loop stimulation in this context, will be considered.

1.5.1 The physiology of ageing

Brain ageing denotes physiologically imposed brain changes, such as reduced density of grey and white matter (atrophy), accumulation of waste substances, and associated functional impairments, such as less efficient cognitive processing (for a review, see Kaup *et al.*, 2011). These alterations can be observed to different degrees in healthy and pathological ageing processes (Fjell *et al.*, 2009).

In a longitudinal 2-year MRI study of cognitively healthy 70-90-year-olds, significant age-related volumetric decrease was found across wide areas of the cerebral cortex (Jiang *et al.*, 2014). Females were found to have thicker cortices, but their atrophy appeared to play out at a more accelerated pace compared to their male counterparts. Another study calculated annual global grey matter loss at -0.91%/year for women and -0.65%/year for men in healthy adults aged 65 years and above (Crivello *et al.*, 2014). Yet neuronal deterioration does not affect similar anatomical brain structures to equal extents (Oh *et al.*, 2014). In a large sample aged 45 to 85 years, increasing age correlated with decreased brain volume in sensorimotor, posterior and anterior cingulate, and one subcortical network, but not the temporal, auditory, and three cerebellar networks (Hafkemeijer *et al.*, 2014). In particular

the (pre-) frontal cortex was discovered to be disproportionally affected by atrophy (Fjell *et al.*, 2009, Raz *et al.*, 1997). Interestingly and in spite of general findings of atrophy, Jiang *et al.* (2014) reported a positive relationship between age and rostral middle frontal regions, suggesting the brain retains some plasticity potential in old age and may engage in regional compensation strategies. Adding to structural changes are findings from functional imaging studies, which consistently point towards altered connectivity patterns. One reliably observed change is the posterior-anterior shift in ageing (PASA), which denotes the combination of decreased occipital and increased frontal activity during wake, believed to be another compensatory mechanism (Davis *et al.*, 2008, Morcom & Johnson, 2015).

The age-related changes detailed so far are likely mediated by further factors, such as alterations in vasculature and circulation, or hormone and neurotransmitter production (Li & Rieckmann, 2014, Topiwala & Ebmeier, 2012, Wyss-Coray, 2016). It is now also thought that the blood-brain barrier, which is responsible for safeguarding cerebral tissue from harmful substances and maintaining an ideal chemical milieu, may break down in old age (Erdö *et al.*, 2017, Iadecola, 2015). The collapse of this protective mechanism has been observed to commence in the hippocampus (Montagne *et al.*, 2015).

As the mechanism tasked with removing waste products from the brain is weakened with advancing age, a build-up of two plaque proteins called tau and β-amyloids in particular have been implicated to play a major role in neuronal and cognitive deterioration (Iliff *et al.*, 2012, Lee *et al.*, 2014, Xie *et al.*, 2013). If not regularly cleared from the brain, these proteins can accumulate and incapacitate neuronal structures; a process which commences years before any symptomatic changes are detected. Once noticeable deposition has commenced, β-amyloid plaques increase by approximately 8% annually in cognitively healthy, elderly individuals (Vlassenko *et al.*, 2011). The degree of such deposition is related to grey matter atrophy in frontal, parietal and temporal areas of the cortex and volumetric hippocampal decline (Erten-Lyons *et al.*, 2013, Oh *et al.*, 2014). In fact, the hippocampus was found to be one of the earliest deposition sites for tau, whereas β-amyloid first begins accumulating in the medial prefrontal cortex (Sepulcre *et al.*, 2013). It is important to note that the presence of plaques in the brain does not necessarily equate to progression towards neurodegenerative pathologies, which depends on various other factors (Vlassenko *et al.*, 2011). The exact causality behind a build-up of plaques remains to be conclusively established. Nonetheless, recent scientific investigations are giving hope for the future development of treatments. Experimental drugs to remove plaques are being trialled in clinical and animal studies, and ultrasound scanning has been reported to successfully remove amyloids and thereby restore memory function in a mouse model (Leinenga & Gotz, 2015, Liao *et al.*, 2018).

1.5.2 Age-related changes in sleep architecture

Sleep in the human brain undergoes quantifiable changes throughout the lifespan. In old age, the micro-level architecture (i.e. oscillatory features) of sleep appears to be more affected by this change than macro-level architecture (i.e. sleep stages) (Schwarz *et al.*, 2017). Nevertheless, the hypnogram in Figure 1.7A (bottom) depicts an example of visibly altered sleep architecture in an elderly individual. Reduced total sleep time, amount of SWS and REM sleep, heightened number of arousal and waking incidents throughout the night and consequent fragmentation of sleep stages and overall cycles are evident when contrasted with a healthy, young adult, as pictured in Figure 1.7A (top) (Carrier *et al.*, 2011, Lo *et al.*, 2014b, Mander *et al.*, 2013, Pace-Schott & Spencer, 2011, Scullin & Bliwise, 2015). More advanced atrophy in the medial temporal lobe has been linked to increased fragmentation of the sleep-wake rhythm in old age (Van Someren *et al.*, 2018). Overall, sleep appears to grow more susceptible to arousal with advancing age due to diminished depth (Ohayon *et al.*, 2004). Such decline in sleep efficiency occurs as part of the healthy ageing process in the absence of age-related pathologies (Schmidt *et al.*, 2012). SWS may even be completely absent in sleep beyond a certain age (Bliwise, 1993). The gradual onset of this decline is thought to occur as soon as in the early 20s. Age-related changes in macro-level architecture further underlie alterations in homeostatic sleep pressure regulation, which appears diminished in older individuals (Mander *et al.*, 2017).

1.5.3 Age-related changes in SOs and spindles

With regard to sleep micro-architecture, SO power, density, and amplitude decrease while slopes flatten in the ageing brain, leading to overall longer de- and hyperpolarised phases, which can be explained by cortical neuronal populations acting in a less synchronised manner (Carrier *et al.*, 2011, Halász *et al.*, 2014, Latreille *et al.*, 2019, Mander *et al.*, 2017), see Figure 1.7B. The resulting marginally out-of-phase activity is reflected in these broader and smaller SOs in the EEG. With respect to topography, Landolt & Borbély (2001) hint at a general age-related power shift in sleep EEG from frontal to central cortical destinations, interpreted as reorganisation in cortical recruitment, but standing in contrast to the previously mentioned PASA shift observed in wake processing. Similar to SOs, KCs were found to occur in lower numbers, as well as decreased density and amplitude with increasing age (Colrain *et al.*, 2010, De Gennaro *et al.*, 2017). It is presently unclear whether the decline in KC and SO activity is caused by impaired generation, less efficient global propagation, or both.

Spindle activity shows a similar decline trajectory in the later years of life. Slow and fast spindle density, amplitude, and duration are all significantly reduced in the older compared to the young adult brain (Mander *et al.*, 2017, Martin *et al.*, 2013). While these changes were observed on a global level, the former two were particularly evident at anterior sites and the latter posteriorly (see Figure 1.7B). Furthermore, spindle frequency was reported to be higher in an older cohort (Crowley *et al.*, 2002), however, Purcell *et al.*

(2017) could not reaffirm this observation in a large meta-analysis.

Lastly, considerable sleep disturbances beyond those experienced in healthy ageing have been reported in individuals affected by mild cognitive impairment (MCI) and dementias. SWS amount, delta power, sleep spindles, and KCs are all greatly reduced in these conditions when compared to healthy age- and sex-matched individuals (Pace-Schott & Spencer,

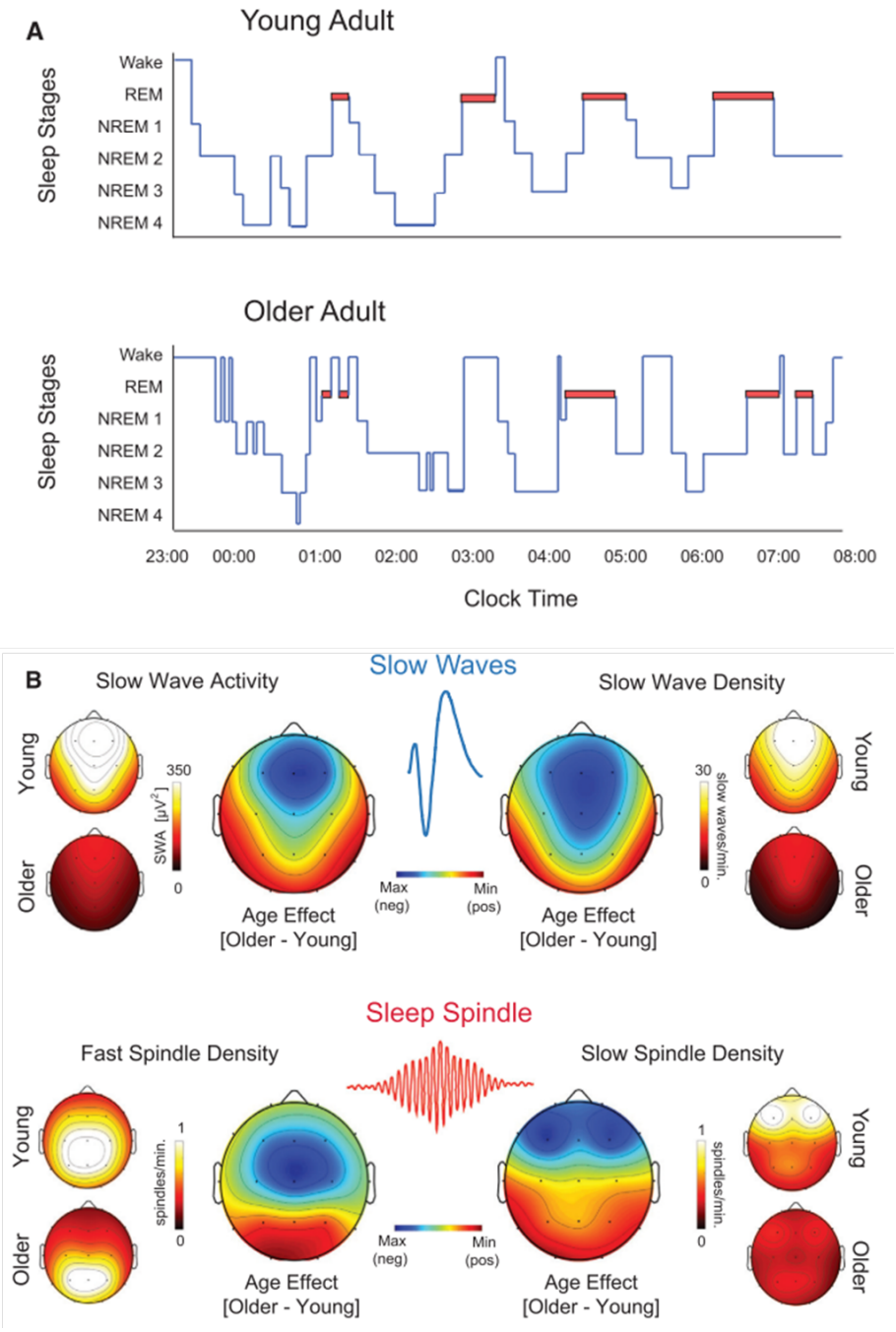

Figure 1.7: Age-induced changes in sleep. **(A)** Sleep stage hypnogram shows sleep architecture for a young (top) and older adult (bottom). With advancing age, time spent in SWS & REM decreases and sleep stages become increasingly fragmented. Nocturnal awakenings increase. **(B)** Changes in oscillatory features of SWS between young to late adulthood. SWA, slow and fast spindle density decrease globally. *Source: Mander* et al. *(2017)*

2011). Based on the trajectory of the sped up decline in sleep quality, pathological ageing may represent an especially accelerated form of the brain ageing process.

1.5.4 Functional implications of age-related brain changes on memory

The age-related brain changes summarised above are reflected in observations of altered functioning across different cognitive domains (Kaup *et al.*, 2011). The frontal cortex has been implicated to play an important role in executive functioning, which covers higher level abilities such as working memory and task switching, and in line with atrophic decline of this particular area it deteriorates with age (Toepper *et al.*, 2014, Wasylyshyn *et al.*, 2011). Attention also declines in age, with older adults showing higher levels of distractibility in experiments, possibly due to an inability to inhibit irrelevant information processing in high perceptual load situations (Healey *et al.*, 2008, Kim *et al.*, 2007, Madden & Langley, 2003). Interestingly, better task-switching performance was linked to longer TST and less wake after sleep onset (Wilckens *et al.*, 2014). Lastly, general processing speed has been found to be slower in the elderly, influencing performance on a number of cognitive tasks (Salthouse, 2000).

One intensively studied cognitive function in relation to age-related decline is memory, which deteriorates with age (Grady, 2012). Different types of memory appear to be affected to varying degrees. For instance, semantic memory is relatively spared from deterioration compared to autobiographical and episodic memory (St-Laurent *et al.*, 2011, Grady, 2012). During the initial encoding of new memories, a functionally impaired hippocampus hampers the initial acquisition and transfer into long-term storage. Wilson *et al.* (2006) reviewed studies on hippocampal performance in animal models of ageing and propose that due to sub-regional changes in this structure, a combination of difficulty in distinguishing between similar new inputs, and insufficient encoding strength account for suboptimal functioning. Prior input appears to obstruct the encoding of new information into the hippocampal hold. A lack of in-depth semantic processing has also been proposed as a causal factor (Craik & Rose, 2012). A study comparing cognitively healthy older adults with varying accumulations of β-amyloids and young controls found that the level of connectivity at encoding related to later memory performance (Oh & Jagust, 2013).

Following encoding, memories undergo consolidation to a certain extent during wake, but also during the hypothesised hippocampal-neocortical dialogue in SWS. Studies have found this consolidation process of declarative memory during sleep to be either diminished or completely absent in older age (Aly & Moscovitch, 2010, Wilson *et al.*, 2006). Another study reported that declarative memory was not enhanced in older participants overnight, as was the case in young adults (Cherdieu *et al.*, 2014). However, they did not find a significant difference in SWS between age groups in their sample, and significant dissimilarities observed in other sleep parameters did not correlate with the age difference in post-sleep performance. These findings indicate that the interplay between sleep and sleep-dependent memory consolidation in age may be more complex than previously as-

sumed. Further covariates, such as sex differences or presently unidentified compensatory mechanisms, are likely to play a mediating role.

Regarding a potential relationship between factors affected by age-related changes, Mander *et al.* (2013, 2015) found a link between the degree of atrophy and β-amyloids in the medial pre-frontal cortex and reduced SWA, which further predicted diminished overnight declarative memory consolidation in an older compared to young adult cohort. Additionally, a significant relationship between medial pre-frontal cortex amyloid pathology and SO density, but not amplitude or negative SO slope was found, indicating that age-related decreases in SWA likely stem from impaired SO generation. Impaired spindle activity and disrupted SO-spindle coupling were also found to explain diminished effects of overnight consolidation (Helfrich *et al.*, 2017, Mander *et al.*, 2014). Similarly to declarative memory, the beneficial post-training consolidation of procedural memory commonly observed in young adults across a sleep period appeared unattainable in older adults (Cherdieu *et al.*, 2014, Fogel *et al.*, 2015, Gui *et al.*, 2017, Spencer *et al.*, 2007).

It is presently unknown whether the detrimental processes of atrophy, amyloid deposition and decreasing SWA stand in unidirectional or circular relationships to one another. Based on the described interactions, different mechanisms are conceivable. Decreases in prefrontal SWA would result in less waste clearance, leading to a build-up in the area. However, if SOs in fact epitomise a protective clearing mechanism, then it remains elusive as to why early amyloid deposition is observed at prefrontal brain sites with maximal slow wave power where any clearance effects should be most pronounced. Alternatively, atrophic decline and increased protein deposition could be consequences of other unexplored processes incapacitating further SO generation through cell death of involved neuronal populations. Such deliberations, however, are purely speculative at this point of investigation and require further attention. In conclusion, the physiological and behavioural changes seen in sleep and memory abilities in the ageing brain appear to be causally linked.

1.5.5 Enhancing sleep in the ageing brain with auditory closed-loop stimulation

Naturally, the above detailed changes in sleep in older age have led to the proposition that targeted manipulation to enhance particular aspects of sleep could lead to an improvement of cognitive functioning. If successful, such manipulation could find large-scale medical application in either preventing or at least decelerating naturally occurring cognitive degeneration in ageing individuals (Pace-Schott & Spencer, 2011). Previous studies have attempted to apply tDCS to boost SWA and associated declarative memory consolidation in older individuals, but with mixed results (Eggert *et al.*, 2013, Ladenbauer *et al.*, 2016, Paßmann *et al.*, 2016).

Given the scale of present-day population ageing, an ideal intervention method would be low-tech, economic, and available for home-use. Auditory closed-loop stimulation presents

one such convenient intervention option and simultaneously provides opportunity to increase our understanding of the dynamic interrelationship between changes in sleep and cognition observed in the ageing brain. Debellemaniere *et al.* (2018), Papalambros *et al.* (2017) provide initial evidence that auditory closed-loop stimulation can be successfully applied to the ageing brain to enhance SWA, and in one case declarative overnight memory consolidation. Further research will need to investigate the exact impact of such stimulation on sleep and associated cognition, particularly if applied longitudinally. Whether changes observed in the central auditory system with increasing age (for a review, see Ouda *et al.*, 2015) could affect the success of auditory stimulation in enhancing sleep and overnight memory consolidation in hearing-impaired individuals also needs to be considered.

1.6 Summary

Sleep is a unique behavioural state, regulated by inherent homeostatic processes. Within sleep, different sleep stages can be identified by distinct sets of oscillatory patterns. These oscillations have captured continued research interest over the past decades as they are believed to be directly linked to and facilitating a variety of vital restorative functions during sleep. Examples range from clearing the brain of waste products to cognitive, metabolic, and endocrine-supportive processes. The most common oscillatory features in SWS, and to a diminished extent stage N2 sleep, are SOs and spindles, which are thought to promote memory processes in the sleeping brain when interference from external sources is minimal. While SHY posits that SOs are involved in maintaining a meaningful signal-to-noise ratio by pruning synapses containing information not worthy of future preservation, the active system consolidation hypothesis theorises that phase-coupled SO and fast spindle activity drives memory consolidation from temporary hippocampal to long-term neocortical memory storage. Despite such propositions on the function of particular oscillations or sleep stages, many questions on their specific dynamics and interactions remain unsolved.

To explore these unknowns, experimental sleep research is investigating ways to manipulate specific oscillations to be able to infer their purpose. Similarly, methods to boost oscillatory activity, which appears to be impaired in a number of situations and pathologies, are being developed. For instance, one such target group could be the ageing population, in which concurrent sleep and memory detriments are particularly evident.

To date, a range of techniques to influence oscillatory features have been trialled. One novel and increasingly popular method is auditory closed-loop stimulation, which fulfils the criteria of being easily administrable and controllable, and has thus far demonstrated promising results. The technique makes use of a particular mechanism in auditory processing of the sleeping brain, which is dependent on the underlying phase of neural oscillations. Applying auditory stimuli during the SO up-state has been shown to increase SWA, phase-coupled fast spindles, and enhance consolidation of memories, amongst other benefits. As such, the method poses great potential to further investigate the dynamics of SOs and

spindles during sleep and to probe their functional implications.

1.7 Research objectives

The overarching aim of the research conducted for this thesis is to further investigate the potential of auditory closed-loop stimulation to enhance oscillatory components of sleep and thereby improve sleep-dependent memory consolidation. In **Chapter 2**, we examine whether this novel stimulation technique could be applied in a population of healthy, late middle-aged adults. Specifically, the possibility of using the technique to counter the decline in sleep quality and memory functioning observed in the ageing brain is of central interest. We first aim to determine the outcome of stimulated sleep compared to endogenous sleep in this group, before contrasting the magnitude of effects with a healthy, young adult cohort. In **Chapter 3**, we turn our attention to sleep spindles by considering the potential of auditory closed-loop stimulation to differentially modulate slow and fast spindle activity. These spindle types show distinct temporal and topographical properties, yet it is unknown whether their differences also relate to separate functions. Fast spindles have previously been ascribed a crucial role in the consolidation of memories overnight, yet studies often turn a blind eye to slow spindles. In this context, we aim to trial a stimulation protocol which would disproportionally enhance slow spindle activity, which, if successful, could be useful for future investigations into the functional implications of these waveforms. Lastly, in **Chapter 4**, we consider whether anticipating auditory closed-loop stimulation and the expected effects thereof on sleep would lead to such changes in a placebo stimulation protocol. The ability of humans to self-enhance selective aspects of their sleep by anticipating such consequences from outside intervention has previously been demonstrated in insomniacs, but has yet to be addressed in healthy sleepers. If existing, such placebo effects could bias the outcome of actual stimulation application by under- or over-estimating the influence of auditory closed-loop stimulation on sleep, hindering a realistic appreciation of the technique's scope.

Chapter 2

Susceptibility to auditory closed-loop stimulation of sleep slow oscillations changes with age

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7^{th} World Sleep Conference (Istanbul, November 2015),
First International Conference on Sleep Spindling (Budapest, May 2016),
Replay@CUBRIC2017 conference (Cardiff, May 2017),
BBSRC DTP symposium (Manchester, June 2017).

2.1 Abstract

Background: Cortical slow oscillations (SOs) and thalamo-cortical sleep spindles hallmark slow wave sleep and facilitate sleep-dependent memory consolidation. Experiments utilising non-invasive stimulation to enhance these oscillations have shown great potential in young subjects. However, to what extent these findings translate to an older population remains unclear.

Objective: We examined the possibility of enhancing sleep SOs, fast spindles, and performance on different memory tasks in an older population using auditory closed-loop stimulation.

Methods: In a within-subject design, subjects (n = 17, 55.7 ± 1.0 years, 9 female) received auditory click stimulation during sleep SO up-states, which was compared to a no-stimulation baseline night. Overnight memory consolidation was assessed for declarative word-pairs and procedural finger-tapping skill. Post-sleep encoding capabilities were tested using a picture recognition task. Additionally, electrophysiological effects of stimulation were compared to those reported previously in a younger cohort (n = 11, 24.2 ± 0.9 years, 8 female).

Results: Auditory stimulation prolonged endogenous SO trains and induced sleep spindles phase-locked to SO up-states in the older population. However, responses were markedly reduced compared to the younger subjects. Also, temporal dynamics of stimulation effects on SO and spindles differed between age groups. Overnight retention and post-sleep encoding performance of the older cohort revealed no beneficial effect of stimulation.

Conclusions: Our findings suggest that the susceptibility to auditory stimulation during sleep drastically changes with age and reveal the difficulties of translating functional protocols from younger to older populations.

Keywords: Sleep, Ageing, Memory, Auditory closed-loop stimulation, Slow oscillations, Sleep spindles

Highlights:

- Auditory closed-loop stimulation induced SOs and sleep spindles in older subjects
- Stimulation effects were reduced and overall susceptibility diminished with age
- Slow oscillation and sleep spindle dynamics in older adults deviated from those in younger subjects
- Auditory closed-loop stimulation did not have a beneficial impact upon memory performance in older subjects

2.2 Introduction

Sleep is an integral part of our lives, and sleep research has accumulated strong evidence on its importance for our bodily health by facilitating a diverse range of restorative and regenerative functions (Grandner, 2017). In particular slow wave sleep (SWS) is thought to facilitate crucial immune-supportive, endocrine, and metabolic functions (Besedovsky *et al.*, 2012, Holth *et al.*, 2019, Morgan & Tsai, 2016, Xie *et al.*, 2013). Hallmarked by <1 Hz slow oscillations (SOs) and 12-15 Hz sleep spindles, SWS not only exhibits a repertoire of brain rhythms which are highly distinct from wakefulness, but the parallel unconsciousness suggests that this sleep stage represents a unique period to support brain-related functions, of which memory consolidation is one of the most important. Accordingly, SOs, sleep spindles, and their intricate interplay are thought to mediate the strengthening of newly acquired declarative and procedural information into long-lasting memories (Rasch & Born, 2013). Likewise, the ability to acquire novel information is contingent on the amount of prior obtained SWS (Antonenko *et al.*, 2013, Diekelmann *et al.*, 2013, Van Der Werf *et al.*, 2009, Yoo *et al.*, 2007).

Sleep quality naturally declines during both healthy and pathological ageing (Mander *et al.*, 2017). Linked to age-related neural atrophy (Mander *et al.*, 2013, Westerberg *et al.*, 2012), the number and amplitude of SOs and delta waves (1-4 Hz) decreases, and overall power in the 0.5-4 Hz slow wave band is strongly reduced (Carrier *et al.*, 2001, Fogel *et al.*, 2012, Landolt *et al.*, 1996). Moreover, age-related deterioration of memory abilities can be predicted by the decline in SWS during later-life brain maturation (Scullin & Bliwise, 2015). In older adult cohorts, the benefit of sleep for the overnight consolidation of declarative and procedural memories is either reduced or non-existent (Cherdieu *et al.*, 2014, Fogel *et al.*, 2017, 2014, Gui *et al.*, 2017). Furthermore, shallower sleep was associated with less successful encoding of novel information post-sleep in a healthy, older cohort (Van Der Werf *et al.*, 2009, Wilson *et al.*, 2006).

With such a crucial role of SWS, experiments to enhance selected sleep oscillations are highly topical and have been successfully trialled in several stimulation modalities (Bayer *et al.*, 2011, Marshall *et al.*, 2006, Pereira *et al.*, 2017, Cellini & Capuozzo, 2018, Perl *et al.*, 2016), and auditory closed-loop stimulation in particular has proven to be an effective and promising technique (Ngo *et al.*, 2013). This stimulation method consists of detecting endogenous SOs, and applying brief auditory stimuli during their positive peaks, which has been shown to prolong ongoing slow oscillatory activity, induce fast spindles phase-locked to the initial elicited positive SO half-wave (up-state), and enhance functionally associated declarative overnight recall in young adults (Bellesi *et al.*, 2014, Leminen *et al.*, 2017, Ngo *et al.*, 2013, 2015, Ong *et al.*, 2018).

Given the debilitating everyday implications of age-related SWS and memory deterioration, the current study investigated whether auditory closed-loop stimulation targeting SOs would likewise boost sleep rhythms and sleep-dependent memory processes in an

older adult cohort. We assessed overnight consolidation of both declarative and procedural memory, as well as post-sleep encoding abilities. By using the setup and protocol of a previously reported dataset in a healthy, young adult cohort (Ngo *et al.*, 2013), we directly compared and contrasted different magnitudes of physiological enhancement in these two age groups.

2.3 Methods

2.3.1 Subjects

An older adult cohort consisting of 17 volunteers (9 female) aged 49 to 63 years (mean ± SEM = 55.7 ± 1.0) with no history of psychological, neurological, or sleep disorders, and no current or recent physiological conditions compromising sleep quality was recruited through newspaper ads and university staff mailing lists. All were non-smoking, native German speakers who did not take any medication known to impact sleep, and had followed a regular sleep/wake schedule for four weeks prior to participation. In a brief initial information and eligibility screening session, subjects were assessed for good hearing (3-digit hearing test) and showed no signs of mild cognitive impairment (indicated by a score $\geq$ 24/30 in the Montreal Cognitive Assessment), nor excessive daytime sleepiness on the Epworth Sleepiness Scale (mean ± SEM score = 10.83 ± 0.91, range = 7-13/24). The experiment had prior ethical approval from the Universities of Tübingen and Manchester, and was conducted in compliance with the Helsinki Declaration. Subjects gave informed written consent before participation and received a monetary reimbursement for their time.

In order to compare electrophysiological effects of auditory closed-loop stimulation in our older cohort to a younger population, we took advantage of a previously published dataset (Ngo *et al.*, 2013). This dataset consists of 11 healthy young adults (eight female, mean ± SEM age = 24.2 ± 0.9 years), who fulfilled matching requirements for participation and were subjects to identical stimulation procedures during sleep.

2.3.2 Experimental design and procedure

An adaptation night accustomed subjects to sleeping in the University of Tübingen sleep laboratory under polysomnographic monitoring. Between adaptation and first experimental night, subjects slept at least one night at home. In a within-subject design, they then spent two experimental nights in the laboratory (see Fig. 2.1A), undergoing one experimental stimulation (Stim) and one Sham (no stimulation) condition each, counterbalanced in order between subjects and at least 7 nights apart. On experimental days, subjects were instructed to wake at 7 am, to not consume any alcohol within 24 hours prior to participation, nor to ingest any caffeine after 2 pm. They presented to the sleep laboratory at 8 pm and were fitted with electrodes for polysomnography. Subjects then performed a psychomotor vigilance task (PVT), followed by a declarative paired associate learning and procedural finger-tapping task. Prior to bedtime at ∼11 pm, they completed the Stanford Sleepiness Scale (SSS). Subjects were awoken the following morning while in light stage 1

or 2 NREM sleep after ∼8 hours of opportunity to sleep. To avoid effects of sleep inertia, subjects completed a questionnaire to assess sleep quality and feeling of being well rested (Görtelmeyer, 2011), as well as the SSS at least half an hour after waking, followed by paired associate learning recall, a post-sleep-only picture recognition task interleaved with a distractor Digit Span Task, and lastly a finger-tapping retest. The order of memory tasks was chosen to minimise interference effects between different types of memory on performance.

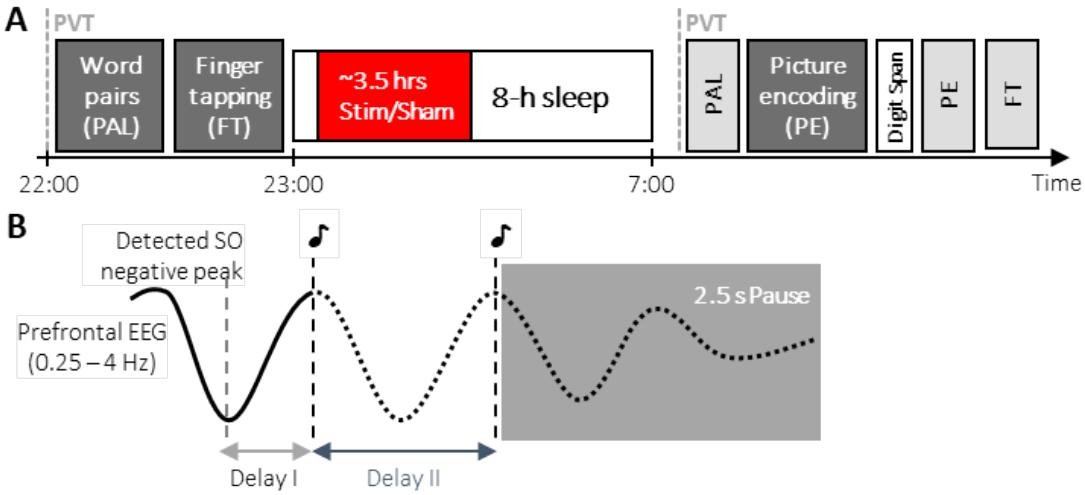

Figure 2.1: Study design for older population. **(A)** Subjects learned word-pairs including an immediate cued recall (PAL) and were then trained on a finger tapping (FT) task. Afterwards subjects were allowed to sleep for 8-hours during which auditory closed-loop stimulation (Stim) or no stimulation (Sham) was applied. The next morning, first, word-pair memory (PAL) was tested, followed by a picture encoding (PE) task, for which the encoding and recognition phases were interleaved by a Digit Span Task. Finally, finger tapping (FT) performance was tested. **(B)** Schematic illustrating the stimulation protocol. Upon detection of a negative SO peak, the first and second click were delivered after two individually adapted delays (Delays I and II) followed by a stimulation pause of 2.5 s.

2.3.3 Memory and control tasks

All tasks were presented on a 19-inch LG Flatron desktop monitor with a screen resolution of 1280 x 1024. Tasks were programmed in E-Prime 2.0 (Version 2.0.10.242, E-Studio 2.0.10.147, Psychology Software Tools, Sharpsburg, USA), unless otherwise stated.

Paired associate learning task. To assess declarative memory, subjects were instructed to learn 80 word pairs of moderately semantically related German nouns (Ngo *et al.*, 2013). Different sets of pairs were used in counterbalanced lists between subjects and nights, with different word pair order in all learning and recall sessions. In evening sessions, subjects were asked to carefully study the word pairs when each was presented once for 4 s on screen with an interstimulus interval of 1 s. A subsequent immediate recall test established baseline retention by presenting the first noun of the word pair and requiring a reply with the second noun. Subjects were given unlimited time and shown the correct response on screen after having verbally provided their answer. The morning recall ses-

sion followed the same procedure but included no feedback. The number of correct pairs retained overnight was calculated as the difference between the scores obtained in the morning and evening (absolute performance). We also examined the relative performance by dividing the overnight change by the number of correctly remembered word pairs in the evening.

Finger tapping task. Procedural memory was assessed on a finger-tapping sequence task (Walker *et al.*, 2002). Following a short practice round, subjects used the four fingers of their non-dominant hand to tap on a computer keyboard a fixed five-digit sequence presented on screen as often and accurately as possible within 30 s intervals. In the evening session, they completed 12 blocks of 30 s, interspaced with 30 s breaks. Feedback in the form of number of correct and overall attempted sequences was shown on screen following each block. The morning retest session followed an identical procedure. Evening and morning performance scores were calculated by averaging the numbers of correctly tapped sequences in the last and first three blocks in the evening and morning sessions, respectively, with their difference representing overnight change.

Picture-encoding task. In the morning sessions only, a picture-encoding task presented subjects with 50 photographs of neutral landscapes and houses for 2.5 s each and then prompted them to indicate via keyboard presses whether the photo depicted a residential house or tropical landscape to aid encoding (Antonenko *et al.*, 2013, Van Der Werf *et al.*, 2009). Picture presentation order was randomised, with a varying interstimulus interval of 0.6-1.4 s. Following the encoding phase, a ∼5 min distractor Digit Span Task was conducted to distract subjects from mentally rehearsing stimuli between encoding and recall. To assess encoding performance, subjects were presented with 100 photographs, 50 of which they had previously been exposed to, and asked to indicate via keyboard presses whether they remembered previously seeing the picture, with answer options of 'yes', 'maybe yes', 'maybe no' and 'no'. Encoding performance was evaluated by combining the former and latter two options respectively, counting cases of correctly remembered items (hit), incorrectly remembered items (false alarm), correctly negated items (correct rejection) and falsely negated items (miss). Accounting for response bias, we then calculated a final score d-prime (d') by subtracting the z-transformed false alarm rate from the z-transformed hit rate.

Digit span task. Subjects were tasked to memorise and immediately verbally relay a number series of increasing length as read out by the experimenter. In the first part of the task and starting at level 1 with 2 trials consisting of 3 digits each, each further sequence increased in length by one digit per level up to level 7, but was ended whenever a subject repeated both trials per level incorrectly. Part two then required the subjects to repeat sequences backwards, with each corresponding level consisting of one less digit than the forward repeat part. Different sequences were used for each experimental night. Scores were calculated per forward, backward, and total number of trials and level reached.

Psychomotor Vigilance Task. A ∼5 min psychomotor vigilance task (PVT, based on Dinges & Powell, 1985) programmed in a custom-made software was used to measure subjects' alertness and vigilance at the beginning of evening and morning sessions. Subjects were instructed to focus on a millisecond counter, which repeatedly appeared at the centre of the screen after a random delay ranging between 2-10 s, and to stop it with a key response as quickly as possible. An average score was calculated from all their responses with delay times below 150 ms and above 800 ms excluded. PVT data from one individual were lost due to a technical error.

2.3.4 Polysomnography recordings

Polysomnography was continuously recorded with a BrainAmp DC amplifier (Brain Products, Gilching, Germany) with scalp electroencephalogram (EEG) electrodes positioned according to the international 10-20 system at F3, Fz, F4, C3, Cz, C4, P3, Pz, P4, all referenced to an average of two mastoid electrodes M1, M2. One ground electrode was placed on the forehead. Impedances of all electrodes were kept below 5 kΩ. EEG data were sampled at 500 Hz and saved without any filters (DC-mode) offline on a computer for later analyses. In addition to the above setup, a further six electrodes were attached to record horizontal and vertical electrooculography, and electromyography in order to detect movement artefacts and facilitate standard sleep scoring.

2.3.5 Auditory closed-loop stimulation

The present study used the same technical setup and stimulation protocol from Ngo *et al.* (2013). A 'Digitimer D360' EEG Amplifier (Digitimer, Welwyn Garden City, UK) and 'Power1401 mk 2' data acquisition interface (Cambridge Electronic Design, UK) were linked to a computer, all separate to the polysomnography. This facilitated real-time filtering between 0.25-4 Hz of the incoming EEG signal streaming from one additional second forehead ground and one EEG electrode placed on Fpz to allow a custom-designed algorithm in Spike2 (Cambridge Electronic Design, Cambridge, UK) to detect whenever the signal value dropped below a previously defined threshold, indicating an SO down-state during SWS (SO trough threshold was set to -80 μV by default, but had to be adjusted to -50 μV and -60 μV for one and two subjects, respectively). Upon detection of such an SO down-state a first click was presented after an individually determined delay (delay I: mean = 583.24 ± 26.50 ms, range = 370-780 ms) to coincide with the subsequent SO up-state (Fig. 2.1B). A second click was delivered after a second individual delay (delay II: mean = 1091.47 ± 21.06 ms, range = 930-1300 ms) concurring with the up-state of the SO induced by the first click presentation. After the second stimulus presentation the detection algorithm paused for 2.5 s before it resumed trough detection. Due to the variant nature of SO parameters in this age group, we individually adjusted delay II for eleven of our 17 older subjects, unlike in the original study where this was kept constant at 1075 ms (Ngo *et al.*, 2013). The number of administered stimulation trials is presented in supplementary Table 2.3 and did not vary statistically between age groups ($t(26) = -1.58$, $P = 0.127$).

Stimuli consisted of 50 ms pink 1/f noise and were delivered binaurally via earbuds (Sony MDR-EX35) at individually adjusted volume levels, which had been previously determined during SWS in the adaptation night (volume range 48-58 dB, mean = 54.53 ± 1.17 dB). Upon questioning in the mornings following experimental nights, only 3 individuals reported hearing sounds, which was ascribed to minor temporary arousals and associated heightened levels of consciousness. Stimulation commenced once subjects had spent ∼5 min in stable NREM sleep and was continued for 3.5 hrs, but stopped manually for arousals or changes in sleep stage. In Sham nights, an identical protocol was followed: below-threshold SO troughs were detected and stimulation markers placed in the recording, but no sounds were delivered. Subjects were blinded on experimental condition order and only debriefed before their final departure from the laboratory.

2.3.6 EEG analysis

All data analysis, for both the older and young cohort, were carried out in Matlab (Version R2016b, 9.1.0.441655, MathWorks, Natick, USA) using custom-made scripts programmed in Fieldtrip (Oostenveld *et al.*, 2011), unless otherwise stated. Following an initial filtering of EEG and EOG between 0.3-30 Hz, and of EMG above 5 Hz, two trained experimenters determined sleep stages across experimental nights according to Rechtschaffen and Kales scoring criteria while blinded to the experimental condition (Rechtschaffen & Kales, 1968). Sleep stages S1, S2, SWS (= S3 + S4), REM sleep, wake, epochs containing movement and other arousals were identified from lights off until waking time, with epochs falling into the latter two categories excluded from analyses beyond sleep statistics. Percentage of time spent in each stage was calculated as time in the respective sleep stage over total sleep time (TST).

To calculate event-related responses time-locked to auditory stimulation, EEG epochs identified as SWS were filtered between 0.3–30 Hz. The signal was then averaged in windows of 5 s, with a -2 s pre-stimulus offset with regard to the first stimulus (at t = 0 s) across subjects per experimental condition. Analysis of evoked fast spindle activity followed the same procedure with an additional bandpass filtering between 12-15 Hz and calculation of the root mean squared signal (RMS) (based on a window of 200 ms) and baseline correction between -2 to -1.5 s before averaging.

To assess spatiotemporal patterns of the overall evoked responses (measured by the large negative component at ∼500 ms post-stimulus) and the fast spindle response, we examined the relative change of the induced responses with respect to the endogenous (initially detected) SO trough for the stimulation conditions in young and older cohorts. To this end, for the overall response, we divided the largest negative amplitude value found 0 to 1 s post-stimulus for the first and second click by the mean baseline value of the endogenous slow oscillation between -1 to 0 s preceding the first click (Fig. 2.3D). Contrarily, for the fast spindle response, we determined the ratio of the largest peak in the fast spindle RMS-

signal between 0.5 and 1.5 s after each stimulus and a mean baseline value between -0.5 and 0.5 s centred around the first click (Fig. 2.4D). Furthermore, to evaluate the impact on spindle refractoriness, we calculated the difference in the mean fast spindle RMS-activity derived early between -2 and -1.5 s and late between 2.5 and 3 s with respect to the 1st click (at t = 0 s) for both groups and experimental conditions (Fig. 2.4E).

Sustained stimulation effects were examined firstly by spectral analysis during SWS using Fast Fourier Transformation (window length 4096 data points with 50% overlap) across the stimulation period (first to last stimulus). Power in the following frequency bands of interest was calculated by the mean of corresponding frequency bins: SO peak frequency between 0.5-1.25 Hz as well as the fast spindle range at 12-15 Hz. SO peak frequency was chosen as a measure over the more conventional 0.5-1 Hz frequency band to fully capture the displayed SO frequency variance in the older adult cohort. Secondly, we additionally detected discrete SOs during SWS epochs across the entire night based on previously described algorithms (Mölle *et al.*, 2011, Ngo *et al.*, 2018). In addition to the 0.3 Hz high-pass filtering, each EEG channel was low-pass filtered at 1.25 Hz (Butterworth, 6th order, two-pass). Then positive to negative zero crossings were identified and all intervals between consecutive zero crossings shorter than 0.8 or longer than 2 s (corresponding to frequencies of 0.5–1.25 Hz) were discarded. Across the remaining intervals the negative peaks and the amplitude from negative to the positive peak were averaged. The resulting mean values were multiplied by 1.25 and served as detection threshold, i.e. intervals were labelled as a SO whenever its negative peak was lower than 1.25 times the mean negative peak value and the amplitude exceeded the 1.25 times the mean amplitude threshold (Ngo *et al.*, 2013). The number of offline detected SO events was then determined for the 3.5 h stimulation/sham period. Furthermore, for the stimulation/sham period, we calculated the SO peak-to-peak amplitude of the offline detected SO events, and phase-locked fast spindle activity was examined by averaging the fast spindle RMS-signal time-locked to the negative SO peak of detected events in a window from -1.25 to 1.25 s with a baseline correction from -1.25 to -1.15 s. In order to assess the temporal interrelationship among SOs during the ∼3.5 h stimulation period, we examined for each offline-detected SO event at electrode Cz the occurrence of pre- and succeeding SOs based on event histograms within 100 ms bins in a ± 3 s time interval (SO trough at t = 0 s). Resulting histograms were normalised by the total number of detected SO events (multiplied by 100) and then the difference between Stim – Sham conditions was calculated and visualised time-locked to the negative peak of detected SO events.

2.3.7 Statistical analyses

All data are shown as mean ± SEM. Statistical analyses were generally based on paired-samples student's t-tests, or repeated-measures analyses of variance (RM ANOVA) with within-subject factors 'condition' (Stimulation vs. Sham), 'topography' (9 EEG channels), and between-subject factor 'age' (young vs. older adults). In these, we focus on reporting significant interactions, and age group main effects. Separate post-hoc ANOVAs were

subsequently conducted for each age group. If necessary, a Greenhouse-Geisser correction for degrees of freedom was applied. Topographical plots were prepared based on channel-wise paired-tests with p-values adjusted for multiple comparison using false discovery rate (FDR) corrections (Benjamini & Hochberg, 1995), unless otherwise stated. The threshold of significance was set to a $p\ value < 0.05$. Bayes Factor (BF) calculations were carried out in JASP (version 0.10.0, JASP Team, Amsterdam, 2018) with default prior settings (Cauchy scale = 0.707) in order to evaluate the evidence of the present behavioural data for both null and alternative hypotheses (Dienes, 2014, Rouder *et al.*, 2009, Jeffreys, 1962). In the case of individual data values missing due to technical error (n = 1 in PVT) or subjects omitting questionnaire items (n = 2 in sleep quality), the respective individuals were excluded from corresponding analyses.

2.4 Results

2.4.1 Memory performance in older adults not enhanced by stimulation

We first examined whether stimulation affected overnight retention across different memory tasks in the older cohort. For the declarative task, the difference in the absolute number of correctly recalled word-pairs obtained in the evening and morning was comparable between the Stimulation and Sham conditions (5.3 ± 1.6 and 8.4 ± 1.2 words for the Stimulation and Sham conditions, respectively; t(16) = 0.803, P = 0.093). Examining the relative change in performance with respect to the number of correct responses in the evening actually revealed a lower performance following a night of stimulation (18.68 ± 6.22% vs. 39.48 ± 8.76% for the Stimulation and Sham condition, respectively, with t(16) = -2.13, P = 0.049, Fig. 2.2A). However, Bayesian analysis indicated the data only lent anecdotal support for any difference between conditions (BF_{10} = 1.496, see Jeffreys (1962)).

Turning to the procedural finger-tapping task, stimulation had no impact on overnight changes in performance in the number of correctly tapped sequences (Stimulation: -0.30 ± 0.56, Sham: 0.33 ± 0.62; t(16) = -1.011, P = 0.328, Fig. 2.2B), with Bayesian analysis showing anecdotal support for the null hypothesis (BF_{01} = 2.577).

Stimulation further did not affect post-sleep encoding capability of pictures (d' = 2.04 ± 0.19 and d' = 1.85 ± 0.17 for Stimulation and Sham condition, t(16) = 1.62, P = 0.125, Fig. 2.2C), although again Bayesian analysis suggests our data to be only of anecdotal support for the null hypothesis ((BF_{01} = 1.355)).

2.4.2 Evoked responses in older cohort are similar to, but weaker than those in younger subjects

In light of these behavioural results, we next set out to examine the electrophysiological responses to the stimulation. We made use of an existing EEG dataset from a young adult cohort (see section 2.3.1 for details), allowing direct contrasting between the two

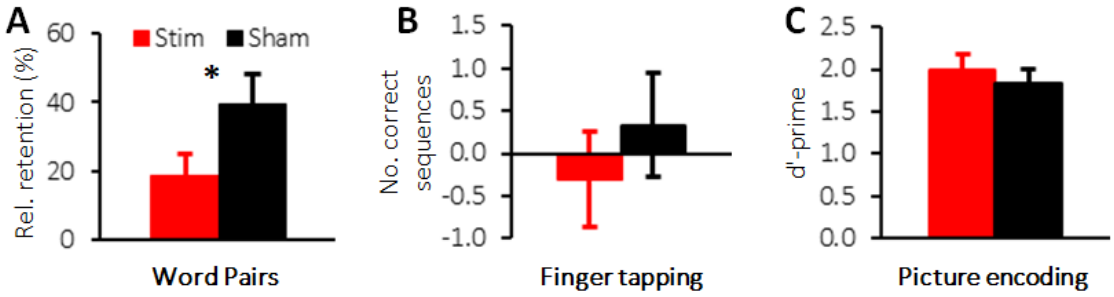

Figure 2.2: Behavioural results for older population. Mean ± SEM of memory performance on the paired associates (relative retention to evening baseline, **A**), finger tapping (**B**) and picture encoding tasks (**C**) for the Stimulation (red) and Sham (black) condition. Asterisk denotes $p < 0.05$.

age groups. As expected, averaging the EEG signal time-locked to the first stimulus revealed that the SO rhythm was prolonged by two additional cycles in our older subjects (Fig. 2.3A). However, a comparison to the younger cohort (Fig. 2.3B) illustrates that these immediate responses are immensely diminished with age. A direct comparison of the stimulation-induced effects relative to the pattern observed in Sham demonstrated markedly greater enhancement in amplitudes in the young adults compared to the older cohort (Fig. 2.3C).

To quantify this observation, we determined the difference in amplitude between the endogenous detected baseline SO trough and the evoked troughs following both the first and second stimulation in the stimulation condition (Fig. 2.3D). A 2x9x2 ANOVA with within-subject factors 'SO trough' (1st vs. 2nd evoked), 'topography' (9 electrodes) and between-subject factor 'age' (young vs. older adults) revealed a main effect of age group ($F(1,26) = 12.174$, $P = 0.002$), and an age group*SO trough interaction ($F(1,26) = 5.907$, $P = 0.022$). Having confirmed that trough amplitudes differed significantly between age groups, we next conducted consecutive 2x9 ANOVAs separately for each age group with within-subject factors 'SO trough' (endogenous vs. 1st or 2nd evoked trough) and 'topography'. The younger cohort showed no difference between the amplitudes of endogenous and elicited SO troughs ($F(1,10) = 0.666$, $P = 0.434$, and $F(1,10) = 0.747$, $P = 0.408$ for the 1st and 2nd induced SO trough, respectively). By contrast, the older group exhibited significantly smaller amplitudes in elicited as compared to endogenous troughs, with elicited troughs decreased down to amplitudes of approximately 50% of the endogenous SO amplitude (main effects of SO trough: $F(1,16) = 46.02$, $P < 0.001$ and $F(1,16) = 62.01$, $P < 0.001$ for the 1st and 2nd induced SO trough compared to the endogenous SO trough, respectively). This pattern of a diminished response in the older but not young adults (Fig. 2.3D) clearly indicates a difference in susceptibility to auditory stimulation during sleep in the older population.

2.4.3 Older subjects exhibit stronger fast spindle refractoriness

Following the notion that fast sleep spindles co-occur with SO up-states, we examined responses in the 12-15 Hz fast spindle frequency range. We extracted spindle RMS-activity

and averaged the signal time-locked to the first auditory stimulation to confirm an increase in spindle power phase-locked to the first induced up-state (Fig. 2.4A). This was absent from the second induced up-state (at t = ∼2 s, see Fig.2.4A/B), mirroring the pattern previously observed in young adults (Fig. 2.4B). However, similar to the overall evoked response shown earlier, the initial increase in fast spindle power (at t = ∼1 s) was diminished in the older population when compared to the younger cohort (see Fig. 2.4C).

To determine the difference between endogenous and elicited fast spindle responses in both age groups, we next calculated the difference between the endogenous fast spindle peak (at the time of the first click presentation t = 0 s, baseline) and the induced fast

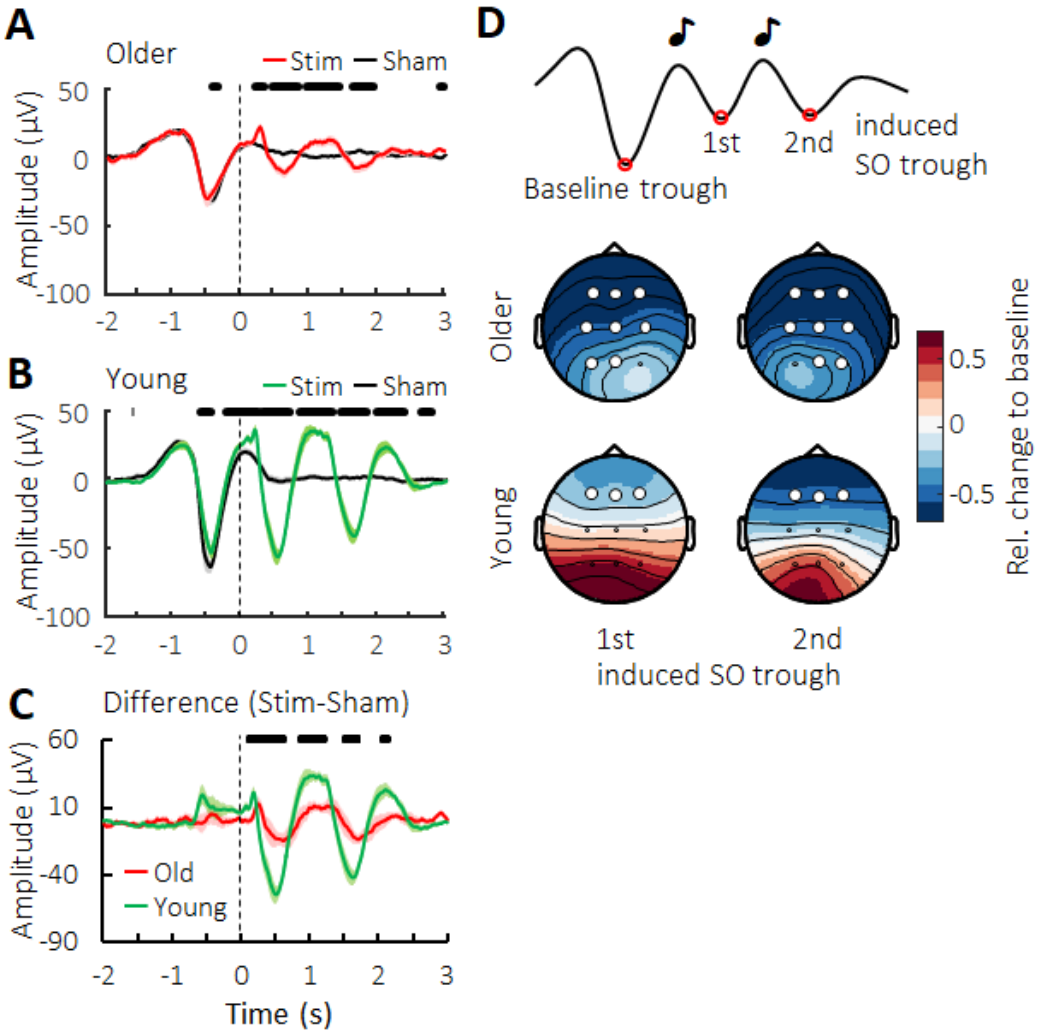

Figure 2.3: Event-related potentials upon auditory stimulation. (**A**) Mean ± SEM EEG-signal from Cz averaged time-locked to the first click for the Stimulation (red) and Sham conditions (black) in older population. Vertical line indicates timing of the first clicks, whereas thick horizontal black lines mark time points of significant difference between conditions. (**B**) Mean ± SEM EEG-signal from Cz averaged time-locked to the first click for the Stimulation (green) and Sham conditions (black) in young cohort. Vertical line indicates timing of the first clicks, whereas thick horizontal black lines at the top mark time points of significant difference between conditions. (**C**) Direct comparison of the stimulation-induced effects (Stim - Sham) between both young (green) and older (red) adults. (**D**) Top schematic illustrates the time points during which trough amplitudes were obtained to determine the relative change shown colour-coded as topographical maps of the evoked response with respect to the endogenous SO. White circles indicate channel location with a significant change from baseline after FDR correction.

spindle peaks (at time t = ∼1 s and t = ∼2 s, respectively) in the Stimulation condition on each electrode. We used a 2x9x2 ANOVA with within-subject factors 'spindle peak' (1st vs. 2nd evoked), 'topography', and between-subject factor 'age' to contrast age differences. This analysis indicated a main effect of age group ($F(1,26) = 4.463$, $P = 0.044$), and a spindle peak*topography interaction ($F(1.99, 51.75) = 3.806$, $P = 0.029$). To explore this interaction, we separately examined the magnitude of fast spindle enhancement at t = ∼1 s in each age group. We thus carried out 2x9 ANOVAs, with the factors 'spindle peak' (endogenous baseline vs. 1st evoked) and 'topography' in each group. This indicated a similar response strength during the endogenous spindle peak and first induced SO up-state for the older group ($F(1,16) = 0.017$, $P = 0.899$, Fig. 2.4D). In the younger group by comparison, this induced spindle response was almost twice the size of the endogenous baseline peak ($F(1,10) = 6.87$, $P = 0.026$). Thus, stimulation led to an enhancement of fast spindle responses on the 1st evoked SO up-states greater than the endogenous response in young adults, but not in our older cohort.

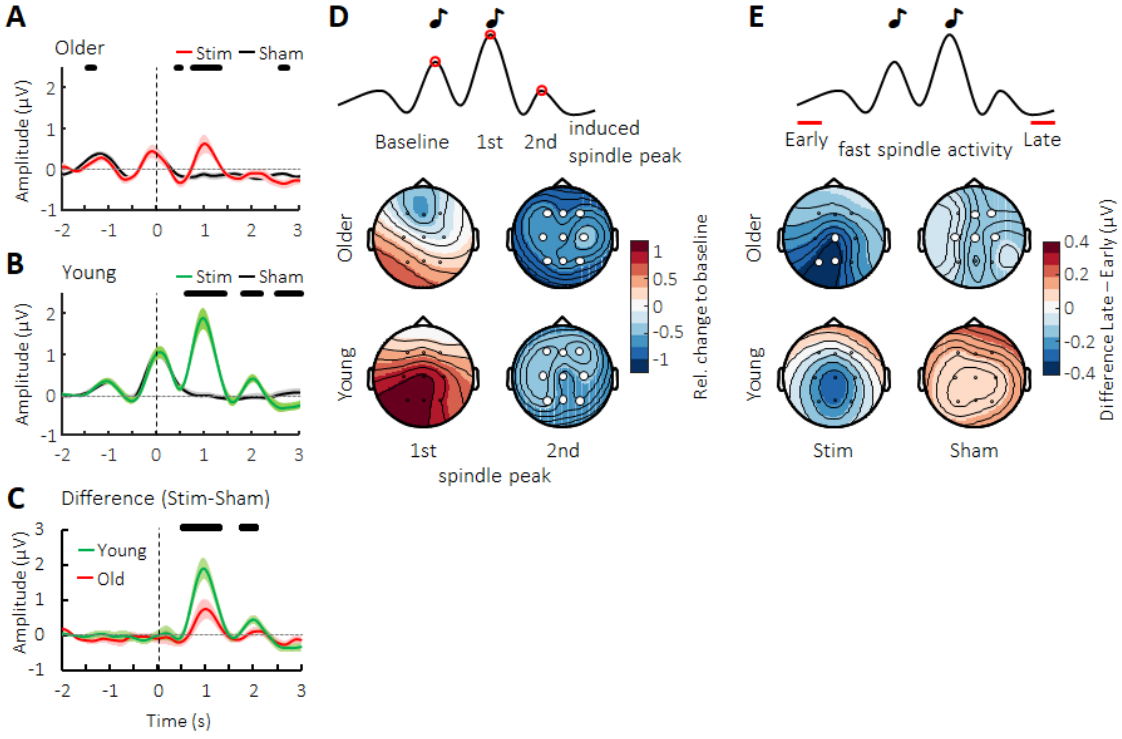

Figure 2.4: Immediate effects on fast spindle activity. Mean ± SEM RMS-signal in the 12-15 Hz spindle-band from Cz averaged time-locked to the first click for Sham (black) and Stim conditions in **(A)** the older population (red) and **(B)** the young adult group (green). Vertical line indicates timing of the first clicks, whereas thick horizontal black lines mark time points of significant difference between conditions. **(C)** Direct comparison of the stimulation-induced fast spindle RMS effects (Stim - Sham) between both young (green) and older (red) adults. **(D)** Top schematic illustrates the time points during which fast spindle peak activity was obtained to determine the relative change shown below colour-coded in the topographical maps of the evoked response with respect to the endogenous SO. **(E)** Topographic distribution of the colour-coded difference in fast spindle activity between two 500-ms intervals early preceding and late after acute stimulation or Sham-trials for the older and younger population, as illustrated in the schematic above. White circles indicate channel location with a significant relative change (D) or difference (E) from baseline after FDR correction.

With regard to the absence of a spindle response following the 2nd stimulation, the older subjects exhibit a pattern similar to the young group in terms of a strong subsequent suppression of fast spindle power. However, a closer visual inspection of the older cohort (Fig. 2.4A) suggests that such suppression is also present in the Sham condition, where no auditory stimulation was performed, and may continue for a longer duration after the first SO than in younger adults. To examine these potential differences in fast spindle refractoriness between age groups in more detail, we contrasted early and late spindle RMS-activity obtained before and after acute stimulation. We compared time windows between -2 to -1.5 s (before stimulation commenced) and 2.5 to 3 s (after stimulation ended and following the 2nd induced spindle peak in Fig. 2.4B), relative to the first stimulation onset (at t = 0) (Fig. 2.4E). A 2x2x9x2 ANOVA with within-subject factors 'time window' (early vs. late), 'condition' (Stim vs. Sham), 'topography', and between-subject factor 'age' returned a spindle window*topography*age group interaction ($F(2.26,38.47) = 5.103$, $P = 0.008$), but no main group effect ($F(1,26) = 2.828$, $P = 0.111$). To investigate the interaction further, we conducted age-group specific 2x9 ANOVAs with factors 'time window' and 'topography' separately for each condition. This showed that while our young subjects did not exhibit spindle suppression in the late time window in either condition (Stimulation: $F(1,10) = 2.64$, $P = 0.135$, Sham: $F(1,10) = 1.25$, $P = 0.291$, Fig. 2.4E), the older population showed a decrease in fast spindle power during the late time window in both Stimulation and Sham conditions ($F(1,16) = 7.81$, $P = 0.013$ and $F(1,16) = 13.96$, $P = 0.002$ for Stimulation and Sham, respectively). This pattern suggests that besides an overall change in susceptibility to auditory stimulation during sleep, the dynamics of spindle-expressing thalamo-cortical networks are altered in the ageing brain, with longer refractory periods observed compared to young adults.

2.4.4 Opposite overall effects of stimulation on SOs and fast spindles between age groups

In order to assess the overall influence of the stimulation irrespective of click presentations, we next turned our attention to spectral power and identified discrete SO events post-hoc within the entire stimulation period.

For SO peak power, a 2x9x2 ANOVA with within-subject factors 'condition', 'topography', and between-subject factor 'age' revealed a main effect for age group ($F(1,26) = 45.508$, $P < 0.001$), as well as significant group*condition ($F(1,26) = 17.671$, $P < 0.001$) and condition*topography ($F(3.84,99.72) = 2.664$, $P = 0.039$) interactions. Decomposing these interactions in separate age-group ANOVAs with within-subject factors 'condition' and 'topography', SO peak power was comparable between Stimulation and Sham conditions in the older subjects ($F(1,16) = 0.99$, $P = 0.334$, Fig. 2.5A, top), whereas the young population showed an increase in SO peak power persisting across the entire stimulation period ($F(1,10) = 22.73$, $P < 0.001$).

Meanwhile, the same previous 2x9x2 ANOVA repeated for 12-15 Hz fast spindle power

across the entire stimulation period returned no significant main group effect or interaction (all P $\geq$ 0.262). However, based on the main effect of condition nearing a trend (F(1,26) = 2.639, P = 0.116), we conducted an exploratory decomposition separately for each age group and revealed a pattern for the fast spindle range that was opposite to that seen in SO power. Thus, while a 2x9 ANOVA with within-subject factors 'condition' and 'topography' showed that fast spindle power was significantly elevated by stimulation

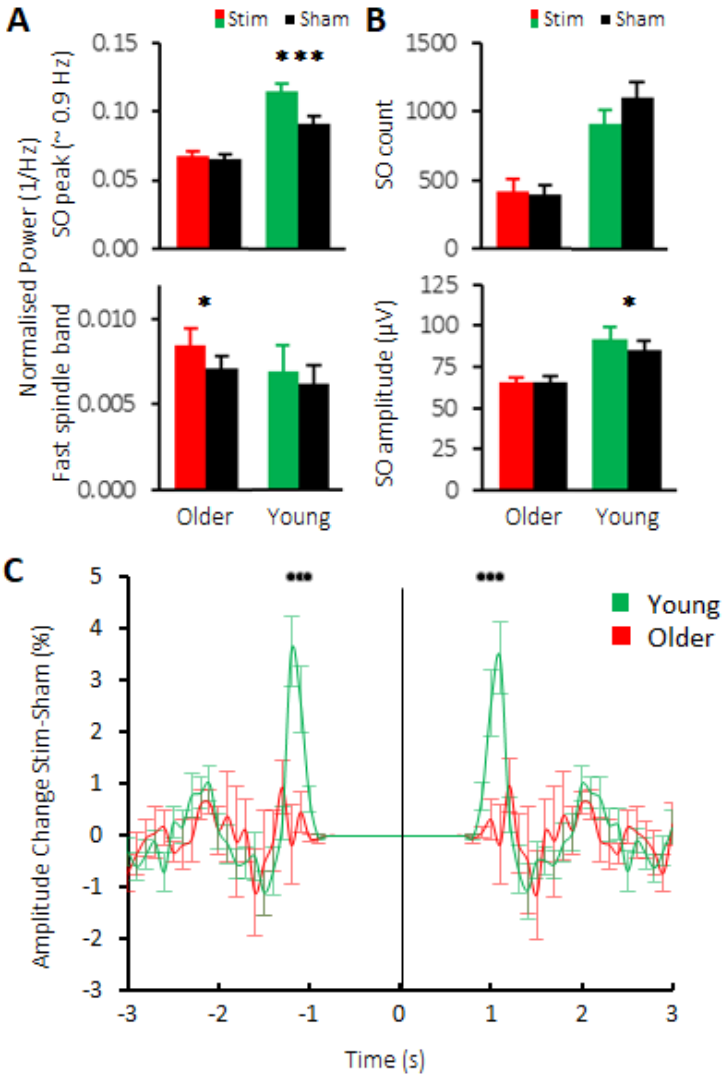

Figure 2.5: Sustained modulation of SOs and fast spindles. **(A)** Global mean ± SEM of the normalised spectral power for the SO peak (top) and fast spindle frequency band obtained across the stimulation period for the stimulation condition in the older (red) and young population (green) and their corresponding Sham conditions. **(B)** Mean ± SEM of SO count (top) and SO amplitude of offline-detected SO events across the stimulation period for the stimulation condition in the older (red) and young (green) cohort and their Sham conditions (black). **(C)** Auto-event histogram of offline-detected SO events reveals no sustained prolonging of SO trains in older subjects. To assess the temporal interrelationship among SOs during the ~3 h stimulation period, we examined for each offline-detected SO event the occurrence of pre- and succeeding SOs based on event histograms within a ± 3 s time interval and 100 ms bins. Resulting histograms were normalised by the total number of detected SO events (multiplied by 100) and then the difference between Stim – Sham conditions was calculated. Mean ± SEM for young (green) and older adults (red) are pictured at representative electrode Cz. Time t = 0 (vertical black line) denotes the negative peak of detected SO events. Black dots denote statistically significant differences between the young and older cohorts (uncorrected).

across the stimulation period in the older subjects ($F(1,16) = 6.104$, $P = 0.025$), the young cohort showed no such difference between Stimulation and Sham conditions ($F(1,10) = 0.147$, $P = 0.710$) (Fig. 2.5A, bottom).

Initial examination of the number of discrete SO events occurring during the stimulation period using a 2x9x2 ANOVA with within-subject factors 'condition', 'topography', and between-subject factor 'age' returned a main effect for group ($F(1,26) = 27.946$, $P < 0.001$) and a group*condition interaction ($F(1,26) = 4.649$, $P = 0.040$). This confirmed an overall lower number of SO events in the older compared to the younger population, however, neither groups showed any difference in SO numbers between experimental conditions (Young: $F(1,10) = 3.168$, $P = 0.105$, Older: $F(1,16) = 0.458$, $P = 0.508$) (Fig. 2.5B, top). Instead, and in line with our spectral results, repeating the previous 2x9x2 ANOVA for SO amplitude (in lieu of SO events) returned a group*condition interaction ($F(1,26) = 8.680$, $P = 0.007$). This demonstrates that compared to Sham, auditory closed-loop stimulation induced higher amplitude SOs (Fig. 2.5B, bottom) in the young but not the older group. Additionally, we visualised the difference of offline detected SO events between experimental conditions per age group in an auto-event correlation histogram, which revealed no sustained prolonging of SO trains by the stimulation in the older compared to the young adults (Fig. 2.5C). Together, these confirm a non-resonant SO response in the ageing brain.

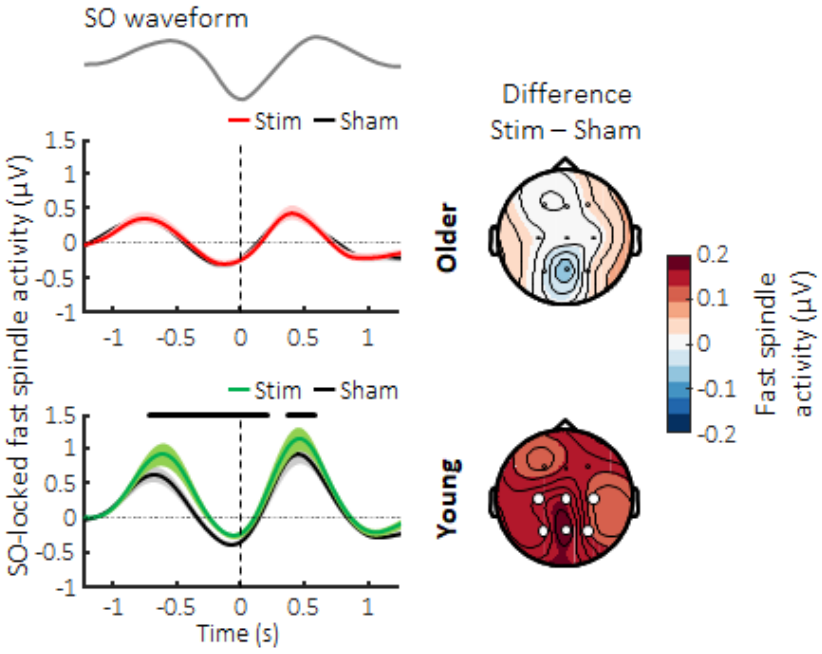

Figure 2.6: Phase-locking of SOs and fast spindles. Fast spindle RMS-activity average time-locked to the negative peak (vertical lines) of offline detected SO events for the older (top) and young population (below) with Stimulation conditions shown in red or green, and Sham conditions in black. Thick horizontal bars mark time points of significant difference between conditions. The corresponding topographical distribution of the difference between conditions over the time intervals -1.25 to 1.25 s (where t = 0 time-locked to the negative SO peak) is shown on the right. White circles indicating channel locations with significant difference in overall phase-locked fast spindle activity between conditions after FDR correction.

Finally, we investigated the cross-frequency coupling between SO and sleep spindles during the stimulation period, given their hypothesized joint contribution to memory consolidation. Averaging the fast spindle RMS-signal time-locked to the down-state of post-hoc detected SOs confirmed increases in fast spindle activity during SO up-states for both older and young adult populations, and in both conditions (Fig. 2.6, left). A 2x9x2 ANOVA with within-subject factors 'condition', 'topography', and between-subject factor 'age' revealed a significant main group effect ($F(1,26) = 16.014$, $P < 0.001$), and a group*condition interaction ($F(1,26) = 7.051$, $P = 0.013$). Subsequent comparison between the conditions in each age group separately in 2x9 ANOVAs with within-subject factors 'condition' and 'topography' showcased a widespread topographical elevation of fast spindle activity during the up-state and down-transition preceding the SO down-state in the Stimulation condition only in young adults ($F(1,10) = 8.10$ with $P = 0.017$), and not in the older cohort ($F(1,16) = 0.06$, $P = 0.808$, Fig. 2.6, right).

2.4.5 Closed-loop stimulation in older age does not alter sleep architecture and other control measures

Table 2.1 contains the general sleep parameters for the older age group (please see supplementary Table 2.4 for younger subjects). As previously reported for the younger subjects (Ngo *et al.*, 2013), paired-samples student's t-tests demonstrated that auditory stimulation did not influence sleep onset ($t(16) = -0.17$, $P = 0.867$), total sleep time ($t(16) =$

	Stim			**Sham**			**P-value**
	mean	±	SEM	mean	±	SEM	
TST (min)	449.32	±	10.05	443.85	±	13.56	0.59
Sleep onset (min)	10.91	±	2.31	11.26	±	2.13	0.87
Stimulation period							
Wake (%)	9.75	±	3.15	11.07	±	3.15	0.55
S1 (%)	3.55	±	0.59	3.84	±	0.60	0.62
S2 (%)	56.95	±	3.64	60.57	±	3.27	0.24
SWS (%)	13.31	±	2.91	11.85	±	2.46	0.46
REM (%)	16.25	±	1.86	12.48	±	2.13	0.19
Arousal index (%)	7.01	±	0.64	8.17	±	1.18	0.39
Entire Night							
Wake (%)	12.07	±	2.97	9.83	±	1.70	0.42
S1 (%)	5.17	±	0.54	5.67	±	0.54	0.38
S2 (%)	56.08	±	2.89	59.13	±	2.10	0.27
SWS (%)	8.17	±	1.64	7.38	±	1.69	0.51
REM (%)	17.89	±	1.52	17.68	±	1.94	0.91
Arousal index (%)	6.64	±	0.77	8.22	±	0.91	0.22

Table 2.1: Sleep architecture during the ∼3.5-hour stimulation period and entire night in the older adults. Stimulation did not alter time spent in any of the sleep stages, total sleeping time, or number of arousals. TST = total sleep time, S1-S2: sleep stages 1 and 2, SWS = slow wave sleep (i.e. S3 + S4), REM = rapid eye movement.

0.55, P = 0.588), nor overall sleep architecture for the stimulation period (all P ≥ 0.193) or the entire night (all P ≥ 0.266) in older adults. Auditory stimulation did not result in disrupted sleep through increased arousals (t(16) = -1.27, P = 0.216).

		Stim			**Sham**			**P-value**
		mean	±	SEM	mean	±	SEM	
SSS	Evening	3.65	±	0.35	4.41	±	0.34	0.10
	Morning	2.59	±	0.24	2.74	±	0.26	0.61
	Difference	-1.06	±	0.39	-1.68	±	0.45	0.33
SQ		3.75	±	0.18	3.81	±	0.17	0.88
Being well-rested		3.73	±	0.16	3.68	±	0.16	0.68
PVT	Evening	349.05	±	9.67	347.01	±	8.83	0.78
	Morning	348.14	±	11.44	350.48	±	13.16	0.77
	Difference	-1.51	±	6.76	3.46	±	9.31	0.64
Digit	Forward	8.88	±	0.49	9.29	±	0.45	0.44
	Backward	7.35	±	0.59	7.53	±	0.50	0.70
	Total	16.24	±	0.88	16.82	±	0.84	0.46

Table 2.2: Control measures in older adult cohort. Stimulation did not impact on Stanford Sleepiness Scale (SSS), subjectively reported sleep quality (SQ) and feelings of being well-rested, or on performance on the psychomotor vigilance (PVT) or Digit Span Tasks.

On sleep questionnaires, our older subjects subjectively reported comparable sleepiness (Evening: t(16) = -1.73, P = 0.103, morning: t(16) = -0.52, P = 0.0.611, and overnight change between conditions: t(16) = 1.00, P = 0.334), sleep quality (t(14) = -0.44, P = 0.664) and feeling of being well rested (t(14) = 0.13, P = 0.895). Furthermore, we found no difference in the performance on the PVT before learning (Stimulation vs. Sham: t(15) = 0.27, P = 0.791), at retrieval sessions (Stimulation vs. Sham: t(15) = -0.30, P = 0.767) or overnight change between conditions (t(15) = -0.48, P = 0.639), nor for the Digit Span Task performed in the morning between the learning and testing phases of the picture encoding task (forward: t(16) = -0.80, P = 0.436 and backwards: t(16) = -0.39, P = 0.704 between the Stimulation and Sham condition, or overall performance total: t(16) = -0.76, P = 0.460). Please see Table 2.2 for an overview of these control measures.

2.5 Discussion

Our data confirm the feasibility of selectively inducing SOs in older subjects using auditory closed-loop stimulation. However, stimulation did not promote performance on any of the assessed memory domains in the older cohort, but rather impaired the retention of declarative memories overnight. Brain responses of older adults were quantitatively diminished and revealed different patterns for SOs and fast spindles in comparison to a younger population, indicating a change in susceptibility to stimulation with age.

The ageing brain shows a distinct physiological response to sounds administered in a

closed-loop manner, in the absence of apparent sleep detriments, i.e. increased arousals. However, when comparing the extent of physiological enhancement between young and older adults, the latter show a markedly reduced response to stimulation. When contrasted with unstimulated baseline potentials, the extent to which SO trough amplitude could be enhanced by stimulation in relation to a preceding, endogenous SO trough was approximately halved in older adults in comparison to the young cohort. Additionally, our older cohort did not demonstrate sustained increases throughout the stimulation period in SO power or amplitudes as observed in young adults, which indicates that stimulation effects on SOs occurred acutely and were more short-lived by comparison. Together, these findings suggest a decreased susceptibility of the older brain to the applied stimulation. Potential reasons for this phenomenon could relate to lower numbers of cortical populations being involved in hyperpolarisations. Furthermore, cells may engage in an un-timely manner due to early neuronal atrophy or an inhibiting build-up of plaques. Finally, decreased plasticity may lead to cardinal sleep rhythms refraining from longer-term resonance due to prolonged cell refractoriness. A combination thereof could prevent the ageing brain from responding to an incoming stimulus as strongly as in younger years (Carrier *et al.*, 2011, Halász *et al.*, 2014). Whether altered SO characteristics in older age hence require a different timing of stimulation, i.e. stimuli which occur at a particular phase 'sweet spot' where greater enhancement could be elicited, remains the subject of future investigations.

In keeping with original findings in the young cohort, stimulation compared to baseline led to an increase in fast spindle activity on the first, but not second induced SO peak in older subjects (Ngo *et al.*, 2015). Similarly to the reduced strength observed in overall evoked responses, the power of both endogenous and induced fast spindles in our older group was roughly half of that measured in young adults. Moreover, whereas fast spindle refractoriness was evident during stimulation in both age groups, signs of stronger spindle refractoriness across subsequent SO cycles were also observed in the Sham condition in older adults compared with the younger cohort. This implies altered thalamo-cortical network dynamics in the ageing brain, which, once a spindle has been expressed, require more time to recover and re-establish baseline levels in cellular reactivity. Surprising in this overall context of a diminished fast spindle response to stimulation was a distinct enhancement in fast spindle power extending across the stimulation period, which was found exclusively in the older cohort. Stimulation hence successfully boosted fast spindle power in the ageing brain. However, age-dependent changes in spindle-expressing networks, i.e. increased spindle refractoriness as observed even during unstimulated conditions, present a limiting physiological factor to this enhancement.

Our analysis on the interplay of SOs and fast spindles indicated increases in fast spindle activity nesting within SO up-states in the young adult group only. In contrast to young adults, where stimulation led to a topographically widespread elevation in fast spindle activity, an insignificant increase remained local in the older cohort. Combined,

these results suggest that while stimulation led to globally boosted, resonant SO-coupled fast spindle activity in younger individuals, older subjects did not show such an effect. This finding provides a conceivable explanation for the stimulation's limited impact on behaviour in the ageing brain. Interestingly, our analyses did not reveal a decoupling between SO and spindles either, i.e. no systematic shift of the preferential phase of sleep spindles occurring within SOs as has been recently reported in elderly subjects (Helfrich *et al.*, 2017, Muehlroth *et al.*, 2019). Given that our older cohort lies in the middle-age range positioned between young and elderly adults, this highlights differential changes accompanying healthy ageing. In our case, these changes were expressed in a strong change in electrophysiological susceptibility but a still intact top-down influence of the SO on subcortical rhythms.

With regard to memory, stimulation did not favourably affect performance on the declarative task in the older group, which is not surprising considering the short-lived increases in SO power and unaltered SO-locked fast spindle activity observed in this cohort. Post-sleep encoding was likewise unaffected by stimulation, presumably due to a lack of increase in SO power during the stimulation period to effectively re-establish hippocampal retention capacity overnight, similar to Ong *et al.* (2018). However, Bayesian analyses suggest the evidence provided by our data to support a difference between conditions on the declarative task, and accept the null hypothesis on both procedural and post-sleep encoding tasks was only weak. Therefore, our data do not lend themselves to any definite conclusions on the effects of stimulation on memory performance in the ageing brain, and this question should be revisited by future studies.

Since a predefined detection threshold must be crossed to trigger stimulation, a decline in naturally occurring endogenous SOs in older age in terms of number, density, and amplitude (Carrier *et al.*, 2001, Fogel *et al.*, 2012, Landolt *et al.*, 1996) thus resulted in fewer stimulation opportunities in simple numerical terms (Garcia-Molina *et al.*, 2018). Stimulation trial numbers of the present study show that our older cohort received on average numerically fewer stimuli than the young adults, but trial numbers did not differ statistically between both age groups. Nonetheless, it is possible that increasing trial numbers in the older cohort could lead to a different behavioural outcome. Adjusting the detection threshold may remedy the situation in such cases; however, lowering it too dramatically would mean targeting oscillations which strictly speaking no longer qualify as SOs due to not meeting the amplitude criterion (Iber *et al.*, 2007). Papalambros *et al.* (2017) found a positive impact of auditory closed-loop stimulation on declarative memory performance when boosting SWA in older individuals. This result is particularly remarkable as their cohort was on average 20 years older than ours. Their phase-locked loop algorithm worked with a threshold which, at -40 μV, was set on average only half as high as ours. Additionally, their experiment administered stimulation throughout the entire night. These factors likely resulted in a proportionally larger fraction of stimulated endogenous SOs. Alternatively, the transfer of novel declarative memories to stable cortical networks could

rely on timely repetition (Inostroza & Born, 2013). This would have been impeded by the prolonged spindle refractoriness found in older adults, and therefore presented a natural limitation to achieving sufficient reiterations. Based on the increasingly variable nature of slow oscillations in older age, a more effective stimulation procedure might determine the timing of stimuli based on the live tracking of SO phase in lieu of applying a fixed delay variable before sound administration. Targeting the ideal stimulation phase with high accuracy may enhance the cross-coupling of SO and spindle frequencies thought to be vital for sleep-dependent consolidation processes. Furthermore, it is possible that differences in hearing abilities between young and older adults constitute a crucial factor in the varying stimulation susceptibility and outcome observed between the two age groups. Hearing abilities during wakefulness decline naturally in older age (Lin *et al.*, 2014, Peelle & Wingfield, 2016). While the auditory threshold was not found to differ between wakefulness and sleep in healthy, young adults (Deacon-Elliott *et al.*, 1987), to our best knowledge it is presently unknown whether this observation would persist throughout later adulthood. The 3-digit hearing test carried out as part of the eligibility screening sessions during recruitment in the present study merely assessed hearing ability in a basic binary manner. We therefore cannot exclude the possibility of a causal relationship between hearing ability of the sleeping brain in older age and our findings of diminished stimulation susceptibility and related behavioural effects. Further research involving more comprehensive hearing tests is required to elucidate this question in future.

To conclude, the present study demonstrates that auditory closed-loop stimulation can be applied to the ageing brain without causing detriments to sleep or altering sleep architecture. In line with previous research, stimulation outcome diverged considerably between the older and younger subjects. This finding suggests the magnitude and nature of inducible enhancement is reduced and altered in the ageing brain, a pattern which has been likewise reported in various studies employing other stimulation techniques (Eggert *et al.*, 2013, Garcia-Molina *et al.*, 2018, Paßmann *et al.*, 2016). Its consequent inability to influence sleep-dependent memory performances is most likely due to a combination of changed characteristics of cardinal sleep rhythms, such as SO and fast spindle-expressing network dynamics, and age-related physiological and cellular constraints. Despite the decreased susceptibility to auditory closed-loop stimulation in older age portrayed in this paper, the fact that similar stimulation efforts in a previous study (Papalambros *et al.*, 2017) have yielded positive behavioural results emphasises the challenge to translate a functional protocol to different age groups. In the light of the present findings, such positive results once more underline that more detailed investigation is required to identify specific factors that might increase and optimise the efficacy of this brain stimulation technique. Despite presenting mixed results, the overall efficacy of auditory closed-loop stimulation of brain activity renders it nonetheless an adaptable and suitable opportunity for developing real-world clinical applications, e.g. to counter decline in healthy ageing, as well as in pathological conditions such as mild cognitive impairment (Ladenbauer *et al.*, 2017), which are strong precursors for Alzheimer's disease and other dementias.

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2.6 Supplementary information

Participant	Older Adults	Young Adults
1	134	705
2	171	325
3	35	509
4	93	348
5	56	384
6	192	128
7	130	74
8	109	247
9	99	401
10	384	319
11	318	235
12	417	
13	176	
14	503	
15	484	
16	99	
17	515	
Mean±SEM	230.29±40.66	334.09±52.50

Table 2.3: Number of stimulation trials in young and older adults in NREM sleep stages 2, 3, & 4.

	Stim			**Sham**			**P-value**
	mean	±	SEM	mean	±	SEM	
TST (min)	413.50	±	6.60	417.60	±	2.80	0.49
Sleep onset (min)	10.90	±	5.40	12.40	±	4.00	0.81
Stimulation period							
Wake (%)	6.30	±	1.60	7.30	±	1.10	0.55
S1 (%)	8.20	±	1.00	7.50	±	1.40	0.66
S2 (%)	48.60	±	2.70	47.30	±	2.20	0.68
SWS (%)	29.10	±	4.10	28.70	±	2.80	0.91
REM (%)	7.70	±	1.60	9.20	±	1.80	0.15
Arousal index (%)	6.70	±	0.40	6.70	±	1.00	0.88
Entire Night							
Wake (%)	5.80	±	1.20	5.40	±	0.90	0.64
S1 (%)	9.60	±	1.00	7.00	±	0.80	0.01
S2 (%)	49.00	±	2.60	50.30	±	1.70	0.59
SWS (%)	20.10	±	2.40	19.00	±	2.00	0.61
REM (%)	15.50	±	1.20	18.30	±	1.80	0.06
Arousal index (%)	7.90	±	0.60	6.70	±	0.50	0.21

Table 2.4: Sleep architecture during the ∼3.5-hour stimulation period and entire night in the young adult cohort. Stimulation did not alter time spent in any of the sleep stages (except for total night S1), total sleeping time, or number of arousals. TST = total sleep time, S1-S2: sleep stages 1 and 2, SWS = slow wave sleep (i.e. S3 + S4), REM = rapid eye movement. From Ngo *et al.* (2013)

Chapter 3

Auditory closed-loop stimulation of sleep slow oscillations differentially modulates slow and fast spindle activity

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3.1 Abstract

Background: Slow oscillations and sleep spindles hallmark stable NREM sleep and are considered vital oscillatory events to facilitate sleep-dependent memory processes. However, slow and fast spindle types differ on their spatial and temporal characteristics, and it is presently unknown whether they serve differential functional purposes. A refractory period of 3-6 s has previously been described for fast spindle generating networks following one single auditory stimulus, but none has been reported for slow spindles to date. Thus, applying a multi-click stimulation protocol during the assumed fast spindle refractory period may present an opportunity to disproportionately enhance slow spindle activity.
Methods: Applying a within-subject design in a healthy, young adult cohort (N = 22, 14 female, aged 18-29 years, mean = 23.09), we investigated the efficacy of auditory closed-loop stimulation as a methodological tool to differentially modulate slow and fast spindle activity in two experimental conditions. Stimulation trials consisting of either one single click or four consecutive clicks were administered to co-occur with up-states of the predominant slow oscillation. We contrasted the effects of both click protocols on slow and fast spindle activity.
Results: Analyses revealed dissimilar dynamics between spindle types and conditions. The four click condition had an enhancing effect on slow but not fast spindle power across stimulation trials compared to a single click. Furthermore, discrete slow spindles demonstrated higher incidence rates in trials, and showed shorter inter-spindle intervals than fast spindles. Sleep quality did not appear to be negatively impacted by the auditory stimulation.
Conclusions: Our findings suggest underlying differences in slow and fast spindle generation dynamics, supporting the possibility of separate functional purposes. Auditory closed-loop stimulation as applied in this study presents a feasible methodological tool to further explore their respective purpose in future investigations.

Keywords: Sleep, Auditory closed-loop stimulation, Slow oscillations, Slow spindles, Fast spindles

3.2 Introduction

Sleep spindles are waxing and waning bursts of oscillatory brain activity thought to originate in the thalamic reticular nucleus. They last between 0.5 to 3.0 s, occupy the sigma frequency spectrum of 9-15 Hz in humans, and occur during stable NREM sleep stages 2 and 3 (Gennaro & Ferrara, 2003, Lüthi, 2014, Mölle *et al.*, 2011). Spindles are further categorised into slow (9–12 Hz) and fast (12–15 Hz) types (Anderer *et al.*, 2001, Diekelmann, 2014). Spindles have been repeatedly linked to a range of memory functions in the sleeping brain. Yet the exact contribution of slow spindles in these consolidation processes, if any, is presently less clear, particularly so because they differ in their spatial and temporal characteristics from fast spindles (Piantoni *et al.*, 2016, Schabus *et al.*, 2007).

Neuroimaging findings suggest both spindle types share a common activation pattern in thalami, paralimbic areas, and superior temporal gyri. But while slow spindles additionally engage the superior frontal gyrus, fast spindles occur in sensorimotor cortices, mesial frontal cortex, and the hippocampus (Schabus *et al.*, 2007). These observations support findings from scalp EEG recordings, which commonly locate slow spindles primarily over (pre-) frontal sites and fast spindles predominantly over centro-parietal topographies (Gennaro & Ferrara, 2003). Temporally, about 60% of fast and 70% of slow spindles co-occur within $\pm$ 3 s of a negative peak of cortical slow oscillations (SOs, $\sim$ 1 Hz) (Mölle *et al.*, 2011), which hallmark stable NREM sleep. Fast spindles dominate the up-state, whereas slow spindles occur on the negative-going slope towards the SO down-state (Klinzing *et al.*, 2016a, Timofeev & Chauvette, 2013).

This spatially and temporally distinct activity pattern may point towards a clear distinction between these oscillations. However, whether both spindle types actually share the same regulation mechanisms or functional purpose is presently unclear. Generally, fast sleep spindles have regularly been linked to intellectual and mnemonic abilities in information processing, and are seen as primers for cortical plasticity and synaptic strengthening (Fogel & Smith, 2011, Holz *et al.*, 2012, Lüthi, 2014, Maier *et al.*, 2018, Ujma *et al.*, 2015, Ulrich, 2016). The active system consolidation hypothesis for example posits that SOs orchestrate the coupling of lower level thalamo-cortical fast spindle and hippocampal ripple activity in a top-down manner (Rasch & Born, 2013, Staresina *et al.*, 2015). This mechanism is thought to facilitate the transfer of novel, labile declarative memories from short-term, hippocampal storage to long-term, neocortical networks (Clemens *et al.*, 2007, Rasch & Born, 2013). A vast number of studies have lent support to this hypothesis by demonstrating increases in SO and fast spindle activity after learning and revealing associations to improved post-sleep behavioural performance (Cairney *et al.*, 2018, Gais *et al.*, 2002, Schabus *et al.*, 2004, Born, 2010, Diekelmann & Born, 2010). Moreover, fast spindles were found to support the consolidation of emotional memories (Cairney *et al.*, 2014), to aid the integration of new information into existing knowledge (Tamminen *et al.*, 2010), to relate to schema maintenance (Hennies *et al.*, 2016), and have recurrently been linked to overnight enhancements in procedural memory (Barakat *et al.*, 2013, Fogel *et al.*, 2015).

In further support, interrelated fast spindle abnormalities and cognitive impairments have been found in healthy ageing (Clawson *et al.*, 2016, Mander *et al.*, 2014, Nicolas *et al.*, 2001), neurodegenerative disorders such as Alzheimer's or Parkinson's (Seibt *et al.*, 2016), schizophrenia (Ferrarelli *et al.*, 2007), as well as sleep apnoea and further sleep disorders (Weiner & Dang-Vu, 2016).

Meanwhile, the purpose of slow spindles is more obscure, and at present only few experiments have demonstrated their role in overnight memory consolidation. For example, decreases in slow spindles were indicative of procedural memory impairments in major depression (Nishida *et al.*, 2016), while experimental enhancement of these waveforms in healthy subjects led to improved declarative overnight memory retention (Marshall *et al.*, 2006). Based on their temporal occurrence shortly prior to SO down-state, fast spindles were postulated to account for thalamo-cortical network coupling to transfer recently encoded memories, while slow spindles could reflect cortico-cortical interactions (Mölle *et al.*, 2011). Correspondingly, a speculative scenario emerges in which slow spindles may play a role in cross-linking information and recruiting frontal areas for optimal long-term integration of memory representations (Astori *et al.*, 2013).

In the light of the prevailing uncertainty about their shared and distinct regulatory and functional properties, slow and fast spindles require further investigation. Recent research efforts have focussed on enhancing (mostly fast) spindle activity through experimental manipulation (for a review, see Astori *et al.*, 2013). A growing number of studies have taken advantage of inert sound processing properties of the sleeping brain by using auditory manipulation, which avoids the caveats of more invasive, transcranial stimulation (Davis, 2014, Horvath *et al.*, 2014). During stable NREM sleep, the degree to which incoming information in the form of auditorily evoked potentials is processed largely depends on ongoing oscillatory activity. SO up-states appear to be brief time windows during which such processing is preferentially supported (Dang-vu *et al.*, 2011, Schabus *et al.*, 2012). Exposed to a non-threatening and non-salient auditory stimulus at this point in time and eager to maintain sleep, the brain typically responds by inducing an immediate neuronal down-state to prevent arousal. By exploiting this inherent mechanism, previous research demonstrated that auditory stimulation timed to this phase could prolong consecutive SO activity, enhance phase-coupled fast spindles during a following SO up-state, as well as improve memory performance over the sleep period (Leminen *et al.*, 2017, Ngo *et al.*, 2013, 2015, Ong *et al.*, 2016). Using a closed-loop setup to anticipate SO up-states by detecting their preceding down-state ensures that such stimulation is delivered at this most responsive phase of endogenous rhythms. Interestingly, in a protocol comprising of multiple SO up-states targeted in succession in a driving, closed-loop manner, only the first sound succeeded in temporarily increasing fast spindle activity during the following SO up-state, but further stimuli showed no such effect (Ngo *et al.*, 2015). Such behaviour suggests an immediate resistance of fast spindle-generating networks to auditory stimulation. This refractoriness of fast spindles is thought to last 3-6 s in duration (Antony *et al.*, 2018).

Notably, the existence of such a refractory period has hitherto not been reported in slow spindles during endogenous activity (Mölle *et al.*, 2011), suggesting they could be underpinned by different neural dynamics. Thus, applying a multi-click auditory closed-loop protocol for the duration of the fast spindle refractory period may present an opportunity to experimentally alter the endogenous ratio between SOs, fast and slow spindles. If successful in disproportionally increasing slow spindles in relation to fast spindles, this protocol would lend itself for future non-invasive and easily controllable applications to shed light on functional differences between spindle types without the need to wholly suppress either type. The aim of the present study is thus to investigate the efficacy of auditory closed-loop stimulation as a method to differentially modulate such activity by exploring probable differences in spindle type dynamics over time. Based on the literature, we hypothesise that by comparing a 4-click to a briefer 1-click protocol, all stimuli in the former multi-click condition will enhance SO and slow spindle activity. Yet, fast spindle activity is expected to be boosted during one SO up-state following the first stimulus only.

3.3 Methods

3.3.1 Participants

Data from 22 healthy adults (14 female, age range 18-29 years, mean = 23.09, SD = 2.86) were collected for this experiment. One additional participant completed the study, but was removed from the dataset due to an inability to reach stable NREM sleep. The study was advertised in departmental emails, local social media interest groups, as well as on posters around the campus of the Cardiff University Brain Research Imaging Centre, UK, where data collection took place.

Adverts re-directed interested individuals to an online eligibility screening questionnaire, which ensured those recruited were between the ages of 18 and 30, habitual non-smokers, had no history of physical, psychological, neurological, or sleep disorders, hypersensitive skin or known contact allergies, and were not taking any medication or substance directly or indirectly influencing sleep quality. We further screened for no daytime napping habits, a regular sleep-wake rhythm and no trouble falling asleep, no transmeridian travel across more than two time zones in the two months prior, and no regular night work in the previous two months. Participants committed to abstaining from alcohol, caffeine, and extreme physical exercise on the day of study, had to be comfortable to have their sleep polysomnographically recorded, able to sleep with in-ear headphones, and did not have any hearing impairments.

The study had prior approval from the School of Psychology's ethics committee (EC.17.01.10.4822A). It was carried out in agreement with the Declaration of Helsinki, with participants giving informed, written consent, and having their rights of withdrawal reiterated before committing to participation.

3.3.2 Procedure

Participants arrived one hour prior to their habitual bedtime to spend one full overnight in the sleep laboratory. Following their arrival and signing consent, participants were wired up for polysomnography, and completed a questionnaire on sleepiness (Stanford Sleepiness Scale). After application of in-ear headphones secured to the ear with a thin strip of medical tape, and sound and signal impedance checks, participants were allowed to go to sleep as desired. Participants had access to a personal alarm to alert the experimenter during the night if necessary. Stimulation was administered throughout the night in stable NREM sleep stages 2 and 3 (henceforth referred to as N2 and slow wave sleep (SWS), respectively). We employed a pseudo-random, within-subject design, with each participant undergoing both experimental conditions (1-click vs. 4-click) within one night.

The following morning, participants were awoken during light sleep at an agreed time, completed questionnaires on sleepiness and sleep quality (SF-A-R, Görtelmeyer, 2011), and reported whether they had perceived any stimulation sounds during the night. They were then wired off, were offered refreshments and a shower before being reimbursed for their time and departing from the laboratory.

3.3.3 Data acquisition

Polysomnographic data were collected continuously throughout the night with a BrainVision BrainAmp MR plus amplifier and visualised with BrainVision Recorder (version 1.21.0102, Brain Products GmbH, Germany). Ag-AgCl electrodes were applied with Grass EC2 cream (Natus Neurology Inc., Middleton, Canada) on sites cleaned with NuPrep exfoliating gel (Weaver & Co., Aurora, USA) according to the 10-20 system at the following standard electroencephalography (EEG) locations: Fpz, F3, Fz, F4, C3, Cz, C4, P3, Pz, P4, O1, and O2. We further attached one ground electrode to the forehead, two electrodes to the left above and right below outer canthus of the eyes for electrooculography (EOG), and recorded two traces for electromyography (EMG) from the chin in order to monitor real-time arousals and facilitate standard sleep scoring. All electrodes were referenced to the combined averaged signal of two mastoid electrodes applied behind the left and right ear. Impedances were kept below 5 kΩ (10 kΩ for EOG & EMG). Data were sampled at a 500 Hz frequency and stored offline for later analysis.

3.3.4 Stimulation protocol

Stimulation commenced once the participant had spent ∼5 min in stable sleep stage N2 or SWS and was administered through in-ear headphones (Sony MDR-EX15LP). The initial volume was set individually per participant to a level that was audible, yet would not cause awakenings (range: 40-60 dB, calibrated with a Benetech Digital Sound Level Meter GM1357); this was manually adjusted throughout the night as required based on the eliciting of evoked responses and signs of arousal. Upon activating the stimulation, a custom-designed Matlab algorithm (version R2016b 9.1.0.441655, MathWorks, Natick,

USA) monitored the EEG signal in real-time as recorded from Fpz and bandpass filtered it between 0.25-4 Hz (slow wave band) for values below a pre-defined stimulation threshold of -80 μV (see Figure 3.1). Once this threshold had been crossed by a down-going slope, indicating the presence of an SO down-state and imminent up-state, stimulation sounds were administered after fixed delays of 0.5 s (first delay) and 1 s (second to fourth delay, applicable in 4-click condition only) after trough detection to approximately coincide with peak timings of the cardinal ~1 Hz slow oscillation.

All auditory stimuli were presented binaurally. They consisted of 50 ms 1/f pink noise, which has previously been shown to elicit the most distinct response in the sleeping brain (Debellemanière *et al.*, in preparation). The noise was ramped up and down during the first and last 5 ms, respectively. Stimulation markers were saved in the EEG for later time-locked analyses.

The stimulation algorithm pseudo-randomly selected one of two conditions (1-click vs. 4-click) for any given stimulation trial while keeping the total number of clicks administered per condition approximately equal. The incentive for this was to balance potential accumulative effects between conditions on reactivity of either spindle type across the

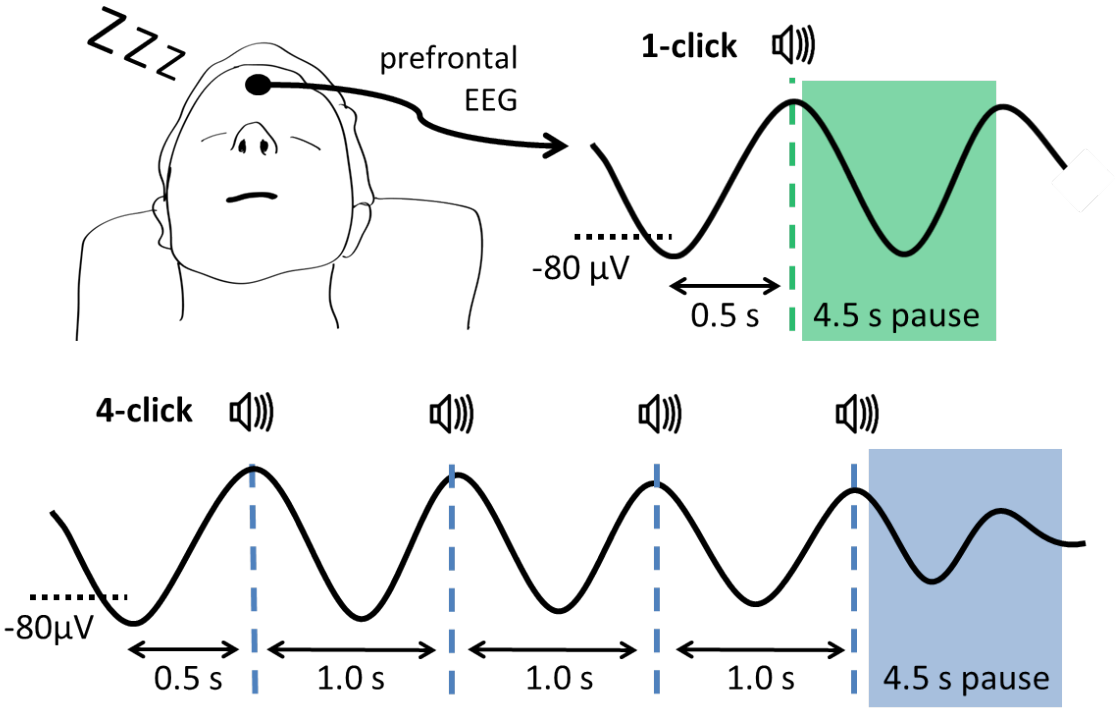

Figure 3.1: Slow wave detection and auditory closed-loop stimulation procedure. An automatic algorithm determined when the pre-filtered 0.5-4 Hz prefrontal EEG signal dropped below -80μV (dotted horizontal lines), indicating an imminent SO up-state. It then pseudo-randomly applied stimulus sounds of 50 ms pink noise (dashed vertical lines) for either condition 1-click (green) or 4-click (blue) according to set delays of 0.5 s between the detected trough and first stimulus, and, 1.0 s thereafter in the 4-click condition. Delay parameters were chosen for clicks to coincide with SO up-states at their predominant ~1 Hz oscillatory frequency. Following the last click in either condition, trough detection was resumed after a fixed 4.5 s pause. (Drawing: 'Sleeper' by Gregory Matesich)

night, which would have resulted in 4 times as many 1-click as 4-click trials. However, owing to a technical error, the algorithm produced only 3 times as many 1-click as 4-click trials (see trial numbers in Table 3.2). Four clicks were chosen for the multi-click condition, as the last click was required to be applied prior to the refractory period of fast spindles ending. Following the last administered stimulus in any trial, the algorithm paused for 4.5 s before resuming detection of the next SO trough. This duration was chosen to result in trial windows of equal lengths between conditions for later EEG analyses. Stimulation was further paused manually whenever any signs of arousals or transitions into NREM stage 1 or REM sleep were observed. The combined hard- and software delays of the closed-loop system used in this experiment were measured and amounted to an approximately constant 100 ms, which were accounted and corrected for by the algorithm.

Auditory closed-loop stimulation is non-invasive and easily controllable, which makes it an ethical and safe brain stimulation technique to administer. Maximal volume limits are required and no overly ambitious persistence should be displayed by the experimenter in instances where participants consistently respond to sounds with arousals or awakenings. When stimulation is applied with due care, any known detriments thus resemble sleep with exposure to recurring environmental noise.

3.3.5 EEG and statistical analyses

Sleep scoring was conducted in SchlafAus© (version 1.5.0.1, Steffen Gais, 2005) on EEG and EOG data bandpass filtered between 0.3-35 Hz, with the EMG bandpassed between 10-100 Hz, and all down-sampled to 200 Hz. Based on guidelines outlined in the AASM Manual for the Scoring of Sleep and Associated Events (Iber *et al.*, 2007), two experimenters (J.S. & D.B., or J.S & P.C.) visually scored sleep stages Wake, N1, N2, SWS, REM, and arousals for each participant on a central EEG channel in continuous 30 s epochs from lights off until the end of night. Additional recording artefacts were marked to be excluded from later analyses. Scorers first worked independently and later compared diverging epochs to reach consensus. Sleep statistics were calculated as percentage of time spent per sleep stage over total sleep time (TST), with sleep efficiency computed as [TST / (TST + wake)]. TST was defined as time between sleep onset and final awakening minus wake, and sleep onset latency as period between lights out and first sleep epoch.

For EEG analyses, the original EEG data were pre-processed by first bandpass filtering continuous recordings between 0.3-35 Hz. Next, channels and their respective time periods of poor signal quality were identified and replaced by interpolated signals based on remaining, clean channels using a weighted linear regression approach. For this interpolation to be meaningful, we ensured that each electrode in question had a minimum of two directly neighbouring (non-diagonal) electrodes with clean signals. Furthermore, only the sections of poor quality were interpolated in order to preserve as much of the original data as possible.

Evoked potentials were calculated by cutting the continuous data into stimulation trials, whose start and end points both fell into the sleep stage(s) of interest, into windows of -1.5 to 5 s around the first stimulus marker (t = 0) in each trial and averaging the filtered signal across participants per electrode and condition with a baseline correction of -1.5 to -0.5 s applied. Computation of spindle power followed the same procedure, but additionally bandpassed the signal between 9-12 Hz and 12-15 Hz for slow and fast spindles respectively, with a zero-phase finite impulse response filter, before calculating root mean square (RMS) activity across sliding time windows of 200 ms, and applying an envelope smoothed over 200 ms. We further detected discrete spindle events with an automated algorithm (Ngo *et al.*, 2018) to estimate mean incidence rates for each spindle type and condition. An individual detection threshold was calculated as mean ± 1.25 * SD of the RMS signal for each participant. Discrete events were consequently identified as any time intervals where the envelope surpassed this threshold for periods between 0.5 to 3 s; single events with intermittent gaps < 100 ms were combined. An additional upper limit threshold (mean ± (1.25 * 5) * SD) for spindle amplitudes was incorporated to discard signal bursts not constituting a spindle. EEG averages were plotted time-locked to the maximal amplitude of identified discrete fast and slow spindles to verify their phase on any ongoing SO. We further assessed inter-spindle intervals (ISIs) by calculating the time interval between any discrete spindle occurring within the 0 to 5 s trial window which was followed by another discrete spindle event within the following 30 s epoch. Moreover, a Fast Fourier Transform with Tukey taper was used to calculate total spectral power for 0.5-4 Hz, 9-12 Hz, and 12-15 Hz frequency bands of interest across trial windows of 0 to 5 s per condition with baseline correction at -1.5 to -0.5 s, results of which were visualised topographically. Lastly, in order to explore the temporal relationship of adjacent sleep frequencies with slow and fast spindles, time-frequency graphs were created per condition by preparing trial segments of -2 to 6 s for Morlet wavelets with a sliding window adapting in width to the respective frequency (width = freq*0.5 cycles, and width < 5 were set to 5 cycles) over frequencies of interest 5-20 Hz in 0.25 Hz steps. Time-frequency representations show the relative change to a baseline interval between -1.5 to -0.5 s.

Individual differences in sleep architecture and stimulation susceptibility of the sleeping brain meant that after exclusion of trials affected by arousal, the overall number of stimulation trials received by participants ranged from 26 to 985 (mean = 392.91, SEM = 58.29) per individual and was rarely split evenly between sleep stages N2 and SWS. Supplementary Table 3.2 further provides an overview of stimuli numbers per participant and sleep stage. Owing to the balancing of total accumulated clicks per condition resulting in 3 times as many 1-click as 4-click trials, we therefore only included participants with at least 30 trials per condition (n = 17) in EEG analyses conducted in stable NREM for meaningful statistical averaging (Boudewyn *et al.*, 2018). For each participant, we counted the number of 4-click trials across the night and then selected a random subset of 1-click trials equal in number. The same trial selection principle was followed for contrasting evoked

responses between N2 and SWS, where the inclusion criterion was set to a minimum of 15 trials for each condition and stage (n = 11; individuals marked * or ⋄ in suppl. Table 3.2 were omitted). We use the term stable NREM to describe the combined sleep stages N2 and SWS (without N1). It is important to note that the sum of N2 and SWS trials does not necessarily equal stable NREM trial numbers. Stable NREM analyses also included trials which began and ended in either N2 or SWS sleep, unlike analyses conducted on a single sleep stage.

All analyses and statistics were computed in Matlab using custom-made scripts with the Fieldtrip toolbox (Oostenveld *et al.*, 2011) to treat all datasets equally. Statistically significant differences between conditions were calculated using two-tailed paired samples t-tests. For spindle incidence rates and ISIs, we conducted repeated measures analyses of variance (RM ANOVAs) with factors condition (1-click vs. 4-click) and spindle type (slow vs. fast spindles). The significance threshold was set to a $p\ value < 0.05$. Where applicable, significances were corrected with false discovery rate (FDR) for multiple comparisons. Unless otherwise denoted, reported values correspond to mean and standard error of the mean (±SEM). In cases of incomplete data (n = 2 in sleep quality questionnaire), those participants were excluded from the respective analysis. When presenting results, we focussed our attention on Cz as a representative electrode to capture both frontal SO and slow spindles, and centro-parietal fast spindles. One participant was excluded from the ISI analysis due to no spindle events within trials being found to be followed by the next discrete spindle within the specified 30 s interval.

3.4 Results

3.4.1 Stimulation does not affect healthy sleep architecture

On average, participants had a sleep efficiency of 97.32±0.66%, and took 13.60±2.50 min to fall asleep. The distribution of sleep stages over total sleep time (TST) and further sleep parameters (Table 3.1) suggest healthy sleep for this age group (Ohayon *et al.*, 2004). The mean percentage of sleep epochs affected by arousal was 14.08±1.17%, and our cohort experienced a mean proportion of 2.68±0.66% wake over total sleep time, indicating arousals did not inevitably entail awakenings. Eleven participants reported hearing stimuli in their sleep, while ten did not, and one individual was unsure. Based on the SF-R-A questionnaire, our cohort rated their sleep quality at 23.80±1.44 (score range 9-32 on a 0-35 scale), and feeling of being well rested at 25.95±1.46 (score range 11-38, on a 0-40 scale). Sleepiness, as assessed by the Stanford Sleepiness Scale, decreased overnight (evening: 4.05±0.23; morning: 2.73±0.18).

3.4.2 Number of SOs evoked by stimulation differs between conditions

To begin with, event-related grand averages of the EEG signal between 0.3-35 Hz were plotted in order to confirm that firstly, stimulus application coincided with SO up-states in both conditions, and secondly, the 4-click protocol resulted in more prolonged SO trains

Parameter	Mean	±	SEM
% Wake	2.68	±	0.66
% N1	6.42	±	0.78
% N2	50.72	±	1.33
% SWS	18.83	±	1.69
% REM	21.35	±	0.91
TST, min	476.43	±	9.86
Sleep onset latency, min	13.60	±	2.50
% Sleep efficiency	97.32	±	0.66
% Sleep epochs with arousals	14.08	±	1.17

Table 3.1: Polysomnographic parameters. Mean±SEM values are presented. N1 = NREM sleep stage 1; N2 = NREM sleep stage 2; SWS = slow wave sleep; REM = rapid eye movement sleep; TST = total sleep time, defined as lights off until last sleep epoch minus wake. Sleep onset latency as time between lights off and first sleep epoch. Sleep efficiency as percentage of time asleep over TST.

compared to 1-click. Figure 3.2A confirms that on average sounds were indeed played during the positive-going slope and/or near the peak of the dominant SO as intended (pictured is the signal at electrode Cz). As the predefined delay parameters of the stimulation were based on an estimate of SOs occurring approximately at a 1 Hz frequency, the increasing distance from the absolute peak for each consecutive click compared to its precursor in the 4-click condition may stem in part from the clicks occurring after fixed delays, but SOs showing natural variability when oscillating. Our algorithm applied only the first click in a closed-loop manner. Thus, in trial cases where intended stimulation and actual SO oscillatory frequencies were not in accord, the ensuing temporal error is likely to have increased over successive SOs, therefore resulting in the observed increased distance between SO peak and stimulus timing. We also confirmed that the 4-click condition resulted in prolonged SO trains (conditions significantly different as shown in Figure 3.2A in a number of time windows, including 1130 – 1360 ms, 1498 – 1850 ms, 2050 – 2080 ms, 2106 – 2418 ms, 2646 – 2874 ms, 3164 – 3446 ms, 3682 – 3928 ms, 4148 – 4156 ms, 4164 – 4202 ms, & 4252 – 4300 ms, at $p < 0.05$, after FDR correction). We subsequently topographically plotted mean slow wave activity (SWA, 0.5-4 Hz) across trial windows of 0-5 s (where the first stimulus was administered at t = 0 s) for both conditions to examine global scope. Figure 3.2B shows the typical (pre-) frontal dominance of slow wave activity in both conditions. Between conditions, 4-click achieved more SWA over the 5 s trial window at all recording sites owing to accumulative power over time (see suppl. Table 3.3 for individual p values after FDR correction per electrode). Further, we observed that the amplitude of the evoked signal decreased from frontal to parietal derivations as previously described in endogenous SOs (see Figure 3.2C, and Massimini *et al.*, 2004).

Furthermore, we assessed whether susceptibility of the brain to the stimulation showed a principally different pattern between sleep stages N2 and SWS, which could subsequently impact spindle activity. Only a subset of participants with a minimum of 15 stimulation trials in each condition per sleep stage (n = 11) were included in this analysis. Comparing grand averages per condition between N2 and SWS (Figure 3.2D), stimulation did not

elicit statistically different responses between the two sleep stages at electrode Cz after FDR correction. Based on the overall similar effects in the appearance of evoked responses, and to ensure adequate statistical power, we thus decided to combine both sleep stages in all further analyses.

3.4.3 Conditions differentially modulate slow and fast spindle activity

In a next step, we turned our attention to spindles and firstly assessed spindle activity. RMS activity for both slow (9–12 Hz) and fast (12–15 Hz) spindles derived from Cz is presented in Figure 3.3A. When visually comparing the temporal pattern of slow and fast

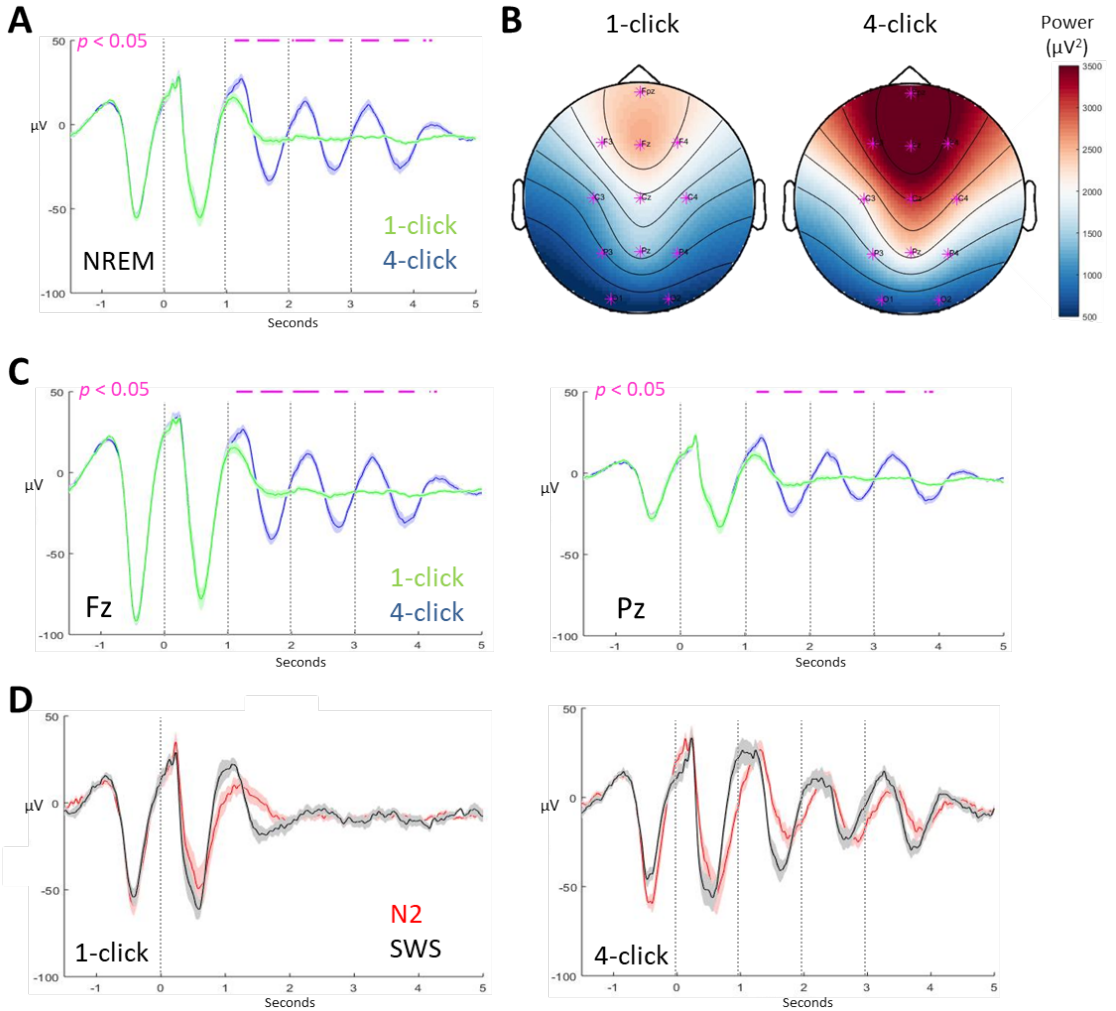

Figure 3.2: Evoked responses and accumulative power in stable NREM sleep. **(A)** Grand averages are plotted for 1-click (green) and 4-click (blue) conditions at electrode Cz. Horizontal magenta-coloured lines at the top indicate statistically significant difference between conditions. Vertical dotted lines mark the timing of stimulation(s); in 1-click only first stimulus at t = 0 s was administered. **(B)** Topographical distribution of mean 0.5-4 Hz SWA power (μV2) in stable NREM for 1-click and 4-click conditions. **(C)** Evoked responses at electrodes Fz (left) and Pz (right) visualise the decrease in amplitude from frontal to parietal locations. **(D)** No differences were detected in grand averages between sleep stages N2 and SWS for a subset of 11 participants at electrode Cz in 1-click (left) and 4-click (right) conditions. Evoked responses are based on a 0.3–35 Hz EEG signal and plotted as mean±SEM. Magenta asterisks and lines mark statistical significance between conditions at $p < 0.05$ after FDR correction. Please note that conditions are compared against one another, as the experiment did not include a sham condition.

spindle peaks, it is noticeable that fast spindle power peaked on average during stimulus application (vertical, dotted lines), which approximately coincided with SO up-states (see evoked responses in Figure 3.2A) in both experimental conditions. Meanwhile, slow spindle activity was increased in between sound clicks, i.e. approximately during up- to down-state transitions, as previously described in endogenous SO trains (Mölle *et al.*, 2011). We confirmed this phase relationship by plotting the averaged signal event-locked to the maximal amplitude of discrete spindle events (suppl. Figure 3.7). Descriptively, the first stimulus (t = 0 s) elicited the strongest response in slow spindles with activity peaking approximately 200 ms later. Slow spindle responses to consecutive stimuli in the 4-click condition showed a numerical trend towards a decreased magnitude thereafter, while activity in the 1-click condition slowly returned to baseline level (horizontal, dotted line) (Figure 3.3B). In contrast, averaged fast spindle power was at its maximum at the time of the second stimulus (t = 1 s) in both conditions, hence suggesting it to be the delayed result of the first stimulus.

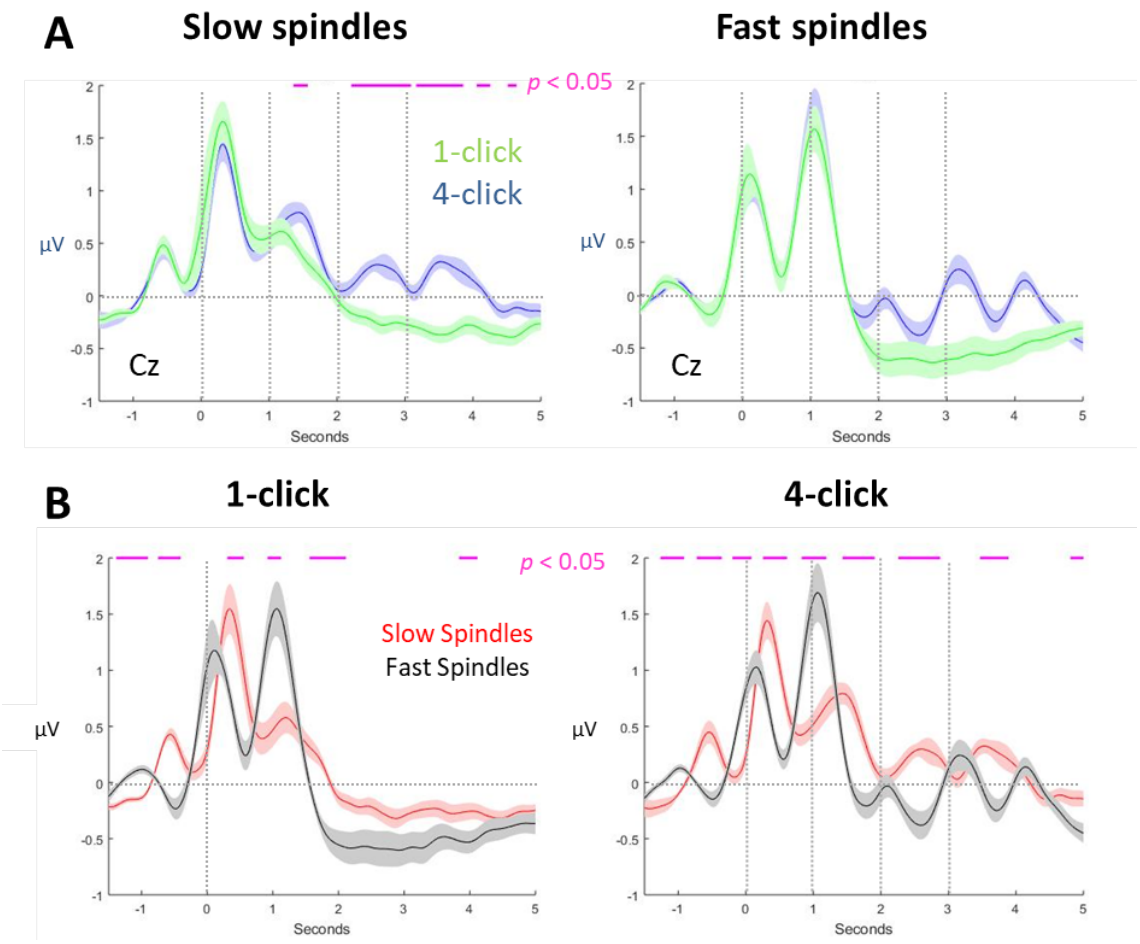

Figure 3.3: Slow and fast spindle power differs by condition. Mean±SEM of slow (9–12 Hz) and fast (12–15 Hz) spindle RMS activity (μV). Comparison between **(A)** 1-click (green) and 4-click (blue) conditions, and **(B)** slow (red) and fast (black) spindle type at electrode Cz. Vertical, dotted lines indicate stimuli; only first stimulus at t = 0 was applied in 1-click condition. Horizontal, dotted lines represent baseline. Magenta bars indicate statistical significance in paired comparisons at $p < 0.05$ after FDR correction.

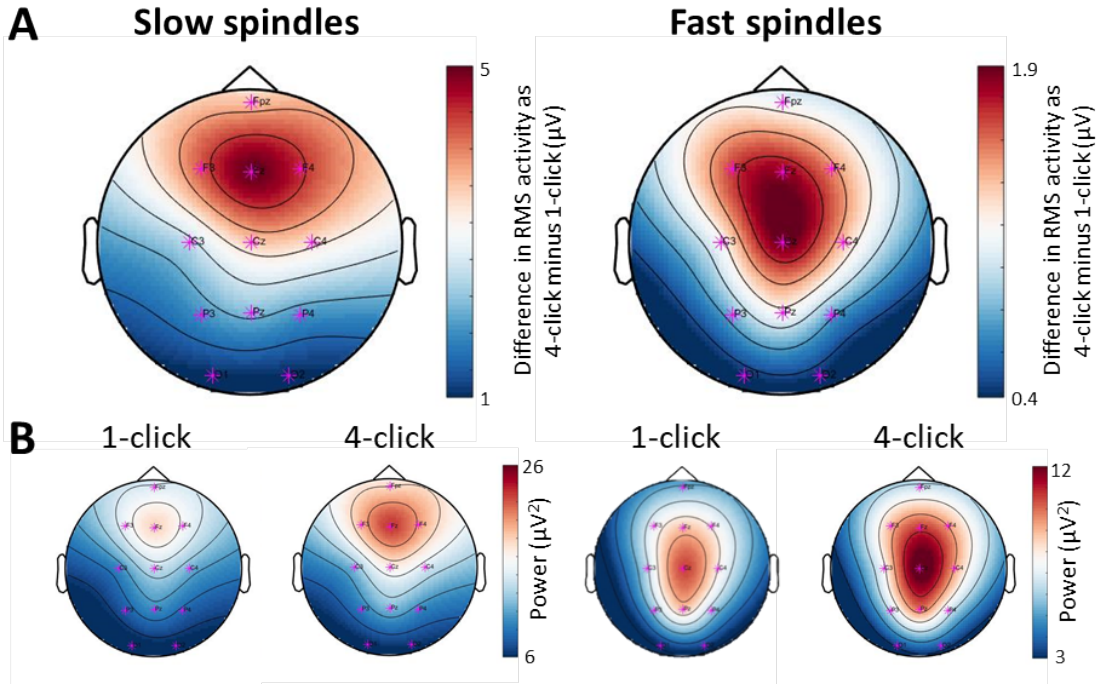

Figure 3.4: **A** Topographical representations for mean slow (9-12 Hz, left) and fast (12-15 Hz right) spindle RMS activity (μV) differences between conditions (4-click minus 1-click) for trial windows of 0 to 5 s with the first stimulus at t = 0 s, and **B** plotted separately per spindle type and conditon. Please note colourbar scales differ. Magenta asterisks denote statistical significance at $p < 0.05$ after FDR correction.

Most interesting is the pronounced suppression of fast spindle activity observed in the 1-click condition after 1500 ms, and mirrored in the 4-click condition despite consecutive clicks, which evoked responses below or marginally above baseline level (Figure 3.3A (right) in fast spindles, t = 2 to 4 s). On the contrary, the activity depicted for slow spindles suggests that although evoked power decreased gradually with successive sounds, it appears to persist above the pre-stimulus baseline in the 4-click condition (horizontal, dotted lines in Figure 3.3A (left)). Between conditions, slow spindle activity was found to be significantly different between the second and third clicks, and following the third click ($p < 0.05$ at 1360 - 1572 ms, 2212 - 3092 ms, 4058 - 4256 ms, and 4516 - 4644 ms, FDR corrected, significances indicated in magenta at electrode Cz, Figure 3.3A). In comparison, experimental manipulation led to no statistical power differences between conditions in fast spindle power.

Following these observations, we calculated total spectral power for both spindle types at all electrodes across trial windows of 0 to 5 s to examine topographical spindle effects. Power differences between 1-click and 4-click conditions per spindle type pictured in Figure 3.4A reveal that 4-click led to increased slow spindle power over primarily (pre-) frontal sites, while fast spindle power was enhanced over more central derivations. Mean trial power differences for each condition (see suppl. Table 3.3 for p values at all electrodes) are visualised in Figure 3.4B, demonstrating that total slow spindle power exceeds fast spindles across the averaged trial.

In order to examine these dynamics in more detail, we detected discrete spindle events to calculate the mean incidence rate of slow and fast types per 0 to 5 s trial window (Figure 3.5A). Statistical analysis revealed a significant main effect of condition ($F(1,16) = 10.66$, $p < 0.01$, $\eta_p^2 = 0.400$) and main effect of spindle type ($F(1,16) = 5.35$, $p < 0.05$, $\eta_p^2 = 0.251$). No significant interaction was found between these factors ($F(1,16) = 2.55$, $p = 0.130$, $\eta_p^2 = 0.138$). Discrete spindles of either type occurred in less than half of the trials. Slow spindles occurred in 30.04±0.03% of 1-click and 38.70±0.06% of 4-click trials; fast spindles in 23.56±0.03% of 1-click and 26.72±0.03% of 4-click trials.

Analysing the inter-spindle interval (ISI) between any one discrete spindle detected within a 5 s trial window starting from the first stimulus and any proximate discrete spindle occurring within the subsequent 30 s epoch, we found a main effect for spindle type ($F(1,15) = 9.14$, $p < 0.01$, $\eta_p^2 = 0.379$), but not condition ($F(1,15) = 0.01$, $p = 0.912$, $\eta_p^2 = 0.001$), and no interaction ($F(1,15) = 0.05$ $p = 0.827$, $\eta_p^2 = 0.003$). Slow spindles had a shorter mean ISI of 9.27±0.71 s in the 1-click, and 9.36±0.62 s in the 4-click condition, whereas fast spindles revealed durations of 11.27±0.62 s in 1-click, and 11.04±0.80 s in the 4-click trials (see Figure 3.5B).

3.4.4 Temporal occurrence of spindle types in relation to neighbouring frequencies

Finally, time-frequency decompositions were conducted to explore the temporal occurrence of oscillatory frequencies adjacent to slow and fast spindle activity (Figure 3.6, pictured is the signal at electrode Cz). In the 1-click condition, theta (5–7 Hz) coincides with slow spindle activity approximately 400 ms after the stimulus, and fast spindle power peaks

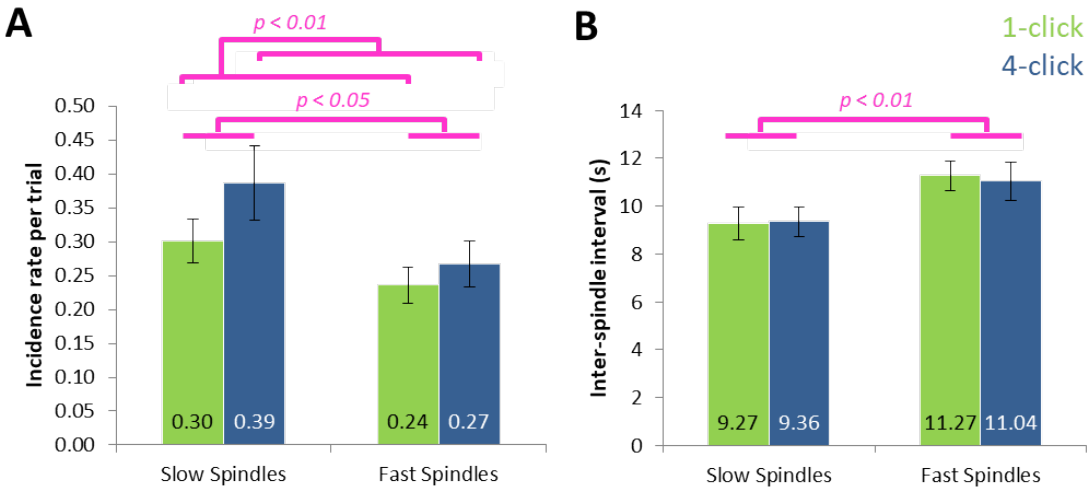

Figure 3.5: Slow and fast spindle incidence rate in trials and inter-spindle intervals. Mean±SEM for slow (9–12 Hz) and fast (12–15 Hz) spindle type in 1-click (green) and 4-click (blue) conditions at electrode Cz of **A** incidence rates, calculated as mean number of discrete spindle event per trial, and **B** inter-spindle intervals (ISI), computed as duration (s) between discrete spindle events occurring during trial window of 0 to 5 s starting with the first stimulus at t = 0, which were followed by another discrete spindle within the consecutive 30 s epoch.

during the following SO up-state (see superimposed evoked responses). A near identical pattern was observed in the 4-click condition; however, the additional three consecutive clicks resulted in heightened theta and slow spindle activity, which appeared approximately comparable in strength to the endogenous bout in the same frequency ranges prior to stimulation onset at –500 ms (see statistical difference between conditions in bottom plot). Furthermore, minor beta activity (low beta, 15–20 Hz) is noticeable following all clicks in both conditions but most strongly responding in the aftermath of the first stimulus. This suggests stimuli may have been followed by brief glimpses of increased alertness and vigilance (Marzbani *et al.*, 2016). Notably, the fact that this frequency is more pronounced after the first stimulus in either condition may suggest some degree of habituation to the sounds is taking place.

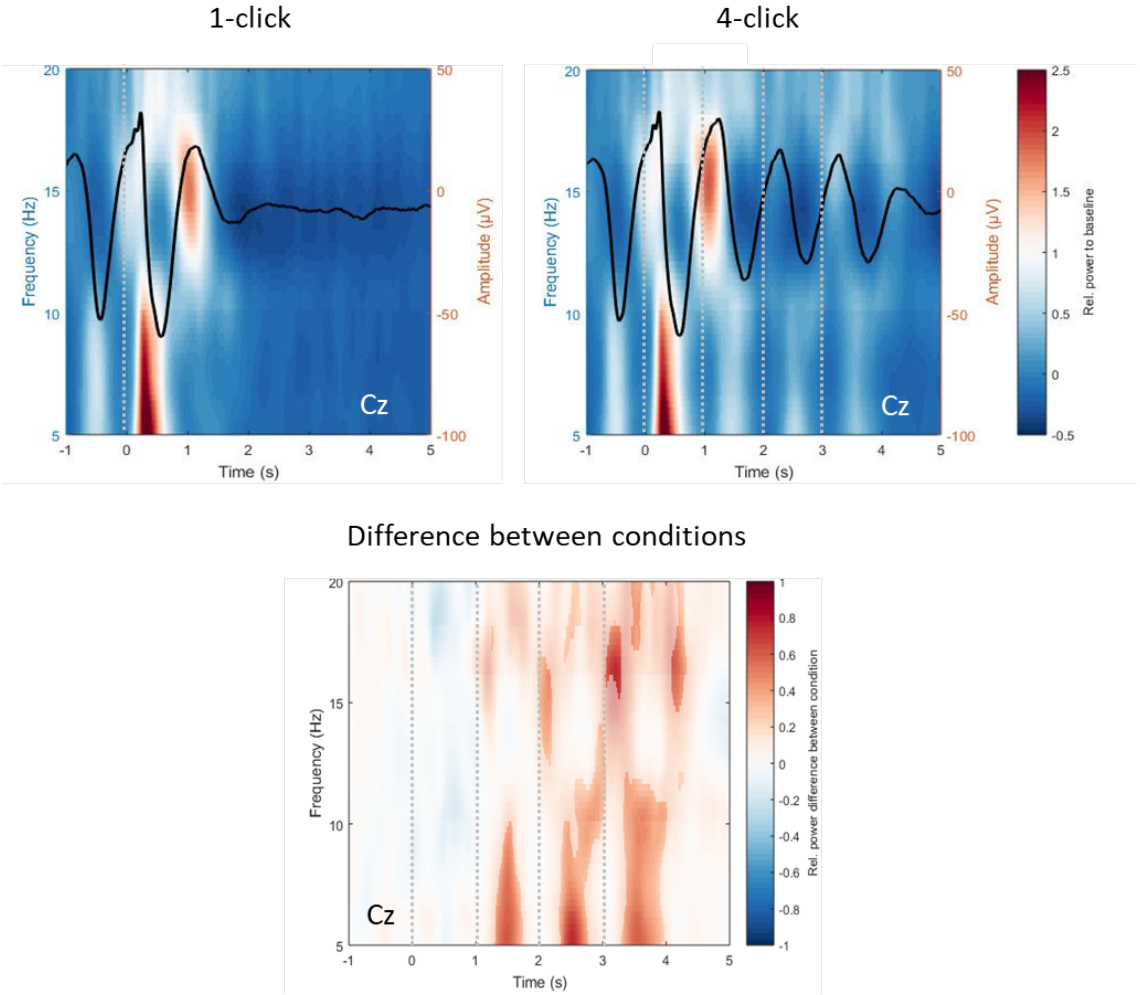

Figure 3.6: Neighbouring oscillatory bands and their temporal relationship to spindles. Plots at top depict spectral decomposition of relative power change to baseline at electrode Cz for 1-click (left) and 4-click (right) conditions with superimposed evoked responses in black, while the bottom plot visualises the relative power difference between condition, with time-frequency clusters not significant at $p < 0.05$ after FDR correction masked. Left and right y-axes show frequency (Hz) and evoked response amplitude (μV), respectively and where applicable. Dotted lines indicate timing of applied stimuli.

3.5 Discussion

In the present study, we demonstrate that auditory closed-loop stimulation has the potential to differentially modulate slow and fast spindle activity in terms of power, incidence rate, and duration of inter-spindle intervals between conditions. These findings hint at underlying differences in slow and fast spindle dynamics, which are discussed below. We also explore limitations of the present study and outline future directives.

Differences in dynamics between spindle types and experimental conditions were evident in power measures. Fast spindle power was suppressed to below baseline levels following the SO evoked by the first stimulus in both conditions, and did not differ significantly between conditions. Based on the principle of fast spindle refractoriness, we speculate that the smaller and brief power increases observed in the 4-click protocol (t = 2 to 4.5 s) may stem from sporadic trials in which an endogenous fast spindle had occurred just prior to stimulation trial onset. In such a case, a varying number of initial auditory stimuli would have fallen into an already on-going refractory period and only elicited a response thereafter. As fast spindle power did not vary between conditions until after application of the second stimulus, it is logical to assume that the maximal peak in fast spindle activity which coincided approximately with the second stimulus represents a delayed response to the first click. This explanation is in line with previously reported patterns in auditory closed-loop protocols (Ngo *et al.*, 2015, 2018). In the same delayed response logic, the fast spindle peak occurring approximately 50 ms after the first stimulus is likely attributed to endogenous spindle activity unrelated to the stimulation.

In turn, slow spindle power differed significantly between conditions at multiple timepoints. It gradually decreased across consecutively administered stimuli in 4-click trials, but remained above baseline level. Meanwhile, slow spindle activity in the 1-click condition demonstrated suppression similar to that of fast spindles, albeit to a lesser degree. The total cumulative power of slow spindles across the averaged trial exceeded that of fast spindles, adding support to hypothesised differences in spindle expressing networks. Whether this discrepancy is based on spindle duration or amplitude remains to be tested in future studies. The most pronounced response in slow spindles was observed immediately after the first stimulus during the transition from up- to down-state of the underlying SO. This phase relationship persisted throughout the multi-click protocol.

Our results on spindle incidence rates within trials suggest that 4-click increased the chances of spindle occurrence in comparison to the 1-click condition, with this effect being numerically more pronounced in slow spindles. In both experimental conditions, the chance of slow spindles occurring was statistically greater than for fast spindles; although all figures indicate spindles only occur in 24-39% of trials. This matches previous reports of higher slow spindle densities (Piantoni *et al.*, 2016). It is unknown whether this incidence rate could potentially be increased by prolonging the inter-trial interval, giving networks more recovery time. The inter-spindle interval was found to be of different

length between spindle types, with slow spindles following one another in closer temporal succession than fast spindles. Considered in sum, these results suggest that slow spindle generating networks show different temporal dynamics than those producing fast spindles and recover more quickly. However, for both spindle types, the durations observed in this study were on average considerably longer than the previously reported refractory period in fast spindles expressing thalamo-cortical networks of 3 – 6 s (Antony *et al.*, 2018). However, the study which described this duration was based on afternoon naps. In this light, it is conceivable that by stimulating all of stable NREM throughout the overnight, spindle-expressing networks were driven too hard and thus responded with suppression periods increasing in duration. Effects of different levels of sleep pressure at night-time could have mediated spindle activity, as previously demonstrated (Himanen *et al.*, 2002). For this reason, we refrain from labelling the inter-spindle intervals observed in the present study as system-inert spindle refractoriness. Introducing longer pauses (> 4.5 s) between stimulation trials could potentially shorten these ISI periods.

Whether the above described differential dynamics in slow and fast spindle activity extend to separate functional purposes requires future investigation. According to the earlier explored speculative scenario in which slow spindles could play a role in cross-linking information and recruiting frontal areas for optimal long-term integration of memory representations (Astori *et al.*, 2013), memories reactivated in this manner and followed by increased slow spindle activity might consequently benefit from such manipulation and be better remembered long-term. Employing an auditory-closed loop protocol which selectively increases slow spindle activity could help test this idea.

Whether sleep stage could affect the number and magnitude of spindles evoked in response to auditory closed-loop stimulation remains to be investigated, as principally, endogenous spindles have been found to occur more frequently in N2 (Mölle *et al.*, 2011). While our evoked responses in the EEG did not show great divergence between sleep stages visually, we cannot assume the same for spindles as our lower sample size did not lend itself to address this hypothesis. Exploratory plots of evoked responses indicate that stimulation elicited marginally larger, consecutive evoked amplitudes in SWS than N2. This could be as a result of the characteristic, underlying neuronal bistability in SWS (Steriade *et al.*, 1993). Further investigation is required to clarify whether this finding translates to spindle activity also.

Our polysomnographic results corroborate the view that the majority of our cohort were able to obtain what would be considered healthy sleep in young adults (Ohayon *et al.*, 2004). While the mean figure of sleep epochs affected by arousals was higher than in similar stimulation experiments (e.g. $14.08 \pm 1.17\%$ compared to $7.9 \pm 0.6\%$ in Ngo *et al.*, 2013), the low figure of wake over total sleep time indicates these were micro-arousals and did not inevitably entail awakenings. It is plausible that stimulating throughout the night and thus into the morning hours with characteristically lighter N2 sleep produced

this arousal figure. Nonetheless, future studies using similar auditory stimulation protocols may benefit from adding an adaptation night to habituate participants to stimuli in order to decrease arousal likelihood and possibly the temporary increases in beta activity suggesting vigilance observed in this experiment. However, habituation could likewise risk diminishing the sleeping brain's evoked responses to familiar sounds, and should therefore be considered carefully.

Small amendments could improve the performance of the protocol used in the present study. Firstly, using a phase-locked loop in lieu of fixed delays might achieve a more precise targeting of SO up-states for stimulation application. Secondly, information processing of external auditory stimuli appears blocked to a certain degree during acute fast spindle activity (Dang-Vu *et al.*, 2010a, Schabus *et al.*, 2012), suggesting a mechanism to be in place which shields ongoing processes from external disruption. Thus, a closed-loop detection algorithm would further benefit from real-time spindle activity monitoring and only commencing stimulus application if no spindle has been detected in a certain preceding interval. Such an approach would also enable control over endogenous spindle activity during stimulation trials. Thirdly, different auditory stimulation protocols have previously attempted to manipulate spindle activity by applying oscillating sound stimuli which mimicked spindle frequencies. Whether these could prove a more efficient way of controlling and evoking spindle activity is as of yet unclear. In one case, a non-immediate spindle enhancement similar to our delayed fast spindle response was observed (Ngo *et al.*, 2018). In another, the chosen protocol only succeeded in affecting 2 s-delayed fast but not slow spindle activity (Antony & Paller, 2017). These studies raise important questions on the optimal type of auditory stimulation to enhance spindles in general. Lastly, online arousal monitoring and terminating automatic stimulation as soon as signs of arousal, e.g. increases in lower beta and EMG activity, are detected, could improve the stimulation experience for both participant and experimenter.

With regard to limitations, the present study did not pay attention to changes in spindle reactivity across the sleep period. Similarly to SWA, sleep spindle (re-) activity is thought to underlie changes in sleep pressure and depth (Andrillon *et al.*, 2011, Himanen *et al.*, 2002), and fast and slow spindles have previously been demonstrated to fare dissimilarly under similar homeostatic control (Knoblauch, 2002). Thus, stimulation at different times during the night could lead to differential outcomes. Conversely, the division of spindles into slow and fast types by frequency is not straightforward. A number of differently defined frequency ranges have been used across previous studies. Various optimal cut-off frequencies of e.g. 12 Hz (Mölle *et al.*, 2011), 13 Hz (Schabus *et al.*, 2007), and 13.5 Hz (Lustenberger *et al.*, 2015) have been proposed to distinguish the two, which makes inter-study comparisons of results challenging. Our time-frequency decompositions support the chosen cut-off value of 12 Hz between spindle types in analyses for this dataset on average. Yet to complicate matters further, the oscillatory activity of spindles exhibits measurable variance between sleep stage bouts and cycles (Himanen *et al.*, 2002, Mölle *et al.*, 2011).

Further, spindle activity has been shown to vary for a range of variables such as age, sex, inter- and intra-sleep cycle changes, menstrual cycle, and is affected by sleep deprivation (Baker & Lee, 2018, Buzsáki, 2015, Cox *et al.*, 2012, Lustenberger *et al.*, 2015, Purcell *et al.*, 2017, Rosinvil *et al.*, 2015). Adding a baseline condition or night to directly contrast endogenous spindles with stimulation effects in future studies could help put some of these variabilities into perspective. Considering and controlling for all these factors was beyond the scope of the current experiment. Thus, it is possible that the definition of frequency ranges for slow and fast spindles imposed in this study did not entirely match the displayed power bands of some individuals in our cohort, which could have introduced some bias. Defining spindle frequencies per individual is a realistic possibility, however, not all individuals have been shown to demonstrate a clear indication of cut-off values between slow and fast spindles (for an example, see Fig.4 in Purcell *et al.*, 2017), which would therefore make this approach more challenging.

To conclude, this study has taken a first step in exploring the efficacy of auditory closed-loop stimulation to differentially modulate slow and fast spindle activity, with demonstrated differences between slow and fast spindle power, incidence rate, and inter-spindle interval lengths. While certainly improvable and expandable to include behavioural testing in future, we provide supporting evidence to suggest slow and fast spindles underlie different neuronal dynamics, which may hint at differential functional purposes. Auditory closed-loop stimulation presents a non-invasive and easily controllable technique to aid in the future investigation thereof.

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J.S., W.E.-D., & P.A.L. conceived and designed the study, A.J.C. & M.N. coded the stimulation algorithm, J.S., D.B., & P.C. performed the study and sleep scored the data, J.S. coded and performed analyses with assistance from H.-V.V.N., J.S., H.-V.V.N., & P.A.L. interpreted results, J.S. wrote the manuscript. M.T. wrote the signal interpolation script.

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3.6 Supplementary information

Participant		**1**	**2 ◇**	**3***	**4 ◇**	**5**	**6***	**7***	**8**
N2	C1	68	30	39	17	48	50	26	81
	C4	21	11	14	4	18	9	3	18
SWS	C1	40	10	325	4	380	282	318	166
	C4	15	4	110	1	126	101	109	64
NREM	C1	118	45	372	21	429	340	348	257
	C4	40	15	124	5	146	111	112	84

Participant		**9**	**10 ◇**	**11**	**12***	**13**	**14**	**15**	**16 ◇**
N2	C1	100	28	111	33	57	149	61	28
	C4	32	10	32	11	20	46	16	8
SWS	C1	415	29	324	102	168	350	149	58
	C4	137	3	121	36	58	120	53	19
NREM	C1	525	59	469	136	236	520	223	94
	C4	172	16	158	47	79	172	72	28

Participant		**17**	**19**	**20***	**21***	**22**	**23 ◇**	**Mean**	**±SEM**
N2	C1	69	56	34	71	226	60	65.55	10.17
	C4	24	17	8	14	67	15	19.00	3.13
SWS	C1	666	328	181	268	476	10	229.50	37.46
	C4	226	111	59	94	163	5	78.86	12.79
NREM	C1	742	387	218	348	714	74	303.41	44.20
	C4	252	129	67	111	233	23	99.82	14.85

Table 3.2: The total numbers of trials (first stimulus counts) are listed per participant and sleep stage after arousal exclusion. For sleep stage specific analyses, only individuals with with $\geq$ 15 trial counts for categories N2 and SWS each were included in EEG analyses. Likewise, only participants with $\geq$ 30 trials in stable NREM were included in all remaining EEG analyses. * marks participants excluded from comparative analyses between sleep stages N2 and SWS, whereas ◇ denotes those excluded from all EEG analyses due to low trial numbers. Mean±SEM of trials are presented across all 22 participants. Participant 18 was excluded after not reaching stable NREM sleep. Please note that numbers for N2 and SWS combined do not necessarily equal NREM trial counts, as the latter also include trials which began in one stage and ended in another, unlike in analyses conducted on a single sleep stage. C1 = 1-click, C4 = 4-click protocol.

Electrode	SWA	Slow Spindles	Fast Spindles
'Fpz'	0.000	0.000	0.000
'F3'	0.000	0.000	0.000
'Fz'	0.000	0.000	0.000
'F4'	0.000	0.000	0.000
'C3'	0.000	0.000	0.000
'Cz'	0.000	0.000	0.000
'C4'	0.000	0.003	0.002
'P3'	0.000	0.000	0.002
'Pz'	0.000	0.001	0.002
'P4'	0.000	0.009	0.002
'O1'	0.000	0.002	0.006
'O2'	0.000	0.004	0.005

Table 3.3: P values from FFT power differences between 1-click and 4-click conditions are listed for slow wave activity (SWA), slow and fast spindles at all twelve EEG electrodes. Significances have been FDR corrected for multiple comparisons within each power band.

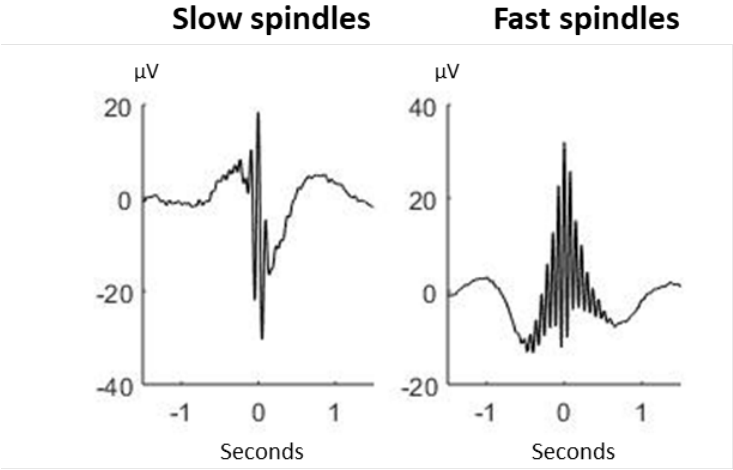

Figure 3.7: Preferred slow wave phase of slow and fast spindles. Averaged EEG signals of identified discrete slow (9–12 Hz, left) and fast (12–15 Hz, right) spindles within undisturbed NREM sleep were plotted time-locked to their respective maximal amplitude (t = 0 s) at electrode Cz to verify their preferred phase of the underlying slow wave rhythm. Slow spindles occurred during the transition from up- to down-state, whereas fast spindles dominated in the up-phase.

Chapter 4

The effects of anticipating nocturnal stimulation on healthy sleep and cognition: a placebo study

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This chapter is based on a manuscript which is being prepared for submission for peer review.

4.1 Abstract

Background: Previous research has attempted to enhance NREM sleep with various methods for greater physiological, psychological, and cognitive gain. The ability of individuals to self-enhance aspects of their sleep by anticipating such effects from outside intervention without actually receiving any has been demonstrated in insomniacs, but remains unexplored in healthy sleepers. In this study, we compare the impact of simulated auditory closed-loop stimulation (placebo) and an undisturbed baseline night on sleep and sleep-dependent cognition in healthy adults.

Methods: Twenty-two (15 female) healthy adults aged 18-28 (mean 23.32 years) were tested in a within-subject crossover design and underwent one night of placebo stimulation and one baseline night. Participants were assessed for overnight change in declarative and procedural memory performance, as well as alertness, and subjective perceptions of sleep quality and affect. We further analysed sleep architecture, spectral slow wave, and slow and fast spindle activity.

Results: Placebo stimulation had no differential impact on sleep architecture or cognitive performance when compared to baseline. However, frontal slow spindle power appeared to be transiently and inconsistently altered in stable NREM sleep stages across different time intervals of the night.

Conclusions: Our findings suggest that anticipating stimulation during sleep impacts on slow spindle activity. We conclude that induction of consistent placebo effects was not possible by simulating an auditory closed-loop protocol, which contrary to our intentions resulted in transient placebo as well as nocebo outcomes. Potential placebo and nocebo effects should thus be given adequate consideration when designing future (auditory) stimulation experiments.

Keywords: Sleep, Memory, Auditory sleep enhancement, Placebo, Sleep spindles, Slow wave activity

4.2 Introduction

Commonly considered a nuisance variable, the placebo effect is a psychophysiological phenomenon which presents an influential factor in treatment outcome. While underlying mechanisms in the central nervous system still warrant more systematic investigation (Rotenberg, 2016), the integrated conceptual framework by Colloca & Miller (2011) posits that an incorporation of available verbal, contextual, and social cues elicits expectancies. These in turn then drive the effect through learnt response behaviour, resulting in measurable behavioural and physiological changes. Placebo effects have been demonstrated in a variety of cases, including e.g. pain and immune disorder treatment, Parkinson's disease, and even sham surgery (Benedetti *et al.*, 2016, Colagiuri *et al.*, 2015, Schedlowski *et al.*, 2015). It has been suggested that the element of human interaction, particularly a warm relationship between patient and care professional, may hold explanatory power (Howe *et al.*, 2017). The extent to which placebo effects are mediated by explicit awareness is under debate (Stewart-Williams & Podd, 2004), but a general anticipatory, yet not fully conscious state may suffice to elicit the effect (Colagiuri *et al.*, 2015).

While the placebo effect is well established in wake, it is unclear whether it could influence aspects of sleep, a behavioural state in which our conscious awareness is fundamentally altered. Human sleep is divided into rapid eye movement (REM) and NREM sleep, with the latter further categorised from shallow to deep slow wave sleep (SWS). Whereas REM sleep with distinctively vivid dream imagery has been ascribed a certain degree of consciousness due to its subjective, phenomenological element (Windt *et al.*, 2016), NREM sleep is thought to be devoid of such due to an effective connectivity breakdown in large-scale functional brain networks required to facilitate a conscious state (Massimini *et al.*, 2005, Tononi, 2008). However, this notion and with it the definition of consciousness in NREM sleep have recently been challenged by the discovery that particular sleep oscillations are able to temporarily and selectively activate one such network (Kaplan *et al.*, 2016, Walker & Robertson, 2016). Moreover, the cyclical nature in which humans repeatedly pass through sleep stages paired with the finding of different stages prevailing simultaneously in separate anatomical structures during sleep stage transitions would make defining the exact degree of consciousness during sleep a complex undertaking (Emrick *et al.*, 2016).

Irrespective of the assumed absence of conscious awareness in NREM, the brain is anything but idle in deep sleep. SWS is characterised by trains of 0.5-4 Hz slow waves, which occur in their highest density and intensity in this sleep stage. Slow waves have been demonstrated to play a driving role in orchestrating phase-locked sleep spindle activity (waxing and waning waveforms between 9-15 Hz) to facilitate overnight consolidation of declarative and procedural memories (Clemens *et al.*, 2007, Diekelmann & Born, 2010, Mölle *et al.*, 2011). Increasing slow waves and sleep spindles has been linked to greater overnight performance gains in both declarative and procedural memory domains (Genzel & Robertson, 2015, Schönauer, 2018). Since these oscillations show disturbed activity in a range of psychobiological pathologies with negative functional implications (Petit *et al.*, 2004),

substantial research efforts have been devoted to the enhancement of SWS, slow waves, spindles, and associated cognition by means of pharmacological, acoustic, somatosensory, visual, electrical, and magnetic manipulation to date (Bayer *et al.*, 2011, Bellesi *et al.*, 2014, España & Scammell, 2011, Marshall *et al.*, 2006, Massimini *et al.*, 2007, Sharon & Nir, 2018). While successful to varying degrees (Feld & Diekelmann, 2015), such interventions require a considerable amount of resources, including sophisticated technical devices and setups, trained personnel for administration and monitoring, or may entail unintended side effects from pharmacological or more invasive stimulation modalities.

Besides such externally driven interventions, an alternative, psychological approach demonstrated that using hypnosis to put young and older adults into a different state of mind was sufficient for increasing SWS duration (Cordi *et al.*, 2014, 2015). However, the favourable result was only apparent in individuals at the higher end of the hypnotic suggestibility spectrum. Interestingly, low suggestibility participants could not self-enhance sleep when asked to simulate effects of hypnotic suggestion (Cordi *et al.*, 2014). Additionally, when overtly instructed to either 'sleep better' or 'sleep worse' in a different study, participants were only able to implement the latter command successfully (Combertaldi & Rasch, 2017). These results raise the question whether and to what extent participants' behavioural compliance during sleep with suggested experimental outcomes can be influenced covertly (instead of overtly). Are participants able to self-enhance aspects of deep sleep by anticipating these effects from an experimental intervention, e.g. through placebo manipulation? Pharmacological placebos have been found to be an effective treatment for insomnia, where they enhanced objective and subjective sleep quality measures (Rogev & Pillar, 2013, Winkler *et al.*, 2015), thus indicating that it is possible to elicit a placebo response in the sleeping brain. However, these results could be due to the nature and pathology of insomnia itself (Akram *et al.*, 2018). Previous studies have reported both placebo and reverse placebo (nocebo) effects in this patient group (Brockner & Swap, 1983). To date, the potential of placebo manipulation in healthy sleepers has not been examined beyond its ability to diminish the first-night effect, a phenomenon of diminished sleep quality in a new environment (Suetsugi *et al.*, 2007).

In the current experiment, we explored whether placebo stimulation, i.e. the prospect of stimulation administration, and an induced anticipation of specific effects on sleep, can alter subjective or objective sleep quality. We further assessed whether this would translate into changes in the domain of sleep-dependent cognition by influencing overnight declarative and procedural memory consolidation. In order to simulate stimulation, setup of auditory closed-loop stimulation, a non-invasive brain stimulation technique available in our lab, was performed; a detailed description of this technique can be found elsewhere (Ngo *et al.*, 2013). If successful, we would expect placebo stimulation to result in an increase in time spent in stable NREM sleep over total sleep time, corresponding changes in spectral power of slow wave and spindle power bands, fewer arousals, subjective reports of perceived increased overnight recovery, and improved performance on memory

tasks. Conversely, if such enhancements cannot be achieved, then this experiment serves to disconfirm bias in stimulation studies where a double-blinded design is not feasible. Finally, the possibility of an anticipation of brain stimulation during sleep having a negative impact on sleep quality should also be considered due to prior nocebo findings in insomniacs. Such an effect could counteract successful stimulation results in part and is therefore equally worth investigating.

4.3 Methods

4.3.1 Participants

Twenty-two healthy participants (15 female, age range 18-28 years, mean = 23.32 years, SD = 3.18) completed this study. Participants were recruited via the departmental participant pool, online advertising on social media, and poster adverts around the campus of the Cardiff University Brain Research Imaging Centre, UK, where this study was performed.

Online eligibility screening verified that all participants were native English speakers with no history of physical, psychological, neurological, or sleep disorders, were following a regular sleep-wake rhythm for four weeks prior to participation, did not habitually nap, had not engaged in any nightshift work, nor undertaken any transmeridian travelling in the preceding two months. We further excluded smokers, consumers of medication or controlled substances known to affect sleep, and individuals with hearing or visual impairments, hypersensitive skin or contact allergies, those who were unwilling to abstain from alcohol for 24 hours, and from caffeinated drinks or extreme physical exercise for 12 hours before every visit to the laboratory, were uncomfortable at the prospect of sleeping with in-ear headphones, or had recently experienced a stressful life event.

Participants had not previously taken part in any sleep study and therefore had no personal experience of receiving auditory closed-loop stimulation during sleep, nor any in-depth knowledge of sleep oscillations. They gave informed written consent prior to participating in this study, were financially reimbursed for their time commitment, and fully debriefed at the end of the study. The experiment had prior approval from the School of Psychology's ethics committee (EC.16.12.13.4805A).

Three additional participants were initially recruited, but withdrew at various stages of the study due to time conflicts (n = 2), and discomfort sleeping with in-ear headphones (n = 1).

4.3.2 Experimental design

An adaptation night was used to accustom participants to sleeping in the laboratory, and to disconfirm the presence of any sleep disorders. We employed a within-subject design, in which all participants spent two experimental nights in the laboratory: one

placebo stimulation (Placebo-Stim) and one baseline (Sham) night in counter-balanced order. Sessions were scheduled one to three nights apart to avoid carry-over effects in sleep pressure between experimental nights. Participants were sent regular reminders before each visit, instructing them to avoid alcohol, caffeine, and extreme physical exercise on study days; compliance was assessed in a brief interview screening at the beginning of each overnight visit.

Participants arrived in the laboratory 2.5 hours before their habitual bedtime (see Figure 4.1). At the beginning of their adaptation visit, participants were briefed on the alleged purpose of the experiment (see Section 4.3.3: Placebo induction procedure). They were wired up for polysomnography (PSG) and filled in questionnaires on sleepiness and affect. Next, participants completed a psychomotor-vigilance test (PVT) to control for alertness, and declarative and procedural memory tasks. On experimental nights, participants then received a sealed envelope informing them of which condition they would be undergoing on said night, and their expectation of the condition's impact on their sleep was recorded. We informed participants of their stimulation condition only at this point in time in order to focus our manipulation efforts solely on sleep, and to not affect prior encoding performance on cognitive tasks as well. In-ear headphones (Sony MDR-EX15LP) were securely attached with a thin strip of medical tape. Once in bed, participants underwent a final impedance and sound volume check. Lights-off time was recorded. Participants had access to a personal alarm to call the experimenter if and when they required attention during the night.

After opportunity to sleep for at least 7 hours, they were awoken the following morning at a previously agreed time by either the experimenter or their personal alarm clock (whichever they were more comfortable with). Experimenters woke participants only from sleep stages N1 or N2; this could not be controlled for in participants who had opted to use their own alarm. Waking method remained identical per participant across visits. We ensured that participants had an equivalent amount of time to sleep, switched lights off and were awakened within an hour between the two experimental nights to control for

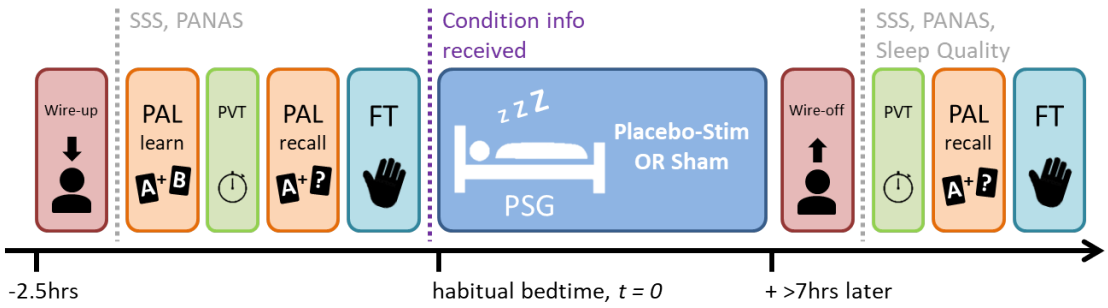

Figure 4.1: Study design. Participants underwent an identical procedure in both experimental overnights. Order of Placebo-Stim and Sham was counterbalanced between participants. PAL = paired-associate learning task; PVT = psychomotor vigilance test; FT = finger-tapping sequence task; SSS = Stanford Sleepiness Scale; PANAS = Positive and Negative Affect Schedule, PSG = polysomnography.

circadian effects. Participants were wired-off, given the opportunity to shower, offered a non-caffeinated beverage and breakfast snack, and once more completed questionnaires to assess sleepiness, affect, and subjective sleep quality scale SF-R-A (Görtelmeyer, 2011). They were once again handed the sealed envelope to report their stimulation experience. At least half an hour after waking to reduce the influence of sleep inertia, they were tested for their post-sleep declarative and procedural memory, as well as alertness.

4.3.3 Placebo induction procedure

The briefing at the beginning of the adaptation night consisted of a short 5-10 min Microsoft PowerPoint© presentation. It comprised information on general healthy sleep structure and stages, the auditory closed-loop stimulation method supposedly used in this study, an example stimulation sound (50 ms of 1/f pink noise), encouraging previous results of applying the method, and the rationale for the present study (slides & script in Appendix A. Participants were free to ask for additional information at any point, and all questions were answered in line with the alleged study purpose as if stimulation would be applied.

Participants were informed that the study's aim was to investigate whether prior awareness of stimulation affects the enhancement of subjective and objective sleep quality and associated cognitive processes. They would be randomly allocated to one of two study sub-groups, only one of which would be made aware of which night they underwent which condition. In order to keep participants from anticipating their condition of the second experimental night, they were further told that all combinations of conditions would be administered, and that they could either receive two sham nights, two stimulation nights, or one of each condition in either order. In reality, all participants received one Placebo-Stim and one Sham night. In line with learning theories, it has been demonstrated that the longer participants learn about expected outcomes, the stronger the placebo response (Colagiuri *et al.*, 2015). For this reason, the desired effects of the stimulation were re-iterated to participants through study advertising, study information sheet, and all relating verbal interaction with the experimenter. While participants were informed that the oscillation targeted with the experimental stimulation generally had functional implications for cognitive processes, it was not specified in which manner these would be affected. Similar suggestions have previously been shown to influence cognitive performance (Draganich & Erdal, 2014), and the aim of this study was to influence cognition not directly but through sleep manipulation.

The nature of interaction between experimenter and participant is thought to be crucial in eliciting a placebo response. Participants interacted with the same experimenter on all visits to enable the latter to establish a rapport of care and trust. Experimenters were briefed to treat all participants equally and to keep the depth of interactions to comparable levels between nights while fostering a warm, caring, and comfortable atmosphere, yet still maintain a professional and scientific attitude (Howe *et al.*, 2017). Experimenters wore

white lab coats to trigger notions of trust, competency, and authority (Brase & Richmond, 2004).

A single-blinded design was used in which the experimenter remained blinded to condition order until all data collection had been completed. For this purpose, the condition information was conveyed to participants in written form in a sealed and coded envelope. Participants were repeatedly reminded not to share any of the information contained in their sealed envelopes, or ask leading questions, to which all duly conformed. The aim of this procedure was to drive any potential placebo response solely on the internally held expectations of the participant, and to exclude the influence of external bias from outcomes anticipated by the experimenter.

In order to simulate stimulation setup, we attached an additional electrode at Fpz on experimental nights, and explained to participants that this was for the detection of to-be stimulated oscillations. Participants were required to wear in-ear headphones throughout all overnights. Prior to lights off on experimental nights, we played a sample stimulation sound consisting of 50 ms 1/f pink noise once headphones had been applied and asked participants to adjust the volume to a level they could still hear but would not be awakened by. It was explained that the stimulation algorithm would automatically adjust the volume accordingly throughout the night if it noticed signs of arousal or movement, or likewise detected no response in the EEG following stimulation. We opted to test for a placebo effect with a device-driven intervention rather than a pharmacological one, as the former has been demonstrated to elicit more pronounced placebo effects in wake experiments (Hróbjartsson & Gøtzsche, 2010).

4.3.4 Ethical considerations

This study involved a crucial element of deception as to its true intent. Since a major factor of the placebo effect is attributed to the participant's faith in the intervention itself and the administering professional, it presents the ethical dilemma of whether it is morally acceptable to deceive an individual about their treatment. Notably, placebo effects have occasionally been observed in non-blinded cohorts (Jakovljevic, 2014, Rogev & Pillar, 2013), however, such effects are even less understood and beyond the scope of the present study. In the specific case of this experiment, we reasoned that the aim being benevolent manipulation and arguably to the participant's own benefit justified an approach involving deception. The cohort consisted of healthy and non-vulnerable adults, and neither experimental condition was expected to cause any harm beyond potentially and temporarily increasing tiredness.

4.3.5 Memory tasks

All cognitive tasks were implemented with Matlab (Version 2016b 9.1.0.441655, MathWorks, Natick, USA), and displayed on 24-inch Asus HDMI desktop monitors in 1920x1080 screen resolution.

Declarative memory: paired-associate learning (PAL) task. In an initial encoding round, participants were instructed to memorise 120 moderately semantically related word pairs, each displayed at the centre of the screen for 3500 ms with an interstimulus interval of 1000 ms. They were informed of forthcoming recall tests. The word pairs were taken from Ngo *et al.* (2013), translated from German into English and crosschecked for difficulty between lists by a native English speaker. Different lists of word pairs were used between visits and participants, with word pair display order randomised in all sessions. In consecutive study rounds, participants were then presented with the first word of every pair and required to recall the missing second word by typing it into a keyboard within a time limit of 25 s. These study rounds included feedback and were repeated until the participant had reached a criterion of at least 60% (72 word pairs in experimental nights) correct responses in one round (Klinzing *et al.*, 2016b). Following the PVT task, participants completed one final of these cued recall rounds without feedback, the performance in which was recorded as their pre-sleep recall score. In the morning, a testing round following the same recall procedure was used to assess post-sleep performance; no feedback was provided. A reduced list of 40 word pairs was used on the adaptation night to allow participants to develop a recall strategy and reduce training effects between sessions. Absolute consolidation was measured through the numbers of correctly recalled pairs as [post-sleep score – pre-sleep score] (a positive score would indicate overnight gains, a negative losses), while relative consolidation performance was computed as [(post-sleep score – pre-sleep score) / pre-sleep score] to take into consideration evening baseline performance. The Matlab toolbox Cogent2000 (version 1.33, developed by the Cogent 2000 team at the FIL and the ICN, and Cogent Graphics developed by John Romaya at the LON at the Wellcome Department of Imaging Neuroscience, UCL, London, UK) was used to visualise this task.

Procedural memory: finger-tapping (FT) sequence task. Using the 4 fingers of their non-dominant hand, participants were asked to type a fixed 5-digit sequence presented on screen as many times and as correctly within a 30 s time interval into the keyboard as they were able to (Walker *et al.*, 2002). A moving arrow indicated their position within the sequence. In the pre-sleep sessions, participants first completed an initial 30 s practice round, followed by 12 blocks of 30 s each, interspaced with 30 s breaks. The numbers of correctly typed and overall attempted sequences were shown at the end of each block for feedback. The post-sleep session followed an identical procedure, with the only difference that 3 blocks of 30 s with a different control sequence were added at the end to demonstrate that any overnight improvement was sequence-specific. Performance was assessed as the mean of the number of correctly tapped sequences in the last and first three blocks in the pre- and post-sleep sessions, respectively. Absolute overnight performance change was calculated as [mean post-sleep sequence number – mean pre-sleep sequence number], and was additionally divided by the pre-sleep sequence number to calculate relative performance changes. The task was programmed using the Matlab extension Psychophysics Toolbox (Version 3.0.13 beta) (Brainard, 1997, Kleiner *et al.*, 2007, Pelli, 1997).

4.3.6 Control task and questionnaires

Psychomotor vigilance test (PVT). In this cued reaction time task (Dinges & Powell, 1985), participants were presented with a black fixation cross in the middle of the screen on a white background. After a random delay interval (between 2 – 10 s), the cross was replaced by an upward counting timer. Participants were instructed to stop this timer as quickly as possible upon appearance by pressing the space key. Feedback on reaction time was provided to encourage motivation. If participants did not respond within 1000 ms, an onscreen message reminded them to please pay attention. The task ran for 10 min. Performances below 100 ms (false start) and above 800 ms (attention lapse) were excluded (Basner & Dinges, 2011), and mean reaction times calculated from all remaining trials. Participants completed this task in both the pre- and post-sleep sessions immediately before the PAL recall test to measure their level of alertness and thus control for potential bias in the comparison of their recall performance between conditions. The task was programmed using the Matlab extension Psychophysics Toolbox.

Questionnaires. As part of the online screening during recruitment, participants completed questionnaires on optimism (Life Orientation Test-Revised, score range 0-24, (LOT-R) (Scheier *et al.*, 1994)), locus of control (Internal Control Index (revised), score range 28-140, (Duttweiler, 1984)), and self-esteem (Rosenberg Self-Esteem Scale, score range 10-40, (Rosenberg, 1965)). In earlier wake experiments, these personality traits were found to hold indicative value over an individual's likelihood to respond to placebos (Brockner & Swap, 1983, Horing *et al.*, 2014, Morton *et al.*, 2009). However, we did not exclusively recruit participants based on their scores on these scales. A further predictor for placebo responding in some trials (Horing *et al.*, 2014) is response expectancy (Morton *et al.*, 2010). Hence expectancy and stimulation experience pre- and post-sleep, respectively, were assessed with a brief questionnaire as part of the sealed envelope procedure (Appendix B).

During their overnight visits, participants completed standardised questionnaires assessing sleepiness (Stanford Sleepiness Scale (SSS, score range 1-7) and affect (Positive and Negative Affect Schedule, PANAS, score range 10-50 per emotional valence) in all evening and morning sessions. In post-sleep sessions, a self-assessment on sleep quality (score range 7-35) and feelings of being well rested (score range 8-40) was carried out (English translation of SF-A/R) (Görtelmeyer, 2011).

4.3.7 Data acquisition, sleep staging, and spectral analyses

PSG was recorded continuously throughout the night with an Embla® N7000^{TM} amplifier and visualised with Embla® RemLogic^{TM} PSG software version 1.1.0.2057 (both Natus Neurology Inc., Middleton, Canada). After electrode sites had been cleaned with NuPrep exfoliating gel (Weaver & Co., Aurora, USA), Ag-AgCl electrodes were attached with Grass EC2 cream (Natus Neurology Inc.) and medical tape according to the 10-20 system at standard locations F3, F4, C3, C4, P3, and P4 on the adaptation night. On experimental nights, electrodes Fpz, Fz, Cz, and Pz were added to increase topographical

coverage. We further recorded two electrooculography (EOG) traces from the left above and right below outer canthus of the eyes, electromyography (EMG) from two chin electrodes, and applied one ground electrode on the forehead. All electrodes were referenced to an average of two mastoid electrodes placed behind each ear, and all impedances were kept below 5 kΩ. Data were sampled at 200 Hz and stored offline for later data processing.

Sleep staging was conducted in SchlafAus© version 1.5.0.1 (Steffen Gais © 2005) on data filtered between 0.3-35 Hz (EEG & EOG) and 10-90 Hz (EMG). Two trained experimenters (J.S. & M.P.) visually scored sleep stages Wake, N1, N2, SWS (N3), REM, and marked arousals for each 30s epoch of sleep on a central EEG channel (C3 or C4, same per participant across nights) from lights off until the end of night according to the AASM Manual for the Scoring of Sleep and Associated Events (Iber *et al.*, 2007). Scorers worked independently and were blinded to participant number and condition. For nights where interrater agreement was below 85%, both scorings on diverging epochs were compared and an agreement reached before final sleep statistics were calculated. Sleep statistics comprise of total sleep time (TST, defined as sleep onset to final awakening minus wake), arousal frequency (% of sleep epochs marked to contain arousal over all sleep epochs), sleep efficiency (percentage of TST /(TST + wake)), percentage spent in each sleep stage over TST, as well as sleep onset latency after lights out.

In preparing for spectral analyses, we first identified electrodes with temporary sections of poor signal in the bandpass filtered EEG channels and substituted these sections with a new virtual signal interpolated through weighted linear regression incorporating all remaining clean EEG signals. We further ensured each new signal was based on a minimum of two directly neighbouring (i.e. non-diagonal) electrodes. Power spectra between 0-30 Hz were computed through Fast Fourier Transformation (FFT) with a Hanning window of 10.24 s (2048 data points) in length and 50% overlap for all 10 EEG signals. As stimulation effects may vary in their magnitude throughout the night (Bayer *et al.*, 2011) due to sleep pressure and sleep cycle dynamics (Achermann *et al.*, 1993), we divided the time window from sleep onset to last sleep epoch into four equal time segments (based on an average sleep time of ∼8 hours in our cohort, and an average adult human sleep cycle lasting up to ∼120 minutes (Carskadon & Dement, 2011)) in order to examine transient spectral power dynamics (relatable results were also found when an hourly dissection into eight ∼1hour segments was used). Analysing the power across the entire night in sleep cycles was not possible, as their number varied between participants. We identified on- and offsets of continuous sleep bouts for sleep stages of interest in each time segment in order to minimise loss of data from later tapering, and based on this information, cut the continuous data into maximum length sleep bouts for the stages of interest, on which the FFT was performed. Finally, power was summed up within prominent NREM sleep oscillatory bands 0.5-4 Hz slow wave activity (SWA), 9-12 Hz slow spindles, and 12-15 Hz fast spindles, before calculating mean scores from EEG channels per participant. Corresponding to the topographically distinct occurrence of these oscillations (Finelli *et al.*,

2001), we based our mean scores for SWA and slow spindles on (pre-) frontal electrodes (Fpz, F3, Fz, and F4), and for fast spindles on centro-parietal sites (C3, Cz, C4, P3, Pz, and P4). In line with the exploratory nature of this study, we chose to focus our attention on these power bands based on their characteristic dominance in stable NREM sleep (denoting combined SWS and N2), and their attested relevance in previous studies applying auditory closed-loop stimulation protocols (Ngo *et al.*, 2013), as well as their hypothesised involvement in overnight memory consolidation processes (Rasch & Born, 2013).

Custom-built Matlab scripts utilising the Fieldtrip toolbox (Oostenveld *et al.*, 2011) were used for all steps in EEG data analyses to treat all datasets equally. Owing to technical difficulties during recording, the EEG data from two participants were excluded from all analyses pertaining to sleep stages and spectral power.

4.3.8 Statistical analyses

IBM© SPSS© Statistics (version 25, IBM Corp, Armonk, NY, 2017) was used for all statistical analyses, unless stated otherwise. Mean ± standard error of the mean (SEM) are reported unless specifically denoted otherwise, e.g. SD for standard deviation. For memory tasks, questionnaires, and sleep stages, dependent student's t-tests and repeated-measures analyses of variance (RM ANOVA) were used to examine mean differences between the two experimental conditions. Where the assumption of normality was violated as indicated by a significant Shapiro-Wilk test, non-parametric Wilcoxon signed-rank tests for pairwise comparison were conducted instead. In cases of missing data (n = 1 in PVT, n = 1 in expectancy questionnaire), the participant in question was excluded from the respective analysis. To examine spectral results, data were $\log_{10}$ transformed to meet the assumption of normality (Field, 2013). Power across the four time segments was compared with RM ANOVAs with factors condition (Placebo-Stim & Sham) and time segment (first, second, third, & fourth) separately for each of the three spectral bands of interest (SWA, slow spindles, and fast spindles). Significant main effects of condition and interactions were followed up with dependent student's t-tests. Where necessary, a Greenhouse-Geisser correction for degrees of freedom was applied. We confirmed that the number of data trials fed into the FFT analysis did not differ significantly between conditions for each segment to eliminate this as a potential source of statistical bias (all $p \geq 0.383$, uncorrected pairwise t-tests, see Table 4.3 for trial statistics). Please note that the number of trials varies naturally between segments across the night.

Pearson's correlations were used to examine the relationship between behaviour, sleep stages, total night spectral results, and recorded predictor variables. Besides the existing predictor of expectancy, we created a new composite variable to best characterise an individual's likelihood of responding to placebos. For this purpose, the discrete scores collected in questionnaires assessing optimism, locus of control, and self-esteem were first z-scored and then combined into a 'personality' predictor through factor dimension reduction using principal component analysis.

The threshold of statistical significance was set to a *p value* < 0.05. Please note that due to the exploratory nature of this study and the number of comparisons required to examine spectral results in sufficient detail, we report all results uncorrected. These should therefore be interpreted with due caution. Where pertinent to the principal research question, effect sizes are reported as Cohen's d or η_p^2. Bayes Factor (BF) analyses were carried out in JASP (version 0.10.0, JASP Team, Amsterdam, 2018) with default prior settings (t-test: Cauchy scale = 0.707; RM ANOVA: fixed effects = 0.5, random effects = 1, covariates = 0.354, auto samples, comparison to null model) in order to evaluate support for both null and alternative hypotheses (Dienes, 2014, Rouder *et al.*, 2009, Jeffreys, 1962).

4.4 Results

4.4.1 Sleep architecture

When assessing mean differences in sleep architecture between experimental conditions, the time spent in any sleep stage in Placebo-Stim did not differ significantly from Sham (all p $\geq$ 0.197, see detailed statistics in Table 4.1), thus the experimental manipulation did not increase the proportion of time spent in deep sleep (SWS: t(19) = -1.17, p = 0.257, Cohen's d = 0.26) as intended, with Bayesian analyses however suggesting only anecdotal support for the null hypothesis (BF_{01} = 2.367, see Jeffreys (1962)). Moreover, neither sleep onset latency after lights off (t(19) = -0.09, p = 0.933), nor total sleep time (Z = -0.48, p = 0.629) showed a differential outcome after Placebo-Stim. Condition did not affect sleep efficiency (Z = -0.69, p = 0.490), or arousal occurrence during sleep (Z = -1.12, p =0.263). Thus, Placebo-Stim does not appear to have had a noticeable impact on sleep architecture when compared to Sham, but require future statistical validation.

Parameter	Placebo-Stim		Sham		p value
% Wake	6.10	± 0.83	4.88	± 0.48	^{W} 0.490
% N1	5.58	± 0.58	5.07	± 0.41	0.245
% N2	47.23	± 1.55	46.49	± 1.21	0.638
% SWS	20.55	± 1.81	21.67	± 1.71	0.257
% REM	20.54	± 0.91	21.87	± 0.78	0.197
TST, hrs	7.95	± 0.18	7.90	± 0.20	^{W} 0.629
Sleep onset latency, min	12.23	± 2.17	12.38	± 2.19	0.933
% Sleep efficiency	93.90	± 0.83	95.12	± 0.48	^{W} 0.490
% Sleep epochs with arousals	5.28	± 0.53	6.44	± 0.73	^{W} 0.263

Table 4.1: Sleep architecture remains unaffected by Placebo-Stim. Mean ± SEM and p values for dependent student's t-tests (two-tailed, uncorrected) for Placebo-Stim and Sham conditions. N1 = NREM sleep stage 1; N2 = NREM sleep stage 2; SWS = slow wave sleep; TST = total sleep time from lights off until last sleep epoch. Sleep onset latency denotes time after lights off to first sleep epoch. Sleep efficiency as percentage of time asleep over TST. ^{W} = Wilcoxon signed-rank test applied after data violated normality assumption.

4.4.2 Spectral power

In order to examine spectral power differences between experimental conditions, an RM ANOVA was conducted for each spectral band of interest (SWA, slow spindles, and fast spindles) with factors condition (Placebo-Stim & Sham) and time segment (first, second, third, & fourth quarter of the night). Significant main effects of condition and interactions were followed up with dependent student's t-tests. Bayesian analyses were additionally conducted to examine the evidence of the data in support of null and alternative hypotheses.

For SWA, the analysis revealed an expected main effect of segment ($F(3, 57) = 99.80$, $p < 0.001$, $\eta_p^2 = 0.84$), but no main effect of condition ($F(1, 19) = 0.31$, $p = 0.584$, $\eta_p^2 = 0.02$) or condition*segment interaction ($F(1.94, 36.86) = 0.54$, $p = 0.584$, $\eta_p^2 = 0.03$, see Figure 4.2A). Bayesian analyses returned a $BF_{01} = 6.284*10^{-36}$ for the segment model, $BF_{01} = 5.845$ for the condition model, and $BF_{01} = 8.438$ for the interaction model, thus suggesting substantial evidence for the null hypothesis for the condition and interaction models, but indicating decisive support in favour of the alternative over the null hypothesis for the segment model.

Examining slow spindles, we again found a significant main effect of segment ($F(3, 57) = 31.36$, $p < 0.001$, $\eta_p^2 = 0.62$) and no main effect of condition ($F(1, 19) = 1.17$, $p = 0.292$, $\eta_p^2 = 0.06$), and a significant condition*segment interaction ($F(3, 57) = 4.767$, $p = 0.005$, $\eta_p^2 = 0.20$, see Figure 4.2B). Bayesian analyses revealed a $BF_{01} = 2.552*10^{-22}$ for the segment model, $BF_{01} = 5.875$ for the condition model, and $BF_{01} = 4.448$ for the interaction model, indicating decisive support for the alternative hypothesis for the segment model, and substantial support in favour of the null hypothesis for the condition and interaction model. Consecutively conducted posthoc comparisons for each segment returned significantly higher slow spindle power in Placebo-Stim in the first quarter of the night ($t(19) = 2.13$, $p = 0.046$, Cohen's $d = 0.06$, $BF_{01} = 0.677$), followed by only numerically less power in Placebo-Stim in the second quarter ($t(19) = -2.07$, $p = 0.052$, Cohen's $d = 0.11$, $BF_{01} = 0.742$). The third quarter revealed significantly less power in Placebo-Stim ($t(19) = -2.63$, $p = 0.017$, Cohen's $d = 0.12$, $BF_{01} = 0.297$), while the last quarter showed no difference between conditions ($t(19) = 0.47$, $p = 0.646$, Cohen's $d = 0.03$, $BF_{01} = 3.901$), with the accompanying Bayes Factor indicating anecdotal evidence in support of the alternative hypothesis for the first and second segments, and substantial support in favour of the alternative hypothesis for the third, and in favour of the null hypothesis for the fourth segment. These findings suggest that the Placebo-Stim condition had a measurable and transient, but inconsistent effect on slow spindle activity.

With regard to fast spindle activity, we found a significant main effect of segment ($F(3, 57) = 8.18$, $p < 0.001$, $\eta_p^2 = 0.30$), but no main effect of condition ($F(1, 19) = 3.69$, $p = 0.070$, $\eta_p^2 = 0.16$) or condition*segment interaction ($F(2.01, 38.11) = 2.28$, $p = 0.116$, $\eta_p^2 = 0.11$, see Figure 4.2C). Bayesian analysis returned a $BF_{01} = 9.872*10^{-5}$ for the segment

model, $BF_{01} = 1.266$ for the condition model, and $BF_{01} = 2.770$ for the interaction model, suggesting anecdotal evidence in favour of the null hypothesis for the condition and interaction models, and decisive support for the alternative hypothesis for the segment model. Hence, fast spindle activity did not appear altered by the experimental placebo condition, but requires future re-examination in more highly powered inquisitions.

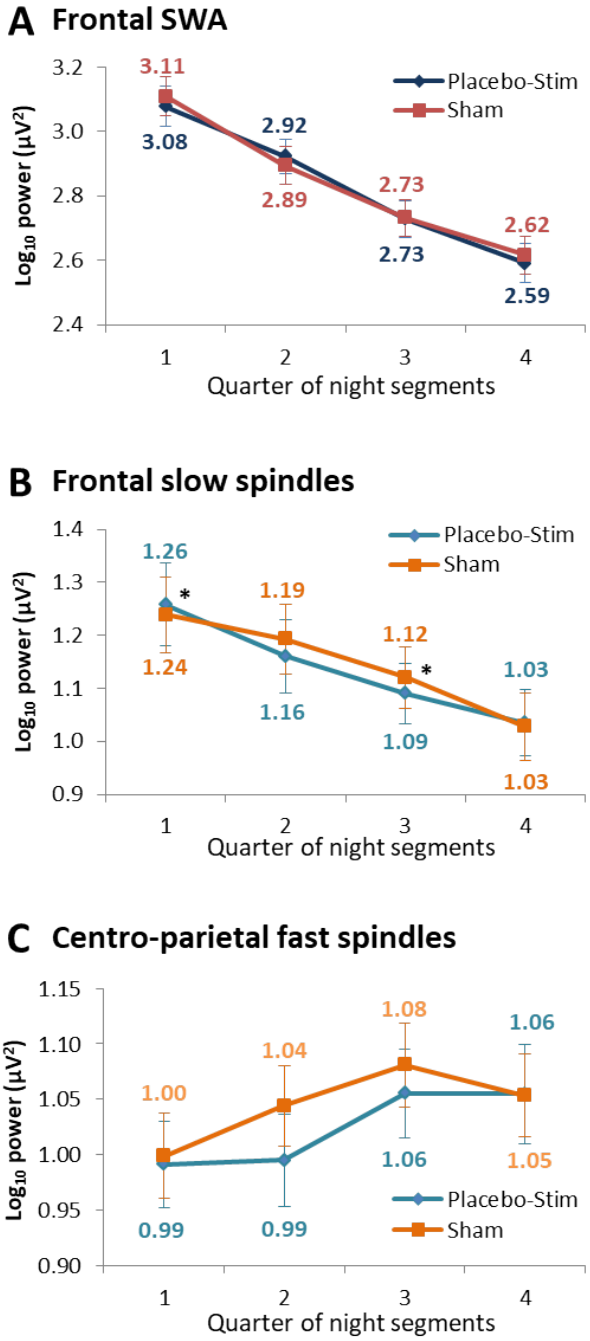

Figure 4.2: Spectral power in stable NREM sleep. Log10 transformed power values are plotted as mean ± SEM error bars. Differences in **(A)** frontal slow wave activity (SWA), **(B)** frontal slow spindles, and **(C)** centro-parietal fast spindles for quarter segments of the night between Placebo-Stim and Sham conditions. Asterisks denote $p < 0.05$ in posthoc pairwise comparisons.

4.4.3 Behaviour and its relationship to sleep stages and spectral power

Declarative memory: paired-associate learning (PAL) task

Baseline evening performance on the PAL task did not differ between conditions (Placebo-Stim: 99.77±1.85, Sham: 102.14±1.58, t(21) = -1.17, p = 0.256), nor did absolute recall performance overnight (Placebo-Stim: 0.68±0.69, Sham: 0.09±0.54, t(21) = 0.64, p = 0.528). Accounting for baseline performance in comparing relative overnight change between conditions confirmed no differential outcome (Placebo-Stim: 0.64±0.72% vs. Sham: 0.10±0.55%, t(21) = 0.56, p = 0.580, see Figure 4.3A), with Bayesian analysis indicating substantial evidence in the data to support the null effect model (BF_{01} = 3.887). Hence Placebo-Stim did not have an impact on declarative overnight memory consolidation. Further descriptive statistics of memory tasks are summarised in Table 4.2.

Procedural memory: finger-tapping (FT) sequence task

For finger-tapping performance, no difference in overnight absolute change was detected between the two conditions (Placebo-Stim: 0.93±1.31, Sham: -0.77±1.19, t(21) = 1.00, p = 0.331). A comparison of relative overnight performance incorporating baseline supported this finding (Placebo-Stim: 0.25±0.22% vs. Sham: 0.01±0.09%, t(21) = 01.08, p = 0.291, positive values represent overnight gain, see Figure 4.3B), however, Bayesian analysis suggests our data only provide anecdotal evidence in favour of the null hypothesis (BF_{01} = 2.664). Participants did not significantly differ at baseline (t(21) = 0.41, p = 0.689). Thus, while our experimental intervention appeared to not have affected procedural memory overnight, our data do not lend themselves to any definite conclusion.

Correlations between behaviour, sleep stages, and spectral power

In the Placebo-Stim condition, the relative overnight change in recalled word pairs on the paired-associate learning task negatively correlated with frontal slow spindle power across the total night (r = -0.51, p = 0.021). Further, relative overnight change on the finger-tapping task negatively correlated with the percentage of REM sleep obtained in the Placebo-Stim condition (r = -0.47, p = 0.035). No other significant associations were found between behavioural performance on the memory tasks and sleep stages or spectral power in either condition (all p ≥ 0.087, uncorrected).

Item	**Placebo-Stim**				**Sham**			
	Evening		Morning		Evening		Morning	
PAL	99.77	±1.85	100.45	±2.07	102.14	±1.58	102.23	±1.66
FT	16.12	±1.30	17.05	±1.61	15.77	±1.23	15.00	±1.48
PVT	337.74	±10.68	326.27	±11.91	340.11	±12.48	322.91	±9.25

Table 4.2: Summary of memory and control tasks. Absolute mean ±SEM are presented across both experimental conditions for pre- and post-sleep testing sessions for the paired-associate learning task (PAL, number of word pairs correctly recalled), procedural finger-tapping sequence task (FT, number of correctly tapped sequences), and psychomotor vigilance test (PVT, reaction time in ms).

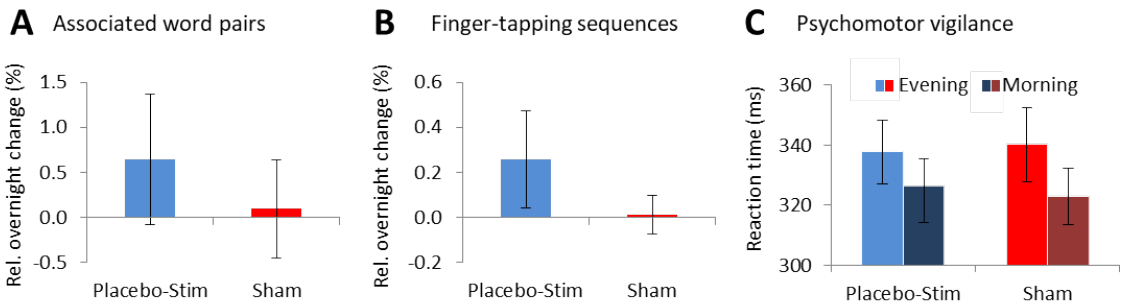

Figure 4.3: Experimental conditions do not have a differential impact on memory performance and alertness. Values represent mean ± SEM error bars. Graphs A and B show overnight change in relation to baseline evening performance; positive values represent overnight gain. **(A)** No significant difference between conditions was found for the relative numbers of correctly recalled word pairs on the paired-associate learning (PAL) task. **(B)** The mean number of correctly tapped sequences in the finger-tapping (FT) sequence task was comparable between Placebo-Stim and Sham. **(C)** Overnight changes in alertness were not affected differently between conditions and sessions as measures by mean reaction times (ms) on the psychomotor vigilance test (PVT).

4.4.4 Control task and questionnaires

Psychomotor vigilance test (PVT)

We assessed alertness prior to PAL recall sessions as a potential influential factor for memory performance. Neither evening baseline ($Z = -0.02$, $p = 0.986$), nor relative overnight change ($Z = -0.26$, $p = 0.794$, see Figure 4.3C), or morning performance levels ($Z = -0.12$, $p = 0.903$) varied between conditions, suggesting alertness levels would not have influencing performance differentially between sessions.

Sleepiness, subjective sleep quality, & feelings of restedness

Participants completed the Stanford Sleepiness Scale (SSS) at the beginning of every cognitive testing session pre- and post-sleep. Results revealed that baseline pre-sleep scores did not differ between conditions (Placebo-Stim: 3.05±0.18 vs. Sham: 3.59±0.31, $Z = -1.50$, $p = 0.135$), nor did post-sleep scores (Placebo-Stim: 2.14±0.15 vs. Sham: 2.32±0.18, $Z = -1.27$, $p = 0.206$). Across both experimental nights, participants felt significantly less sleepy in the morning compared to evening (Placebo-Stim: $Z = -2.92$, $p < 0.005$; and Sham: $Z = -3.67$, $p < 0.001$), as expected. The degree to which sleepiness decreased overnight did not differ between conditions (Placebo-Stim: 0.91±0.24 vs. Sham: 1.27±0.24, $Z = -0.97$, $p = 0.330$).

The absence of statistical differences in sleepiness was mirrored in responses gathered with the SF-R-A subjective sleep quality questionnaire, which assessed subjective sleep quality as well as feelings of restedness. Completed each morning, there was no difference in the ratings for subjective sleep quality (Placebo-Stim: 28.55±0.93 vs. Sham: 30.32±0.75, $t(21) = -1.44$, $p = 0.165$), or feelings of being well rested (Placebo-Stim: 28.56±0.93 vs. Sham: 28.48±0.60, $t(21) = 0.084$, $p = 0.934$) between conditions. Participants hence felt equally rested, and did not perceive their subjective sleep quality any differently between experimental conditions.

Affect

Emotional valence was assessed pre- and post-sleep with the PANAS questionnaire. There was no baseline evening difference between conditions for either emotional valence (positive: Placebo-Stim: 28.73±1.35, Sham: 27.05±1.79, Z = -1.26, p = 0.207; negative: Placebo-Stim: 10.41±0.14, Sham: 10.36±0.22, Z = -.30, p = 0.763). Overnight change between conditions was not significantly different for positive (Placebo-Stim: -2.36±1.27, Sham: -3.36±1.06, Z = -0.45, p = 0.653) or negative (Placebo-Stim: 0.05±0.12, Sham: 0.14±0.24, Z = -0.05, p = 0.957) affect.

Predictor questionnaires

High optimism, low locus of control, and low self-esteem have previously been credited with predictive value for an individual's increased likelihood of responding to placebos. Our cohort exhibited a mean score of 18.65±0.45 (range 15-23 of 0-24 possible) for optimism, 103.70±1.94 (range 82-118 of 28-140 possible) for locus of control, and 31.95±0.96 (25-40 of 10-40 possible) for self-esteem. Thus, our participants were generally situated towards the higher ends of the optimism, self-esteem, and locus of control spectra. The new z-scored personality predictor score had a SEM of ±0.22, ranging from -2.00 to 2.00. Personality predictor score did not correlate with any of the observed transient power differences between conditions (all $p \geq 0.385$). Thus, contrary to evidence from previous wake experiments, these measures do not appear to hold value for predicting placebo responding in sleep.

Prior to lights off, participants were asked what type of impact they expected the forthcoming condition to have on their imminent night's sleep. On the Placebo-Stim night, 12 participants had positive, six negative expectations of the stimulation, and four did not anticipate any changes, whereas on the Sham night, only one participant reported positive expectations, with the remainder of the cohort anticipating no impact (response from 1 participant missing). Interestingly, two participants of the total 22 reported having heard stimulation sounds in their sleep following the Placebo-Stim night. Whether this was the result of a true placebo effect or perhaps related to dream content is unknown, however none of the dream accounts collected as part of the sleep quality questionnaire featured the stimulation. No reports of hearing stimuli during sleep were made following the Sham overnight. The expectancy scores did not correlate with power measures in either condition, nor with the observed significant power differences between them (all $p \geq 0.170$), and thus proved to be of little predictive value for placebo/nocebo effects during sleep.

4.5 Discussion

In the present study, brain stimulation during sleep was simulated to explore whether healthy sleepers would be able to self-enhance aspects of NREM sleep by anticipating these effects from experimental intervention. We also examined whether such outcome would translate into improved performance on sleep-dependent memory tasks. Our find-

ings would suggest that it is not possible to induce consistent placebo effects in healthy sleepers by using the described method. On the contrary, anticipation of placebo stimulation led to placebo and nocebo effects in the form of transient in- and decreases in slow spindle power in stable NREM sleep across the night. However, sleep architecture, overnight memory consolidation and subjective perceptions of sleep quality, sleepiness, and affect were not affected. Thus, anticipation of experimental brain stimulation during sleep appears to have affected selected aspects of NREM sleep, however, existing statistical limitations require the present results to be validated experimentally before any definite conclusions are drawn.

Spindles hallmark stable NREM sleep and are thought to be vital for sleep-dependent cognitive processes, such as overnight consolidation of declarative and procedural memory (Diekelmann *et al.*, 2009, Fogel *et al.*, 2015, Rasch & Born, 2013). The observed, temporary power in- and decreases in slow spindles through experimental manipulation in the present study suggest placebo stimulation may have affected the quality of stable NREM sleep at certain time points across the night. Previous research has demonstrated that spindle oscillations possess sleep-maintaining properties, with external sound processing found to be highly limited during spindle activity (Dang-vu *et al.*, 2011, Schabus *et al.*, 2012). In this light, we speculate whether the sleeping brain may have first increased and later reduced these frequencies in anticipation of auditory stimulation during NREM sleep. This could be considered a precautionary and natural behavioural response in an anticipatory state of uncertainty, i.e. with no prior personal experience of stimulation.

One potential explanation regarding this anticipatory state of uncertainty concerns the situational novelty of the experimental stimulation. It is possible that any changes caused by placebo stimulation would only be present in the first night in which an individual anticipates the stimulation, after which situational habituation would set in. In this respect, the observed effects could be similar to the recognised 'first night effect', which describes impaired sleep quality in otherwise healthy sleepers on their first night spent in a new environment (Agnew *et al.*, 1966, Toussaint *et al.*, 1995). It is therefore conceivable that sleep could be altered by placebo or actual stimulation when experienced or anticipated for the first time. At present, experimental sleep research has yet to determine whether stimulation during sleep yields comparable outcomes between the first and a later application time, as initial research suggests (Debellemaniere *et al.*, 2018).

The experimental impact on spindles was not consistent throughout the night, as is evident by the initial slow spindle increase during the first quarter of the night followed by later decreases in this spindle band. Nor were any induced changes of sufficient magnitude to have an impact on sleep-dependent cognition, although correlational analyses suggest higher slow spindle power reduced declarative overnight consolidation, and a larger percentage of time spent in REM sleep was indicative of less procedural consolidation across the night. In part, the observed transient changes could stem from an interaction of in-

duced effects with sleep pressure, which is strongest at the beginning of the night and dissipates towards the morning hours. It should be noted that a human sleep cycle lasts approximately 90-120 min (Carskadon & Dement, 2011), and is dominated by SWS in the first half of the night and N2 and REM sleep in the latter half. Therefore, not all time segments will contain comparable amounts of stable NREM sleep between them, particularly amounts of respective N2 and SWS sleep stages, which could have influenced our results. Furthermore, healthy human nocturnal sleep comprises of a number of brief awakenings following each sleep cycle. Nocturnal awakenings could potentially be associated with temporarily increased situational awareness. We presently cannot exclude the possibility that these waking occurrences were temporally linked to the timing of power differences between conditions. As the exact function of slow spindles in the sleeping brain is presently still unclear, the initial placebo result of increased slow spindles is difficult to interpret, but could speculatively relate to the anticipatory state induced in participants immediately prior to bedtime, and associated cognitive processes of consciousness and expectancy.

The absence of the intended overall sleep-enhancing placebo effect in the present study begs the question whether obtaining such outcomes more constantly in healthy sleepers is realistically possible, contrary to our present findings. While slow spindle activity was found to be temporally enhanced in the first quarter of the night, this effect showed reversal in power decreases in the third quarter. The circumstances of the intended enhancement may prove to be crucial; for instance, prior first-hand experience of the effects to be attained may be necessary in order to facilitate the ability to self-induce particular outcomes. Our cohort had no self-reported knowledge of sleep oscillations, nor personal experience of auditory closed-loop stimulation. Finally, it is probable that a more suggestible cohort would have fared differently in this experiment. Our findings add to Cordi *et al.* (2014), who previously described no sleep enhancement after overt psychological induction of sleep improvements in a low suggestible group of healthy sleepers.

Our findings do not alleviate the concern of placebo/nocebo effects introducing bias in studies applying (auditory) stimulation during sleep. On the contrary, the present results suggest that anticipating auditory stimulation during sleep may entail inconsistent temporary in- and decreases in slow spindles. However, a number of limitations require consideration. Firstly, we chose to report our findings uncorrected due to the exploratory nature of this study. Therefore, any results need to be interpreted cautiously with a chance of type I errors in mind and are required to be validated by further research before any definite conclusions on the subject matter are drawn. Based on the present results it is conceivable that the placebo stimulation may have had an impact on sleep in our placebo stimulation condition, however, Bayesian analyses suggest some of the evidence presented by our data to only provide anecdotal support. Therefore, more highly powered future re-examinations are required. Secondly, future research could likewise address anticipation-mediated effects on other sleep oscillations and stages, e.g. theta and REM sleep, for a more conclusive picture. Lastly, while we have attempted to characterise the

difference between placebo stimulation and baseline, comparing the previous two conditions to actual stimulation will help determine the relative scale of anticipation-induced power changes. Adding this additional condition was beyond the scope of, and the resources available for the present study, and requires future investigation.

In sum, we conclude that induction of consistent placebo effects does not appear to have been possible by simulating auditory closed-loop stimulation. Contrary to our intentions, the experimental manipulation resulted in transient placebo and nocebo outcomes in slow spindle power. We suggest anticipation of auditory brain stimulation during sleep may have a positive and/or negative impact on oscillatory activity in stable NREM sleep. Until more details about the underlying psychophysiological mechanisms of such effects emerge and our findings are validated, placebo and nocebo effects remain a possible factor in sleep studies involving (auditory) stimulation protocols. In the light of the present findings, they should be given adequate consideration in methodological design to avoid unnecessary bias in future investigations.

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4.6 Supplementary information

NREM	**Placebo-Stim**		**Sham**		**p value**
	mean	±SEM	mean	±SEM	
Total Night	3792.30	±93.23	3727.90	±114.04	0.474
Seg. 1	1167.10	±49.44	1159.05	±43.70	0.833
Seg. 2	974.65	±35.44	956.70	±35.61	0.647
Seg. 3	889.50	±40.22	885.90	±38.76	0.941
Seg. 4	758.00	±35.59	722.75	±41.14	0.383

Table 4.3: Number of FFT trials per segment for power analysis. Absolute mean±SEM of data trials included in FFT analyses are presented across both experimental conditions. The four segments denote macro time bins of ∼120 min length across the total night. Uncorrected, pairwise t-tests confirm that the number of data trials did not differ in any segment between conditions and thereby preclude this as a potential source of statistical bias.

Chapter 5

General Discussion

The principal objective for conducting the research presented in this thesis was to further explore the potential of auditory closed-loop stimulation as a method to enhance oscillatory features of sleep and thereby improve associated cognitive functions, such as sleep-dependent memory consolidation. Auditory closed-loop stimulation is a relatively novel brain stimulation technique. Over the past few years, it has gained considerable popularity in sleep research for being easily administrable and controllable, yet non-invasive. The application possibilities of this technique are manifold and present many exciting opportunities for filling gaps in the existing literature on the neural dynamics and functional purpose of specific brain oscillations during sleep.

To date, studies applying this technique in human participants focussed mostly on modulating SOs and spindles in NREM sleep. A consensus emerging from these suggests that on an oscillatory level, when stimuli were applied approximately in phase with SO upstates, subsequent SWA and phase-coupled fast spindles can be enhanced. On a functional level, boosting these oscillations has been shown to facilitate improved performance on behavioural tasks assessing overnight memory processes, as well as SWS-related immune and autonomic nervous system functions. While stimulation has been attempted in individuals of advanced age, whether it is as effective as in a young adult brain remained unclear. Such information is needed for further optimising and developing the technique for possible clinical application to combat age-related decline in sleep and associated cognition. Similarly, the extent to which spindle frequencies could be purposefully and differentially modulated by auditory closed-loop stimulation had not yet been addressed. Equally unexplored was the psychological component of receiving auditory closed-loop stimulation during sleep, and whether such could introduce bias to measured outcomes. These open questions in the literature inspired and were addressed by the experiments presented in this thesis. In investigating these, we drew on methodological combinations of behavioural measures, PSG, and variations of auditory closed-loop stimulation protocols.

In this final chapter, I will begin by summarising key findings emerging from the experiments presented in this thesis. Next, I will attempt to integrate results, both in terms of oscillatory and functional outcomes of stimulation in the young and older brain, and with regard to psychological factors pertaining to the application of the technique.

Throughout, I will reflect on limitations, highlight open questions, and suggest possible future research directives to address these.

5.1 Summary of findings

In Chapter 2, we examined in the first instance whether auditory closed-loop stimulation could successfully be applied in a population of healthy, late middle-aged adults. This was of central interest in respect of the known decline in sleep quality and impaired memory functioning observed in older age (Gui *et al.*, 2017, Mander *et al.*, 2017). Secondly, we aimed to compare and contrast the impact of stimulation in this older group to a young adult cohort. We here took advantage of a previously published seminal study in such a group, which had used an identical stimulation setup (Ngo *et al.*, 2013). Our analyses revealed that while the ageing brain clearly responded to the stimulation, as evidenced by prolonged SO trains and induced phase-locked fast spindles, these oscillatory responses were markedly reduced in comparison to those measured in young adults. Moreover, older adults showed impaired overnight declarative memory retention, but no effect on procedural memory consolidation or post-sleep encoding abilities after stimulation compared to a baseline night. These findings suggest that the susceptibility to auditory closed-loop stimulation is altered in the ageing brain, and changed neural dynamics result in differential and less favourable behavioural outcomes.

In Chapter 3, we contemplated the potential of auditory closed-loop stimulation to differentially modulate slow and fast spindle activity. Both spindle types show distinct temporal and topographical properties, but whether they serve different functional purposes remains unknown (Cox *et al.*, 2017, Mölle *et al.*, 2011). Fast spindles have been ascribed a vital role in facilitating the consolidation of sleep-dependent declarative and procedural memories, but there is little information on the function of slow spindles. In this context, we aimed to trial an auditory closed-loop protocol to disproportionately enhance slow spindle activity, with the hope that this would encourage future investigations on behavioural implications of this waveform. We found that a 4-click protocol indeed enhanced slow spindle activity to a greater extent than fast spindles. Our analyses further revealed higher incidence rates and shorter inter-spindle intervals in slow spindles, suggesting slow and fast sleep spindles may underlie different neural dynamics in their generation. Auditory closed-loop stimulation will thus be useful as investigative, non-invasive tool to probe possible functional differences between the two spindle types in future.

Lastly, in Chapter 4, we considered the psychological impact associated with receiving auditory closed-loop stimulation during sleep. Past research suggests that insomniacs could self-enhance aspects of their sleep by expecting such effects from outside intervention (Rogev & Pillar, 2013, Winkler *et al.*, 2015), but this possibility had not yet been examined in healthy sleepers. By instilling anticipation of benevolent brain stimulation prior to sleep as part of a placebo protocol in healthy young adults, we investigated whether this psychological manipulation would lead to any measurable changes in sleep architecture,

slow wave and spindle activity, as well as declarative and procedural overnight memory consolidation. While cognitive performance, sleep architecture, and frontal slow wave and centro-parietal fast spindle activity remained unaltered after a placebo stimulation night compared to sham, frontal slow spindle power appeared to be inconsistently altered across different time intervals in the placebo night. An initial increase in slow spindle power in the first quarter of the night was found, followed by a decrease in the third quarter in slow spindles. Thus, contrary to our intention of eliciting a consistent placebo effect, our experimental protocol resulted in inconsistent and transient placebo and nocebo outcomes. Our findings suggest that anticipating auditory closed-loop stimulation may entail consequences for selected sleep oscillations, which, once experimentally validated due to statistical limitations, will require careful consideration in future studies.

5.2 Auditory closed-loop stimulation and SWA

Across the growing number of studies which have used the technique (see Table 1.1), auditory closed-loop stimulation has been shown to induce oscillatory responses in the slow frequency band. This effect is based on inherent sound-processing mechanisms of the brain, which are aimed at maintaining sleep in the face of non-salient or non-threatening stimuli (Jahnke *et al.*, 2012, Velluti, 2008). With varying slow frequency bands analysed in studies, 0.5-1 Hz SO, 1–4 Hz delta, and 0.5-4 Hz SWA enhancements have previously been reported to occur immediately in the form of enhanced amplitudes following acute stimulation. Since the brain in deep SWS demonstrates a strong tendency to engage in an alternating de- and hyperpolarisation pattern due to underlying neuronal bistability (Steriade *et al.*, 1993), stimulation thus led to prolonged slow oscillatory trains (Ngo *et al.*, 2015). Anecdotally, auditory closed-loop stimulation targeting SO activity results in more prolonged SO trains in SWS than N2 sleep due to the bistable state of neurons in the former. Yet, whether stimulation in these two sleep stages differs with respect to physiological or functional outcomes still requires experimental investigation. In Chapter 3, we found no difference between evoked responses in SWS and N2, but this statistical insignificance may be attributable to the small sub-sample size in the respective analysis, and should therefore not form the basis of any conclusions on this matter.

Responses evoked by multi-click stimulation protocols in our experiments in Chapter 2 (2-click stimulation contrasted to sham) and Chapter 3 (4-click stimulation protocol compared to 1-click) exhibit this effect of prolonging SO trains. Similarly to Ngo *et al.* (2015) in their driving stimulation paradigm, we observed that the momentum in terms of amplitudinal enhancement gradually decreases with each successive stimulus across a trial when multiple sounds were administered. This suggests a limiting factor may exist for any SO enhancement by auditory closed-loop stimulation. However, unpublished data from our lab (Debellemanière et al., in preparation) show the bistable momentum can be maintained by stimulation for up to ten consecutive SOs in healthy young adults. Moreover, another study appears to have previously employed a longer multi-click protocol which enhanced SWA in middle-aged adults, but unfortunately provides insufficient information

on the average number of stimuli applied consecutively (Garcia-Molina *et al.*, 2018). Notably, in our late middle-aged cohort, the stimulation-induced momentum was lost more quickly across consecutive SOs, but this can partly be ascribed to the previously discussed general decrease in SO amplitudes in older age and its cellular causes (Carrier *et al.*, 2011). Papalambros *et al.* (2017) show a similar gradual decline in evoked responses in their cohort of healthy, elderly individuals, which received stimulation on five consecutive SOs. The extent to which slow oscillatory activity can be enhanced and what factors determine how the momentum of such enhancement is best maintained remains to be investigated. It is theoretically imaginable that a stimulation 'sweet spot' exists on SOs, which could be temporally more limited than the broader SO up-state where more detailed auditory processing seems supported (Batterink *et al.*, 2016, Schabus *et al.*, 2012). For example, administered stimuli could elicit larger responses if applied just in time when the ongoing SO has gained a certain momentum, but prior to the onset of any phase-coupled fast spindle, which are thought to partially block sound processing (Dang-Vu *et al.*, 2010a), or other activity. Post-hoc analyses currently underway in our lab on data pooled from different auditory closed-loop stimulation experiments are investigating this question of a 'sweet spot' and attempting to determine the factors which predict a most favourable stimulation outcome based on the morphology of the initial endogenous SO which triggered stimulation. Determining the possible existence of such a window will hopefully also shed some clarity on whether phase-monitoring algorithms are to be preferred over those working with fixed-delays after threshold detection, as the latter are less likely to purposefully hit an exact 'sweet spot' in phase due to the natural variability of SO characteristics within sleep bouts and across sleep cycles (Halász *et al.*, 2014). Moreover, whether this window would vary in young and older adults is of central interest, and if so may help explain the reduced impact of stimulation in the ageing brain detailed in Chapter 2.

Interestingly, acute SWA increases during stimulation trials do not necessarily translate into similar increases across the total stimulation period or night, as was the case in our older adult group in Chapter 2. In such cases, it may be assumed that experimentally induced increases are subsequently followed by endogenously regulated decreases in the previously enhanced power band(s) during stimulation-free intervals, which either diminishes or nullifies any net enhancements across the total night statistically. It is unclear whether driving stimulation harder, e.g. by opting to decrease the detection threshold to allow smaller endogenous waves to trigger stimulation, decreasing inter-trial pauses in algorithms, or stimulating throughout the entire night, would make a difference in this respect. It is notable that despite stimulating smaller slow waves throughout the night, Papalambros *et al.* (2017) still found no overall SWA enhancement in older adults. However, such overnight enhancement has previously been reported in young participants (Santiago *et al.*, 2019), thus whether the trade-off effect only exists in an older brain or depends on specific stimulation parameters is unclear.

A possible inherent physiological mechanism which limits SWA in the brain appears plau-

sible in the light of the principles of balance proposed by SHY. Likewise, it raises the question of whether enhancing solely SWA is an actually desirable aim when applying auditory closed-loop stimulation. SHY posits that the primary purpose of SWA is to downscale synaptic connections which were previously subjected to potentiation during wake in order to maintain a meaningful signal-to-noise ratio and avoid oversaturation over time (Tononi & Cirelli, 2003, 2014). Exclusively increasing SWA experimentally could disproportionately affect other frequencies which ordinarily co-occur, as these may underlie different refractory mechanisms, as is the case for fast spindles (Antony *et al.*, 2018, Ngo *et al.*, 2015). According to the active system consolidation hypothesis, phase-coupled activity between SOs and fast spindles is needed to support memory consolidation processes in the sleeping brain (Diekelmann *et al.*, 2009, Latchoumane *et al.*, 2017). Thus, a disproportional frequency ratio alteration could arguably result in increased down-scaling and weaken memory traces. Such unintended consequences could partially help explain why stimulation in our late middle-aged cohort in Chapter 2, in which fast spindle activity increases did not co-occur together with enhanced SOs, did not result in enhanced overnight memory consolidation. In future investigations, auditory closed-loop stimulation could be used to investigate the differential functional implications of enhancing SWA singularly or concurrently boosting cross-frequency coupled activity. Amending stimulation algorithms to not only take into account endogenous activity of SOs, but also spindles and other frequencies of interest to contrast the functional implications of enhancing SWA exclusively by stimulating during or outside of refractory periods of associated frequencies, may help provide answers to this question in future.

5.3 Auditory closed-loop stimulation and sleep spindles

Targeting SOs with auditory closed-loop stimulation has previously been shown to enhance fast spindles phase-locked to the up-state of one immediately following SO only (Grimaldi *et al.*, 2019, Leminen *et al.*, 2017, Ngo *et al.*, 2015, 2013, Ong *et al.*, 2016, Santostasi *et al.*, 2015). Consecutively applied stimuli no longer elicit fast spindles over a certain consecutive time period of a few seconds due to refractoriness of spindle-expressing thalamo-cortical networks (Antony *et al.*, 2018, Ngo *et al.*, 2015). In Chapter 2, we found a similar, but prolonged refractory period in our late middle-aged cohort in both stimulation and sham conditions in comparison to a young adult population. This suggests that the dynamics governing thalamo-cortical networks are altered in the ageing brain, where more time for recovery is needed following spindle expression, an observation which is supported by past research noting lower spindle density in older age (Nicolas *et al.*, 2001), and speedier recuperation cannot be tempted by stimulation. Likely reasons for this change in spindle activity in older age could relate to disrupted temporal dynamics at generation, or underlying age-related physiological and cellular constraints (Clawson *et al.*, 2016). Interesting and a little counter-intuitive in this context is that auditory closed-loop stimulation in our older adults in Chapter 2 led to a fast spindle enhancement across the ∼3.5-hour stimulation period. While our older adults did not exhibit the previously described fast spindle de-coupling effect with SOs in old age (Helfrich *et al.*, 2017), stimulation did not enhance

such coupling either as it did in the younger group. We can therefore only speculate that stimulation in combination with an already naturally prolonged refractory period in the older brain led to a re-bound expression of fast spindles during stimulation-free intervals. Such temporal dynamics should be further examined in future studies, as they pose a defining limiting factor to any possible experimental enhancement of fast sleep spindles in the ageing brain.

While Chapter 2 focussed on fast spindles, in Chapter 3 we turned our attention to the difference between slow (9-12 Hz) and fast (12-15 Hz) spindle subtypes. To our knowledge, this was the first study to use varied protocols of auditory closed-loop stimulation to differentially modulate slow and fast spindle activity. Prior experiments have attempted to manipulate fast spindles by either using auditory stimuli mimicking their peak frequency (Ngo *et al.*, 2018), or administering stimulation immediately upon detecting an endogenous fast spindle (Antony *et al.*, 2018, Choi *et al.*, 2018), but none of these approaches successfully enhanced fast spindle activity acutely as intended. Slow spindles are commonly ignored or neglected in present day sleep research, which is due to the uncertainty about their functional purpose (Cox *et al.*, 2017). We demonstrated in Chapter 3 that the likelihood of slow spindles occurring can be disproportionally enhanced by employing a multi-click protocol which exploits the nature of fast spindle refractoriness, adding further evidence to the clear distinction in temporal dynamics between these two adjacent power bands. It is hoped that this experiment will inspire future investigation to use non-invasive manipulation techniques such as the method trialled in our study to probe into the functional implications of such disproportionate enhancement with respect to overnight memory consolidation or further sleep-dependent cognitive processes. Based on their temporal occurrence shortly before the SO down-state and following on from fast spindles, slow spindles have been suggested to reflect cortico-cortical interactions (Mölle *et al.*, 2011). If true, such an interaction could speculatively point towards a role in cross-linking information to integrate information long-term into existing networks (Astori *et al.*, 2013). The pre-frontal cortex, where slow spindles occur most prominently, has previously been linked to partake in memory processes by exerting cognitive control through selection, monitoring, and inhibition during wake (Simons & Spiers, 2003). Whether this is also the case during sleep, where conscious, executive functions are not thought to be available, is unknown but an interesting preposition, particularly in the light of lacking clarity on how memories considered worthy of preservation are tagged during wake and later selected for consolidation during sleep. Experimentally, this hypothesis could be tested by adding a behavioural task to the study described in Chapter 3 to assess consolidation and integration of novel memories longitudinally. By utilising a closed-loop TMR protocol similar to Göldi *et al.* (2017) and replacing the first stimulus in our 4-click protocol with a brief, semantically meaningful stimulus, a specific memory could be reactivated. Long-term tracking could then establish whether a memory will be more successfully recalled across time in comparison to a trace reactivated in a 1-click trial with lower chances of consecutive slow spindle activity for example.

Beyond the respective relationship of slow or fast spindle activity to SO phase, an additional factor which has been often ignored to date concerns the nature of the temporal interrelationship between these two waveforms. Mölle *et al.* (2011) describe how slow and fast spindle events correlate, with slow spindles following their faster counterpart. However, it is unclear whether this co-occurrence with a small temporal lag is necessary for a certain functional purpose, and whether solitary events of either waveform would have a different effect. Studies which experimentally and exclusively boosted fast spindles showed consequent behavioural improvements (Ketz *et al.*, 2018, Ladenbauer *et al.*, 2017). However, it is possible that by not considering slow spindles at all or separately in their analyses, interrelations between the two spindle types may have been missed as a result. Casting a closer look at this temporal interrelationship, either through directly manipulating their separate occurrence experimentally, or post-hoc examination of trials during which specific memories were reactivated with TMR, could help elucidate this question.

Finally in Chapter 4, we discovered that anticipating auditory closed-loop stimulation during sleep could have an impact on spindle activity, with slow spindles found initially in- and later decreased across the night. Interestingly, these changes occurred in the absence of any apparent SWA alterations. We assume these spindle effects relate to experimental manipulation and associated cognitive processes of anticipation or environmental factors, such as participants experiencing the stimulation for the first time, as they are unlikely to be natural and endogenous fluctuations due to the counter-balanced design. However, statistical limitations as well as the lack of an actual stimulation condition make validation of these experimental findings a future necessity.

5.4 Using auditory closed-loop stimulation to influence behaviour

From the summary of previous studies presented in Table 1.1 of this thesis, it is evident that the large majority successfully enhanced behavioural performance by administering auditory closed-loop stimulation. Despite testing for different memory domains, experiments in which stimulation was beneficial for declarative, procedural, working, and recognition memory, as well as word and category fluency have been presented (Choi *et al.*, 2018, Diep *et al.*, 2018, Göldi *et al.*, 2017, Leminen *et al.*, 2017, Ngo *et al.*, 2013, Ong *et al.*, 2016, Papalambros *et al.*, 2017, Shimizu *et al.*, 2018). In this light, it is surprising that applying the stimulation in our late middle-aged cohort in Chapter 2 did not support overnight consolidation of declarative or procedural memory, nor improve encoding abilities post-sleep, but instead impaired relative consolidation of word pair memory. This is particularly the case since Papalambros *et al.* (2017) previously demonstrated such a beneficial effect in an older cohort, albeit with a different phase-locked stimulation protocol. This contradiction in findings highlights a few important points. Firstly, simply boosting SWA may not be sufficient to yield a behavioural effect, as previously discussed. A more detailed under-

standing of the intricate interplay between different co-occurring frequencies is needed to explain such diverging results. Secondly, as noted, the fact that our stimulation impaired overnight declarative memory in older individuals, but enhanced it in the young adult cohort emphasises the challenge of translating a functional auditory closed-loop protocol to a different age group. Future work should clarify whether optimising specific parameters or settings of the stimulation would help achieve a beneficial behavioural effect in the ageing brain. Thirdly, it is conceivable that in order to affect behaviour, any experimental alteration of oscillatory patterns must be more profound and may have been countered by the aforementioned possible trade-off effect, which diminished induced SWA increases by consecutive endogenous decreases. Similarly, boosting spindles without concurrent SWA enhancements may not lead to desired functional outcomes. The latter explanation of comprehensive enhancements being required would also be supported by our findings in Chapter 4, where a placebo stimulation protocol impacted inconsistently on spectral measures within time intervals throughout the night, but did not affect total night power of these frequency bands (besides a minor trend in decreased fast spindles), nor declarative or procedural overnight memory. Notwithstanding, alternative explanations for this effect, such as the sample tested in Chapter 4 performing too close to ceiling (as was the case on the declarative word-pair memory task, with an average of approximately 100 out of 120 word pairs recalled correctly in the pre-sleep testing session), are viable.

A wealth of research evidences an apparent direct relationship between specific sleep parameters and overnight memory consolidation processes to date. Yet it is vital to remark at this point that a recent study drawing on a sample of 900+ healthy, young adult participants found not a single correlation between any of the assessed sleep parameters (% time spent in SWS or REM; SWA and theta activity; spindle density) and performance on a commonly used episodic memory task (Ackermann *et al.*, 2015), mirroring the relative lack of correlations between behaviour and spectral results found in Chapter 4. The authors stress that their insights do not necessarily question the importance of sleep for memory processes, but instead explain how the view of a positive association between these variables which prevails in the field could be based on a combination of a large number of inadequately powered studies with small samples sizes, overestimation of true effects, and lack of adequate statistical analysis, such as transparently correcting for multiple comparison. When paired with an academic climate in which it is easier for researchers to publish positive over negative or null findings (Joober *et al.*, 2012), a certain degree of unintended misrepresentation of the real causal relationships between sleep oscillations and memory seems probable. Modern approaches aimed at combatting publication bias, such as open science and preregistration, and a combination of frequentist and Bayesian analyses may help prevent such bias in future and help to clarify the exact role of sleep for the overnight consolidation of different types of memory. It has also been suggested that to fully appreciate the details which determine successful consolidation, a bigger picture beyond a few single sleep measures is desperately needed (Mantua, 2018), urging researchers to explore their data in greater detail and describe it with a range of measures in lieu of focussing

on a few selective metrics.

5.5 Auditory closed-loop stimulation in the ageing brain

With increasing age, changes in sleep physiology become apparent. Older individuals spend less time in SWS, with less SWA and spindles (Mander *et al.*, 2017). Sleep becomes increasingly shallow and fragmented by more frequent arousals, resulting in common sleep complaints in the elderly (Carrier *et al.*, 2011). Concurrently, cognitive abilities such as memory are regularly impaired in the ageing brain, which has been related to structural and functional changes during the ageing process (Scullin & Bliwise, 2015), and attempts have been made to determine the exact causal relationships between the observed alterations. It has been proposed that pre-frontal atrophy was a likely factor in impaired SO generation, which in turn affects the process of sleep-dependent memory consolidation thought to be facilitated by these waveforms (Mander *et al.*, 2013). Furthermore, the cross-coupling of SO and fast spindle frequencies thought to be involved in the consolidation process appears to be disrupted (Helfrich *et al.*, 2017, Muehlroth *et al.*, 2019). In this light, the principal aim of applying auditory closed-loop stimulation in the ageing brain was to enhance SWA and phase-locked fast spindles, and in doing so to lend support for sleep-dependent memory consolidation processes to occur. Our results in Chapter 2 demonstrate that despite the employed algorithm being successful in this respect in a young adult group (Ngo *et al.*, 2013), stimulation failed to improve performance on all assessed memory domains in a sample of healthy, late middle-aged adults. This is in contrast to previous research suggesting auditory closed-loop stimulation to improve declarative memory overnight in an elderly cohort (Papalambros *et al.*, 2017). A number of considerations are necessary in the light of these inconsistent outcomes.

Firstly, it is possible that our stimulation was not successful in facilitating improved memory consolidation due to physiological limitations in our sample. Despite careful examination of our participants' cognitive abilities in eligibility screening sessions during recruitment, we did not collect any structural or functional measures assessing levels of atrophy, plaque deposition, or connectivity, and therefore cannot exclude the possibility that such heterogeneous factors may have influenced our results. It has been pointed out that older participants should be screened more thoroughly, as the commonly applied dichotomous terms of healthy and pathological are insufficient to comprehensively describe an individual's physiological and cognitive ageing status and trajectory (Kaup *et al.*, 2011). Structural and functional changes often commence years before any related cognitive impairments can be readily recognised in screenings (Beason-Held *et al.*, 2013). Thus, such aspects may have negatively impacted on the success of the stimulation applied in our older cohort and should be considered in future studies recruiting older individuals.

Secondly, the idea of applying auditory closed-loop stimulation in late middle-aged adults may not be fully thought through. As evidenced in the comparison of evoked responses between young and older adults in Chapter 2, SO amplitude and count were lower in our

middle-aged cohort, suggesting the age-related decline in certain sleep parameters had already begun in our sample. Yet, we showed that the ageing brain was still susceptible to stimulation, albeit to a smaller degree. In order for auditory closed-loop stimulation to have a greater impact in either slowing down or precluding this decline in ageing altogether, it may be necessary to commence administration earlier and apply it longer-term in order to prevent initial deterioration to a point where only a limited impact can be achieved by stimulation. Our incomplete contemporary understanding of all elements involved and their respective dynamics highlights that it would be premature to use the stimulation technique in its current form for treating individuals further along the ageing trajectory, such as those affected by MCI or AD. Prior experimentation and optimisation in healthy, older cohorts should be prioritised in the first instance, particularly since potential consequences of long-term administration of this stimulation have yet to be explored. Alternatively, perhaps the full impact of stimulation in an older brain could be restored by administering auditory closed-loop stimulation in combination with supplementary treatment, such as interventions aimed at removing plaques which are currently being developed and trialled (Leinenga & Gotz, 2015, Liao *et al.*, 2018), or by using blood transfusions from younger donors to rejuvenate cognitive function and plasticity (Villeda *et al.*, 2014).

Lastly, with the stimulation modality being auditory, it should be noted that hearing abilities during wakefulness decline naturally in older age (Lin *et al.*, 2014, Peelle & Wingfield, 2016). While the auditory threshold was not found to differ between wakefulness and sleep in healthy, young adults (Deacon-Elliott *et al.*, 1987), to our best knowledge it has yet to be investigated whether this persists throughout later adulthood. A possible relationship between hearing ability of the sleeping brain in older age and stimulation outcome will need to be examined in future. One advantage of auditory closed-loop stimulation is that it does not appear to increase arousals during sleep irrespective of age, and as such does not impact negatively on sleep macrostructure. In our experiment in Chapters 2, our older participants were screen for normal hearing during wake and then asked to choose the initial stimulation volume themselves; this was later adjusted manually by the experimenter throughout the stimulation period in response to evoked responses, arousals, or sleep stage transitions. Perhaps incorporating automatic monitoring of such responses into a stimulation algorithm, which could then adjust the volume in a more continuous and consistent manner, would optimise the impact of stimulation in older, as well as possibly younger participants (Bellesi *et al.*, 2014).

5.6 Psychological aspects of stimulating the sleeping brain

In Chapter 4, we considered the psychology of stimulation administration to the sleeping brain. By simulating auditory closed-loop stimulation in a placebo protocol, we discovered that anticipating the stimulation did have an inconsistent impact on frontal slow spindles across the night. However, contrary to our intention and expectation of inducing an overall placebo outcome, the effect was both positive and negative with respective transient

in- and decreased power in this band. These results would suggest that effects observed in Chapters 2 and 3 are not entirely unlikely to be partially biased by a placebo/nocebo effect. On the other hand, the placebo and nocebo results in Chapter 4 could imply that the true magnitude of stimulation impact in Chapters 2 and 3, as well as in any other study where auditory closed-loop stimulation is administered and fully blinding participants is not feasible, may be underestimated for selected oscillations.

In the light of the exploratory nature and consequent statistical limitations detailed by frequentist and Bayesian analyses in Chapter 4, we recommend experimental validation of the present findings. Until then, future research should not eliminate the possibility of placebo or nocebo effects affecting their experimental manipulation, and to therefore include adequate control conditions to assess bias in their own investigation and moreover the validity of our findings. Additionally, it is conceivable that the directionality of the majority of such unintended effects (i.e. placebo or nocebo) depends on the stimulation modality used, and the degree to which positive or negative notions thereof are held by the participant. We did not query our participants as to their perception of auditory closed-loop stimulation per se, and should therefore entertain the possibility that auditory stimuli would have literally been perceived as 'noise' despite reassurances made during the initial participant briefing, thus eliciting notions of sleep disruption and increasing personal wariness of stimuli.

While different personality factors had previously been attested a predictive value for eliciting placebo effects (Horing *et al.*, 2014), we found none of the personality measures collected for the experiment in Chapter 4 predicted the oscillatory changes during sleep, nor did the participant's own expectation of stimulation outcome. Thus, if our placebo/nocebo effects are replicated in future, further testing for factors aiding the predictability of such effects should be conducted in order to help investigations estimate the risk of placebo/nocebo bias in their chosen sample populations. Furthermore, it is unclear whether age could be a mediating factor in placebo/nocebo effects. Placebo mechanisms have been hypothesised to be partially driven by expectancy and related processes of executive function thought to be located in (pre-) frontal cortical regions (Colagiuri *et al.*, 2015, Funahashi & Mario, 2013). One study noted how older individuals with AD showed a diminished placebo effect (in this case a reduced analgesic treatment efficacy during wake), which was attributed to reduced functional connectivity in pre-frontal brain areas (Benedetti *et al.*, 2006). Hence, it is conceivable that individuals with impairments, such as pre-frontal atrophic decline or decreased connectivity, may not present sufficiently preserved neural and cognitive mechanisms required to elicit placebo or nocebo responses.

On the whole, sleep is a complex behavioural state whose mechanisms are far from fully understood. As demonstrated by past research as well as the results presented in Chapter 4, it can be influenced and impacted on by circumstantial factors, such as the sleeping environment and conditions, and an individual's perception of these. Research has so far

largely ignored the psychological aspect that comes with the application of nocturnal brain stimulation. In future, more detailed testing is needed to explore and separate the true impact of such stimulation from psychological factors causing confounds, e.g. by applying double-blinding, and incorporating appropriate baseline, sham, or placebo conditions in designs.

5.7 General reflections on the future use of auditory closed-loop stimulation during sleep

As an easily administrable, amendable, and non-invasive brain stimulation method, auditory closed-loop stimulation has seen a rise in popularity over the past few years. In contrast to other brain stimulation modalities, such as tACS/tDCS or TMS, auditory closed-loop stimulation does not enforce rhythms on the sleeping brain in an unnatural way, nor does it add artefacts to the EEG signal during acute stimulation. In the worst case, applying the technique would result in sleep akin to such spent in an environment with recurrent noise disruption. While certainly not ideal or intended, one-off application is presently not thought to entail long-term negative consequences. However, with a heavy investigative focus on enhancement, whether auditory closed-loop stimulation also entails any negative outcome, whether in terms of oscillatory dynamics or psychologically, has yet to be explored in more detail. In the meantime, timing stimuli in a closed-loop manner helps control when sounds are administered, thereby targeting the brain's inherent receptiveness to external input during specific oscillatory phases, minimising the likelihood of causing arousals, while at the same time maximising stimulation impact (Bellesi *et al.*, 2014, Bergmann, 2018). Anecdotally, it also appears that when administering sounds during an overnight, participants regularly show fewer arousals to stimuli once they have completed their first sleep cycle and experienced their first REM bout. Whether this observation is related to gradual habituation of the participant to sounds during sleep, individual differences in acoustic arousal thresholds in connection with sleep pressure levels, or is due to specific properties of REM sleep, and will uphold when assessed statistically will require future investigation.

Based on the underlying mechanisms of sound processing during stable NREM sleep (Dang-Vu *et al.*, 2010a), studies have so far focused on enhancing slow wave and phase-coupled (fast) spindle frequencies. Despite the growing number of studies using auditory closed-loop stimulation, it is as of yet unclear how much driving of the stimulation would be required depending on the desirable outcome (e.g. consolidation or forgetting). Future research is likely to explore whether, and to what extent, this technique can be adapted in terms of stimulus choice, frequency, intensity, duration, or to impact on different frequencies of interest as well. At present, 50 ms of pink noise are most commonly used as stimuli (see research summary in Table 1.1), although other noise spectra, sound types, and sound oddball paradigms have been trialled, but proven less successful (Debellemanière et al., in preparation). Moreover, with a breadth of possibilities, it would be interesting to examine

whether characteristic events of other sleep stages, such as theta rhythms or phasic eye movements in REM sleep, would also respond to auditory stimulation administered in a closed-loop manner. Importantly, this will likely require some experimentation with different stimulus types. Another idea would be to compose stimuli to the same frequency as the targeted oscillatory frequency, as previously attempted for fast spindles (Ngo *et al.*, 2018). However, the human hearing range is thought to begin at ∼20 Hz (Dobie & Van Hemel, 2005), which would make it difficult to use this type of stimulus to entrain rhythms below this limit.

While the future of auditory closed-loop stimulation as an investigative tool is unlimited, its immediate use for clinical application is uncertain. At present, too many unknown factors persist, making the overall impact difficult to assess. Similarly, to date no study has investigated the impact of administering the technique longitudinally in young or older adults. While one study demonstrated that stimulation effects did not vary across ten overnights (Debellemaniere *et al.*, 2018), more data are needed to arrive at a definite conclusion, including information on potential structural and functional changes following habitual stimulation. In this light, the number of home devices available for private purchase should be seen as a product of well-intended, but possibly slightly pre-mature business ventures. A more detailed understanding and further optimisation of stimulation algorithms to rule out negative side effects of long-term use of such devices are urgently needed. Given the intricate interrelationship between different sleep frequencies, it is absolutely vital to comprehensively explore the functional implications of auditory closed-loop stimulation protocols before commencing any systematic manipulation of human sleep on a larger scale.

5.8 Conclusions

Sleep is a complex behavioural state in which we spend approximately one third of our lives. While present-day sleep research is still exploring the exact reasons behind this commonly adopted behaviour of living organisms, novel brain stimulation methods are aiding our quest to investigate the intricate oscillatory and functional details of this curious state we find ourselves in every night. This thesis explored the potential of one such novel stimulation method, called auditory closed-loop stimulation, to enhance oscillatory features in sleep, and thereby improve sleep-dependent memory consolidation processes in samples of healthy young and older adults. SO and sleep spindles hallmark stable NREM sleep and display intricate, cross-frequency interactions. We demonstrated that by considering their neural dynamics, these oscillations can be separately and purposefully manipulated by auditory closed-loop stimulation in healthy, young individuals. While the ageing brain also appears receptive to sounds administered in this manner during sleep, their susceptibility to the stimulation is markedly decreased, and shows a differential and less favourable, functional outcome. Psychologically, merely expecting auditory stimulation during sleep can impact on oscillatory activity. It is hoped that by presenting new ideas for protocol variations, as well as defining limitations of auditory closed-loop stimulation administra-

tion, the work presented in this thesis has highlighted the potential of this technique, but also raised important questions and will inspire future investigations into the intricate world and functional purpose of different sleep oscillations.

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Appendices

Appendix A: Placebo induction briefing

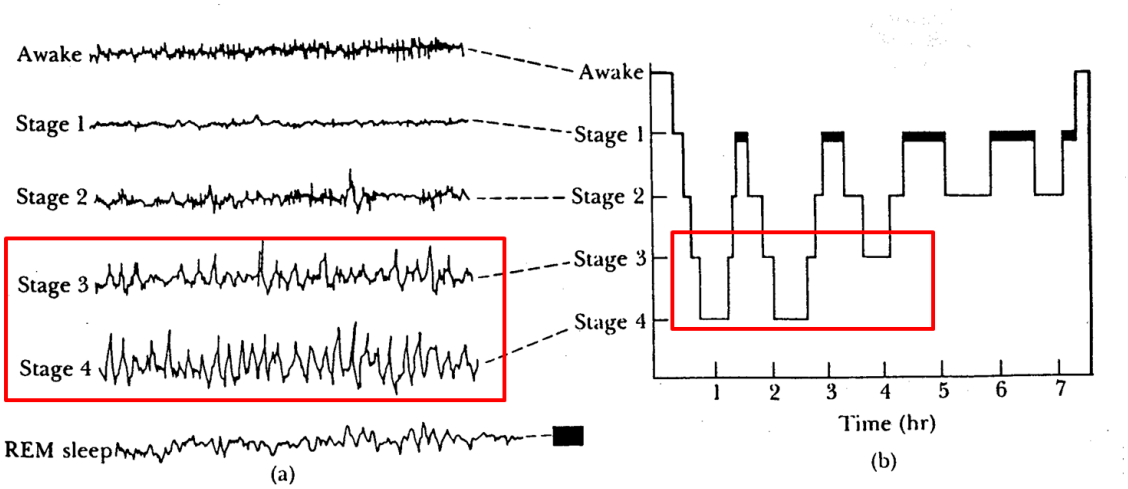
Does prior awareness influence the outcome of offline stimulation?

Introductory Participant Briefing

This project has been approved by the Cardiff University School of Psychology Ethics Committee. (Project Code EC.16.12.13.4805)

Experimenter: *"I will now give you a quick mandatory briefing on the experiment and the stimulation we will apply to make sure you know what you're getting into and what to expect. As I talk you through these slides, please do stop me at any point if you would like more information or have any questions, ok?"*

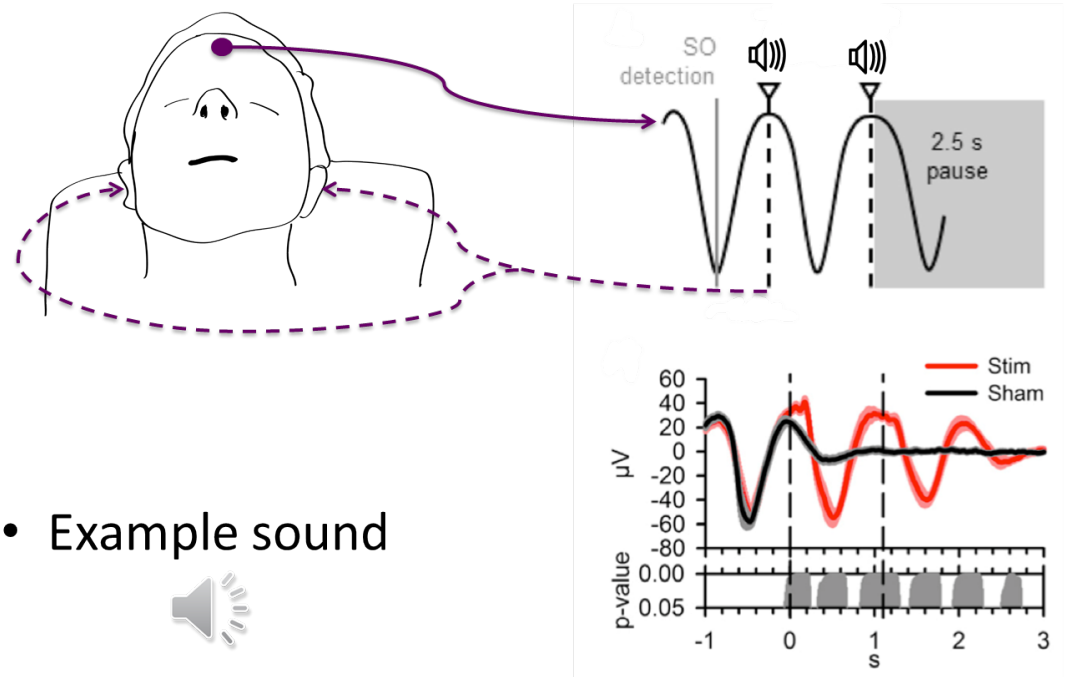
Sleep stages and structure



Experimenter: *"I'll start off by telling you a little bit about sleep to explain what exactly we will be manipulating. There are 4 different sleep stages which you repeatedly cycle through in one night of sleep. You can see these cycles in the hypnogram on the right of the graph. The left side of the graph here shows you what your brain activity looks like when we record it with electrodes on your scalp, as we will do in this experiment. In wake, when all your different brain areas are busy carrying out a multitude of different tasks, you see a low amplitude, wiggly line. Once you have just about fallen asleep, the activity becomes a lot less busy in early light sleep (Stage 1). The deeper your sleep becomes, the more high amplitude and low frequency activity can be detected on your scalp. Stages 3 and 4 form the deepest stage of sleep. You get this pattern of slow oscillations because now that the brain is no longer busy monitoring and engaging with the outside world, it can finally finish up all the processing it didn't get done during the day and clear out the rubbish, and to get these tasks done, large areas of brain cells engage simultaneously in the same firing patterns. Imagine it like large, sweeping waves travelling across your brain from your forehead towards the back of your head.*

Over the past few decades, sleep researchers have discovered that it is these slow oscillations which are absolutely vital for having a good night of sleep, maintaining the homeostatic balance of your brain, restoring resources, making sure you remember what is important for you and are able to forget the rest. People who have sufficient deep sleep wake up feeling refreshed, and usually show good cognitive abilities. Unfortunately, in many sleep disorders, deep sleep is impaired, which negatively affects daily functioning. We're therefore very interested to investigate brain stimulation techniques which help enhance deep sleep."

Auditory closed-loop stimulation



- Example sound

Experimenter: *"The type of stimulation we apply in this project is called auditory closed-loop stimulation. It has a lot of benefits, in that, it's not invasive in any way, does not interfere with natural brain activity, and has no known negative side effects. The way it works is that we feed the brain activity of a forehead electrode into an algorithm, which automatically registers when you are in deep sleep, then detects the trough of an on-going slow oscillation and plays you a short sound at the very peak of the same oscillation. The sound is 50msec of pink noise, which sounds like this (CLICK DEMO NOISE). Through internal sleep promoting mechanisms, your brain will briefly check that the sound does not pose any kind of threat, and then deepen your sleep by enhancing the following slow oscillations. The algorithm will immediately stop any stimulation if it notices that you transition into another sleep stage, and can also lower the volume if it notices the sounds leading to any kind of brief arousal. This stimulation therefore increases the number of slow oscillations, and thereby further deepens your sleep. In previous studies, we found that if we apply this stimulation, you will spend more time in restorative, deep sleep across a night of sleep, which has an impact on your cognitive functioning as well."*

The current study

- Does being aware of the stimulation enhance its effects?

- Random sub-groups allocation:
  1. Aware in which experimental night stimulation is applied
  2. Unaware when stimulation is applied

- Experimenter is <u>not allowed</u> to know which sub-group you have been allocated to, or which night you undergo which condition – please don't tell them ☺

Experimenter: *"While this type of sound stimulation does not disturb your sleep in any way, or even wake you up, one of the unknown factors is the extent to which the brain processes the sound, and whether this can, paired with being aware of stimulation being applied, enhance the effect of stimulation itself. To find out, we will randomly allocate every participant to one of two sub-groups. In one group, participants will be explicitly informed in which of their two experimental nights they receive the stimulation. The other group will undergo both the experimental nights without knowing their order. Please note that you could receive two sham nights, two stimulation nights, or one of each because we need to control for other effects as well. We will then be able to compare the magnitude of stimulation effects between the two groups.*

In this design, it is very important that the experimenter does not know which group you are in, or which condition you are undergoing on either night, as this could lead to an external expectancy effect. You will be informed which sub-group you have been allocated to, and - if applicable - which condition you undergo which night by letter at the beginning of your first and second experimental night. The experimenter will therefore follow an identical procedure on both nights. You are welcome to ask any questions about the stimulation and the experiment during the adaptation night, but please do not make any reference that could lead the experimenter to infer your group/condition thereafter.

Do you have any remaining questions?"

Appendix B: Condition, expectancy & experience letter

Dear Participant,

You have been assigned to the **awareness cohort** in this experiment, and will therefore be made aware of which night you undergo which condition (i.e. auditory stimulation or no stimulation).

Please remember that this study is based on a blind design and your experimenter **cannot** know which condition you are undergoing which night. **Please do not talk to them about it.** Thanks ☺

Participant No.	**X**
Experimental Night	**1st/2nd**
Tonight's Condition	**Stimulation/No Stimulation**
Code written on Envelope	**_____** *(participant to complete)*
Today's Date	**___ / ___ / 2017** *(participant to complete)*

Please ignore the sections below for now.
Your experimenter will prompt you to answer them at a particular time in the experiment.

Section 1: Your awareness and expectations of tonight

I expect that the condition which I am undergoing tonight will have the following impact on my sleep:

□ **positive** – my sleep will be enhanced and I will wake up feeling more rested than usual

□ **negative** – my sleep will be disrupted and I will wake up feeling less rested than usual

□ **neutral/not changed** – my sleep will not be affected and I will wake up feeling just as rested as usual

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Section 2: Your actual experience of the condition

Last night, I heard stimulation sounds during my sleep: □ **yes** □ **no**

Thank you ☺