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Charles University in Prague

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Dissertation Thesis

Zpětnovazebná sluchová stimulace pomalovlnného spánku: aplikace a mechanismy fungování

Closed-loop auditory stimulation of slow-wave sleep: applications and mechanisms of function

Supervisor: PhDr. Jana Kopřivová, PhD.

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Abstract

Non-pharmacological manipulations of sleep represent an important tool to study the function of sleep causally. Auditory stimulation, and closed-loop auditory stimulation (CLAS) in particular, represent an innovative, easy to apply, and effective method to alter sleep. This thesis introduced our original research in CLAS application (Study 1) and mechanisms of action (Study 2) in a chronic insomnia population for the first time. Study 1 assessed the feasibility and efficacy of CLAS for improving sleep quality and memory consolidation in chronic insomnia. This crossover, sham-controlled study involved 27 participants undergoing two nights of either CLAS or sham stimulation, monitored via polysomnography to measure sleep parameters, along with scales for assessing subjective sleep quality and a word-pair memory task for measuring overnight memory consolidation. Initial findings from 7 participants with sufficient amount of stimulations indicated that while CLAS significantly increased slow oscillation (SO) amplitude and power during slow-wave sleep, it did not alter sleep-dependent memory consolidation, overall sleep architecture, number of arousals, discrete sleep spindles, or subjective sleep quality. Additionally, Study 2 explored CLAS mechanisms with their effects on SOs on a subset of 9 participants from Study 1, comparing the phase-locked loop (PLL, PLL-XOR and PLL with an integral part) and fixed-step stimulus methods using our streamed sleep data. The fixed-step method proved more reliable and practical than the PLL methods. Importantly, we found significant phase synchronization of SOs, suggesting a possible mechanism of action of CLAS altering existing SOs rather than generating new ones. Despite its feasibility in insomnia patients, high variability in stimulation efficacy in our sample highlights the need for optimized and more tailored protocols to discover potential benefits of CLAS for sleep structure, memory consolidation, and subjective sleep quality in such clinical settings.

Abstrakt

Nefarmakologické modulace spánku představují důležitý nástroj pro kauzální studium funkcí spánku. Sluchová neboli akustická stimulace, a zejména zpětnovazebná sluchová stimulace (closed-loop auditory stimulation, CLAS), je inovativní, snadno aplikovatelnou a účinnou metodu pro ovlivňování spánku. Tato práce představuje naše originální výzkumy týkající se aplikace CLAS (Studie 1) a mechanismů jejího působení (Studie 2) u pacientů s chronickou nespavostí. Studie 1 hodnotila proveditelnost a účinnost CLAS pro zlepšení subjektivní kvality spánku a konsolidace paměti u chronické nespavosti. Tato křížová, placebem kontrolovaná studie zahrnovala 27 účastníků, kteří podstoupili dvě noci buď s CLAS, nebo s falešnou stimulací, přičemž byli monitorováni polysomnografií, škálami pro hodnocení subjektivní kvality spánku a testem párového asociačního učení pro měření konsolidace paměti přes noc. Počáteční výsledky od 7 účastníků ukázaly, že i když CLAS významně zvýšila amplitudu a sílu pomalých oscilací během NREM3, neměla vliv na konsolidaci paměti závislé na spánku, celkovou architekturu spánku, počet probuzení, výskyt spánkových vřetének nebo subjektivní kvalitu spánku. Studie 2 zkoumala mechanismy CLAS a její účinky na pomalé oscilace u podmnožiny 9 účastníků ze Studie 1, přičemž porovnávala metody stimulace s fázově uzamčenou smyčkou (phase-locked loop, PLL: PLL-XOR a PLL s integrální složkou) a metodu s pevným krokem (fixed-step). Metoda s pevným krokem se ukázala být spolehlivější a praktičtější než metody PLL. Důležitým zjištěním byla významná fázová synchronizace pomalých oscilací, což naznačuje možný mechanismus působení CLAS spíše skrze modifikaci existujících SO, než prostřednictvím generování nových. Navzdory proveditelnosti u pacientů s nespavostí vysoká variabilita účinnosti stimulace v našem vzorku zdůrazňuje potřebu optimalizovaných a lépe přizpůsobených protokolů stimulace, aby bylo možno posoudit jejich potenciální přínosy pro strukturu spánku, konsolidaci paměti a subjektivní kvalitu spánku u klinických populací.

List of Abbreviations

AASM	American Academy of Sleep Medicine
ADHD	Attention Deficit Hyperactivity Disorder
AI	Artificial Intelligence
aMCI	Amnestic Mild Cognitive Impairment
BAI	Beck Anxiety Inventory
BDI-II	Beck Depression Inventory-2
CBT-I	Cognitive Behavioural Therapy for Insomnia
CLAS	Closed-Loop Auditory Stimulation
CL-TMR	Closed-Loop Targeted Memory Reactivation
CZ-PAL	Czech Version of Paired-Associates Learning Task
EEG	Electroencephalography
EMDR	Eye Movement Desensitization and Reprocessing
ERP	Event-Related Potential
ESS	Epworth Sleepiness Scale
FDR	False Discovery Rate
FIR	Finite Impulse Response
fMRI	Functional Magnetic Resonance Imaging
GABA	Gama-Aminobutyric Acid
IC	Inferior Colliculus
ICSD-3	International Classification of Sleep Disorders, Third Edition
ICx	Shell of the Inferior Colliculus
IRR	Infinite Impulse Response
ITPC	Inter-Trial Phase Clustering
KC	K-complex
LC	Locus Coeruleus
M	Mean
Mdn	Median
MEG	Magnetoencephalography
MEQ	Morningness-Eveningness Questionnaire
MGd	Medial Geniculate Dorsal
MGm	Medial Geniculate Caudo-Medial
MGv	Medial Geniculate Ventral
MRF	Midbrain Reticular Formation
n	Number of Participants
NGC	Nucleus Gigantocellularis
NIMH-CZ	National Institute of Mental Health, Czechia
NREM	Non-Rapid Eye Movement Sleep
OLAS	Open-Loop Auditory Stimulation
PLL	Phase-Locked Loop
PLL-XOR	Phase-Locked Loop - XOR Gate
PRF	Pontine Reticular Formation

PSG	Polysomnography
PTSD	Post-Traumatic Stress Disorder
RCT	Randomized Controlled Trial
REM	Rapid Eye Movement Sleep
Sag	Sagulum
SC	Superior Colliculus
SD	Standard Deviation
SE	Sleep Efficiency
SEM	Standard Error Of Mean
SHAM	Sham Stimulation Condition
SHY	Synaptic Homeostasis Hypothesis
SO	Slow Oscillation
SOL	Sleep Onset Latency
ST	Spinothalamic Tract
STFT	Short-Time Fast Fourier Transform
STIM	Stimulation Condition
SWA	Slow-Wave Activity
SWR	Sharp Wave Ripple
SWS	Slow-Wave Sleep
tACS	Transcranial Alternating Current Stimulation
TC	Thalamocortical
tDCS	Transcranial Direct Current Stimulation
TMR	Targeted Memory Reactivation
TMS	Transcranial Magnetic Stimulation
TOPOSO	Topographic Targeting of SOs
TRN	Thalamic Reticular Nucleus
TST	Total Sleep Time
VCO	Voltage-Controlled Oscillator
WASO	Wakefullness After Sleep Onset

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1. Introduction

1.1. Overview of non-invasive methods of stimulating sleep

Non-invasive sleep stimulation represents a unique way to alter sleep rhythms, thereby allowing researchers to shed light on causal mechanisms of sleep functions. Non-invasive methods of stimulating sleep have been seminally explored since 2004, offering insights into how sleep, particularly slow oscillations (SOs), can be both enhanced and suppressed through various techniques including electrical, magnetic, and sensory stimulation (Marshall et al. 2006; Marshall et al. 2004; Massimini et al. 2007; Ngo et al. 2013b; Rasch et al. 2007). This approach to sleep science highlights the potential to manipulate sleep rhythms and architecture directly and to study their effects on brain and cognitive functions (Esfahani et al. 2023).

Among the sensory modalities targeted for sleep stimulation—including visual, somatosensory, and olfactory—auditory stimulation has been found particularly effective. It reliably elicits K-complexes, a type of brain activity that occurs during non-rapid eye movement (NREM) sleep and is integral to sleep-based neurophysiological modulation (Esfahani et al. 2023). Compared to other stimulation methods such as transcranial direct/alternating current stimulation (tDCS/tACS) or transcranial magnetic stimulation (TMS), auditory stimulation offers several advantages: it is cost-effective, non-invasive, easy to apply, and causes fewer artifacts in the recording signal, making it less intrusive and more comfortable for the subjects (Wunderlin et al. 2021).

This thesis focuses primarily on the auditory stimulation of slow-wave sleep (SWS), specifically on closed-loop auditory stimulation approaches, and provides a deeper summary of potential clinical applications as well as known mechanisms of action. However, to give a wider context, subsequent sections of this thesis will first briefly overview other various non-invasive stimulation methods.

1.1.1. Electric and magnetic stimulation

Transcranial electrical stimulation techniques like tDCS and tACS utilize weak electric currents applied via scalp electrodes to modulate brain activity, particularly to entrain slow oscillations in the cortex important for sleep-associated memory consolidation. This method is thought to align the brain's natural oscillations by delivering currents that mimic the natural frequency of sleep oscillation of interest. tACS has been shown to enhance slow-wave activity and

declarative memory in both healthy younger and older individuals, as well as those with mild cognitive impairment, by synchronizing intrinsic brain oscillations to the natural frequencies of slow-wave sleep (Ketz et al. 2018; Ladenbauer et al. 2016; Ladenbauer et al. 2017; Lustenberger et al. 2016; Marshall, et al. 2006). However, challenges in measuring electrophysiological data due to artifacts caused by tACS/tDCS complicate the evaluation of these techniques' effectiveness in real-time brain activity modulation, with current methodologies struggling to accurately capture spectral information, SOs and sleep spindles (Fehér et al. 2021).

Transcranial magnetic stimulation (TMS) utilizes a time-varying magnetic field to pass through the skull and generate an electric current in brain structures aligned with the coil orientation (Wagner et al. 2007). This TMS-induced current can modify neuronal excitability or trigger action potentials, with the neuronal response being contingent on the targeted neuronal population's state, thus influencing specific regional brain dynamics (Johnson et al. 2010; Kobayashi and Pascual-Leone 2003). In Massimini, et al. (2007), TMS was used to directly initiate SOs in NREM sleep that propagated across the cortex, mirroring naturally occurring sleep oscillations. Modulated SOs were linked to improved memory performance and thus showed potential therapeutic value for disorders like insomnia and depression (Massimini, et al. 2007).

TMS offers one major advantage over transcranial electrical stimulation or auditory stimulation due to its spatial precision, which allows for a more targeted investigation of slow wave modulation in specific brain regions and local effects of SO modulation (Fehér, et al. 2021).

Despite its precision and potential, only a handful of studies applied TMS during sleep, as it is technically challenging and limited to brief sessions rather than all-night studies. To ensure accurate coil positioning, participants often must sleep in a reclining chair, which complicates the setup for both the experimenter and the participant (Fehér, et al. 2021). Typically, studies utilize block designs that compare periods of active stimulation to periods without stimulation or wakefulness.

1.1.2. Non-auditory sensory stimulation

Non-auditory sensory stimulation, including olfactory, visual, and somatosensory modalities, has been previously utilized in sleep and memory research, particularly for targeted memory

reactivation (TMR) where memories are systematically reactivated during sleep to bias sleep-dependent memory consolidation processes (see details in 1.1.3.1).

For example, olfactory cues during slow-wave sleep were shown to reactivate memories and improve the retention of hippocampus-dependent declarative memories the next day (Rasch, et al. 2007). The odour reexposure during SWS enhanced frontal delta and parietal spindle power and was specific to the odour being presented during learning (Rihm et al. 2014). Visual stimulation cues were used in animal models as well as in humans. Research involving animal models suggests that visual stimulation, like dim red light, can enhance slow-wave activity without disrupting sleep patterns (Thankachan et al. 2022). Nevertheless, studies like those by Danilenko et al. (2020) indicate that the effectiveness of increasing delta power density during NREM sleep may not always be consistent in comparison with auditory stimulation.

Somatosensory stimulation was also used in both humans (Bayer et al. 2011; Perrault et al. 2019) and animal models (Kompotis et al. 2019) in a series of so-called rocking bed experiments. This stimulation used gentle rocking of the bed (0.25 Hz) to facilitate transitions from wake to sleep and enhance sleep stability by boosting slow oscillations, NREM2 and NREM3, and spindle activity all the while decreasing arousals (Bayer, et al. 2011; Perrault, et al. 2019). This method was shown also to improve memory consolidation during sleep (Perrault, et al. 2019), although practical application outside controlled settings remains a challenge.

In summary, non-auditory sensory stimulations using olfactory, visual, and somatosensory cues have been effectively utilized in sleep research, particularly for enhancing memory consolidation processes during sleep. Overall, these diverse sensory stimulation techniques showed their valuable contributions to our understanding of sleep's impact on memory, however, they are generally limited by their ease of application and practicality for which auditory stimulation seems to be a better candidate.

1.1.3. Auditory stimulation

Auditory stimulation, also known as acoustic stimulation, encompasses a variety of approaches aimed at directly modifying endogenous sleep rhythms. These methods include both open and closed-loop approaches, utilizing a spectrum of sounds from meaningful, longer audio clips to brief bursts of white or pink noise lasting approximately 50ms. Despite overall increasing interest in the literature and the potential of applications in a range of areas, a recent meta-

analysis indicated a relatively low effect size for the overall effect of auditory stimulation on episodic memory, with a Hedges' g of 0.25 at a trend-level of significance ($p = 0.07$) (Wunderlin, et al. 2021). However, it is important to point out that within the auditory stimulation field, there are numerous approaches (mentioned in further sections in more detail) and research suggests that the effectiveness of these individual approaches can somewhat vary.

1.1.3.1. Targeted memory reactivation (TMR)

Targeted memory reactivation, TMR, also known as cued memory reactivation, is a well-established technique designed to selectively stimulate specific memories during sleep using sensory cues that were linked to learning before sleep. As briefly mentioned above, this technique involves presenting specific cues, such as odours or sounds, during the learning so they become associated with the material being learned. These same cues, or a subset thereof, are then reexposed during subsequent sleep to facilitate memory reactivation and consolidation. TMR operates independently of brain state, typically delivering stimulation during stable periods of NREM sleep, most commonly NREM3. Specifically, TMR works without synchronizing with instantaneous brain activities or to specific brain oscillations, i.e. it may target different phases and different frequencies of oscillations by chance during stable NREM3 and not be necessarily in phase with SOs (Carbone and Diekelmann 2024; Esfahani, et al. 2023). Rudoy et al. (2009) in their well-known Science paper were pioneers in applying TMR using auditory stimuli, demonstrating its feasibility and effectiveness in influencing individual selective memories.

The application of TMR using auditory cues spans various types of memories, including declarative, procedural, and emotional (Carbone and Diekelmann 2024). Early studies primarily focused on enhancing these memories through TMR (Rasch, et al. 2007; Rudoy, et al. 2009; Schönauer et al. 2014). More recent research has expanded to explore other dimensions such as the long-term TMR effects over extended periods (Rakowska et al. 2021; Rakowska et al. 2022), the role of interference between different cues (Schechtman et al. 2021a), and even the potential to induce memory forgetting (Schechtman et al. 2021b; Schechtman et al. 2020). Additionally, intriguing developments have been observed in the use of TMR during REM sleep, particularly its impact on dream content (Schwartz et al. 2022). The broader applications of TMR also extend into fields such as mental health, education, and home-based settings, showcasing its versatility and potential for broader societal benefits (Göldi et al. 2019;

Neumann et al. 2020; Talamini and Juan 2020; van der Heijden et al. 2022; Whitmore et al. 2022).

To evaluate this method overall, a meta-analysis by Hu et al. (2020) estimated the overall effect of TMR, finding a relatively small effect size with a Hedges' g of 0.29 across sleep stages N2 and N3. This statistical measure underscores the modest yet significant impact of TMR on enhancing memory processes, affirming its potential as a tool for memory enhancement and therapeutic applications.

1.1.3.2. Open-loop auditory stimulation (OLAS)

Open-loop auditory stimulation (OLAS), also referred to in the literature as non-phase locked stimulation (NPLAS), follows a predetermined schedule that does not adjust based on the individual's current physiological state or responses. In OLAS protocols, auditory stimuli are delivered rhythmically but without being synchronized or phase-locked to specific neural oscillations. Typically, stimulations are applied at fixed-time intervals during NREM sleep (Diep et al. 2020; Choi et al. 2019; Weigenand et al. 2016).

Despite the simplicity and potential applications of OLAS, it is generally assumed that closed-loop approaches may be more effective and less disruptive to natural sleep processes. This assumption is based on the idea that stimulating out-of-sync with the natural up-state of sleep oscillations could potentially disrupt endogenous SO trains (Wunderlin, et al. 2021). Such disruptions could interfere with the natural dynamics and functions of sleep, particularly in terms of memory consolidation and neural plasticity.

The comparative lack of effectiveness of OLAS is underscored by its estimated low impact, with research such as that by Wunderlin, et al. (2021) indicating a minimal effect size (Hedges' g) of .03, which was statistically non-significant ($p = 0.9$). This result highlights the critical distinctions between open-loop and closed-loop stimulation methods, suggesting that while OLAS offers a straightforward approach to auditory stimulation during sleep, it might not be the most efficacious method for manipulating sleep rhythms. As a result, OLAS is considered less effective than CLAS and remains an understudied area within the field of auditory stimulation of sleep.

1.1.3.3. Closed-loop auditory stimulation (CLAS)

The primary focus of this thesis is on closed-loop auditory/acoustic stimulation (CLAS), also commonly referred to in the literature as ACLS (auditory/acoustic closed-loop stimulation). The term refers to a process of feeding the manipulated signal back into the system, thereby "closing the loop." This method contrasts with open-loop systems, which follow a predetermined schedule and do not adapt to the individual's current state or responses after stimulation (Esfahani, et al. 2023).

Auditory closed-loop stimulation is therefore a brain state-dependent method that considers and evaluates instantaneous brain activity. The stimulation is precisely timed to target, for example, the up-state of the SO, which researchers identify as the optimal time window for the target phase of CLAS in both neutral and semantically meaningful stimuli (Batterink et al. 2016; Göldi, et al. 2019; Ngo, et al. 2013b; Schabus et al. 2012). This precise targeting allows not only specific brain waves to be stimulated but also their specific phases, enhancing the specificity and accuracy of the method. This technique is utilized to probe the causal role of sleep oscillations in various functions, including cognitive functions, endocrine regulation, immunological processing, and glymphatic brain clearance (Esfahani, et al. 2023).

An ongoing debate in the field questions the authenticity of some closed-loop approaches, particularly concerning whether the detection algorithms are informed by a stimulated and thus altered endogenous signal versus relying solely on the endogenous signal itself without adjustments from the re-informed outcomes, for example when implementing off periods or refractory periods (Wunderlin, et al. 2021). Overall, the strengths of CLAS include its low cost, non-invasiveness, ease of application, and capability to control the timing and phase of target oscillations. Furthermore, the artifacts induced by stimulation in the online signal remain minimal compared to other types of stimulation, such as electrical (tDCS/tACS) or magnetic (TMS) methods (Esfahani, et al. 2023; Wunderlin, et al. 2021).

The effectiveness of CLAS is still under investigation. Recent meta-analyses shed light on its impact: Wunderlin, et al. (2021) reported a low but significant effect size (Hedges g of .36-.44), while Stanyer et al. (2022) found a medium significant effect in younger adults ($g = 0.68$, $p = 0.031$) but no significant effect in adults over age 35 ($g = -0.83$, $p = 0.223$). Harlow et al. (2023) then included additional two trials and observed a declining trend in effect size, with the publication date being the sole predictor of declining effect size. The same authors concluded

a lack of significant pooled effect (Glass´ estimator $d_{\delta} = -0.39$) and attributed this to the „decline effect"—a phenomenon in psychological and other sciences driven by factors such as underpowered initial studies, unpublished findings, selective reporting, as well as low-task reliability (Harlow, et al. 2023; Pietschnig et al. 2019; Schimmack 2020; Schooler 2011).

1.1.3.4. Closed-loop targeted memory reactivation (CL-TMR)

The latest development in the field of auditory stimulation of sleep is closed-loop targeted memory reactivation (CL-TMR), also termed real-time TMR. This innovative approach synthesizes the principles of both CLAS and traditional TMR, extending their capabilities to optimize individual memory consolidation during sleep. Recent studies have successfully integrated TMR with closed-loop systems (e.g. (Göldi, et al. 2019; Ngo and Staresina 2022; Shimizu et al. 2018).

CL-TMR focuses on the timing of cue presentation in the naturally occurring slow oscillations (SO) and spindle activities during sleep. The critical insight from this approach is that the impact of TMR is profoundly influenced by the precise synchronization of cues relative to ongoing oscillatory brain activity (Göldi, et al. 2019; Ngo and Staresina 2022). Recognizing the importance of this synchronization, recent research suggests that future TMR studies must consider the dynamics of SO and spindle activities to maximize the reactivation and consolidation of memories (Carbone and Diekelmann 2024).

By leveraging the natural rhythms of the brain during sleep, CL-TMR not only enhances the efficacy of memory reactivation but also aligns it more closely with the brain's intrinsic processes, potentially leading to more robust and durable memory consolidation effects. This cutting-edge approach holds promise for further exploration and could prove more effective in comparison to basic TMR approach.

1.2. Sleep rhythms implicated in sleep-dependent memory consolidation

The intricate relationship between sleep and memory consolidation is significantly mediated by specific sleep rhythms, which are especially prevalent during SWS. The hallmark rhythms implicated in both SWS and sleep-dependent memory consolidation include SOs, sleep spindles, and sharp wave-ripple complexes (SWRs). Understanding how these rhythms function and interact provides essential insights into the mechanisms that facilitate the reinforcement and stabilization of memories. This section delves into the characteristics of each

rhythm while the following section 1.3 explores their collective impact on the consolidation processes that occur during sleep.

1.2.1. Slow oscillations (SOs)

Slow oscillations (SOs), also sometimes referred to as slow waves, are large amplitude, low-frequency waves integral to the architecture of NREM sleep, particularly noted for their role in sleep-dependent memory consolidation. These oscillations generally range from 0.1 to 1.5 Hz, though they are most commonly considered to occur at frequencies below 1 Hz (Massimini et al. 2004; Muehlroth et al. 2019; Ngo et al. 2019; Schneider et al. 2020). For instance, specific studies have considered SO frequencies between 0.75–1.25 Hz (Marshall, et al. 2006) and 0.5-1.25 Hz (Ngo, et al. 2019; Schneider, et al. 2020), with some extending the range up to 2 Hz (Morgan et al. 2021).

At the cellular level, SOs are associated with the activity of pyramidal neurons in the sensory, motor, and association cortex. These neurons display a pattern of depolarized (active up-states) and extended hyperpolarized states (silent down-states) (Esfahani, et al. 2023; Steriade et al. 1993). Originating primarily in the cortex, electroencephalographic (EEG) source localization studies have pinpointed hotspots for slow waves in areas such frontal medial, orbitofrontal areas and ventral limbic cortex. Specifically, SO sources have been associated with large currents in the medial frontal gyrus, the middle frontal gyrus, the inferior frontal gyrus, the anterior cingulate, the precuneus, and the posterior cingulate, or areas known as a major connectional backbone of the cortex and integral parts of the default network (Murphy et al. 2009). Additional research identified the ventral limbic cortex, including the medial temporal and caudal orbitofrontal cortex, as origins for SOs, with the thalamus also playing a significant role in their generation (Morgan, et al. 2021; Sheroziya and Timofeev 2014).

SOs are considered a travelling wave, originating at specific sites in prefrontal-orbitofrontal regions and propagating in an anteroposterior direction over the scalp at speeds estimated between 1.2-7.0 m/sec (Massimini et al., 2004). These waves are known to interact with and trigger other sleep rhythms such as sleep spindles and delta waves, often occurring in trains which are sequences of subsequent SOs. Targeted manipulation of these SO trains through CLAS aims to promote their occurrence, thereby enhancing sleep protection, neural plasticity (1.3.1), and memory consolidation processes (1.3.2) (Esfahani, et al. 2023).

It is important to distinguish the terminology used when discussing SOs; they are considered different from both slow wave activity (SWA, 0.5-2Hz), and from delta activity or delta waves (0-4Hz), according to standards set by the American Academy of Sleep Medicine (AASM 2020). This specificity in defining SOs will aid in clarifying their unique contributions to sleep and cognitive functions, e.g. Kim et al. (2019).

1.2.2. Sleep spindles

Sleep spindles, a hallmark rhythm of N2 sleep, are recognized as one of the most heritable EEG signatures and are critically implicated in memory processes (Fernandez and Lüthi 2020). These distinct brain waves are characterized by their waxing and waning amplitude that forms into a spindle shape, typically lasting between 0.5 to 3 seconds, and are most prominent in central brain derivations (AASM 2020). Spindles generally fall within the sigma frequency band (10-15 Hz), though there are varying opinions on the exact frequency range, with some sources citing 11-16 Hz (AASM 2020; Antony et al. 2019), and 12-14 Hz as typical (AASM 2020).

There are two primary types of spindles—slow and fast. Slow spindles range from 9-12 Hz and are predominantly observed in frontal regions, while fast spindles, occurring from 12-15 Hz, are more prominent centro-parietally (Fehér, et al. 2021). Additionally, spindles can be categorized as local or global events based on their distribution across recording sites, with local spindles being present on only a few sites. In general, spindles appear to be a mostly local sleep event (Andrillon et al. 2011; Fernandez and Lüthi 2020). Approximately two-thirds of local spindle events are observed to couple with local SOs, with the strongest synchronization seen in fast centroparietal spindles (Andrillon, et al. 2011). These local sleep spindles display a wide distribution across the cortical surface, varying in characteristics and density depending on the specific brain region and lobe. More global spindle activities tend to occur predominantly in frontal-temporal regions, suggesting a preferred pathway for spindle propagation (Fernandez and Lüthi 2020).

The generation of sleep spindles involves complex interactions within cortico-thalamic (CT) and thalamo-cortical (TC) networks. The gridlike thalamic reticular nuclei (TRNs) play a pivotal role as pacemakers in this process, initiating spindles through synaptic GABAergic inhibition that is both broad and powerful (Fernandez and Lüthi 2020). This initiation triggers a sequence of synaptic activities: TRN burst discharges (i.e. action potentials in bursts of high frequency) inhibit TC cells, which then produce a rebound burst that intensifies during the

spindle and diminishes as TRN intensifies its inhibitory discharge. This activity typically spans 0.5-3 seconds and is followed by a refractory or silent period lasting 3-10 seconds, during which the recurrence of spindles is limited (Antony et al. 2018; Fernandez and Lüthi 2020).

The development of sleep spindles across the lifespan exhibits a characteristic profile that parallels cortical maturation, evolving from early postnatal periods through adolescence and into ageing (Fernandez and Lüthi 2020).

Sleep spindles have been studied not only for their neurophysiological properties but also for their functional implications. They play a protective role similar to K-complexes, with higher densities of spindles correlating with greater resilience to external disturbances during sleep (Fernandez and Lüthi 2020). Furthermore, spindles serve as a biomarker for various cognitive functions such as intelligence, and for disorders leading to cognitive dysfunction, such as dementia, making them useful for diagnostic purposes and monitoring the inner workings of TC loops. Importantly, their involvement in plasticity and memory functions ties them to both procedural and declarative learning, underscoring their importance in cognition (Fernandez and Lüthi 2020).

1.2.3. Sharp wave ripples (SWRs)

Sharp wave hippocampal ripples (SWRs) are fast depolarizing events characterized by bursts of activity that primarily occur during SWS but can also appear during non-exploratory wakefulness. These events are distinguished by their high frequency, typically ranging between 100-300 Hz, and play a crucial role in the processes of memory consolidation (Rasch and Born 2013).

At the cellular level, SWRs originate in the hippocampus, specifically generated in the CA3 region. This activity becomes superimposed by ripple activity originating from CA1. The ripples represent high-frequency local field potential oscillations. The generation of ripples involves intricate interactions between inhibitory interneurons and pyramidal cells. These interactions are mediated through synaptic (both glutamatergic and GABAergic) connections and gap junctions, facilitating the complex neural dynamics underlying these events (Rasch and Born 2013).

Functionally, SWRs are critically involved in the consolidation of hippocampus-dependent memories during sleep. These ripple events are thought to support the reactivation of recent

memories and aid in their transfer from hippocampal to neocortical networks (Rasch and Born 2013). Further details on the function and impact of SWRs in memory consolidation can be found in section 1.3.2.

1.3. Sleep-dependent memory consolidation: theoretical pillars

Sleep is fundamentally intertwined with neural plasticity and memory consolidation, serving as a crucial period for the enhancement and stabilization of memory. Memory consolidation refers to the process through which memories are strengthened, stabilized, and integrated within the brain's neural architecture (Wunderlin, et al. 2021). This process ensures that new information acquired during wakefulness is retained and seamlessly woven into the existing memory network. This section will delve into the theoretical underpinnings of how sleep orchestrates these transformative processes, discussing the key mechanisms and interactions at play in memory consolidation during sleep.

1.3.1. The synaptic homeostasis hypothesis (SHY)

Neural plasticity during sleep is on the neural level explained by the synaptic homeostasis hypothesis (SHY), first proposed by Tononi and Cirelli (2006). This theory posits that sleep, particularly SWS and its associated SOs, plays a crucial role in rebalancing the brain's synaptic strengths. Throughout waking hours, learning and experiences lead to synaptic potentiation, which can saturate neuronal networks, consuming significant energy and space. During sleep, specifically through the alteration of SO up- and down-states, the brain undergoes a process of synaptic downscaling. This downscaling not only improves the signal-to-noise ratio in neural circuits but also conserves energy and frees up space, allowing the brain to return to baseline and prepare for new learning experiences the following day (see Figure 1 for a graphical representation) (Tononi and Cirelli 2006).

This principle of synaptic homeostasis is vital for maintaining an optimal distribution of synaptic strengths, preventing the saturation of neuronal networks, and ensuring the effective coding of new memories. Further supporting this, research within the field of neural plasticity has suggested that cortical SO down-states are implicated in the induction of long-term potentiation during sleep, which plays a significant role in supporting overnight memory consolidation (Chauvette et al. 2012).

Although SHY provides a major theoretical backdrop for understanding neural plasticity during sleep, it is not the primary theoretical framework for this thesis. However, it is important to discuss to ensure a comprehensive understanding and to provide a broader theoretical context.

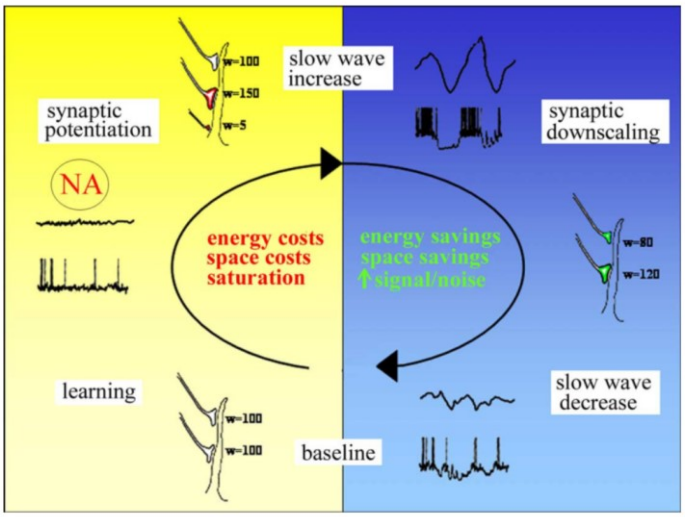


Figure 1. Taken from Tononi and Cirreli (2006). The synaptic homeostasis hypothesis postulates the differences between wake (yellow) and sleep (blue) states and their effects on the balance of synaptic strengths. Wake state is characterised by learning processes accompanied by synaptic potentiation which costs energy and space. During the sleep state, energy and space are saved by increasing the signal-to-noise ratio with synaptic downscaling during SWS.

1.3.2. The active systems consolidation hypothesis

The active systems consolidation hypothesis presents a theoretical model for understanding hippocampal-neocortical dialogue during sleep, extensively reviewed in the literature since 2007, e.g. Rasch and Born (2007); Rasch and Born (2008); Rasch and Born (2013). This model synthesizes elements from both the dual-process view, which suggests that different sleep stages support the consolidation of different types of memory (i.e., SWS supports declarative memories, while REM sleep supports non-declarative memories), and the sequential hypothesis, which emphasizes the importance of the cyclical pattern of NREM followed by REM sleep for memory consolidation. These stages are viewed as complementary in their functions within memory consolidation processes (Rasch and Born 2013).

Central to the hypothesis of active systems consolidation is the role of sleep in episodic memory consolidation, which relies heavily on the hippocampus. It describes the transfer of memory traces from temporary to long-term brain storage through a dynamic process of reactivation and integration. Specifically, the process involves fragile, newly acquired memories stored in the

hippocampus being repeatedly reactivated within the neuronal network of the specific memory trace, eventually integrating these memories into the existing neocortical knowledge network. As a result, the dependency of the memory on the hippocampus decreases while its reliance on neocortical areas increases, thereby stabilizing the memory long-term (Rasch and Born 2013).

SWS is particularly crucial to this process due to the high occurrence of slow oscillations (SOs), which are integral because of their distinct depolarized up-states and hyperpolarized down-states. During these SOs, the nesting and synchrony of various rhythms, including SOs, sleep spindles, and SWRs, facilitate the transfer of memory (Figure 2). The temporal coordination of these rhythms is vital for successful memory consolidation. Specifically, sleep spindles occurring during SO up-states and fast SWRs nested within the trough of the sleep spindle are associated with effective memory consolidation (Mikutta et al. 2019; Muehlroth, et al. 2019; Wunderlin, et al. 2021). Both spindles and SWRs induce lasting plastic changes in cortical areas, which are believed to stabilize memory during the synaptic consolidation processes that are presumed to occur during subsequent periods of REM sleep (Rasch and Born 2013).

This hypothesis forms the basis for most of the work in this thesis, providing a framework within which we explore the intricate mechanisms of CLAS affecting sleep rhythms and sleep-related memory consolidation in the context of chronic insomnia.

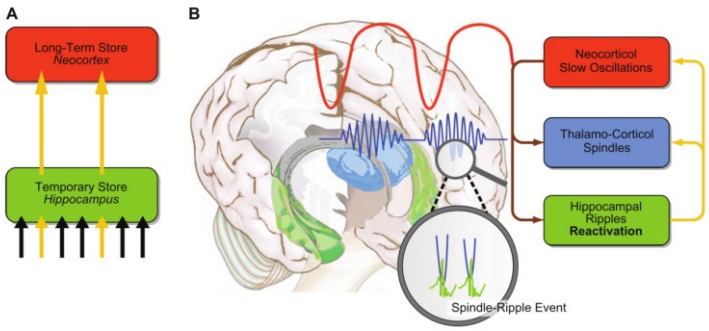


Figure 2. Taken from Rasch and Born (2013). A model of active systems consolidation in SWS describes how newly encoded declarative memories in a temporary hippocampal store are repeatedly reactivated. The memory reactivations gradually shift these memories to a long-term store of the neocortex (A). Systems consolidation during SWS thus involves a dialogue between the neocortex and the hippocampus, which is regulated by neocortical slow oscillations (in red). The depolarizing up phases of these slow oscillations trigger the repeated reactivation of hippocampal memory representations along with sharp wave ripples (in green) and thalamo-cortical spindles (in blue). This temporal synchrony facilitates the creation of spindle-ripple events, where sharp wave-ripples and the reactivated memory content become integrated into long-term neocortical storage sites (B).

1.3.3. Principle of memory reconsolidation

Memory reconsolidation is a process involving the re-stabilization of memories after they have been reactivated or retrieved from long-term storage. This re-stabilization is necessary for memories to persist over time (Nader and Hardt 2009). Within the framework of the "reconsolidation" concept, memories are understood to exist in either an active or inactive state. Initially, memories are active but unstable; through consolidation, they transform into a passive but stable form. Reactivation of these memories, however, renders them susceptible once again to interfering influences. This destabilization, as highlighted by Rasch and Born (2013), can be highly adaptive, offering an opportunity to update memories in light of new experiences.

Sleep, particularly, has a beneficial role in the reconsolidation of remote memories, acting at a faster rate than the consolidation associated with wakefulness. The mechanisms underlying this enhanced reconsolidation are likely related to SWS-related replay events, which facilitate the integration of new information with established memories (Klinzing et al. 2016). This process not only helps in reinforcing old memories but also ensures that they remain relevant and up-to-date, thereby enhancing the overall adaptability and functionality of memory storage.

1.3.4. Note on wake-dependent memory consolidation

Besides sleep-dependent memory consolidation, it is also important to acknowledge the significant body of research dedicated to wake-dependent memory consolidation. Similar to processes observed during sleep, wake-dependent consolidation involves memory reactivations, which are often facilitated by hippocampal cell firing patterns associated with SWRs. Although SWRs are more frequent during sleep, they also occur during wakefulness, albeit to a lesser extent. During wakefulness, replay of memories can occur in both forward and backward directions, contributing to the reinforcement and updating of memory traces (Rasch and Born 2013).

The replay is modulated by the theta rhythm as seen on MEG, which in turn predicted working memory performance (Fuentemilla et al. 2010). This highlights that while sleep provides an ideal state for consolidating new memories or reconsolidating old ones due to specific neural conditions, memory consolidation during wakefulness remains a crucial and prevalent process. It underscores the continuous and dynamic nature of memory consolidation that occurs across different states of consciousness, ensuring that memories are not only retained but also remain adaptable and functional in response to new information and experiences.

1.4. Note on used terminology

To ensure clarity and understanding in the introductory overview, presented findings and discussions in this thesis, it is essential to specify the terminology used throughout this work. Given the specific yet sometimes discrepant terms used in CLAS and memory consolidation literature, the following definitions are provided below.

1. **Auditory/Acoustic Stimulation**: These terms are used interchangeably in this text to refer to auditory closed-loop stimulation (CLAS), unless specified otherwise. Both terms are widely used in the literature without a clear consensus on preference (Esfahani, et al. 2023).

2. **Up Wave/Down Wave**: These terms refer to the rising and falling phases of a wave, respectively, with the dividing point being the peak (maximum of the wave). The rising phase or up-wave is defined as the portion before the peak, ascending from the minimum, while the falling phase or down-wave is the portion after the peak, descending toward zero and extending negatively to the next minimum.

3. **Down-State vs. Up-State**: These refer to the negative and positive halves of an EEG wave, respectively. The dividing point is zero; values below zero are considered the down-state, and values above zero are considered the up-state, often referenced to a neutral position such as the mastoids. These can also be referred to as the negative trough and positive peak.

4. Terms including **SOs, SWA, SW, K-Complexes, delta** and **SWS** are somewhat overlapping yet sometimes without clear distinction within the literature. For each definition as used in this work, see Table 1.

These definitions aim to standardize the use of terms within this thesis and aid the reader in understanding the nuanced distinctions and applications within the field of sleep and sleep-dependent memory consolidation neuroscience.

Type of oscillation	Definition
Slow oscillation (SO)	<1 Hz frequency
Slow wave activity (SWA)	0.5 - 2 Hz frequency
Delta waves	1 - 4 Hz frequency
Slow waves (SW), K-complex	Sometimes used interchangeably with SOs depending on frequency specification; they represent individual wave components within the broader spectrum of slow-wave sleep phenomena (Fehér, et al. 2021)

Slow wave sleep (SWS)	A sleep stage, commonly known as NREM3, characterized predominantly by high-amplitude slow waves

Table 1. Definitions for SO, SWA, SW, delta, SWS as used in this work. Each term denotes a different aspect of NREM sleep phenomena.

1.5. Applications of auditory stimulation

Auditory stimulation, particularly CLAS, offers a versatile tool for exploring the causal and functional roles of sleep across a broad spectrum of both basic research areas and clinical applications. By targeting slow oscillations (SOs) or other specific sleep rhythms, CLAS enables researchers to manipulate sleep patterns directly and observe their causal effects on various biological processes. This method has been utilized in a wide range of studies focused on memory consolidation, emotion regulation, glymphatic brain clearance, immunological processing, energy metabolism, and endocrine regulation (Esfahani, et al. 2023).

1.5.1. Memory research

The primary and original objective of CLAS was to enhance memory consolidation processes that are dependent on sleep, a field where it has garnered the most significant attention. By specifically targeting SOs, which are crucial for hippocampal-neocortical dialogue (detailed in section 1.3.2), CLAS can effectively modulate memory consolidation. This modulation may be facilitated through increased coupling of SOs and sleep spindles (Ngo, et al. 2013b).

Additionally, research has indicated a certain mechanism of spindle refractoriness under CLAS, which prevents the overdrive of SO activity, thus maintaining the integrity and effectiveness of memory processing during sleep (Ngo et al. 2015). Beyond merely enhancing memory consolidation, CLAS has also been explored for its potential to improve memory encoding. A combined EEG-fMRI study demonstrated that increased SOs and sleep spindle activity following CLAS were correlated with heightened hippocampal activation during the encoding phase of memory formation (Ong et al. 2019).

1.5.2. Somatic research

CLAS may have profound implications for understanding and modulating basic biological processes including endocrine regulation, immunological responses, and glymphatic brain clearance. The interaction between sleep, especially SWS with its SWA, and these

physiological processes underscores the integral role of sleep in various systemic functions such as cardio-metabolic health, endocrine balance, and immune functionality.

Research has demonstrated that CLAS can influence these areas. For instance, it has been shown to reduce cortisol levels, suggesting a potential for stress response reduction and improved endocrine function (Besedovsky et al. 2017; Grimaldi et al. 2019). Additionally, CLAS has been found to modulate the immune system by reducing blood T and B cell counts, indicating its potential in managing immune responses (Besedovsky, et al. 2017). However, it is important to note the negative outcomes of CLAS on glucose homeostasis, food intake, and energy expenditure, highlighting the complexity of its effects on bodily functions (Santiago et al. 2019).

1.5.3. Work-setting applications

Interestingly, the applications of CLAS extend beyond medical and biological research into practical uses in work settings, where it can enhance employee sleep and subsequently influence work behaviours. Barnes et al. (2023) found that CLAS was associated with higher levels of work engagement, improved task performance, and increased organizational citizenship behaviour. Positively, it did not significantly affect counterproductive workplace behaviour. The study also noted that the effectiveness of CLAS varied with age; younger employees benefited more from the use of sleep-enhancing headbands compared to their older counterparts. For a more detailed discussion on the implications of ageing on CLAS effectiveness, please refer to section 5.4.2 of this thesis.

These findings highlight the potential of CLAS to improve overall workplace well-being and productivity, thereby making it a prospective tool in organizational health management strategies.

1.5.4. Clinical populations

CLAS has begun to make inroads into clinical research, particularly in populations experiencing sleep disruptions, which are common in many neurological and neurodevelopmental disorders. This emerging interest is grounded in the potential of CLAS to non-pharmacologically address sleep-related disturbances across various conditions including epilepsy, amnestic mild cognitive impairment (aMCI), attention deficit hyperactivity disorder (ADHD), post-traumatic stress disorder (PTSD), and nightmares.

Fattinger et al. (2019) and Klinzing et al. (2021) have explored the impact of CLAS on patients with epilepsy. Fattinger, et al. (2019) found that CLAS neither affected spike activity nor sleep quality but did enhance the duration of N3 sleep. In a study of benign epilepsy with centrotemporal spikes by Klinzing, et al. (2021), CLAS was shown to suppress spike activity, potentially by inducing TC refractoriness, which could help in managing this condition.

In aMCI patients, while CLAS has been shown to increase SWA, its effects on memory performance were mixed, benefiting only some patients (Papalambros et al. 2019). Preliminary research by Prehn-Kristensen et al. (2020) in ADHD patients indicated that CLAS can improve procedural and working memory, although it did not appear to influence declarative memory. CLAS was also used in patients suffering from schizophrenia and showed increases in slow waves and temporally coupled sleep spindle activity during CLAS. However, no effect on the overnight declarative memory consolidation was observed (Weinhold et al. 2022).

An ongoing trial in PTSD is investigating CLAS as an adjunct therapy to eye movement desensitization and reprocessing (EMDR) therapy for PTSD. Early findings suggest good tolerability and effective targeting of SOs, despite typically reduced SO power in this population (de Boer et al. 2020; van der Heijden, et al. 2022; Van Marle et al. 2017). Further examples of possibilities for CLAS applications include suppressing nightmares (Juan et al. 2023).

While the clinical outcomes of CLAS have been modest so far, these studies underscore its feasibility and potential utility in clinical populations (Esfahani, et al. 2023). Importantly, our research extends these findings by introducing CLAS to a new study population of individuals with chronic insomnia, aiming to elucidate further the potential of this innovative therapeutic approach.

1.5.5. Chronic insomnia disorder

1.5.5.1. Definition and symptoms of insomnia

According to the International Classification of Sleep Disorders-3 (ICSD-3) (AASM 2014), chronic insomnia disorder is characterized by ongoing problems with initiating, maintaining, consolidating, or achieving quality sleep, despite having sufficient opportunity and a conducive environment for sleep. This condition leads to some form of daytime impairment. The definition of insomnia therefore inherently includes three key elements: persistent difficulties

with sleep, adequate opportunities for sleep, and resultant daytime dysfunction, all of which we generally understand as insomnia.

To diagnose chronic insomnia, several criteria must be met (AASM 2014). In adults, the patient must report difficulties such as trouble falling asleep, staying asleep, or waking up too early. The nighttime issues should lead to daytime consequences like fatigue, attention or memory problems, impaired social or professional performance, mood disturbances, excessive sleepiness, decreased motivation, and a higher likelihood of making mistakes or accidents. The sleep complaints should not be due to inadequate sleep opportunities or conditions, occur at least three times per week, persist for at least three months, and not be attributable to any other sleep disorder. Notably, the diagnosis is based purely on subjectively perceived symptoms, with no requirement to provide polysomnography in laboratory settings (AASM 2014).

In older literature and various diagnostic manuals, this condition has been referred to by several other names including chronic insomnia, primary insomnia, secondary insomnia, and comorbid insomnia most commonly. The latest ICSD-3 insomnia classification represents a significant shift from previous editions in both its conceptual approach and simplicity. Earlier versions distinguished between primary insomnia and secondary insomnia—the latter being linked to underlying psychiatric, medical, or substance abuse disorders. However, the overlap in symptoms between primary and secondary insomnia has often proven impractical and rarely reflected distinct diagnostic entities in clinical settings. Over time, even secondary insomnia tends to persist independently of its primary cause and may continue to affect the outcome of comorbid conditions if not treated. This has led to the recognition of insomnia as a comorbid disorder that benefits separate treatment attention and a more recent diagnostic term of chronic insomnia (AASM 2014).

1.5.5.2. Prevalence and impact of insomnia

Insomnia disorder is a common condition, affecting about 30% of the general population at some point, while an additional 20% experience occasional symptoms (Morin and Jarrin 2022), with variations depending on the specific criteria and populations studied. Population-based studies across various countries consistently find that around 30% of adults report experiencing at least one symptom of insomnia (Ancoli-Israel and Roth 1999). However, when diagnostic criteria include perceived daytime impairment or distress and duration of more than 1 month,

so the formal criteria for chronic insomnia are met, the prevalence estimate drops to about 6-10% (Morin and Jarrin 2022; NIH 2005; Ohayon 2002).

Insomnia tends to be a chronic issue, persisting at a rate of 40% over five years (Morin et al. 2020). The COVID-19 pandemic has further exacerbated insomnia and related psychological symptoms, with higher rates of persistence than remission during the pandemic's first year (Meaklim et al. 2024; Morin et al. 2021). Certain demographics and factors, including age, gender, lower socioeconomic status, medical and psychiatric conditions, and shift work all elevate the risk of developing chronic insomnia (Morin and Jarrin 2022).

Chronic insomnia not only leads to absenteeism, frequent accidents, and memory issues but also increases healthcare utilization significantly. Among the most severe impacts of insomnia is a heightened risk of developing depression (Roth and Roehrs 2003). Given its widespread prevalence and severe repercussions, insomnia represents a significant public health issue that necessitates targeted clinical interventions and broader sleep health initiatives at the population level.

1.5.5.3. Treatment approaches to insomnia

Cognitive-behavioural therapy for insomnia (CBT-I) is universally recommended as the first-line treatment for chronic insomnia in adults, applicable across all age groups, including those with comorbid conditions (Riemann et al. 2023). CBT-I strategically targets the maladaptive sleep habits, irrational beliefs about sleep, and hyperarousal that often characterize insomnia. The therapy employs a variety of techniques aimed at improving sleep quality and hygiene, including psychoeducation, sleep hygiene education, relaxation training, sleep restriction therapy to optimize sleep efficiency, and stimulus control therapy to reinforce positive sleep associations (Riemann, et al. 2023). Additionally, cognitive techniques such as cognitive control ("putting the day to rest" before bedtime), paradoxical intention (abandoning the effort to sleep), imagery training (active visualization exercises), and cognitive restructuring (challenging negative thoughts) are integral components of CBT-I (Riemann, et al. 2023).

Despite the evidence-based preference for CBT-I, pharmacotherapy remains widely utilized, with around 60% of insomnia patients (in 2008-2009) receiving benzodiazepines or related medications (Hoebert et al. 2012). Pharmacological interventions should be considered when CBT-I does not lead to sufficient improvement. Short-term treatments include benzodiazepines, benzodiazepine receptor agonists, daridorexant, and low-dose sedating antidepressants,

generally limited to a duration of up to four weeks due to the potential for developing tolerance (Riemann, et al. 2023; Winkler et al. 2014). In some cases, longer-term treatments may be considered, weighing the advantages and disadvantages carefully.

Additionally, orexin receptor antagonists and prolonged-release melatonin are options for periods extending up to three months, especially in older patients. However, antihistaminergic drugs, antipsychotics, fast-release melatonin, ramelteon, and phytotherapeutics are generally not recommended for treating insomnia due to limited supportive evidence (Riemann, et al. 2023).

Adjunct therapies such as light therapy and exercise interventions are also seen as beneficial when combined with CBT-I. These alternatives, including light (blocking) therapy (Shechter et al. 2018; van Maanen et al. 2016) and exercise modalities like tai chi (Amiri et al. 2021; Han et al. 2022), and music therapy (Chen et al. 2021) show promise in complementing standard treatments, though the efficacy of other non-conventional treatments like homeopathy and acupuncture remains uncertain due to the limited quality of the supporting studies (Baglioni et al. 2020; Ernst et al. 2011; Cheuk et al. 2012). Given the available evidence, or the lack thereof in some cases, it is premature to recommend these treatments as standalone interventions for insomnia. However, integrating elements of exercise and light therapy with CBT-I may offer additional benefits, enhancing the overall therapeutic outcome for individuals suffering from insomnia (Riemann, et al. 2023). Further high-quality studies are needed to better define the effectiveness and applicability of these treatments.

1.5.5.4. Non-invasive stimulation in insomnia

In the pursuit of novel effective treatments for insomnia, several non-invasive brain stimulation techniques have been explored. These include rTMS (Sun et al. 2021), tDCS/tACS (Ma et al. 2021), vagus nerve stimulation (Wu et al. 2022; Zhang et al. 2023) and brain cooling (Roth et al. 2018). In the realm of auditory stimulation approaches, a recent study by Bressler et al. (2024) conducted a randomized controlled trial (RCT) on the efficacy of alpha phase-locked auditory stimulation for treating sleep onset insomnia. The results showed a significant effect on SOL, with a reduction of 10.5 ± 15.9 minutes. Authors suggest that phase-locked acoustic stimulation could serve as a viable non-pharmaceutical alternative to accelerate sleep onset in individuals experiencing prolonged sleep onset latencies (Bressler, et al. 2024).

The abovementioned methods are tested for their potential to alleviate various symptoms of insomnia, offering alternatives to widely used pharmacological approaches. However, the overall body of research on non-invasive brain stimulation for insomnia treatment often faces methodological challenges. Issues such as the lack of blinding and insufficient sham controls frequently limit the interpretability of these studies. As highlighted by Krone et al. (2023) and Riemann, et al. (2023), the therapeutic benefits of current non-invasive brain stimulation protocols are likely overestimated and at present, there is no consensus recommending the use of these brain stimulation approaches as a definitive treatment strategy for insomnia. Further, high-quality RCTs are needed to better understand the usefulness of these alternative approaches.

Importantly, our research contributes to this body of research on novel alternative approaches by introducing CLAS in chronic insomnia for the first time, aiming to explore the feasibility and effectiveness of CLAS during SWS in insomnia.

1.6. Mechanisms underlying auditory stimulation: neural and technical perspectives

1.6.1. Brain processing of auditory stimuli during sleep

Auditory stimuli are processed through two distinct neural pathways: the lemniscal (primary) and non-lemniscal (secondary) pathways (Figure 3). The lemniscal pathway transmits signals sequentially through the brainstem, thalamus, and auditory cortex, a process that can be observed in EEG recordings (Atienza et al. 2001). In contrast, the non-lemniscal pathway merges auditory signals with other sensory inputs within the reticular formation to determine their priority. For conscious awareness to occur, these two pathways must meet at the level of the thalamus. Although the lemniscal pathway operates similarly during both sleep and wakefulness, the non-lemniscal pathway's ability to communicate is restricted during sleep, which limits the integration of combined sensory data for conscious perception (Atienza, et al. 2001; Hu 2003). A recent study using simultaneous EEG and MEG has illustrated that during CLAS, auditory information is relayed to the ventral frontal lobe regions via the non-lemniscal pathways, where SOs are generated and propagated (Jourde et al. 2024).

Additionally, studies utilizing simultaneous EEG and functional magnetic resonance imaging (fMRI) have revealed that auditory stimulation during sleep not only modulates sleep oscillations but also induces extensive activity across cortical areas involved in cognitive processing (Czisch et al. 2009; Dang-Vu et al. 2008; Dang-Vu et al. 2010; Fang et al. 2019;

Schabus et al. 2007). Despite the finding that regional brain activation during auditory processing in sleep differs from wakefulness, there is evidence suggesting humans can still differentiate between sounds of varying subjective importance while asleep (Legendre et al. 2019; Portas et al. 2000). This indicates a sophisticated level of auditory processing that can persist even during sleep, highlighting the brain's remarkable capacity to handle auditory sensory information under different states of consciousness.

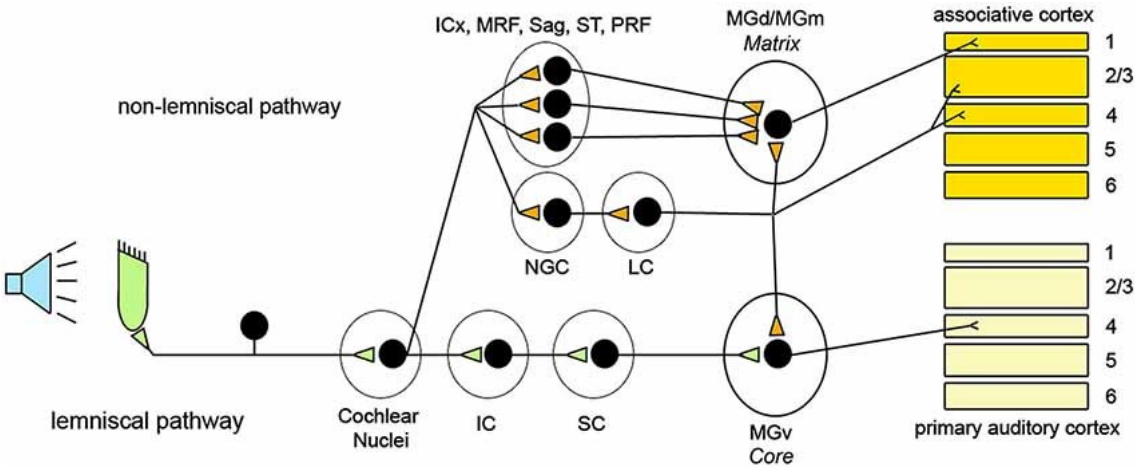


Figure 3. Taken from Bellesi et al. (2014). Schematic depiction of the structure of the ascending lemniscal and non-lemniscal auditory pathways and their associated targets in the thalamus and cerebral cortex. During CLAS, auditory stimuli are relayed via the non-lemniscal pathway (Jourde, et al. 2024). ICx: shell of the inferior colliculus; MRF: midbrain reticular formation; Sag: sagulum; ST: spinothalamic tract; PRF: pontine reticular formation; NGC: nucleus gigantocellularis; LC: locus coeruleus; IC: inferior colliculus; SC; superior colliculus; MGd: medial geniculate dorsal; MGm: medial geniculate caudo-medial; MGv: medial geniculate ventral.

1.6.2. SOs vs. K-complexes

The differentiation of spontaneous and evoked K-complexes versus SOs continues to be a subject of ongoing scientific debate (Esfahani, et al. 2023) and is of relevance to this thesis. K-complexes are characterized by near-simultaneous depolarization across widely distributed cortical areas, triggered by diffuse cortical projections (Amzica and Steriade 2002; Fehér, et al. 2021). They are defined as sharply delineated negative waves immediately followed by a positive component, distinctly standing out from the background EEG activity, with a duration of at least 0.5 seconds and typically most prominent in frontal derivations during NREM2 (AASM 2020).

The generation of evoked K-complexes involves a combination of non-specific brain responses to sensory stimuli and specific sensory responses observed in the primary sensory cortex

(Czisch, et al. 2009; Numminen et al. 1996). While K-complexes and SOs may share underlying cellular mechanisms (Steriade, et al. 1993), K-complexes in contrast to SOs are singular cortical biphasic waves characterized by a down-state followed by an up-state.

In scalp EEG, intracranial SOs are often represented as K-complexes, functioning as a protective mechanism in sleep by shielding against external noise, thereby preventing unnecessary arousals and awakenings (Esfahani, et al. 2023). Additionally, while SOs during N2 sleep are sometimes referred to as K-complexes, it is suggested that K-complexes and the SOs observed in N3 sleep may originate from two distinct synchronization processes that involve different subcortical and intracortical mechanisms (Fernandez and Lüthi 2020). This distinction implies that there could be sleep-stage-dependent differences in how SO-spindle couplings are mechanistically generated and how they contribute to memory formation.

In the context of CLAS, literature also differentiates the N550–P900 complexes, which are specific types of evoked SOs observed during N2 and N3 sleep stages that appear approximately 550 to 900 milliseconds following the sensory input (Bellesi, et al. 2014; Latreille et al. 2020; Riedner et al. 2011). These complexes are closely associated with, and frequently identified as, K-complexes, though K-complexes may also occur independently of external stimuli (Jourde, et al. 2024).

1.6.3. CLAS setup: methodological and technological aspects

The standard setup for CLAS involves a combination of methodological precision and advanced technology to study and/or enhance sleep function. The typical experimental schema for CLAS includes real-time recording of scalp EEG, which is instrumental in monitoring brain activity to detect specific sleep oscillations such as SOs. During the stable NREM3, upon successfully detecting an SO, the system either administers an auditory stimulus in the stimulation condition or places sham markers in the sham condition without actual stimulation of sounds being played. The process involves setting a threshold based on amplitude or other predictive methods to identify the trough of an oscillation, which is then marked for stimulation or sham stimulation release.

From the technical perspective, three key components are therefore essential for successful CLAS administration as mentioned in Esfahani, et al. (2023): 1) access to ongoing brain activity through continuous EEG monitoring, 2) detection algorithm which analyses the ongoing EEG

data to pinpoint the sleep rhythms of interest, 3) stimulation device responsible for delivering the sound pulse.

To ensure the effectiveness and reliability of CLAS, each study must tailor its methodological setup based on specific experimental conditions, which include the laboratory equipment available, the studied population, and the computer processing power at hand. Additionally, adaptation nights are often recommended to optimize individual parameters (Esfahani, et al. 2023). Only this comprehensive approach ensures that CLAS not only adheres to rigorous scientific standards but also adapts flexibly to meet the diverse research needs of various studied populations.

1.6.3.1. Access to brain activity using EEG

EEG monitoring in auditory stimulation studies, particularly those involving CLAS, requires real-time and continuous observation, with a crucial understanding of potential delays at various stages. These delays include: 1) from the brain to the amplifier, where the initial EEG signals are captured; 2) from the amplifier to the server, where the data are transmitted for processing; 3) during processing, which encompasses the detection algorithm and filtering; 4) in the triggering of the stimulus; and 5) within the stimulus pathway itself, from the moment the stimulus is released to when it reaches the speakers and ultimately the participant's ears. Accurately measuring these delays is essential, as they must be carefully considered in the design and execution of the study. Understanding these time lags is critical for interpreting the effects of CLAS, as any misalignment could skew results and lead to incorrect conclusions (Esfahani et al., 2023). For a more in-depth discussion of delays in the present work, please refer to section 5.3.3.

1.6.3.2. Different detection algorithms

The detection of sleep oscillations such as SOs for stimulation employs a variety of algorithmic approaches, each with its method of identifying oscillations of target:

1. **Amplitude-Based Thresholds**: This is the simplest and most direct approach, utilized in studies 1 and 2 of this thesis. It involves filtering the EEG signal within a specific target frequency range (e.g., 0.25-1.5 Hz for SOs). When the amplitude within this range crosses a predefined threshold (e.g., -75 microvolts), the algorithm identifies it as an SO, detects its minimum, and then triggers the sound stimulus. While this method is computationally fast and

simple, it does not adapt to overall variations in EEG amplitudes, which can affect its accuracy in hitting the correct phase of an SO.

2. **Prediction-Based Thresholds**: These more advanced approaches model the sinusoidal wave of SOs and predict their occurrence. From the literature, three examples and the most common approaches were chosen to be overviewed and included:

1) Cox approach utilizes a Hilbert-transform to extract instantaneous SO and fits a sine wave to the most recent data segment (Cox et al. 2014). A similar method using endpoint-corrected Hilbert transform is applied to faster sleep rhythms such as αCLAS (Hebron et al. 2024).

2) Phase-locked loop (PLL) approach continuously tracks phase alterations of SOs on filtered data. This approach is a topic of Study 2 in this dissertation, with more details provided in section 3.2.5.

3) Talamini approach employs a sine fitting procedure on raw data, modelling the full oscillatory dynamics of the signal, which helps avoid signal distortions from filtering and other data transformations (Juan, et al. 2023; Pathak et al. 2021; Talamini and Van Poppel 2019). This method is assumed to accurately target any phase within the 360-degree range of the target oscillation (Esfahani, et al. 2023).

3. **Topographic Thresholds**: This novel approach employs topographic targeting of SOs (TOPOSO). This method detects SO activity in specific cortical regions based on the correlation between instantaneous and precomputed voltage maps typical for SO up- and down-states (Fehér et al. 2023; Ruch et al. 2022). Its strength lies in its high specificity, ensuring the detected SOs are indeed true SOs. It was shown to reliably target up-waves over frontal, sensorimotor, and centro-parietal regions. On the other hand, this method offers less sensitivity as it detects fewer events (Esfahani, et al. 2023).

4. **Faster Frequency Detections**: This umbrella term for methods targeting faster frequencies includes envelope-based detections and other types of detections. These methods are ideal for rhythms with specific profiles such as sleep spindles (Hassan et al. 2022; Choi and Jun 2022; Choi, et al. 2019) or faster rhythms such as REM theta (Harrington et al. 2021a). Development of these algorithms is ongoing, targeting faster sleep rhythms with a specific profile. These methods require significant computational power to achieve the necessary precision (Esfahani, et al. 2023).

1.6.3.3. Stimulus release

The release of auditory stimuli during CLAS experiments is managed through a stimulation device, which generally comprises a sound card and an output medium such as speakers or earphones. The device receives a signal from the detection algorithm and administers the stimulus directly to the participant.

In terms of delivery, stimuli can be administered using in-ear or out-of-ear headphones, or through external speakers. Each method has its advantages and practical considerations. Headphones, whether in-ear or out-of-ear, provide direct delivery of sound to the ears and allow for precise control over the volume and administration of the stimulus. However, using headphones introduces the need for additional equipment—such as cables and earphones—that participants must wear, which can potentially affect comfort, movement during sleep, and overall sleep quality. Moreover, securing the headphones to ensure they do not dislodge or fall out during the night is often a necessary precaution in laboratory settings. As a side note, there are headphones specifically designed for sleep that might offer better comfort and stability compared to standard models (see section 3.1.4. for specifics used in our laboratory).

Conversely, using external speakers eliminates the discomfort associated with wearing headphones and the need to manage cables and other equipment, enhancing participant comfort. However, this setup sacrifices some control over the volume and simultaneous and more precise delivery of the stimulus to both ears. The sound from external speakers may not be as consistently delivered bilaterally, potentially affecting the efficacy of the stimulus.

Overall, the choice between headphones and speakers largely depends on the specific requirements of the study and the balance between participant comfort and the precision of stimulus delivery. Each setup requires careful consideration of how it might influence both the scientific outcomes and the participant's sleep in an undesired way.

1.7. Introducing Study 1: CLAS in chronic insomnia

Insomnia ranks as the second most prevalent mental disorder in Europe, affecting approximately 10% of the population and significantly impacting public health (Ohayon 2002; Wittchen et al. 2011). This condition not only heightens the risk of cognitive impairments and mood disturbances but also predisposes individuals to serious mental disorders, including depression (Baglioni et al. 2011). While CBT-I remains the first-line treatment, complemented

by various pharmacotherapies, a notable portion of the patient population either does not respond or finds these treatments insufficient (Murtagh and Greenwood 1995; Okajima et al. 2011). Furthermore, certain interventions like sleep restriction can be demanding and entail undesirable side effects, paralleling some challenges associated with pharmacotherapy (Atkin et al. 2018; Kyle et al. 2014).

The diagnosis of insomnia typically relies on subjective complaints, yet objective measurements such as increased sleep fragmentation, decreased SWS, and reduced REM sleep have been consistently observed in insomnia sufferers through polysomnography (Baglioni et al. 2014). Both SWS and REM sleep are crucial for memory processing; disruptions in these sleep stages can significantly affect daytime functioning due to impairments in memory consolidation and emotional processing (Backhaus et al. 2006; Cellini et al. 2014; Nissen et al. 2011; Wassing et al. 2019). Insomnia is also associated with continuous cortical activation indicative of autonomic and central nervous system hyperarousal, which manifests as elevated activity in faster EEG frequencies and reduced delta activity (Riemann et al. 2010; Svetnik et al. 2017).

Given these complexities, non-invasive brain stimulation techniques, particularly those targeting specific sleep characteristics or aiming to reduce arousal, emerge as promising alternatives (Geiser et al. 2020). Recent research suggests that while CLAS may not alter sleep macrostructure, it can modulate specific sleep dynamics, promoting slow sleep oscillations, sleep spindles, and memory consolidation (Ngo, et al. 2013b; Papalambros et al. 2017; Stanyer, et al. 2022). Moreover, CLAS could potentially diminish sensitivity to external noise, thereby protecting sleep (Pathak, et al. 2021).

1.8. Introducing Study 2: Excitation of SOs using CLAS

Sleep serves as a crucial platform for memory consolidation, particularly during deep sleep phases, where SOs dominate (details in sections 1.2. – 1.3.). SOs, oscillating within a frequency range of 0.5 Hz to 1.0 Hz, play a pivotal role in the neocortical-hippocampal dialogue that facilitates the replay and redistribution of memories into long-term neocortical stores (Rasch and Born 2013). This process underscores the importance of enhancing SOs during sleep to boost memory consolidation.

Historically, various stimulation methods including electrical, olfactory, and notably, acoustic stimulation, have been explored to augment SOs and thereby improve memory consolidation

(Marshall, et al. 2006; Marshall, et al. 2004; Ngo, et al. 2013b; Rasch, et al. 2007). The technique of synchronized auditory stimulation has shown particular promise in modulating SOs and enhancing memory consolidation by targeting the up-phase of SOs (Ngo et al. 2013a; Ngo, et al. 2013b). This phase of the SO wave is considered optimal for auditory stimulation due to its potential to influence memory processing positively (Cox, et al. 2014; Ngo, et al. 2013b).

Pioneering studies, such as those by Ngo, et al. (2013a); Ngo, et al. (2013b) and Besedovsky, et al. (2017), utilized two-phase controlled auditory stimulation, where an initial negative peak of an SO was detected, followed by precisely timed auditory stimuli. These studies have laid the groundwork for subsequent research exploring the synchronization of auditory stimulation with EEG signals to facilitate memory consolidation. Techniques like the PLL method have been adapted for EEG signal stimulation, providing a fine-tuned approach to enhancing the synchronization between auditory stimuli and the natural oscillatory dynamics of sleep (Ong et al. 2016; Papalambros, et al. 2017; Papalambros, et al. 2019).

Research has consistently aimed to refine these stimulation methods to maximize their efficacy across various demographic groups, including the elderly, and those with psychiatric and cognitive disorders (section 1.5.4). The precision of stimulation timing has been a focal point of recent studies, seeking to optimize the modulation of SOs for therapeutic outcomes (section 5.5.2.2).

Our study extends this research trajectory by focusing on chronic insomnia—a condition prevalent among the elderly and characterized by significant sleep disruption and a consequential impact on life quality. Chronic insomnia not only affects up to 30% of the adult population at some point during their life (Morin and Jarrin 2022) but also poses risks for several health complications (Roth and Roehrs 2003). The disorder is often marked by a decrease in SWA (Merica et al. 1998), making it a prime candidate for SO stimulation strategies.

2. Aims and Hypotheses

2.1. Study 1: CLAS in chronic insomnia

The aim of Study 1 was to assess, for the first time, the feasibility of CLAS in insomnia and its effect on sleep macro- and microstructure. We hypothesized there would be no alterations of sleep macrostructure after CLAS, but that the CLAS would enhance the SOs and SWS activity as well as the duration, density, and amplitude of sleep spindles. We hypothesised these changes were related to the improvement in a declarative memory task and increased subjective sleep quality. As a part of the exploratory analysis, we aimed to assess changes in alpha and beta EEG bands to see the possible effect of CLAS on arousal during sleep.

2.2. Study 2: Excitation of SOs using CLAS

The aims of the Study 2 were twofold. Firstly, we aimed to quantitatively compare three currently most widely used CLAS methods (fixed-step, two versions of phase-locked loop, PLL). Specifically, we studied which of the three methods was most precise in detecting SOs and their target phase. As part of the first aim, we further developed new technical CLAS solutions for additional testing. Secondly, to elucidate the mechanisms behind the CLAS effect on SOs, we aimed to answer an important question of whether CLAS induces new SOs or modulates naturally occurring and already existing SOs.

3. Methods

3.1. Study 1: CLAS in chronic insomnia

3.1.1. Participants

A group of 27 volunteers suffering from chronic insomnia was gathered through recruitment at the Department of Sleep Medicine of the National Institute of Mental Health, Czech Republic (NIMH-CZ), as well as online advertisements. To be included in the study, participants had to meet specific criteria: (a) a confirmed diagnosis of chronic insomnia based on the criteria outlined in the ICSD-3 (AASM 2014), (b) self-reported difficulties with maintaining sleep, and (c) being at least 18 years old. Exclusion criteria included: (a) the use of sleep-affecting medications, (b) significant psychiatric, neurological, or somatic comorbidities, (c) consumption of cognitive stimulants (such as caffeine, tobacco, or energy drinks) within 6 hours before the study, and (d) extreme chronotype. The research protocol was approved by the Ethics Committee of the NIMH-CZ, and all participants provided informed consent before their involvement in the study.

3.1.2. Design and procedure

A study employing a within-subject, randomized, crossover, sham-controlled design was conducted. Following the completion of a screening questionnaire to determine eligibility, participants spent two experimental nights at the NIMH-CZ sleep laboratory, with a minimum interval of 7 days between the experimental nights. During the experiment, participants were subjected to either verum stimulation (CLAS) or sham stimulation (SHAM), with the order of conditions randomized and balanced across participants. Participants were blinded to the assigned condition during the experiment. In the SHAM condition, the same SO detection procedure was followed as in the stimulation condition, but no sound was delivered through the headphones. Participants received instructions to abstain from napping, alcohol, caffeine, and drugs on the days of the experiments.

Before and after each experimental night, participants completed sleep quality scales, questionnaires, and a word-pair memory task (Dudysova et al. 2016). Lights were turned off at approximately 10:00 p.m., with slight variations to accommodate individual differences in sleep preparation times. Each participant was allotted an 8-hour opportunity to sleep during which polysomnography (PSG) was recorded. They were awakened at approximately 6:00 a.m.

3.1.3. Polysomnography

Overnight PSG was conducted, capturing an array of physiological signals. Each recording comprised EEG channels following the 10/20 standard system, along with electrooculography, electromyography (three submental electrodes), electrocardiography, and video monitoring. The EEG channels encompassed seven sites (Fpz, F3, F4, C3, C4, P3, and P4), referenced contralaterally to mastoids (M1 and M2) on the scalp. Data acquisition utilized the Brainscope PSG system (M&I spol. s.r.o., Czech Republic), employing a band-pass filter (0.1–200 Hz) and a sampling rate of 1000 Hz.

Two independent raters visually assessed all recordings following the criteria outlined by the American Academy of Sleep Medicine (AASM 2020). Analysis and calculation of sleep macrostructure parameters, such as duration (in minutes) and proportion (as a percentage) of total sleep time (TST), wakefulness after sleep onset (WASO), number of arousals, and time spent in each sleep stage (NREM1, NREM2, NREM3, REM), were performed using the 'EEG Viewer' software, version 2019 (Unimedis s.r.o., Czech Republic). Arousal events (3-15 s) were identified visually for both experimental conditions, and the arousal index (number per hour) was computed.

3.1.4. CLAS

Following the original methodology outlined by Ngo, et al. (2013b), acoustic stimulation was administered during the initial two sleep cycles occurring in SWS. Upon visual detection of the SWS stage by the experimenter, while ensuring SWS stability for a minimum of 3 minutes, the stimulation protocol was initiated manually. In the event of movement, awakening, or transition to a different sleep stage, the stimulation was manually halted by the investigator until the next stable occurrence of SWS.

A Python application was utilized for the real-time identification of slow oscillations (SOs) from the averaged signal at the F3 and F4 electrodes. The threshold for detecting SOs was established at -80 μV for participants aged ≤ 30 (as per Ngo, et al. (2013b), and at -40 μV for participants over 30 years old (as per Papalambros, et al. (2017), ensuring sufficient stimulation across all ages.

CLAS was synchronized and targeted with the SO up-states. Following the detection of the negative half-wave peak of the SO, two consecutive pink noise pulses (1/f) were administered,

each lasting 50 ms. Pink noise was chosen for its softer and more comfortable auditory perceptual characteristics, commonly utilized in sleep research (Ngo, et al. 2013b; Papalambros, et al. 2017). The first stimulus (pulse) coincided with the predicted up-phase of the detected SO, followed by the second stimulus 1.075 s later. Following these pulses, a 2.5-second pause ensued before the detection process resumed. Auditory stimuli were presented binaurally through soft, all-rubber headphones suitable for sleep (Maxrock, model: EL-273707). The sound intensity was individually adjusted by each participant before each experimental night to a level that was detectable but non-disruptive.

The detection procedure during the SHAM condition remained the same, but no auditory stimuli were delivered. The timing of predicted stimulations (sham stimuli) was annotated in the PSG recording for subsequent analysis.

3.1.5. Acute effect of CLAS analysis

All analyses were conducted using MATLAB, R2019a software (MathWorks Inc., Natick, Massachusetts, 2015). An identical analysis procedure was applied to both CLAS and SHAM recordings. To ensure the accuracy of CLAS, a phase analysis was performed utilizing the Matlab toolbox FieldTrip (Oostenveld et al. 2011). Data underwent finite impulse response (FIR) bandpass filtering (0.5 to 4.0 Hz) with a demean filter. The mean of the F3 and F4 electrodes was computed. Outliers were identified on the segments/trials level, with entire segments defined as outliers if they exceeded three interquartile amplitude ranges above the upper quartile (75%) or below the lower quartile (25%).

Four-second segments (1 s before and 3 s after the first stimulation) were extracted, and phase values were derived using the Hilbert transformation for both the 1st and 2nd stimulation during both SHAM and STIM conditions. Relative spectral powers were calculated as a ratio of the power of individual bands to the total power within the range of 0.5 to 30.0 Hz.

The analysis of relative power regarding the effect of CLAS followed a consistent procedure, i.e. filtering between 0.5 and 30.0 Hz (with a demean filter), and the same criteria were applied for outliers. Relative power spectral analysis was conducted on 4-second segments of on and off intervals of EEG recordings, with on intervals starting 1 second before detection and off intervals beginning 1 second after the 2nd stimulation. The multitaper method with a Hanning window was utilized for power-spectra estimation, focusing on SOs (0.5 to 1.0 Hz) and delta bands (1.0 to 4.0 Hz).

The effect of CLAS was analysed based on EEG segment amplitudes for both CLAS and SHAM conditions. Segments were aligned around the CLAS/SHAM time-point, and analysis was performed on the Fpz electrode. Amplitude and baseline-corrected amplitude were assessed using a non-parametric statistical test with cluster-based correction for multiple comparisons (Maris and Oostenveld 2007). Outliers exceeding an amplitude of 300 μV or falling below 10 μV were excluded from the analysis.

3.1.6. EEG power spectral analyses

All analyses were conducted using MATLAB, R2019a software (MathWorks Inc., Natick, Massachusetts, 2015). The same analysis protocol was applied to both CLAS and SHAM EEG recordings. Manual visual artefact rejection, addressing movement and electrode artefacts, was performed in 5-second segments. Segments containing any artefact were discarded entirely, irrespective of whether the artefact affected only a portion or the entirety of the segment.

Initially, power spectral analyses focused on all SWS episodes throughout the entire CLAS vs. SHAM night. The EEG analysis encompassed frequency bands such as slow oscillations (SOs, 0.5-1.0 Hz), delta (1-4 Hz), and sigma (12-15 Hz, corresponding to the frequency of fast sleep spindles). Both absolute and relative power spectral values were computed following the methodology outlined by Mander et al. (2013), with relative power calculated as a proportion of total power.

Subsequently, analyses were separately conducted on the first two sleep cycles, as CLAS was administered then. Additionally, to ascertain whether CLAS affected faster EEG activity indicative of heightened arousal, alpha (8-12 Hz), beta1 (15-20 Hz), and beta2 (20-30 Hz) bands were checked. These analyses were performed across all electrodes (C3, C4, F3, F4, P3, and P4) during SWS.

3.1.7. Sleep spindle detections and analysis

To investigate sleep spindles, we employed an automated detection method adapted from prior research (Ferrarelli et al. 2007), focusing on the frequency band of fast sleep spindles (12-15 Hz). The analysis encompassed six channels (F3, F4, C3, C4, P3, and P4) across all SWS intervals throughout the night. Sleep spindles were examined for their density (#/min), the average duration (s), and the power-integrated spindle amplitude (μV/min).

3.1.8. Memory task

We utilized a Czech version of the word-pair association learning (CZ-PAL) task, as described by Dudysova, et al. (2016), to evaluate overnight declarative memory performance during both experimental nights. This methodology has been employed in various research examining declarative memory and its relationship to sleep (Marshall, et al. 2004; Payne et al. 2012), as well as in investigations exploring the impact of acoustic stimulation during sleep on memory consolidation (Ngo, et al. 2013b; Papalambros, et al. 2017).

Each participant completed two parallel versions of the test, presented in a randomized and balanced order. The task comprised 120 moderately semantically related word pairs (e.g., apple-peach, consciousness-brain), presented in random order during each testing session. Participants viewed all 120 word pairs on a computer screen for 4 s each, with a 1-second inter-stimulus interval. The first word served as a cue, while the second word required later recall (target word). Participants were instructed to memorize as many pairs as possible.

Following the learning phase, participants underwent a recall test with feedback, where the same word pairs were presented in a different random order. This test phase allowed for both new learning and confirmation of already learned pairs. After a 5-minute break, participants completed a second recall test without feedback, with the word pairs presented in a different order. The fourth and final phase of the task took place in the morning, at least 30 minutes after participants woke up and proceeded in the same fashion as Evening Recall.

Overnight memory consolidation was quantified as the difference between the number of correctly recalled pairs in the morning and evening. This difference was then divided by the number of correctly recalled pairs in the evening and multiplied by 100.

3.1.9. Self-reported scales and questionnaires

A set of questionnaires was administered to assess various aspects of an individual's subjective sleep. The Epworth Sleepiness Scale (ESS) (Johns 1991) was utilized to assess daytime sleepiness levels, while the Morningness – Eveningness questionnaire (MEQ) (Horne and Ostberg 1976) was employed to identify and exclude extreme chronotypes. Furthermore, the Beck Depression Inventory-2 (BDI-II) (Beck et al. 1961) and Beck Anxiety Inventory (BAI) (Beck and Steer 1993) were utilized to monitor the severity of self-reported symptoms of depression and anxiety.

To assess subjective sleep quality, participants also answered 3 questions during the experimental nights. In the morning following the experiment, participants rated their perceived level of restfulness on a 3-point Likert scale (completely rested – partly rested – not rested at all). The second question required participants to evaluate the quality of their sleep during the experimental night using a 4-point Likert scale (very bad – bad/superficial – pretty good – good/refreshing). Lastly, participants indicated the number of hours of sleep they perceived to have obtained.

3.1.10. Statistical analysis

A statistical analysis of the sleep macrostructure was performed via the Wilcoxon signed-rank test with false discovery rate (FDR) correction for multiple comparisons. The evaluation of the acute effect of CLAS was performed by the paired non-parametric Wilcoxon test with cluster-based correction for multiple comparisons. The CircStat toolbox (Berens 2009) for MATLAB was used for the descriptive statistical evaluation of the phase targeting, including the mean and spread for detection, SHAM and both CLAS pulses. For the whole night power spectral analyses, we performed via the Wilcoxon signed rank test with FDR correction. The relative overnight memory consolidation was used for a comparison between CLAS and SHAM conditions also via Wilcoxon signed ranks test.

For all statistical analyses, we reported means (M), standard deviations (SDs), and effect sizes (r) where appropriate. All analyses were done using the IBM Statistical Package for the Social Sciences (SPSS®; IBM Corp., Armonk, NY, USA) and MATLAB software (MathWorks Inc., Natick, MA, USA).

3.2. Study 2: Excitation of SOs using CLAS

3.2.1. Participants and experimental design

A subset of participants from Study 1 was used as a basis for analyses of Study 2. Therefore, the design and procedure apply as mentioned above (see sections 3.1.1.-3.1.4 for details).

The analysed dataset in Study 2 comprised 18 recordings obtained from 9 subjects (aged 20–52, M = 25.67, SD = 10.10, 3 women). As mentioned above, each participant underwent both CLAS and SHAM sleep EEG recordings during 2 nights. Pink noise stimulations/pulses were administered to subjects during the CLAS night, with individual sound levels determined subjectively by each participant to ensure the stimulus was audible yet not disruptive. The fixed-

step method of stimulation (Ngo, et al. 2013b) was utilized. The SHAM night replicated these conditions, with subjects wearing headphones but no sound was presented.

The duration of all included EEG recordings averaged 7.84 ± 0.12 hours (M ± SEM). The mean number of detected SOs in real-time measurement was 142, with a minimum of 2 and a maximum of 413 detections. Recordings were included in the analysis if they exhibited at least 50 detections.

3.2.2. Comparison of CLAS methods - simulations of real-time CLAS

Simulations of real-time CLAS involving offline EEG data re-streaming, were conducted using MATLAB software, release 2020a (The MathWorks, Inc., Natick, MA, USA). These simulations aimed to compare different stimulation methods, specifically, the fixed-step stimulation (Ngo, et al. 2013b; Ngo, et al. 2015) method and the PLL method (Santostasi et al. 2016). Original EEG recordings sampled at 1000 Hz were utilized for this purpose, as illustrated in Figure 4. Data was imported into MATLAB using the easys2matlab toolbox (Piorecka 2021).

For subsequent analysis, uninterrupted segments of NREM2 and NREM3 EEG sleep recordings were selected. These NREM stages were chosen based on expert scorers' assessments of individual sleep phases. Only segments with a duration of at least 5 minutes were included in the analysis. The average duration of the NREM segments was 5.58 ± 0.22 hours (M ± SEM). Table 2 provides the total number of detection and stimulation events for both the PPL-XOR method and the fixed-step method. The objective of this analysis was to assess the fixed-step and PLL stimulation methods and to examine their detection and stimulation phases.

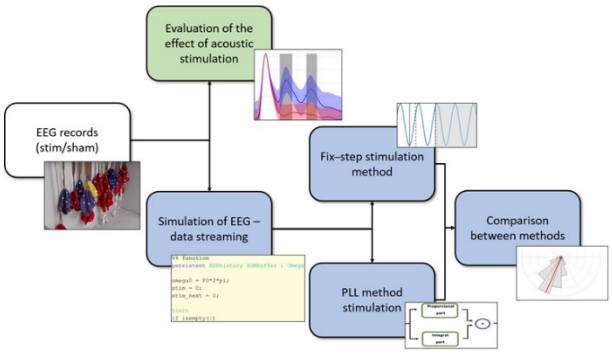


Figure 4. A diagram of analytical procedure: Real EEG recordings were utilized for two distinct analyses. The initial green branch assesses stimulation during real-time EEG recordings, focusing on the verum immediate impact of CLAS on SWA. The second blue branch illustrates re-streamed data and provides a quantitative comparison of both stimulation methods (Piorecky et al. 2021).

	Total Number of Events		
	Minimum	**Maximum**	**Mean**
Fixed-step	255	1889	1064
PLL-XOR	278	2597	1530

Table 2. A total number of events (detection/stimulation) during the restreaming of data of simulating both stimulation methods across 9 subjects.

3.2.3. Detection of SOs

Stimulating slow waves during their ascending phase is crucial for enhancing the SOs (Ngo, et al. 2013b). Real-time SWA stimulation relies on identifying the optimal phase for CLAS, illustrated in Figure 5. Consequently, the SO trough was initially detected to facilitate this process.

As previously mentioned in Section 3.1.4, SWA detection was applied to the reference signal, which was derived from the mean of F3 and F4 channels re-referenced to the mastoids (M1 and M2 electrodes). Before SWA detection, the reference signal underwent low-pass filtering. An infinite impulse response (IIR) low-pass filter, 3rd order, with a cutoff frequency of 4 Hz was utilized. The choice of an IIR filter was driven by the need for rapid processing of real-time signal and by the need to avoid instability. SWA, known for its substantial amplitude (Kurth et al. 2010), was detected when the negative voltage of the EEG wave exceeded -80 µV (Ngo, et al. 2013b; Ngo, et al. 2015). This minimum was defined as $index(min(x_{-1}, x_0, x_{+1})) = 0$.

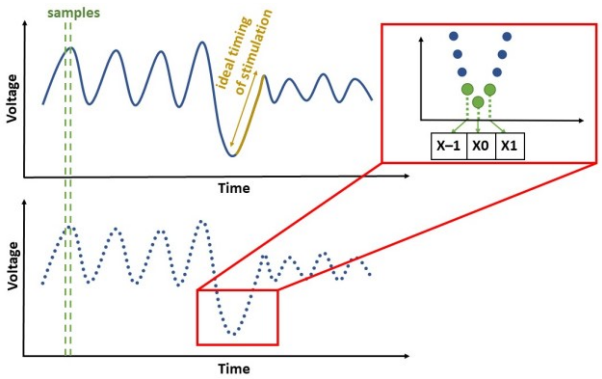


Figure 5 illustrates the slow-wave detection principle. A representative EEG signal is illustrated by the blue curve, while the optimal stimulation time is indicated by the yellow curve, corresponding to the rising phase of the wave. Three subsequent samples serve as reference points for minimum detection (Piorecky, et al. 2021).

3.2.4. Fixed-step CLAS method

The original method involved stimulating the SWA at fixed time intervals (Ngo, et al. 2013b; Ngo, et al. 2015). The first stimulation was timed to occur 0.350 s after detection, as depicted in Figure 6. Subsequently, the second stimulation was scheduled for 1.075 s after the first one (Ngo, et al. 2013b). Following the second stimulation, a pause lasting 2.500 s was enforced. These parameter settings were informed by SWA characteristics and the requirement for stimulation to coincide with the rising phase of the wave.

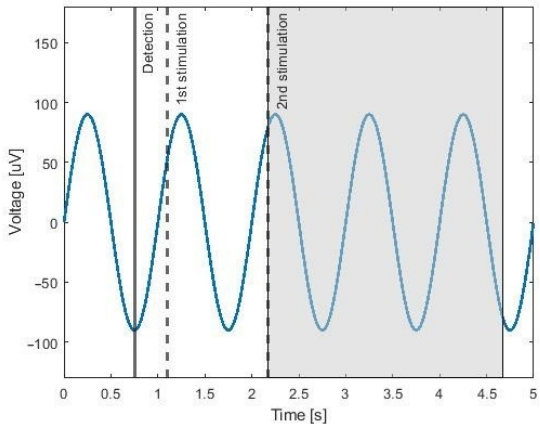


Figure 6 illustrates an example of EEG data with a 1 Hz sine wave (blue curve). The black line indicates the detection point at the signal's minimum. Dashed lines denote the timings of the first and second stimulations, while the shaded area indicates the pause of 2.5 s preceding the subsequent detection (Piorecky, et al. 2021).

3.2.5. Phase-locked loop CLAS method

Phase-locked loops (PLLs) are closed-loop feedback systems comprising of analog and digital components, including a voltage-controlled oscillator (VCO). They serve to generate an output signal synchronized, or "locked," to a reference input frequency. Digital PLLs typically consist of four key components: a phase detector, loop filter, VCO, and divider (Best 2007). The phase detector produces a signal responsive to phase differences, while the loop filter attenuates high-frequency components. The VCO generates a periodic output signal (Behzad 1996).

In the context of stimulating sleep, different types of PLLs have been employed in previous studies (Ong, et al. 2016; Santostasi, et al. 2016). The Study 2 implemented two types of PLLs. The first one, known as the PLL-XOR method, was selected for its robustness. The second type was PLL implementation involving an integral part.

Both PLL implementations generated an artificial harmonic signal with known instantaneous frequency and phase. Stimulation was applied at a specific point in the rising phase, ensuring consistency across waves with varying frequencies. Stimulation occurred when the PLL signal fell within a predefined interval, corresponding to values indicative of the appropriate phase for stimulation. This target interval ranged from 310° to 360°, extending beyond the original target phase of 340° as described in a previous study (Papalambros, et al. 2017).

3.2.5.1. PLL-XOR implementation

The XOR gate is a basic yet effective tool in signal analysis (Abramovitch 2003), particularly useful in aligning frequencies in brain signals. It operates by comparing the directions (positive or negative) of two signals, disregarding their strength. This simplification allows for straightforward calculations without the need to adjust for signal amplitude (Abramovitch 2003).

In the present research, the XOR gate's outputs, representing the immediate comparison results, are stored in two types of memory buffers: one for current values and another for recent past values. This dual-memory setup enables the tracking of signal changes over time. A mathematical formula (see Piorecky, et al. (2021) for details) then uses these stored values to calculate the frequency difference between the current and past signals, effectively updating the signal's frequency to reflect real-time changes accurately.

More details including the specific parameters of our PLL-XOR implementation may be found in the original publication of (Piorecky, et al. 2021).

3.2.5.2. PLL implementation with the integral part

In this implementation, we started with a basic version that used both integral and proportional components (Behzad 1996; Scher 2021). Initially, the EEG signal was filtered through a low-pass FIR filter. Then, the filtered signal was adjusted by a proportional gain in one part and integrated over time with an integral gain in another part. These components were combined to correct signal errors and adjust the phase and amplitude of the simulated PLL signal accordingly. The process included calculating the phase from the error signal and the amplitude using the sine function, based on the VCO's native frequency and the current time. Mathematical formulas for these computations may be found in Piorecky, et al. (2021).

Optimizing the PLL's parameters was crucial due to the system's sensitivity. This optimization involved simulating the PLL with various gain settings on a training dataset of 5 recordings to identify the best parameters. We defined three criteria for optimization: phase criterion, time-phase criterion, and fixed-time criterion, each considering the difference between the targeted and actual phases or times of the physiological signals. This comprehensive approach aimed to fine-tune the PLL settings to accurately simulate and adjust to the EEG signals, ensuring a balanced response to the natural variations in brain activity.

3.2.6. The accuracy of SO detections and stimulations

The EEG data was processed using MATLAB software, 2020a version (MathWorks, Inc., Natick, MA, USA) and we also utilized tools from the Fieldtrip toolbox (Oostenveld, et al. 2011). For pre-processing, we focused on the averaged signals from electrodes F3 and F4 and prepared the data by referencing them against M1 and M2. Next, we applied an Infinite Impulse Response (IIR) low-pass filter with a cutoff at 4.00 Hz.

To assess the accuracy of our signal detection and the timing of our stimulations, we analysed the phases of the EEG signals at detections and stimulations using the Hilbert transform. We then displayed the phase values using polar histograms, which were divided into 20 bins to calculate and show the average phase. For this part of the analysis, we relied on the CircStat toolbox (Berens 2009) to provide a statistical evaluation and visualisation of the phase values.

3.2.7. The effect of CLAS on SOs

In the pre-processing stage, we first filtered our data in frequencies between 0.25 and 4.00 Hz, based on the zero-phase finite impulse response (FIR) method. We chose to focus on the mean signals from the frontal electrodes, specifically Fpz (Papalambros, et al. 2017), and only included data from participants who had over 50 detections in their recordings (n = 7). Any outlier data segments that were too noisy, either too high above 300 μV or too low below 10 μV, were excluded. Initially, we examined 7s segments (2 s before, 5 s after a sound stimulus). However, to avoid the boundary effect caused by close subsequent stimulations in our analysis, we then adjusted the interval to 4 s total (1 s before and 3 s after the stimulus).

To evaluate the immediate effect of CLAS on SO morphology, we carried out our analysis in three parts. First, we looked at the EEG signal's response to the first sound stimulus, comparing the average waveforms from both CLAS and SHAM conditions as previously shown in (Ngo,

et al. 2013b; Ngo, et al. 2015; Papalambros, et al. 2017). This part mixed both amplitude and inter-trial phase synchronisation of the brain response. Second, we applied the Hilbert transform to analyse changes in the signal's amplitude over time, averaging these changes across multiple trials for each participant, and then statistically compared these averages between the CLAS and SHAM conditions. This step helped us measure the signal strength independently of the phase and inter-trial phase synchronisation of the brain response. Third, we calculated the inter-trial phase synchronisation of the brain response across different trials using inter-trial phase clustering (ITPC) between the CLAS and SHAM conditions. This measure reflected brain response independent of signal amplitude. Detailed description of the ITPC analysis may be found in Piorecky, et al. (2021). The criterion for the minimum and therefore adequate number of stimulation trials was set to 50 (see Figure 7 for details).

To visualise the brain response to CLAS, we used a time-frequency plot, measuring both the ITPC and power across frequencies from 0.25 Hz to 25.00 Hz over 7s raw data segments. This approach used a short-time fast Fourier transform (STFT) filter with a Hanning window, adjusting time in 0.1s steps for frequencies between 0.1 - 25 Hz. The analysis window's duration varied to match the frequency, from 1 s at the lowest to 0.1 s at the highest frequency.

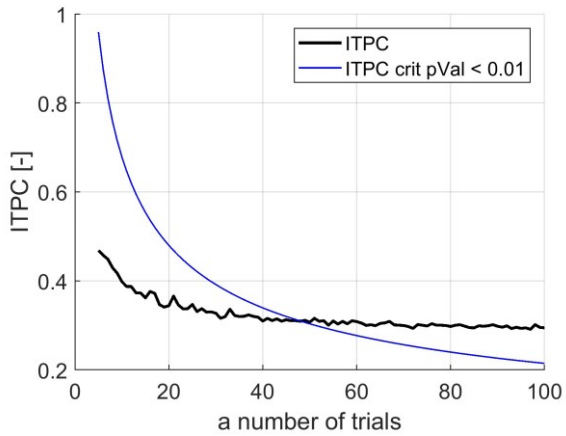


Figure 7 shows the critical ITPC values determined for a p-value of 0.01 using the Rayleigh Z approximation (represented by a thin blue line) alongside the ITPC values calculated from seven subjects (depicted by a thick black line). Both sets of data were plotted to compare changes as the number of trials increased. The minimum number of trials required, including detections and stimulations, was established at 50 (Piorecky, et al. 2021).

3.2.8. Statistical analysis

We used non-parametric statistical tests that included cluster-based correction for multiple comparisons (Maris and Oostenveld 2007) to analyse all three metrics mentioned in the

previous section (3.2.7). Before performing the statistical tests, we baseline-corrected the data for average waveform, signal amplitude, and power. This baseline interval occurred between 0.5 s and 0.35 s prior to the first stimulus. To correct the averaged waveform and amplitude, we subtracted the average signal value recorded during this baseline period. We normalised the signal power to this baseline, statistically comparing the relative change in power, measured in decibels, before and after the stimulus. Additionally, the ITPC was consistently aligned in all scenarios due to the precise and identical detection of SWA across both conditions, which ensured uniform phase synchronization during the baseline.

4. Results

4.1. Study 1: CLAS in chronic insomnia

4.1.1. CLAS was feasible in chronic insomnia patients

To test the effectiveness of CLAS in chronic insomnia patients, we assessed both the dropout rates and the extent of stimulation administered. Out of an initial group of 27 participants (aged 20-59 years, M_{age} = 36.6 years, SD_{age} = 14.0), several were excluded from further analyses: four because there were not enough opportunities for CLAS, three due to poor quality of PSG recordings, one due to an anxiety disorder, and one because of previously unknown medication use affecting sleep. Additionally, one participant opted out after experiencing discomfort during the first night of the experiment. Hence, 17 participants were included in the main analyses, whose demographic and clinical details are summarized in Table 3.

The frequency of stimulation cycles per night (i.e. SO detection, 1^{st} + 2^{nd} stimulation) varied widely, ranging from 2 to 413 cycles, which equated to 4 to 826 stimulations per night. For meaningful analysis of the sleep architecture changes after CLAS, we only considered participants who received a significant amount of stimulation—set at a minimum of 50 stimuli based on (Debellemaniere et al. 2018; Piorecky, et al. 2021). This criterion resulted in a final group of 7 participants (4 men, aged 20-59, M_{age} = 28 years, SD_{age} = 13.86) for further detailed analysis. See Table 3 for further demographic and clinical information. We found no significant age difference between the participants excluded due to insufficient stimulation (n = 14, M_{age} = 39.29 years, SD_{age} = 12.73) and those included with adequate stimulation (n = 7, M_{age} = 28.0 years, SD_{age} = 13.86), Z = -1.572; p = 0.128.

Sample	Initial analysis (n = 17)	Sufficient stimulation (n = 7)
Age (years)	33.53 (13.99)	28.0 (13.86)
Sex (females/males)	10 / 7	3 / 4
ESS	8.31 (4.03)	5.33 (2.21)
MEQ	55.31 (10.97)	51.83 (4.74)
BDI-II	11.56 (7.57)	8.50 (3.55)
BAI	8.13 (5.63)	7.17 (3.80)

Table 3. Demographic and clinical details for both the initial sample (n = 17) and a smaller subset that received an adequate number of stimulations (n = 7) are outlined. The table includes average scores (M) and standard deviations (SD) for various questionnaires. These questionnaires included ESS (Epworth Sleepiness Scale), MEQ (Morningness – Eveningness

Questionnaire), BDI-II (Beck Depression Inventory-II), and BAI (Beck Anxiety Inventory) (Dudysová et al. 2024).

4.1.2. CLAS had immediate effect on SOs

To assess the precision of our CLAS approach, polar histograms were used to illustrate the angles at which detection and stimulation occurred during the SWS sleep phase, as depicted in Figure 8. Typically, the detection of SOs was accurate, occurring at 225° (SD = 21°) past their through (180°). The initial sound pulse was effectively timed to coincide nearly with the peak (360/0°) of the SO's upward phase (M = 351°, SD = 50°). Similarly, SHAM stimulations were also precisely timed during the SO's upward phase, close to its peak (M = 354°, SD = 45°). However, the second pulse generally occurred just after the peak, during the transition from the upward to the downward phase of the SOs (M = 37°, SD = 66°), which was delayed and thus deviated from our intended target phase.

To assess the immediate impact of CLAS, we analysed changes in amplitude and power spectra surrounding the sound stimulation events. Figure 9 displays the average waveform amplitude in slow-wave sleep (SWS) for both STIM and SHAM conditions. Notably, CLAS significantly increased the amplitude of the downward phase of the SOs in the two waves following the sound stimuli.

CLAS notably improved the average relative power in the SO band (on interval: 0.361, off interval: 0.354, $Z = 3.40$, $p < 0.001$). However, there was no significant change in the relative power within the delta band following CLAS (on interval: 0.559, off interval: 0.551, $Z = 1.740$, $p = 0.080$).

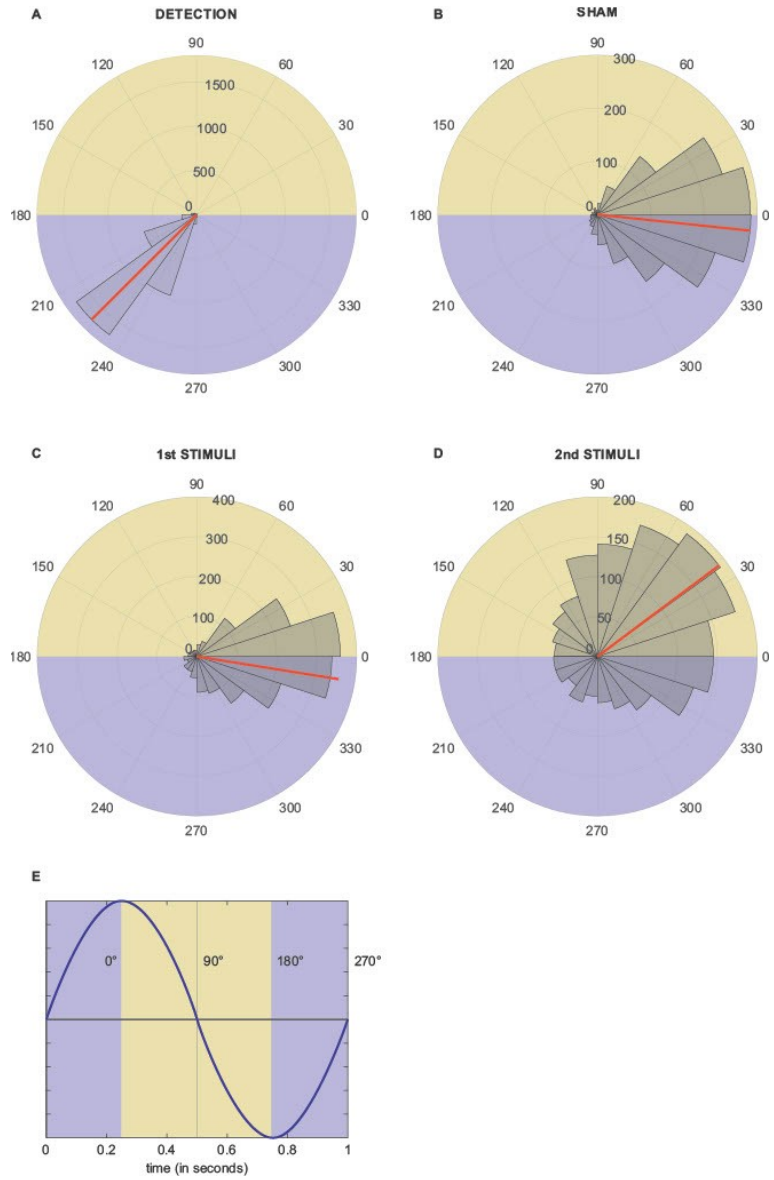


Figure 8 shows polar histograms for various stimulation conditions (detection (A), SHAM (B), first (C), and second stimulation (D)) during the NREM3 sleep phase in a group that received sufficient stimulation (n = 7). A red line marks the average phase, while the grey marks the distribution of phases across 30-degree intervals for all stimulations. In the SHAM condition (B), the phase is marked even though no sound was emitted. A graphic showing the phase angles (E) uses yellow and purple to represent the down- and up-phases of the SOs, respectively. We aimed to apply stimulations during the SOs' up-phase. The SHAM and first stimulations correctly occurred during this up-phase, whereas the second stimulations were delivered during the down-phase, deviating from our intended target phase (Dudysová, et al. 2024).

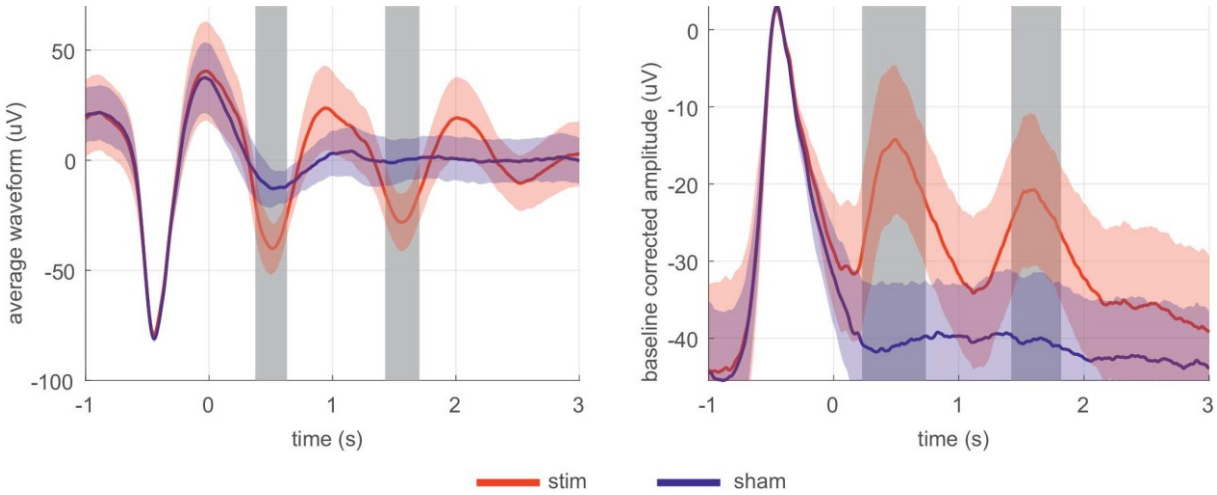


Figure 9 illustrates the amplitude parameter for a group of 7 participants. The point labelled '0' on the timeline marks the time of the first sound stimulus. The graph displays the grand average of the waveform amplitudes for each subject, with standard deviation bands around the curves, in blue for the SHAM condition and red for the stim condition. Any corrected significant differences between the STIM and SHAM conditions are highlighted with grey bars (Dudysová, et al. 2024).

4.1.3. CLAS did not alter sleep macrostructure

Analysis of PSG recordings showed that there were no statistically significant differences in sleep macrostructure characteristics—like sleep onset latency (SOL), total sleep time (TST), sleep efficiency (SE), the duration and proportion of various sleep stages, and the number of arousals—between the STIM and SHAM conditions (refer to Table 4 for details). Therefore, CLAS did not affect any aspects of the sleep macrostructure.

	STIM	SHAM	Z	p	r
SOL (min.)	23.93 ± 5.72	24.71±14.71	-1.01	0.310	-0.381
TST (min.)	411.68 ± 30.28	405.78 ± 58.10	-0.16	0.866	-0.060
SE (%)	84.33 ± 8.62	83.18 ± 9.41	-0.16	0.554	-0.060
Wake (min.)	54.96 ± 23.15	60.67 ± 50.62	-0.33	0.735	-0.125
Wake (%)	11.69 ± 4.54	12.95 ± 10.45	-0.50	0.612	-0.189
Arousals (#)	66.66 ± 32.94	52.50 ± 18.57	-1.18	0.237	-0.446
Arousal index (#/h)	9.37 ± 4.44	8.23 ± 3.12	-0.52	0.600	-0.197
NREM 1 (min.)	12.71 ± 9.26	12.42 ± 6.60	-0.17	0.865	-0.064
NREM 1 (%)	2.69 ± 1.92	2.67 ± 1.30	-0.16	0.866	-0.060

NREM 2 (min.)	217.85 ± 44.60	203.14 ± 39.54	-0.76	0.446	-0.287
NREM 2 (%)	46.47 ± 8.05	43.69 ± 8.34	-0.67	0.499	-0.253
NREM 3/SWS (min.)	93.28 ± 46.21	94.71 ± 31.04	-0.16	0.866	-0.060
NREM 3/SWS (%)	20.29 ± 10.58	20.39 ± 6.57	-0.16	0.866	-0.060
REM (min.)	88.07 ± 29.32	95.78 ± 37.56	-0.42	0.672	-0.159
REM (%)	18.85 ± 6.00	20.41 ± 7.32	-0.50	0.612	-0.189

Table 4 presents the objective sleep macrostructure parameters for both the STIM and SHAM nights, covering a group of 7 participants with sufficient stimulation. The data are presented as Mean ± SD. The Wilcoxon signed ranks test was utilized to analyse the differences. The abbreviations include SOL (sleep onset latency), TST (total sleep time), and SE (sleep efficiency).

4.1.4. CLAS increased the power of SOs and decreased delta and sigma power

EEG spectral analysis of SWS across an entire night indicated a notable increase in the relative power of slow oscillations (SOs) and a decrease in delta power, as depicted in Figure 10. This suggests that CLAS might enhance SOs potentially by reducing delta power, especially since the overall absolute power showed no change. Additionally, there were decreases in both absolute and relative sigma power at F4 (absolute sigma: STIM M = 0.679, SD = 0.399; SHAM M = 0.820, SD = 0.461; Z = -2.197, p = 0.028; relative sigma: STIM M = 0.009, SD = 0.009; SHAM M = 0.012, SD = 0.012; Z = -2.197, p = 0.028).

We furthermore analysed the first two sleep cycles where stimulations occurred. The spectral analyses similarly revealed reductions in sigma activity, specifically at C3 and F4, with absolute sigma activity decreasing during stim condition at C3 (STIM M = 0.610, SD = 0.317; SHAM M = 0.736, SD = 0.407, Z = -2.366; p = 0.018) and F4 (STIM M = 0.689, SD = 0.391, SHAM M = 0.850, SD = 0.465, Z = -2.197, p = 0.028). Correspondingly, the relative sigma power at F4 decreased (STIM M = 0.008, SD = 0.008, SHAM M = 0.011, SD = 0.009, Z = -2.197, p = 0.028). Consistent with the overall night's SWS findings, absolute SO power was higher at F4 during the stim condition (STIM M = 57.222, SD = 36.692, SHAM M = 52.570, SD = 34.950, Z = -2.366; p = 0.018). Additional significant changes in relative SO and delta power are detailed in Figure 10. All significant findings were adjusted using FDR correction for multiple comparisons.

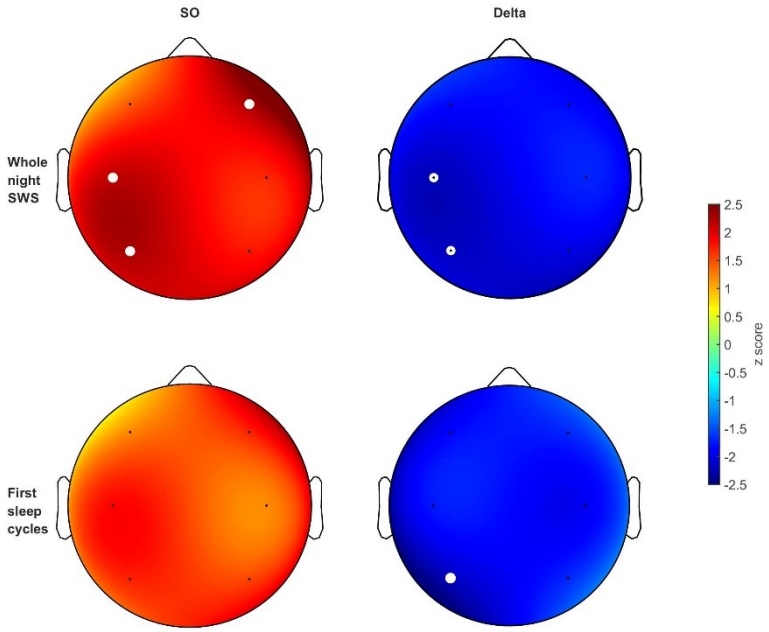


Figure 10 features topoplots that illustrate the increase in relative SWS slow oscillation (SO) power shown in red, and the decrease in relative SWS delta power depicted in blue, following CLAS throughout the entire night (upper topoplots) and during the first two sleep cycles (lower topoplots) where stimulations occurred. White dots on the topoplots indicate locations of statistically significant results, which have been corrected for multiple comparisons using the FDR method (Dudysová, et al. 2024).

4.1.5. CLAS did not alter discrete sleep spindles

We next looked at discrete spindle events detected using an automated method by Ferrarelli, et al. (2007). In a comparison between the stimulation and sham nights, we observed a slight reduction in the average amplitude of sleep spindles at the C3 electrode during slow-wave sleep (SWS) on the stimulation night (STIM M = 10.064; SD = 2.865, SHAM M = 10.786; SD = 3.079, Z = -2.197; p = 0.028, corrected). However, CLAS did not significantly affect other characteristics of SWS sleep spindles, such as their amplitude (at electrodes besides C3), density, frequency, or duration across all electrodes.

4.1.6. CLAS did not change subjective sleep quality nor overnight declarative memory consolidation

There was no significant difference in the subjective total sleep time (TST) between stimulation (M = 400.31 min., SD = 67.68) and sham nights (M = 375 min., SD = 83.42), t(15) = 1.587, p = 0.133, r = 0.138. Additionally, participants did not feel more rested after the stimulation night (Mdn = 2) relative to the sham night (Mdn = 2), T = 18, p = 1.000, r = 0. There was also no

difference in subjective sleep quality between the stimulation (Mdn = 2) and sham nights (Mdn = 2), T = 20, p = 0.405, r = -0.132.

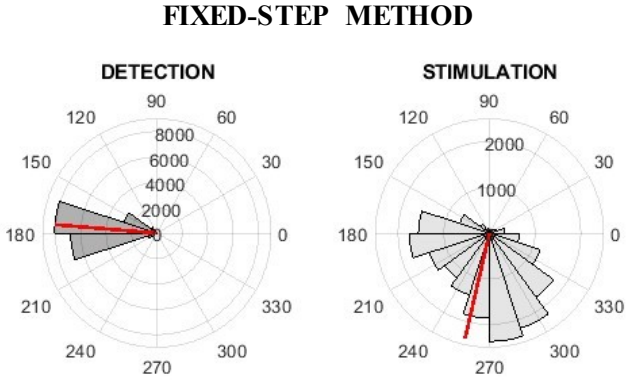
Performance on the overnight memory consolidation task showed no significant difference following the stimulation (STIM: M = -3.86; SD = 7.56; SHAM: M = -3.25; SD = 5.56), Z = 0.00, p = 1.000.

4.2. Study 2: Excitation of SOs using CLAS

4.2.1. Fixed-step method was more efficient than PLL-XOR

To evaluate the phase values across subjects, we calculated and depicted the phase values at the time of detection and stimulation using polar histograms, which can be seen in Figure 11. The results showed the average phase value of detections was virtually the same for both methods, just past the SO trough: 175.30 degrees for the fixed-step stimulation method, and 175.57 degrees for the PLL-XOR method. The average phase values of stimulations were similar on average, occurring in the targeted up-wave: 256.97 for the fixed-step method, and 244.29 for the PLL-XOR method. However, the variance was notably larger in the PLL-XOR method case suggesting a lower consistency, less robustness, and a higher tendency to stimulate during the down-phase of SO.

The detailed descriptive statistics of detection and stimulation are presented in Table 5. The PLL implementation with an integral part could not be tuned due to oscillations that did not converge in one value and were unstable on the group level. Thus, the PLL with integral part results are provided in the next section.



PLL-XOR METHOD

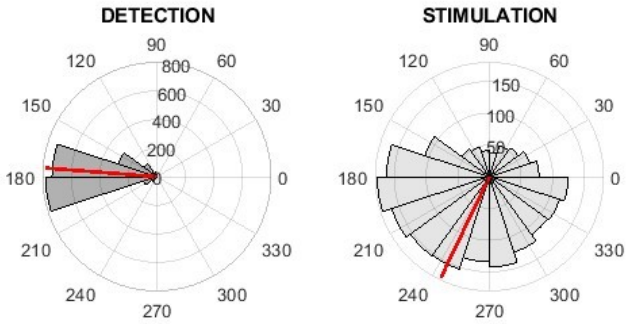


Figure 11 presents the analysis of phase values across subjects using real EEG records streamed digitally. It features polar histograms that show the phase values at the time of detection (left) and during the first stimulation (right) using the fixed-step stimulation method (top) and the PLL XOR implementation method (bottom). The red line in each histogram depicts the mean of the phase values. 0°-180° denotes the up-wave/rising phase of the wave. 180°-360°/0° denotes the down-wave/falling phase of wave (Piorecky, et al. 2021).

Method	M	Variance	SD	SEM	Skewness [−]	Kurtosis [−]
Detection phase						
Fixed-step	175.30	2.18	15.79	0.11	0.01	0.86
PLL-XOR	175.57	3.45	19.87	0.43	0.01	0.79
Stimulation phase						
Fixed-step	256.97	27.18	55.81	0.40	0.09	−0.03
PLL-XOR	244.29	41.48	68.94	1.50	−0.10	−0.04

Table 5 displays descriptive statistical parameters for a comparison of two chosen stimulation methods, for both detection and stimulation phases. All values are displayed in degrees, except for the skewness and kurtosis coefficients. M = mean, SD = standard deviation, SEM = standard error of mean

4.2.2. The optimal parameters for PLL with integral part were diverse

The PLL featuring an integral component was assessed using the training dataset. Variations in the number of detections/stimulations led to the development of diverse parameters, corresponding to three optimal settings for three different PLL tuning methods: phase-based criterion, fixed-time-based criterion, and time-phase-based criterion. The phase-based criterion modelled the PLL wave according to the phase of the streamed signal, e.g. if the streamed signal had a 90° phase, the PLL algorithm modelled a 90° wave as well. The fixed-time-based criterion considered the time/frequency of the wave instead. For if the streamed signal lasted 1 s (corresponding to 1 Hz), the PLL wave was modelled in 1 s/1 Hz accordingly. This criterion included the specification of the delay between detection and stimulation. Finally, the combined

approach of the time-phase-based criterion considered both the time interval and the phase of the streamed signal and adapted the PLL frequency according to the fixed-time approach and the current phase of the PLL according to its phase.

Table 6 summarizes results for the number of detections/stimulations simulated based on the three criterion methods. The resulting variation in the number of detections/stimulations was influenced by the frequency characteristics of the PLL signal and the amplitude difference, which acted as the threshold for reinitiating detection following pacing (i.e. pause in stimulation). Here, the phase-based criterion method resulted in the most detections/stimulations (147.6 on average), followed by the fixed-time-based criterion (56.2 detections/stimulations on average). We found the least amount of detections/stimulations using the time-phase-based criterion (26.6 on average).

Figure 12 then illustrates the results for the average and spread of phase values across the training dataset for detections and stimulations in the three tuning methods. Regarding detections, we found very similar results in the average phase and spread for all three methods. However, the three methods differed in stimulation phases. We found the phase-based method resulted in most variation and instability, perhaps in part due to the largest amount of stimulations. The method resulted in stimulations past the peak on average, corresponding to the down-wave of the SO. The other two methods resulted in more precise and stable results regarding the target phase of stimulation, with similar average phases, yet past the peak of the SOs in the down-wave.

Finally, Table 7 presents the average values for the spectral range of the simulated PLL signal, the maximum spectral power, and the values of the most optimal parameters, G1 and G2 for each of the three tuning methods. There was a large individual variance in all of the tuning methods, thus we were unable to tune PLL with the integral part properly.

Training dataset subject ID	**Phase-based criterion**	**Time-phase-based criterion**	**Fixed-time-based criterion**
subject 1	52	106	86
subject 2	344	3	2
subject 3	43	13	74
subject 4	165	1	110
subject 5	134	10	9
mean	147.6	26.6	56.2
SD	121.6	44.7	48.1

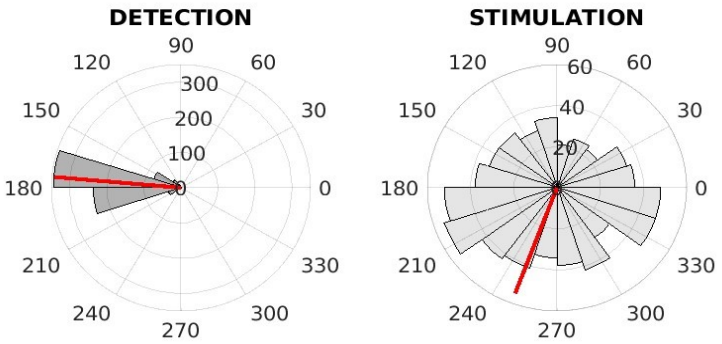
Table 6 presents a comparison of the number of stimulations for three different tuning methods (phase-based, time-phase-based, and fixed-time-based criteria) applied to the PLL with an integral part. The phase-based criterion resulted in the largest amount of detection/stimulation events. Note the large individual variance amongst all criteria, which makes means difficult to interpret.

Tuning Version	Spectral Range [Hz]	Maximum spectral power [Hz]	G1 [−]	G2 [−]
Phase-based	0.38–1.36	0.86	0.0008	0.0007
Time-phase-based	4.63–4.75	4.75	0.0092	0.8555
Fixed-time-based	11.82–14.68	12.48	1.0000	0.5356

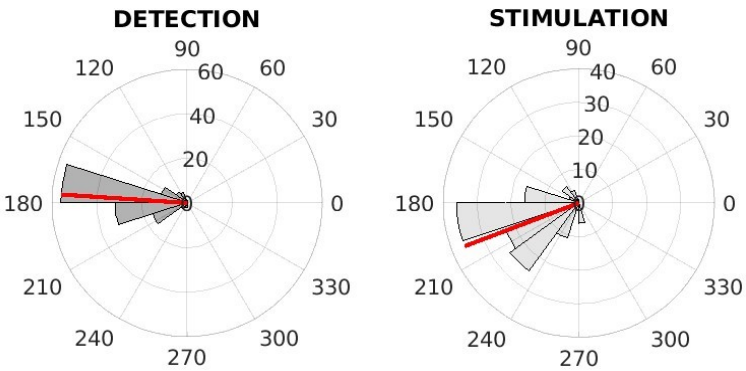
Table 7. A comparison of parameters including spectral range, maximum spectral power, and the two most optimal parameters (G1, G2) for the three different tuning methods in the case of PLL with the integral part. Note the large differences in all of the tuning methods making tuning universal parameters difficult.

PLL WITH INTEGRAL PART METHOD

PHASE-BASED CRITERION



TIME-PHASE-BASED CRITERION



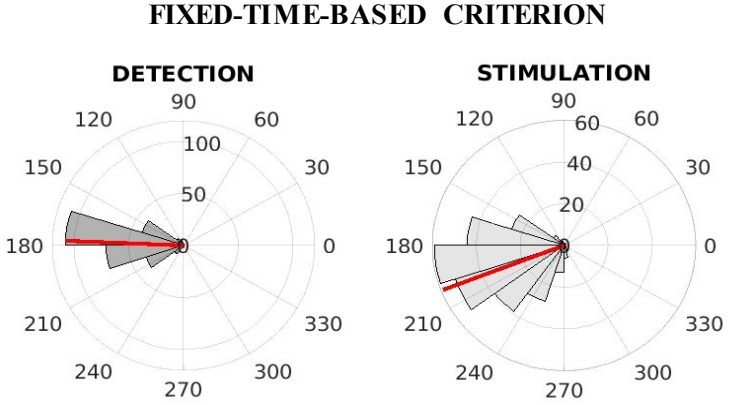


Figure 12. The analysis of inter-subject phase values from the training dataset is shown using polar histograms. These histograms display the phase values at the time of detection (left) and at the time of the first stimulation (right) for the PLL with an integral part, using the 3 criteria, phase-based (top), time-phase-based (middle), and fixed-time based (bottom) criterion. The red lines indicate the mean phase values. 0°-180° denotes the up-wave/rising phase of the wave. 180°-360°/0° denotes the down-wave/falling phase of the wave (Piorecky, et al. 2021).

4.2.3. CLAS caused phase synchronization of SOs

Our analysis indicated that the ITPC was more responsive to changes in brain activity within the SWA frequency band following a sound stimulus compared to the average waveform and amplitude metrics. The ITPC showed a significant increase in phase synchronization from 0.6 s to 2.5 s after the initial stimulus, as detailed in Figure 13. Meanwhile, the average amplitude (waveform) showed a significant increase during the interval from 1.4 s to 1.7 s (Figure 14), and the baseline corrected amplitude saw significant increases from 0.2 s to 0.7 s and from 1.4 s to 1.8 s (Figure 15).

A time-frequency data analysis confirmed a marked increase in ITPC specifically within the frequency range of 0.5 Hz to 4.0 Hz (Figure 16), with the effect on phase synchronization extending from about 0.2 s to 2.5 s post-stimulus. This finding aligns with the statistics of the ITPC time course in Figure 13.

Moreover, the time-frequency representation of signal power, as shown in Figure 17, revealed that the most notable changes between stimulation and sham conditions occurred broadly across frequencies. The impact of sound stimulation did not confine to slow waves. Notably, there were increases in delta and theta wave activities from 0.0 s to 0.7 s and from 1.2 s to 1.7 s. These findings are in line with the results above on time-specific amplitude increases shown in

Figure 15. Additionally, there was an increase in the power within the sleep spindle band and beta band from 0.7 s to 2.5 s, highlighting a broad, multi-frequency response to the stimulation.

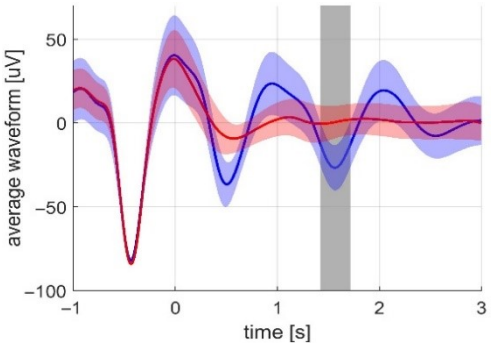


Figure 13 presents the overall average of amplitude values across subjects for the stimulation (in blue) and sham (in red) conditions. The shaded areas around the average curves show the standard deviation. The grey bars denote the time intervals with the significant differences between the stimulation and sham, i.e. 1.4 – 1.7 s after stimulus presentation (Piorecky, et al. 2021).

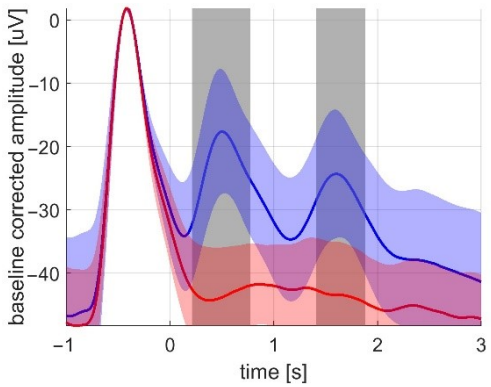


Figure 14 shows the grand average of the waveform across subjects for the stimulation (in blue) and sham (in red) conditions. The shaded bands around the average curves represent the standard deviation. The grey bars mark the period where there was a significant difference between the stimulation and sham conditions, corresponding to 0.2 - 0.7 s and 1.4 – 1.8 s post-stimulus (Piorecky, et al. 2021).

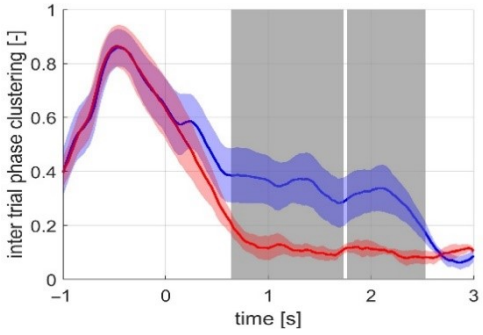


Figure 15 displays the overall average of the ITPC values across subjects for both the stimulation (in blue) and sham (in red) conditions. The shaded bands surrounding the average curves illustrate the standard deviation. Notably, the grey bars highlight the time intervals where the differences between the stimulation and sham conditions were significant. Specifically, the significant intervals corresponded to 0.6 – 2.5 s after stimulus (Piorecky, et al. 2021).

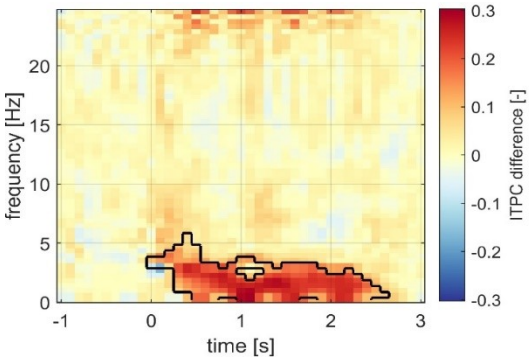


Figure 16 illustrates the time-frequency representation of the ITPC differences between the stimulation and sham conditions. A specific time-frequency regions between 0.2 - 2.5 s after stimulus mark the areas where the differences are most notable. This difference in ITPC is distinctly associated with slow frequency bands until 4 Hz (Piorecky, et al. 2021).

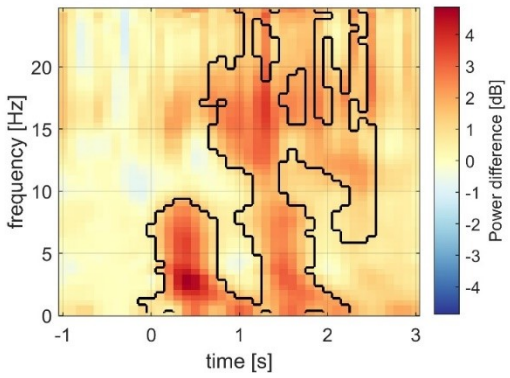


Figure 17 displays the time-frequency representation of the relative power difference in decibels between the stimulation and sham conditions. Significant differences are highlighted within the specified time-frequency regions. The power variation predominantly shifts towards higher frequencies, with notable differences observable across various frequency bands including delta, theta, beta, and sigma (i.e. sleep spindles) (Piorecky, et al. 2021).

5. Discussion

5.1. Study 1: CLAS in chronic insomnia

This study was to our knowledge the first to assess the effect of closed-loop acoustic stimulation (CLAS) during sleep in participants with chronic insomnia and among first it expands our knowledge of the clinical applications of CLAS. Chronic insomnia is characterised by cortical hyperarousal (Riemann, et al. 2010), which may increase vulnerability to external stimuli and decrease waking thresholds (Thacher et al. 2006). We investigated whether CLAS would increase the frequency of awakenings and other signs of arousal in this clinical population. Our analyses did not show any differences in the number of arousals, awakenings, duration and proportion of wakefulness, nor enhanced fast EEG activity after CLAS, suggesting that CLAS does not disturb sleep in insomnia patients. In contrast, the immediate effect of CLAS on slow oscillations was similar to the effect previously reported in healthy volunteers (Ngo, et al. 2013b). The slow oscillations showed increased amplitude following the stimuli and higher relative power in ON (stimulation) versus OFF intervals. Additionally, SO activity was enhanced throughout the entire SWS period in the stimulation condition compared to the sham condition, consistent with studies on healthy participants. Interestingly, relative delta activity in SWS was reduced after stimulation compared to sham, possibly favouring the slow oscillations as the overall absolute power remained unchanged.

Our findings align with those of Feige et al. (2018), who found no evidence of a reduced awakening threshold in response to auditory stimuli in insomnia compared to good sleepers. Additionally, a recent ERP study by the same group showed that insomnia patients do not exhibit altered acoustic stimuli processing in NREM sleep, although they are prone to sleep disturbances following acoustic stimuli presented in phasic REM sleep (Feige et al. 2021). In this context, CLAS delivered during deep sleep appears to be a feasible approach for treating insomnia. However, it is important to note that more than half of our participants received only a small number of stimuli during sleep, which could reduce the efficiency of the stimulation protocol. This may have been due to several factors, including technical issues during recordings, temporarily worsened signal quality, or factors related to different sleep morphology in insomnia, such as lower power and amplitude of slow oscillations and delta waves (Hogan et al. 2020; Zhao et al. 2021), lower amounts of SWS, increased sleep fragmentation, and hyperarousal (Baglioni, et al. 2014; Riemann, et al. 2010; Svetnik, et al. 2017). Although we used two amplitude thresholds for stimulation based on the age of our

participants (Ngo, et al. 2013b; Papalambros, et al. 2017), our results indicate that the threshold for stimulation needs to be further adapted and less strict for patients with insomnia to achieve sufficient CLAS opportunities in this patient population.

Further individualization of the CLAS protocol could include adjustments for presenting multiple subsequent sound stimuli. In our study, the initial sound pulses were delivered upon detecting a hyperpolarizing SO minimum, while the second sound stimuli followed after a fixed interval of 1.075 s (Ngo, et al. 2013b). However, due to the fixed time step, the second stimulation could not account for the variable frequency of the subsequent SO or the potentially different characteristics of SOs in insomnia, such as lower amplitude or higher frequency. Our results indicated that the second stimulation often occurred during the descending phase of the slow oscillation. Since down-phase stimulation can inhibit slow waves (Ngo, et al. 2013b), our second stimulation might have inadvertently reduced the efficacy of CLAS for enhancing SOs and sleep-dependent memory consolidation. Future studies should ideally personalize the intervals between subsequent stimuli or use a single sound stimulus to target the desired phase of the wave.

In this study, we did not observe any differences in sleep spindle density, frequency, or duration between the CLAS and sham conditions. However, our analyses did reveal a lower spindle amplitude at one electrode and a decreased relative sigma power following CLAS, which might indicate a potential suppression of spindle activity throughout the night. Research on spindle activity in insomnia has yielded inconsistent results (Weiner and Dang-Vu 2016), making it challenging to draw specific conclusions about this population. A previous study reported enhanced spindle activity in insomnia, possibly indicating an increased level of sleep-protecting mechanisms in response to heightened arousal (Spiegelhalder et al. 2012). Additionally, a fast spindle rate has been associated with sleep pressure (Knoblauch et al. 2002). The reduced spindle activity observed in our study could thus reflect increased sleep pressure due to lowered arousal following CLAS, consistent with the increased SO activity after CLAS.

Consistent with previous research, our study did not find any differences in sleep macrostructure (Ngo, et al. 2015; Papalambros, et al. 2017). Total sleep time (TST) and sleep efficiency, as well as sleep-staging characteristics in terms of both minutes and percentage, remained unchanged. Additionally, CLAS did not affect subjective sleep quality, corroborating earlier findings (Leminen et al. 2017; Papalambros, et al. 2017). However, in the context of

treating insomnia, this raises questions about whether acoustic stimulation leads to sleep changes that result in clinically meaningful improvements. Despite this, current evidence indicates that CLAS does not negatively impact sleep quality. Similarly, there was no difference in declarative memory performance between the stimulation and sham conditions. Our negative findings might be due to limitations in our stimulation approach, such as a low number of stimulations in some participants, potentially blunting the effect of the second stimulus and a small overall sample size. These memory findings contrast with the majority of research (Wunderlin, et al. 2021), although similar inconsistencies can be found in other studies (Diep, et al. 2020; Schneider, et al. 2020).

The present study has several limitations and offers multiple opportunities for future research. Firstly, the study did not include a control group, which would have allowed a comparison of the effects of CLAS on participants with insomnia disorder and healthy subjects using the same stimulation protocol under identical experimental conditions. A direct comparison between insomnia patients and healthy individuals could provide insights into the reasons for the low overall amount of stimulation in the insomnia group, and whether this issue is related to the algorithm used or is specific to insomnia. Secondly, our stimulation approach relied on manual detection of stable SWS, which was limited by the experimenters' ability (e.g., expertise in visually detecting SWS online, vigilance level, and signal quality). This manual step affected the start and end of stimulation windows and thus could have influenced the amount of stimulation and the overall efficiency of the stimulation protocol. Thirdly, although the study was balanced in terms of the order of the stimulation and sham conditions, providing participants with an adaptation night to eliminate the first night effect of the sleep laboratory environment on sleep quality would be beneficial.

Additionally, due to the high variability in the number of stimulations among our participants and the lack of knowledge about the minimum number of stimuli needed to elicit the effects of CLAS, the main analyses were performed only on subjects receiving at least 50 stimuli, reducing our sample to seven subjects. This is another limitation of the study. A relatively low success rate in stimulated subjects may be due to our SO detection algorithm being too strict for this clinical group, as the character of SOs is less pronounced than in healthy controls (Hogan, et al. 2020). Further research is needed to study the characteristics of SOs in insomnia in more detail and to determine an optimal CLAS algorithm to assess whether CLAS may be an effective approach to ameliorating insomnia.

In summary, our study provides the first evidence that CLAS during sleep is feasible for individuals with chronic insomnia. This method does not increase arousal or wakefulness after sleep onset but can influence sleep dynamics. Specifically, CLAS in our study led to an immediate increase in the amplitude of slow oscillations, enhanced SO activity, and reduced delta and sleep spindle activity during slow-wave sleep throughout the entire night. It remains unclear whether these changes signify enhanced sleep consolidation. Future studies could benefit from adjusting the stimulation threshold and employing a more precise stimulation algorithm. Additionally, it is essential to investigate whether multiple nights of stimulation could further improve sleep structure and subjective sleep quality in patients with insomnia.

Further research should aim to refine the CLAS protocol, potentially incorporating personalized stimulation parameters to optimize effectiveness. Exploring the long-term effects of CLAS on sleep quality and overall well-being in individuals with chronic insomnia is also crucial. By addressing these aspects, future studies may determine the full therapeutic potential of CLAS and its applicability as a treatment for insomnia. Expanding the sample size and including control groups for comparison could provide conclusions that are more robust and help clarify the mechanisms underlying the observed effects of CLAS on sleep architecture and memory consolidation.

5.2. Study 2: Excitation of SOs using CLAS

Research on real-time stimulation of SOs is advancing, with several teams having already reported their methodologies and findings (Ngo, et al. 2013b; Ngo, et al. 2015; Papalambros, et al. 2017). However, it is still unclear what specific detection methods are more appropriate, and what mechanisms underlie the SO excitation. Our study brought forward evidence of underlying mechanisms and principles of stimulation.

In SO detection literature, there is a growing consensus favouring the replacement of the fixed-step method (Ngo, et al. 2013a) with PLL methods to enhance subsequent SO stimulation, not just the first wave after detection. Previous research included various implementations of PLL, such as (Kumar and Kumar 2016; Santostasi, et al. 2016; Scher 2021). Our study implemented two PLL types commonly used: a PLL-XOR method and one with an integral part.

Santostasi, et al. (2016) also utilized a PLL method incorporating an integral component for stimulating SWA. Although the authors provided a comprehensive description of their methodology, certain aspects proved challenging for us to replicate. Specifically, the cut-off

frequency for their low-pass filter was set to 0.03 Hz, a parameter that was not feasible with standard filters during our real-time processing. Our IIR filters at this cut-off frequency demonstrated instability, and using an FIR filter was not an option due to its delayed response. Consequently, we adopted the PLL method with an integral part based on the Scher implementation (Scher 2021), with appropriately tuned parameters. This approach largely mirrored the one in the Santostasi, et al. (2016) study, with both implementations involving a low-pass filter post-phase detection and using an integral form to translate the filtered signal into the current phase of the PLL signal. Additionally, our PLL strategy incorporated a proportional form in the calculations. While there are minor differences between our method and the one previously described in Santostasi, et al. (2016), the similarities in their foundational algorithms make them sufficiently comparable for analysis purposes.

We observed high sensitivity of PLL behaviour to its settings, leading us to favour the fixed-step method due to its robustness. We explored three approaches for optimizing PLL parameters. The first approach, the phase-based method resulted in oscillations at frequencies too low (lower than 0.5 Hz) for a typical SWA band (0.5-4 Hz), fitting slow drifts in EEG data that we could not effectively filter in real-time. Specifically, it was not possible to apply an FIR filter for real-time stimulation due to its very high order, and the IIR filter was not stable in this case. Although we mitigated the slow drifts, it was not possible to eliminate them.

The second approach, the time-phase-based method addressed some shortcomings of the phase-based approach by eliminating the ambiguity that led to fitting these slow drifts. With this revised method, the PLL signal exhibited a higher frequency oscillation; with a peak of around 5 Hz in the power spectral density. The stimulations were timed to occur during the rising phase of the actual signal. However, this method encountered an issue with the PLL frequency being set too high, resulting in both the first and second stimulations occurring within the same rising phase of the real EEG data. This high frequency, significantly above the target frequency, could lead to inaccurate PLL fits. Specifically, while the output frequency of the PLL was around 5 Hz, the target frequency for the SWA was about 0.5 Hz, indicating a substantial discrepancy that could affect the effectiveness of the stimulations.

The third fixed-time-based method aimed to minimize the influence of noise by specifying the delay between detection and stimulation. We designed this method to prevent the phase estimation noise, common in both the phase-based and time-phase-based methods. However,

adhering strictly to a noise-free criterion also led to challenges, particularly with overfitting the PLL parameters. Even slight adjustments to the pre-set delay significantly altered the PLL's behaviour, as evidenced by the high mean frequency of the PLL output observed in our study. This method produced the highest frequency of the three approaches tested, and the phase at which stimulation occurred did not align with the intended adjustment for the PLL, illustrating a misalignment with the target phase settings.

Overall, we observed that the PLL method exhibited complex behaviour that the traditional optimization metrics previously employed in other studies may not adequately capture. An over-fitted PLL can lead to a polar histogram with limited range, yet the output signal may not effectively fit the natural EEG data. Consequently, this can result in a PLL that operates spuriously, producing misleading results.

For instance, it is critical to maintain a very narrow interval for stimulation during the rising phase of the PLL output. If the PLL oscillates too quickly, the stimulation may be omitted, as discussed in more detail in Piorecky, et al. (2021). Consequently, there may be few stimulation events, potentially leading to an inaccurate PLL fit due to unevenly distributed weights among subjects. To address this issue, we broadened the stimulation interval during the PLL's rising phase, which increased the number of stimulations and aligned the PLL signal more closely with the spectral characteristics of SWA. However, this adjustment led to stimulations being dispersed across the wave, including during the down-phase. This dispersion suggested suboptimal synchronization between the PLL and EEG signals.

Overall, our analyses indicate that while both stimulation methods were applied to the same artificially streamed dataset, the fixed-step method typically stimulated more frequently compared to the PLL-XOR method. This was primarily because the PLL-XOR method included stimulation intervals, which were too long, missing some SOs to stimulate during the off/pause interval. In many instances, the fixed-time pause, designed for the lowest frequency of 0.5 Hz, was shorter than the pauses set by the PLL method.

The fixed-step method exhibited less variance, suggesting a uniform distribution of stimulation positions. This consistency likely reduced the frequency of stimulations occurring during the falling phase of the wave, as the transition from the mean value of the stimuli to the rising phase was smoother. In contrast, the PLL-XOR method displayed a higher kurtosis value, indicating a greater presence of extreme values in the phase distribution compared to the fixed-step

method. Both methods showed low skewness values, pointing to a relatively symmetrical distribution of stimulation phases. However, the fixed-step method demonstrated positive skewness, while the PLL-XOR method showed negative skewness. Negative skewness was more beneficial in this context implying that outlier values were more frequent early in the distribution, reducing the likelihood of stimulation during the falling phase, provided the phasing was accurate. This characteristic potentially gave the PLL-XOR method an advantage in ensuring that stimulations were more optimally timed.

The mean value of the PLL-XOR stimulation position and that of the fixed-step method are similar, both approximately 250°, i.e. just past the transition from the down-to-up state. However, the PLL-XOR method exhibits greater variance and a higher amount of stimulation during the down phase of SOs. Our study indicates that PLL cannot be easily adapted for universal use across different populations and individuals. When we optimized PLL parameters for each recording separately, they naturally varied among individuals. Thus, attempting to find a common set of parameters for all individuals reveals the limitations and certain boundaries of PLL in adapting to our requirements. Conversely, the fixed-step method rarely stimulates during the down phase of the SWA, making it a more robust and effective method with better stimulation position results.

In this study, we proposed a combination of ITPC and amplitude analysis to examine the immediate effects of acoustic stimulation on brain activity, using the fixed-step approach during sleep. Previous studies primarily used the averaged signal across stimulation or sham trials to demonstrate the effects (Ngo, et al. 2013b; Ngo, et al. 2015; Santostasi, et al. 2016). However, we found that ITPC is more sensitive to these effects compared to the commonly used averaged signal. Because ITPC is amplitude-independent, it specifically addresses the phases of the SO, unlike the averaged signal, which contains information about both amplitude and phase. For the first time, we have shown that acoustic stimulation increases the phase synchronization of the SWA. Notably, rather than prolonging SWA due to stimulation, ITPC measures reflected the phasing effect directly, without the influence of amplitude. This finding enhances our understanding of the actual effects of acoustic stimulation by providing evidence for alterations of existing endogenous brain activity rather than inducing new SWA. This finding could lead to new theoretical advancements in this area.

Additionally, ITPC represents an initial step towards a rigorous interpretation of cross-frequency coupling (CFC) (Cohen 2014). CFC is increasingly observed in EEGs during sleep, with phase-amplitude coupling (PAC) being the most common form. However, for PAC to be accurately interpreted, ITPC must be understood to eliminate potentially spurious couplings resulting from stimulus presentation. The contribution of an evoked response to the observed changes in SWA due to an actual sound stimulus remains an open question. We believe that calculating ITPC can help differentiate between these two mixed phenomena, enabling a more precise use and interpretation of advanced methods.

We discovered that the time-frequency representations of ITPC and signal power are less similar than expected. Rather than aligning, ITPC and signal power yielded complementary results. ITPC increased within a frequency-specific band over a broad period, while signal power changes occurred in a more time-specific manner and spread across a broad frequency band. Notably, ITPC showed a significant increase in the SWA band with a longer duration compared to the changes in signal power.

In sum, combining ITPC and power time-frequency representations provided a comprehensive method for analysing the effects of acoustic stimulation, as ITPC is amplitude-independent and power is phase-independent. This approach is effective in distinguishing between evoked and induced changes. For instance, our results indicated that acoustic stimulation altered signal power in the spindle band without affecting phase synchronization. This suggests that spindle activation is unlikely to be due to an evoked response to the acoustic stimulus. The specific latency of these observed changes also offers additional insights that can be compared with known evoked phenomena in EEGs. By utilizing this proposed approach and integrating the obtained data, we can better differentiate between evoked and induced changes caused by acoustic stimuli, thereby supporting rigorous theories explaining the effects of this promising method.

To conclude and summarise the second paper of this thesis, the aim was to explore the acoustic stimulation of slow-wave activity (SWA) during sleep and its impact on spontaneous brain activity. We could not achieve optimal stimulation placement with the PLL methods since the ideal parameters varied significantly among individuals, making PLL highly sensitive and unsuitable for universal application. On the other hand, the fixed-step method yielded satisfactory results for chronic insomnia patients, leading us to conclude that this simpler

approach was more appropriate for experiments with insomnia patients in comparison to the PLL method.

We found that purely phase-based quantification of SWA was the most sensitive method for distinguishing between stimulation and sham conditions. The ITPC and signal power metrics, which quantified the effects of acoustic stimulation, complemented each other well. Our findings indicated that the stimulation synchronizes spontaneous brain activity without eliciting a new SWA response in terms of amplitude. Therefore, moving the stimulation closer to detection appears more appropriate. This contrasts with the previous belief that the stimulation should be timed as close to the wave maximum as possible. Sending the pulse earlier increases the likelihood of phasing the waves, thereby enhancing the average SWA amplitude. Shortening the fixed time interval makes it more likely for the stimulation to occur during the rising (up) phase. SWA minimum detection using the fixed-step method is simple, robust, and efficient for real-time stimulation, with its accuracy primarily dependent on the sampling frequency; in our case, a satisfactory performance was achieved at a frequency of 1 kHz.

5.3. Strengths and limitations: reflections on design and methods of Study1 and 2

5.3.1. CLAS volume setup

Setting the volume or loudness of stimuli for CLAS is a complex issue that requires careful consideration to optimize its effectiveness while minimizing any adverse effects on sleep in the form of arousals or awakenings. There are several methods to determine the appropriate volume for CLAS, each with its strengths and limitations.

First, the **subjective method** was chosen for Study 1 (Dudysová, et al. 2024) and 2 (Piorecky, et al. 2021). It involved participants setting up their own comfortable volume before sleep (e.g. (Henin et al. 2019; Papalambros, et al. 2017; Papalambros, et al. 2019). We instructed the participants to adjust the volume so that the sound was comfortable and not disruptive. This method was easy to use and allowed for personalised comfort. However, a limitation was that participants set the volume right before sleep, i.e. in conditions where the laboratory environment tends to be noisier than later at night. This might lead to a tendency for the volume to be set too high and may prove disruptive at night resulting in arousal and awakenings during sleep. Therefore, from a practical standpoint, it is recommended that future research incorporating this subjective setup should allow for manual volume control from the control

room. Manual volume control would enable researchers or technicians to make necessary adjustments if the volume is set too high and causes undesired awakenings or arousals.

An alternative second approach is the **hearing threshold-based method**, where the participant's hearing threshold is first measured, and then the volume or amplitude is set slightly higher (by -5-15 dB/0.5 microV respectively), e.g. Leminen, et al. (2017); Wunderlin et al. (2023). This objective measurement ensures that the volume is individualised for each participant's hearing ability, making it suitable for both clinical and non-clinical populations where hearing ability can vary widely. This method is particularly advantageous as it tailors the volume to the participant's specific auditory sensitivity, thereby optimizing the stimulation's effectiveness while minimizing the risk of disturbing sleep. On the other hand, this method is limited practically by a more complicated setup for the measurement of each individual's hearing threshold. Nevertheless, automated algorithms have also been used (e.g. Diep, et al. (2020)).

Another third method involves setting **the same volume for all** participants, typically in the range of 45-60 dB (Ngo, et al. 2013b; Ngo, et al. 2015). This approach is straightforward to implement and ensures standardization across individuals. However, it is more suitable for young adults or more homogenous study populations and less suitable for populations with expected variable hearing abilities, such as the ageing population, as it does not account for individual differences in auditory sensitivity. This lack of customization can lead to potential sleep disturbances, thereby affecting the efficacy and comfort of the CLAS.

Lastly, a more individualized approach involves **testing several sound volumes** during one or multiple nights (Diep, et al. 2020; Lustenberger et al. 2022). If several volumes are to be tested within one night, the initial volume is then increased or decreased based on the online occurrence of arousals. This approach is beneficial for individualizing volume for each participant; it is however limited by the necessity of vigilant and experienced sleep technicians, who need to identify arousals and awakenings after too loud stimuli online as participants sleep, although automated devices have been previously used also (Diep, et al. 2020).

If multiple nights with several volumes is tested, it is possible to directly compare night event-related potentials (ERPs) between sham and several stimulation volumes, researchers can determine which volume causes arousals or awakenings. The goal is to identify the highest volume that does not disrupt sleep for each individual. While this method is also highly tailored

and minimizes the risk of arousals or awakenings, it is more complex and requires multiple night recordings for both sham and stimulation conditions. This individualized setup, though demanding, can provide the most precise adjustment of stimulus volume, ensuring both effectiveness and comfort in CLAS.

In sum, while various methods are routinely used to set the volume for CLAS, each with distinct advantages and challenges, future research should keep in mind the target population and aim to balance ease of use with the need for individualized settings to maximize the therapeutic potential of CLAS without compromising sleep quality.

5.3.2. Visual vs. automatic detection of SWS

An important aspect of CLAS methodology involves the choice between a visual control of the sleep stages by technicians/experimenters versus the use of automatic online sleep scoring algorithms to turn on and off the detection algorithm for SOs. Each approach has its merits and challenges.

In our two abovementioned studies, visual control was the chosen method for monitoring SWS, awakenings, and arousals. This method was also previously used elsewhere (e.g. Ngo, et al. (2013b); Ngo, et al. (2015)). This approach involved manual detection of SWS by experienced technicians who then manually activated or deactivated the SO detection algorithm based on observed changes in sleep stages. This method required highly trained staff, leading to higher costs and broad training requirements. Notably, despite proper training, differences between scorers and less reliability connected to online sleep staging may have arisen. However, this approach offered significant advantages too. Manual control allowed for greater sensitivity and control over the technical quality of the recordings. Technicians could identify artifacts and poor data quality, enabling them to manually override the system and reduce the likelihood of false positives in CLAS activation. This hands-on approach allows for flexible adjustments to CLAS based on the technical conditions of each recording session.

Conversely, automatic detection algorithms operate without the need for continuous staff presence, making them well-suited for home-based or longitudinal studies. Several studies have shown its effectiveness (e.g. Lustenberger, et al. (2022); Wunderlin, et al. (2023)). These algorithms automatically detect SWS and related changes, thereby controlling the SO detection algorithm to appropriately time the delivery of stimuli. The strength of this method lies in its consistency and scalability; it ensures that SOs are detected uniformly across all participants

and facilitates the management of larger datasets over multiple nights. However, the automated approach does have limitations. It may not always accurately identify the correct sleep stage, and there are challenges with detecting slow-moving artifacts like sweat, which could be mistakenly classified as SOs.

In summary, the choice between visual and automatic control in CLAS methodology depends on the specific requirements and constraints of the study, including the need for accuracy, the scale of the study, and the available resources. Each method offers distinct benefits and entails particular drawbacks, underscoring the need for careful consideration in designing future CLAS study protocols.

5.3.3. Adjusting for delays

As mentioned and discussed in introductory section 1.6.3.1, it was necessary to know and adjust for delays resulting in network, processing, and filtering to provide correct analyses and interpretation of data. In our sleep laboratory, the tests were facilitated by a device, which converted the audio signal into an electrical signal sent directly to the amplifier. This setup allowed the recording to display a marker representing the time the sound was sent from the algorithm and a signal indicating when the sound was played. The tests of network delays revealed that a delay primarily arose during the transmission of information from the algorithm control placed in the control room to the sound card placed in the sleepers' room. Our detection algorithm accounted for this delay by approximately 95 milliseconds, which was the average delay across this and other following projects.

The second type of delay we considered was a delay associated with the signal processing itself, which was negligible in our detection algorithm, as it was not computationally demanding. Our results and analyses were therefore not influenced by the network delay as it was adjusted for already in the detection stage nor by signal processing delay that was insignificant.

However, another type of delay we have encountered and have not adjusted for in our studies involved filter delays due to the phase shifting of the signal when using filters. Specifically, with IIR filters used for real-time signal processing in our studies, the delay increased with the number of coefficients—the filter order. For instance, for the project regarding this thesis (Study 1 and 2), we were using filters with four orders causing a shorter delay in comparison to other subsequent projects where our setup involved a filter with six coefficients, resulting in a longer delay. The exact delay in milliseconds varied because the IIR delay differed for each

frequency. Thus it can be characterized only by calculating the group delay response of the filter, which indicates how much the signal is delayed at specific frequencies (Figure 18). Unfortunately, our studies have not been adjusted for this type of delay and could have influenced our analyses and results, which is a limitation to consider.

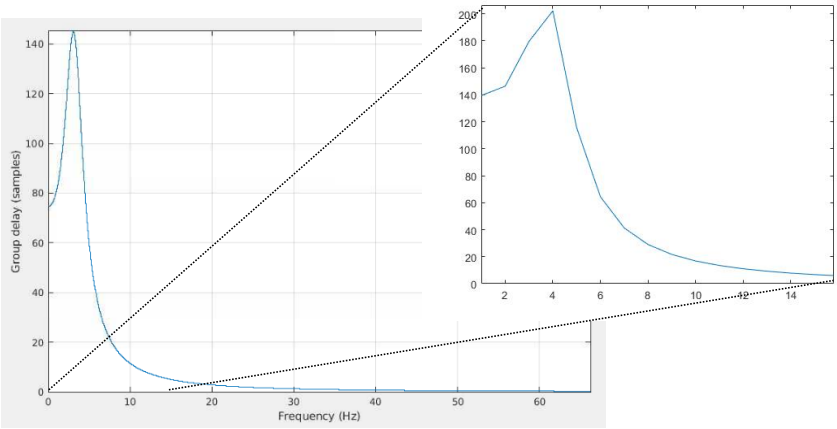


Figure 18. Group delay response of the 4-order filter used in Study 1 and 2. Notably, the delay was the largest in the slow frequencies. More specifically, in the frequencies of our interest, the delay was approximately 140ms long.

5.3.4. Blinding

The necessity for rigorous double-blinding procedures in sleep studies is paramount to negate any potential biases that could influence the outcomes due to important confounding variables (Esfahani, et al. 2023). In single-blinding setups, participants remain unaware of the condition—either sham or stimulation—they are subjected to during the experimental nights. This approach generally suffices as participants are typically unconscious and unaware of any auditory stimuli unless the stimulation is overly loud to awaken them, bringing the noise into their conscious awareness. However, while single blinding controls participant bias, it does not account for potential biases from researchers.

Double blinding extends beyond preventing participant bias, also limiting potential biases among researchers involved in the study. In this more robust setup, neither the participant nor the experimenters are aware of the condition being administered. Implementing double blinding among researchers is more complex and necessitates careful attention at various stages of the research process, including during interactions with the participant, data collection, and crucially, during data processing and post-processing. In the double blinding procedure,

researchers need to score sleep data and analyse results without any knowledge of the experimental conditions to which the participants were assigned.

In studies 1 and 2, our methodology was limited to single blinding, where the lab technicians and researchers were aware of the conditions being set up during the data collection phase and could have thus inadvertently influenced participants in their behaviour. Additionally, the markers in the recordings made it evident to researchers which conditions the participants had undergone during data processing. Recognizing these limitations, our newer experiments, particularly those involving home-based EEG systems in insomnia patients, have adopted a more stringent double blinding procedure. This adjustment aims to enhance the validity of our findings in the future, by thoroughly controlling for any experimenter biases that could potentially influence the study's outcomes.

Recently, discussions in the field, as highlighted by Esfahani, et al. (2023) and Harlow, et al. (2023), have raised concerns about the effectiveness of blinding procedures. It is estimated that approximately 34% of subjects in studies are not blind to the study condition as they report hearing acoustic stimulation during the stim condition (Harlow, et al. 2023). The awareness of the stimuli can significantly influence the outcomes of the study. To mitigate this issue, future studies could employ a random-phase stimulation condition, where stimuli are presented at random phases, serving as an additional placebo control to enhance blinding effectiveness (Harlow, et al. 2023). At the very least, it is essential to document whether participants heard the sounds in both sham and stim conditions to control the effect post-hoc during the analysis stages. This thorough approach in future methodologies will strengthen the reliability and validity of findings by ensuring that experimenter and participant biases are comprehensively managed.

5.4. General discussion: synthesis of results, general limitations of the field

5.4.1. Terminological discrepancy in literature

One of the overarching limitations within the field of CLAS and auditory stimulation more generally and memory consolidation research is the lack of standardized terminology and consistent methodological frameworks. This inconsistency often extends to the fundamental understanding and application of CLAS and OLAS. While CLAS is designed to adaptively respond to changes in sleep states by feeding back into the ongoing neural processes (Ngo, et al. 2015), OLAS typically follows a predetermined, non-adaptive stimulation pattern (Antony,

et al. 2018). This distinction is crucial yet often blurred in literature, leading to potential confusion and misinterpretation of study outcomes and mechanisms (Antony et al. 2022).

Moreover, the field is limited by a certain fragmentation in the terminology used to describe phenomena about sleep waves. Terms like "up-wave," "down-wave," "down-state," and "up-state" are defined variably across studies (details on definitions in introductory section 1.3.4), which complicates cross-study comparisons and may hinder collaborative efforts. Establishing a definite consensus on terminology and its consistent use across studies would enhance clarity, allowing for more precise communication and more robust comparative analyses within the research field.

5.4.2. Inter-individual and population differences in responsivity to CLAS

Understanding how different individuals and populations respond to CLAS is pivotal for optimizing its application in therapeutic and research settings. As research continues to explore the effectiveness of CLAS, it becomes increasingly important to identify why some individuals or populations respond more favourably to this method than others.

A notable point of discussion is the distinction between **responders and non-responders**. Studies have previously reported significant individual differences in how subjects' SWA is enhanced by CLAS. This issue was first openly discussed by Diep, et al. (2020), who observed that individuals with a greater enhancement of SWA exhibited larger improvements in cognitive functions such as verbal fluency and working memory compared to non-responders. Thus, significant positive associations were found between increased SWA and enhancement in these executive function outcomes. Additionally, factors such as the time of day and the cognitive abilities of subjects have been shown to affect responsiveness to CLAS (Koo-Poeggel et al. 2022). This variability suggests that not only do intrinsic individual characteristics play a role in the efficacy of CLAS but also that the timing and context of its application may be crucial.

In this context, our data from Study 1 also hint at a broad range within the responsiveness to CLAS as the number of effective stimuli delivered across participants varied considerably, suggesting that some may be naturally more receptive to auditory cues during sleep than others. Understanding this variability could be the next key to maximizing the therapeutic benefits of CLAS in insomnia and other populations, particularly in terms of memory consolidation.

Ageing is also increasingly recognized as a critical factor influencing the effectiveness of CLAS. Research has shown mixed results among middle-aged and older populations, with some studies reporting a positive impact on memory (Papalambros, et al. 2017) and others noting negative results (Diep, et al. 2020; Schneider, et al. 2020). Notably, the influence of CLAS appears to differ significantly with age: older adults tend to exhibit short-lived benefits compared to younger individuals, where the effects are more enduring. Additionally, while CLAS enhances the coupling of SOs and sleep spindles in younger adults, this effect does not consistently manifest in older adults (Schneider, et al. 2020). This discrepancy was attributed to age-related neural changes, including neuronal degradation, diminished connectivity between the thalamus and cortex, and slower cellular refractory periods (Schneider, et al. 2020).

Moreover, the optimal timing for auditory stimulation seems to narrow with increasing age (Navarrete et al. 2020), underscoring the necessity of understanding SO dynamics across different age groups and contexts. Overall, the current body of research suggests that CLAS may be more effective in younger populations, although these findings are preliminary due to the limited number of studies focusing on older adults. For instance, the effect sizes reported for younger versus older populations show a difference—Hedges' g values of .31 in younger adults versus .11 in older adults—though these are not statistically significant (Wunderlin, et al. 2021).

Parallel to the findings on age-related differences in responsiveness to CLAS, certain **clinical populations** may prove more amenable to sleep stimulation techniques aimed at enhancing sleep and memory functions. It was suggested that population groups such as individuals with ADHD, autism, MCI, early stages of Alzheimer's, and patients suffering from depression or schizophrenia could potentially benefit from CLAS or other forms of auditory stimulation (Esfahani, et al. 2023). These populations, characterized by specific neurological profiles and sleep disturbances, may exhibit unique responsiveness to auditory cues that align with their particular neural and sleep patterns.

The question remains, however, whether insomnia patients represent an appropriate target group for such interventions. Drawing parallels with the literature on ageing and its associated changes in SWA power, one might speculate that the smaller effects observed in older adults could predict similarly modest outcomes in individuals with insomnia. However, unlike the general ageing population, insomnia sufferers typically experience disruptions in sleep without

age-related morphological brain changes. Conducting case-control studies that compare individuals with insomnia to healthy volunteers could thus provide valuable insights into the efficacy of CLAS in insomnia.

A last factor, which may play some role in responsiveness to CLAS, is a possible effect of **gender**. Physiological variations in men and women in SWS, specifically greater SWA power in women compared to men (Carrier et al. 2001; Ehlers and Kupfer 1997; Mourtazaev et al. 1995) might influence outcomes of CLAS. This physiological difference could hypothetically make women more responsive to CLAS, potentially enhancing the efficacy of such treatments in female populations. Despite these possibilities, systematic investigation into gender effects on the responsiveness to auditory stimulation is notably lacking in the current literature.

Contrary to this hypothetical expectation, a recent meta-analysis conducted by Stanyer, et al. (2022), which considered the number of males in studies as a potential moderator, found no significant gender effect on the outcomes of CLAS in young adults. This finding indicates that, at least in this age demographic, gender does not significantly influence the effectiveness of CLAS. Nonetheless, the absence of gender as a covariate in many studies underscores a gap in the research and may prove insightful in future research.

To sum up, factors such as individual differences, age, clinical population, and gender may significantly influence how individuals respond to auditory stimulation, underlining the necessity for tailored therapeutic approaches. The evidence suggests that while younger individuals and both healthy and certain clinical groups may experience benefits from CLAS, older adults and possibly individuals with insomnia might exhibit more modest responses. Additionally, there is an understudied potential for gender-related differences although gender effects may be absent (Stanyer, et al. 2022). Future studies should strive to address these gaps by incorporating a broader range of demographic and clinical characteristics to better understand the full spectrum of responses to CLAS. This will not only enhance the generalizability of the findings but also enable the development of more effective, personalized CLAS strategies for improving sleep and cognitive functions across diverse populations.

5.4.3. Underpowered research field

The field of auditory stimulation research, particularly in the contexts of OLAS and CLAS, is significantly limited by underpowered studies. Typically, studies include relatively small sample sizes, with the most common sample sizes ranging from 8 to 30 participants (16 on

average) (Wunderlin, et al. 2021). Given the modest effect size associated with auditory stimulation (combined effect size of .25 for OLAS and CLAS), such sample sizes result in a power of only about 15%. To achieve a more acceptable power level of 80%, studies would need to include approximately 127 subjects. Even for studies observing larger effects, particularly in younger populations or with CLAS-specific methodologies, the required sample sizes would still need to range between 42 and 62 participants to ensure sufficient statistical power (Wunderlin, et al. 2021).

This persistent issue of underpowered studies has been suggested to contribute to the observed decline in overall effect sizes of CLAS approaches (Harlow, et al. 2023). The small sample sizes across studies not only limit the statistical power but also lead to inconclusive findings and discrepancies in the literature. On the other hand, the practicality of including larger sample sizes of 40-120 participants remains a challenge due to technical, financial, and logistical constraints. As the field progresses, especially with the advancement of out-of-laboratory methods and more accessible auditory stimulation technologies, there may be potential to overcome this substantial limitation. Such developments could facilitate larger-scale studies that provide more definitive insights and contribute to a more robust understanding of auditory stimulation's effects on sleep and cognitive functions in both healthy and clinical populations.

5.4.4. Memory consolidation research discrepancy

The field of research on the relationship between auditory stimulation and memory consolidation has several factors which considerably limit the generalizability of findings and are presented further:

5.4.4.1. Discrepancies across different studied groups

This is characteristic not only for different age groups as mentioned in 5.4.2, but also within younger populations. In younger groups, several studies report positive associations between CLAS and enhanced memory consolidation (Leminen, et al. 2017; Ngo, et al. 2013b; Ngo, et al. 2015; Ong, et al. 2016; Prehn-Kristensen, et al. 2020). However, equally numerous studies have failed to establish any association between auditory stimulation and memory improvements (Harrington et al. 2021b; Henin, et al. 2019; Choi, et al. 2019; Ngo, et al. 2019; Ong, et al. 2019; Weigenand, et al. 2016). Similarly, our Study 1 (Dudysová, et al. 2024) also found no significant relationship between overnight memory consolidation and CLAS, further highlighting the inconsistent findings within this demographic.

5.4.4.2. Opposing roles of SOs and delta waves

One of the explanations for this discrepancy in findings might be the complex and potentially opposing roles that SOs and delta waves play in memory processes (Wunderlin, et al. 2021). Previous research in an animal model suggests that while SOs facilitate memory consolidation, delta waves may play a role in forgetting (Kim, et al. 2019). This dual role could explain some of the conflicting results observed in human studies. For example, in our investigations, we noted that CLAS increased relative SO activity but simultaneously decreased relative delta activity. This interaction might implicate a nuanced interplay between memory enhancement and forgetting mechanisms that CLAS could inadvertently influence. Despite the increased SO activity, there was no corresponding improvement in memory processing observed in our study. This finding underscores the necessity to examine the specific frequencies and interactions of SOs versus delta waves more closely, as their interrelation might significantly affect the outcomes of auditory stimulation studies. Current detection algorithms often do not distinguish between these oscillations, nor do they investigate delta waves in detail, which could lead to overlooked variables critical for understanding the full impact of CLAS on memory consolidation (Esfahani, et al. 2023; Ngo and Born 2019).

5.4.4.3. More SO types

Another explanation for the discrepancies seen in memory consolidation studies might stem from the detection and stimulation of different types of SOs. Recent insights from Navarrete et al. (2023) proposed that there are multiple types of SOs, each with unique topographical characteristics and neural synchronization. They categorized SOs into three distinct types: 1) steep-slope slow waves with a global spread, 2) flat-slope waves with localized spread and a homeostatic decline over time, and 3) multiple-peaked flat-slope events with a global spread. Each of these types responded differently to CLAS (Navarrete, et al. 2023). This diversity in SO types suggests that if CLAS targets one specific type of SO preferentially or by chance over another, it could lead to inconsistent results across different studies. This variation in SO response to stimulation may underpin some of the incongruities reported in the literature concerning the efficacy of CLAS on memory processes.

5.4.4.4. Limits of traditionally used memory tasks

Another potential issue contributing to the discrepancies in memory research relates to tasks traditionally used to measure overnight memory consolidation. Firstly, there is a discussion

over the hippocampal dependence of them in the context of active systems consolidation processes (see 1.3.2 for details). While many studies employ tasks like word-pair associations, it can be argued that these tasks might not be solely dependent on hippocampal processes due to their semantic components. Tasks such as word-nonsense-pair tasks (Diep, et al. 2020), spatial navigation tasks (Henin, et al. 2019), or face-name association tasks (Leminen, et al. 2017), which are considered to rely more directly on hippocampal function, could provide a stronger measure of memory consolidation in the context of CLAS. Yet, even with the use of these hippocampus-dependent tasks, studies have reported negative findings (Diep, et al. 2020; Henin, et al. 2019; Leminen, et al. 2017).

A second discussion point on the traditionally used tasks such as semantically related word pairs, also used in Study 1, pertains to their psychometric unreliability. The statistical challenge arises because pre- and post-sleep scores are highly correlated, typically around r = 0.949 (95% CI 0.920, 0.979) (Harlow, et al. 2023). This high correlation diminishes the reliability of the difference scores and implies that observed changes in word-pair retention overnight might be influenced heavily by measurement error rather than genuine cognitive changes, thereby biasing effect sizes and reducing statistical power (Harlow, et al. 2023).

To address this limitation, it has been suggested that the reliability of these measures could be improved by increasing the number of testing sessions, both for sleep and memory assessments. Providing repeated measurements would enhance test-retest reliability and help separate true variance from noise. Additionally, employing multiple forms of memory tasks that engage similar physiological circuits could help validate findings across that specific cognitive domain. If consistent results are obtained across these varied tasks, it would strengthen the generalizability of the findings (Harlow, et al. 2023).

5.4.4.5. Discrepancy in other memory types

Furthermore, there is not only discrepancy within the declarative hippocampus-dependent memory literature but also within literature testing other memory types. For instance, one study compared the outcomes of four different memory tasks and found that only the word-pair task showed improvement following CLAS, while other tasks such as procedural finger tapping, picture recognition, and face-name association did not exhibit any significant effects (Leminen, et al. 2017). This pattern is echoed in a meta-analysis where CLAS was associated with significant improvement in declarative memory among young adults (Hedges' g = 0.87, p =

0.014), but it showed no effect on non-declarative memory tasks (Hedges' g = -0.25, p = 0.357) (Stanyer, et al. 2022).

5.4.4.6. Discrepancy in other cognitive domains

Various studies have included measures for domains such as attention, verbal fluency, working memory, planning, inhibition, and navigation, among others. The majority of these studies report negative findings except a few positive outcomes (Wunderlin, et al. 2021). For instance, working memory and verbal fluency improvements were observed post-CLAS but only among high responders (Diep, et al. 2020). Furthermore, attention measures in healthy subjects were found to improve following two consecutive nights of stimulation (Diep et al. 2021). Interestingly, working memory improvements were noted in ADHD patients following CLAS but not in healthy controls (Prehn-Kristensen, et al. 2020).

5.4.4.7. Interactions with endocrine system

A last factor to ponder when discussing memory research discrepancies is considering higher-order interactions with endocrine functions. For instance, CLAS has been demonstrated to influence cortisol levels, which are intricately linked with cognitive performance, mental health, and sleep-mediated memory functions (Besedovsky, et al. 2017; Diekelmann and Born 2010). This suggests that the impact of auditory stimulation extends beyond the direct modulation of sleep architecture to encompass broader physiological and neuroendocrine responses. The influence of cortisol, a stress hormone known to affect memory consolidation adversely, underscores a complex interplay where CLAS might simultaneously modulate both sleep and systemic hormonal levels. Thus, the observed effects of CLAS on memory could be the result of a multifaceted interaction between neural, cognitive, and hormonal changes. This complexity hints that CLAS may be impacting a broader range of biological systems than previously appreciated, potentially explaining why its effects vary across different studies and cognitive tasks.

To summarize, the research area on CLAS and memory consolidation is characterized by a certain disparity in findings. While some studies demonstrate that auditory stimulation can enhance hippocampus-dependent memory functions in certain populations, other memory and cognitive tasks yield mixed results, suggesting that the benefits of auditory stimulation may not universally apply across all populations, cognitive domains or even within one domain. This discrepancy highlights the need for more nuanced and sufficiently powered investigations to

pinpoint the specific conditions under which auditory stimulation can be advantageous. It is essential to consider that the relationship between CLAS and memory consolidation may involve higher-order factors beyond the memory task employed, potentially including endocrine interactions that affect cognitive outcomes. Such insights could pave the way for refining CLAS and other auditory stimulation methods to enhance their effectiveness across diverse cognitive functions and clinical applications.

5.5. Further directions in CLAS research

5.5.1. Clinical applications of CLAS in insomnia: future research recommendations

As CLAS continues to be explored for its therapeutic potential in insomnia and other clinical settings, future research should focus on refining and optimizing the technique for improving clinical applicability. Here are several recommendations for advancing the clinical applications of CLAS in insomnia:

5.5.1.1. Detection algorithm development

Designing more sophisticated and ideally automated algorithms tailored specifically for clinical populations, such as those with notable alterations in SWS architecture, is crucial. Such algorithms should accommodate the distinct physiological characteristics of insomnia patients to improve the efficacy of CLAS.

5.5.1.2. Healthy control comparative studies

Conducting comparative studies between insomnia patients and healthy volunteers will provide insights into whether the responses to CLAS are consistent across these different groups or whether the insomnia population reacts to CLAS specifically. Given the lack of healthy controls in our experiment of Study 1, we were unable to compare the effects of CLAS in insomnia vs. healthy subjects directly. This comparison will be vital for understanding the unique or shared CLAS effects.

5.5.1.3. Multicentre studies

Implementing multicentre studies can help accumulate a sufficient number of participants, ensuring robust statistical power and more generalizable findings across diverse populations (including insomnia) and geographic locations.

5.5.1.4. Adjunct therapy

Investigating the potential of CLAS as an adjunct therapy to existing treatments, such as CBT-I could open new avenues for enhancing treatment outcomes. There is promising potential, as indicated by an ongoing trial with PTSD patients enhancing the effects of EMDR therapy (de Boer, et al. 2020; van der Heijden, et al. 2022; Van Marle, et al. 2017).

5.5.1.5. Integrating CLAS approaches

Exploring the combination of different CLAS protocols within a single treatment session could potentially amplify its therapeutic effects. For instance, merging alpha phase-locked auditory stimulation, which targets sleep onset symptoms (Bressler, et al. 2024), with CLAS targeting SO/SWS enhancement (Dudysová, et al. 2024), might provide a more comprehensive approach to ameliorate insomnia symptoms and improve overall sleep quality.

5.5.2. Mechanisms of CLAS: Optimal CLAS parameters and their mechanistic effect on SOs and memory

In exploring the efficacy of CLAS, it is crucial to undertake systematic investigations into the functioning of slow oscillations (SOs) and the parameters of CLAS. Understanding these dynamics is essential for identifying the most effective stimulation parameters and elucidating mechanisms that either enhance or inhibit the intended effects. Such studies will not only refine our understanding of how CLAS interacts with neural oscillations but also establish a more solid foundation for potential clinical applications.

5.5.2.1. New vs. endogenous SOs

To elucidate the mechanisms of function in CLAS research, further studies have yet to answer an imperative question of whether CLAS elicits new SO activity (that would otherwise not be there) or whether the endogenous (and already existing) activity is simply altered. In our investigation of scalp EEG data (Piorecky, et al. 2021), we found the first evidence that endogenous SOs were synchronised by CLAS rather than new SOs were generated.

A study by Jourde, et al. (2024) was not able to distinguish between elicited (evoked) and endogenous brain activity due to the nature of their design but they interestingly captured both endogenous and evoked activity using combined EEG and MEG. The authors confirmed that the primary generator with the strongest SOs is the ventral frontal area and showed that the information flows from the orbitofrontal cortex (OFC) in the direction of the primary auditory

cortex (AC) bilaterally in the SWA range. The excitability state of OFC then significantly affected the success of CLAS to generate N550-P900 complex (an evoked SO about 550 to 900 ms after sensory input (Bellesi, et al. 2014; Riedner, et al. 2011)). Thus, there is a common origin of evoked and endogenous SOs in ventral frontal brain regions. This suggests that CLAS-related memory consolidation is activated through stimulation in the same areas as spontaneous consolidation.

However, these mechanisms need to be tested experimentally in a direct comparison of spontaneous versus evoked sleep rhythms and memory replay processes (Jourde, et al. 2024). For example, animal models or computer neural modelling methods could shed light on this pertaining question.

5.5.2.2. Optimal phase for stimulation

Determining the optimal phase for auditory stimulation within the SO cycle remains an open point of discussion among researchers, with precise timing being essential to maximizing the effects of CLAS on sleep and memory enhancement (Wunderlin, et al. 2021). Several approaches have been documented in the literature all commonly targeting the SO up-state, however each meaning a different ideal phase during the SO up-state. Although more approaches may be existing, they are broadly categorized into three groups as follows:

1. **Targeting up-wave:** Several studies targeted the ascending phase of SOs (anywhere between the minimum and maximum) to facilitate the SO enhancement and associated memory benefits (Cox, et al. 2014; Navarrete, et al. 2020). Conversely, stimulating during or at the beginning of the down-wave has been shown to disrupt SO continuity, undermining the effectiveness of CLAS (Cox, et al. 2014; Ngo, et al. 2013b).

2. **Targeting zero crossing**: Some methodologies regard the negative-to-positive transition point as optimal for stimulation, which capitalizes on the natural shift in neuronal activity. For example, a modelling study found the transition from down-to-up state as the most sensitive period for effective stimulations. It influenced the spatial and temporal distribution of the slow waves while optimizing memory replay. Conversely, stimulation during the up-states did not significantly affect the slow waves or post-sleep memory performance (Wei et al. 2020).

3. **Targeting around the peak:** Some authors advocate for stimulation close to the peak of the SO. This may involve both conditions just before (up-wave) and behind (down-wave) the peak

(Ngo, et al. 2013a; Ngo, et al. 2013b; Ngo, et al. 2015). This targeting led to increased spindle power, reduced memory forgetting and effective CLAS protocols.

The discussion on the optimal target phase of CLAS may be clued in by findings from similar fields. In CL-TMR research, mixed results regarding the phase-specific effects on memory consolidation were found. For instance, while one study has found that cueing during the up-state enhanced the consolidation process (Ngo and Staresina 2022), another had reported no significant difference between up-state and down-state cueing, suggesting that the general reactivation processes during NREM sleep may facilitate memory consolidation irrespective of the SO phase (Wang et al. 2022). Furthermore, experimental evidence using EEG-guided TMS during NREM sleep demonstrated that stimulation near the peak of the SO led to increased evoked responses compared to stimulation just before the trough of oscillation (Bergmann et al. 2012).

Importantly, age and possibly other unidentified factors may affect the ideal stimulation phase, suggesting that different clinical populations might require tailored approaches. A promising direction involves a more localized stimulation during the up-state in ventral frontal regions, which are critical for generating N550–P900 complexes and associated spindles. This approach would require high-density EEG (HD-EEG) capable of source-localizing SOs in real-time, coupled with advanced computing power and possibly machine learning techniques to optimize the timing and location of auditory cues (Jourde, et al. 2024).

In summary, identifying the optimal phase for stimulation within the SO cycle is crucial for enhancing the mechanistic efficacy of CLAS, particularly in contexts involving memory consolidation. Generally, the depolarized active upstate is considered an optimal phase for releasing CLAS, however, it is still an open question what phase exactly within the upstate is found most effective. Future studies need to explore these dynamics more comprehensively to establish the most effective and tailored auditory stimulation strategies for both the general population and specific clinical populations.

5.5.2.3. Optimal number of sounds

One understudied aspect of CLAS research involves determining the optimal number of auditory cues that should be presented during sleep. The question arises whether more stimulation is always better, or is there a point beyond which additional sounds no longer contribute to—or could even hinder—sleep-dependent memory consolidation processes?

Firstly, the concept of neural homeostasis suggests that the brain strives to maintain a balanced state, and artificially boosting SWA might potentially lead to adverse effects if overdone (Esfahani, et al. 2023). This is especially pertinent given that the brain has an inherent capacity to regulate its neurophysiological activities, including SOs. While under-stimulation might be insufficient to yield significant effects, over-stimulation could disrupt the natural sleep architecture or lead to diminishing returns in terms of memory benefits.

Sparse empirical evidence suggests that there may indeed be an upper limit to the beneficial effects of increased SWA. While some CLAS studies have successfully enhanced total SWA (Diep, et al. 2020; Garcia-Molina et al. 2019; Santiago, et al. 2019), others have observed that SWA levels plateau after a certain amount of stimulation (Ngo, et al. 2015; Papalambros, et al. 2017; Schneider, et al. 2020). This indicates that there might be a threshold beyond which additional stimulation does not translate into further gains and could be counterproductive. What this threshold is and how we may measure it is up to future investigations to find.

Moreover, the characteristic of refractoriness of sleep spindles highlights a physiological limit to how frequently auditory cues can effectively be presented. Research indicates that the thalamic networks expressing spindles undergo a refractory period lasting between 3 to 10 seconds after spindle activity, during which time these networks are less responsive (Antony, et al. 2018; Fernandez and Lüthi 2020; Ngo, et al. 2015). Memory improvements were found more pronounced when auditory cues were timed to follow this refractory period (Antony, et al. 2018). Stimulating within this window may fail to induce additional spindle activity coupled with SOs, thereby nullifying or even potentially counteracting the benefits on memory enhancement.

A second crucial point of discussion regarding the optimal amount of stimulation concerns the efficacy of various stimulation protocols using various numbers of clicks/sounds after one detected SO. Is it better to present one, two, or multiple subsequent sound clicks within a single SO train? Can it significantly influence the outcomes of CLAS? Each protocol variant comes with its theoretical benefits and practical challenges and may be broadly classified into two areas:

1. **Fixed-step protocols** are predominant in auditory stimulation studies, where stimulation is triggered based on the predicted timing of an upcoming SO up-wave, followed by a pause to assess the impact of the stimulation (Besedovsky, et al. 2017; Cox, et al. 2014; Diep, et al.

2020). These studies often involve a single click to enhance a wave (Henin, et al. 2019; Leminen, et al. 2017) or a double-click strategy where the first sound enhances the initial SO and a second sound targets the next following enhanced SO (Dudysová, et al. 2024; Ngo, et al. 2013b). While the two-click method aims to strengthen consecutive SOs, if not precisely timed for each individual's SO rhythm, the second click may disrupt the natural oscillation as was in our case (Dudysová, et al. 2024), suggesting a single click might be more beneficial by reducing the risk of phase misalignment during the second following SO.

2. **Block-wise protocols**, as employed in studies by Papalambros, et al. (2017); Papalambros, et al. (2019) and Ong, et al. (2016); Ong, et al. (2019), administer stimuli in intermittent 'on' vs. 'off' blocks. These can involve sequences such as five consecutive stimuli (Papalambros, et al. 2017) or time intervals of stimulation followed by equal durations of rest (e.g., 10 seconds on followed by 10 seconds off, Huwiler et al. (2022). The theoretical advantage of this approach is that it could sustain or even entrain an ongoing train of SOs, providing a rhythm that might enhance overall SWA dynamics. However, empirical support for this hypothesis remains limited and the results are mixed, highlighting the need for more robust and systematic investigations focusing directly on this issue.

The absence of direct comparisons between these stimulation strategies underscores a substantial gap in the literature. For now, the best educated guess for the most efficient approach would be to use a single click/sound stimulation for one detected SO and then to detect and stimulate another SO after a refractory period of about 6-10 seconds. However, future studies are needed to systematically evaluate what amount of stimulation yields the most robust and consistent enhancements in SO activity and related memory consolidation functions.

5.5.2.4. Optimal type, length, and volume of sounds

Understanding the impact of sound characteristics on the efficacy of CLAS is another important open area that is pivotal for optimizing CLAS protocols. This involves exploring variations in the type of sound, its duration, and volume to determine the most effective parameters for enhancing SO activity without disrupting sleep.

1. **Type of sound:** The choice between pink and white noise, among other sounds, is a fundamental consideration. Pink noise, characterized by a 1/f power spectrum, is generally preferred in auditory stimulation studies due to its softer, more natural sound profile which is less likely to cause sleep disturbances. Empirically, it has been shown to elicit more pronounced

event-related potentials (ERPs) and recruit a broader range of cortical areas compared to white noise (Debellemaniere et al. 2022). Despite this, the same research suggests that any type of sound can generally trigger an SO response and that varying the types of sounds used may also help in reducing habituation effects during the night (Debellemaniere, et al. 2022).

2. **Length of sound:** The duration of auditory stimuli typically ranges from 50 milliseconds to 500 milliseconds in CLAS studies. The standard 50 ms duration is widely used as it has been proven effective in inducing the necessary ERP responses. However, the question remains whether different sound lengths might influence oscillatory brain activity differently or vary in their impact on sleep architecture. There has been no formal comparison within the field to assess how these variations might affect the effectiveness of CLAS, suggesting an area for further investigation.

3. **Loudness of sound:** The intensity of auditory stimuli is another formally understudied critical factor that influences the effectiveness of CLAS. While louder sounds may initially evoke a stronger brain response, there is a threshold beyond which they may become counterproductive, potentially arousing the participant and disrupting sleep-dependent memory consolidation. More details on the experimental planning about this issue may be found in the earlier section "CLAS Volume Setup" (5.3.1.). Overall, formally investigating the sound intensity may help future investigations optimize CLAS in all experimental setups and potential clinical applications.

5.5.2.5. Optimal detection/stimulation algorithms

The development and refinement of detection and stimulation algorithms are crucial for the effective implementation of CLAS in basic and applied research. As discussed earlier in the introduction, numerous approaches exist for detecting and stimulating SOs, each with unique advantages and potential limitations (see section 1.6.3.2 for more details).

Despite the variety of existing methods, there is a notable lack of direct comparisons between these algorithms, which poses a challenge in identifying the most effective approach (Esfahani, et al. 2023). Findings from our research indicate that amplitude-based thresholds might outperform PLL approaches due to their simple, robust, and efficient nature (Piorecky, et al. 2021). However, the efficacy of the Talamini approach and other methods remains to be thoroughly evaluated. Systematic research comparing these different strategies is essential to ascertain which is most beneficial for enhancing sleep-dependent memory consolidation.

An additional layer of complexity is introduced by the possibility of different types of SOs, as earlier highlighted in section 5.4.4.3. Navarrete, et al. (2023) suggested the distinction between at least three SO types, characterized by variations in topography and dynamics. Recognizing and targeting specific SO types could potentially optimize the effects of CLAS, addressing some of the discrepancies observed in the literature regarding the impact of auditory stimulation on memory consolidation. Future detection algorithms should be capable of identifying these specific SO types online to target the SOs most relevant to memory processes, potentially minimizing complex endocrine interactions (details in 5.4.4.7).

5.5.2.6. Night vs. nap CLAS effect

One more unknown CLAS aspect concerns the question of whether CLAS is equally effective during naps as it is during nocturnal sleep and is a function of sleep duration. Considering the homeostatic pressure peaks in the evening it seems plausible that night-time CLAS could provide more frequent stimulations and thus be more effective in enhancing SO amplitude and sleep spindle activity and consequently overnight memory. The stimulation's impact may be then less pronounced during naps due to shorter sleep duration and potentially lower homeostatic pressure (Koo-Poeggel, et al. 2022). Moreover, the role of SOs is likely influenced by both circadian rhythms and homeostatic sleep pressure (Koo-Poeggel, et al. 2022) and research also must consider the facilitative contribution of REM sleep to synaptic downscaling and memory processes (Niethard and Born 2019) which may not be as present in nap studies.

Conversely, findings from studies using the TMR method suggest that the duration of sleep may not significantly affect the benefits of memory cues. A meta-analysis indicated that TMR enhanced memory consolidation similarly, whether cues were presented during afternoon naps or nocturnal sleep (Hu, et al. 2020). This suggests the possibility that CLAS could be equally effective during naps as it is during overnight sleep.

However, to date, no formal studies have directly compared the effects of CLAS between naps and overnight sleep. It is crucial for future research to conduct empirical investigations or, ideally, perform meta-analyses to consolidate findings across different study designs and populations. This would provide a more definitive understanding of how sleep duration and time of day influence the efficacy of CLAS in enhancing sleep-dependent memory consolidation and other cognitive functions.

5.5.3. CLAS in the home environment

The future of auditory stimulation research is likely to embrace the development of systems for use in home settings, promising significant advancements in how we utilize CLAS. Home-based systems enable long-term observations and within-subject assessments of CLAS efficacy, offering a non-invasive and potentially fully automated approach to sleep enhancement. Some existing studies have already demonstrated the feasibility and effectiveness of enhancing SWA in such settings (Ferster et al. 2022; Ferster et al. 2019; Garcia-Molina, et al. 2019; Lustenberger, et al. 2022).

Several commercial products are emerging such as SleepLoop (University of Zurich, Switzerland) and SmartSleep Deep Sleep Headband (Philips, USA). Notably, one promising device and SO detection algorithm currently under testing in our insomnia CLAS studies is developed by Lucia Talamini and colleagues at the University of Amsterdam. It utilizes a commercial EEG headband called Zmax (Hypnodyne, Bulgaria) and wireless transmission of data to a computational unit (PC/tablet) that processes signals in real time and applies stimulation as per the detected sleep oscillations (Talamini et al. 2016; Talamini and Korjoukov 2018). Regardless of the type of device, a cautious adoption is advised due to the current lack of comprehensive evidence supporting their everyday use (Wunderlin, et al. 2021).

To fulfil the potential of home-based CLAS systems, several challenges in their development must be addressed. First, it will be essential to refine algorithms that can reliably detect sleep stages while minimizing errors caused by common artifacts like sweat, which could lead to false stimulations. Secondly, home-based systems must be designed to ensure comfort and ease of use for individuals without prior experience with such technology. This will help prevent discomfort associated with long-term use and increase the willingness of participants and patients to engage with the technology regularly (see the following section 5.5.4 for more details). Thirdly, the research in home-based systems must also overcome the caveats associated with the non-standard EEG montages and a requirement for a certain computational power for effective signal processing and wireless transmission.

The line of research in home-based systems will help determine the dose-dependent effects of CLAS which has important implications for further development of clinical applications. Lustenberger, et al. (2022) as first observed a dose-dependent response in SWA over a two-week protocol of active CLAS versus a sham condition. Future research needs to explore

whether numerous stimulation nights lead to additive enhancements in brain response and whether these dose-related changes translate into perceptible improvements in sleep quality, alterations in sleep macrostructure, or memory consolidation.

5.5.4. Research on negative/side effects of CLAS

One area of complete lack of research attention is the exploration of potential side effects associated with CLAS. Alike to the scrutiny given to any new therapeutic intervention, it is crucial to study CLAS in this context. As advancements in technology allow for the application of CLAS over multiple consecutive nights, understanding and mitigating any associated risks become increasingly important.

To date, there have been no specified negative side effects of CLAS reported in the literature. However, possible adverse effects could arise from the physical setup used in studies, particularly when multiple nights are involved. Possible adverse effects include:

1. Continuous use of EEG equipment can cause physical discomfort, such as indentations in the skin or skin irritations. It is essential to use hypoallergenic materials and to screen participants for allergies to any materials used, e.g. during adaptation nights.

2. Although no studies have reported changes in sleep macrostructure, such as alterations in REM or NREM stages due to CLAS (Stanyer, et al. 2022), there is a possibility of arousals or awakenings if the sound volume is too high. This aspect has not led to observable negative impacts in short-term studies, but its effects in longer-term, more intensive protocols warrant careful monitoring. A notable concern highlighted by Lustenberger, et al. (2022) is that while a two-week protocol of CLAS increased SWA, it also led to a decrease in mood associated with alterations in REM duration. This finding suggests that while CLAS can enhance certain aspects of sleep architecture, it may inadvertently impact other facets of sleep and mental well-being.

3. The broader biological implications of long-term or intensive CLAS usage have yet to be thoroughly investigated. Potential systemic effects on immunity, the cardiovascular system, mood, and overall health need to be carefully evaluated in future longitudinal studies to ensure the safety and efficacy of CLAS as a therapeutic tool. This oversight is critical to ensure that while pursuing the benefits of CLAS, the overall health of participants and patients remains protected.

5.5.5. Outlook for new theoretical advancements

The active systems consolidation hypothesis (details in 1.3.2.), while foundational, does not comprehensively account for the variances observed in auditory stimulation research. This underscores a need for more refined theories that can integrate new findings and resolve inconsistencies noted across studies, both in sleep research generally and more specifically in auditory stimulation. The success of future endeavours in this domain depends significantly on the quality of the theoretical frameworks employed; without robust theories, it becomes challenging to apply innovative hypotheses and construe well-designed, methodologically sound experiments.

One general area that remains underexplored is the process by which memories are identified and tagged as significant during encoding, labelling them for later reactivation and consolidation during sleep. This tagging of memories for their salience suggests that not all learned information receives equal treatment during the consolidation process. According to Brodt et al. (2023), salient information, likely relevant for future adaptation, is prioritized at the encoding stage. The differential effects of CLAS on various types of memory tasks observed further illustrate this point. Tasks that are perceived as relevant or meaningful, such as learning meaningful word pairs, may be more likely to benefit from CLAS compared to those considered less relevant, like word-nonsense pairs. This differentiation could be driven by the intrinsic value assigned to the memory content at the time of learning.

In this line of thought, the motivation of participants and their perception of the information's relevance may significantly influence the efficacy of CLAS. If participants recognize the learned information as useful for their future needs, it could potentially enhance the memory consolidation effect of auditory stimulation. For example, some studies indicate that emotional stimuli—considered more salient—are often prioritized over neutral stimuli (Nishida et al. 2009; Payne et al. 2008). This interaction between memory salience, emotional significance, and participant motivation highlights complex dynamics at play, which future theoretical advancements must address to fully elucidate the mechanisms and optimal conditions under which auditory stimulation can be beneficial.

Additionally, the process of tagging salient memories intersects with emerging research on how sleep facilitates the abstraction of memories, specifically the formation of generalized, schematic representations distilled from prior experiences. This aspect of memory processing

highlights sleep's role in enhancing our ability to abstract rules and regularities from complex information. Several studies have demonstrated that sleep significantly aids in the improvement of abstract rule extraction (Lerner and Gluck 2022; Lutz et al. 2018; Sanders et al. 2019; Wagner et al. 2004), suggesting that sleep contributes to higher-order cognitive processes by reorganizing and synthesizing memories into more useful schemas. However, this enhancement is not consistently observed across all studies as some research has reported an absence of this effect (Brodt et al. 2018; Talamini et al. 2022).

On general level, advancing theoretical frameworks may greatly benefit from integrating findings from animal models, which can offer unique insights into the neural mechanisms underlying memory processing during sleep. For example, Kim, et al. (2019) proposed the novel concept of the opposing roles of SOs and delta waves in memory processing. This paradigm shift suggests that while SOs may facilitate memory consolidation, delta waves might be involved in memory suppression. To further explore this hypothesis, future human studies could employ specific frequency targeting within the slow wave band—stimulating at 1 Hz to target SOs and at 2 Hz, 3 Hz, or 4 Hz to explore the effects of different delta frequencies on overnight memory consolidation and concomitant sleep alterations in specified frequencies. Such experiments would clarify how varying frequencies within the slow wave spectrum distinctly influence memory consolidation or suppression. Moreover, while the abovementioned research underscores the importance of translating animal model findings to human contexts (Esfahani, et al. 2023), there also remains a gap vice versa, in directly applying human studies to animal models and validating them (Brodt, et al. 2023).

New theoretical advancements in the field are emerging already. For example, an opinion article by Niethard and Born (2019), has begun to challenge and expand upon existing models like the SHY (visited in section 1.3.1.), suggesting that REM sleep may play a more significant role in global synaptic downscaling than SWS. An additional theoretical update also encompasses a more specified role of sleep spindles in memory reprocessing. According to Antony, et al. (2019), memories may not only be reinstated by spindles but further processed during spindle refractory periods, which protect memory reprocessing from interference. This framework posits that while localized spindle events optimize oscillatory interactions necessary for systems consolidation, the temporally coordinated spindle refractory periods across local networks may be pivotal in consolidating rich, multimodal memory representations. These insights highlight the complex interplay between different sleep stages and neural processes.

5.5.6. Precision approach – tailoring CLAS to each individual

The fields of neuroscience and sleep medicine will likely increasingly embrace the concepts of 'precision neuroimaging', 'precision/personalised medicine' and 'person-specific methods'. This approach integrates information on individual differences in brain function and behaviour through symptom clusters, polysomnographic phenotypes, physiological endotypes, and neuroscientific indicators such as brain activation or connectivity (Boland et al. 2019; Light et al. 2019; Liu et al. 2023; Michon et al. 2022). The precision approach highlights the importance of considering factors like genetic predispositions, lifestyle habits, environmental influences, and underlying health conditions to tailor diagnostic and therapeutic strategies effectively (Garbarino and Bragazzi 2024).

Technological advancements are pivotal in this paradigm, with wearable devices, mobile health applications, and sophisticated diagnostic tools playing crucial roles. These technologies facilitate the continuous collection and analysis of detailed sleep data, while the integration of machine learning and artificial intelligence (AI) significantly improves the personalization of treatment plans (Garbarino and Bragazzi 2024). CLAS and similar methods could be an integral part of this approach. In practice, this precision approach could lead to not only tailoring detection algorithms to suit an individual's unique neural signatures but also to automatic adjusting of stimulation parameters such as volume in response to detected EEG features (e.g. arousals), thus enhancing both comfort and effectiveness.

Ultimately, personalized sleep medicine is likely to revolutionize the management of sleep disorders, offering more accurate diagnoses, targeted treatments, and proactive disease management. This shift towards personalized care promises significant improvements in the quality of life (Garbarino and Bragazzi 2024). However, the development of personalized medicine in sleep health will largely depend on improved research standardization and enhanced international collaboration to better delineate phenotype-genotype interactions and their impact on individualized sleep disorder treatments (Grunstein 2010).

The adoption of the precision medicine concept in sleep science, especially with CLAS in insomnia, will enhance our understanding of brain function and behaviour. Technologically innovating CLAS will support this personalized approach, allowing for real-time adjustments to the method based on detailed sleep data and AI-enhanced analyses. This shift towards tailored care is poised to improve outcomes significantly for patients and the general population

likewise, emphasizing the need for robust international collaboration and standardized research to enlighten the benefits of precision sleep medicine fully.

6. Conclusion

The present thesis aimed firstly to explore the feasibility of CLAS in chronic insomnia, examining its impact on sleep structure, overnight declarative memory performance, and sleep quality, hypothesizing no macrostructural changes but enhancements in SOs, SWA activity, and sleep spindle characteristics (Study 1). Next, we aimed to compare three existing CLAS methods—fixed-step and two versions of a phase-locked loop (PLL)—to identify the most precise in detecting and stimulating SOs and to develop new technical solutions, while also investigating whether CLAS generates new SOs or modulates existing ones (Study 2).

Our findings from Study 1 showed CLAS to be feasible in chronic insomnia, influencing sleep dynamics without increasing arousal or wakefulness during sleep. CLAS notably increased the amplitude and activity of SOs while reducing relative delta and sleep spindle activities during SWS. Furthermore, CLAS did not alter sleep-dependent memory consolidation, sleep macrostructure characteristics or subjective sleep quality. Study 2 findings brought important contributions to the study of CLAS mechanisms. We found that the fixed-step method of SO detection produced satisfactory outcomes for patients with chronic insomnia, suggesting its superiority over PLL methods. We encountered challenges in achieving optimal stimulation placement using the PLL methods due to considerable variability in ideal parameters across individuals, rendering PLL unsuitable for a universal application. Additionally, our results demonstrated that the stimulation synchronized spontaneous brain activity without inducing new SO responses in amplitude.

Future research in CLAS applications could improve CLAS outcomes in insomnia by fine-tuning the stimulation thresholds and utilizing a more targeted stimulation algorithm. It would also be beneficial to examine the effects of sustained, multi-night stimulation on sleep structure and subjective quality in home settings. Studying the extended effects of CLAS on sleep quality and general well-being as well as any potential side effects in those with chronic insomnia will be imperative. Increasing the study sample sizes and incorporating healthy control groups could yield more definitive conclusions about how CLAS benefits sleep architecture and memory consolidation. Additionally, future efforts should also test CLAS as an adjunct therapy to already existing treatments, ideally in multicentre efforts to provide larger sample sizes and more statistical power. Such efforts should fully reveal CLAS's therapeutic potential in chronic insomnia management.

From the CLAS mechanisms perspective, future research should focus on the neural mechanisms underlying stimulation and on the study of optimal stimulation parameters including optimal phase, number of sounds, and sound characteristics including their amount, type, length, and volume. It will be also important to further empirically evaluate the optimal detection and stimulation algorithms in both night and nap settings.

In sum, our research brings important contributions to the field of auditory stimulation of sleep. For the first time, we showed CLAS feasibility in a new clinical setting of chronic insomnia and provided more broadly applicable insights into CLAS mechanisms of functioning by bringing evidence that CLAS alters endogenous sleep activity rather than generates new SOs and evidence supporting the use of a fixed-step detection algorithm.

7. Summary

This thesis explored the applications and mechanisms of closed-loop auditory stimulation (CLAS) in a clinical setting of individuals with chronic insomnia. Study 1 focused on assessing the feasibility of CLAS as a non-invasive sleep modulation method, exploring its effects on sleep quality, overnight memory consolidation, and overall sleep architecture. Using polysomnography, subjective quality assessments, and a memory task Study 1 showed increases in slow oscillatory (SO) activity, decreases in delta, and sleep spindle dynamics during slow-wave sleep. However, these changes did not consistently translate into improved sleep-dependent memory consolidation or subjective sleep quality. In Study 2, we evaluated different acoustic stimulation methods like the phase-locked loop (PLL) and fixed-step approaches and found evidence in favour of the fixed-step approach. Importantly, we brought the first insights that CLAS caused SO phase alignment and thus altered existing endogenous activity rather than induced new SOs.

The research within this thesis contributed to the understanding of CLAS from applied and mechanistic perspectives. It confirmed the feasibility of CLAS in insomnia, with a focus on optimizing stimulation parameters to enhance its effectiveness. Presented work showed that simpler stimulation approaches like the fixed-step method may be more suitable for insomnia than more complex algorithms like PLL, due to their robustness and ease of application in diverse populations, both patient and non-patient.

Looking ahead, advancing the personalization and better tailoring of CLAS protocols could potentially improve the efficacy of CLAS in insomnia and other clinical settings. Developing CLAS systems and adjusted parameters could facilitate long-term statistically more powerful studies and provide more comprehensive insights into the cumulative effects of auditory stimulation on sleep, its quality, and cognitive functions. The ultimate goal would be to establish standardized, effective CLAS applications that could be integrated into routine clinical practice, enhancing sleep and its quality in those suffering from sleep or memory disturbances.

8. Souhrn

Tato dizertační práce zkoumala aplikace a mechanismy zpětnovazebné sluchové stimulace (closed-loop auditory stimulation, CLAS), poprvé u klinické skupiny lidí s chronickou nespavostí. Studie 1 se zaměřila na posouzení proveditelnosti CLAS jako neinvazivní metody modulace spánku, zkoumala její účinky na kvalitu spánku, noční konsolidaci paměti a celkovou architekturu spánku. Pomocí polysomnografie Studie 1 ukázala zvýšení aktivity pomalých oscilací a snížení výkonu v pásmu delta a spánkových vřetének během pomalovlnného spánku (NREM3). Tyto změny však nevedly konzistentně ke zlepšení konsolidace paměti závislé na spánku nebo ke změně subjektivní kvality spánku. Ve Studii 2 jsme hodnotili účinnost a přesnost různých metod sluchové stimulace, jako jsou metody PLL a metody s pevným krokem (fixed-step), a našli jsme důkazy ve prospěch metody s pevným krokem. Přinesli jsme první evidenci, že CLAS způsobila sfázování pomalých oscilací a tím změnila existující endogenní aktivitu místo indukce nových pomalých oscilací.

Výzkum v této disertační práci přispěl k pochopení CLAS z hlediska mechanismů jejího účinku i aplikačního využití. Potvrdil proveditelnost CLAS u nespavosti se zaměřením na optimalizaci parametrů stimulace za účelem zvýšení její účinnosti. Prezentovaná práce ukázala, že jednodušší metody stimulace, jako je metoda s pevným krokem, mohou být vhodnější než složitější algoritmy jako PLL, kvůli jejich robustnosti a jednoduchosti aplikace. Do budoucna by pokročilé přizpůsobení protokolů CLAS mohlo zlepšit účinnost metody u nespavosti i v dalších klinických kontextech. Budoucí vývoj CLAS systémů a jejich parametrů by také mohl usnadnit dlouhodobější a statisticky silnější studie a poskytnout komplexnější pohled na kumulativní účinky stimulace, kvalitu spánku a kognitivní funkce. Konečným cílem by bylo nalezení standardizovaných účinných aplikací CLAS, které by mohly být integrovány do běžné klinické praxe a mohly by zlepšit spánek i jeho kvalitu u lidí trpících poruchami spánku nebo paměti.

9. References

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10. Publications overview

1. Original research articles *in extenso*, related to the thesis

a) With impact factor

DUDYSOVÁ, D., K. JANKŮ, M. PIORECKÝ, V. HANTÁKOVÁ, et al. Closed-loop auditory stimulation of slow-wave sleep in chronic insomnia: a pilot study. J Sleep Res, 2024, e14179. IF_{2023} = 3.400

PIORECKY, M., V. KOUDELKA, V. PIORECKA, J. STROBL, et al. Real-Time Excitation of Slow Oscillations during Deep Sleep Using Acoustic Stimulation. Sensors, 2021, 21(15), 5169. IF_{2021} = 3.847

b) Without impact factor

2. Original research articles *in extenso*, unrelated to the thesis

a) With impact factor

ANYZ, J., BAKSTEIN, E., **DUDYSOVA, D.**, VELDOVA, K. et al. No wink of sleep: Population sleep characteristics in response to the brexit poll and the 2016 U.S. presidential election. Social Science & Medicine, 2018, 222, 112-121. IF_{2018} = 3.087

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b) Without impact factor

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11. Appendix

Original research articles in extenso, related to the thesis