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Does one size fit all? - Towards the optimization and personalization of non-invasive brain stimulation paradigms to enhance motor learning

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par

Laurijn Rianne DRAAISMA

Acceptée sur proposition du jury

Prof. S. Micera, président du jury
Prof. F. C. Hummel, directeur de thèse
Prof. L. Cohen, rapporteur
Dr I. Violante, rapporteuse
Prof. N. Wenderoth, rapporteuse

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Abstract (English)

The acquisition and re-acquisition of skills is an important aspect of daily life and in the recovery after a stroke. Non-invasive brain stimulation (NIBS) is a technique that is used to improve motor learning and enhance motor recovery in stroke survivors. Although the current results are promising, the outcomes are heterogeneous with responders and non-responders. This thesis aimed to investigate multiple NIBS strategies to enhance stimulation efficacy and work towards protocol personalization that could ultimately improve stroke recovery. These alternative strategies include targeting other areas of the motor network than the primary motor cortex (M1), targeting the brain as a network with the use of multifocal stimulation, and using a variety of stimulation techniques (TMS, tACS, tDCS) to study the effects of conceptually different stimulation protocols.

Study 1 explored the methodological implications of TMS for measuring inhibitory and excitatory neurotransmissions. With the use of TMS, short intracortical inhibition (SICI) and intracortical facilitation (ICF) were measured in healthy young adults. SICI and ICF were studied with different stimulators, waveforms, and current directions using interstimulus intervals at 1, 3, 10, and 15 ms. Our findings indicated high comparability among the different stimulation paradigms, except for SICI at 3 ms.

Study 2 measured the effect of 50 Hz tACS applied to the cerebellum (CB) on a novel motor learning task in healthy young adults. Targeting the CB with NIBS is a relatively new field of research with open questions that need to be addressed. Therefore, we explored for the first time the effect of CB-tACS on a motor learning task. Our results did not show an improvement of 50 Hz CB-tACS. We argue that this might have been related to the task and/or the stimulation frequency. Therefore, this stimulation paradigm requires further optimization to be effective for motor learning.

Study 3 compared multifocal sequential stimulation to monofocal tDCS on motor learning in stroke patients. The multifocal paradigm consisted of an orchestrated M1 – CB stimulation setup, the monofocal paradigm targeted the M1. Our results indicated a significant effect of multifocal M1-CB stimulation, mainly driven by CB-tDCS during the early phase of learning. Moreover, baseline performance and neurophysiology were related to stimulation responsiveness that could potentially lead to biomarkers to predict stimulation efficacy in stroke patients.

Study 4 measured the effect of personalized bifocal theta tACS applied to the frontoparietal network (FPN) on motor learning in healthy older adults. Sequence learning has a cognitive component related to working memory (WM) capacity, a process mediated by the FPN. We hypothesized that targeting the FPN might be beneficial for motor learning. tACS to the FPN improved performance when WM load was high, but not when WM load was low. Therefore, we conclude that the FPN is a promising new target to enhance motor learning which might be most beneficial for individuals with decreased WM capacity due to age or stroke.

In conclusion, this thesis demonstrates that targeting alternative motor network areas, and multifocal stimulation is promising. These results expand on the current knowledge of NIBS and identified open questions that require further examination but could ultimately lead to enhanced efficacy and the personalization of study protocols.

Abstract (French)

L'acquisition et la ré-acquisition de compétences est un aspect important de la vie quotidienne et de la récupération après un AVC. La stimulation cérébrale non invasive (NIBS) est une technique qui est utilisée pour améliorer l'apprentissage moteur et renforcer la récupération motrice chez les survivants d'un AVC. Bien que les résultats actuels soient prometteurs, les résultats sont hétérogènes avec des répondeurs et des non-répondeurs. Cette thèse a pour but d'étudier plusieurs stratégies de NIBS afin d'améliorer l'efficacité de la stimulation et de travailler à la personnalisation du protocole qui pourrait finalement améliorer la récupération après un AVC. Ces stratégies alternatives comprennent le ciblage d'autres zones du réseau moteur que le cortex moteur primaire (M1), le ciblage du cerveau en tant que réseau avec l'utilisation de la stimulation multifocale, et l'utilisation d'une variété de techniques de stimulation (TMS, tACS, tDCS) pour étudier les effets de protocoles de stimulation conceptuellement différents.

L'étude 1 a exploré les implications méthodologiques de la SMT pour mesurer les neurotransmissions inhibitrices et excitatrices. Grâce à la SMT, l'inhibition intracorticale courte (SICI) et la facilitation intracorticale (ICF) ont été mesurées chez de jeunes adultes en bonne santé. La SICI et l'ICF ont été étudiées avec différents stimulateurs, formes d'onde et directions de courant en utilisant des intervalles interstimulus de 1, 3, 10 et 15 ms. Nos résultats ont indiqué une grande comparabilité entre les différents paradigmes de stimulation, sauf pour le SICI à 3 ms.

L'étude 2 a mesuré l'effet de la tACS 50 Hz appliquée au cervelet sur une nouvelle tâche d'apprentissage moteur chez de jeunes adultes en bonne santé. Le ciblage du cervelet avec les NIBS est un domaine de recherche relativement nouveau, avec des questions ouvertes qui doivent être abordées. Nous avons donc exploré pour la première fois l'effet du CB-tACS sur une tâche d'apprentissage moteur. Nos résultats n'ont pas montré une amélioration du CB-tACS 50 Hz. Nous pensons que cela pourrait être lié à la tâche et/ou à la fréquence de stimulation. Par conséquent, ce paradigme de stimulation nécessite une optimisation supplémentaire pour être efficace pour l'apprentissage moteur.

L'étude 3 a comparé la stimulation séquentielle multifocale à la tDCS monofocale sur l'apprentissage moteur chez les patients ayant subi un AVC. Le paradigme multifocal consistait en une configuration orchestrée de stimulation M1 - CB, le paradigme monofocal ciblait la M1. Nos résultats indiquent un effet significatif de la stimulation multifocale M1-CB, principalement dirigé par la tDCS CB pendant la phase précoce de l'apprentissage. De plus, les performances de base et la neurophysiologie étaient liées à la réactivité de la stimulation, ce qui pourrait conduire à des biomarqueurs permettant de prédire l'efficacité de la stimulation chez les patients victimes d'un AVC.

L'étude 4 a mesuré l'effet de la tACS thêta bifocale personnalisée appliquée au FPN sur l'apprentissage moteur chez des adultes âgés en bonne santé. L'apprentissage de séquences a une composante cognitive liée à la capacité de la mémoire de travail (MM), un processus médié par le FPN. Nous avons émis l'hypothèse que le ciblage du FPN pourrait être bénéfique pour l'apprentissage moteur. Le tACS appliqué au FPN a amélioré les performances lorsque la charge de la mémoire de travail était élevée, mais pas lorsque la charge de la mémoire de travail était faible. Par conséquent, nous concluons que le FPN est une nouvelle cible prometteuse pour améliorer l'apprentissage moteur qui pourrait être plus bénéfique pour les personnes ayant une capacité WM réduite en raison de l'âge ou d'un accident vasculaire cérébral.

En conclusion, cette thèse démontre que le ciblage de zones alternatives du réseau moteur et la stimulation multifocale sont prometteurs. Ces résultats élargissent les connaissances actuelles sur les NIBS et ont identifié des questions ouvertes qui nécessitent un examen plus approfondi mais qui pourraient finalement conduire à une efficacité accrue et à la personnalisation des protocoles d'étude.

Keywords

Non-invasive brain stimulation, motor learning, stroke recovery, alternative strategies, TMS, tACS, tDCS, multifocal stimulation, personalization.

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1. Introduction

1.1 Motivation

The ability to learn new skills is important to be able to adapt to changes and challenges of daily life and to keep up with fast-changing societies. The process of motor learning can be hampered due to aging or neuropathological reasons such as after a stroke. Motor impairments are the most common symptoms after stroke, affecting 80% of stroke survivors in the acute phase and more than 40% in the chronic phase (Lee et al., 2015). Motor impairments or a reduced capacity to learn new skills gravely diminishes the quality of life and imposes a burden on the affected person, loved ones, and society as a whole (Tatemichi et al., 1994; Feigin et al., 2017; Kapoor et al., 2017). Therefore, stroke rehabilitation focuses on the recovery of impaired movements. In this regard, motor learning paradigms have been used as a strategy to improve recovery (Krakauer, 2006). The mechanism of motor learning is based on the ability of the brain to exhibit practice-dependent functional plasticity. This plasticity allows for the acquisition, retention, and relearning of motor skills. It is also the main underlying mechanism of relearning skills after brain damage caused by stroke, trauma, or other pathologies. It has been shown that at an older age or after neurological impairment, such as a stroke, the brain still shows plasticity and the ability to reorganize brain networks and establish compensatory mechanisms (Johansson, 2000; Reuter-Lorenz and Cappell, 2008; Park and Reuter-Lorenz, 2009; Winstein et al., 2016). Therefore, effective strategies that enable the acquisition and the re-acquisition of new skills play a pivotal part in motor improvements and stroke recovery (Krakauer, 2006). However, although rehabilitation strategies have improved, most stroke patients do not fully recover with less than 20% being able to go back to their normal life (Di Carlo, 2008). This highlights the need for the development of strategies to augment the effect on motor learning and improve recovery after stroke (Krakauer, 2006; Raffin and Hummel, 2018)

An increasingly popular technique to improve motor learning and enhance motor recovery is the use of non-invasive brain stimulation (NIBS) (Hummel and Cohen, 2005; Dayan et al., 2013). In the past ~15 years, different NIBS techniques have been studied to test its efficacy to improve motor learning, enhance cognition, and as an adjuvant rehabilitation strategy after stroke (Hsu et al., 2012; Wessel et al., 2015; Raffin and Hummel, 2018; Draaisma et al., 2020a; Hara et al., 2021). Results are promising showing improvements in motor learning in healthy young, older adults and patients with neurological deficits. However, recently it has become more and more clear that the findings are rather heterogeneous with varying effect sizes with responders and non-responders to the intervention (Hsu et al., 2012; Elsner et al., 2016; O'Brien et al., 2018; Hara et al., 2021). Factors that play a role in the heterogeneity of these outcomes are the varying characteristics of the individuals (e.g., age, gender, etc.), and stroke-related (e.g. lesion location and size, functional impairment, etc.). These parameters can affect the responsiveness to neuromodulation and they might not all benefit from the same type of stimulation (Hummel et al., 2008; O'Shea et al., 2014). Therefore, more research about the precise underlying mechanisms is of pivotal importance to move away from a "one-size-fits-all" strategy and move towards the personalization of stimulation paradigms tailored to the needs of the individual (Hummel et al., 2008; Wessel and Hummel, 2018).

The main aim of this thesis is to expand the scope of current knowledge by evaluating the efficacy of a multitude of alternative NIBS strategies to enhance motor learning in healthy adults and/or stroke patients. A larger choice of stimulation strategies will increase the possibility to tailor stimulation paradigms to the specific features of the individuals, which ultimately might lead to a maximized effect of the intervention. The different strategies consist of:

1) Alternative stimulation targets. Currently, most of the NIBS studies to enhance motor learning focus mainly on the primary motor cortex (M1) (Seidler et al., 2012; Wessel et al., 2015; Dupont-Hadwen et al., 2019). However, studies have shown that other brain areas, such as the cerebellum (CB) and the dorsolateral prefrontal cortex (DLPFC), are relevantly involved in motor learning too (Hikosaka et al., 2002; Floyer-Lea and Matthews, 2005; Hardwick et al., 2013). Therefore, the conventional M1 target might benefit some, but not all. Motor learning is implemented in a large cortico-subcortical network comprising brain areas such as the CB and frontoparietal regions. The CB, an important secondary motor area, plays a relevant role in motor learning and motor control (Hardwick et al., 2013; De Zeeuw and Ten Brinke, 2015). The CB is a promising stimulation target for healthy adults and stroke patients due to its dense connections to cortical motor and cognitive areas (Manto, 2006; Bostan et al., 2013) and the rich variety of inherited plasticity mechanisms (Carey, 2011). Moreover, because the CB is well connected to the neocortical areas, it could serve as a non-lesioned entry in the network in case of a supratentorial stroke (Wessel and Hummel, 2018). Other promising stimulation targets are frontal and parietal areas that have been convincingly related to motor learning (Dayan and Cohen, 2011; Doyon et al., 2018; Pollok et al., 2020). Based on neuroimaging studies, it has become clear that the DLPFC and the parietal cortex are involved in different phases of motor learning (Floyer-Lea and Matthews, 2005; Dayan and Cohen, 2011). The DLPFC shows higher activation during the fast learning phase, while the parietal cortex shows increased activation as learning progresses. Moreover, the frontoparietal network (FPN) is an important network for working memory (WM), which is crucial for the practice of challenging motor learning tasks (Dayan and Cohen, 2011; Marek and Dosenbach, 2018; Maruyama et al., 2021). Although the FPN has been related to motor learning, its potential as a stimulation target to enhance motor learning and motor recovery has not yet been determined.

2) Multi-focal brain stimulation. Motor learning is a process that requires multiple functionally involved brain areas that work together like a well-orchestrated network (Sporns et al., 2004; Hardwick et al., 2013). Therefore, rather than targeting a single brain area, it might be beneficial to focus on targeting networks with the use of multi-focal stimulation. Applying NIBS to multiple areas could enhance aspects of natural brain processing such as joint network-related activation in connected areas (Wessel et al., 2021b). Bifocal M1 stimulation has shown positive effects on motor learning compared to sham stimulation (Lindenberg et al., 2010). However, due to the lack of adequate control conditions, the additional effects of multifocal stimulation compared to monofocal stimulation remains unclear. As mentioned above, the M1 has been by far the most targeted brain area for motor learning. However, including other brain areas that are relevantly related to motor learning could potentially have additive effects. A core component of the motor network for motor learning is the motorcortico-cerebellar loop (Hardwick et al., 2013). Therefore, targeting the CB as well as the M1 could lead to network-related enhancements of motor learning. For example, the M1 has been related to online learning, while the CB has shown to be effective in the offline learning phase (Zimerman et al., 2013; Wessel et al., 2016). Therefore, targeting brain areas that are involved in different phases of learning could lead to synergistic effects. Moreover, motor learning does not rely exclusively on motor areas but also shows activation in frontal and parietal areas. Therefore, targeting these areas as networks with the use of multifocal stimulation could have a beneficial effect on motor learning.

3) Stimulation techniques. An important goal of this thesis was to gather more knowledge to enhance stimulation efficacy and define possible biomarkers that could ultimately lead to the personalization of study protocols. Therefore, a variety of different techniques have been used to measure differences in stimulation effects based on task specificity, study cohort, and stimulation type. For this reason, we studied the efficacy of NIBS on two different types of motor learning tasks. Previous studies have shown

that stimulation effects can be task-specific and that careful consideration of task-type combined with the stimulated location is necessary (Buch et al., 2017). Moreover, personalizing tasks based on the physical or cognitive capacities of the individual might enhance stimulation efficacy. Multiple types of NIBS have been used that allow to measure and induce different neuronal aspects such as inhibitory and facilitatory mechanisms, cortical excitability, and target ongoing cortical oscillatory activity. Corticomotor excitability and intracortical inhibitory mechanisms play an important role in neuronal plasticity and have been related to motor learning capacity (Hummel et al., 2009; Stagg et al., 2011a; Mooney et al., 2017). Measuring these plasticity mechanisms can increase our knowledge about the underlying mechanisms and might point toward biomarkers for stimulation-associated effects. Finding effective biomarkers can lead to the prediction of stimulation efficacy and ultimately aid personalization.

The thesis consists of four different studies that use one or more of the above-mentioned strategies to study motor learning and is built up in the following way. The first study was a preparatory study with the aim to achieve a more methodological understanding of intracortical inhibitory and facilitatory neurotransmission. Specifically, with the use of transcranial magnetic stimulation (TMS), short intracortical inhibition (SICI) and intracortical facilitation (ICF) were determined to evaluate markers of GABAergic and glutamatergic neurotransmission (Kujirai et al., 1993; Chen, 2004). SICI and ICF have been used to measure neurological conditions and to evaluate the effect of NIBS interventions combined with motor learning paradigms on inhibitory and excitatory mechanisms (Zimerman et al., 2012; O'Shea et al., 2014). However, different technical setups and stimulation protocols have been used which may result in heterogeneity in outcomes due to technical differences. Therefore, to see whether different TMS setups show comparable results to induce SICI and ICF, two frequently used types of TMS devices were used to assess and compare different stimulation protocols. Moreover, these results were used to determine the optimal TMS parameters for possible use in the following studies. The succeeding studies focused on the use of alternative NIBS strategies to enhance motor learning with the ultimate goal of translation into stroke recovery.

The second study was focused on targeting the CB to enhance motor learning. The CB as a neuromodulation target is a relatively new field of research with several open questions that need to be addressed leading to better understanding, development, and successful application (Wessel and Hummel, 2018). Proof-of-principle studies in healthy adults have shown motor learning improvements using transcranial direct current stimulation (tDCS) to the CB (Galea et al., 2011; Hardwick and Celnik, 2014; Cantarero et al., 2015; Wessel et al., 2016). With this study, I aimed to expand on the current knowledge by testing the efficacy of an alternative stimulation paradigm, namely cerebellar transcranial alternating current stimulation (CB-tACS) during a novel motor task in healthy young adults. The use of tACS has been suggested to selectively target specific neuronal oscillations in the CB. Cerebellar oscillations in the gamma frequency have been linked to motor improvements in a previous study (Naro et al., 2017). This study of my thesis evaluated whether cerebellar gamma tACS can enhance the effects of motor learning. To determine underlying mechanisms complex TMS approaches were used to determine the impact of CB-tACS on motor cortical inhibitory (SICI) and faciliatory (ICF) mechanisms.

In the third study, the efficacy of multi-focal stimulation applied to the M1 and the CB as target locations was investigated. The stimulation was applied in conjunction with the practice of a motor learning task in a cohort of stroke patients with upper-limb impairments. The stimulation was applied sequentially to the M1 and to the CB to achieve specific learning phase enhancements. More specifically, tDCS applied to the M1 has previously shown efficacy during the online learning phase (Zimerman et al., 2012), while cerebellar stimulation affected the offline learning phase (Wessel et al., 2016). Therefore, in separate training sessions, both areas were targeted to enhance phase-specific learning mechanisms. In addition, monofocal M1 stimulation was compared to bifocal M1- CB stimulation to define the possible

additive effects of multifocal stimulation over monofocal stimulation.
In a final study, the effect of multifocal stimulation to the frontoparietal areas during the practice of a motor learning task was evaluated. This strategy was based on the fact that neuroimaging studies have shown the involvement of frontoparietal areas during motor learning. However, current NIBS studies have focused predominantly on the motor network (Zimerman et al., 2012; Pollok et al., 2015; Wessel et al., 2015; Krause et al., 2016). Studies have shown that cognitive processes, such as WM, are involved in motor learning too (Shea et al., 2006; Bo and Seidler, 2009). WM is a cognitive process that is often diminished in healthy older adults and stroke patients who suffer from cognitive impairments. Moreover, WM impairments have been related to decreased performance on motor learning tasks (Bo et al., 2009). Therefore, targeting the cognitive areas to improve WM performance might have a beneficial effect on motor learning, especially in people with WM impairments. Earlier studies have shown improvements in WM performance in healthy adults with the use of multifocal tACS to the FPN (Polania et al., 2012; Violante et al., 2017). Based on the correlational evidence for the involvement of the FPN in motor learning and because WM capacity has been related to motor learning, we aimed to provide causal evidence of the role of the FPN in motor learning. Therefore, we tested whether multifocal theta tACS to the FPN improved motor learning in healthy older adults.

The following section provides background information about the main topics of this thesis. It starts with an in-depth explanation of motor learning, its important structural and functional substrates, and relevant cognitive and aging-related aspects. Then, the different types of NIBS that are used in this thesis will be explained as well as the mechanisms of action. Finally, a general introduction of stroke, the underlying mechanisms of recovery, and NIBS for stroke recovery will be covered.

1.2 Motor learning

Motor learning is omnipresent in every aspect of life. It encompasses all actions and movements animals and humans perform, consciously or unconsciously. Motor learning is the main topic of interest in many professions such as athletes, musicians, coaches, and physical therapists. Moreover, it is of great theoretical and experimental interest focusing on understanding the underlying mechanisms, important neural substrates, possibilities to improve motor learning, and the search for effective rehabilitation strategies when motor functioning is impaired. The definition of motor learning is a practice-dependent process, where movements are performed quicker and more accurately (Willingham, 1998). It is an umbrella term for different types of motor learning, such as sequence learning, de novo learning, and adaptation (Krakauer et al., 2019). Although all types of motor learning are important in their way, the main focus in this thesis is on sequence learning.

1.2.1 Sequence learning

In daily life, any action or task consists of a sequence of actions that need to be successfully completed. This can range from getting dressed in the morning, typing on a keyboard, or playing a musical instrument. Generally, the separate movements of the action have already been learned. It is the selection of the separate movements in the correct sequence that has to be learned to perform that one specific action. Sequence learning is a process where independent movements are associated, eventually resulting in a multi-element sequence, which can be performed quickly and accurately (Seidler et al., 2012; Krakauer et al., 2019). In experimental settings, sequence learning can be studied in a controlled manner with the use of different tasks such as the serial reaction time task (SRTT) (Robertson, 2007), the sequential finger-tapping task (SFTT) (Karni et al., 1995), or force modulation tasks (Reis et al., 2009). The SRTT requires individuals to respond as fast as possible to targets that are shown in a specific sequence (Robertson, 2007). The reaction time is the general outcome measure that will decrease when the sequence is learned (Robertson, 2007; Krakauer et al., 2019). This type of task is often portrayed as implicit sequence learning because the participants are not informed that the targets appear in a sequential matter. During sequence learning tasks like the SFTT and force modulation tasks, participants are often required to reproduce a sequence generally between 4 – 9 elements and are instructed to perform the task “as quickly and accurately as possible”. The difficulty of these tasks is maintained because increased speed directly relates to increased difficulty. Continuously increasing difficulty aids learning as a certain amount of error is necessary to update and improve motor skills (Guadagnoli and Lee, 2004; Zimerman et al., 2013; Krakauer et al., 2019; Maruyama et al., 2021). The tasks have different outcome measures such as the number of correct sequences, completion time, or the speed-accuracy trade-off, where an improvement can be quantified as an increase of speed without a decrease in accuracy (Reis et al., 2009). In the case of the SFTT, sequences are commonly performed with simple movements, such as button presses, much like playing the piano or typing on a computer keyboard (Krakauer et al., 2019). Force modulation tasks often consist of pinch or grip force modulations. In most cases, the order of the sequence is explicitly provided during the task which reduces cognitive load (Krakauer et al., 2019). The fast execution of sequences leads to the grouping together of elements into “chunks”. This chunking behavior is thought to reduce mental load and consequently increase execution speed (Verwey, 1996; Bo and Seidler, 2009; Maceira-Elvira et al., 2021). In this thesis, a novel force modulation task was evaluated using grip force. This was done as an attempt to accommodate stroke patients with upper limb impairments who might not be able to execute fine differential finger movements like keypresses but can open and close their hands, for details please see the methods section of chapter 4.

1.2.2 Online and offline effects

Learning a motor skill is only achievable through practice, with different phases of learning that relate to the amount of practice during a prolonged period (Korman et al., 2003). Online learning effects are within-session improvements that can occur during the training session. Online learning can be divided into a fast and slow learning phase with the fast learning phase showing immediate improvements that are often large and are related to the novelty of the movements (Korman et al., 2003; Doyon and Benali, 2005; Reis et al., 2009). The slow learning phase can continue for days or even weeks during the repeated practice of the task until performance reaches a ceiling level (Korman et al., 2003). Motor memories are strengthened through consolidation, which refers to two processes: memories becoming resilient against perturbation or performance improvements between training sessions (offline learning) (Robertson et al., 2004a). The offline improvements can occur during rest periods within a session (micro-offline learning) (Bönstrup et al., 2019) or after longer periods of rest, between sessions (Shadmehr and Brashers-Krug, 1997; Savion-Lemieux and Penhune, 2005). An important factor for offline learning is sleep. Studies have shown that sleep improved performance to a larger extent than an equal period of rest spent awake (Walker et al., 2002; Korman et al., 2003). The sleep-dependent improvements are specific to explicit learning paradigms such as sequence learning tasks (Fischer et al., 2002; Walker et al., 2002). Skills that are learned unconsciously through implicit learning do not show offline learning that is sleep-dependent (Robertson et al., 2004b). Finally, the learned motor skills can be retained over long periods in varying degrees. Long-term retention is dependent on the amount of practice and the delay of reassessment (Savion-Lemieux and Penhune, 2005). Long-term retention can last up to months after the initial training of the sequences (Shadmehr and Brashers-Krug, 1997). Although decrements in performance do occur depending on the amount of delay (Savion-Lemieux and Penhune, 2005).

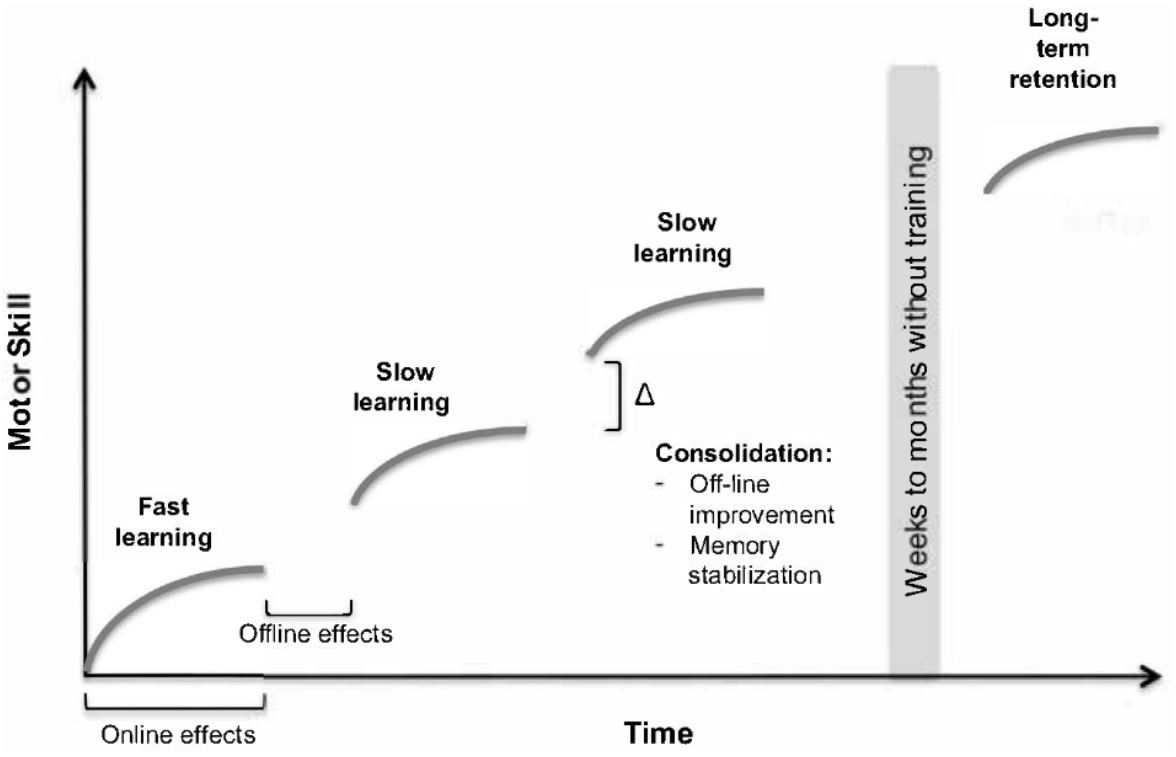

Figure 1. Motor learning. Schematic depiction of the different phases of motor learning. Adapted from Wessel, et al. (2015).

1.2.3 Structural and functional anatomy of motor learning

A vast amount of research has shown the involvement of multiple brain areas during the process of motor learning, which can differ based on the specific type or phase of motor learning (Shadmehr and Holcomb, 1997; Doyon et al., 2003; Hardwick et al., 2013). A meta-analysis including 70 different studies showed a consistent activation in the M1, supplementary motor area (SMA), dorsal premotor cortex (dPMC), the primary somatosensory cortex (S1), superior parietal lobule (SPL), thalamus, putamen, and CB (Hardwick et al., 2013). Thereby suggesting a core motor network, which varies in activation patterns and additional brain areas depending on the type of task. This meta-analysis confirmed a previously proposed model of two core neuroanatomical loops involved in motor learning, the cortico-striatal (CS) and the cortico-cerebellar (CC) loop (Hikosaka et al., 2002; Doyon and Benali, 2005). The CS and the CC are both involved in the early stages of motor learning (e.g., fast learning phase), when the skills are well learned the contribution of the two different motor networks differs depending on the type of task. The original model of Doyon and Ungerleider stipulates that the CS is mainly involved in sequence learning while the CC is mainly involved in adaptation learning (Doyon and Ungerleider, 2002). Since then, additional research has shown the involvement of these networks in the formation of motor routines to learn new skills. For example, both networks are involved in implicit and explicit sequence learning (Aizenstein, 2004; Doyon and Benali, 2005), as well as the acquisition of self-paced finger movements and adaptation tasks (Imamizu et al., 2003; Wu et al., 2004). This interaction between the two networks during motor learning has been further confirmed by clinical studies. For example, Parkinson's patients rely more heavily on CB activation than healthy individuals during a sequence learning task, which implies that the CC can compensate for the clinical impairment of the nigrostriatal pathway (Mentis et al., 2003). Although the two networks interact and show compensatory mechanisms in the early learning phase, the model of the two distinct systems for sequence learning and adaptation learning does seem to hold for the so-called automatization phase of learning, when performance becomes close to perfect (Doyon et al., 2003).

Hikosaka and colleagues made a distinction between two striatum-cerebellar networks including different brain areas that are specialized in different features of sequence execution. The frontoparietal associative striatum-cerebellar network is involved in learning the spatial coordinates, the specific location of the items in the sequence, while the M1-sensorimotor striatum-cerebellar loop supports learning the motor coordinates, the execution of movements (Hikosaka et al., 2002). Learning the spatial coordinates of sequences has been shown to require more cognitive resources, such as attention and executive functions than learning the motor coordinates. These resources are provided by the frontoparietal regions of this loop (Miller and Cohen, 2001; Hikosaka et al., 2002). Diedrichsen and colleagues show similar results by looking at the neural representation of motor sequences. By measuring the neural activity patterns during three different levels of sequence representation (single finger, chunk, sequence), they found that individual movements were uniquely represented in the M1 and S1, while chunks and full sequences representation was seen in the premotor cortices and the parietal cortex. They argue that the distinct cortical separation has a temporal component as the individual finger movements require fast execution, which suits the intrinsic properties of the M1. However, the time for chunks or sequences requires a longer representation, which may be better sustained by the premotor and parietal areas (Yokoi and Diedrichsen, 2019). Although the models differ in explaining the underlying mechanisms involved in sequence learning, all models point toward a network-related interaction of multiple brain regions including the CB and frontoparietal areas to facilitate learning.

The CB and sequence learning

The CB has been shown to play an important part in motor control and is related to the performance of smooth and accurate movements (Ito, 2000) by cerebellar brain inhibition (CBI) mechanisms. CBI is mediated by projections of the Purkinje cells (PC) to the dentate-thalamic pathways, which in turn connect to the M1 (Daskalakis et al., 2004; Naro et al., 2017). Causal proof of cerebellar-mediated inhibition of the M1 was given with the use of bifocal TMS applied to the CB and the M1 (Ugawa et al., 1995). Inhibitory mechanisms are important for motor execution by supporting selective activation of target muscles by the inhibition of nearby non-target muscles (Beck and Hallett, 2011). Surround inhibition is a process involved in the execution of individual finger movements and the amount of inhibition is enhanced depending on the task difficulty (Beck and Hallett, 2011). The CB is more involved in sequence learning than what was originally thought. However, the specific role of the CB is less known for sequence learning than its role in adaptation learning (Hardwick et al., 2013). The study of Inhoff and colleagues showed that patients with cerebellar dysfunction showed increased response onset time and slower inter-key presses within the sequences compared with healthy participants (Inhoff et al., 1989). This could be due to a decrease in coordination and accuracy. Inhoff and colleagues suggest that the CB is involved in the translation of a programmed sequence into execution before the onset of the movement. Another study showed the involvement of the CB in the storage and retrieval of well-learned motor sequences by inactivating the dentate nucleus in monkeys (Lu et al., 1998). More specifically, inactivating the dorsal and central part of the dentate nucleus resulted in a significant increase in errors in previously well-learned sequences. Neuroimaging studies have shown a shift in activity patterns during different learning phases. A shift from the fast learning phase to the slow learning phase is marked by a decrease in cerebellar activation pointing toward a phase-specific role (Dayan and Cohen, 2011). The CB is convincingly involved in motor sequence learning and is, therefore, a promising stimulation target for NIBS studies to enhance motor learning.

The frontoparietal network and sequence learning

The main focus of studying motor sequence learning has been on motor cortical areas, especially the role of the M1 (Karni et al., 1995; Dupont-Hadwen et al., 2019). However, it has been convincingly established that sequence learning relies on multiple areas and networks that interact during different phases of learning (Hikosaka et al., 2002; Doyon et al., 2003; Hardwick et al., 2013). For example, fast sequence learning modulates brain activity in the dorsolateral prefrontal cortex (DLPFC), the M1, and the pre-SMA (Floyer-Lea and Matthews, 2005), with a decrease in activation in later stages of learning. On the other hand, activation in the premotor cortex (PMC), SMA, striatum, CB, and parietal regions increased while learning progressed (Floyer-Lea and Matthews, 2005; Dayan and Cohen, 2011). These activation patterns show the involvement of non-motor brain areas such as the prefrontal and parietal regions during sequence learning. A meta-analysis has shown activation in the frontoparietal network (FPN) in studies during the execution of sequences on different visually or self-paced finger tapping tasks (Witt et al., 2008) as well as during movement preparation of sequential movements during a learning task (Maruyama et al., 2021). The FPN is an important network related to cognitive processes such as working memory (WM) (Pascual-Leone et al., 1996; Verwey, 2001; Hikosaka et al., 2002). WM is the process of temporarily storing and manipulating information in the mind (Baddeley and Hitch, 1974). The process of sequence learning and the execution of sequences requires the capacity to keep the parts of the sequence in mind. Therefore, WM capacity is needed for successful sequence learning (Seidler et al., 2012). An important process during sequence learning is the grouping of elements of the sequence in “chunks”. It has been proposed that sequence learning consists of two mechanisms: buffer loading and a dual-processor. Buffer loading is the process of programming sequence chunks before they are executed. The dual-processor consists of a cognitive and a motor

component. The cognitive component is responsible for learning chunked motor representations and selecting these chunks for execution, while the motor component performs the movements in parallel (Verwey, 2001). The exact role of the FPN during sequence learning is currently unclear and evidence for its involvement is correlational using neuroimaging techniques and behavioral studies (Floyer-Lea and Matthews, 2005; Witt et al., 2008; Dayan and Cohen, 2011; Maruyama et al., 2021). This highlights the necessity for studies to explore the causal role of the FPN on sequence learning with the use of NIBS.

1.2.4 Age-related differences in sequence learning

Healthy aging leads to structural and functional changes in the brain, which can lead to reduced cognitive and motor performance (Bishop et al., 2010; Seidler et al., 2010). Due to this reason, older adults show diminished performance on implicit as well as explicit sequence learning tasks compared to young adults (Shea et al., 2006; Voelcker-Rehage, 2008; Maceira-Elvira et al., 2021). Multiple factors contribute to age-related differences in sequence learning. The difference in performance can partially be explained by physical aspects such as aging-related muscle atrophy, reduced spinal motoneurons, or reduced muscle elasticity (Thompson, 1994; Aagaard et al., 2010). On a neuronal level, there are volume decreases in cortical areas and the corpus callosum, which results in coordination deficits and general movement slowing (Seidler et al., 2010; MacDonald and Pike, 2021). Moreover, aging is related to changes in brain metabolism, such as a depletion in catecholaminergic neurotransmitters (i.e., dopamine, noradrenaline), which results in slower processing speed and is related to a decrease in motor and cognitive functions (Li et al., 2000; Nieoullon, 2002; Cools and D'Esposito, 2011). A recent study has compared the performance of an SFTT in young, middle-aged, and older adults. They found that the three groups significantly differed in the speed which consistently improved over multiple training sessions. However, a different dynamic in accuracy improvements is visible between young and older adults. The young adults showed a steep increase in accuracy and reach a plateau during the early stages of training while the older adults showed a gradual increase in accuracy after multiple training sessions (Maceira-Elvira et al., 2021). This is in line with other studies that show decreased and slower learning in older adults compared to young adults (Howard and Howard, 1992; Curran, 1997; Howard et al., 2004). This suggests a difference in learning strategy between the two groups. Differences between young and older adults on motor sequence learning tasks can be seen in chunking behavior. Shea and colleagues found that older adults did not develop clear chunking patterns while young adults did (Shea et al., 2006). A different study, with multiple training sessions, could show that chunking behavior was indeed different from young adults during the first training day. However, throughout 5 training sessions during one week, the chunking behavior of the older adults became "young-like" (Maceira-Elvira et al., 2021). It has been hypothesized that due to the difference in speed between young and older adults, young people could practice the sequence more on the first training day, which led them to generate motor chunks quicker than old people. This difference could also be related to the age-related decline in cognitive functions, such as WM (Verhaeghen and Cerella, 2002; Park and Reuter-Lorenz, 2009). This age-related decline in WM is thought to be a factor in the diminished performance on explicit sequence learning tasks (Shea et al., 2006; Bo et al., 2009). WM capacity has been related to performance on sequence learning tasks in terms of chunking behavior (Bo and Seidler, 2009). They showed that younger adults were able to make larger chunks within the sequence than older adults (Bo et al., 2009). These suggest a correlation between age, working memory capacity, and sequence learning performance. Multiple factors can be related to the decrease in performance on sequence learning tasks. Based on the decreased performance on motor sequence learning tasks and the possible correlation with WM capacity, enhancing WM capacity with the use of FPN stimulation could lead to positive effects on motor sequence learning.

1.2.5 Stroke related difference in sequence learning

Motor impairments are one of the most common symptoms after stroke (Rathore et al., 2002). These include loss of dexterity and coordinated movements, abnormal muscle tone, and decreased sensory input (Lang et al., 2013). The severity of these symptoms depends on the size and location of the stroke and varies largely among individuals (Ingram et al., 2021). The level of motor impairment influences drastically the ability to perform motor tasks, in both the affected and unaffected hand, compared to healthy controls (Ingram et al., 2021). Motor sequence learning is an important part of stroke recovery as they both rely on the same neuroplasticity processes (Krakauer, 2006). However, due to the heterogeneous character of a stroke, it is difficult to precisely define how a stroke influences motor learning. Studies have shown that stroke patients can learn new sequences, confirming the neuroplasticity capacity necessary for recovery. However, stroke patients show deficits in sequence learning in comparison to healthy age-matched controls. Fleming and colleagues argue that this indicates a need for more training sessions (Fleming et al., 2018). However, Hardwick and colleagues argue the opposite, showing that stroke patients can reach the same performance level as the untrained baseline performance of less impaired individuals, but reach a learning plateau that could not be enhanced by further practice (Hardwick et al., 2017). The process of sequence learning is network-related, involving multiple brain areas during different phases of learning (Hikosaka et al., 2002). Therefore, the location of the lesions can relevantly influence the acquisition, consolidation, or retention of sequences (Dahms et al., 2020).

1.3 Non-invasive brain stimulation

The use of NIBS has become increasingly popular because of its potential to influence neuroplasticity, cortical excitability, and behavior (Hummel and Cohen, 2005; Dayan et al., 2013). The most common techniques are transcranial magnetic stimulation (TMS) and transcranial electrical stimulation (tES) (Hummel and Cohen, 2006; Hallett, 2007). In this thesis, NIBS is used for two main reasons: the evaluation of inhibitory and excitatory mechanisms in the motor cortex and the modulation of excitatory or inhibitory plastic changes in the brain, which can aid motor learning and neurorehabilitation (Hummel and Cohen, 2005). NIBS is widely used in the field to identify causal links between brain areas and their specific cognitive or motor functions, to provide insight into neuronal network organization, to aid practice-dependent plasticity, and for rehabilitation after cognitive or motor impairments (Hummel and Cohen, 2012; Wassermann and Zimmermann, 2012; Dayan et al., 2013; Wessel et al., 2015; Draaisma et al., 2020a). In the following sections, the techniques will be explained in light of their use in this thesis, namely TMS as an evaluation technique for cortical excitability and tES for neuromodulation of plasticity.

1.3.1 Transcranial magnetic stimulation

TMS is a versatile NIBS technique that is frequently used as a research tool to study neurophysiology, but it also has clinical utility (Hallett, 2007). For stimulation, a brief high-current pulse is run through the magnetic coil. This produces a magnetic field perpendicular to the coil and the brain tissue. This brief magnetic field lasts around 100 µs and can reach up to 2 Tesla. This, in turn, induces an electrical field perpendicularly to the magnetic field, parallel to the coil in the nearby brain tissue, see figure 2. The electric field can cause a fast depolarization of cell membranes, resulting in action potentials in neurons. The extent of the neuronal activation depends on the intensity of stimulation. In general, the stimulation does not activate the corticospinal neurons directly but rather through synaptic input (Hallett, 2000, 2007; Farzan, 2014). TMS coils vary in shape and size but are most typically round or figure-of-eight shaped. The induced current flows in loops parallel to the plane of the coil. In a round coil, the current of the loops is the strongest near the circumference of the coil and the weakest near the center of the coil. With a figure-of-eight coil, the strongest current is at the intersection of the two round components, in the middle of the coil. The round coil is typically more powerful, while the figure-eight coil has more focality (Cohen et al., 1990). TMS is most often applied to the M1 as it can induce muscle twitches called motor-evoked potentials (MEPs), which can be measured with the use of electromyography (EMG) and have enabled to map functional representations in the motor cortex (Wassermann et al., 1992). The amplitude of the MEP depends on the intensity of the stimulation and the corticospinal integrity. It is commonly measured from the negative trough to the positive peak and referred to as “peak-to-peak amplitude”, see Figure 2. Most of the detailed information about the stimulation mechanisms comes from M1 stimulation, although it is likely that they are similar in other parts of the brain (Hallett, 2007). In terms of stimulation mechanisms, studies have shown that the induced action potential causes descending volleys in the corticospinal tract which can include D-waves (direct waves) followed by I-waves (indirect waves) (Di Lazzaro et al., 2001). TMS does typically not directly activate descending axons, which causes an early D-wave. The mechanism is rather based on an indirect activation of excitatory synapses of the pyramidal neurons in layer 5 of the motor cortex, producing I-waves, see Figure 2. Depending on the directionality of the induced current, different MEP amplitudes can be produced. In general, the largest MEP is produced by a posterior-anterior direction producing an I1- wave as the first wave. A lateral-medial direction can produce a D-wave, and the anterior-posterior direction typically produces an I3-wave. The amplitude of the MEP is also dependent

on whether the muscle is at rest or contracting. Higher MEPs are measured in contracting muscles due to the higher level of activity in the motor neurons (Hallett, 2000). TMS has multiple stimulation paradigms but is most commonly applied in single, paired, or repetitive pulses (Dayan et al., 2013). These paradigms can be used to measure different aspects of cortical excitability, corticospinal tract integrity, or modulate cortical plasticity (Hallett, 2007).

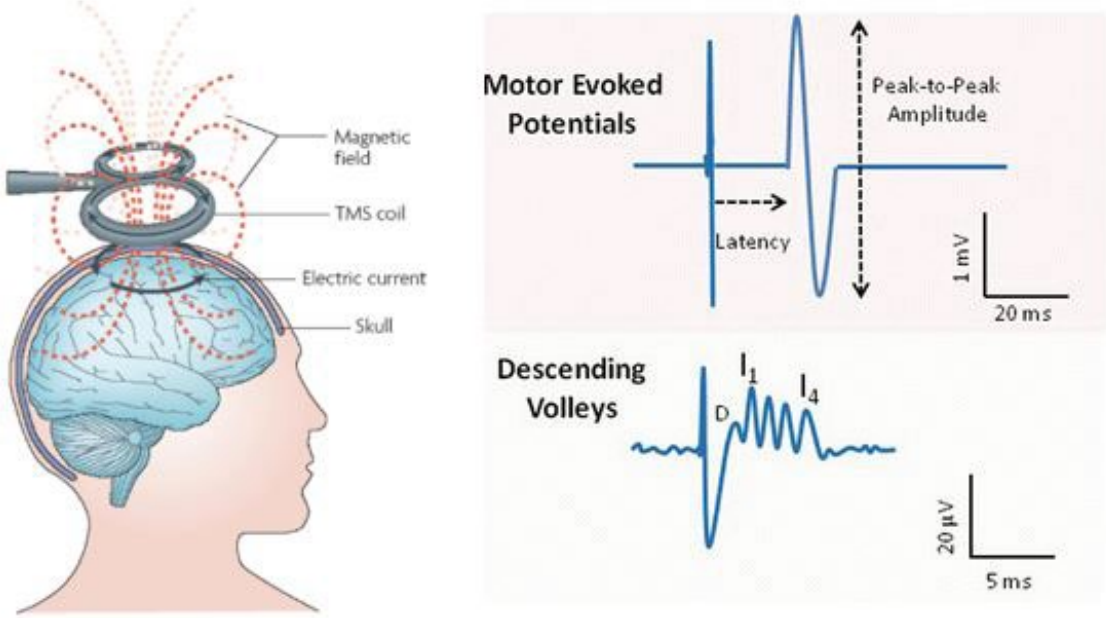

Figure 2. Mechanism of TMS. Visual depiction of a MEP and the descending volleys after M1 stimulation. Adapted from Ridding and Rothwell (2007) and Farzan (2014).

Neurophysiology measures

TMS has been used to quantify different parameters of cortical excitability in the M1, such as resting motor threshold (RMT), recruitment curves, MEP latency, short intracortical inhibition (SICI), and intracortical facilitation (ICF) (Farzan, 2014). RMT is the neuronal threshold for producing an MEP during rest, which reflects cortical excitability (Hallett, 2007). The RMT can be determined with the relative frequency method by finding the minimum amount of intensity to induce an MEP of ≥50 µV in 5 out of 10 trials (Farzan, 2014). Alternative methods exist for the definition of RMT, although this lies outside the scope of this thesis as the relative frequency method was used. RMT is a measure of axonal excitability. For example, the blockage of voltage-gated sodium and calcium channels, which are important for the regulation of axonal excitability, increased the RMT. Moreover, NMDA antagonists have been shown to reduce RMT, while GABA-ergic drugs do not affect RMT (Ziemann et al., 1996a, 1996b). The variance in RMT can be attributed to multiple factors such as the strength of the corticospinal projections, the size of the cortical representation, and the difficulty to access the muscle with TMS. For example, the RMT is lower for the hand areas compared to lower limbs (Chen et al., 1998). RMT can be affected by diseases that cause changes in the integrity of the CST, such as stroke. Therefore, it can be used to characterize lesion severity and the integrity of unaffected areas (Liepert, 2003).

The recruitment curve is defined by the different MEP amplitudes depending on the increase of the stimulation intensity (Hallett, 2007). Recruitment curves can assess the excitability of neurons that are

less excitable or more distant from the center of the TMS pulse (Chen et al., 2008). The shape of the recruitment curve is defined by a slope and a plateau, which varies depending on the tested muscle (Devanne et al., 2002). The measures have been related to the strength of the corticospinal projections and are influenced by increased levels of GABA. For example, Lorazepam has been shown to decrease the slope of the recruitment curve (Boroojerdi et al., 2001). Based on the fact that GABA-ergic drugs did not affect RMT, the two measures appear to be complementary to each other (Farzan, 2014). In stroke patients, changes in recruitment curves after a stroke have been used to quantify lesion location and severity (Chen et al., 2008).

The MEP latency is a measure of corticomotor conduction time and reflects the time between the TMS pulse and the peripheral MEP response. The latency is affected by distance, muscles closer to the stimulation site (facial muscles) have a shorter latency than muscles in the lower extremities (leg muscles) (Hallett, 2007; Farzan, 2014). MEP latencies are reflective of the integrity of white matter fibers depending on the fiber diameters or the myelin sheet thickness (Farzan, 2014).

Paired-pulse stimulation paradigms have been used to measure cortical excitability and physiology. With the use of a mono-focal double pulse applied over the M1, it is possible to quantify neuroplastic changes in the inhibitory and excitatory motor networks and reflect interneuronal influences (Ziemann et al., 1996c). Short intracortical inhibition (SICI) and intracortical facilitation (ICF) are induced with a paired-pulse paradigm (Kujirai et al., 1993). More specifically, a subthreshold conditioning stimulus (CS) is given, which is big enough to activate cortical neurons but too small to induce a MEP. This is followed by a suprathreshold test stimulus (TS) after a short interval. The CS influences the amplitude of the TS depending on the inter-stimulus interval (ISI) between the two pulses. A short interval between 1-6 ms has an inhibitory effect on MEP amplitude (SICI) while longer intervals between 8-30 ms facilitate the MEP amplitude (ICF), please see Figure 3. (Ziemann et al., 1996c; Hallett, 2007). SICI has been proposed to be mediated by GABAergic and ICF by glutamatergic mechanisms (Chen, 2004). SICI is a complex phenomenon that involved a balance between inhibitory and excitatory circuits. For example, MEP facilitation has been measured while using the parameters of the SICI protocol, especially at high stimulation intensities. This facilitation might be related to short-interval intracortical facilitation (SICF), which is seen at similar ISI's (Chen et al., 1998). The modulation of SICI has been relevantly related to motor learning as a measure of motor cortical plasticity (Stagg et al., 2011a). Decreases in SICI levels have been related to increased facilitation of plasticity (Floyer-Lea et al., 2006). Moreover, SICI levels can be modulated by NIBS techniques such as tDCS (see below). This modulation of SICI in response been to tDCS has shown to aid motor learning (Stagg et al., 2011a). In stroke patients, this modulation of SICI during movement preparation is significantly reduced (Hummel et al., 2009). Reductions in SICI levels have been seen in well-recovered stroke patients and have been related to compensatory mechanisms (Butefisch et al., 2008). Moreover, baseline SICI values have been related to motor gain in response to anodal tDCS in stroke patients. Indicating the possible predictive value of SICI as a biomarker for the efficacy of NIBS in stroke patients (O'Shea et al., 2014).

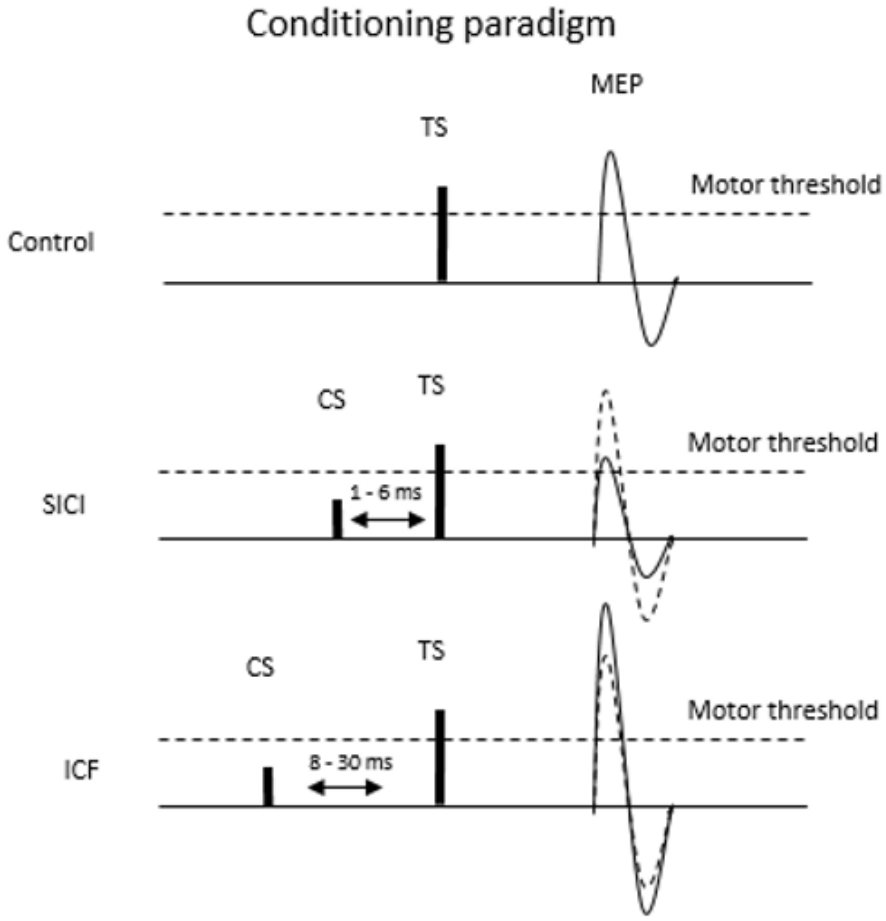

Figure 3. Conditioning paradigm for SICI and ICF. Top: the single-pulse control MEP. Middle: double-pulse SICI paradigm showing a decreased MEP amplitude. Bottom: double-pulse ICF paradigm with an increased MEP amplitude. Abbreviations: Conditioning stimulus (CS), Test stimulus (TS), Motor evoked potential (MEP), Short intracortical inhibition (SICI), Intracortical facilitation (ICF).

Modulation

With the use of repetitive TMS (rTMS) Inhibitory and excitatory circuits can be modulated with effects outlasting the time of stimulation. Depending on the frequency of stimulation the effect is either inhibitory (0.2 Hz – 1 Hz) or facilitatory (≥ 5 Hz) (Pascual-Leone et al., 1994b; Chen et al., 1997; Hallett, 2000; Dayan et al., 2013). This relates to mechanisms of increasing synaptic strength (long-term potentiation (LTP)) or decreasing it (long-term depression (LTD)) (Hallett, 2007). Due to the modulatory mechanisms rTMS has been used as a rehabilitation strategy in stroke patients to reduce maladaptive plasticity (Takeuchi et al., 2007). The stimulation paradigms have focused on either inhibiting the contralesional hemisphere with low frequencies or facilitating the ipsilesional hemisphere with high frequencies. Multiple reviews have shown varying effects of rTMS on the improvement of motor function in stroke patients (Hao et al., 2013; Fisicaro et al., 2019). Targeting the contralesional hemisphere has shown to be more effective than the ipsilesional hemisphere (Fisicaro et al., 2019). Based on the heterogeneous results more research is required to better understand the parameters that relate to the efficacy of rTMS in stroke rehabilitation.

1.3.2 Transcranial electrical stimulation

The most commonly used techniques of tES are transcranial direct current stimulation (tDCS) and transcranial alternating current stimulation (tACS). Although both techniques are different in terms of underlying mechanisms, they share common features. Both techniques are modulatory by applying a weak electrical current (< 4 mA) between electrodes on the scalp (Hummel and Cohen, 2005). As opposed to TMS, tDCS and tACS are not used to induce action potentials by a fast depolarization of neuronal membranes, but rather to change neuronal excitability or neuronal oscillatory activity (Nitsche and Paulus, 2011; Antal and Paulus, 2013).

Transcranial direct current stimulation

During tDCS, a continuous, direct current often between 1-2 mA is applied to the scalp. The main mechanism of action is by subthreshold modulation of neuronal membrane potentials. More specifically, this causes polarization of the underlying tissue that modulates spontaneous neuronal excitability by depolarization or hyperpolarization of resting membrane potential. As the effects of the modulation are subthreshold, the polarization of the neuron will not cause an action potential on its own. However, the polarization can modulate the probability of synaptic firing when activated by different neuronal inputs. Thus, the resting state potential of a cell can be increased by excitatory tDCS which reduced the threshold to produce an action potential compared to that same cell without tDCS stimulation (Antal and Herrmann, 2016). The effects of tDCS are dependent on the underlying physiological state of the target neurons (Fritsch et al., 2010). Basic animal studies have shown that tDCS alone did not induce LTP-like effects in the M1 of mice. However, when the input was combined with another input such as thalamocortical stimulation, large LTP-like effects were seen. This resulted in the conclusion that in humans, tDCS combined with the behavior it intends to modulate might be more effective (Fritsch et al., 2010). Thus, tDCS effects are likely dependent on the baseline brain state at the time of application. Other biological effects, induced by the electrical field are changes in neurotransmission, effects on glial cells, and modulation of inflammatory processes (Woods et al., 2016). The effects of tDCS depend on the polarity, current strength, electrode size and shape, and stimulation time (Nitsche and Paulus, 2000). tDCS stimulation is most often bipolar comprising an anode and cathode current. The anode electrode is the positive current, while the cathode electrode is the negative current. For a depiction of anodal and cathodal tDCS current shapes, please see Figure 4. Whether the stimulation is excitatory or inhibitory depends on the polarity and the placements of the electrodes. In most cases, current flows from the anode to the cathode electrode induce excitatory activity, the inverse has an inhibitory effect. The electrodes are placed in such a way that the target electrode is placed over the target brain areas and the return electrode is placed over another region, not necessarily over a brain area (Wessel et al., 2015). “Classical” tDCS uses relatively large sponge electrodes with a saline solution which has low focality (Nitsche et al., 2007). This results in the stimulation of not only the target area but also neighboring areas in an uncontrolled fashion (Lang et al., 2005). This is generally one of the main limitations of using tDCS, as low focality of stimulation results in difficulty to conclude which area contributed to the outcome (Woods et al., 2016). To overcome this limitation, high definition tDCS (HD-tDCS) with the use of a small target electrode, surrounded by 4 small return electrodes (4x1 montage) or ring electrodes resulting in a less diffuse electric field, see figure 5 (Minhas et al., 2010). Both classical tDCS and HD-tDCS have shown positive effects on motor learning, motor rehabilitation, and cognition, in healthy adults and stroke survivors (Hummel and Cohen, 2005; Reis et al., 2009; Wessel et al., 2015; Hill et al., 2016; Morya et al., 2019) In stroke patients, the target strategy has been to either inhibit the contralesional hemisphere or facilitate the ipsilesional hemisphere based on the interhemispheric competition model (See below in section: 1.3.3). It is becoming more evident that the interhemispheric competition model is only effective for a selective group of patients that are mildly impaired. Therefore, current studies show heterogeneous results with moderate effect sizes but large variance (Kang et al., 2016). Therefore, although tDCS is a

promising technique to enhance motor recovery in stroke patients, it is of paramount importance to increase the knowledge about tDCS efficacy to work towards more personalized treatment.

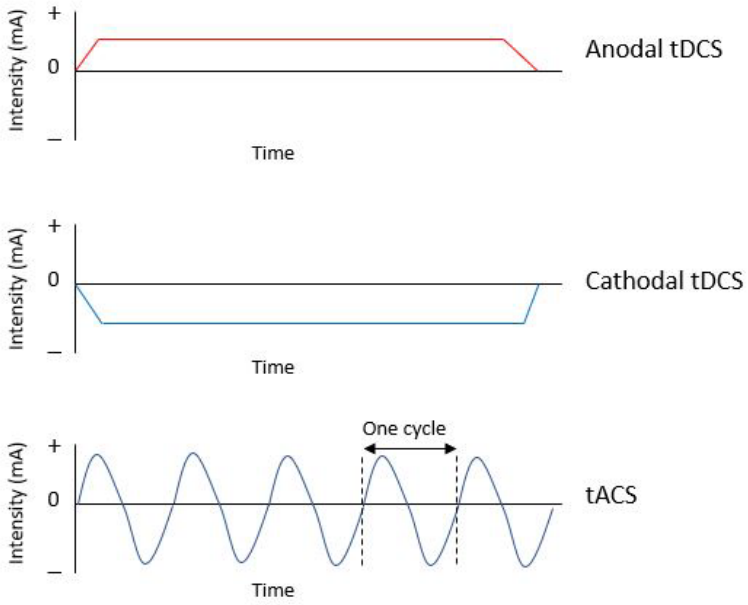

Figure 4. tDCS and tACS current shapes. Example of anodal and cathodal tDCS, showing the direct continuous current with a positive (anodal) and negative (cathodal) current. The bottom shows the tACS current, oscillating from positive to negative in one cycle.

Transcranial alternating current stimulation

tACS is a relatively recent technique that makes use of an alternating electrical current compared to a direct current with tDCS, please see Figure 4. The working mechanisms of tACS rely on the interference of ongoing cortical oscillations in the brain. Cortical oscillations play a crucial role in motor and cognitive functions. The exogenous induced cortical oscillations are believed to be able to entrain or synchronize neuronal oscillations (Antal et al., 2008). The most commonly used waveform for stimulation is sinusoidal, although other waveforms are possible such as rectangular. Important parameters for tACS are the frequency, intensity, and phase of stimulation (Antal and Paulus, 2013). tACS can be applied in a wide frequency range but most conventionally between 0.1 and 80 Hz. The effects of the used frequencies might differ depending on the read-out parameters and the underlying brain state (Antal and Paulus, 2013). For example, alpha tACS to the M1 did significantly improve implicit sequence learning while the same frequency reduced MEP amplitude (Antal et al., 2008). Gamma oscillations to the CB have been associated with increased MEP amplitude and improvement in motor tasks (Naro et al., 2017). On the other hand, theta tACS to frontal and parietal areas has been linked to cognitive improvements on attentional and WM processes (Sauseng et al., 2007; Polania et al., 2012; Violante et al., 2017). The intensity of the stimulation has been shown to have effects on the outcome. For example, low intensity of 0.4 mA had an inhibitory effect on MEP amplitude while 1 mA facilitated the MEP amplitude (Moliadze et al., 2012; Antal and Paulus, 2013). Synchronization of oscillations is one of the key mechanisms of information transfer among brain areas (Buzsáki and Draguhn, 2004). Multi-focal tACS has recently been introduced as a technique to influence the phase relationship of the endogenous oscillations among different brain areas. With this type of stimulation,

it is possible to exogenously synchronize (in-phase) or desynchronize (out-phase) cortical oscillations between distant areas which are thought to improve or decrease performance (Polania et al., 2012; Saturnino et al., 2017; Violante et al., 2017). For example, improvement in WM performance was induced after the synchronization of theta oscillations in the frontal and parietal regions (Polania et al., 2012; Violante et al., 2017). However, desynchronization of theta oscillations in these regions resulted in decreased WM performance (Polania et al., 2012). The type of electrodes used, and the electrode placement can have different effects on the focality of the electric field. Similar to tDCS, high definition tACS (HD-tACS) electrodes have recently emerged. Saturnino and colleagues have compared multiple electrode setups, showing that the focality can differ significantly for the different setups as well as for the stimulation phase. The use of ring electrodes resulted in good electric field focality for both in-phase and out-phase stimulation (Saturnino et al., 2017). For an example of focality differences, please see Figure 5.

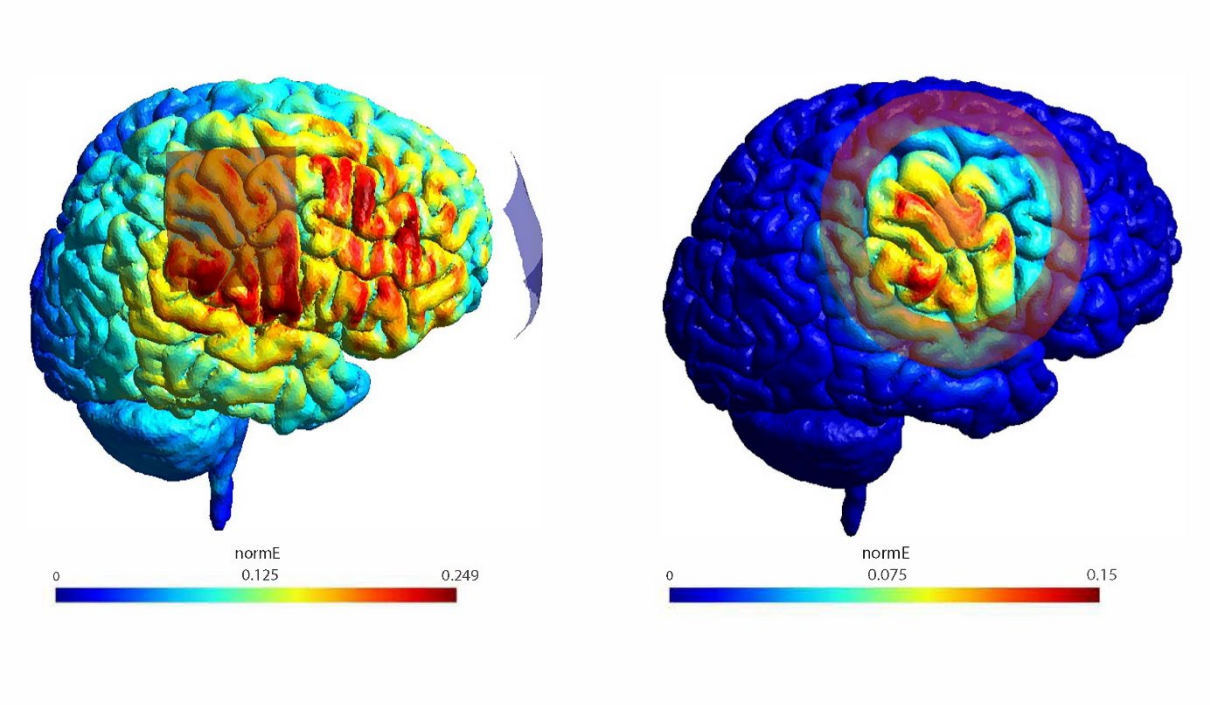

Figure 5. Simulation of tDCS stimulation with 5x5 cm sponge electrodes on the left, or ring electrodes with centre electrode size diameter: ca. 20 mm, area: ca. 3 cm^2 and ring electrode size diameter: out 100 mm/ in 70mm, area: ca. 40 cm^2. Stimulation intensity was set at 2mA. Simulations were done in SimNibs with the use of a template head.

1.4 Stroke

Stroke is a leading cause of long-term disability worldwide (Feigin et al., 2017). One of the most common symptoms is cognitive and motor impairments (Rathore et al., 2002; Jokinen et al., 2015). In the acute phase after a stroke, between 70-90% of patients show some degree of cognitive impairment with around 50% of those patients showing impairments in multiple cognitive domains (Nys et al., 2005; Blackburn et al., 2013; Jokinen et al., 2015). Moreover, it is estimated that around 80% of stroke survivors in the acute phase suffer from motor impairments, especially in the upper limbs (Rathore et al., 2002). Recovery from motor or cognitive impairments is of paramount importance for daily life activities and regaining independence (Langhorne et al., 2009). Due to the natural plasticity of the brain, there is spontaneous recovery to some extent in both the cognitive and motor domains (Kwakkel et al., 2003; Langhorne et al., 2011; Ramsey et al., 2017). After the acute medical treatment, most of the neurorehabilitative strategies consist of extensive cognitive or physical training (physiotherapy, vocational therapy, cognitive behavioral therapy, physical exercise) (Cicerone et al., 2011; Cumming et al., 2012; Di Pino et al., 2014; Draaisma et al., 2020b). However, in most cases, spontaneous recovery and the current treatment strategies are not sufficient for full recovery, leading to long-term impairments and difficulty to reintegrate into daily life activities (Kolominsky-Rabas et al., 2001; Langhorne et al., 2009; Pollock et al., 2014; Winstein et al., 2016; Nijsse et al., 2017). This highlights the importance of the development and/or improvement of strategies that can add to the already existing rehabilitation approaches. One of these strategies that have shown promising results is the use of NIBS for stroke rehabilitation, both for cognitive and motor rehabilitation (Hummel et al., 2005; Hummel and Cohen, 2006; Wessel et al., 2015; Draaisma et al., 2020a). In this thesis, the focus lies on motor impairments and the use of NIBS to enhance motor functions. Therefore, the following background information refers to the underlying mechanisms of motor rehabilitation.

1.4.1 Underlying mechanisms of recovery

The time after a stroke can be divided into common stages from acute (0 – 7 days) to early subacute (< 3 months), late subacute (< 6 months) followed by the chronic stage (> 6 months) (Bernhardt et al., 2017; Grefkes and Fink, 2020). The stages indicate different phases of recovery. Within hours after a stroke, plasticity-enhancing mechanisms induce dendritic growth, axonal sprouting, and the formation of new synapses (Grefkes and Fink, 2020). The largest and most rapid changes in natural recovery take place in the first weeks to months after the stroke for most people (Bernhardt et al., 2017). After 6 months, natural recovery is often at its limit and further improvements are dependent on continued effective rehabilitation strategies (Cramer, 2008). Stroke patients show generally differing recovery patterns that result in heterogeneous outcomes (Langhorne et al., 2011). The general assumption is that stroke patients with mild initial deficits will recover better than severely impaired patients. The ‘proportional recovery rule’ defines that patients can improve on average by around 70% (±15%) and that this is independent of the intensity of the rehabilitation therapy (Winters et al., 2015; Stinear, 2017; Grefkes and Fink, 2020). However, this has been criticized as results seem to rely on over-estimations and because of a relevant amount of “non-fitters” to the rule (Hope et al., 2019; van der Vliet et al., 2020). It has become more evident that stroke recovery is not linear and that recovery profiles vary between individuals (van der Vliet et al., 2020). Therefore, the definition of biomarkers for motor recovery and rehabilitation is of pivotal importance to allow for accurate predictions of recovery and personalize treatment to the specific features of the patients (Stinear, 2017).

Although it is not the only biomarker that predicts recovery, the severity of impairment based on the Fugl-Meyer assessment of the upper extremity (FM-UE) has been instrumental for modeling spontaneous recovery (Stinear, 2017; van der Vliet et al., 2020). As mentioned above, the proportional recovery rule has been criticized due to over-estimations of recovery and rigidity of outcomes. However, a longitudinal study examining a mixture-model of FM-UE recovery did show a strong relation between FM-UE score and motor recovery after 3-6 months (van der Vliet et al., 2020). Not fitting to the proportional recovery rule has been related to the integrity of the cortical spinal tract (CST) after stroke (Feng et al., 2015; van der Vliet et al., 2020). Cross-sectional studies confirm the importance of CST integrity as a biomarker for stroke recovery (Stinear et al., 2012; Boyd et al., 2017).

Neurophysiological measures such as GABA-ergic and glutamatergic signaling are important for plasticity and recovery after stroke (Boyd et al., 2017). Reduced levels of GABA at rest measured with TMS, have been related to good recovery (Butefisch et al., 2008; Hummel et al., 2009). Reduced inhibition and therefore enhanced resting-state excitability have been related to a compensatory mechanism in stroke (Liepert et al., 2000). Moreover, the modulation of GABA levels during movement preparation has been shown to change due to stroke (Liuzzi et al., 2014). Measures of GABA-ergic neurotransmission could be a promising biomarker for compensating excitatory mechanisms in the motor cortex.

Studies focusing on the neural correlates of stroke recovery have shown alterations in motor-related neural activation during the acute and chronic phases of recovery (Rehme et al., 2012). For example, moving the contralesional hand (affected) resulted in bilateral activation patterns in the motor network. These bilateral activation patterns were lesion-related as movements of the ipsilesional hand (unaffected) did not result in the same activity patterns (Ward et al., 2003). Further research showed that patients who showed more recovery also showed a more lateralized activation pattern than patients with more severe remaining impairments (Ward, 2004). The role of the more widespread brain activation in patients with more severe impairments is still under debate. Two opposing models are used to explain adaptive and maladaptive mechanisms. The interhemispheric competition model describes a maladaptive disbalance in inhibitory influence from the unaffected to the affected hemisphere (Murase et al., 2004). Studies have shown that the over activation of the intact hemisphere can have a detrimental effect on the lesioned hemisphere through transcallosal connections from the M1 of the intact hemisphere (M1intact) to the M1 of the lesioned hemisphere (M1lesioned). The vicariation model states the opposite, the overactivation of the intact hemisphere is thought to be a compensatory mechanism to make up for lost function in the affected hemisphere (Riecker et al., 2010; Bradnam et al., 2012; Carrera and Tononi, 2014; Bajaj et al., 2016). Di Pino and colleagues' postulate that the balanced interhemispheric inhibition is a predictor of good recovery for patients with high "structural reserve", measured by the quantity of strategic neural pathways that are spared and could compensate for lost function. In patients with low structural reserve the imbalanced interhemispheric inhibition is rather adaptive (Di Pino et al., 2014).

Networks are important for the recovery of stroke and changes in structural and functional activity patterns between relevant areas have been important markers for motor recovery and functioning after stroke (Rehme and Grefkes, 2013). Among other areas, the CB and the frontoparietal tracts have been positively correlated to motor function after stroke showing that these areas might have a relevant role in network reorganization and compensatory mechanisms (Schulz et al., 2015b). The structural integrity of the cerebellar-cortical pathway has been related to residual motor output in chronic stroke patients (Schulz et al., 2015a; Koch et al., 2021). Moreover, lesions in the cerebellar-cortical pathway can cause a disbalance in cerebellar brain inhibition (CBI) which plays a relevant role in motor control. Targeting this disbalance with the use of NIBS could therefore be a promising treatment strategy for stroke

rehabilitation (Wessel and Hummel, 2018). Furthermore, Koch and colleagues emphasized the importance of the frontoparietal motor network, including the M1, PMv, SMA, and parietal areas such as the intraparietal sulcus and superior parietal gyrus, by showing that connectivity between these areas was crucial for favorable outcomes (Koch et al., 2021). Additionally, an increase in activation patterns in the FPN has been shown after a stroke (Rehme et al., 2012). Consistent excitatory interactions between the frontoparietal areas and the ipsilesional M1 were seen during the movement of the affected limb, pointing towards a compensatory mechanism (Rehme and Grefkes, 2013). These results highlight the importance of other areas than the M1 in the recovery of motor impairments. Therefore, the modulation of these areas with the use of NIBS strategies could be a promising strategy for stroke rehabilitation.

1.4.2 Summary of NIBS and stroke recovery

NIBS-based interventions have been increasingly studied for stroke rehabilitation, to enhance the beneficial and reduce the maladaptive mechanisms of recovery (Ovadia-Caro et al., 2019). Both tES and rTMS techniques have been used (Wessel et al., 2015; Zhang et al., 2017). This thesis will focus on tES techniques as this was the technique used in our studies. The interhemispheric competition model has inspired NIBS studies to either inhibit the unaffected hemisphere or facilitate the affected hemisphere in stroke patients (Hummel and Cohen, 2006; Di Pino et al., 2014). The first tDCS studies showed positive effects of anodal tDCS on the affected M1 or cathodal tDCS on the unaffected M1 on motor function, please see figure 6 (Fregni et al., 2005; Hummel and Cohen, 2005; Hummel et al., 2005). However, the results are heterogeneous and are most beneficial to mildly impaired patients (Hao et al., 2013; Sung et al., 2013; Kang et al., 2016; Bertolucci et al., 2018; O'Brien et al., 2018). More severely impaired patients seem to benefit more from facilitatory stimulation to the unaffected hemisphere, based on the vicariation model (Riecker et al., 2010; Bradnam et al., 2012; Carrera and Tononi, 2014; Bajaj et al., 2016). Therefore, the bimodal-balance recovery model incorporates both models and moves toward more individualized therapy (Di Pino et al., 2014). In this model, the "structural reserve" defines whether patients would benefit more from rebalancing interhemispheric inhibition or facilitating the unaffected hemisphere (Di Pino et al., 2014). This model promotes individualization and potentially provides biomarkers for the most effective neuromodulation targets, tailored to the patient (Di Pino and Di Lazzaro, 2020). Indeed, when only severely affected patients were studied, facilitating the unaffected hemisphere resulted in performance improvement while a meta-analysis showed that rebalancing interhemispheric inhibition was more effective in mildly impaired patients (Bertolucci et al., 2018; McCambridge et al., 2018). Currently, most studies focused on ipsi- or contralesional M1 stimulation. However, stimulating lesioned areas might induce confounding effects, such as shunting of electrical current. Moreover, targeting the non-lesioned M1 with inhibitory stimulation might affect the unaffected hand (Wessel et al., 2015). Therefore, based on the importance of other brain areas such as the CB and the FPN in stroke rehabilitation, targeting these areas could be a potential alternative strategy (Wessel et al., 2015; Koch et al., 2021). Promising results have shown the efficacy of cerebellar stimulation on standing balance and gait in stroke patients (Picelli et al., 2015; Zandvliet et al., 2018, 2019). However, studies combining motor learning with cerebellar or FPN stimulation in stroke patients are currently missing. This highlights the need for investigation of the feasibility of these stimulation targets to enhance motor learning, which is the aim of this thesis.

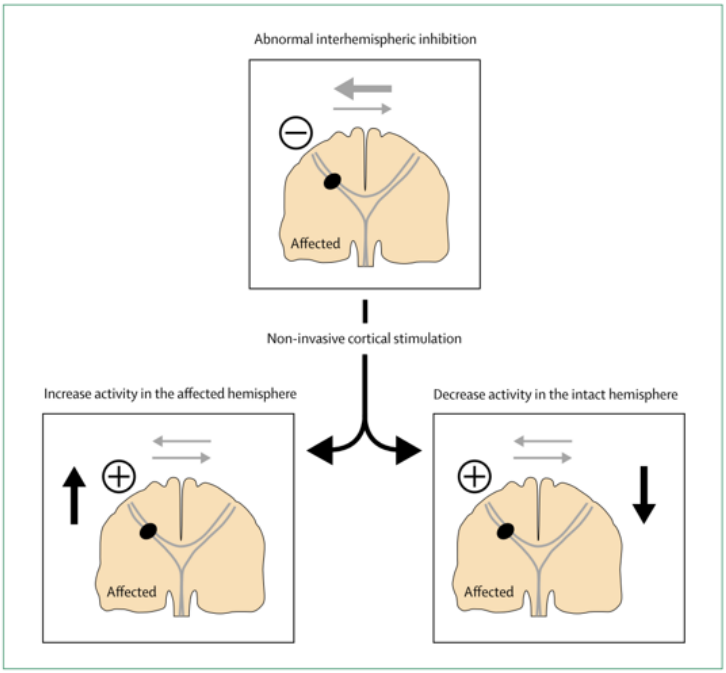

Figure 6. Interhemispheric competition model. Figures indicate the abnormal inhibition of the unaffected hemisphere to the affected hemisphere. NIBS can be used to either decrease the activity in the unaffected hemisphere or increase the activity in the affected hemisphere, taken from Hummel & Cohen (2006).

1.4.3 NIBS paradigm development

As mentioned, upper-limb impairments are one of the most common symptoms of stroke, and even after rehabilitation, less than 20% of the patients recover to the same level as they were before the stroke (Di Pino et al., 2014). Therefore, strategies to improve rehabilitation after stroke, such as NIBS, are studied. Currently, the outcomes of NIBS studies are too heterogeneous and therefore not ready for clinical translation. A possible reason for this is that a one-size-fits-all strategy does benefit some but not all patients. To achieve more homogenous results strategies that are tailored to the needs of the patients might be more effective. Therefore, the development of alternative NIBS paradigms that can expand on the current knowledge and ultimately allow for individualization is the main aim of this thesis. Multiple factors could aid in optimization including multiple stimulation sessions, which have been shown to have additional effects compared to a single session (Boggio et al., 2006, 2007). As mentioned above, the M1 is the most conventional target for stroke rehabilitation. However, convincing neuroimaging evidence has shown that stroke recovery is network related which also involves other areas than the M1 (Koch et al., 2021). Therefore, targeting other brain areas than the M1 could be beneficial (Plow et al., 2015; Wessel and Hummel, 2018). Proof-of-principle studies in healthy adults have targeted secondary motor areas such as the PMC and CB with promising results (Galea et al., 2011; Vollmann et al., 2013; Hardwick and Celnik, 2014; Wessel et al., 2016). Moreover, the FPN has been related to motor learning mechanisms and is a crucial network for motor recovery (Hardwick et al., 2013; Koch et al., 2021). Therefore, these areas could provide alternative targets within the motor learning network. Finally, the process of motor learning is not restricted to one single brain area but rather integrated into a well-orchestrated network (Hikosaka et al., 2002; Sporns et al., 2004; Floyer-Lea and Matthews, 2005; Dayan and Cohen, 2011). However, most of the conventional paradigms target one brain area

exclusively. The use of multi-focal NIBS paradigms might further enhance efficacy in stroke patients (Singer, 1999; Wessel et al., 2015).

1.5 Thesis at a glance

Following the general introduction, this thesis consists of 4 different articles that were completed during my doctoral studies. Study 1 is a methodological study comparing the effectiveness of two different TMS stimulators in eliciting SICI and ICF responses and whether these responses were comparable, in a cohort of healthy participants. The following three studies were aimed at evaluating alternative NIBS strategies to improve motor learning. Study 2 assessed the effectiveness of 50 Hz tACS applied to the CB during a novel motor sequence learning task in healthy young participants. Study 3 evaluated the efficacy of a sequential bifocal tDCS paradigm, applied to the M1 and the CB, on motor learning in a cohort of stroke patients with upper-limb impairments. Study 4 tested a personalized bifocal tACS paradigm applied to the frontoparietal network to determine a causal relationship between WM and motor sequence learning in healthy older adults.

1.6 Personal contribution

At the time of writing, study 1, study 2, and reviews 1-3 have been published. Study 3 is in preparation and study 4 is currently under revision. Please find below the list of included studies in this thesis and the published reviews that have not been included.

Study 1: *The Effects of Stimulator, Waveform, and Current Direction on Intracortical Inhibition and Facilitation: A TMS Comparison Study* (2019) - Wessel M.J.*, **Draaisma L.R.***, Morishita T., Hummel F.C., *Frontiers in Neuroscience*, *13*(703), Article 703. https://doi.org/10.3389/fnins.2019.00703

*Authors contributed equally

Personal contribution: Study design, recruitment, data acquisition, analysis, writing, and editing of the manuscript.

Study 2: *Cerebellar transcranial alternating current stimulation in the gamma range applied during the acquisition of a novel motor skill* (2020), Wessel, M. J.*, **Draaisma, L. R.***, de Boer, A. F. W., Park, C.-H., Maceira-Elvira, P., Durand-Ruel, M., Koch, P. J., Morishita, T., & Hummel, F. C., Scientific Reports, 10(1), 11217. https://doi.org/10.1038/s41598-020-68028-9

*Authors contributed equally

Personal contribution: Study design, recruitment, data acquisition, analysis, writing, and editing of the manuscript.

Study 3: *Multi-focal stimulation of the cortico-cerebellar loop during the acquisition of a novel hand motor skill in chronic stroke patients.* Wessel M.J.*, **Draaisma L.R.***, Durand-Ruel M., Maceira-Elvira P., Moyne M., Turlan J.-L., Mühl, A., Léger, B., Chauvigné, L., Park C., Koch P.J., Morishita T., Guggisberg A.G. & Hummel F.C. (in preparation)

*Authors contributed equally

Personal contribution: Recruitment, data acquisition, analysis, writing, and editing of the manuscript.

Study 4: *Targeting the frontoparietal network using multifocal personalized transcranial alternating current stimulation to enhance motor sequence learning in healthy older adults.* **Draaisma, L.R.**, Wessel, M.J., Moyne, M., Morishita, T. & Hummel, F.C. (under revision), Brain Stimulation, DOI: 10.1101/2022.02.16.480660

Personal contribution: Conceptualization of the idea, study design, recruitment, data acquisition, analysis, writing, and editing of the manuscript.

Published articles not included in thesis

Review 1: *Non-invasive brain stimulation to enhance cognitive rehabilitation after stroke.* **Draaisma, L. R.***, Wessel, M. J.*, & Hummel, F. C. (2020a). *Neurosci Lett*, *719*, 133678. https://doi.org/10.1016/j.neulet.2018.06.047

*Authors contributed equally

Personal contribution: Writing and editing the review

Review 2: *Neurotechnologies as tools for cognitive rehabilitation in stroke patients.* **Draaisma, L. R.**, Wessel, M. J., & Hummel, F. C. (2020b). *Expert Review of Neurotherapeutics*. https://doi.org/10.1080/14737175.2020.1820324

Personal contribution: Writing and editing the review

Review 3: *Mini-review: Transcranial Alternating Current Stimulation and the Cerebellum.* Wessel MJ, **Draaisma L.R.**, Hummel FC (2022) The Cerebellum. https://link.springer.com/10.1007/s12311-021-01362-4

Personal contribution: editing the review

2. Study 1: *The Effects of Stimulator, Waveform, and Current Direction on Intracortical Inhibition and Facilitation: a TMS Comparison Study*

Maximilian J. Wessel[1,2]*, Laurijn R. Draaisma[1,2]*, Takuya Morishita[1,2] & Friedhelm C. Hummel[1,2,3*]

*Authors contributed equally

[1] Defitech Chair of Clinical Neuroengineering, Center for Neuroprosthetics (CNP) and Brain Mind Institute (BMI), Swiss Federal Institute of Technology (EPFL), Geneva, Switzerland.

[2] Defitech Chair of Clinical Neuroengineering, Clinique Romande de Réadaptation, Center for Neuroprosthetics (CNP) and Brain Mind Institute (BMI), Swiss Federal Institute of Technology (EPFL Valais), Sion, Switzerland.

[3] Clinical Neuroscience, University of Geneva Medical School, Geneva, Switzerland

*Correspondence:
Prof. Dr. Friedhelm C. Hummel
Defitech Chair of Clinical Neuroengineering
Center for Neuroprosthetics (CNP)
Swiss Federal Institute of Technology (EPFL)
Campus Biotech,
Rm H4.3.132.084
9, Chemin des Mines
1202 Geneva
email: friedhelm.hummel@epfl.ch
Tel: +41 21 69 35 440

2.1 Abstract

Background: Cortical function is dependent on the balance between excitatory and inhibitory influences. In the human motor cortex, surrogates of these interactions can be measured in-vivo, non-invasively with double-pulse transcranial magnetic stimulation (TMS). To compare results from data acquired with different available setups and bring data together, it is inevitable to determine whether different TMS setups lead to comparable or differential results.

Objective: We assessed and compared short intracortical inhibition (SICI) and intracortical facilitation (ICF) testing four different experimental conditions.

Methods: SICI and ICF were studied with different stimulators (Magstim BiStim² or MagVenture MagPro X100), waveforms (monophasic or biphasic), current directions (anterior-posterior or posterior-anterior) at interstimulus intervals (ISIs) of 1, 3, 10, 15 ms.

Results: The conditions led to comparable results for SICI and ICF, except for SICI tested at 3 ms in which the anterior-posterior current direction led to stronger modulation.

Conclusions: SICI and ICF data sets obtained with two different, commonly used simulators (Magstim BiStim² or MagVenture MagPro X100) with conventionally used stimulation parameters are largely comparable. This suggests that SICI and ICF results obtained in different studies can be well compared and allows the combination of data sets in an open science view.

Keywords:

Transcranial magnetic stimulation, short intracortical inhibition, intracortical facilitation, Magstim, MagVenture, comparison.

2.2 Introduction

Inhibitory and excitatory interactions are key components of cortical processing (Kirkwood, 2015). With the use of double-pulse transcranial magnetic stimulation (dpTMS), it is possible to assess correlates of these interactions within the human motor cortex in-vivo and non-invasively, first described by Kujirai and colleagues (Kujirai et al., 1993; Chen, 2004). It pairs a subthreshold conditioning stimulus (CS) with a subsequent suprathreshold test stimulus (TS). The test response, mainly measured via the amplitude of motor evoked potentials (MEPs), is inhibited at shorter interstimulus intervals (ISIs) of 1-6 ms, this effect is commonly termed short intracortical inhibition (SICI). At longer ISIs of 8-30 ms the test response is facilitated, here referred to as intracortical facilitation (ICF). SICI and ICF have been widely used to study motor cortex physiology in healthy subjects and neurological disorders, e.g., (Hummel et al., 2009; Heise et al., 2010, 2013). SICI has been associated with GABAA and ICF with glutamatergic neurotransmission (Ziemann et al., 1998; Di Lazzaro et al., 2000). Importantly, this neurotransmission has been linked to various aspects of human behavior, such as the regulation of learning, memory, cognition, and emotions (Ende, 2015).

SICI and ICF are frequently used to study neurological conditions and to validate neurotechnological interventions, such as transcranial direct current stimulation, e.g., (Zimerman et al., 2012). However, as different devices and protocol parameters are used, it is important to determine whether these different devices and protocols lead to comparable data. This would be a crucial prerequisite to judging whether results derived from different experimental setups are comparable. Furthermore, this knowledge will pave the way to comprehensively combine data sets from different sources, e.g., towards open science approaches. Even more importantly, it is a necessary basis for the potential of SICI and ICF as diagnostic tools or biomarkers to predict recovery and treatment response.

Comparative studies of different devices have been conducted to assess motor thresholds, MEP amplitudes, MEP latencies, or TMS-evoked potentials (Kammer et al., 2001; Van Doren et al., 2015). For example, Van Doren and colleagues reported a higher magnetic field strength, a shorter magnetic flux duration, lower motor threshold, shorter recovery time from the TMS artefact, a shorter MEP latency, and a reversed first artefact trajectory comparing the MagVenture MagPro with two other devices (the Magstim Rapid and the Deymed DuoMag XT-100 stimulator) operating in biphasic mode (Van Doren et al., 2015).

To the best of our knowledge however, the effects of the parameter interactions between stimulator, waveform, and current direction have not been studied yet for SICI and ICF. Therefore, our objective was to compliment the available literature by investigating the effects of stimulator, waveform, induced current direction, and ISI on SICI and ICF. We compared two commonly used TMS stimulators, the Magstim BiStim2 stimulator (Whitland, UK) and the MagVenture MagPro X100 stimulator (Farum, Denmark). In addition to the commonly used monophasic waveform, we tested a biphasic waveform, which may provide the benefit of lower values for motor thresholds (Sommer et al., 2006). We assessed the effects of the induced current direction, since on the contrary to the most commonly applied posterior to anterior (PA) currents, single-pulse anterior to posterior (AP) currents and SICI rather influence later I-waves (Nakamura et al., 1997; Sakai et al., 1997). Furthermore, we assessed different ISIs to conclude on phase-specific effects.

The aim of the present study was to make inferences about setup related confounds and emphasize between center and study comparability, when assessing SICI or ICF in future.

2.3 Materials and Methods

Participants

Fifteen young, healthy, right-handed participants were recruited for the study (eight female, mean age 25.20 years, mean laterality quotient Edinburgh handedness inventory 87.63 (Oldfield, 1971). The inclusion criteria were as follows: ≥ 18 and < 35 years, right-handedness, normal values of Mini-mental state examination (>26/30), absence of contraindication for transcranial electric stimulation (tES), transcranial magnetic stimulation (TMS) or magnetic resonance imaging. The exclusion criteria were: presence of neuropsychiatric diseases, history of seizures, intake medication that potentially interacts with tES or TMS, musculoskeletal dysfunction that compromise finger movement, pregnancy, professional musician or intense professional usage of a computer keyboard, intake of narcotic drugs, request of not being informed in case of incidental findings. The study was carried out in accordance to the Declaration of Helsinki (World Medical Association, 2013). Written informed consent was obtained from all participants. Approval was obtained from the cantonal ethics committee Vaud, Switzerland (project number 2017-00765).

Experimental design

The objective was to assess the effects of different TMS conditions on SICI and ICF. The following conditions were tested and compared: A.) Magstim BiStim2 stimulator (Whitland, UK) with a monophasic waveform and a posterior-anterior current direction (MS PA). B.) Magstim BiStim2 stimulator (Whitland, UK) with a monophasic waveform and an anterior-posterior current direction (MS AP). C.) MagVenture MagPro X100 stimulator (Farum, Denmark) with a biphasic waveform and an anterior-posterior to posterior-anterior current direction (MV AP-PA). D.) MagVenture MagPro X100 stimulator (Farum, Denmark) with a monophasic waveform and a posterior-anterior current direction (MV PA), please see also Fig. 1. The current direction is indicated as induced in the underlying brain tissue throughout the manuscript. The assessments were grouped into one session per stimulator. The order of the respective configurations and sessions followed a pseudorandomized sequence.

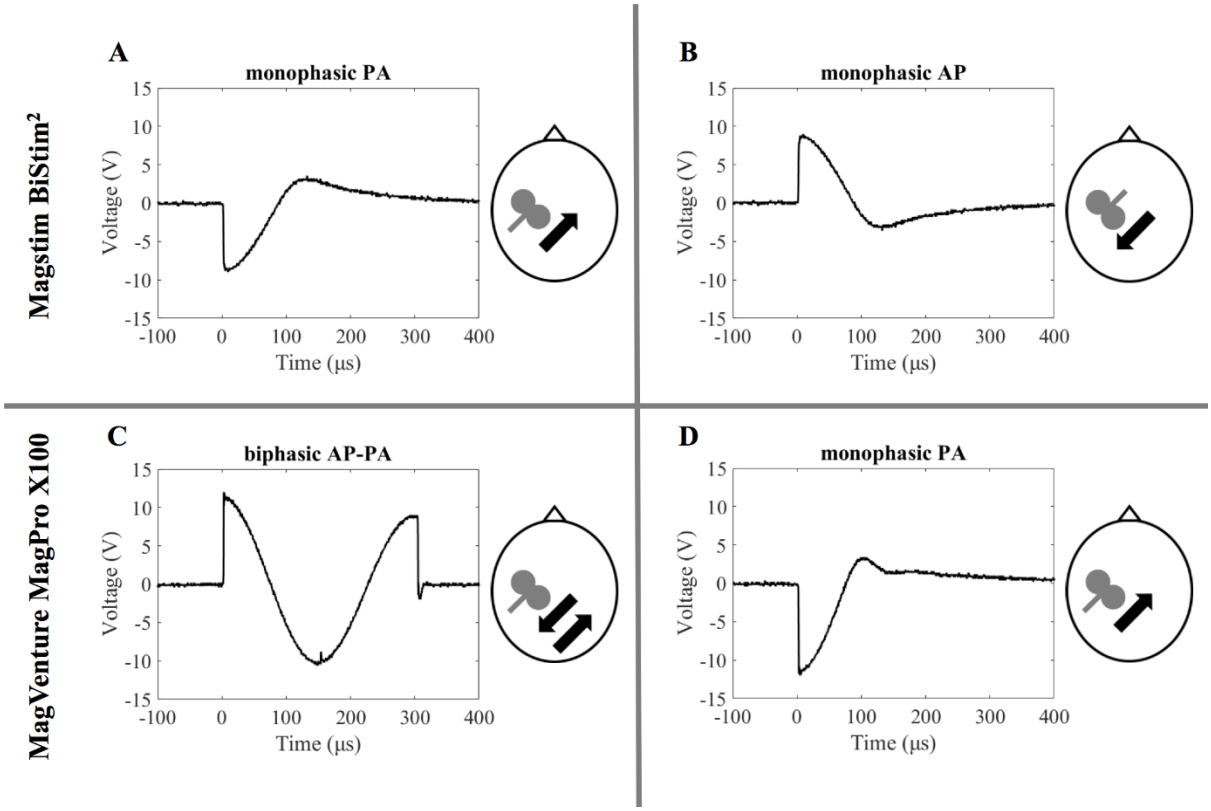

Fig. 1: Experimental setup. *Depicted are the four experimental conditions. The waveforms were measured with a probe fixed above at the coil wire intersection and a single TMS pulse applied at 50% of maximum stimulator output (MSO). Arrows indicate the current directions as induced in the underlying brain tissue, either anterior-posterior (AP) or posterior-anterior (PA).*

Transcranial magnetic stimulation

A double-pulse protocol was utilized to assess SICI and ICF at rest (Kujirai et al., 1993). In this protocol, a subthreshold conditioning stimulus (CS) was followed by a suprathreshold test stimulus (TS). SICI was tested at ISIs of 1 and 3 ms. ICF at ISIs of 10 and 15 ms, except for MV PA in which technical limitations of the stimulator restrained us from testing a 1 ms interval. The CS was adjusted to 80% of resting motor threshold (RMT) (Kujirai et al., 1993). The RMT was defined as the minimal output of the stimulator that elicited MEPs with peak-to-peak amplitude of ≥ 50 µV in at least 5 out of 10 consecutive trials (Groppa et al., 2012). The TS was adjusted to evoke MEPs of ~ 1 mV (Sanger et al., 2001). The TMS pulses were applied over the left motor hotspot with a figure-of-eight coil. For the MagVenture setup, we used a MC-B70 Butterfly Coil and for the Magstim setup a D70 Alpha Flat Coil, for a comparison of coil specifications please see Tab. 1. The coil was oriented that the handle pointed backwards and ~ 45° to the midsagittal line. Twenty trials were recorded for the TS and for each double-pulse paradigm with at an inter-trial-interval of 7 s ± 25 %. The order followed a pseudorandom sequence, except for the first two participants in which we used a block randomization for MV PA, due to an earlier version of our trigger setup.

Technical specifications of utilized TMS coils

Manufacturer	Coil type	Averaged inductance	Focality	Stimulation depth	Coil wing external diameter	Angle	Wire loops overlap
Magstim	D70 Alpha Flat Coil (uncoated)	16 µH	14.8 cm^2	1.41 cm	90 mm	180°	No
MagVenture	MC-B70 Butterfly Coil	11.9 µH	13.9 cm^2	1.35 cm	97 mm	150°	Yes

Table 1: *The inductance values were provided by the respective manufacturer. Most prominent difference is the slight bend of the surface of the MC-B70 Butterfly Coil and the overlapping wire loops, which leads to a slight increase in focality.*

EMG recording

MEPs were recorded from the right first dorsal interosseous (FDI) muscles via surface electrodes positioned in belly-tendon montage. The signal was recorded with a Noraxon DTS Receiver (Scottsdale, AZ, USA) (gain 500, sampling rate 3000 Hz, high-pass filter: 10 Hz analog Sallen-Key, low-pass filter: 1000 Hz digital FIR 128th order Butterworth) and for further processing transferred and saved on a laptop via CED Signal software (version 6.05a, Cambridge, UK).

TMS pulse characterization

The applied TMS waveforms were characterized using a MagVenture MagProbe 3D (Farum, Denmark), with the probe fixed on the intersection of the respective figure-of-eight coil. For the recording of the pulse shapes, the stimulator output was set to 50% of maximum stimulator output (MSO).

Normalization of RMT between stimulators

In order to compare the stimulator intensities used for the RMT and to reach a 1 mV TS, the values were normalized by the square root of the maximum energy (W) stored in capacitor (Kammer et al., 2001). For Magstim W = 578.1 joules and for MagVenture W = 300 joules, as provided by the respective manufacturer.

Data processing

The data were analyzed offline. The EMG time series were exported to MATLAB (version 2018a, Natick, MA, USA) and analyzed using a custom-designed graphical user interface. All trials were visually inspected. Trials with muscle pre-activation exceeding ± 25 µV from baseline < 100 ms and/or ± 100 µV from baseline 500-100 ms before the TMS pulse (Hallett, 2007), trials with technical artefacts, no clear MEPs for the TS and ICF conditions (in analogy to Rossini criterion: peak-to-peak amplitude < 50 µV, for review please see (Groppa et al., 2012)), or with documented suboptimal coil placement were rejected from further analysis. The MEP peak-to-peak amplitude was computed in a response window of TMS pulse + 20 ms to + 50 ms. The resulting peak-to-peak amplitude was averaged per condition. To indicate inhibitory or excitatory modulation, the SICI and ICF conditions were contrasted to the average TS MEPs amplitude and expressed as mean(CS+TS) / mean(TS) * 100.

Statistical analysis

The statistical analysis was performed with the R software environment (version 3.5.1., 2018) (R Core Team, 2013). Statistical significance was assumed at $p < 0.05$. The normality of the distributions was checked with the Shapiro-Wilk test. Normally distributed data were analyzed with a repeated measures analysis of variance (RM-ANOVA), applying pairwise t-test post-hoc comparisons, Bonferroni-corrected. Non-normally distributed data were analyzed with a Friedman test, with Wilcoxon-signed rank post-hoc tests, Bonferroni-corrected. For all the analyses that involved the MS AP condition the data of one participant has been not considered for further statistical processing due to missing values (high motor threshold). Differences between conditions were tested for every ISI separately. Secondly, we tested for differences in effectiveness between SICI and ICF within every condition. Thirdly, the MEP amplitudes of SICI and ICF were compared with the test-pulse amplitude to see if there was effective modulation in the dpTMS protocols. Lastly, we tested for differences of TS amplitude between conditions. All values in text, figures, and tables are depicted as mean ± SEM.

2.4 Results

Condition comparisons

We tested whether there was a difference in the MEP amplitude between the four different conditions for the different ISIs (SICI and ICF). Analysis of SICI 1 showed a significant condition effect $\chi2(2) = 7.00$, $p = 0.030$. However, post-hoc pairwise comparisons did not show any significant differences. There was a significant difference between conditions for SICI 3 $\chi2(3) = 20.14$, $p < 0.001$. Post-hoc analysis showed that MS PA (56.78 ± 12.94 %) was significantly larger than MS AP (9.52 ± 1.70 %, $p = 0.005$). MS AP was significantly smaller than MV AP-PA (49.17 ± 11.11 %, $p = 0.002$) and smaller than MV PA (42.05 ± 10.84 %, $p = 0.007$). There were no overall significant differences between conditions for ICF 10 $\chi2(3) = 6.43$, $p = 0.093$ or between conditions for ICF 15 $\chi2(3) = 0.94$, $p = 0.815$. In summary, for most of the ISIs the different conditions resulted in comparable effects on the MEP amplitude, only SICI assessment at an ISI of 3 ms with an unconventional anterior-posterior current direction achieved a larger decrease of the MEP amplitude, for details please see Fig. 2.

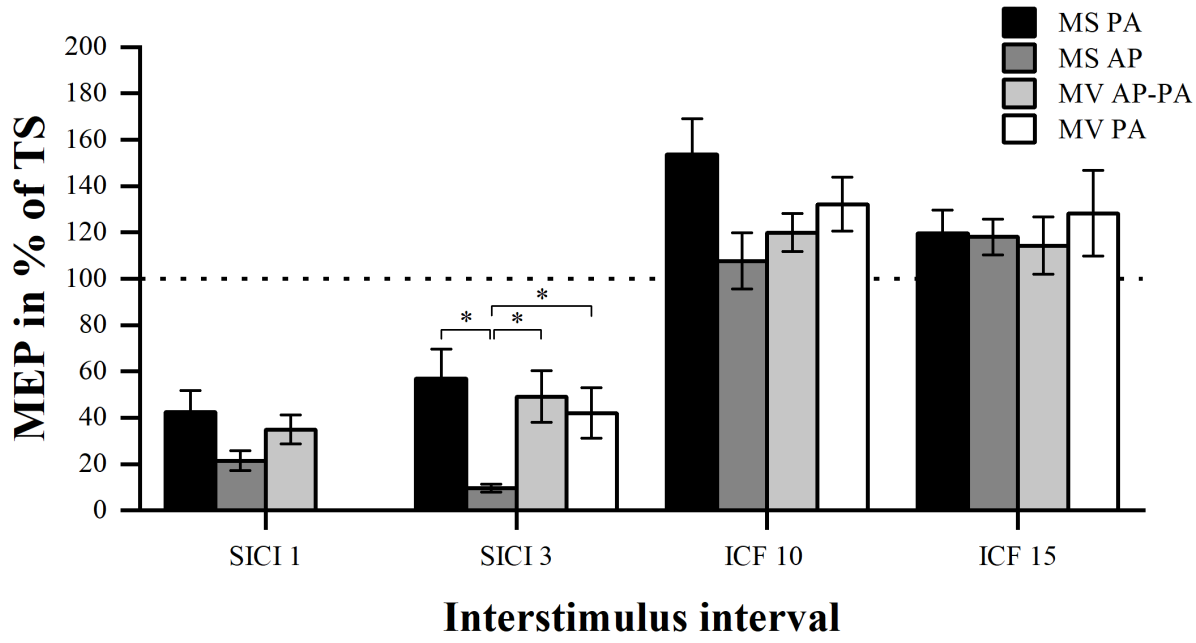

Fig. 2: Modulation compared per condition. *Comparison of MEP modulation induced by the four different stimulator current direction conditions sorted by ISI (1, 3, 10, 15 ms). * $p < 0.05$.*

Effectiveness of stimulation paradigms

For every condition, we tested whether the four different ISIs resulted in differential MEP modulation. Furthermore, we compared the two inhibition and facilitation paradigms within the conditions to see whether one of the two ISIs was more effective, please see Fig. 3.

As expected, in all conditions the comparison between the four different paradigms showed significant overall differences in MEP modulation. Results are for MS PA: $\chi2(3) = 30.44$, $p < 0.001$, MS AP: $\chi2(3) = 34.71$, $p < 0.001$, MV AP-PA: $\chi2(3) = 31.24$, $p < 0.001$ and MV PA: $\chi2(2) = 19.60$, $p < 0.001$, respectively. Post-hoc comparisons showed that there was no significant difference in MEP magnitude between the two inhibitory paradigms in all the conditions. Post-hoc comparisons within the facilitation paradigms did also show that there were no significant differences. Overall, different ISIs did not result in significantly different MEP modulation within the inhibitory or facilitatory paradigms.

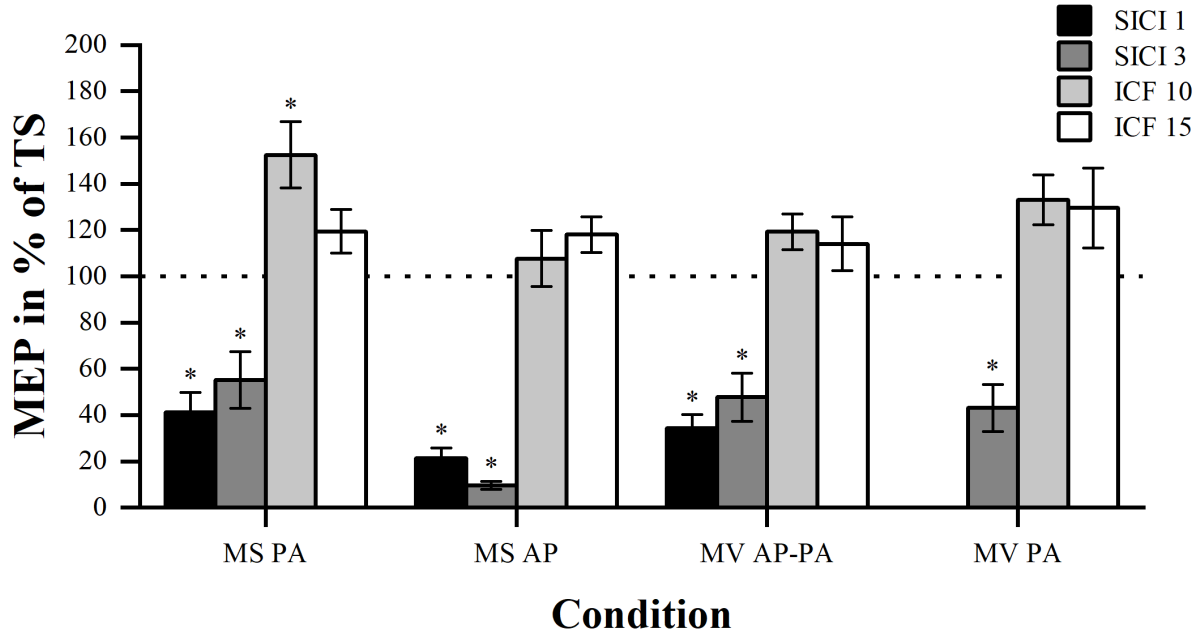

Fig. 3: Modulation compared per ISI. *Comparison of MEP modulation induced by SICI and ICF at the four different ISIs (1, 3, 10, 15 ms) sorted by condition. Asterisk indicates significant modulation compared with TS alone. * $p < 0.05$.*

Modulation effect

We tested whether the MEP amplitudes assessed at different ISIs were significantly different from the TS, to show whether modulation was present. The results showed that all the inhibitory paradigms resulted in significant modulation for all conditions, please see Tab. 2. However, for the facilitatory paradigms only ICF 10 in MS PA resulted in a significant modulation.

Modulation effect

Condition	**SICI 1** *p*-value	**SICI 3** *p*-value	**ICF 10** *p*-value	**ICF 15** *p*-value
MS PA	0.003*	0.034*	0.034*	1.000
MS AP	0.001*	0.001*	1.000	0.245
MV AP-PA	< 0.001*	0.012*	0.302	1.000
MV PA	n/a	0.003*	0.288	0.332

Table 2: *Modulation effect for every ISI compared with the TS. Significant results depict that there was significant inhibition or facilitation compared with the TS. * $p < 0.05$.*

Auxiliary analysis

To compare the RMT between stimulators, the threshold was normalized to the maximal energy stored in the stimulator (Kammer et al., 2001). There was a significant effect of condition $F(3, 39) = 79.01$, $p < 0.001$. Post-hoc comparisons showed that MS PA (1.70 ± 0.07) was significantly smaller than MS AP (2.34 ± 0.11, $p < 0.001$). MS PA was smaller than MV PA (2.28 ± 0.11, $p < 0.001$), MS AP was larger than MV AP-PA (1.67 ± 0.07, $p < 0.001$) and MV AP-PA was smaller than MV PA ($p < 0.001$), see Fig. 4A.

The achieved TS amplitudes are depicted in Fig. 4B. Results showed that there were significant differences between the four conditions in terms of TS amplitude $F(3, 39) = 3.02$, $p = 0.041$. However, post-hoc pairwise comparisons did not show any significant differences between the conditions.

The stimulator output intensities to achieve a TS MEP of ~ 1 mV were normalized to the maximal energy stored in the stimulator. The four conditions turned out to be significantly different from each other $F(3, 39) = 77.99$, $p < 0.001$. Post-hoc comparisons showed that MS PA (2.01 ± 0.10) was significantly smaller than MS AP (2.76 ± 0.13, $p = 0.001$), MS PA was smaller than MV PA (2.79 ± 0.14, $p = 0.001$), MS AP was larger than MV AP-PA (2.05 ± 0.15, $p = 0.003$) and MV AP-PA was smaller than MV PA ($p = 0.002$), see Fig. 4C.

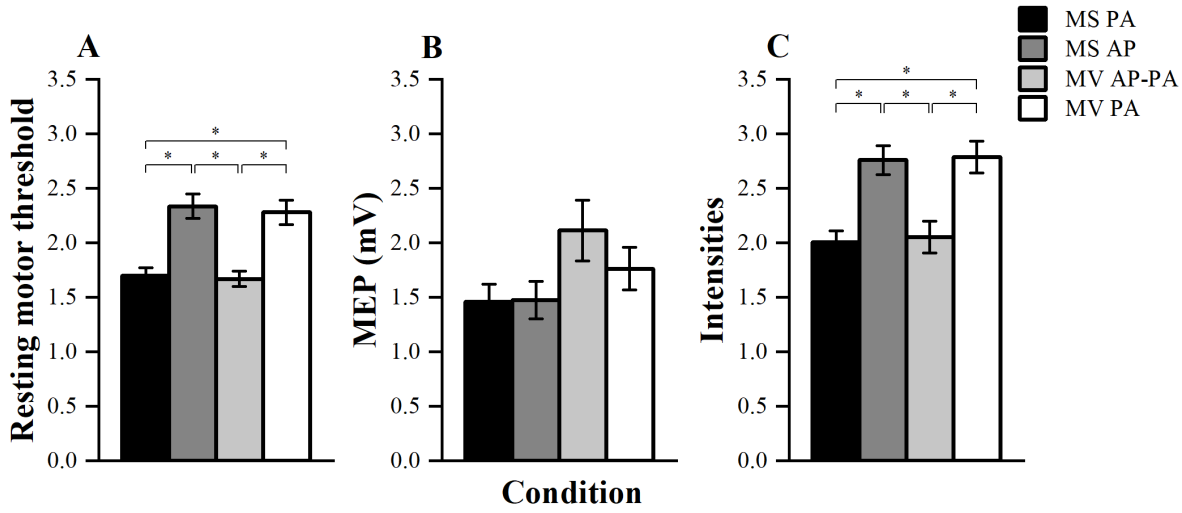

Fig. 4: Resting motor threshold, TS amplitude, stimulator intensities. *A. Normalized resting motor threshold (RMT) across conditions. B. Achieved peak-to-peak amplitude for the TS across different conditions. C. Normalized stimulator output intensity at the achieved TS amplitude (~ 1 mV) across conditions.*

2.5 Discussion

In summary, the present study demonstrated that different stimulators (Magstim BiStim2 versus MagVenture MagPro X100), waveforms (monophasic versus biphasic), and current directions (posterior-anterior versus anterior-posterior) resulted in comparable changes of the MEP during SICI and ICF protocols. This suggests that results are comparable from studies using these different parameters. The only exception in the present study was SICI tested at an ISI of 3 ms with a monophasic waveform and an anterior-posterior current direction, which led to stronger inhibition, a protocol rather rarely used.

Effect of current direction

The current direction dependent effect for SICI demonstrated here has been described previously in the literature with the largest difference at an ISI of 3 ms (Hanajima et al., 2008). A proposed mechanism may be that SICI affects mainly later I-waves (Nakamura et al., 1997) and these are mainly targeted by AP currents. In contrast, PA currents mainly evoke I1-waves (Sakai et al., 1997). Depending on the specific research questions, it might be useful to study SICI with different current directions including the unconventional AP direction. This can provide additional information, e.g., when assessing underlying mechanisms of neurological conditions (Hanajima et al., 2008, 2011). A practical technical note to be mentioned is that when utilizing the Magstim BiStim2 stimulator with a standard D70 alpha flat coil the AP technique is manually more demanding, when the equipment does not provide a switch-option to change the current direction within the coil. Furthermore, the AP condition requires higher stimulation intensities for RMT and 1 mV MEP (Kammer et al., 2001), which could limit its application in specific conditions with increased thresholds, such as in healthy aged populations (Sale et al., 2016) or within neurological disorders such as stroke (McDonnell and Stinear, 2017).

Effect of interstimulus interval

For SICI two different phases, an early at ~ 1 ms and a late at ~ 2.5 ms, have been reported (Fisher et al., 2002). These phases seem to have different thresholds and a differential susceptibility towards voluntary muscle activation. Furthermore, they show a low correlation and are most likely mediated by different inhibitory circuits (Roshan et al., 2003). For a comparable CS intensity as used in our study (80% of RMT) a monophasic waveform with a PA current direction has resulted in comparable levels of inhibition for both phases (Roshan et al., 2003). The present study was able to replicate these previous findings. For the AP current direction, we found a trend for stronger inhibition at an ISI of 3 ms compared with 1 ms. Moreover, we found a trend for more facilitation at the 10 ms ISI compared with the 15 ms for the ICF paradigm in the Magstim PA condition. Both may be explained by different threshold levels of the underlying neuronal circuits.

Effect of waveform

The effects of waveforms on SICI and ICF was recently investigated by Davila-Pérez and colleagues (Davila-Perez et al., 2018). They found less inhibition for a biphasic pulse when compared with a monophasic pulse at a 3 ms ISI in their post-hoc testing, without a significant main effect. We were not able to replicate these results. Small effects size (no significant main effect) and difference in TS adjustment (120% of RMT versus adjusted to ~ 1 mV MEP) may have contributed to the differential findings. Furthermore, Davila-Pérez and colleagues reported significant less facilitation for ICF with monophasic waveform and a PA current direction, when compared with the biphasic waveform. These findings were not apparent in our current data, though measured at a different ISI (12 ms versus 10 or 15 ms). A possible explanation for the similarity of the induced effects for the monophasic PA and biphasic AP-PA condition, in our data, could be the similar pattern of supposedly recruited descending volleys (Di Lazzaro et al., 2001).

No consistent effect of ICF

It is of note, that we could not find significant facilitation for the ICF paradigm when compared to TS for most conditions, except for the conventional Magstim PA condition at a 10 ms ISI. This complements available literature, which reports low reliability for ICF (Boroojerdi et al., 2000; Maeda et al., 2002; Fleming et al., 2012). Discussed underlying biological sources of variability are asynchrony and phase cancellation of descending volleys, inherent changes in cortical excitability (Boroojerdi et al., 2000), and different thresholds for SICI and ICF (Hermsen et al., 2016).

Limitations

We have identified a few limitations of our study. Our adjustment of the TS amplitude tended to be larger than the aimed 1 mV peak-to-peak amplitude (MS PA: 1.46 ± 0.16 mV, MS AP: 1.48 ± 0.17 mV, MV AP-PA: 2.11 ± 0.28 mV, MV PA: 1.76 ± 0.19 mV). However, our amplitudes were well in the comparable range for SICI (Sanger et al., 2001; Garry and Thomson, 2009). The impact for ICF paradigms might be larger, since the effect of ICF seems to decrease at higher TS amplitudes (assessed target amplitude 4 mV) (Sanger et al., 2001). However, the range around 4 mV is much higher than in our study. We cannot exclude that suboptimal TS adjustment may have contributed to the inconstant facilitation we found for ICF. Moreover, the Magstim and MagVenture coils differ in design, e.g., inductance, angle of the surface, overlap of the wire loops, please see Tab. 1. Though, they share comparable values for focality and stimulation depth (Deng et al., 2013) and seem to trigger similar physiological effects (Thielscher and Kammer, 2004). Lastly, we did not use a coil tracking system, which may improve coil positioning (Washabaugh and Krishnan, 2016). Although, for motor-cortex centred SICI assessments hand-held and navigated approaches have shown to result in comparable reliability (Fleming et al., 2012).

In summary, we obtained comparable results for SICI and ICF modulation, when using different stimulators (Magstim BiStim2 and MagVenture MagPro X100) and conventional TMS pulse-configurations. This opens the opportunity to combine data sets sampled with different experimental setups, supports conduction of multi-center trials, and enables between study comparisons towards open science.

References:

References are listed in the common reference list of this thesis.

3. Study 2: *Cerebellar transcranial alternating current stimulation in the gamma range applied during the acquisition of a novel motor skill*

Maximilian J. Wessel[1,2]*, **Laurijn R. Draaisma[1,2]***, Anne F. W. de Boer[1,2], Chang-Hyun Park[1,2], Pablo Maceira-Elvira[1,2], Manon Durand-Ruel[1,2], Philipp J. Koch[1,2], Takuya Morishita[1,2] & Friedhelm C. Hummel[1,2,3]

*Authors contributed equally

[1] Defitech Chair of Clinical Neuroengineering, Center for Neuroprosthetics (CNP) and Brain Mind Institute (BMI), Swiss Federal Institute of Technology (EPFL), Geneva, Switzerland.

[2]Defitech Chair of Clinical Neuroengineering, Clinique Romande de Réadaptation, Center for Neuroprosthetics (CNP) and Brain Mind Institute (BMI), Swiss Federal Institute of Technology (EPFL Valais), Sion, Switzerland.

[3] Clinical Neuroscience, University of Geneva Medical School, Geneva, Switzerland

*Correspondence:
Dr. Maximilian J. Wessel
Defitech Chair of Clinical Neuroengineering
Center for Neuroprosthetics (CNP) and Brain Mind Institute (BMI)
Swiss Federal Institute of Technology (EPFL)
Campus Biotech,
Chemin des Mines 9
1202 Geneva
email: maximilian.wessel@epfl.ch
Tel: +41 21 695 50 90

3.1 Abstract

The development of novel strategies to augment motor training success is of great interest for healthy persons and neurological patients. A promising approach is the combination of training with transcranial electric stimulation. However, limited reproducibility and varying effect sizes make further protocol optimization necessary.

We tested the effects of a novel cerebellar transcranial alternating current stimulation protocol (tACS) on motor skill learning. Furthermore, we studied underlying mechanisms by means of transcranial magnetic stimulation and analysis of fMRI-based resting-state connectivity.

N = 15 young, healthy participants were recruited. 50 Hz tACS was applied to the left cerebellum in a double-blind, sham-controlled, cross-over design concurrently to the acquisition of a novel motor skill. Potential underlying mechanisms were assessed by studying short intracortical inhibition at rest ($SICI_{rest}$) and in the premovement phase ($SICI_{move}$), intracortical facilitation at rest (ICF_{rest}), and seed-based resting-state fMRI-based functional connectivity (FC) in a hypothesis-driven motor learning network.

Active stimulation did not enhance skill acquisition or retention. Minor effects on striato-parietal FC were present. Linear mixed effects modelling identified $SICI_{move}$ modulation and baseline task performance as the most influential determining factors for predicting training success. Accounting for the identified factors may allow to stratify participants for future training-based interventions.

Keywords

Cerebellum; tACS; motor learning; SICI; ICF; MRI

3.2 Introduction

Neurostimulation in combination with behavioural training is a promising strategy for restoring normal function after neuronal damage or for functional enhancement in healthy individuals (Lane, 2013). Currently, numerous interventional studies are implementing this strategy by combining transcranial electric stimulation (tES) with motor training. First evidence for this approach provided promising results, for review please see e.g., work from Wessel and colleagues or Buch and colleagues (Wessel et al., 2015; Buch et al., 2017). Proof-of-principle studies could show that the application of transcranial direct current stimulation (tDCS) to the primary motor cortex (M1) could enhance the learning of a novel motor skill in healthy individuals or chronic stroke patients (Reis et al., 2009; Zimerman et al., 2012). However, several challenges, which limit further translation of this approach remain, such as limited reproducibility, varying and non-satisfactory effect sizes, and a lack of mechanistic understanding (Buch et al., 2017).

To partially address these challenges, we strived to validate potential effects of a novel cerebellar transcranial alternating current (CB-tACS) protocol (Naro et al., 2016) on the acquisition of a novel motor skill in healthy individuals. We based our research approach on three main assumptions. Firstly, the cerebellum is considered as a core node of the motor learning network (Doyon and Benali, 2005; Dayan and Cohen, 2011) and it has a high potential to undergo neuroplastic changes (Marr, 1969; Ito, 2002). These plastic changes can be modulated by tES techniques (Galea et al., 2009; Zuchowski et al., 2014; Wessel et al., 2016). Secondly, tACS, when compared with steady-state stimulation protocols such as tDCS, offers the benefit of exerting its effects more selectively on targeted neuronal elements, which have an "Eigenfrequency" close to the stimulation frequency (Antal and Herrmann, 2016; Naro et al., 2017). Furthermore, oscillatory activity in cerebellar cortex has been linked to various aspects of neuronal processing (De Zeeuw et al., 2008). We specifically chose a 50 Hz stimulation frequency, based on the work from Naro and colleagues, who have demonstrated facilitatory effects on different aspects of motor function (Naro et al., 2016, 2017), namely repetitive finger opposition movements or some items of the Wolf Motor Function Test (WMFT) such as turning a key in a lock. It is of note however, that the field of cerebellar tACS is just evolving and more research and validation also studying other stimulation frequencies is needed. Thirdly, we investigated potential underlying mechanisms by studying intracortical interactions in M1 with double-pulse transcranial magnetic stimulation (dpTMS) to determine intracortical inhibitory and facilitatory neurotransmission. Specifically, we chose to investigate short intracortical inhibition (SICI) and intracortical facilitation (ICF) allowing us to evaluate markers of GABAergic and glutamatergic neurotransmission (Chen, 2004), which have been linked to memory formation and motor learning (Rioult-Pedotti et al., 1998; Trepel and Racine, 2000; Reis et al., 2008). Furthermore, SICI and ICF interact with the cerebello-cortical output drive (Daskalakis et al., 2004), which has shown to be responsive to modulation via 50 Hz tACS (Naro et al., 2016). We assessed seed-based functional connectivity (FC)(van den Heuvel and Hulshoff Pol, 2010) in a hypothesis driven motor learning network (Dayan and Cohen, 2011) with resting-state functional magnetic resonance imaging (rsfMRI), allowing us to extend our research perspective towards also studying effects on larger scale brain network interactions. We strived to combine this multi-modal data to create predictive models for the responsiveness towards our intervention or training, which may allow us to stratify participants in responders and non-responders in future studies and hereby reduce variability and stabilize effect sizes.

In the present study, we hypothesized that: (i) active cerebellar tACS applied concurrently to the training phase enhances the acquisition of the novel motor skill, and (ii) a combined predictive model including multi-modal parameters from behavioural motor tests, dpTMS, and rsfMRI assessments will allow to predict the learning success.

3.3 Methods

Participants

We recruited N = 15 young, healthy, right-handed participants for the study applying a cross-over design (N = 7 female, mean age ± s.d.: 26.20 ± 3.34, mean laterality quotient Edinburgh handedness inventory 89.47 (Oldfield, 1971)). 24 data sets were considered for the analysis including behavioural measures, the remaining data sets were excluded due to application of a preliminary version of the motor learning task (N = 5) and technical difficulties during recording (N = 1). Our inclusion criteria were: ≥ 18 and < 35 years, right-handedness, normal values of Mini-mental state examination (>26/30), absence of contraindication for transcranial electric stimulation (tES), transcranial magnetic stimulation (TMS), or magnetic resonance imaging (MRI). Our exclusion criteria were: presence of neuropsychiatric diseases, history of seizures, intake medication that potentially interacts with tES or TMS, musculoskeletal dysfunction that compromise finger movement, pregnancy, professional musician or intense professional usage of a computer keyboard, intake of narcotic drugs, request of not being informed in case of incidental findings. The study was carried out in accordance to the Declaration of Helsinki (World Medical Association, 2013). Written informed consent was obtained from all participants. Approval was obtained from the cantonal ethics committee Vaud, Switzerland (project number 2017-00765).

Experimental design

The study followed a double-blind, sham-controlled, cross-over design. At session T0 participants were screened, filled in baseline questionnaires, and were characterized with a set of behavioural tests, including pinch-, key-, grip-force assessments, the nine-hole peg test (9HPT), and the box and block test (BBT). At session T1, we acquired a baseline MRI scan. Session T2 consisted of the motor training with concurrent tACS. The training session was embedded in dpTMS assessments (preT2 and postT2). Task retention was assessed 1 day and circa 10 days after the training phase (T3 and T4). Additionally, at T3 a follow-up MRI assessment (preT3) and at T4 a follow-up dpTMS assessment (postT4) were conducted. The minimum time between T4 before cross-over and T1 after cross-over was set to two weeks. See also Fig. 6A.

Motor learning task

As a motor skill learning task, we used a computerized sequential grip force modulation task (SGFMT) adapted from Reis and colleagues (Reis et al., 2009), implemented in MATLAB (The MathWorks, Inc., Natick, MA, USA), see Fig. 6B. The grip forces were sampled with a fibre optic grip force sensor (Current designs, Inc., Philadelphia, PA, USA). The participants had to control an onscreen cursor via the modulation of grip forces using their non-dominant, left hand. The cursor moved upwards in vertical direction with increasing forces. The participants were instructed to navigate the cursor between a home zone (H) and 5 target zones, which were scaled to individual maximum force. A rightward pointing arrow (bookmark) indicated which target had to be reached next. The instruction was to perform the task as accurately and as quickly as possible. In the training session (T2), the participants were firstly familiarized to the task (simplified task version, only 3 target zones). Following the familiarization, they conducted a 90 s baseline without tACS. The actual training with concurrent tACS lasted circa 20 min and consisted of 9 blocks. The follow-up sessions consisted of 3 blocks. The blocks lasted 90 s and were separated by breaks. The targets followed the sequential order A or B counterbalanced between the active and sham stimulation condition, see also Supplementary Tab. S3. However, in Block 5 the targets were arranged in a pseudorandom, untrained order, to assess for effects on simple motor performance.

Cerebellar tACS

tACS was applied to the left cerebellum utilizing a DC-stimulator plus (neuroConn GmbH, Ilmenau, Germany). The stimulation protocol was adapted from Naro and colleagues (Naro et al., 2016) and was defined by following parameters: sinusoidal waveform, intensity 2 mA (peak-to-peak), fade-in/out interval 2 s (100 x 2π cycles), duration (i) active 20 min (60000 x 2π cycles) (ii) sham 30 s (cycles: 1500 x 2π), 5 x 5 cm rectangular sponge-covered conductive rubber electrodes soaked in saline solution. The active electrode was placed 3 cm lateral to the inion and the return electrode over the ipsilateral buccinator muscle (Galea et al., 2009), please see Fig. 6C.

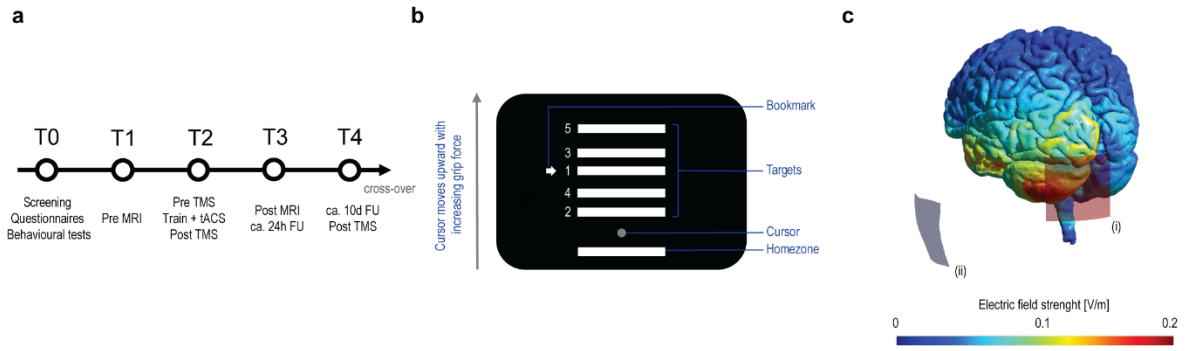

Figure 6. *Experimental setup. (a) Timeline: tACS was applied in a double-blind, sham-controlled cross-over design concurrently to the training (Train) of a novel motor skill (T2), skill retention was assessed at a circa 24h and circa 10d follow-up (T3, T4), dpTMS and MRI assessments were incorporated in the experimental protocol (T1, T2, T3, T4). (b) Sequential grip force modulation task (SGFMT): participants had to navigate a cursor by modulating their applied grip force between a home zone and five target zones following a sequential order. (c) Computer simulation of the applied stimulation protocol implemented in SimNIBS* (Thielscher et al., 2015)*, the active electrode was placed 3 cm lateral to the inion over the left cerebellar hemisphere (i) and the return electrode over the ipsilateral buccinator muscle (ii)* (Galea et al., 2009)*, depicted is the electric field strength (norm E) at π/2 phase.*

Double-pulse transcranial magnetic stimulation (dpTMS)

DpTMS was used to study GABAergic and glutamatergic circuits in the motor cortex (Chen, 2004) at baseline and their modulation after stimulation and during the time course of learning. We assessed short intracortical inhibition at rest ($SICI_{rest}$), during movement preparation ($SICI_{move}$) and intracortical facilitation at rest (ICF_{rest})(Kujirai et al., 1993; Hummel et al., 2009). Methods are described in detail in our prior published work (Hummel et al., 2009; Wessel et al., 2019). In brief, monophasic TMS pulses inducing a posterior to anterior current direction in the underlying brain tissue were applied using a MagPro X100 stimulator connected to a MC-B70 coil (MagVenture, Farum, Denmark). The coil was placed over the hotspot of the first dorsal interosseous muscle (FDI) with its handle pointing backwards circa 45° to the midsagittal line. The coil positioning was guided throughout the experiment with the support of a neuronavigation system (Localite, Bonn, Germany). The conditioning pulses (CP) were

adjusted to 80% of resting-motor threshold (RMT), defined as the lowest stimulus intensity that produced a motor-evoked potential (MEP) with a peak-to-peak amplitude ≥ 50 μV in 5 out of 10 consecutive trials (Groppa et al., 2012). The test pulse (TP) was adjusted to elicit a MEP of circa 1 mV in the relaxed FDI. Stimulator output intensities for TP and CP were readjusted before each session to measure SICI/ICF magnitude modulation in a comparable part of the respective recruitment curves (Sanger et al., 2001; Garry and Thomson, 2009), with the aim to control for the confound of potential changes in cortico-spinal excitability. SICI and ICF were assessed with the following parameters and tested together with test pulse only trials following a pseudorandom order: inter-stimulus interval (i) SICI 3 ms (ii) ICF 10 ms, number of trails per condition (i) rest 18 (ii) $SICI_{move}$ 24, inter-trial-interval (i) rest 7 s ± 25% (ii) $SICI_{move}$ 5.5, 6.5, 7.5, 8.5 s. During the $SICI_{move}$ assessments, the participants performed a simple reaction time task (RT)(Hummel et al., 2009), which was implemented in Presentation software (Neurobehavioral Systems, Inc., Berkeley, CA, USA). The participants had to perform left index finger abductions as fast as possible after visual cue (circle). During the inter-trial-interval they we instructed to fixate a fixation cross with their eyes. The TMS pulses were applied at 20 and 90% of individual median RT. The electromyography (EMG) data as sampled with a Noraxon DTS Receiver (Scottsdale, AZ, United States) with the following settings: gain 500, sampling rate 3000 Hz, high-pass filter 10 Hz analog Sallen-Key, low-pass filter 1000 Hz digital FIR 128th order Butterworth. The signal was transferred for further processing and saved on a laptop via Signal software (Cambridge Electronic Design, Ltd., Cambridge, UK).

Magnetic resonance imaging (MRI)

The MRI data were obtained with a Prisma 3T scanner (Siemens Healthcare AG, Erlangen, Germany). Structural T1-weighted images were obtained with following parameters: 208 slices, voxel size 1x1x1 mm, 8° flip angle, repetition time (TR) 2300 ms, echo time (TE) 2.26 ms, 256 mm field of view. Whole brain echo-planar images (EPI) were acquired at resting-state with following acquisition parameters: duration 7:11 min, 66 slices, 420 volumes, voxel size 2x2x2 mm, 50° flip angle, TR 1000 ms, TE 32 ms, 225 mm field of view. During the acquisition of the EPI the participants were instructed to keep their eyes open and fixate a fixation cross.

Data processing

The behavioural motor learning data were processed with an in-house script implemented in MATLAB. Our a priori primary outcome was defined as the area under the curve (AUC) of the movement trajectory for correctly performed sequences. For further processing the motor learning data was averaged per block.

The MEP data were visually inspected and processed by using an in-house MATLAB-based graphical user interface, which automatically documented trial rejections and processing steps, for trial rejection criteria please see Supplementary Information. The peak-to-peak MEP amplitude was measured in a response window of TMS pulse + 20 ms to + 50 ms and was averaged per condition. DpTMS conditions were contrasted to the corresponding TS only condition and expressed as mean (conditioned MEPs) / mean (unconditioned MEPs) ∗ 100.

The pre-processing of the rsfMRI data and the 1st level analysis was done using CONN toolbox (Whitfield-Gabrieli and Nieto-Castanon, 2012). We calculated seed-based connectivity (SBC) by using each area in a hypothesis driven motor learning network (Dayan and Cohen, 2011) as a seed, for further details see Supplementary Tab. S4.

Statistical analysis

Statistical significance was assumed at p-values < .05. Normality of the data were confirmed by assessing their skewness, which range was in-between 1 and -1 (Gravetter and Wallnau, 2014). The statistical analysis of the behavioural data, TMS data, and for possible biomarkers of training success

was implemented in RStudio (version 1.1.456, 2018). Linear Mixed-Effects Models were fitted using the lme4 package (Bates et al., 2015). Statistical testing was done with use of the likelihood ratio test (Winter, 2013). To further examine potential null results of main behavioural outcomes as suggested by the frequentist analysis, we added in addition a Bayesian approach by computing Bayesian ANOVAs in JASP software (Version 0.9.1, 2018)(JASP Team, 2018). For the predictive model the goodness of fit was determined by the Akaike information criterion (AIC). The statistical analysis of the SBC data was done by calculating RM-ANOVAs and was implemented in SPM12 (Friston, 2007), statistical significance was determined at a cluster-level threshold of FDR-corrected p-value < .05 with a voxel-level threshold of uncorrected p-value < .001. Post-hoc analysis for the modulation of functional connectivity was done with paired t-tests, Bonferroni corrected. Effect sizes for the linear mixed-effect models were expressed as Cohen's f^2 (≥ 0.02 small, ≥ 0.15 medium, ≥ 0.35 large) based on the approach of Selya and colleagues (Selya et al., 2012) and as Cohen's *d* (≥ 0.2 small, ≥ 0.5 medium, ≥ 0.8 large) for the t-tests. The values in figures are shown as mean ± standard error of the mean (s.e.m.).

3.4 Results

tACS-associated sensations and effectiveness of blinding

All participants tolerated the tACS application well, there were no adverse effects. Minor stimulation-associated sensations were present, see Supplementary Tab. S1. The participants' ability to distinguish active from sham stimulation was not significantly different from chance level. Please see also Supplementary Information and Supplementary Tab. S1.

Behavioural data

The analysis of the training phase revealed a significant effect of BLOCK $\chi(7) = 36.65$, $p < .001$, $f^2 = .071$, but not of STIMULATION $\chi(1) = 3.14$, $p = .076$, $f^2 = .008$, or BLOCK x STIMULATION interaction $\chi(7) = 0.76$, $p = .998$, $f^2 = .001$, indicating that our learning task - the sequential grip force modulation task (SGFMT) - served as a valid learning model. The potential null result for STIMULATION suggested by the frequentist analysis was further examined by additionally conducting a Bayesian-based analysis. The Bayesian repeated measures ANOVA indicated a Bayes factor for STIMULATION of $BF_{01} = 5.85$, meaning that the data was 5.85 times more likely to occur under the null-hypothesis than the alternative hypothesis. This is considered a moderate effect. The Bayes factor for BLOCK was $BF_{01} = 1.95$, which corresponds to small effect. Additionally, the Bayes factor for INTERACTION between BLOCK and STIMULATION was $BF_{01} = 649.59$, meaning that was 649.59 times more likely that the null-hypothesis is true. This is considered a strong effect. The combined statistical approaches indicate that no clear stimulation associated effects were present during the training phase, see Fig. 1a. The pseudorandom block (B5) did not significantly differ from the neighbouring blocks, B4 vs. B5 $t(23) = 1.28$, $p = .214$, $d = 0.26$, B5 vs. B6 $t(23) = 1.10$, $p = .283$, $d = 0.22$, implying that also sequence-independent learning was present.

In order to avoid carry-over effects there was a wash-out period between the before and after cross-over sessions of M: 50.8 (SD: 32.43) days with a range between 25 and 103 days. The comparison of baseline performance before and after cross-over did not indicate major carry-over effects $t(21.28) = 0.22$, $p = .826$, $d = 0.09$.

Retention at T3 and T4 were analysed as compound measures (RETENTION) using the average of the three blocks per session divided by the last training block (B9). The analysis of the retention phase indicated a significant effect for STIMULATION $\chi(1) = 6.50$, $p = .011$, $f^2 = .084$, but not of RETENTION $\chi(1) = 0.26$, $p = .609$, $f^2 = .003$, or RETENTION x STIMULATION interaction $\chi(1) = 0.71$, $p = .399$, $f^2 = .007$ for the whole group analysis, see Fig. 1b. However, the significant STIMULATION effect did not persist after performing an influential point analysis (leave-one-out approach) – STIMULATION $\chi(1) = 2.72$, $p = .099$, $f^2 = .044$. With the same reasoning as for the training data, we performed a Bayesian ANOVA on the retention data to investigate the potential null result in more depth. The result showed a Bayes factor for STIMULATION of $BF_{01} = 1.73$, meaning that it was 1.73 times more likely that the null-hypothesis (no stimulation effect) is true. This is considered a small effect, which indicates that the analysis does not provide substantial evidence for the null hypothesis (no effect of stimulation on retention) considering the commonly accepted threshold of $BF_{01} > 3$(Jeffreys, 1998). However, under the assumption of minor, slight stimulation-associated effects on retention these were rather in the direction of task disturbance. Moreover, the Bayes factor for RETENTION was $BF_{01} = 3.34$, showing that it was 3.34 times more likely that the null hypothesis (no effect of time) was true, this is considered a moderate effect. The interaction between STIMULATION x RETENTION resulted in a Bayes factor of $BF_{01} = 14.83$, rendering it 14.83 times more likely that the null-hypothesis was true over the alternative hypothesis. In conclusion, we observed a slight indication for disturbance of skill retention by 50 Hz CB-tACS, which however could not be confirmed after correcting for an influential data point.

Next, we proceeded by analysing the magnitude of the skill reduction at the follow-up assessments by contrasting their session average to the last training block (B9). Active and sham groups showed a significant loss when compared with B9 $\chi(2) = 6.38$, $p = .041$, $f^2 = .058$. There was no significant effect of STIMULATION $\chi(1) = 1.23$, $p = .289$, $f^2 = .013$ or SESSION x STIMULATION interaction $\chi(2) = 1.46$, $p = .481$, $f^2 = .012$, see Fig. 1c.

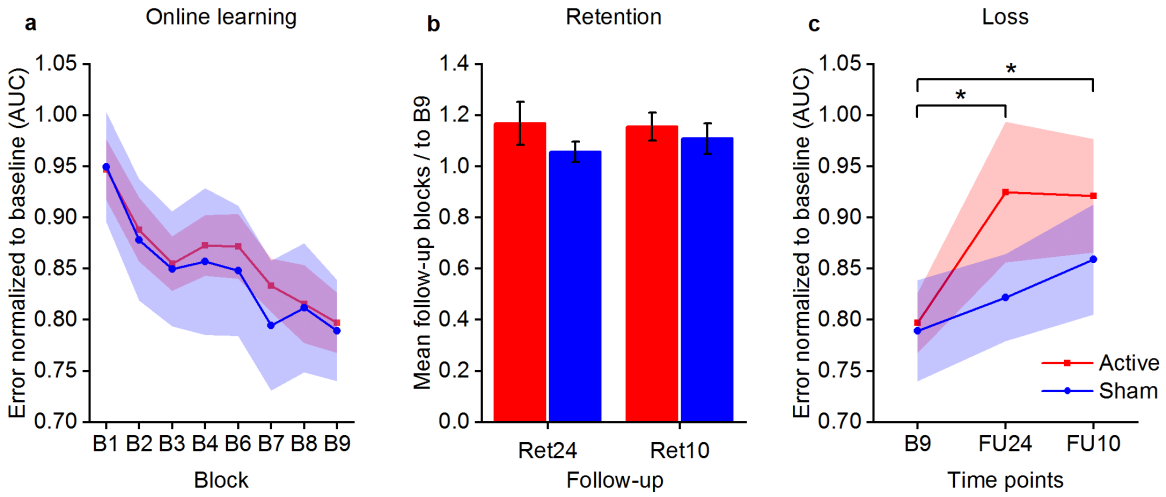

Figure 1. *Behavioural data. (a) Training performance during the motor task normalized to baseline, quantified as area under the curve (AUC) of the movement trajectory of correctly performed sequences. Block B5 (not shown) was used as a performance probe, applying a pseudorandom, untrained sequence. (b) Retention measured circa 24h (Ret24) and circa 10 days (Ret10) after training. Retention was calculation by contrasting the average the follow-up blocks to the last training block (B9). (c) Figure depicts performance during the two follow-up sessions (FU24 and FU10) compared and the last block of training (B9). Follow-up performance was averaged per session. Margin of errors are depicted as standard error of the mean (s.e.m.).*

Short intracortical inhibition ($SICI_{rest}$) and intracortical facilitation (ICF_{rest}) at rest

Test pulse (TP) peak-to-peak amplitudes at rest and required stimulator output (MSO) were not significantly different between stimulation conditions or assessment time points, confirming a stable TP adjustment throughout the course of the experiment, for details please see Tab. 1. The analysis of the $SICI_{rest}$ data revealed no effect of STIMULATION $\chi(1) = 0.01$, $p = .916$, $f^2 = <.001$, SESSION $\chi(2) = 2.05$, $p = .359$, $f^2 = .013$ or STIMULATION x SESSION interaction $\chi(2) = 2.30$, $p = .316$, $f^2 = .014$, see Fig. 2a. To achieve homogeneity of variance the ICF_{rest} data were log-transformed. The analysis of ICF_{rest} indicated no effect of STIMULATION $\chi(1) = 1.30$, $p = .254$, $f^2 = .007$, for SESSION $\chi(2) = 0.31$, $p = .857$, $f^2 = .002$ or for STIMULATION x SESSION interaction $\chi(2) = 0.62$, $p = .732$, $f^2 = .003$, see Fig. 2b. In summary, our dataset failed to reveal any significant differences in the modulation of intracortical interactions in M1 at rest by phase of learning or stimulation type.

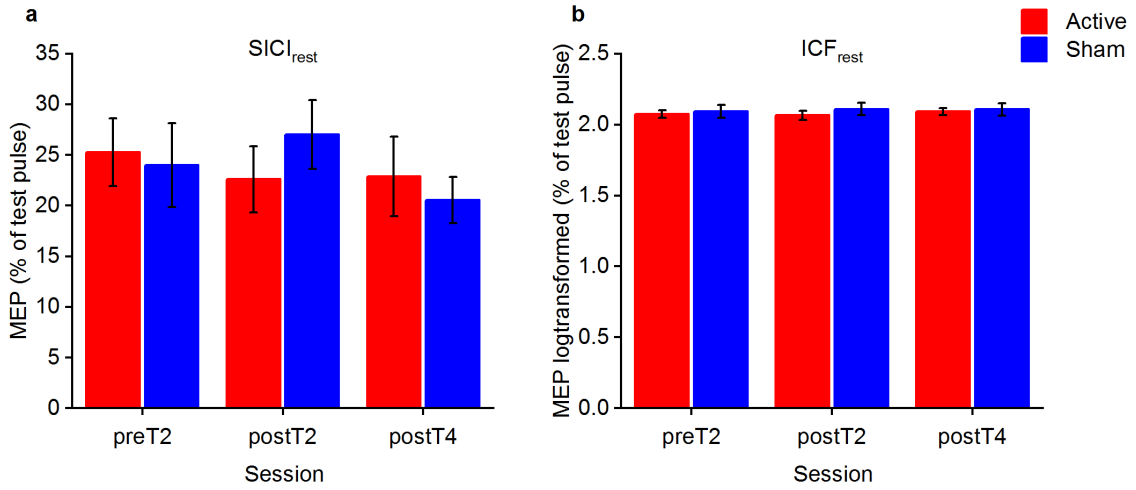

Figure 2. *$SICI_{rest}$ and ICF_{rest}. (a) SICI measured with dpTMS during rest. MEP is related to average test pulse. (b) ICF measured during rest. Data was log-transformed to achieve homogeneity of variance. MEP is related to average test pulse. Error bars are depicted as standard error of the mean (s.e.m.).*

Table 1

Stimulation	**PreT2**	**PostT2**	**PostT4**	**Statistics**
Peak-to-peak amplitudes of TP_{only} MEPs (mV)				
Active	1.44 (0.16)	1.46 (0.17)	1.46 (0.15)	STIMULATION $\chi(1)$ = 0.40, p =.529; f^2 = .002; SESSION $\chi(2)$ = 2.71, p =.258, f^2 = .015; STIMULATION x SESSION interaction $\chi(2)$ =0.89, p =.640, f^2 = .005
Sham	1.24 (0.13)	1.47 (0.12)	1.47 (0.16)	
Required % of MSO to reach ~ 1mV adjusted MEPs (% of MSO)				
Active	56.07 (4.27)	56.60 (3.94)	58.40 (4.09)	STIMULATION $\chi(1)$ = 0.94, p =.332, f^2 = 0.001; SESSION $\chi(2)$ = 2.63, p =.268, f^2 = .002; STIMULATION x SESSION interaction $\chi(2)$ =0.39, p =.823, f^2 < .001
Sham	55.80 (3.71)	56.33 (3.61)	56.93 (3.66)	

Table 1. *Averages of peak-to-peak amplitudes of single pulse MEP's at resting state, measured in millivolts (mV) and the percentage (%) of maximum machine output intensity (MSO) that was used to induce the single pulse MEPs, s.e.m. are depicted between brackets.*

Short intracortical inhibition during movement preparation ($SICI_{move}$)

The analysis of the event-related $SICI_{move}$ assessment indicated a significant effect of TIMING $\chi(1) = 48.08$, $p < .001$, $f^2 = .174$, confirming prior research (Hummel et al., 2009; Heise et al., 2013), which suggests disinhibitory dynamics of SICI towards movement onset. Subsequently, we analysed the modulation dynamics of $SICI_{move}$. These were quantified by computing the difference of $SICI_{move}$ assessed in late (90% of reaction time (RT)) and early premovement phase (20% of RT). We found a trend for effect of SESSION $\chi(2) = 5.37$, $p = .068$, $f^2 = .013$. There was not a significant effect of STIMULATION $\chi(1) = 1.23$, $p = .267$, $f^2 = .057$ or SESSION x STIMULATION interaction $\chi(2) = 2.37$, $p = .305$, $f^2 = .024$, see Fig. 3.

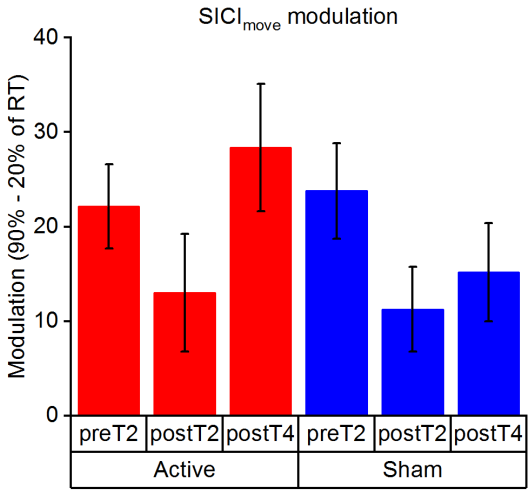

Figure 3. *Modulation of SICImove, quantified by computing the delta between 90% and 20% of RT, during the time course of the protocol. Error bars are depicted as standard error of the mean (s.e.m.).*

$SICI_{move}$ modulation and training performance

To further investigate underlying mechanisms, we assessed potential associations between $SICI_{move}$ modulation and training success. For preT2 the results showed that there was a significant effect of MODULATION $\chi(1) = 5.73$, $p = .017$, $f^2 = .296$ showing that less modulation was associated with larger training success. There was no effect of STIMULATION $\chi(1) = 1.38$, $p = .241$, $f^2 = .064$ or MODULATION x STIMULATION interaction $\chi(1) = 0.87$, $p = .351$, $f^2 = .040$. During postT2 there was no significant MODULATION $\chi(1) = 0.15$, $p = .699$, $f^2 = .007$, STIMULATION $\chi(1) = 0.89$, $p = .345$, $f^2 = .040$ or MODULATION x STIMULATION interaction $\chi(1) = 0.02$, $p = .897$, $f^2 < .001$. At postT4 there was no significant effect of MODULATION $\chi(1) = 0.82$, $p = .364$, $f^2 = .036$, STIMULATION $\chi(1) = 0.36$, $p = .547$, $f^2 = .016$ or MODULATION x STIMULATION interaction $\chi(1) = 1.31$, $p = .252$, $f^2 = .058$. Please see Fig. 4.

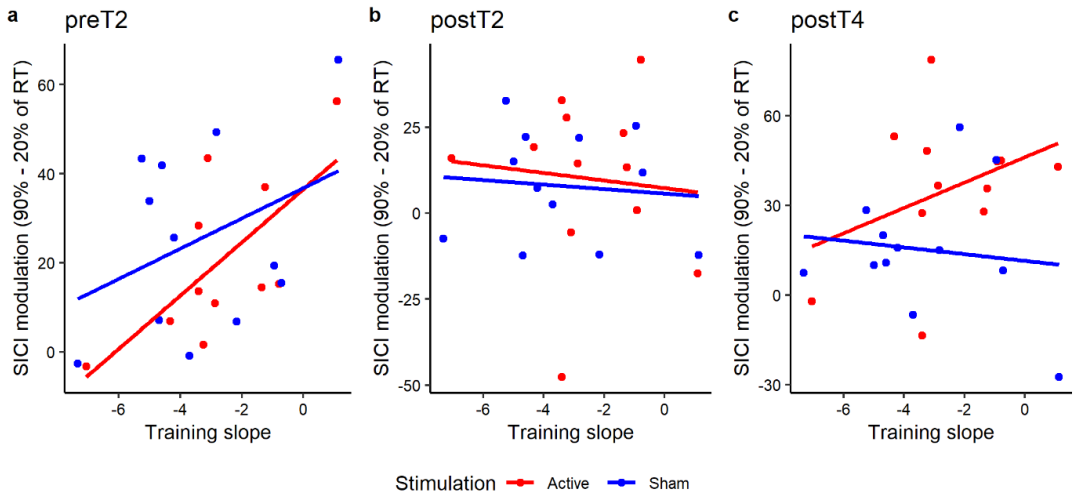

Figure 4. *Association between SICImove modulation and online learning, quantified by the linear slope fitted though the AUC data of the training blocks, for the protocol time points – (a) pre-training (preT2), (b) post training (postT2), (c) post follow-up 2 (postT4). Lower values for training slope depict better learning. SICImove modulation is quantified by computing the delta between 90% and 20% of RT.*

Rs-fMRI-based connectivity

The analysis of seed based functional connectivity (SBC) revealed a significant TIME x STIMULATION interaction for a cluster of 226 voxels located in the right caudate with the seed in posterior parietal region (PPC), please see Fig. 5. Post-hoc analysis suggested a significant reduction of SBC in the active group compared to the sham group in the 1st follow-up session (T3), see Tab. 2. This reduction of SBC in the active group in T3 was also significantly different from baseline (T1). Taking also into account the influential point analysis, our dataset failed to reveal clear associations between the modulation of SBC between T1 and T3 and behavioural output (online learning, 24h and 10d retention), please see Supplementary Tab. S2. For all other seeds, we did not find a significant TIME x STIMULATION interaction.

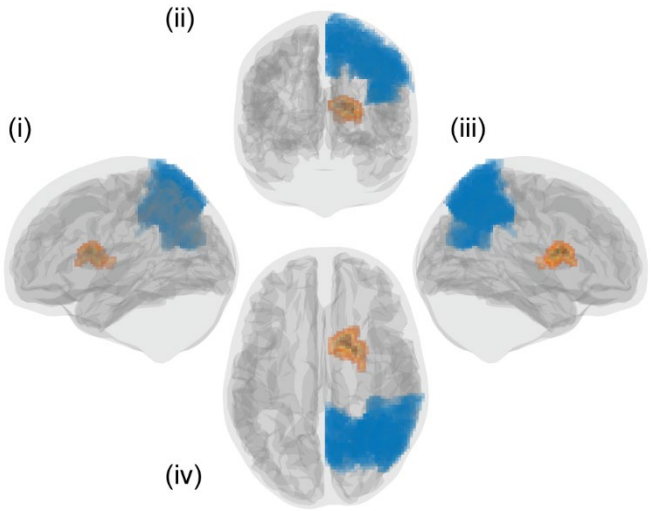

Figure 5. *Effect on striato-parietal FC. Depicted is a cluster of voxels located in the right caudate (orange), which showed a significant TIME x STIMULATION interaction with a seed located in the parietal region (blue). (i) left, (ii) posterior, (iii) right, and (iv) superior view.*

Table 2

Comparison	T (df)	*p*-value, uncorrected	*p*-value, Bonferroni-corrected	Cohen's *d*
Pre_active vs. pre_sham	1.28 (14)	.222	0.89	0.33
Post_active vs. post_sham	-6.97 (14)	<.001	<.001	1.80
Pre_active vs. post_active	6.22 (14)	<.001	<.001	1.61
Pre_sham vs. post_sham	-2.16 (14)	.048	.192	0.56

Table 2. *Modulation of striato-parietal FC. Post-hoc testing for the modulation of seed-based FC for the significant cluster of voxels located in the right caudate and the PPC seed.*

Predicting training success. In order to study the predictive potential of behavioural, dpTMS, and rsfMRI measures for the dependent variable training success, we computed a linear mixed effects model. Training success was quantified by fitting a linear slope through the AUC data of the training phase. At first, we included the following fixed effects in the model: (i) BASELINE – performance in the SGFMT at the baseline block, (ii) MOTOR - compound motor score of the applied behavioural tests (pinch-, key-, grip force test, nine-hole peg test (9HPT), and the box and block test (BBT)) defined by the first two components extracted by principal component analysis, (iii) $SICI_{rest}$ at preT2, (iv) ICF_{rest} at preT2, (v) ΔSICImove MODULATION - defined as the difference in $SICI_{move}$ modulation between 90% and 20% of RT at preT2, and (vi) mean z-transformed correlation coefficient between the significant cluster in the right caudate and the PPC seed at T1. Participants were included as random effects to account for the repeated measures. The best model was selected based on the Akaike information criterion (AIC). The final model was able to explain 47.68% (R^2) of the variance in training success. It included the factors BASELINE, $SICI_{rest}$, ICF_{rest} and $\Delta SICI_{move}$ MODULATION. BASELINE ($F(1,23) = 6.85$, $p = .015$) had a significant influence on training performance, with every unit increase in baseline AUC the linear slope became more negative by - 0.03, indicating more learning. ΔSICImove MODULATION had a significant influence on training performance ($F(1,23) = 7.90$, $p = .010$), with every unit increase in modulation the linear slope became more positive by + 0.05, indicating less learning. Please see also Tab. 3.

Table 3

	Model statistics				**Confidence interval (95%)**		**ANOVA statistics**	
	Variance (SD)	Para-meters estimate (SE)	T	*p*-value	Lower	Upper	F (df)	*p*-value
Random effects								
Participants	0.00 (0.00)							
Residuals	2.52 (1.59)							
Fixed effects								
Intercept		0.78 (1.98)	0.39	0.70	-3.26	4.82		
BASELINE		-0.03 (0.01)	-2.62	.015 *	-0.05	-0.01	6.85 (1,23)	.015*
$SICI_{rest}$		-0.04 (0.03)	-1.52	.143	-0.09	0.01	2.31 (1,23)	.143
ICF_{rest}		0.00 (0.01)	0.10	.921	-0.02	0.02	0.01 (1,23)	.921
$\Delta SICI_{move}$ MODULATION		0.05 (0.02)	2.81	.010 *	0.01	0.08	7.90 (1,23)	.010*

Table 3. *Results of final predictive model for training success. Linear mixed effects model analysis was conducted to identify the most influential factors for predicting training success. The full model included following fixed factors: (i) BASELINE – SGFMT performance in the baseline block, (ii) MOTOR - compound motor score of the applied behavioural tests (pinch-, key-, gripforce test, 9HPT and BBT) defined by the first two components extracted by principal component analysis, (iii)* $SICI_{rest}$ *at preT2, (iv)* ICF_{rest} *at preT2, (v)* $\Delta SICI_{move}$ *MODULATION - defined as the difference in SICImove modulation between 90% and 20% of RT at preT2, and (vi) mean z-transformed correlation coefficient between the significant cluster in the right caudate and the PPC seed at T1. The final model was selected based on the AIC criterion and identified* $\Delta SICI_{move}$ *MODULATION and BASELINE as the most influential factors.*

3.5 Discussion

The main finding of the study was that 50 Hz tACS applied to the ipsilateral cerebellum did not enhance the acquisition or retention of a novel motor skill based on sequential grip force control. This contrasts earlier research, which has reported beneficial effects of a similar stimulation protocol on different aspects of motor function, specifically the adaptation to frequency variations during finger tapping or to some aspects of the WFMT (Naro et al., 2016, 2017). Several factors may explain our null results. When implementing the present learning task, we formulated two core requirements. It should have considerable similarity to activities of daily living, such as grasping of objects, and should have a translational potential for future studies recruiting patients with motor constraints. The SGFMT fulfilled these criteria. However, the task is likely less specific to cerebellar resources as classical cerebellum-dependent tasks, such as motor adaptation or finger-tapping-based paradigms (Penhune and Doyon, 2002; Shadmehr et al., 2010). In fact, grip force execution and their modulation rely additionally to the sensorimotor cortex and the cerebellum on frontal, prefrontal, supplemental motor, cingulate motor, and parietal areas (Ehrsson et al., 2001; Ward et al., 2008). This potential neuronal non-specificity may have prevented us from detecting constricted beneficial effects.

It is possible that the chosen stimulation frequency was suboptimal to facilitate the underlying neuronal processing of the task. It is assumed that Purkinje cells (PC) are a core responsive element for tES (Galea et al., 2009; Naro et al., 2016) and that they are crucially involved in motor learning (Ito, 2000; Nguyen-Vu et al., 2013). Indeed, PCs show oscillatory dynamics in the gamma range, however 50 Hz oscillations might not be the most relevant component. For instance, the peak of the frequency distribution of PCs simple spikes during upper limb movements is rather located at higher frequencies ranges (circa 100 Hz) (Thach, 1968). Furthermore, a considerable proportion of PCs shows an intrinsic trimodal firing pattern, which is characterized by an monotonical increase of the firing rate from the beginning of the monotonic towards the start of the burst period (circa 40 Hz to circa 100 Hz) (Womack and Khodakhah, 2002). In fact, a set of recent cerebellar tACS studies tested higher frequency ranges and provided evidence that 70 Hz cerebello-motorcortical tACS can induce beneficial effects, when studying an isometric force modulation task (Miyaguchi et al., 2018, 2019). We found rather an indication for disturbing effects of the 50 Hz stimulation frequency, when assessing task retention (whole group level analysis). This combined evidence points towards the direction that 50 Hz cerebellar tACS protocols may be suboptimal for boosting performance in motor tasks relying on sequential modulation of grip forces. Future research should address potential effects of task specificity and different stimulation frequency ranges in greater detail. Furthermore, the stimulation site slightly differed from prior work from Naro and colleagues (Naro et al., 2016, 2017) – 3 cm lateral to the inion over the left cerebellar hemisphere versus 1-2 cm below and 3-4 cm lateral to the inion over the right cerebellar hemisphere. When designing the study protocol, we modified the electrode montage according to the Celnik et al. configuration (Galea et al., 2009) as it had shown efficiency in a similar motor task applying consecutive cerebellar tDCS (Cantarero et al., 2015). Subsequently, our chosen montage may have influenced the magnitude of potential behavioural effects. However, when considering the focality range of conventional cerebellar tES protocols (Rampersad et al., 2014), we consider this effect rather as negligible, see also Fig. 6C.

An additional point to consider is the site of the cerebellar hemisphere, which was stimulated (right versus left). We chose to train the left hand and stimulate the left cerebellar hemisphere to allow more room for improvement in right-hand dominant participants. It is important to note, that the majority of tES protocols targeted the right cerebellar hemisphere so far – e.g., the majority of studies identified in the recent cerebellar tDCS meta-analysis from Oldrati and colleagues (Oldrati and Schutter, 2018) stimulated the right cerebellar hemisphere. However, some authors have suggested that motor learning

functions are lateralized towards the left cerebellar hemisphere (van Mier et al., 1998; Hu et al., 2008), which was targeted in our study. Certainly, more research is needed to identify potential hemispheric differences for responsiveness towards cerebellar neuromodulation protocols.

Furthermore, we studied potential underlying mechanisms mediated by GABAergic and glutamatergic motor-cortical circuits by assessing $SICI_{rest}$ and ICF_{rest}. The effect of motor training protocols on $SICI_{rest}$ and ICF_{rest} is discussed controversially. For $SICI_{rest}$, a reduction (Garry et al., 2004; Rosenkranz et al., 2007) or no changes (Cirillo et al., 2010; Zimerman et al., 2015) after motor training have been reported. Influential factors might be the nature of the applied training task, the utilized hand (dominant vs. non-dominant) (Garry et al., 2004), or the phase of learning (Rosenkranz et al., 2007). We were not able to detect any learning or stimulation associated effects on $SICI_{rest}$. This absence of effects for $SICI_{rest}$ ($GABA_A$ surrogate (Chen, 2004)) extends prior work from Naro and colleagues, who found no effects of the applied protocol on the long-interval intracortical inhibition ($GABA_B$ surrogate (Chen, 2004))(Naro et al., 2016). For $ICF_{rest,}$ the available data on motor training-related effects is more sparse, mainly no clear effects have been reported (Liepert et al., 1998; Perez et al., 2004; Berghuis et al., 2015). This is in agreement with our study, in which ICF_{rest} was not significantly modulated by training or stimulation.
We were able to show disinhibitory dynamics of $SICI_{move}$ towards movement onset confirming prior research (Hummel et al., 2009; Heise et al., 2013; Dupont-Hadwen et al., 2019). Our analysis suggested that these disinhibitory dynamics might be modulated by the phase of training, pointing towards a reduced modulation of $SICI_{move}$ in the immediate post training phase. Contrary to our finding, Dupont-Hadwen and colleagues did not detect learning phase-dependent effects on $SICI_{move,}$ when studying a ballistic thumb abduction task (Dupont-Hadwen et al., 2019). The differential results might be explained by task-dependent (simple reaction time vs. ballistic thumb abduction), or timing-dependent effects (timing of late premovement assessment 90% vs. 75%), or the nature of the provided feedback. However, in line with Dupont-Hadwen we were able to associate the magnitude of event-related disinhibition with the amount of learning, linking weaker $SICI_{move}$ modulation with greater training-related improvement. We speculate that less disinhibitory dynamics in premovement phase are a correlate of a well-tuned motor system with effective inhibitory control and high susceptibility towards training success. Conversely, Heise and colleagues were able to link stronger event-related modulation of $SICI_{move}$ with better motor performance in a life span cohort (Heise et al., 2013), which however might be interpreted as a correlate of a compensatory mechanism to diminish age-related constraints (Hummel et al., 2009; Gleichmann et al., 2011; Heise et al., 2013).

To study potential effects on the underlying brain networks, we assessed seed-based FC with rsfMRI. Modulations of resting state networks in the post-training phase have been previously characterized. For instance, Sami and colleagues have reported an engagement of the sensory-motor network in the late post-training phase (> 6h) studying the serial reaction time task (Sami et al., 2014). In our sample, the combination 50 Hz cerebellar tACS and motor training reduced FC between a cluster of voxels, located in the right caudate, and the PPC seed. Functionally, preserved synchrony between cortical and striatal areas including parietal regions, has been associated with sleep-dependent memory consolidation (Debas et al., 2014). Based on these considerations, one is tempted to speculate that the limited retention (whole group analysis) in the active stimulation group might have been a correlate of perturbed striato-parietal connectivity. However, in the present data, no clear association between the magnitude of reduced FC and task retention was found.

Baseline performance in the SGFMT and $SICI_{move}$ modulation before training appeared as most influential determining factors for subsequent training success. Specifically, these results indicated that

worse baseline performance was associated to better training performance. This could be either explained by the phenomenon that higher motor variability can result in better motor performance due to an increase in exploratory behaviour (Wu et al., 2014). Conversely, it could point towards possible ceiling effects, as the initially better performers might be limited for further improvement, due to constraints of the task. The second influential factor was the modulation of $SICI_{move}$. The results showed that less $SICI_{move}$ modulation before training was associated with larger training success. This result is in contrary to prior research, which has linked stronger event-related modulation of inhibition with better motor performance (Heise et al., 2013). This discrepancy might be explained by task- or cohort-specific effects and should be further addressed in future work. As discussed above, we currently speculate that weaker modulation of inhibitory dynamics is a correlate of an efficient and well-tuned motor system and by this means a favourable prerequisite for efficient motor learning.

A few limitations of the present work have to be mentioned. The studied sample size is rather small. However, the participant number is in the same range as similar prior proof-of-principle work in the field (Reis et al., 2009; Wessel et al., 2016). Moreover, our chosen cross-over design provides the advantage of higher statistical power as e.g., the referenced work. We would like to emphasize that the SGMFT is based on modulation of grip forces in a constrained range and the results should not be overgeneralized to other motor tasks. It is of note, that other motor learning entities, such as motor adaptation paradigms, have shown responsiveness towards different non-invasive cerebellar stimulation paradigms. For instance, Koch and colleagues could show that cerebellar theta-burst stimulation (TBS) can enhance the learning in a visuomotor adaptation task in healthy participants (Koch et al., 2020). Importantly, the aforementioned cerebellar TBS protocol has shown to improve gait and balance functions in chronic stroke patients (Koch et al., 2019) pointing towards a considerable potential for successful further clinical translation. Lastly, it is important to note that fMRI-based FC studies have a poor test-retest reliability (Noble et al., 2019). The described timing-dependent modulation of striato-parietal FC should be interpreted with caution.

Overall, cerebellar 50 Hz tACS did not enhance the acquisition or retention of a novel motor skill. Minor effects on striato-parietal FC were present. By means of linear mixed effects modelling, we were able to explain circa 48% of the variance in training success and identified baseline task performance and the modulatory dynamics of $SICI_{move}$ as the most influential determining factors. Accounting for the identified most influential factors may allow to stratify participants for future training-based interventional studies.

References:

References are listed in the common reference list of this thesis.

4. Study 3: *Multi-focal stimulation of the cortico-cerebellar loop during the acquisition of a hand motor skill in chronic stroke patients*

Wessel M.J.*, Draaisma L.R.*, Durand-Ruel M., Maceira-Elvira P., Moyne M., Turlan J.-L., Mühl, A., Léger, B., Chauvigné, L., Park C., Koch P.J., Morishita T., Guggisberg A.G. & Hummel F.C.

*: authors contributed equally

1. Defitech Chair of Clinical Neuroengineering, Center for Neuroprosthetics (CNP) and Brain Mind Institute (BMI), Ecole Polytechnique Fédérale de Lausanne (EPFL), Geneva, Switzerland
2. Defitech Chair of Clinical Neuroengineering, CNP and BMI, EPFL Valais, Clinique Romande de Réadaptation, Sion, Switzerland
3. Department of Neurology, University Hospital Würzburg, Würzburg, Germany
4. Department of Clinical Neurosciences, Geneva University Hospital (HUG), Geneva, Switzerland

Correspondence:

Prof. Dr. Friedhelm C. Hummel

Defitech Chair of Clinical Neuroengineering

Center for Neuroprosthetics (CNP) and Brain Mind Institute (BMI)

Swiss Federal Institute of Technology (EPFL)

9 Chemin des Mines, 1202 Geneva

Email: friedhelm.hummel@epfl.ch

Telephone: +41 21 69 35 440

4.1 Abstract

Impairment of motor hand function is a common consequence after a stroke and a critical influencing factor for regaining a self-determined life. An important current rehabilitation strategy is task-specific training, i.e., the repeated, challenging practice of functional, goal-directed activities. The approach aims at facilitating favorable plastic changes within the motor learning network associated with specific phases of the learning process. It has been suggested that concurrent application of transcranial direct current stimulation (tDCS) to the motor cortex (M1) can further enhance the effects of motor training in stroke survivors. However, a convincing clinical translation of the stimulation strategy has not yet been achieved. A shortcoming of conventional stimulation approaches is that the brain network-based architecture, necessary for mediating motor learning processes, especially the dynamic interactions within the cortico-striatal and cortico-cerebellar system, has not been adequately considered.

Here, we tested whether the application of a sequential multifocal tDCS protocol targeting the cortico-cerebellar loop can further enhance motor functions during motor training. N = 11 chronic stroke survivors were recruited for the study. Anodal tDCS was applied simultaneously to a hand skill-based motor training during four training sessions distributed equally over two days. The following stimulation sequences were applied and compared: (i) sequential multifocal stimulation (M1-CB-M1-CB) vs. (ii) a monofocal control condition (M1-Sham-M1-Sham). Skill retention was assessed 1 and ca. 10 days after the training phase. Multimodal systems neuroscience data (clinical, paired-pulse transcranial magnetic stimulation) was recorded to characterize stimulation response determining features.

The application of CB-tDCS boosted motor behavior in the early training phase in comparison to the control condition. However, we did not detect faciliatory effects on the late training phase, skill retention, or the learning rate of the individual sessions. The findings suggest a learning phase-specific role of the cerebellar cortex during the acquisition of a motor skill. These findings might be a consequence of the stimulation-induced recruitment of cerebellar cortical structures, in particular the PC that are involved in the mediation of error-based learning, which is more relevant during the early phase of learning. In agreement with previous neuroimaging work, the neuronal processing at later stages of motor learning appear to substantially rely on structures, such as deep cerebellar nuclei or the cortico-striatal system, which were not targeted by the applied stimulation protocol.

In summary, network-based stimulation strategies may allow to further decipher mechanisms underlying motor learning after a stroke and might allow for tuning behavior during motor practice towards enhancing the effects of rehabilitative treatment.

4.2 Introduction

Stroke is a frequent neurological disorder and a major cause of disability worldwide (GBD 2016 Neurology Collaborators, 2019). Many stroke survivors experience problems with their hand motor function, which imposes a challenge for regaining a self-determined life (Hummel and Cohen, 2006). An important and current rehabilitation strategy for alleviating constraints in hand motor function is task-specific training, i.e., the repeated, challenging practice of functional, goal-directed activities (Winstein et al., 2016). An important substrate of the gained behavioral improvement is training-induced plasticity within the cerebral motor learning network (Krakauer, 2006; Hardwick et al., 2013). Core components of this network are the cortico-striatal and cortico-cerebellar systems and their dynamic interactions, which shape the evolution of novel or re-acquired motor memory traces (Hikosaka et al., 2002; Doyon and Benali, 2005; Dayan and Cohen, 2011).
One experimental approach that allows for further investigating and potentially supporting motor learning processes is the application of transcranial direct current stimulation (tDCS) in conjunction with behavioral training (Reis et al., 2009; Zimerman et al., 2012, 2013; Buch et al., 2017; Wessel et al., 2021b). For example, it could be demonstrated that tDCS of the primary motor cortex (M1) during hand-based motor training activities may enhance learning processes (Reis et al., 2009; Zimerman et al., 2012). The approach has gained interest in translational neuroscience research and has been investigated in several follow-up studies, for review see e.g., Wessel et al. 2015 (Wessel et al., 2015). However, the strategy has so far yielded mixed results with variable response rates within and across studies, making it difficult to predict stimulation effects on the level of the individual patient (Buch et al., 2017; Wessel et al., 2021a). A convincing clinical translation of the conventional M1-based stimulation strategies has not been achieved yet (Lefaucheur et al., 2017). A clear limitation of previous work is that, for the most part, the dynamic brain network-based architecture, necessary for mediating motor learning processes, has not been adequately considered. In other words, conventional strategies were largely based on a "one-node-only" stimulation approach.
To address this ongoing research challenge, we evaluated a sequential “dual-node” stimulation strategy focusing on the cortico-cerebellar model system and characterized its impact on different subcomponents of learning. The model was chosen based on the first reports of successful modulation of motor learning behavior through monofocal cerebellar tDCS applications (CB-tDCS) in young healthy subjects (Cantarero et al., 2015; Wessel et al., 2016), the intactness of the target region in the majority of strokes cases (“non-lesioned entry to the system”) and the involvement of the cortico-cerebellar loop in stroke-related pathophysiological processes (Wessel and Hummel, 2018).

In the here presented study, we compared a sequential multifocal stimulation approach (stimulation sequence M1-CB-M1-CB) to a monofocal control condition (stimulation sequence: M1-sham-M1-sham). The stimulation protocol was applied during a hand motor training and distributed over four training sessions on two consecutive days (D1S1-D1S2-D2S1-D2S2). We hypothesized that the application of sequential multifocal stimulation of the cortico-cerebellar loop would boost motor behavior and learning with respect to a monofocal control condition. Furthermore, multimodal data, including clinical, transcranial magnetic stimulation (TMS), were acquired to search for features that allow characterizing stimulation response variability.

4.3 Methods

Participants

Twelve chronic stroke survivors were recruited for the study. One subject dropped out of the study after the screening session due to scheduling difficulties. The demographic characteristics of the remaining subjects, who participated in the study, are listed in Tab. 1.

#	Lesion location	Time since stroke [months]	Gender	Age	FMA-UE [max. 66]	NIHSS [max. 42]	MMSE [max. 30]	SIS [max. 100]
1	Paramedian pontine left - subtentorial	22	m	75	65	0	30	87
2	MCA deep branches right - supra	37	f	74	59	1	28	61
3	MCA inferior division right - supra	53	m	82	59	2	28	79
4	MCA frontal operculum, insula, temporo-parietal right - supra	44	f	77	44	2	29	86
5	Paramedian pontine left - sup	48	m	60	65	0	29	69
6	MCA frontal operculum right - supra	97	f	72	61	0	29	83
7	MCA left -supra	14	m	72	58	2	27	68
8	MCA deep branches left - supra	66	m	61	40	1	28	67
9	MCA left - supra	83	m	63	62	1	30	65
10	MCA right - supra	126	m	60	61	1	30	88
11	Thalamus right - supra	13	f	72	61	1	29	64
Mean	n/a	54.82	4/11 f	69.82	57.73	1.00	28.82	74.27
SD	n/a	35.70	n/a	7.59	8.14	0.77	0.98	10.36

Tab. 1. *Patient characteristics. Columns include assessment scores of FMA-UE (Fugl-Meyer Assessment Upper Extremity), NIHSS (National Institute of Health Stroke Scale), MMSE (Mini-Mental State Examination), SIS (Stroke impact scale).*

The inclusion criteria were: ≥ 18 years of age, first-ever stroke (determined by clinical assessment), ictal event ≥ 6 months, motor deficit, normal values of Mini-mental state examination (> 26/30), absence of contraindication for non-invasive brain stimulation. The exclusion criteria were: limited capacity to consent, multiple clinical apparent strokes, cerebellar stroke, concurrent neuropsychiatric diseases, history of seizures, intake of medication that potentially interacts with non-invasive brain stimulation, high degree of spasticity (Ashworth > 2), musculoskeletal dysfunction that compromised

finger movement, pregnancy, professional musicians or intense professional usage of a computer keyboard, intake of narcotic drugs, request of not being informed in case of incidental findings. The study was conducted in accordance with the Declaration of Helsinki. All subjects gave their written informed consent. The study protocol was approved by the cantonal ethics committee Vaud, Switzerland (project number 2017–00765).

Experimental design

The study followed a randomized, double-blind, sham-controlled, cross-over design. The following two experimental conditions were tested and compared: (i) sequential multifocal stimulation following the stimulation sequence M1-CB-M1-CB (in the following specified as MF-stimulation) and (ii) a monofocal control condition respecting the sequence M1-sham-M1-sham (in the following specified as control). In general, the subjects participated in 13 sessions. For an illustration of the timeline please see Fig. 1A. During session 0 after obtaining informed consent, the stroke survivors were characterized by conducting a set of scores and scales. At the beginning of visit 1 an electrophysiological baseline assessment was conducted utilizing paired-pulse TMS (ppTMS) techniques (see below). Afterwards, during visits 1 and 2, the stroke survivors conducted a motor training of two sessions on each of two consecutive days (D1S1, D1S2, D2S1, D2S2). There was a break of about 90 minutes between the training sessions within a day. Motor task retention was assessed at a 1 day and about 10 days follow-up (FU1, FU2). This was followed by a washout phase of an average of 35 (range 13-51) days and a cross-over to the other experimental condition. After the cross-over, sessions 1 to 6, were repeated and are labelled as sessions 7 to 12.

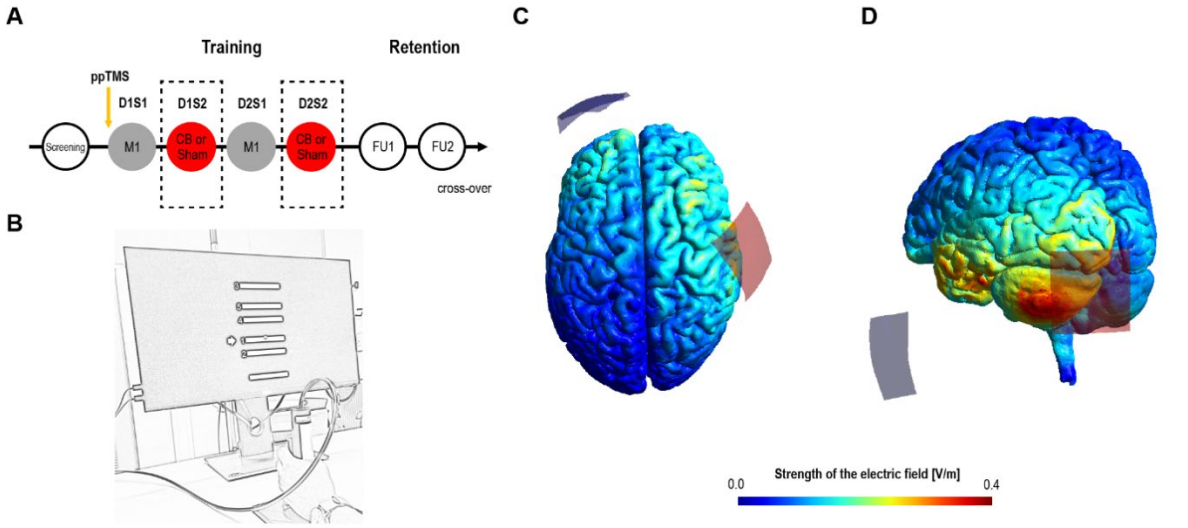

Fig. 1. Experimental setup

(A)Timeline of the experiment. (B.) Illustration of sequential grip strength modulation tasks. The subjects had to navigate a cursor to target zones via the modulation of grip force using their affected hand. The order of targets followed a pre-defined sequence. (C.&D) Depiction of the utilized tDCS montages, M1 stimulation (C.), CB stimulation (D.) and respective electric field simulations implemented in the SimNIBS platform (Thielscher et al., 2015)*. The montage depicted illustrates the setup for a patient with a right hemispheric lesion.*

Motor learning task

A computerized sequential grip force modulation task (SGFMT) served as the motor learning task. For details please see also our prior work Wessel & Draaisma et al. 2020 (Wessel et al., 2020). Prior to the baseline assessments and training the subjects were briefed on the task procedures both verbally and in writing and conducted a simplified familiarization version of the task. The subjects were instructed to conduct the task as quickly and as accurately as possible. During the task, the patients had to control a grip-force sensor (Current designs, Inc., Philadelphia, PA, USA) with their paretic hand with the aim to navigate an on-screen cursor between a home zone and five target zones via modulation of their grip force. The force range was scaled to the individual maximum grip force. The order of the targets followed two pseudorandom, complexity-matched sequences A and B, which were randomized across subjects and stimulation conditions. The task difficulty was adjusted to the subject's pre-baseline performance level. The subjects were allocated to the respective difficulty level based on the number of correctly performed sequences in a pre-baseline session (< 1: easy, 1: moderate, > 1: difficult task version). The pre-baseline block was succeeded by a 90 s baseline block at the allocated difficulty level to which all the subsequent motor learning data were normalized (see data processing below). The actual training consisted of 9 blocks of 90 s each separated by a 45 s break. In block 5 an additional pseudorandom, complex-matched target sequence was tested to assess for potential effects on sequence-independent motor performance. During the behavioral follow-up sessions, the subjects performed the motor task for 3 blocks, which were also separated by 45 s breaks.

Transcranial direct current stimulation (tDCS) protocol

The currents for tDCS were generated via a DC-stimulator plus *(neuroConn GmbH, Ilmenau, Germany)* and applied transcranial via rectangular (5 x 5 cm) sponge-like electrodes soaked in a saline solution that contained an electrode pad made of conductive rubber. The stimulation protocols targeted at modulating the neuronal activity in the cortico-cerebellar system were adopted from our prior work (Wessel et al., 2021b). The active M1 stimulation protocol was defined by the following parameters: polarity - anodal stimulation, intensity - 1 mA, duration - 20 min, fade-in/out interval- 8 s, target electrode - TMS M1 hotspot contralateral to the affected hand, frontal edge oriented 45° to the midsagittal line, return electrode - supraorbital region ipsilateral to affected hand (Hummel et al., 2005), for a depiction of the montage see Fig. 1C. The active CB stimulation protocol was defined by the following parameters: polarity - anodal stimulation, intensity - 2 mA, duration - 20 min, fade-in/out interval - 8 s, target electrode - 3 cm lateral of the inion over the Cerebellum ipsilateral to the affected hand, return electrode - over ipsilateral buccinator muscle (Galea et al., 2009; Wessel et al., 2016), for a depiction of the montage see Fig. 1D. The sham stimulation was applied in the cerebellar stimulation configuration using the same stimulation parameters as indicated in the active CB stimulation protocol, except that the current was already ramped down after 30 s of stimulation (Galea et al., 2009; Wessel et al., 2016).

Paired pulse transcranial magnetic stimulation (ppTMS)

PpTMS was utilized to assess GABAergic and glutamatergic neurotransmission linked to M1 at baseline (Chen, 2004). The procedures are described in detail in our prior work Wessel & Draaisma et al. 2019 and Wessel & Draaisma et al. 2020 (Wessel et al., 2019, 2020). In brief, a MagPro X100 stimulator connected to an MC-B70 coil *(MagVenture, Farum, Denmark)* was used to deliver monophasic TMS pulses with posterior to anterior current direction in the underlying brain tissue. The coil was placed on the motor hot spot contralateral to the affected hand and oriented so that the handle pointed backward with an approximate angle of 45 degrees to the midsagittal line. The coil positioning was guided using a neuronavigation system *(Localite, Bonn, Germany)*. Specifically, we assessed short intracortical inhibition at rest (SICI rest), during movement preparation (SICI move), and intracortical

facilitation at rest (ICF rest) (Chen, 2004; Hummel et al., 2009). The motor-evoked potential (MEP) data were sampled from the first dorsal interosseous muscle (FDI) using a belly-tendon montage. The test pulse (TP) was adjusted to elicit a MEP of approximately 1 mV in the relaxed FDI. The conditioning pulses (CP) were adjusted to 80% of the resting-motor threshold (RMT), defined as the lowest stimulus intensity which produced a MEP with a peak-to-peak amplitude ≥ 50 μV in 5 out of 10 consecutive trials. SICI was evaluated at an inter-stimulus interval of 3 ms and ICF of 10 ms. To assess SICI modulation in the pre-movement phase subjects performed a simple reaction time task responding to a visual cue with index finger abduction, while the TMS pulses were applied at about 20% and 90% of their reaction time, for further details about the MEP sampling, SICI and ICF procedures and SICI modulation sampling, please see our prior work (Hummel et al., 2009; Wessel et al., 2019, 2020).

Data processing

The behavioral motor learning was sampled and pre-processed via custom written MATLAB scripts (The MathWorks, Inc., Natick, Massachusetts, United States). The a priori defined motor performance metric was the area under the curve (AUC) of the movement trajectory for correctly performed sequences (Wessel et al., 2020). For further analysis, the motor learning data were averaged per block. The motor learning score per block was normalized by subtraction to baseline. The MEP data inspection and processing were performed offline using a custom MATLAB graphical user interface (GUI), which automatically checked for rejection criteria. The final decision for rejected trials had to be manually confirmed by the investigator. Trials were excluded when: 1) the root mean square (RMS) value of the baseline EMG activity (100 - 0 ms before TMS pulse) was outside the Mean ± 2 SD of all stimuli (van de Ruit and Grey, 2016); 2) Overlap of voluntary muscle activation with the MEP (for SICI move condition); 3) MEP amplitude smaller than 50 μV, except for the SICI trials. The MEP amplitude was quantified by its peak-to-peak value measured in the time window of 20 - 50 ms after stimulation. The modulation of the ppTMS conditions was calculated as a percentage of the TS only condition and defined as: mean (conditioned MEPs) / mean (unconditioned MEPs)*100.

Statistical analysis

The statistical analyses were conducted using Rstudio (version 1.4.1717). The normality of data was visually checked with Q-Q plots and histograms of residual values and further verified by the assessment of their skewness ranging between -1 and 1 (Gravetter and Wallnau, 2014). Statistical significance was assumed at p-values < .05. The equality of the two baseline groups was verified using Bayesian statistics by computing a Bayesian paired-samples t-test in JASP software (version 0.16.0.0). The data were analyzed using linear mixed-effects models included in the "lmerTest" package in Rstudio (Kuznetsova et al., 2017). Omnibus tests were performed with type II ANOVA of the model. Effect sizes were calculated as partial eta squared using the "effectsize" package (Ben-Shachar et al., 2020). Post-hoc analyses were done by pairwise comparisons using the "emmeans" package (Lenth, R., 2020). Simple two-group comparisons have been analyzed using a paired samples t-test, Bonferroni corrected. The motor learning data were analyzed using the mean AUC output as the dependent variable and the training sessions (D1S1, D1S2, D2S1 & D2S2) and the stimulation conditions (MF-stimulation vs. control). To conduct a responder analysis, subgroups of the preselected metrics (baseline task performance (motor ability), SICI, ICF, SICI move), were derived by a median split procedure.

4.4 Results

Effect of CB-tDCS on motor behavior during the training phase

The analysis of the training sessions (D1S1, D1S2, D2S1, D2S2) indicated a significant main effect of STIMULATION $F(1, 609.1) = 15.31$, $p < .001$, $\eta_p^2 = .02$ and of SESSION $F(3, 609.2) = 3.61$, $p = .013$, $\eta_p^2 = .02$, but no STIMULATION x SESSION interaction $F(3, 609.1) = 1.80$, $p = .146$, $\eta_p^2 = .008$. Specifically, during MF-stimulation, the subjects demonstrated a globally better motor performance (smaller AUC of the movement trajectory) in comparison to control (Fig. 2A). To further quantify the effects of the study intervention, in the next step, we analyzed the sessions with cerebellar stimulation (active vs. sham CB-tDCS) separately, namely D1S2 and D2S2. The results indicated a significant effect of STIMULATION $F(1, 300.2) = 8.21$, $p = .004$, $\eta_p^2 = .03$, of SESSIONS $F(1, 300.3) = 7.79$, $p = .006$, $\eta_p^2 = .03$ and a significant SESSIONS x STIMULATION interaction $F(1, 300.3) = 5.27$, $p = .022$, $\eta_p^2 = .02$. Post-hoc pairwise comparisons showed that the effect of STIMULATION was significant for SESSION D1S2 $t(287.9) = 3.66$, $p = .002$ but not for D2S2 $t(286.7) = 0.4$, $p = .979$, which suggests a learning-phase specific effect of CB-tDCS. Furthermore, the effect of SESSION was significant for the sham stimulation group $t(49.2) = 3.69$, $p = .003$ but not for the real stimulation group $t(5.8) = 0.34$, $p = .986$. Indicating a CB stimulation effect during the early stages of motor learning that remains stable over time and an eventual a “catching-up” in performance in the control group.

Retention was measured over three follow-up blocks at 1 day and ~ 10 days after the training session. The data were normalized by subtraction to the last block of the last training session to ensure a comparison of actual retention of the learned sequence in respect to the end of the training phase. Results showed no significant effect of STIMULATION $F(1, 99.4) = .244$, $p = .622$, $\eta_p^2 = .002$, or of FU $F(1, 5.5) = 0.04$, $p = .845$, $\eta_p^2 = .007$, or a STIMULATION x FU interaction $F(1, 98.3) = 0.19$, $p = .667$, $\eta_p^2 = .002$, see Fig. 2B.

To mitigate carry-over effects, after crossing-over to the remaining stimulation condition, a wash-out-phase was respected (mean: 35 days, range: 13 to 51 days). Additionally, we used Bayesian statistics to check for equality between the baseline results before and after cross-over. The analysis indicated a Bayes factor of BF01 = 3.12, meaning that it was 3.12 times more likely that the baseline results are equal than different. Thus, we can rule out as considerable carry-over effect. In addition to aggregated whole group data, exemplary movement trajectory data of a single participant sampled in the early and late training phase are depicted in Fig. 2C&D.

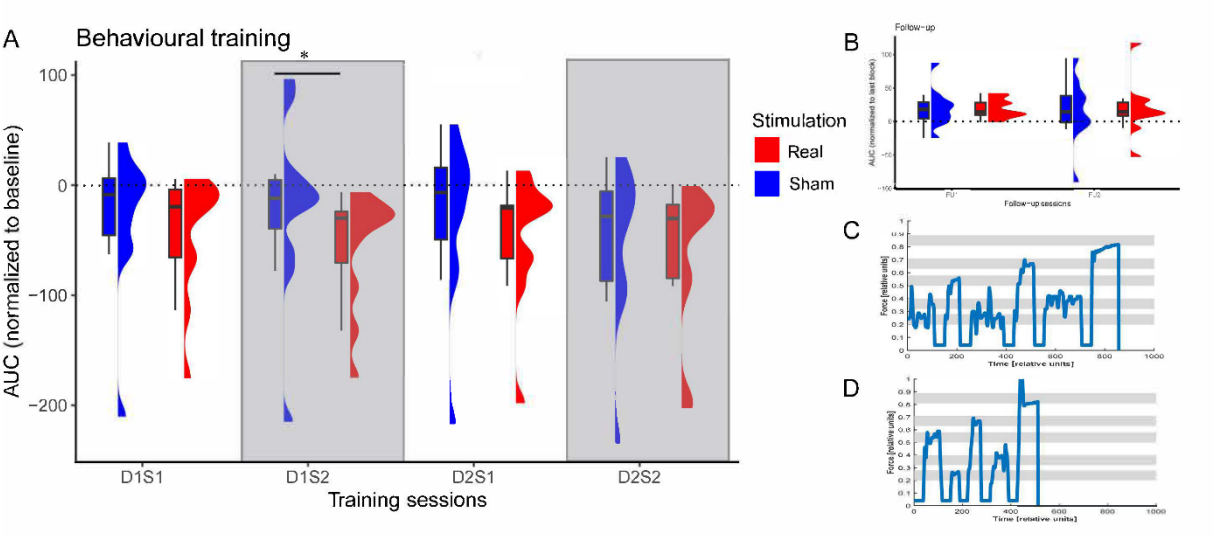

Fig 2. Results of behavioral training
*(A) Training sessions separated by stimulation group. In the MF-stimulation condition the stimulation sequence followed the order of active-M1, active-CB, active-M1, active-CB and was applied during the four consecutive training sessions (D1S1, D1S2, D2S1, D2S2). During the control condition the stimulation sequence was active-M1, sham-CB, active-M1, sham-CB. "Real" depicts the MF-stimulation group and "Sham" the control group. The grey background delineates the CB-stimulation sessions. * indicates significant difference between the stimulation groups. (B) Results of the follow-up session after 1d and ~10 days after the last training session. (C) Individual movement trajectory of one patient, who completed one sequence during the early stage (C.) or during a later stage of the training phase.*

Analysis of temporal subcomponents of learning

To analyze offline learning, the within-day offline analysis was separated from the overnight offline analysis. This was done because of different stimulation paradigms (M1 vs. CB) and the additional factor of sleep during the overnight offline learning (Walker and Stickgold, 2004). The analysis of the within-day offline learning between D1S1 and D1S2, and between D2S1 and D2S2 showed no significant effect of STIMULATION $F(1, 33) = 0.002$, $p = .962$, $\eta_p^2 = .003$, or of TIMING $F(1, 33) = 0.095$, $p = .760$, $\eta_p^2 < .001$, nor an interaction between STIMULATION x TIMING $F(1, 29) = 0.27$, $p = .607$, $\eta_p^2 = .009$. The overnight offline learning between session D1S2 and D2S1 showed no effect for STIMULATION $F(1, 8.5) = 2.01$, $p = .192$, $\eta_p^2 = .19$.

Impact of baseline motor ability on stimulation response

To investigate, if motor ability at baseline impacts subjects' stimulation response variability, the patients were separated by a median split, into low - vs. high performers based on their baseline performance. With the use of a linear mixed-effects model we included behavior as the dependent factor, the independent factors were TIMING (D1S1, D1S2, D2S1. D2S2) and PERFORMANCE (high vs. low). The results showed a significant main effect for TIMING $F(3,32.74) = 4.18$, $p = .013$, $\eta_p^2 = .28$, for PERFORMANCE $F(1, 10.87) = 10.14$, $p = .009$, $\eta_p^2 = .48$ and a trend for an interaction between TIMING x PERFORMANCE $F(3, 270.73) = 2.52$, $p = .059$, $\eta_p^2 = .03$. Post-hoc pairwise comparisons showed that MF-stimulation resulted in a stronger enhancement of motor behavior in the low performer group compared with the high performers in training session D1S1 $t(15.4) = 2.59$, $p = .02$, in D1S2 $t(15.7) = 2.26$, $p = .038$, in D2S1 $t(15.9) = 3.13$, $p = .006$ and D2S2 $t(15.8) = 3.2$, $p = .006$. Pointing towards an ability dependence of the induced effect, please see figure 3A.

To further explore the stimulation sensitivity, the active vs. sham stimulation conditions during the CB stimulation sessions were compared. The high vs. low performers were related to their respective stimulation conditions, creating four separate groups for comparison: “Active - High”, “Active - Low”, “Sham - High” and “Sham - Low”. The results showed a significant main effect for TIMING F(1, 303.26) = 9.64, p = .002, η_p^2 = .03 and for GROUPS F(3, 27.58) = 14.17, p <.001, η_p^2 = .61, and an interaction effect for TIMING x GROUPS F(3, 393.36) = 5.49, p = .001, η_p^2 = .05. There was a significant difference in performance in response to active stimulation compared with sham stimulation in the low performers during D1S2 t(297.9) = -6.25, p <.001; this effect did not remain in D2S2 t(297.4) = -2.37, p =.085. However, there was no effect of active vs. sham stimulation in the high performers during D1S2 t(295) = 0.45, p = .970 and during D2S2 t(295.9) = 1.48, p = .452. This indicates that the CB-tDCS effect on early training on the group level was driven by a high protocol susceptibility in patients with lower baseline motor ability, please see figure 3B.

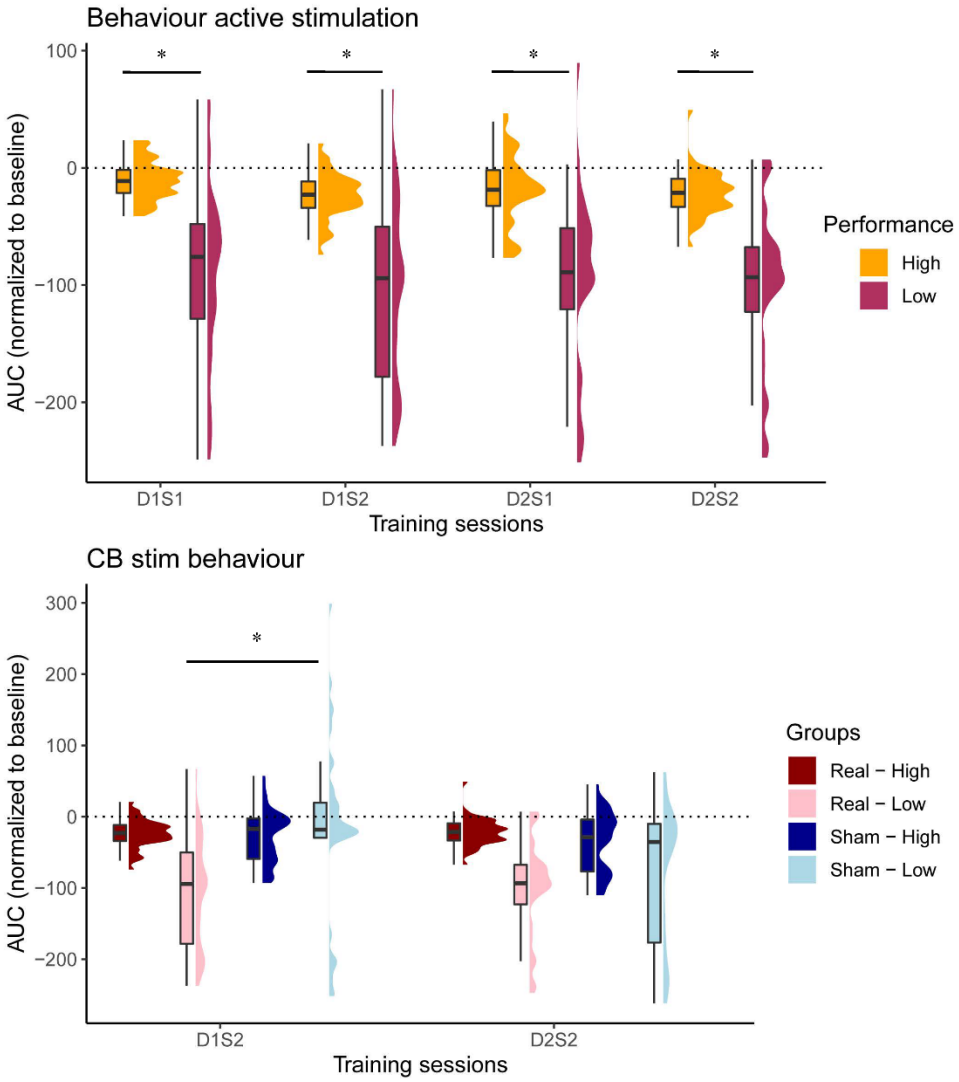

Fig. 3. Motor ability-dependent effects of CB-stimulation.
A) The performance on the behavioral task during the active stimulation sessions only. The groups have been separated in high vs low performance based on the baseline performance. B) the performance during the cerebellar stimulation sessions only. Groups are divided into real vs. sham stimulation and by high vs. low performance during the preceding baseline session.

Impact of intracortical inhibition and facilitation of motor cortex and stimulation response rate
The TMS-based metrics measured at the beginning of D1S1 showed no significant differences for the test pulse (TP) peak-to-peak amplitudes at rest and the percentage of maximal stimulator output (MSO) between the before and after cross-over sessions, for details please see Tab. 4. This points towards a reliable adjustment of the TMS parameters, which assured that the metrics were obtained at a comparable range of the respective recruitment curves.

Parameter	Before cross-over	After cross-over	Statistics
TP_{only} MEP (mV) Peak-to-peak amplitude	0.59 (0.08)	0.5 (0.09)	t(7) = -1.8, *p* = .116
Maximal stimulator output (%)	71.25 (5.66)	72.88 (5.3)	t(7) = 1.24, *p* = .256
SICI rest	79.65 (20.11)	81.52 (19.69)	t(7) = -0.24, *p* = .815
ICF rest	142.32 (24.91)	139.04 (20.9)	t(7) = 0.3, *p* = .771
SICI move	71.67 (5.72)	64.13 (5.08)	t(7) = -0.17, *p* = .869

Tab 3. Overview on the archived adjustment of the TMS parameters. *Table shows the mean and SEM in brackets of the different TMS parameters before and after cross-over. Paired samples t-test comparisons between before and after cross-over sessions is shown in the statistics column.*

To evaluate if the assessed metrics contain information that determine the subsequent response to stimulation, the data was divided into two groups based on a median split. Only the CB stimulation sessions have been taken into account for active vs. sham stimulation comparisons. Following this procedure, we obtained two subgroups per assessed TMS metric, low vs. high inhibition for SICI, low vs. high facilitation in ICF, and large vs. small modulation for SICImove. These factors have been grouped with active vs. sham stimulation during the CB stimulation sessions. Resulting in 4 groups: Real - high, Real - low, Sham - high, Sham – low.
For SICI the results indicated a significant main effect for TIMING $F(1, 222) = 5.35$, $p = .022$, $\eta_p^2 = .02$, and for GROUPS $F(3, 224.88) = 25.29$, $p < .001$, $\eta_p^2 = .25$. There was no interaction between TIMING x GROUPS $F(3, 220) = 1.19$, $p = .314$, $\eta_p^2 = .02$. Post-hoc pairwise comparisons showed a better performance for high vs. low inhibition with active stimulation during session D1S2 $t(219.8) = 5.69$, $p < .001$ and D2S2 $t(220.4) = 4.63$, $p < .001$. There was better performance for high vs. low inhibition with sham stimulation during D1S2 $t(222.9) = 7.4$, $p < .001$ and D2S2 $t(222.6) = 6.84$, $p < .001$. The patients with low inhibition performed significantly better with active vs. sham stimulation during D1S2 $t(220.3) = -3.56$, $p = .011$, but not during D2S2 $t(218.4) = -2.61$, $p = .159$. There was no difference between real vs. sham stimulation in the high inhibition group during D1S2 $t(215.2) = -1.29$, $p = .903$ or during D2S2 $t(215.2) = 0.7$, $p = .997$, please see Fig. 4A.
The ICF results show a significant main effect for TIMING $F(1,221.91) = 5.55$, $p = .019$, $\eta_p^2 = .02$ and for GROUPS $F(3, 208.6) = 3.23$, $p = .024$, $\eta_p^2 = .04$, but not interaction between TIMING x GROUPS $F(3, 221.92) = 1.37$, $p = .252$, $\eta_p^2 = .02$. On visual inspection there seemed to be an indication that the patients with high facilitation perform better than the patients with low facilitation in the active but not the sham conditions. However, post-hoc comparisons showed no significant differences between any of the groups or for any of the timings, please see figure 4B.

The results of the SICI move analysis revealed a significant main effect for TIMING $F(1, 222.03) = 5.76$, $p = .017$, $\eta_p^2 = .03$, and for GROUPS $F(3, 224.63) = 10.39$, $p < .001$, $\eta_p^2 = .12$, but there was no significant interaction between TIMING x GROUPS $F(3, 222.03) = 0.92$, $p = .433$, $\eta_p^2 = .01$. On visual inspection the patients with high SICI modulation seemed to perform better than in the low SICI modulation group during active stimulation. However, post-hoc comparisons did not show significant differences during D1S2 $t(219) = -1.76$, $p = .647$ or D2S2 $t(218.4) = -1.82$, $p = .605$. There was an opposite pattern in the sham group, indicating a better performance for the low SICI modulation group. However, post-hoc comparisons showed no significant difference during D1S2 $t(219) = 2.45$, $p = .222$ but there was a difference during D2S2 $t(218.9) = 3.72$, $p = .006$. The low SICI modulation group seemed to perform better after sham compared with active stimulation. Analysis showed no significant difference during D1S2 $t(216) = 2.39$, $p = .251$, but there was a significant difference during D2S2 $t(216) = 3.74$, $p = .006$. Pointing towards a disturbing effect of CB-stimulation on patients with low SICI modulation prior to training in the later training phase, please see figure 4C.

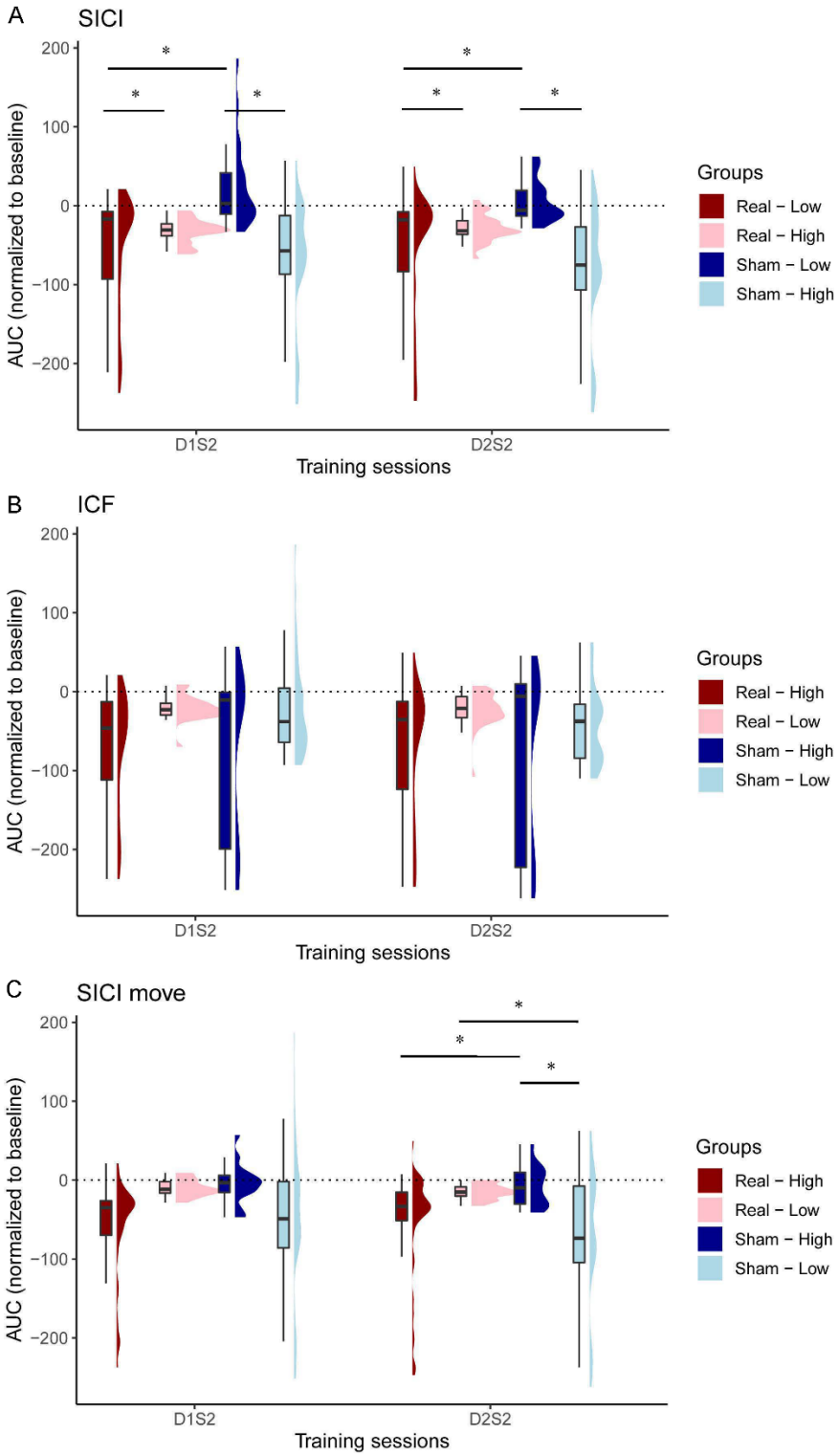

Fig. 4. Relationship of ppTMS-derived metrics and stimulation response variability.
Groups were separated based on high vs. low inhibition, facilitation or modulation. Groups were separated by the real vs. sham condition, resulting in 4 segregated groups. Only CB-stimulation conditions were taken into account for real vs. sham comparisons. (A) Baseline SICI high vs. low inhibition in relation to task performance. (B) Baseline ICF high vs. low facilitation in relation to task performance. (C) Baseline SICI high vs. low modulation during movement preparation in relation to task performance.

Impact of corticospinal tract integrity

As an exploratory sub-analysis, we have separated the patients by their corticospinal tract (CST) integrity, quantified by the presence or non-presence of MEPs from the affected limb. We have analyzed the training effect during the 4 separate training sessions and the effect of stimulation on the subgroup with MEPs and the subgroup without MEPs (No-MEP). It should be taken into account that the No-MEP group consisted of N = 2 patients and the MEP group of N = 9 only. Therefore, this uneven divide in groups rendered the data unbalanced with small sampled subgroups. Thus, we have refrained from a formal statistical analysis. The descriptive statistics suggest that the averages of the performance during the training sessions demonstrated a difference between the active in respect to the sham stimulation in the no-MEP group, with an faciliatory effect of active stimulation on training. This was not as evident in the MEP group (Fig. 5). This might indicate a higher sensitivity for CB-stimulation in patients with a no-MEP status.

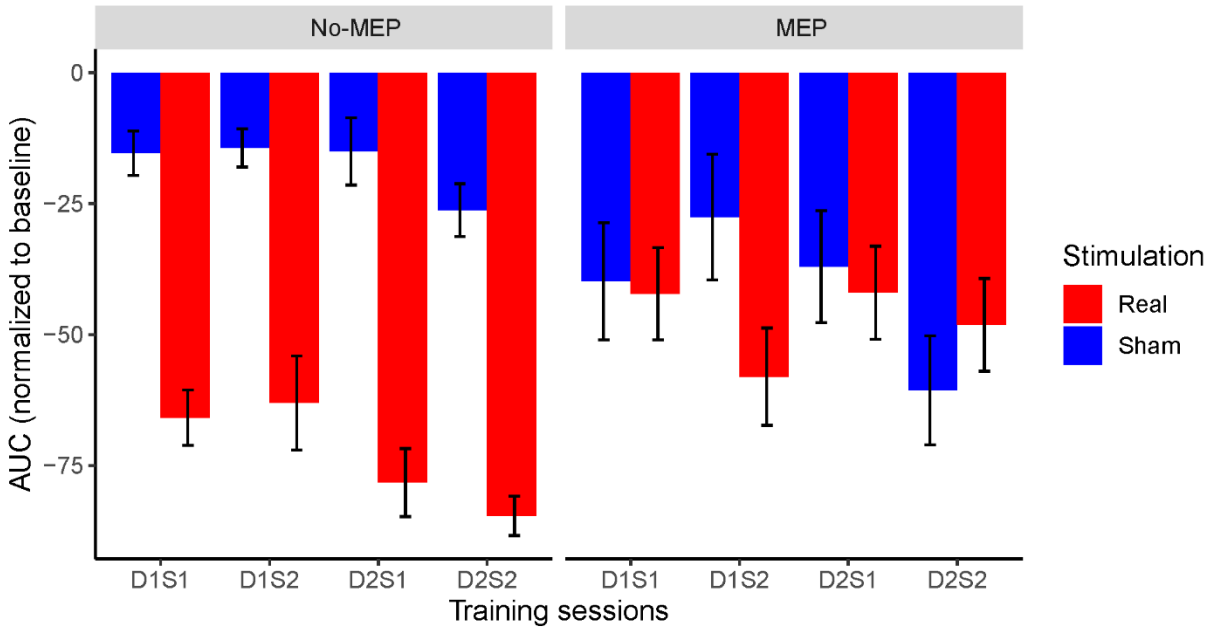

Fig. 5. Exploratory responder analysis based on the presence of MEPs.
Training performance shown as AUC normalized to baseline. Negative values indicate better performance. The tDCS effect seemed to be pronounced for patients with a no-MEP status.

4.5 Discussion

The main finding was that bifocal tDCS sequentially applied to the M1 and CB improved motor performance in a hand-based, sequential motor task in chronic stroke survivors. The effects were mainly driven by active CB stimulation during the first training day (D1S2), indicating stimulation efficacy during the early phase of learning. Furthermore, several features that were associated with the subjects' stimulation response variability such as baseline motor performance (motor ability), level of SICI, or CST integrity were detected.

CB-tDCS boosts motor behavior in the early training phase

Several neurobiological models have been developed in systems neuroscience to describe the involvement of distinct neuronal structures underlying the process of motor skill learning (Hikosaka et al., 2002; Doyon and Benali, 2005; Dayan and Cohen, 2011; Doyon et al., 2018). The core assumptions are that the cortico-striatal and the cortico-cerebellar system represent crucial neural substrates and that the engagement of the different subregions is learning phase-dependent. In addition, an intrinsic, phase-dependent shift of neural representations has been described for the targeted cerebellum. For instance, Doyon and colleagues were able to characterize the evolution of the brain activation pattern during a motor sequence learning task within the cerebellum by using functional magnetic resonance imaging (Doyon and Ungerleider, 2002). This was characterized by a pronounced activation of the cerebellar cortex during the early learning stage. As learning progressed, the level of cortical activation decreased and activity at the dentate nucleus level increased. Moreover, the shift of engagement from the cortex to the level of the deep nuclei is supported by theoretical circuit-based models of cerebellum-mediated motor learning. For example, Mauk predicted a sequence of distributed plasticity across the cerebellar circuitry (Mauk, 1997). The model suggests that during the early learning phase plasticity mainly occurs at the parallel fibre-Purkinje cell synapses in cortex. The paired presentation of a respective sensory context (mossy fibres) and error signals (climbing fibres) during the learning process induces postsynaptic long-term depression (LTD) and subsequently a disinhibition of PC output. This in turn results in long-term potentiation (LTP) at mossy fibre-nucleus cell synapses at the later stage.

We speculated that anodal CB-tDCS application may have supported these inherit processes through promotion of LTD-like plasticity at the early learning state, namely the D1S2 session (Fritsch et al., 2010; Ferrucci and Priori, 2014; Rohan et al., 2020) (see also Fig. 2A). Conversely at a later stage of learning (D2S2), a major part of the underlying plasticity was already transferred to deeper cerebellar structures and other systems, which were not directly targeted thus not sufficiently modulated by the CB-tDCS protocol. A complementary explanation for the phase-specific CB-tDCS effect could be that learning in the early training phase largely relied on an error-based mechanism, which is driven by the mismatch of intended and perceived motor outcome (sensory-prediction error) and which strongly involves the cerebellum (Spampinato and Celnik, 2021). It is possible that a stronger weight was set on other learning mechanisms at the later learning stage, which mainly recruited neuronal processing in other brain areas. For example, that rather reinforcement-based (basal ganglia), use-dependent (M1), or strategy-based learning (prefrontal cortex) processes were recruited. Thus, it can be speculated that these alternative learning mechanisms, which have a different topography profile, were not responsive to CB-tDCS to a similar degree.

No effects of multifocal M1-CB stimulation on the overall training success and skill retention

Current evidence suggests that different tDCS protocols exert their effects via modulation of distinct temporal components of motor learning (Buch et al., 2017). For example, in their seminal work Reis and colleagues could show that anodal tDCS applied to M1 during the acquisition of task that required young healthy subjects to execute and learn a sequence of pinch forces was able to enhanced the total

learning in respect to a sham control and that this effect was driven by and enhancement of offline effects (Reis et al., 2009). However, other studies emphasized online effects of anodal M1 tDCS protocols, when employing different motor learning paradigms (Nitsche et al., 2003; Stagg et al., 2011b; Zimerman et al., 2013). Likewise, the most susceptible temporal components to CB-tDCS appears to be task-specific (Cantarero et al., 2015; Wessel et al., 2016). Despite this apparent dependence of the most susceptible components on the applied task, the site of stimulation, and the cohort studied, an objective of the present study was to investigate whether the sequential reinforcement of different learning components through stimulation of different targets could increase the overall effect size. The study was designed in a sequential fashion in the order M1- followed by CB-stimulation based on previous in-house data, which indicated that anodal tDCS of M1 mainly exerted it effects via modulation of online and CB-tDCS mainly via the modulation of offline effects (Zimerman et al., 2013; Wessel et al., 2016). We hypothesized that this sequential engagement of different mechanisms underlying motor skill learning boosts the overall training success. The acquired data did not validate this hypothesis. At the end of the training (D2S2 session), we were not able to detect differences across stimulation groups (see Fig. 2A). As discussed above the CB-tDCS effect was phase specific and boosted primarily the performance in the early training phase (D1S2). This could be potentially explained by ceiling effects of the task and a consecutive catch-up of performance of the control group. Finally, we did not detect stimulation-associated effects on task retention, which further strengthens the notion that the applied multifocal stimulation protocol was able to modulate single individual components of learning in the early training phase (enhanced motor performance during D1S2, a trend for modulation of over-night offline learning), but the "boosting" effects were not retained at later evaluations.

Responder- / non-responder analysis

Retrospective analyses revealed a high degree of response variability towards tDCS-aided motor learning-based interventions within and across studies in stroke survivors (Kang et al., 2016). Several factors that have been associated with stimulation response, such as lesion location, time since stroke or level of impairment have been identified, for further reading see e.g. Wessel et al. 2018 and Wessel & Egger et al. 202. Based on this emerging responder / non-responder pattern for tDCS protocols in general, we investigated the effect of a priori determined possible influencing factors of stimulation response.

As a first step, we investigated the effect of baseline motor performance (motor ability). This we approached by dividing the patients into a low and a high performer group based on their baseline performance in the task. The analysis indicated that the CB-tDCS-mediated effect on the early learning phase was driven by a high susceptibility of patients with low baseline motor ability. A possible mechanism underlying this finding could be that patients in the low-performer group, who performed less accurate movement trajectories, received a stronger error signal while conducting the task at the early learning stage. It is possible that this increased error signal, which was mediated by climbing fibre input and which was further processed at the level of the cerebellar cortex (Ito, 2000), this provided a crucial point of action for CB-tDCS.

As a second step, we evaluated for a possible relationship of the tested ppTMS metrics, SICI, ICF, SICI move at baseline and stimulation response variability. We have looked exclusively at the CB-stimulation sessions to be able to compare active vs. sham conditions. Our findings indicated that strong SICI (inhibited state) was related to better performance, both during sham and active stimulation. Furthermore, our results indicated that active CB-stimulation significantly improved performance in patients with weak initial SICI levels (disinhibited state) compared to sham stimulation (see Fig. 4A). At first sight, this seems to be in contrast to a previous study showing a relationship between higher GABA levels (more inhibited state) and better performance in response to anodal tDCS of M1 (O'Shea et al., 2014). Furthermore, the result challenges available electrophysiological models linking the cerebellar output tone (quantified via the TMS-based assessment of cerebellum brain inhibition – CBI,

for details see (Ugawa et al., 1995)) with the state of SICI in motor cortex (Daskalakis et al., 2004). This model suggests that a stronger CBI is related to weaker SICI values (disinhibition). Strengthening of CBI, which would be the most likely consequence of anodal CB-tDCS, would not impact on a system which does not have sufficient room for further disinhibition. These considerations render it unlikely that the CB-tDCS induced effect was to a significant extent mediated via a modulation SICI in motor cortex. The ICF measurements at rest showed more variable results, which could not be distinguishably related to motor performance or stimulation effects. This is comparable to our previous findings for ICF during a similar task in healthy young adults (Wessel et al., 2020).

Furthermore, we evaluated the impact of SICI move modulation during premovement phase and stimulation response variability. A reduction of the modulation of SICI move has been reported in stroke patients (Hummel et al., 2009) and its magnitude has been related with the recovery of hand motor function (Liuzzi et al., 2014). Our findings indicate that low SICI modulation was linked to better performance in the sham conditions compared to high SICI modulation. This relates to the findings of our previous study in which lower pre-training SICI modulation was associated with training success (Wessel et al., 2020) and the findings of Dupont-Hadwen and colleagues who showed that a reduced release of SICI related to motor training improvements (Dupont-Hadwen et al., 2019). Therefore, maintained pre-movement inhibition could be a compensatory mechanism and a measure of better movement control, which has facilitated skill learning. Although not significant, the active CB stimulation indicated a shift in training response, showing increased performance for the high SICI modulation compared to low.

As a third step, we assessed for possible effects on stimulation response variability related to an electrophysiological measure of cortico-spinal tract integrity. In this exploratory analysis, we quantified the CST integrity based on the presence vs. absence status of MEPs recorded from the affected limb. Our exploratory analysis demonstrated larger training success in response to multifocal stimulation compared to monofocal stimulation in the patients with no MEP compared to the patients with MEP. It is of note, that the sample size of the no-MEP subgroup was small, which indicates that this hypothesis generating finding has to be tested in future research.

Limitations and future perspective

The present study has some limitations. The sample size is rather small for statistical comparisons. Yet, the sample size is within the range of other NIBS studies recruiting stroke patients (Wessel et al., 2021a). Moreover, a cross-over design was used to increase statistical power. One of the main limitations of conventional tDCS is the lack of focality of stimulation. Due to the relatively large electrode sizes the electric field is more dispersed (Saturnino et al., 2017) (see also Fig. 1C&D). Therefore, it might well be that adjacent brain areas have been simultaneously stimulated, because of the large induced electric field. Different shapes of electrodes, such as concentric electrodes have shown to increase focality, suggested to be used in future studies (Saturnino et al., 2017). Another limitation might be the difficulty of the task. Although the task was adjusted to the baseline performance level of the patients, there is a significant difference in task performance between low and high performers measured at baseline. This could point towards a ceiling effect of high performing patients. Moreover, the task has more temporal restrictions than other motor tasks such as the sequential finger tapping task. The grip force modulation task required the patients to hold force and remain in the target for a specific amount of time before returning to the home zone. This results in a limited possibility to increase speed during the task, which may have reduced the possibility to improve. However, using tests like the sequential finger tapping tests might result in a skewed image of motor learning in stroke patients as only mildly impaired patients would be able to perform the task. Therefore, using grip force modulation, which comprises more gross movements, allows testing sequence learning in a larger variety of stroke patients. Another limitation lies within the current study design. To reduce the amount of time to acquire the data and to not make the experiment too lengthy and straining for the patients, we decided not to measure SICI and ICF values post-training. However, in the light of the current results, we can argue

that the additional TMS measures would have been informative. Future studies should consider including post-training TMS measurements, while considering time and comfort of the patient.

Conclusion

In summary, our results indicate that it is possible to modulate hand motor performance of chronic stroke survivors through CB-tDCS application. The effect was driven by a selective enhancement of task performance in the early training phase. The subsequently conducted responder analysis indicated that stroke survivors with low baseline motor ability and a maintained disinhibited resting-state SICI in the chronic phase benefited the most from the intervention. It seems possible, that especially these patients relied on error-based learning mechanism, which have been linked to neuronal processing at the cerebellar cortex. It is of note, that the CB-tDCS associated facilitation of behavior at the early training phase, did not translate into an enhanced overall training success or skill retention, which could be related to ceiling effects of the applied motor learning task.

References:

References are listed in the common reference list of this thesis.

5. Study 4: *Targeting the frontoparietal network using multifocal personalized transcranial alternating current stimulation to enhance motor sequence learning in healthy older adults*

Draaisma, L.R.[1,2], Wessel, M.J.[1,2,3], Moyne, M.[1,4], Morishita, T.[1,2] & Hummel, F.C. [1,2,4]

5. Defitech Chair of Clinical Neuroengineering, Center for Neuroprosthetics (CNP) and Brain Mind Institute (BMI), Ecole Polytechnique Fédérale de Lausanne (EPFL), Geneva, Switzerland
6. Defitech Chair of Clinical Neuroengineering, CNP and BMI, EPFL Valais, Clinique Romande de Réadaptation, Sion, Switzerland
7. Department of Neurology, University Hospital Würzburg, Würzburg, Germany
8. Department of Clinical Neurosciences, Geneva University Hospital (HUG), Geneva, Switzerland

Correspondence:

Prof. Dr. Friedhelm C. Hummel

Defitech Chair of Clinical Neuroengineering

Center for Neuroprosthetics (CNP) and Brain Mind Institute (BMI)

Swiss Federal Institute of Technology (EPFL)

9 Chemin des Mines, 1202 Geneva

Email: friedhelm.hummel@epfl.ch

Telephone: +41 21 69 35 440

Declaration of interest: none

5.1 Abstract

Background
Healthy older adults show a decrease in motor learning capacity as well as in working memory (WM) performance. WM has been suggested to be involved in motor learning processes, such as sequence learning. Correlational evidence has shown the involvement of the frontoparietal network (FPN), a network underlying WM processes, in motor sequence learning. However, causal evidence is currently lacking. Non-invasive brain stimulation (NIBS) studies have focused so far predominantly on motor related areas to enhance motor sequence learning while areas associated with more cognitive aspects of motor learning have not yet been addressed.

Hypothesis
In this study, we aim to provide causal evidence for the involvement of WM processes and the underlying FPN in successful motor sequence learning by using a theta transcranial alternating current stimulation (tACS) paradigm targeting the FPN during motor sequence learning.

Methods
In a cohort of 20 healthy older adults, we applied bifocal tACS in the theta range to the FPN during a sequence learning task. With the use of a double-blind, cross-over design, we tested the efficacy of active compared with sham stimulation. Two versions of the motor task were used: one with high and one with low WM load, to explore the efficacy of stimulation on tasks differing in WM demand. Additionally, the effects of stimulation on WM performance were addressed using an N-back task. The tACS frequency was personalized by means of EEG measuring the individual theta peak frequency during the N-back task.

Results
The application of personalized theta tACS to the FPN improved performance on the motor sequence learning task with high WM load (p <.001), but not with low WM load. Active stimulation significantly improved both speed (p <.001), and accuracy (p =.03) during the task with high WM load. In addition, the stimulation paradigm improved performance on the N-back task for the 2-back task (p = .013), but not for 1-back and 3-back.

Conclusion
Motor sequence learning can be enhanced with the use of personalized bifocal theta tACS to the FPN when WM load is high. This indicates that the efficacy of this stimulation paradigm is dependent on the cognitive demand during the learning task and provides further causal evidence for the critical involvement of WM processes and the FPN in motor sequence learning in healthy older adults. These findings open exciting new possibilities to counteract the age-related decline in motor learning capacity and WM performance.

Keywords: ***tACS, motor learning, healthy aging, working memory, fronto-parietal network, personalization.***

5.2 Introduction

The ability to acquire new motor skills is important in daily life. Motor learning is a practice-dependent process in which consequence movements are performed quicker and more accurately (Willingham, 1998). A vast amount of research has contributed to an increased understanding of the neural substrates and underlying mechanisms involved in the acquisition, consolidation and retention of new motor skills. Neuroscientific studies have focused predominantly on the motor network and the pivotal role of the primary motor cortex (M1) (Karni et al., 1995; Seidler et al., 2012; Dupont-Hadwen et al., 2019). This is especially the case for non-invasive brain stimulation (NIBS) studies that attempt to improve motor learning by combining the practice of a challenging motor task with a stimulation paradigm (Pollok et al., 2015; Wessel et al., 2015; Krause et al., 2016; Buch et al., 2017). However, studies have suggested that challenging motor tasks, such as motor sequence learning (MSL), do not rely exclusively on motor related processes, but also on cognitive processes, such as working memory (WM) processes (Maxwell et al., 2003; Anguera et al., 2010; Seidler et al., 2012). Surprisingly, WM related brain areas have not been a target for NIBS paradigms intended to study MSL.

MSL is a process where independent movements are associated, eventually resulting into a multi-element sequence that can be performed quickly and accurately (Seidler et al., 2012; Krakauer et al., 2019). Studies have shown the involvement of WM in MSL (Shea et al., 2006; Bo and Seidler, 2009; Bo et al., 2009). WM refers to the ability to temporarily store and manipulate information in the mind (Baddeley and Hitch, 1974). Inter-individual variability in WM consist of the number of items that can be held and worked with (Seidler et al., 2012). This is important for MSL especially during the process of grouping elements of the sequence together in "chunks". This chunking process results into quicker execution of the movements (Pascual-Leone et al., 1996; Verwey, 2001; Hikosaka et al., 2002). Many studies have shown that healthy older adults show a decline in the ability to learn motor sequences (Shea et al., 2006; Bo et al., 2009). Moreover, aging decreases cognitive functions including WM (Verhaeghen and Cerella, 2002). Therefore, an interaction among age, WM capacity and MSL has been recently suggested (Bo et al., 2009), though causal evidence in favour of this suggestion remains limited.

A promising neurotechnology to provide causal evidence is the use of NIBS such as transcranial alternating current stimulation (tACS) (Kuo and Nitsche, 2012; Antal and Paulus, 2013; Herrmann et al., 2013; Draaisma et al., 2020b). This technique allows to exogenously interfere with ongoing oscillatory activity and to target specific networks, such as the fronto-parietal network (FPN), to enhance or decrease specifically respective cognitive functions, such as WM processes (Polania et al., 2012; Violante et al., 2017). The FPN, a network related to WM, has shown to be activated during motor sequence tasks (Honda and Shibasaki, 1998; Hikosaka et al., 2002; Floyer-Lea and Matthews, 2005; Lin et al., 2012; Pammi et al., 2012). Cognitive processes rely on coordinated interactions within and among brain networks, implemented in the brain by oscillatory activity (Varela et al., 2001; Fries, 2005). For example, efficiency is increased by oscillatory synchronization of neuronal firing, which creates ensembles of neurons that carry out specific computational functions (Fries, 2005, 2015). The main working mechanism of tACS is to entrain with or synchronize neuronal networks (Antal and Paulus, 2013; Fröhlich, 2016). The stimulation frequency is adjusted to match the endogenous oscillatory frequency and its brain state. More specifically, tACS allows to exogenously interact with ongoing oscillations, which can result in enhanced coherence within networks with the respective behavioural impact (Kuo and Nitsche, 2012; Antal and Herrmann, 2016; Fröhlich, 2016). Neuronal oscillations in the theta range (4-8 Hz) are engaged in WM tasks, with an increase in theta power during increased WM load (Sauseng and Klimesch, 2008; Fell and Axmacher, 2011; Constantinidis and Klingberg, 2016). Polania and colleagues and Violante and colleagues have shown a causal relationship

between the synchronization of theta oscillations with a relative 0º phase difference in the FPN and the improvement of WM performance (Polania et al., 2012; Violante et al., 2017). However, knowledge about the effects of tACS induced synchronization of theta oscillations in the FPN and MSL is lacking.

In this study, we aimed to determine a causal relationship between WM and MSL in healthy older adults. To do this, personalized theta tACS was applied to the right dorsolateral prefrontal cortex (DLPFC) and the posterior parietal cortex (PPC) intended to improve MSL by means of the training of the sequential finger tapping task (SFTT) (Karni et al., 1995). To evaluate the importance of WM during MSL and how this is affected by the FPN stimulation, two versions of the SFTT were used. The versions differed in terms of low vs. high WM load. WM load was kept low by explicitly showing the sequence on a screen during the task (Zimerman et al., 2015). In the high WM load version, the sequence had to be memorized prior to the task and was not shown during the task. This online maintenance of the sequence while performing the movements relies relevantly on WM processes (Haith and Krakauer, 2018). In addition, we verify whether the present stimulation paradigm improves WM with the use of an N-back task (Violante et al., 2017). With this study, we introduce the FPN as an additional stimulation target location for motor learning enhancement and shine a light on the importance of taking cognitive processes into account during MSL paradigms.

5.3 Methods

Participants

In this study, we recruited N = 21 healthy, older, right-handed participants (N = 11 female, mean age ± sd: 69.6 ± 4.4, mean laterality quotient Edinburgh handedness inventory 85.03 ± 17.3 (Oldfield, 1971). The data of N = 20 participants were considered due to a drop-out of one participant caused by an unrelated change in physical health. Inclusion criteria were: ≥ 60 years (Rogasch et al., 2009; Hummel et al., 2010; Todd et al., 2010), right-handed and absence of contraindications for transcranial electrical stimulation (tES). Exclusion criteria were: neuropsychiatric diseases, history of seizures, medication that potentially interacts with tES, musculoskeletal dysfunction that impairs finger movements, professional musician, intake of narcotic drugs. All participants have signed an informed consent. The study was performed in accordance to the declaration of Helsinki (World Medical Association, 2013). Ethical approval was obtained from the cantonal ethics committee Vaud, Switzerland (project number: 2017-00765).

Experimental design

The design of this study was double blind, sham-controlled, cross-over. It consisted of two sessions before cross-over and two sessions after cross-over. During the session on day 1, the participants were informed, screened and asked to fill in three different questionnaires (tES safety questionnaire, Edinburgh Handedness Inventory (EHI), Center for Epidemiological Studies Depression Scale (CES-D)) (Oldfield, 1971; Radloff, 1977). Afterwards the participants performed an N-back test with EEG acquisition for peak frequency analysis. Following the EEG measurement, the participants did the motor training and the cognitive training with concurrent tACS. The next day the participants performed only the motor training with tACS. The stimulation condition was kept the same on both consecutive days and was changed after cross-over. The order of stimulation was defined in a pseudo-randomized fashion by an experimenter not involved in the data acquisition. The blindness for stimulation condition of both the participant and experimenter was ensured by an additional experimenter who set the parameters and turned on the stimulators during the experiment. Between the before and after cross-over sessions there was a minimum time period of two weeks, based on our previous work (Wessel et al., 2020). The same tasks, with different sequences, were repeated after cross-over, excluding the questionnaires. Please see figure 1 A for the timeline of the study design.

Motor learning task

Participants executed two different versions of the SFTT based on the SFTT task used in earlier studies (Karni et al., 1995; Wessel et al., 2021). They were asked to perform a 9-item sequence with their non-dominant left hand. The non-dominant hand was used to allow for a larger range of improvement (Wessel et al., 2020). They were orally instructed to continuously tap the same sequence as fast and as accurately as possible on a four-button keyboard (Current Designs, Philadelphia, PA, USA). The sequences consisted of 4 digits from 2 to 5, which corresponded to the four fingers from index (2) to the little finger (5) of the left hand. A cursor underneath the displayed sequence moved in response to every finger tap to identify the target digit, regardless of whether or not the button was pressed correctly. Different sequences were used for the baseline and the training measurements. All sequences were matched in complexity verified with the Kolmogorov complexity test (Lempel and Ziv, 1976). The baseline measurement consisted of one block of 90 seconds, the training measurement consisted of seven blocks of 90 seconds, with 90 seconds breaks after every block which lasted 20 minutes in total. The task was implemented in Presentation software (Neurobehavioral Systems, Berkeley, CA, USA). Participants performed a low and a high WM load SFTT version. The low WM load version displayed the sequence on the screen asking participants to execute the sequence without prior familiarization. A

cursor underneath the displayed sequence moved in response to every finger tap to identify the target digit. This version is referred to as the "non-memorized" version. For the high WM load version, participants had to memorize the sequence before the task started. They received the sequence on a paper and were asked to learn the sequence by heart, without practicing it on the button keyboard. With the use of a distractor task, during which they had to spell random words in a reversed order sufficient memorization of the sequence was verified. More precisely, participants had to spell backwards 3 words in a row and recall the sequence out loud afterwards. After 3 times correct, the sequence was deemed sufficiently learned (Zimerman et al., 2014). During the memorized version of the task, the participants could not see the sequence. Displayed on the screen was a sequence of 9 "X's" with the moving cursor underneath to identify the target digit. Participants performed both versions divided over day 1 and day 2 in a randomized order. This order of versions was reversed after cross-over, see figure 1 C.

Cognitive task

Participants were asked to perform the N-back task to verify whether this stimulation paradigm enhanced WM performance. The task was implemented in Matlab (The MathWorks Inc.,Natick, Massachusetts, USA) and was based on the single N-back task used by Jaeggi and colleagues (Jaeggi et al., 2010). The script was adapted from Quent, A.J. (Quent, 2021) in terms of language (French & English), length and difficulty level. The task consisted of a sequence of visual stimuli that were shown on a computer screen. The participants had to respond by clicking the right "Control" button on a computer keyboard when the stimulus was the same as the stimulus presented N positions back. Participants should not respond when a different stimulus was presented. The visual stimuli consisted of 10 random shapes, eight 8-points shapes (number 14, 15, 17, 18, 20, 22, 23, and 27) and two 12-points shapes (number 20 and 24) taken from Vanderplas and Garvin (Vanderplas and Garvin, 1959). The stimuli were presented for 500 ms each with a 2500 ms interstimulus interval. The participants were required to respond within the response window that starts at the onset of the stimulus until the end of the interstimulus interval (3000 ms). The task consisted of 1 until 3-back levels, in that order. The task was divided into a baseline and training session, with the baseline session consisting of 1 block per n-back level (3 blocks in total) and the training session of 3 blocks per level (9 blocks in total). Every block consisted of 20 + n trials, with 6 targets and 14 + n non-targets. The reaction times, hits, misses, false alarms and correct rejections were measured. Please see figure 1 B for a schematic illustration of the task. We had to exclude N = 9 before cross-over N-back task data sets due to an error in the response recording. A total of N =31 N-back data sets were considered.

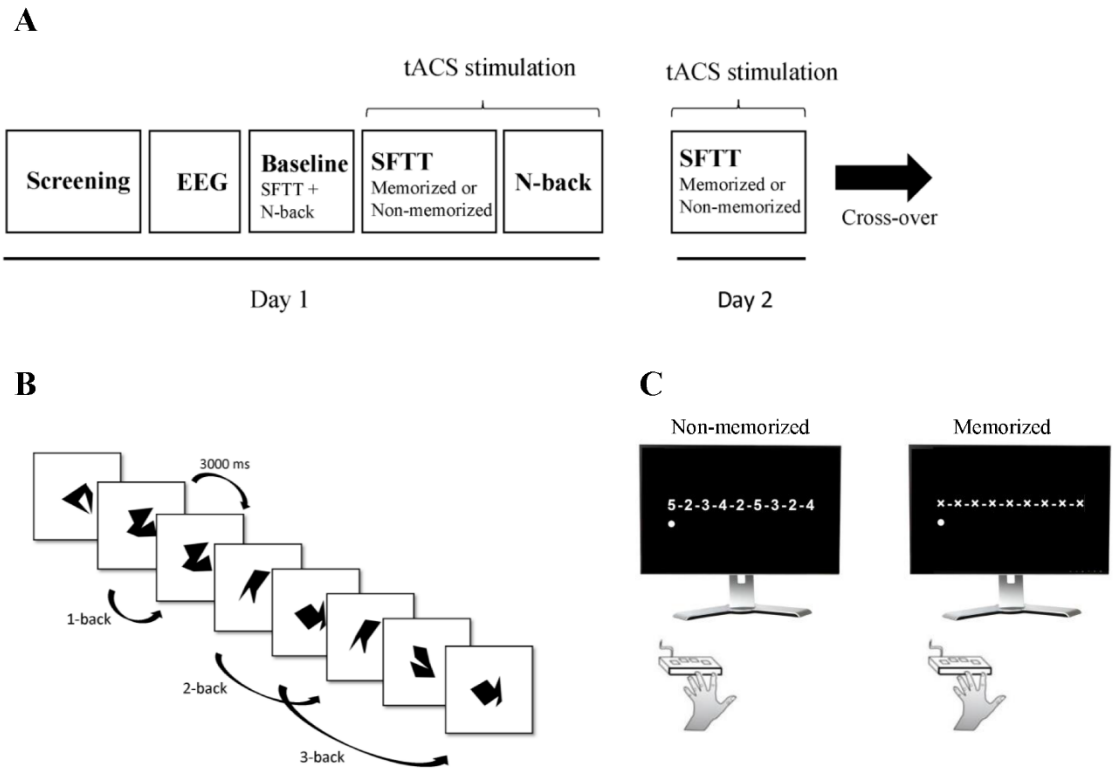

Figure 1. *Experimental design.* ***A)*** *timeline of study design.* ***B)*** *Example of N-back test with the three difficulty levels shown.* ***C)*** *Example of the non-memorized and the memorized version of the SFTT. Please note that with each key press advancing point indicates in both conditions just the position within the sequence*

Transcranial alternating current stimulation

Multifocal tACS was applied to the right FPN using two neuroConn DC plus stimulators to enable bifocal stimulation (neuroConn GmbH, Ilmenau, Germany). Participants received both real (30 min) or sham (30 seconds) stimulation in randomized order, before or after cross-over (Gandiga et al., 2006; Polania et al., 2012; Violante et al., 2017). The stimulation protocol consisted of the following parameters: in-phase (0º phase lag), intensity 2 mA (peak-to-peak) was gradually ramped up/down with an interval of 8 seconds. The in-phase stimulation between the two stimulators was assured by a repeated trigger from stimulator A to stimulator B after every completed cycle to signal the start of a new cycle (Salamanca-Giron et al., 2020). The stimulation frequency was adjusted to the personal theta peak frequency, which was recorded during an EEG recording while performing a pre-baseline N-back test of 1 block per level. Rubber concentric electrodes were used: centre electrode size diameter: ca. 20 mm, area: ca. 3 cm2 and ring electrode size diameter: out 100 mm/ in 70mm, area: ca. 40 cm2. Electrode location was defined with the use of a standard 64 channel, EEG actiCAP with 10/20 system (Brain Products GmbH), targeting F4 corresponding to the dorsolateral prefrontal cortex (DLPFC) and P4 corresponding to the posterior parietal cortex (PPC). The paste used for conductivity with adequately low impedance was SAC2 electrode cream (Spes Medical Srl, Genova, Italy). This paste was adhesive which ensured stable electrode placements. The electrode placement and the electric field distribution were visualised with the use of standard template in SimNIBS (Version 3.2) (Thielscher et al., 2015). The script to implement bifocal stimulation with ring electrodes was adapted from the open access Matlab script (© G. Saturnino, 2018). A template head model was used to simulate the electrode

placement and electric field distribution. For the electrode placement and electric field distribution, please see figure 2 A & B. At the end of the last stimulation session, we investigated whether the stimulation was well tolerated and if there was a significant difference in experienced sensations between the real and sham condition. Moreover, we asked the participants to indicate whether they thought they had received real or sham stimulation during the before and after cross-over sessions. The stimulation sensations were described with the use of a structured interview (Antal et al., 2017). We checked for the following sensations: itching, pain, burning, metallic/iron taste in mouth, warmth, fatigue, other. With the possibility to respond: "none", "mild", "moderate", "strong".

EEG

All EEG recordings were done in a shielded faraday cage. A customized electrode set-up with 9 electrodes was used, Frontal (Fp1, Fp2, F3, Fz, F4), parietal (Cz, P3, Pz, P4), please see figure 2 C. Using a 64-channel ANT Neuro EEG cap with eego^{tm}mylab software (ANT Neuro, Netherlands). EEG was recorded during the performance of the N-back task. With markers, the beginning and the end of every separate N-back level were defined. Recordings were done during 3 N-back blocks resulting in approximately 3 minutes of recording time. The peak frequency in the theta range (4 – 8 Hz) was calculated using a custom Matlab script (The MathWorks Inc., USA) adapted from the script used by Salamanca-Giron and colleagues (Salamanca-Giron et al., 2020) and made suitable for theta frequency analysis during N-back task performance. The target electrodes F4 & P4, which are the same as the stimulation locations show small variance in recorded theta frequency. The average theta frequency for the F4 electrode was 4.71 (range 4.12 – 7.77) and for the P4 electrode 4.97 (range 4.11 – 6.84).

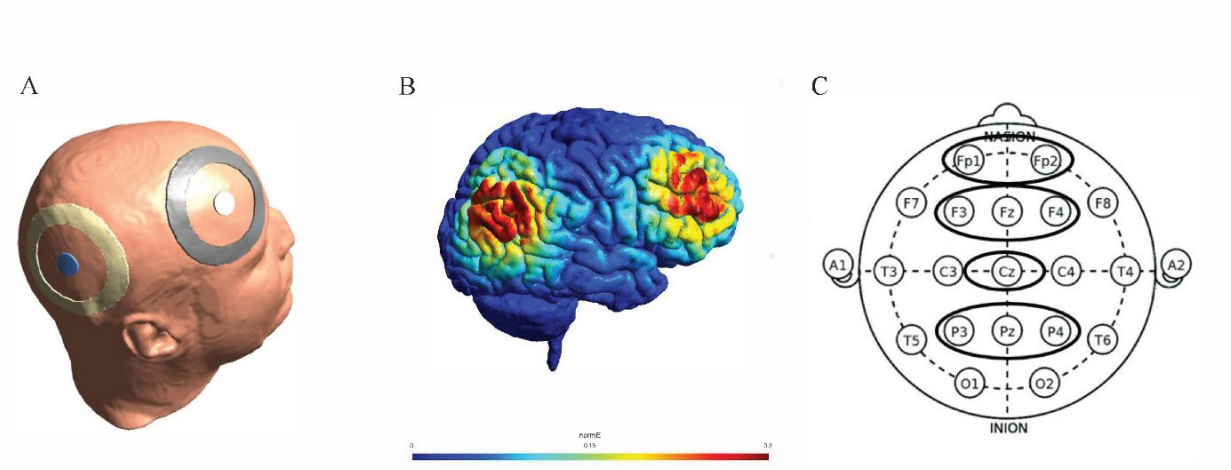

Figure 2. *Bifocal tACS application and EEG recording*. **A**) *Bifocal electrode placement for tACS with concentric electrodes placed on F4 and P4. Image created with the use of SimNIBS software. Head is derived from a standard template provided.* ***B)*** *Simulation of the electric field distribution of tACS set-up created with the SimNIBS software. Label indicates strength of electric field (V/m). Brain is derived from a standard template provided in the program. Stimulation parameters are adjusted to the current study.* ***C)*** *EEG recording sites for the determination of the individual peak frequency during the working memory task.*

Data analyses

Normality of the data was visually checked with histograms and Q-Q plots of residual values and confirmed by verification of skewness ranging between 1 and -1 (Gravetter and Wallnau, 2014). P-values of <.05 indicate statistical significance. Pre-processing of the behavioural data of the SFTT was done with an in-house script implemented in Matlab. Main output measures were: correct sequences, total completed sequences and correct sequences / completed sequences. Pre-processing of the individual N-back data was done with RStudio (version 1.4.1717, 2021)(RStudio Team, 2021). Individual data were combined in one main file using Microsoft Excel. For analysis, the data was normalized by subtraction to the baseline block related to the stimulation condition. The baseline blocks were compared in R using paired-samples t-tests. The equality of the baseline blocks was verified using Bayesian statistics by computing a Bayesian paired-samples t-test with the use of JASP software (version 0.16.0.0). All other analysis of the SFTT and the N-back data were done in Rstudio (version 1.4.1717). Data were analysed with the use of Linear mixed-effects models that were fitted with the "lmerTest" package. Output was type III anova table with p-values for F-tests (Kuznetsova et al., 2017). Effect size was determined using partial eta squared with the "effectsize" package. Post-hoc analysis was done by pairwise comparisons, using the estimated marginal means and Tukey correction. Analysis of the tACS stimulation sensations and blinding responses were analysed with JASP (version 0.8.5.1) (JASP Team, 2019). Responses to the real vs. sham stimulation estimations were analysed using a binomial test. The stimulation sensations were analysed using contingency tables with chi-squared analysis to control for differences between the real and sham stimulation conditions.

5.4 Results

Sequential finger tapping task

The two SFTT's have been analysed separately as they differ in the amount of WM-load (high and low WM load). Prior to the main analysis, the baseline performance between active and sham stimulation was compared and was not significantly different for both the memorized condition t(19) = 0.72, p = .48, d = 0.16, and the non-memorized condition t(19) = 0, p = 1, d = 0 . To further analyse the null-result and to confirm equality of the groups active vs sham groups were compared in both conditions using Bayesian statistics. The analysis indicated for the memorized condition BF01 = 3.41, meaning it is 3.4 times more likely that the baseline results are equal than different. The non-memorized condition indicated BF01 = 4.3, therefore is it 4.3 times more likely that the baseline groups are equal.

In this study, online learning is defined as a significant improvement of behaviour within the training session. A significant effect of stimulation on learning is defined by a change in improvement dynamics during the training. With the use of a linear mixed effects model the analysis of the amount of correct sequences of the memorized version of the SFTT showed a significant effect for blocks F(6, 247) = 18.57, p < .001, ηp2 = 0.31 indicating a large effect size, as well as a significant effect for stimulation F(1, 247) = 18.83, p <.001, ηp2 = 0.07 with a medium effect size, but no blocks x stimulation interaction F(6, 247) = 0.77, p = 0.59, ηp2 = 0.02. To further define the effect of stimulation on learning, we determined the difference between the conditions at the end of the training, which showed a strong trend for a significant difference t(19) = -2.07, p = .052, d = -0.46. The results of the non-memorized version show a significant effect for blocks F(6, 247) = 16.00, p <.001, ηp2 = 0.28 (large effect), but no stimulation F(1, 247) = 0.46, p = .499, ηp2 = 0.002 or interaction effect F(6, 247) = 0.36, p = .901, ηp2 = 0.009. Indicating that in both conditions, participants learned significantly but only in the memorized condition the tACS stimulation affected learning compared to sham, see figure 3. To further investigate the results on the SFTT and appreciate the variance in performance the individual trajectories of the participants are indicated in the supplementary material, supplementary figure 1.

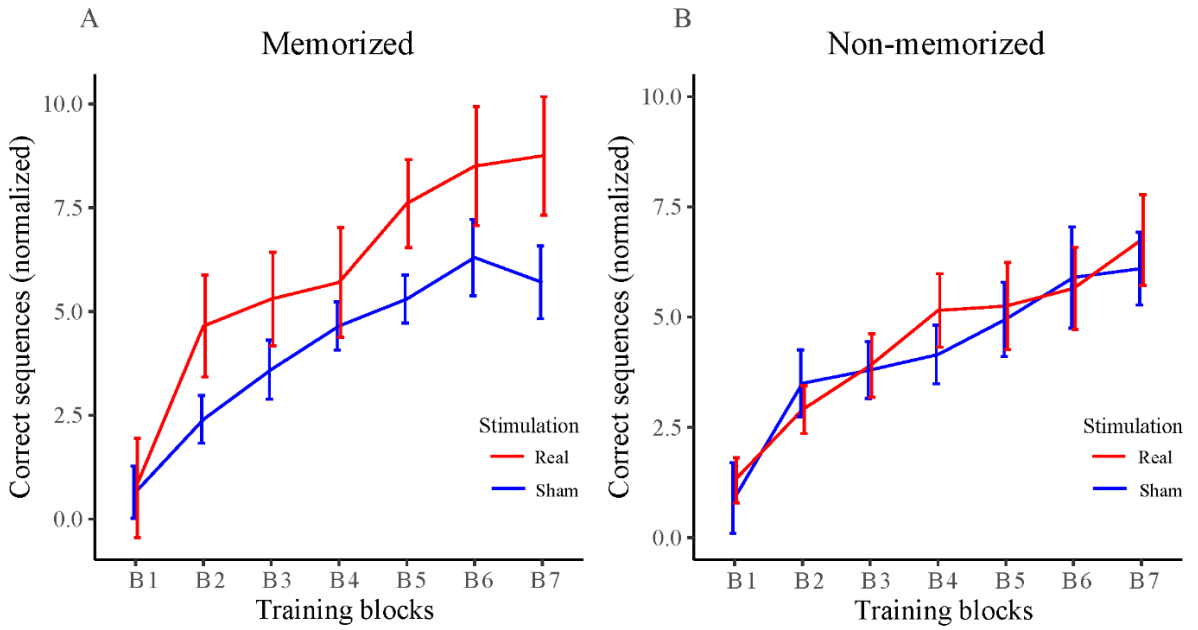

Figure 3. *Plot of the correct sequences of the SFTT, results are normalized to baseline by subtraction. Error bars show standard error of the mean (SEM). On the left, the results of the memorized version. On the right, the results of the non-memorized version. Please note a significant stimulation effect with enhanced behavioural improvement in the memorized version (left graph).*

Speed and accuracy

To further investigate the results of the memorized condition, the total amount of completed sequences were analysed as a measure of speed. The results showed a significant block effect $F(6, 247) = 28.21$, $p < .001$, $\eta p2 = 0.41$ (large effect) and a significant stimulation effect $F(1, 247) = 15.92$, $p < .001$, $\eta p2 = 0.06$ (small effect), but no interaction effect $F(6, 247) = 0.23$, $p = 0.968$, $\eta p2 = 0.006$, see figure 4A. Although the active stimulation group is faster compared to the sham group, the similar pattern of improvement points towards a performance rather than a learning effect.

In order to see whether the increased number of correct sequences was driven by faster sequence execution or by a simultaneous increase of accuracy, we analysed the ratio between the total amount of sequences and the correct sequences as an accuracy measure. Upon inspection, the real stimulation group shows different dynamics in accuracy than the sham group. The real stimulation group demonstrates a steep significant increase in accuracy between the first and the second training block while the sham group's increase is more gradual $t(19) = -2.68$, $p = .015$. The accuracy between the groups during the 1st training block was not significantly different $t(19) = 0.85$, $p = .404$. Therefore, to visualize the difference in dynamics we measured the difference in accuracy with regard to block 1. Results showed a significant block effect $F(6, 247) = 3.47$, $p = .003$, $\eta p2 = 0.08$ (medium effect), and a significant stimulation effect $F(1, 247) = 18.31$, $p < .001$, $\eta p2 = 0.07$ (medium effect), but no block x stimulation interaction $F(6, 247) = 0.85$, $p = .529$, $\eta p2 = 0.02$, see figure 4B. In an additional analysis the comparison of behavior on block 7 shows a significant difference between verum and sham $t(19) = -2.31$, $p = .032$, $d = -0.51$. Indicating that accuracy significantly improved with stimulation in the early stage of training.

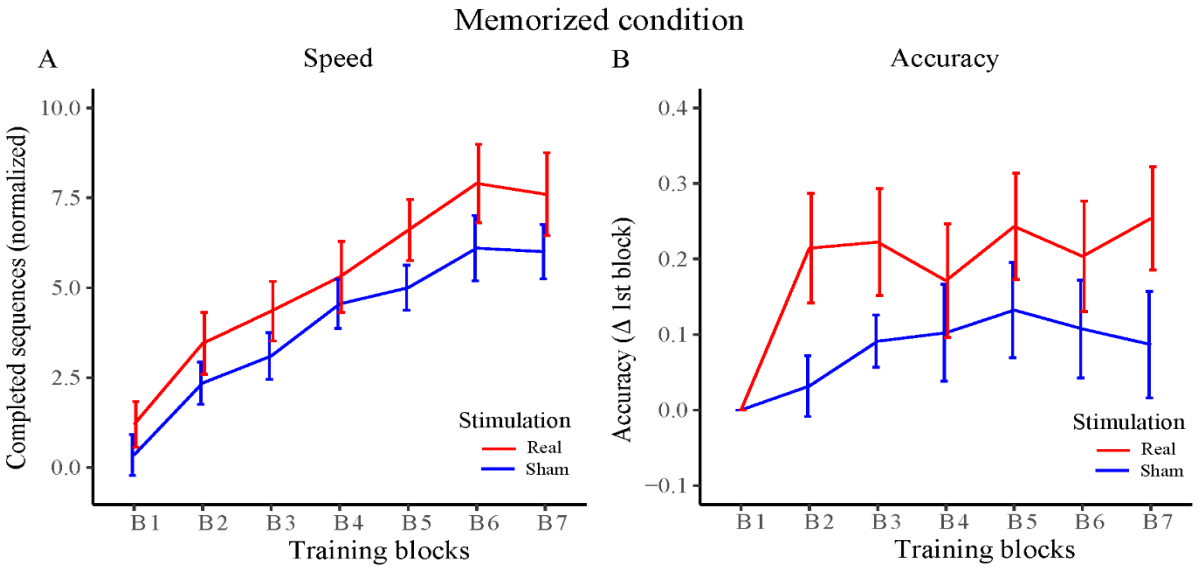

Figure 4. *Plots of the speed and accuracy of the SFTT in the memorized condition. The error bars indicate standard error of the mean (SEM). Plot* ***A)*** *shows the speed determined by the total amount of completed sequences. Higher numbers depict better performance. Plot* ***B)*** *Shows the difference in accuracy as determined by the ratio of correct sequences divided by completed sequences, with regard to the first block. Please note the tACS significantly enhanced both, speed (A) and accuracy (B).*

N-back task

The N-back task performance was analysed by the following outcomes: hits, false alarms, accuracy (hits – false alarms), and reaction time for hits. All parameters were analysed separately using linear mixed effects models. The stimulation conditions (real vs. sham) and the three N-back difficulty levels were included as independent variables in the model. Two separate analyses were performed, one model included difficulty levels 1 and 2 to mimic the conditions comparable to the study of Violante *et al.* (2017), additionally we added difficulty level 3 to the model to test for a stimulation effect on the task with higher cognitive demand (Violante et al., 2017).

Reaction time

We were able to replicate the results of Violante and colleagues for the parameter reaction time with a significant effect of stimulation $F(1, 47.10) = 5.33$, $p = .025$, $\eta_p^2 = 0.1$ (medium effect), as well as an effect for difficulty level $F(1, 35.77) = 44.61$, $p < .001$ $\eta_p^2 = 0.55$ (large effect), and an interaction effect $F(1, 34.54) = 4.83$, $p = .035$, $\eta_p^2 = 0.12$ (medium effect). Post-hoc analysis with Tukey correction showed a significant difference between sham and real stimulation during difficulty level 2, $t(42.1) = 3.22$, $p = .013$, but not for level 1 $t(42.6) = 0.20$, $p = .997$. Both conditions showed a significant increase in reaction time between level 1 and level 2, which was more prominent in the sham condition $t(36.4) = -6.16$, $p < .001$ than the real condition $t(36.4) = -3.25$, $p = .013$ Adding the 3-back difficulty level to the model resulted in no effect for stimulation $F(1,78.24) = 1.61$, $p = .209$, a significant effect for difficulty level $F(2, 67.65) = 34.99$, $p < .001$ and no interaction effect $F(2, 67.65) = 0.75$, $p = .478$, see figure 5.

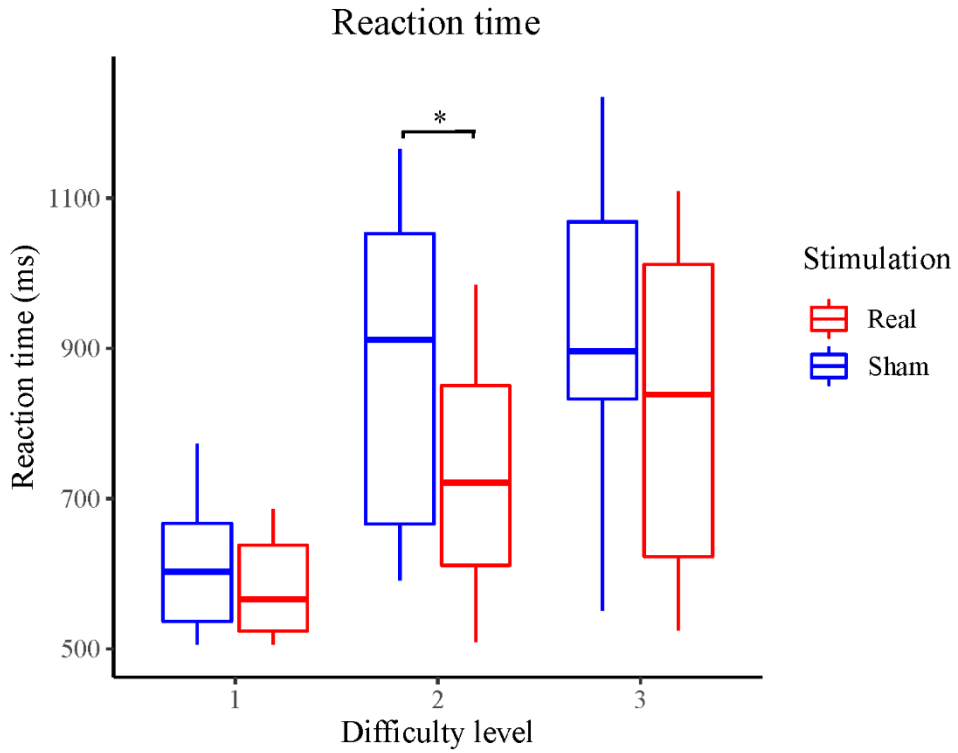

Figure 5. *Figure of the reaction time for the hit responses on the N-back task. Difficulty levels 1-, 2- and 3-back included. The box ranges from Q1 (the 25th percentile) to Q3 (the 75th percentile), the bar shows the median. Results show better performance in reaction time during level 2-back for the real stimulation condition compared to sham.*

N-back performance parameters (hits, false alarms, accuracy)

The analyses did not show a main effect of stimulation on any of these parameters for the 2 and the 3 level of difficulty models. There was a significant main effect of difficulty level. Indicating a significant decrease in performance with increasing n-back levels on all parameters. There were no stimulation x difficulty interaction effects. Please see table 1 for statistical results.

Table 1. **N-back parameters**

Parameter	**Statistics**		
Model 1	**Stimulation**	**Difficulty**	**Interaction**
Hits	$F(1, 40.4) = 0.80$, $p = .375$	$F(1, 34.5) = 39.65$, $p < .001$	$F(1, 33) = 0.49$, $p = .488$
False alarms	$F(1, 33.1) = 0.54$, $p = .467$	$F(1, 29.4) = 56.78$, $p < .001$	$F(1, 29.4) = 0.87$, $p = .357$
Accuracy	$F(1, 32.1) = 0.12$, $p = .733$	$F(1, 25.9) = 85.12$, $p < .001$	$F(1, 25.9) = 0.05$, $p = .823$
Model 2	**Stimulation**	**Difficulty**	**Interaction**
Hits	$F(1, 75.4) = 0.70$, $p = .407$	$F(2, 65.2) = 69.46$, $p < .001$	$F(2, 65.2) = 2.05$, $p = .137$
False alarms	$F(1, 76) = 1.05$, $p = .309$	$F(2, 65.8) = 42.26$, $p < .001$	$F(2, 65.8) = 0.43$, $p = .651$
Accuracy	$F(1, 75.5) = 0.02$, $p = .892$	$F(2, 65.4) = 65.38$, $p < .001$	$F(2, 65.4) = 1.57$, $p = .215$

Statistical results of all the n-back parameters. Columns show the main effect and the interaction effect of the independent variables. Model 1 shows the analysis with difficulty level 1 and 2, model 2 additionally includes level 3.

Peak frequency analysis

During the EEG measurements data from 9 electrodes were acquired during the performance of the pre-baseline measurement of the N-back task. The individual average peak frequency of the 3 N-back levels combined was used as the personalized theta stimulation frequency for the rest of the study. Results of the overall average peak frequency showed a group mean of 4.5 (sd = 0.28) with a range between 4.1 and 5.4. For more details, please see table 2.

Table 2. **Peak frequency analysis**

	Mean Theta	1-back	2-back	3-back
Mean (sd)	4.48 (0.28)	4.42 (0.35)	4.46 (0.46)	4.51 (0.65)
Minimum	4.1	4	4	4
Maximum	5.4	5.7	6	7.8
Missing	0	0	0	1

Descriptive statistics of the peak frequency analysis measured during the three levels of the pre-baseline N-back measurements. The results show the overall means from the three levels combined and the means per N-back level. For the personalized stimulation paradigm, the individual mean peak frequency of the 3 N-back levels combined was used.

Stimulation sensations & blinding

Based on the stimulation sensation interview, there were no adverse effects due to the tACS stimulation and only minor tACS sensations were reported. Most participants responded either with "none" or "mild". Moreover, there was no significant difference between the stimulation and sham condition for any of the perceived sensations, see table 3.

Participants were not able to discriminate between real and sham stimulation. A binomial test indicated that the proportion of correct answers during session 1 was 0.4, which was not significantly different than the chance level (0.5), $p = .503$. For session 2, the proportion of correct answers was 0.6, which was not significantly different than chance, $p = .503$, see table 4.

Table 3. **Stimulation sensations**

	None		**Mild**		**Moderate**		**Strong**		**Statistics**	
	Real	**Sham**	**Real**	**Sham**	**Real**	**Sham**	**Real**	**Sham**	**Chi-square**	***p*-value**
Itching	75	80	20	15	5	5	0	0	0.18	.916
Pain	85	85	5	15	10	0	0	0	3.00	.223
Burning	80	80	10	20	10	0	0	0	2.67	.264
Warmth	70	90	30	10	0	0	0	0	2.50	.114
Metallic/iron taste	100	100	0	0	0	0	0	0	n/a	n/a
Fatigue	85	80	15	20	0	0	0	0	0.17	.677
Other	80	80	15	15	5	5	0	0	0.00	1.000

tACS sensations shown in percentages of participants who chose that specific response option for the intensity of the sensation. Statistics show comparison between real and sham stimulation

Table 4. **Descriptive statistics for the tACS blinding.**

Session	**Answer**	**Frequency**	**%**
Before cross-over			
	Correct	8	40
	Incorrect	12	60
	Total	20	100
After cross-over			
	Correct	12	60
	Incorrect	8	40
	Total	20	100

The frequencies of correct and incorrect distinctions between real and sham stimulation before and after cross-over.

5.5 Discussion

The main outcome of this study is that personalized and synchronized tACS to the right FPN can enhance performance on the SFTT with high WM load in healthy older participants. This stimulation paradigm did not affect the performance on SFTT with low WM load. These findings indicate that the efficacy of bifocal theta tACS applied synchronously to DLPFC and PPC is dependent on the underlying cognitive state during the task. This result is further supported by the findings that tACS also improved N-back task performance specific to a difficulty level that was demanding enough.

Motor task

The present results support the view of a causal effect of synchronised bifocal theta frequency oscillations applied to the right FPN on MSL. Correlative evidence of the activation of the FPN during finger tapping tasks has been previously shown using neuroimaging (Witt et al., 2008; Maruyama et al., 2021). The meta-analysis of Witt and colleagues (2008) has shown that visually or self-paced finger tapping tasks induce concordant activity in the right DLPFC and the right inferior parietal cortex (Witt et al., 2008). However, to the best of our knowledge, this was the first time the FPN was used as a target for a tACS paradigm with the intend to improve MSL.

We were able to demonstrate an improvement in MSL with this approach, but exclusively for the SFTT condition with high WM load (memorized condition). Therefore, the efficacy of the present orchestrated stimulation paradigm on motor behaviour was dependent on the amount of WM load during the task. This is in line with the study of Violante *et al.* (2017) that showed that theta tACS to the right FPN improved performance on a WM task, but only for the task with higher WM load (Violante et al., 2017). This might be explained by the fact that the FPN shows more coherence in the theta range during WM tasks with high WM load (Sauseng et al., 2005). With the use of tACS it is suggested to be able to exogenously enhance coherence by the entrainment of the cortical oscillation between distant regions (Antal and Paulus, 2013). Although we did not verify network coherence with the use of EEG or other neuroimaging measures, we hypothesize that exogenously induced theta oscillations might have amplified the ongoing oscillations engaged in WM processing, which in turn has supported the training of the motor task with high WM load, but not with low WM load. Another possible explanation is that the involvement of the FPN is related to a specific sub-process of WM. WM can be roughly divided into three sub-processes: encoding, maintenance and retrieval (Kim, 2019). The non-memorized SFTT condition required the participants to learn the sequence while performing the movements, which falls under the encoding phase. During the memorized SFTT condition, the participants needed to maintain and retrieve the previously learned sequence while performing the movements. A recent meta-analysis has shown that during the transition from encoding to maintenance and retrieval stages, the involvement of the FPN progressively increases. Therefore, it can be well hypothesized that the memorized SFTT condition benefits more from the FPN as a target while the non-memorized SFTT condition profits more from stimulation of other brain regions. For instance, the acquisition phase relies heavily on the dorsal attention network (DAN), which predominantly includes the frontal eye fields and the intraparietal sulci (Kim, 2019; Chabran et al., 2020). Moreover, studies have shown a high involvement of the M1 during the early stages of learning, with a reduction of activity to baseline when a sequence becomes explicitly known (Pascual-Leone et al., 1994a; Dayan and Cohen, 2011).

The involvement of the frontal and parietal areas during MSL has been well established, however their precise functional role is less clear (Dayan and Cohen, 2011; Hardwick and Celnik, 2014; Doyon et al., 2018; Pollok et al., 2020). MSL can be divided into three different learning phases: stage 1 for acquisition, stage 2 for consolidation and stage 3 for retention. The early learning phase relies more

heavily on cognitive processes such as WM, showing an activation in the prefrontal cortex and parietal areas (Anguera et al., 2012; Janacsek and Nemeth, 2013; Heinzel et al., 2017). In this study, the efficacy of targeting the FPN to enhance performance on the MSL task is most likely specific to WM load. Studies that focused on the WM processes found that the FPN is associated with the maintenance and manipulation of information when theta oscillations were in synchrony between the two brain areas (Sauseng et al., 2005; Polania et al., 2012). This might explain why the performance on the motor sequence task only improved during the high WM load task, where the participants had to perform the sequence from memory. Moreover, both accuracy and speed improved significantly in the memorized condition due to the tACS stimulation. However, the real stimulation induced a sharp increase in accuracy, while the sham group improved more gradually. Thereby showing a difference in improvement dynamics, which was not driven by a difference in initial performance. The speed measure did not show differences in improvement dynamics, even though the effect of stimulation was significant. We argue that the improvement in online learning was mainly driven by an improvement in accuracy, but that the improvement in speed rather points towards a performance improvement. Both components have played a role in the overall dynamics of the behavioral improvements and the respective stimulation effect. Similar results have been shown in a study comparing real vs. sham anodal transcranial direct current stimulation (atDCS) applied to the M1 on a SFTT. Different age groups were compared and older adults showed a sharp increase in accuracy in the real stimulation group and a gradual increase in the sham group (Maceira-Elvira et al., 2021). They argue that the active M1 stimulation facilitated the encoding and storage of the sequence in memory. In the current study, the stimulation target was the FPN and was effective in the memorized condition when the sequences were already learned. This result could be driven by an enhanced capacity to maintain and retrieve the previously learned sequence, due to the synchronization of theta oscillations in the FPN (Kim, 2019).

This study aimed to extend previous studies that have targeted the FPN with bifocal theta tACS to improve WM performance (Polania et al., 2012; Alekseichuk et al., 2017; Violante et al., 2017; Röhner et al., 2018) by using a similar setup to study the effects on MSL. This has been the first time that both the DLPFC and the PPC have been targeted with the use of bifocal theta tACS during a motor sequence learning task. The main aim was to target the FPN as a network that has shown to be important for WM and has shown activation MSL (Honda and Shibasaki, 1998; Hikosaka et al., 2002; Floyer-Lea and Matthews, 2005; Lin et al., 2012; Pammi et al., 2012; Polania et al., 2012; Alekseichuk et al., 2017; Violante et al., 2017; Röhner et al., 2018). Although we were able to show that bifocal tACS to the FPN was effective when WM-load was high, we cannot exclude that this effect might have been generated by a monofocal stimulation of either the DLPFC or the PPC. This study did not intend to compare the efficacy of monofocal to bifocal theta tACS on MSL. However, based on the positive effects of targeting these areas with bifocal theta tACS to improve MSL more research is necessary to define the exact working mechanisms and to determine the effects of monofocal stimulation to either of the two areas separately. Due to the lack of comparative studies, no final conclusive statement can be made about the beneficial effects of bifocal FPN stimulation over targeting one single of the target brain areas. Further research in upcoming studies will have to address this open question in detail.

Personalized tACS

This study has used personalized tACS stimulation in the theta range on MSL and cognitive function. This approach was based on a study of Reinhart and Nguyen who showed beneficial effects of personalized fronto-temporal theta tACS compared to a standard theta tACS on a WM task in healthy older adults (Reinhart and Nguyen, 2019). Individual peak frequencies were measured while participants performed the N-back task to determine the individual stimulation frequency, though a comparison between personalized and standard theta was not in the scope of the present study. We assume that individualizing stimulation paradigms might be important due to a more effective peak

frequency as suggested by e.g., Reinhart and Nguyen, but also based on the differential functional effects of low theta frequencies (4-4.5 Hz) compared to high theta frequencies (7 Hz) on WM performance (Wolinski et al., 2018; Bender et al., 2019; Jones et al., 2019). More specifically, 4 Hz tACS to the right parietal cortex improved WM capacity, while 7 Hz tACS reduced WM capacity in healthy young adults (Wolinski et al., 2018; Bender et al., 2019; Jones et al., 2019). Jones et al. compared bifocal 7 Hz tACS to 4.5 Hz tACS applied to the FPN and found positive effects for 4.5 Hz, but not 7 Hz stimulation on WM performance (Jones et al., 2019). However, there are also reports which did not show effects of personalization such as in a current TMS study (Brownjohn et al., 2014). The average stimulation frequency in our study was 4.5 Hz, which fits with the abovementioned low theta frequencies relevant for WM. However, as the comparison between personalized and standard theta was not in the scope of the present study, we cannot assure that personalization here is more effective than non-personalised bifocal tACS in the theta range, an interesting question that has to be addressed in upcoming studies.

N-back task

The reason for the use of the N-back task was twofold. First and foremost, as a way to measure the individual theta frequency while performing a WM task. Second, it was used as an additional control experiment to verify that the stimulation was indeed directed to the FPN and modulates a key function processed by the FPN. The behavioural results of the WM task support the notion that theta tACS to the FPN enhances WM performance (Polania et al., 2012; Violante et al., 2017). As there was no neuroimaging data to confirm that the FPN was indeed targeted, a behavioural difference in WM performance provides correlational evidence.

In this study, we could replicate the observations of Violante et al. showing that exogenous synchronization of cortical oscillations in the theta range improved WM performance when cognitive demands were moderately high (2-back level) (Violante et al., 2017). We have extended the results with showing that this was only applicable to level 2-back and not the more difficult 3-back level. The efficacy of the stimulation paradigm seems to follow an inverted u-shape in relationship to the difficulty of the task. The present study cohort were healthy older adults. Although it is currently unclear whether young adults would still benefit from the oscillatory synchronization during the 3-back task, one could speculate that the inverted u-shape with the peak at the 2-back task is age related.

Studies that have compared performance on WM tasks between young and healthy older adults have shown age related reduction in performance especially in tasks with high cognitive demand (Nyberg et al., 2009; Nagel et al., 2011). In response to high WM load, older adults show a relative hypoactivation in fronto-parietal regions compared to young adults (Rajah and D'Esposito, 2005; Nagel et al., 2011). The "Compensation-Related Utilization of Neural Circuits Hypothesis" (CRUNCH) provides a framework for this phenomenon; age-related hyperactivations are seen during tasks with low WM load due to reduced neural efficiency, with hypoactivation for tasks with high WM load due to reduced neural capacity (Reuter-Lorenz and Cappell, 2008). Showing that older adults use compensatory mechanisms already with low WM load tasks (1-back) and are therefore not able to recruit the necessary neural resources during high WM load tasks (3-back) (Nyberg et al., 2009; Nagel et al., 2011). Heinzel and colleagues hypothesized that the change in neuronal activity is due to a decrease in FPN coupling; they showed that fronto-parietal connectivity decreased in older adults during 2-back and even more during 3-back tasks (Heinzel et al., 2014, 2017). This could indicate that the difference in efficacy of stimulation between the 2-back and the 3-back tasks is related to the degree of deficient coupling of the FPN within these tasks and that the interventional approach with tACS could only sufficiently compensate these mechanisms for the 2-back task, but not any more for the 3-back task. The lack of improvement during the 1-back condition could indicate that the natural compensatory mechanisms are not sensitive to the effects of this stimulation paradigm. This points towards a specific efficacy that is

dependent on the brain state caused by the amount of WM load.
The results of the N-back task showed a specific effect on reaction times, and not on hit-rates, false alarms, and accuracy. These findings are similar to previous studies by Polania et al. (2012), Violante et al. (2017), and Alekseichuk et al. (2017) that used theta tACS to target the FPN. Synchronized tACS decreased reaction times (Polania et al., 2012; Violante et al., 2017), while desynchronized tACS increased the reaction time on a visual WM task (Polania et al., 2012; Alekseichuk et al., 2017). The exact reason for the effect on reaction times, but not on other parameters remains elusive. Violante et al. showed a relation between increased parietal BOLD activation and decreased reaction times (Violante et al., 2017). Evidence suggests a critical role of the parietal area in WM maintenance (Pessoa et al., 2002). Therefore, Violante et al. suggest that the increase in neural activation in the parietal areas might have interacted with the mechanisms related to reaction times (Violante et al., 2017). However, Alekseichuk et al. argue that the improved reaction times are network-related, as they found increased reaction times after desynchronization of the prefrontal areas from the parietal areas (Alekseichuk et al., 2017). They argue that this is due to a decline of information uptake, reflected in the outlasting theta rhythm desynchronization in the cortex (Alekseichuk et al., 2017). Although the results seem to point towards specific effects of synchronized theta tACS on reaction times, the exact mechanisms remain unclear. Further analysis is necessary to disentangle the exact physiological mechanisms of responses during WM tasks.

Future steps

The present study was a proof-of-principle study with the aim to investigate the involvement of the FPN in motor sequence learning. This study has a few limitations, which are discussed by means of suggestions for future studies. Firstly, multiple training sessions and/or a follow up session will enhance our understanding about the consolidation and possible retention of behavioural improvement. Motor learning encompasses multiple processes such as online and offline learning. Online learning is the improvement during the training of the task; offline learning happens after training and is a vital part of the consolidation of learned behaviour (Robertson et al., 2004a, 2005; Reis et al., 2009; Dayan and Cohen, 2011). Multiple sessions will allow to investigate whether improvement continues with multiple training sessions and whether it retains during longer periods. Secondly, future studies should additionally include a standardized frequency (e.g., 6 Hz) as used in many other tACS studies, to compare with a personalized frequency (Kuo and Nitsche, 2012; Polania et al., 2012; Violante et al., 2017). Comparing the standardized to a personalized stimulation paradigm could provide more conclusive results about the importance of personalization to endogenous oscillatory activity. Lastly, to further personalize the approach future studies should personalize the placement of electrodes to the individual brain based on simulations. In the current study the electrode placement was defined by standardized locations using an EEG cap with the 10/20 system. We have used concentric electrodes and each montage consisted of a small circular centre electrode surrounded by a larger return electrode. This set-up has shown to improve focality compared with other electrodes such as the 5 x 5 cm rectangular electrodes or ring electrode set-ups with the return electrode on a separate region (Saturnino et al., 2017). For a simulation of the electric field distribution, please see figure 2B. This improved focality highlights the importance of precision of the electrode placement as the stimulation is most effective close to the centre of the electrodes (Nitsche et al., 2007). The currently used technique based on the 10-20-electrode system has been widely used in NIBS studies (Woods et al., 2016). However, this is a standardized electrode placement system based on anatomical landmarks that can vary across participants (Herwig et al., 2003). A recent study of Scrivener and Reader compared the locations of the electrode placements with the use of an EEG cap with MRI images of the same participants. They found that the electrode placements deviated from the actual cortical locations with the smallest SD of 4.35 mm in frontal areas and the largest SD of 6.25 mm in the occipital and parietal areas (Scrivener

and Reader, 2021). These deviations are unlikely to result in any behavioural differences due to the focality of the stimulation. However, it does show that there is room for improvement in terms of precise definition of target locations and consistency in electrode placement. A way to improve precision is by using neuronavigation techniques guided by structural neuroimaging or with the use of functional MRI to pinpoint the exact target locations for stimulation (Woods et al., 2016).

Conclusion

In conclusion, in this study, we were able to show a causal relationship between stimulating the FPN and improvements on MSL. Moreover, we were able to show distinctive efficacy of FPN synchronization for motor tasks with low- and high WM load, resulting in significant enhancement of performance in the motor task with high WM load, but no stimulation effects on the motor task with low WM load. The mechanisms of action point towards an effect of the stimulation paradigm on an improved capacity to maintain and manipulate the sequences. The current knowledge about using tACS to target frontal and parietal areas to improve MSL is limited. However, these results indicate that targeting the FPN as a network using personalized bifocal oscillatory stimulation is a promising approach. In addition, the present study showed that theta tACS applied to the FPN improved WM performance. This reveals an important interplay between the motor and cognitive domain pointing to it as a promising target for interventional strategies based on NIBS. However, to do this successfully, it is critically important that such an approach might only be effective when then cognitive load of a respective task is significantly high as demonstrated here by the WM load.
Taken together, personalized orchestrated bifocal tACS applied to the FPN might be a promising strategy to enhance motor sequence learning in healthy older adults and potentially in neurological patients showing deficits in motor learning.

6. General discussion

The main aim of this thesis was to acquire a better mechanistic understanding of neuronal processes induced by NIBS, which could enhance performance on motor learning tasks and ultimately improve rehabilitation strategies for upper-limb impairments. In this regard, the efficacy of alternative NIBS strategies on motor learning tasks was investigated. These alternative strategies included: targeting other brain areas of the motor network than the M1, using multifocal stimulation to target the brain as a network, and exploring the effect of different NIBS techniques to modulate neuronal activations. In addition, we used TMS-based methods to determine underlying electrophysiological mechanisms of the effects of NIBS applied concomitantly to motor learning, such as intracortical inhibition (SICI) and intracortical facilitation (ICF). With these three different studies (Draaisma et al., 2022 *(under revision*); Wessel & Draaisma et al., 2020; Wessel & Draaisma et al., 2022 (*in preparation)*), we explored the effects of conceptually different stimulation concepts impacting motor learning. Before the interventional studies, we performed a methodological study to determine the most suitable TMS-based methods (paired-pulse TMS) to evaluate underlying mechanisms based on intracortical inhibition and facilitation (Wessel & Draaisma et al., 2019). Our findings showed promising effects of the novel interventional protocols expanding the current knowledge of the field. In stroke patients, orchestrating stimulation to the M1 and CB during the training of a motor task resulted in positive effects on learning compared with M1 stimulation only. Moreover, targeting the FPN in healthy older adults did enhance motor performance. tACS in the gamma range, applied to the CB did not enhance motor learning in healthy young adults. This thesis provides useful building blocks for the improvement of stimulation effects and the possibility to ultimately work towards a more personalized treatment strategy. In the following section, I will discuss the results of the studies based on the alternative strategies

6.1 Alternative stimulation targets

Most NIBS studies to enhance motor learning in healthy individuals and stroke patients have predominantly focused on targeting the M1 (Wessel et al., 2015; Dupont-Hadwen et al., 2019). However, results have been heterogeneous with responders and non-responders. Motor learning and stroke recovery are embedded in a large network comprising brain areas besides the M1, such as frontoparietal regions, including secondary motor areas, somatosensory and prefrontal areas, as well as subcortical areas like the CB (Dayan and Cohen, 2011; Hardwick et al., 2013; Koch et al., 2021). Therefore, these areas are promising targets for neurostimulation. This thesis focused on the inclusion of the CB and the FPN as stimulation targets modulated by specific brain stimulation protocols. We were able to show that both the CB and the FPN are promising targets for neuromodulation to enhance motor learning. The findings of the thesis further point toward and support the view that stimulation protocols for neuromodulation must be applied in a specific way adjusted to the cognitive functions, tasks, and cohort, which should be modulated to achieve large and reliable effects.

Cerebellum stimulation

The CB is a secondary motor area that is involved in motor learning and motor control (Hardwick et al., 2013). The dense connections to cortical motor areas and the rich variety in plasticity mechanisms make the CB a promising target for NIBS studies (Carey, 2011; Bostan et al., 2013). The CB as a stimulation target is a relatively new field with many unanswered questions. In this thesis, we aimed to answer some of these questions by targeting the CB during a motor learning task with the use of 50 Hz tACS. At the time of data acquisition, the effect of 50 Hz CB tACS had not yet been studied on motor

learning. In summary the present findings did not show a significant effect on motor learning in healthy young individuals.

The current study was inspired by previous studies of Naro and colleagues who did find positive effects of 50 Hz CB-tACS on the performance of motor movements (Naro et al., 2016, 2017). Naro *et al.* showed improvements on measures such as fine-tuned finger movements (e.g. picking up a paperclip, turning a key in a lock) and grip strength. The task of our study consisted of grip force modulation movements, that are less accurate than fine individual finger movements. The reason for this was that the current task was designed with two core goals: to be similar to common daily-life movements such as grasping and to be suitable for studies with patients with motor impairments. A possible explanation for the lack of a stimulation effect might be that the CB is more involved in the execution of accurate and fine-tuned movements (Ito, 2000) than in gross whole hand movements. As the current task depended on grip force modulation of the hand it might have been less sensitive to fine-tuned accuracy driven by the CB.

The PC in the CB are the core responsive component of tES (Galea et al., 2009; Naro et al., 2016) and are relevantly involved in motor learning processes (Ito, 2000; Nguyen-Vu et al., 2013). An animal study has shown that these PC peak frequency spikes during upper limb movements are rather at higher frequencies (100 Hz) (Thach, 1968). Moreover, a study using magnetoencephalography (MEG) analysis showed high gamma frequency >65 Hz in the CB during a self-paced finger movement task (Dalal et al., 2008). We argue that the present negative result might be related to gamma frequencies that were too low. Indeed, later studies have shown significant effects of CB-tACS in a higher frequency range (70 Hz) on an isometric force tracking task and a bimanual motor performance task (Miyaguchi et al., 2018, 2020, 2022). Moreover, a recent study has shown a decrease in performance on a motor sequence learning task due to 50 Hz CB-tACS (Giustiniani et al., 2021). Based on our and the recent findings, we argue that 50 Hz CB-tACS might not be the optimal stimulation frequency and that future studies should investigate a more optimal range of gamma frequency.

The modulation of SICI plays an important role in motor learning and can be influenced by brain stimulation (Butefisch et al., 2000; Ziemann et al., 2001; Floyer-Lea et al., 2006; Stagg et al., 2011a; Amadi et al., 2015). We were able to correlate low SICI modulation to better performance. Our findings are not in line with earlier results that show that more disinhibition towards the movement correlates with better motor performance (Heise et al., 2013). However, these results stem from a lifespan cohort ranging between 20-88 years. Therefore, increased modulation might have been an age-related compensatory mechanism (Gleichmann et al., 2011). We speculate that less disinhibition in the pre-movement phase might signify a well-tuned motor system with effective inhibitory control in healthy young adults. The results of this study do not allow to make conclusive inferences about the function of disinhibitory mechanisms after motor learning and require further research to better define the exact mechanisms of action.

In summary, we tested for the first time the effect of 50 Hz tACS during a sequential force modulation task in a cohort of healthy adults. Although we did not significantly enhance motor performance, we do believe that the used study protocol is promising and that the results of this study provide useful information to pave the way to adapt this concept in terms of choice of task and stimulation frequencies to achieve significant modulatory changes.

Frontoparietal network stimulation

The use of NIBS to enhance motor learning has predominantly focused on motor areas. However, neuroimaging studies have convincingly shown the involvement of frontoparietal areas, such as the

DLPFC and the PPC (Floyer-Lea and Matthews, 2005; Dayan and Cohen, 2011). Moreover, motor learning and especially motor sequence learning does not exclusively rely on motor functions, but includes cognitive processes such as WM related processes (Seidler et al., 2012). WM relates to the capacity to temporarily store information in the mind (Baddeley and Hitch, 1974). The process of sequence learning and the execution of sequences requires the capacity to keep the sequence in mind. Therefore, WM capacity is needed for successful sequence learning (Seidler et al., 2012). Although WM is important for sequence learning, brain areas involved in WM performance, such as the frontoparietal areas, have not yet been targeted with NIBS to enhance motor learning. Study 4 of this thesis has for the first time explored the involvement of the FPN during motor sequence learning by targeting it with bifocal, synchronized tACS in the theta range. . The study included healthy older adults, because WM capacity shows an age-related decrease. Moreover, WM impairments have been related to decreased motor learning (Bo et al., 2009). Therefore, we hypothesized that older adults might benefit from FPN stimulation concurrent to the training of a motor tasks to enhance motor learning. Our results showed significant effects of active stimulation on a sequence learning task with high WM load, but not for a task with low WM load.

The differentiation between the effectiveness of stimulation during the task with high WM load compared with low WM load, compatible with the hypothesis, shines a light on the underlying mechanisms of the role of the FPN during motor sequence learning. Our results indicate that stimulating the FPN was exclusively effective during the task with high WM load. This points towards a specific efficacy of this stimulation paradigm, which is dependent on the underlying brain state. This can be related to the study of Violante *et al.* who used a similar FPN theta tACS paradigm to enhance WM, and found positive results when WM load was high (Violante et al., 2017). Sauseng *et al.* have shown the theta coherence in the FPN increases during challenging tasks with high WM load (Sauseng et al., 2005). We speculate that the externally applied theta oscillations to the FPN might have increased theta coherence, which could have aided the performance during the challenging WM task. No coherence analysis was done in the current study; therefore, this hypothesis requires further analysis. It has been suggested that a confounding factor of tACS is that it rather has a somatosensory effect and does not directly affect cortical activity due to shunting of the current (Vöröslakos et al., 2018). However, a recent animal study has shown similar effects on local field potentials (LFP), when applied with and without anaesthetic cream (Vieira et al., 2020). Therefore, we do not expect that this effect was solely driven by somatosensory input. Moreover, Violante *et al.* found evidence of the neuronal correlates by showing increased functional connectivity after theta tACS to the FPN while performing a WM task (Violante et al., 2017).

We chose to include healthy older adults because it has been shown that WM capacity diminishes with age, which results in a reduction in performance on WM tasks, especially with high WM load (Nyberg et al., 2009; Nagel et al., 2011). More specifically, due to reduced neural efficiency older adults show hyperactivation of the FPN during low WM load tasks, and hypoactivation during high WM load tasks due to decreased neural capacity compared to healthy young adults (Rajah and D'Esposito, 2005; Reuter-Lorenz and Cappell, 2008). Therefore, the effect of stimulation might have induced a possible restoration of the changed activity patterns in the FPN in healthy older adults. Moreover, studies have shown an age-related decrease in FPN coupling during the performance of a WM task, which continuously decreased based on WM load (Heinzel et al., 2014, 2017). With the use of tACS, it is possible to externally induce synchronized oscillations that can entrain the endogenous oscillations (Salamanca-Giron et al., 2020; Fröhlich and Riddle, 2021) and might therefore restore deficient FPN coupling in healthy older adults. However, further research is warranted to investigate whether bifocal tACS within the current setup impacts on functional coherence associated with respective behavioral changes and whether benefits of stimulation to the FPN network are indeed age-related.

In summary, bifocal theta tACS applied to the FPN did enhance the performance on a sequence learning task in healthy older adults. Both abovementioned studies were fundamentally different from each other, which hampers the possibility to draw common conclusions. However, both studies do relevantly add to the currently existing knowledge of NIBS to enhance motor learning. The relative novelty of both stimulation paradigms enlarged the choice of paradigms that could be effective for motor learning and ultimately motor rehabilitation.

6.2 Multifocal stimulation

Motor learning is represented in a large cortico-subcortical network with different crucial hubs and their interaction adding to the implementation and success of learning novel motor acts or skills. The main hubs that have been addressed individually by means of NIBS are the M1 or the CB (O'Brien et al., 2018; Wessel and Hummel, 2018; Wessel et al., 2022). However, based on the learning-related network interactions and the different roles of these hubs during the learning process (Hardwick et al., 2013), one can hypothesize that applying NIBS not only to one region, but several hubs in an orchestrated fashion might enhance interregional interactions, or adapted to the specific parts of the learning process might lead to much larger behavioral effects. Thus, targeting the brain as a network rather than single sites has been one of the main alternative stimulation strategies and has been studied in study 3 and 4 of this thesis. With the use of two different types of multifocal stimulation paradigms, sequential vs. simultaneous, we were able to show positive results on motor sequence learning tasks in stroke patients and healthy older adults, which will be discussed separately.

In study 3, we compared an orchestrated sequential M1 and CB anodal tDCS paradigm to monofocal M1 stimulation in a cohort of stroke patients. Our findings indicated that multifocal stimulation significantly improved motor performance. The choice of orchestrating M1-CB stimulation was based on earlier results, showing the efficacy of M1 tDCS on the online learning phase (Zimerman and Hummel, 2010; Zimerman et al., 2012, 2013) and positive results of CB tDCS on the offline learning phase (Wessel et al., 2016). We hypothesized that sequentially targeting both learning phases could both boost online and offline learning, and therefore lead to a synergistic, additive, or supra-additive effect on motor learning. Our findings indicated online learning effects during CB stimulation, which was specific for the early learning phase. However, these findings were different from our hypothesis. There are different factors that might have led to the present results, e.g., being related to the type of learning task used or cerebellar phase-dependency. Wessel *et al.* found significant offline learning effects during a motor synchronization continuation task, which has a large temporal component that is known to be processed in the CB. Our study used a grip force modulation task, which addresses a different aspect of motor learning. Therefore, it might be that the CB involvement differs between the two tasks. This does fit the results of Cantarero *et al.,* who found online, but not offline learning effects using a task that is more similar to our task, an isometric grip force task (Cantarero et al., 2015). The difference in CB stimulation effects on the specific learning phases discussed in the above studies, suggests that the learning-phase-related effects might be task-specific. Therefore, it is of pivotal importance to consider, which stimulation paradigm could be combined with what type of task. An additional explanation is the phase-dependent involvement of the CB in learning. Doyon *et al.* characterized the CB activation patterns during motor sequence learning with the use of fMRI (Doyon and Ungerleider, 2002). The cerebellar cortex showed increased activity during the early stages of learning and a decrease when learning progressed. Therefore, CB-stimulation might have aided the phase-specific involvement of the CB during sequence learning.

Interestingly, we found performance specific stimulation effects for both, M1 and CB stimulation. The stroke patients that were classified as "low performers" during the baseline measurement of the task performed significantly better during active stimulation. This effect can be partially explained by the

fact that the low performers have a larger margin to improve than the high performers. Another explanation could be stimulation-specific sensitivity of the low performers. Further analysis confirms this hypothesis by showing a performance-related sensitivity for active CB stimulation compared to sham. Only the low performers showed a significant stimulation effect, which was not apparent in the high performing group. The reason for this might be that low performers are likely to show less accurate and controlled movements in the early phase of training. The CB has been related to fine-tuning accurate movements (Naro et al., 2017). Moreover, the CB is involved in error-dependent learning mechanisms (Ito, 2000), which might be more relevant for low performers than high performers. Therefore, CB stimulation might have aided error-dependent learning mechanisms and more accurate movements that were more beneficial for the low performers than the high performers. This hypothesis can be partially confirmed by a recent study using sequential M1 – CB stimulation in healthy young adults (Wessel et al., 2021b). The multifocal stimulation did not result in additional effects over single-site M1 stimulation. We argue that healthy young adults without motor impairments benefit less from CB stimulation than stroke patients with motor impairments. Based on these results, it seems that the efficacy of stimulation protocols is dependent on multiple variables such as study cohort, type of task, learning phase, and baseline performance. Nonetheless, the current results show beneficial effects of the sequential paradigm compared to the monofocal paradigm.

Study 4 showed significant stimulation effects during a bifocal simultaneous stimulation paradigm. The working mechanism of this paradigm is different as it allows to target network-related mechanisms such as interregional coherent activity between two distant areas rather than targeting separate phases of learning as was done with the sequential stimulation paradigm (Antal and Paulus, 2013). Our findings showed positive effects on motor learning, which have been discussed in more detail above (section "frontoparietal network"). Targeting the FPN with tACS to improve motor sequence learning had not been studied before in healthy young, healthy older adults or stroke patients. Moreover, monofocal tACS to either the frontal or parietal areas to enhance sequence learning has not yet been studied. Therefore, we cannot exclude that monofocal stimulation to the DLPFC or PPC might have led to comparable effects. That being said, increased rhythmic synchronization across a brain network is thought to improve network efficiency (Fries, 2005). Motor and cognitive processes depend on coordinated interactions of a network. Along these lines, Miyaguchi *et al.* have used bifocal gamma tACS to target the M1 and CB to facilitate network coherence. They showed a significant error reduction on the performance of a grip force task during bifocal M1-CB tACS, but not for the single-site M1 or CB stimulation conditions (Miyaguchi et al., 2018). Therefore, the ability of multifocal tACS stimulation to exogenously entrain oscillatory activity between functionally related distal areas of a network renders it a promising stimulation technique to enhance motor or cognitive performance (Antal and Paulus, 2013).

Both studies show that multifocal stimulation is a promising NIBS strategy. The sequential multifocal tDCS paradigm was more effective than the monofocal paradigm. However, due to the current setup of this study, it is not possible to disentangle whether these results are mostly due to the early phase CB stimulation or whether it was an orchestrated mechanism. Future studies could include a monofocal CB condition which would allow making more conclusive inferences about the effect of sequential M1-CB tDCS efficacy in stroke patients. The multifocal tACS does currently not allow to conclude higher effectiveness of multifocal tACS compared to monofocal tACS. This open question lies beyond the scope of this thesis and has to be addressed in future studies. However, the current paradigm is an interesting new stimulation target to enhance motor learning, by including the importance of cognition in the process of motor learning.

6.3 Personalization

In this thesis, efforts have been made towards personalization by finding potential biomarkers that can predict stimulation responsiveness and by adjusting the stimulation frequency to the endogenous oscillations of the healthy older adults in study 4.

Finding potential biomarkers could ultimately lead to more effective stroke rehabilitation strategies by identifying candidate stroke patients that are likely to benefit. Our findings indicated potential biomarkers based on baseline performance, baseline neurophysiology, and CST integrity. The baseline performance of stroke patients could generally be related to sensitivity for active M1 and CB stimulation. Moreover, active CB stimulation was more effective in the low performers compared to sham stimulation (for details, please refer to the section "multifocal stimulation"). These results suggest that baseline performance could be a potential predictive marker for CB stimulation efficacy in stroke patients. Due to the setup of this study, we only tested active M1 stimulation without sham M1. Therefore, no inferences can be made about stimulation sensitivity for M1 stimulation in the low performers.

With the use of TMS, inhibitory (SICI) and facilitatory (ICF) neurotransmission was measured to study the underlying mechanisms of learning and stimulation response. In our third study, we did not find any changes or potential biomarkers based on ICF values. However, the baseline level of inhibition (SICI) during rest could be related to performance and stimulation effects. We compared the active vs. sham stimulation condition of CB tDCS to see whether SICI could be related to stimulation responsiveness. Our findings indicate a rather specific effect where indeed the overall performance is better in the patients with high SICI levels (more inhibition). This was independent of active vs sham stimulation. Whereas significant stimulation effects are seen in the patients with low SICI levels (disinhibition). This differs from the results of O'Shea et al. who found that high baseline SICI values were related to behavioral improvements during anodal M1 tDCS (O'Shea et al., 2014). Moreover, our results challenge the model of Daskalakis et al. linking the cerebellar output, measured by cerebellum brain inhibition (CBI)(for details see (Ugawa et al., 1995)) with SICI (Daskalakis et al., 2004). This model suggests that a stronger CBI is related to weaker SICI values (disinhibition). Strengthening of CBI, which would be the most likely consequence of anodal CB-tDCS, would not be beneficial for patients with less room for disinhibition. In this study, we did not measure the SICI values after CB-stimulation and motor training. Therefore, it is not possible to define whether CB-stimulation indeed caused a disinhibition. To make conclusive statements about the working mechanisms of CB-tDCS on SICI measures further analysis is warranted. Nonetheless, we argue that high SICI levels might be a potential biomarker for training success. However, based on the current results low SICI levels might be a potential biomarker for CB-tDCS responsiveness in stroke patients.

As a final biomarker for training success and stimulation susceptibility in stroke patients, we measured the CST integrity by the manifestation of MEP. CST integrity is an important biomarker for recovery in stroke patients (Stinear et al., 2012, 2017; Hordacre et al., 2021). Our findings pointed towards more sensitivity for active stimulation in the patients with no MEP compared to patients with MEP. The sample size, and especially the division between MEP vs. no MEP groups did not allow to do statistical analysis. Therefore, these results are merely exploratory and require additional studies to confirm these findings.

In the final study, the tACS frequencies were adjusted to the endogenous neural oscillations of the individuals. The reasoning for this was that the inter-individual neuronal oscillations vary (Fröhlich and Riddle, 2021), and adjusting stimulation frequencies to the endogenous oscillations increased the power of oscillations (Boyle and Fröhlich, 2013; Reinhart and Nguyen, 2019; Fröhlich and Riddle, 2021). Moreover, the entrainment theory suggests that when tACS oscillations are close to the natural

frequency, the endogenous oscillations can be synchronized and amplified by the stimulation frequency (Antal and Herrmann, 2016). The individual peak frequencies were measured during the performance on a WM memory task. This was done to acquire brain-state dependent frequencies rather than the theta frequency at rest. Our results have shown variances in the measured theta frequency in the individuals. Although the mean theta frequency variance was relatively small 4.1 Hz – 5.4 Hz, we argue that selecting one frequency for all participants might induce heterogeneity in the results. However, the current study did not compare the effect of non-personalized tACS frequencies to personalized frequencies. Therefore, more research is needed for more conclusive statements about the efficacy of personalized tACS frequencies.

The findings of this section show the potential of predictive biomarkers and techniques that aid to work towards the eventual personalization of stimulation paradigms based on the needs of the individual. Extensive research is needed to precisely define the specificity of these biomarkers and the possible improved efficacy of personalizing stimulation frequencies.

6.4 Clinical translation

The present section will discuss the possible clinical translation of the neuromodulation concepts by means of NIBS, such a CB-tACS and targeting the FPN for motor learning in the view of neurorehabilitation of stroke patients. 50 Hz CB-tACS did not enhance motor learning performance in healthy young adults. As discussed, this might be due to the task requiring less accurate movements that did not rely as much on cerebellar input as the earlier study of Naro *et al.* (Naro et al., 2017). For stroke patients the motor impairments cause less accurate and less fine-tuned movement executions in the affected hand (Ingram et al., 2021) and therefore possibly a larger reliance on the cerebellum than young healthy adults. Stroke patients show less disinhibition during movement preparation than age-matched controls (Hummel et al., 2009). However, more disinhibition of the ipsilesional M1 was related to better motor performance and is thought to be a mechanism of stroke recovery. Naro *et al.* argue that 50 Hz tACS perturbed PC activation which enhances M1 excitability (Naro et al., 2017). Stroke patients might be more responsive to 50 Hz CB-tACS due to differing disinhibitory dynamics than healthy young adults (Hummel et al., 2009; Liuzzi et al., 2014). The stimulation could therefore be an adjuvant to the natural compensatory mechanisms by increasing ipsilesional M1 excitability. Extensive research is needed to investigate this possible efficacy of CB-tACS in stroke patients.

Based on study 4, multifocal tACS to the FPN or other brain areas might be beneficial for the rehabilitation of stroke patients. Increased FPN coupling has been related to patients with severe CST damage measured (Hordacre et al., 2021). More specifically, stroke patients with no MEP showed increased FPN coupling compared to patients with MEP's. Interestingly, increased FPN coupling was associated with better upper-limb performance in patients with CST damage (Hordacre et al., 2021). Pointing towards a crucial role of the FPN in motor recovery, especially for patients with more severe damage. Moreover, changes in the coherence between other brain areas than the FPN have been related to motor recovery. Westlake *et al.,* found that motor recovery could be predicted by increased alpha-band connectivity in the ipsilesional somatosensory area, supplementary motor area, and the CB, with reduced connectivity of contralesional motor areas (Westlake et al., 2012). Furthermore, increased beta-band connectivity between ipsilesional M1 and the premotor cortex is related to larger motor gains during rehabilitation (Wu et al., 2015). Moreover, bilateral M1 beta-band coherence is positively correlated with motor function in subacute and chronic stroke (Pichiorri et al., 2018). Therefore, not only does oscillatory connectivity provide valuable biomarkers for the prediction of rehabilitation after stroke, it also points towards their potential as targets for innovative interventional strategies, such as

multifocal frequency specific tACS to enhance the effects of neurorehabilitation towards better recovery.

In stroke recovery, the phase of recovery (acute, sub-acute or chronic) might play an important role in the efficacy of the NIBS paradigm as they are marked by different pathogenic mechanisms (Grigoras and Stagg, 2021). Different neurophysiological biomarkers in the acute phase have been related to recovery in later stages. For example, ipsilesional loss of power in the alpha frequency band and an increase in power in the beta-band within the first 2 weeks has been related to poor recovery (Finnigan and van Putten, 2013). As mentioned above, bilateral beta-band coherence in the subacute phase has been related to motor improvements (Nicolo et al., 2015; Pichiorri et al., 2018). Moreover, with the use of TMS measurements, it has been shown that the acute and sub-acute phase is marked by reduced intracortical inhibition while the chronic phase often shows a normalisation of intracortical inhibition (Grigoras and Stagg, 2021; Liuzzi et al. 2014). Changes in GABA-ergic neurotransmission have been related to improvements in function during rehabilitation (Blicher et al., 2015). These neurophysiological biomarkers can be used to define the NIBS protocol depending on the recovery phase. However, it has been stated that the decreased GABA-ergic inhibition in the ipsilesional hemisphere in the acute phase plays an important role in the support of neuroplasticity, which is related to increased spontaneous recovery, when this normalises during the chronic phase it also decreases the amount of motor recovery (Grigoras and Stagg, 2021). The higher levels of spontaneous recovery during the sub-acute phase have been one of the main confounding factors for NIBS as it is not clear how to disentangle stimulation effects from spontaneous recovery effects (Winters et al., 2015). Therefore, most studies have focused on the chronic phase for NIBS interventions when spontaneous recovery reaches a plateau (Kang et al., 2016; Parikh et al., 2021). However, studies that did use NIBS during the sub-acute phase showed similar effect sizes as during the chronic phase (Kang et al., 2016). Therefore, extensive research is needed to define a most optimal time window for NIBS stimulation in stroke patients.

Based on the current results of this thesis, future studies could aim to incorporate the alternative strategies that were used and work towards a stratification study tailored to the needs of the patients. For this to be possible, there are remaining unanswered questions that need to be studied first. For example, whether stroke patients would be aided by targeting the CB with tACS in the gamma range and additionally whether multifocal tACS applied to the M1 and CB would have additive effects to monofocal tACS to the CB or M1. Moreover, it would be interesting to base the training task on the baseline performance. It might well be that patients with low baseline performance benefit from training the SGFMT combined with CB NIBS, while high performers might benefit more from a task with more accurate finger movements such as the SFTT in combination with CB NIBS. Finally, it might be interesting to consider the level of WM impairments in stroke patients to define whether patients might be aided more by tACS to the FPN or to motor related areas such as CB and M1. A future study could consist of a comparison between a stratified stimulation condition based on the initial WM and motor impairments to a non-stratified condition.
Further investigation of the feasibility of personalizing stimulation and motor learning paradigms is necessary before conclusive statements can be made about optimal strategies. However, this thesis has added additional possibilities for stratification strategies by proposing alternative stimulation locations and paradigms which could ultimately lead to enhanced efficacy.

7. Conclusion

The aim of this thesis was to investigate multiple strategies of NIBS to enhance stimulation effects on motor learning. The tested protocols were all based on fundamentally different concepts and mechanisms, therefore direct comparisons between the studies are not possible. However, the studies do fit in an overarching idea of expanding the current knowledge about NIBS potential in regard of novel targets, efficacy and working towards the personalization of stimulation paradigms that can ultimately improve stroke rehabilitation.

Study 1 aimed to get a more methodological understanding of different TMS paradigms to induce inhibitory and excitatory measures. Our findings showed comparable results for SICI and ICF measures using different TMS stimulators, waveforms (monophasic vs. biphasic), and current directions. This suggests that SICI and ICF measures obtained from different studies could be combined to allow for larger data sets and multicentre research collaborations.

Study 2 explored the effects of 50 Hz tACS applied to the CB in healthy young adults. This stimulation paradigm did not affect the performance on a SGFMT in healthy young adults. We speculate that the task was not challenging enough for healthy young adults and that the CB was not the optimal stimulation site for this motor task. CB stimulation has been shown to improve the accuracy of fine-tuned movements, which were less required in this task. Moreover, stimulation frequency optimization might increase efficacy. Therefore, although we did not find significant stimulation effects, the study has provided insights for possible improvements based on stimulation frequency and task choice.

Study 3 investigated an orchestrated stimulation paradigm to enhance specific learning-phase mechanisms in stroke patients. Our findings indicated significant effects of a multifocal M1 – CB tDCS application on the performance on a SGFMT in stroke patients. Sequential multifocal tDCS was more effective than monofocal M1 stimulation, which points towards the efficacy of orchestrating stimulation by targeting multiple important hubs of a network. However, we were not able to show the learning phase-specific enhancements as we hypothesized. We were able to relate baseline performance and SICI parameters to stimulation effects. Although these possible biomarkers require further investigation, they provide opportunities for possible stratification measures to enhance stimulation effects in stroke patients.

Study 4 indicated that personalized bifocal theta tACS to the FPN enhanced performance on a motor task with high WM load, but not for a motor task with low WM load in a cohort of healthy older adults. We argue that the specificity of the stimulation effects is due to the fact that tasks with high WM load results in increased theta coherence in the FPN. tACS might have externally enhanced interareal interactions (e.g., by means of increased coherence), thereby enhancing the performance on motor learning tasks with high WM load. Moreover, we propose the FPN as an interesting target location for stroke patients, as FPN coupling has been related to better stroke recovery.

In conclusion, targeting other brain areas than the M1 has shown promising results. Moreover, we could highlight the importance of network interactions for motor learning by showing that the application of multifocal stimulation resulted in positive effects. In addition, we identified potential biomarkers for stimulation efficacy that require further examination but could ultimately lead towards the personalization of study protocols. Exploring these alternative stimulation strategies to enhance motor learning has provided useful insights. Most importantly, it has shown that stimulation effects are highly specific and dependent on multiple factors that warrant careful consideration when designing a NIBS study. Further increasing our understanding can lead towards constructive and fact-based ideas for improvements of stimulation protocols that could ultimately lead to the translation of NIBS paradigms in clinical practices to improve rehabilitation after stroke. The fact that has been omnipresent throughout these studies is that one size does indeed not fit all.

8. References

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9. Supplementary material

9.1 Supplementary material study 2

Transcranial alternating current stimulation (tACS) associated sensations

Active versus sham stimulation distinction. Participants could not effectively distinguish the active from the sham stimulation during the two sessions. The proportion of correction distinctions before cross-over was .467 and was not significantly different from chance level $p = 1.00$. The proportion of correction distinctions after cross-over was .600 and not significantly different from chance level $p = 0.607$. The respective statistical analysis was implemented in JASP (JASP Team, 2019).

Reported sensations. After the final training session, the participants were asked about their perceived sensations during the stimulation for both training sessions applying a structured questionnaire, adapted from Antal and colleagues(Antal et al., 2017). We checked for the following sensations: itching, pain, burning, metallic/iron taste in mouth, warmth, fatigue, other. Response options were: "0" = none, "1" = mild, "2" = moderate, "3" = strong. Most of the responses for all of the sensations were either "none" or "mild". None of the reported sensations differed significantly between active and sham stimulation, please see also table below. The respective statistical analysis was implemented in JASP (JASP Team, 2019).

	None		**Mild**		**Moderate**		**Strong**		**Statistics**	
	Active	**Sham**	**Active**	**Sham**	**Active**	**Sham**	**Active**	**Sham**	**Chi-square**	***p*-value**
Itching	33.3	33.3	53.3	60	13.3	6.7	0	0	0.39	.822
Pain	80	73.3	20	26.7	0	0	0	0	0.19	.666
Burning	53.3	66.7	40	20	0	13.3	6.7	0	4.22	.238
Warmth	60	40	33.3	60	6.7	0	0	0	2.74	.254
Metallic/iron taste	100	100	0	0	0	0	0	0	n/a	n/a
Fatigue	80	86.7	6.7	13.3	13.3	0	0	0	2.37	.305
Other	40	53.3	26.7	26.7	33.3	20	0	0	0.79	.675

Numbers correspond to percentages of participants, who chose the response option for the respective stimulation condition.

Supplementary Table S1. tACS-associated sensations depicted for the active and sham stimulation condition separately.

No association of modulation of striato-parietal FC and behavior

	SBC			**SBC x STIMULATION Interaction**		
	χ(df)	***p*-value**	f^2	χ(df)	***p*-value**	f^2
Online learning	*3.60(1)*	*p* = .058	f^2 =*.169*	*0.94(2)*	*p* = .624	f^2 =*.017*
Retention 24h	*0.24(1)*	*p* = .621	f^2 =*.009*	*7.27(2)*	*p* = .026	f^2 =*.094*
Retention 24h (influential point analysis)	*0.35(1)*	*p* = .552	f^2 =*.016*	*1.66(2)*	*p* = .437	f^2 =*.069*
Retention 10d	*0.01 (1)*	*p* = .913	f^2 =<*.001*	*6.54(2)*	*p* = .038	f^2 =*.028*
Retention 10d (influential point analysis)	*0.01(1)*	*p* = .904	f^2 =<*.001*	*2.60(2)*	*p* = .273	f^2 =*.018*

Supplementary Table S2. We calculated three separate linear mixed-effect models for online learning, ca. 24h retention and ca. 10d retention to assess whether the change in SBC and SBC x STIMULATION interaction had a significant influence. The behavioral data was taken as the dependent variable and the delta between SBC T1 and T3 was used, as well as the stimulation effect. In line with the analysis reported in the main manuscript, an influential point analysis was conducted for the retention variables.

Applied sequences in the sequential grip force modulation task (SGFMT)

Block	**Sequence**
Baseline A	H-4-H-3-H-1-H-2-H-5
Baseline B	H-5-H-4-H-2-H-3-H-1
Training A	H-3-H-1-H-4-H-2-H-5
Training B	H-2-H-1-H-5-H-3-H-4
Pseudorandom A	H-1-H-5-H-2-H-4-H-3
Pseudorandom B	H-4-H-2-H-5-H-1-H-3
H: homezone, numbers correspond to bar position on the screen counting from the home zone upwards.	

Supplementary Table S3. The order of targets followed predefined, complexity-matched sequences. Sequence set A or B was allocated to one of the two stimulation conditions following a pseudorandom order.

TMS trial rejection criteria

Our trial rejection criteria were as follows: muscle pre-activation exceeding ± 25 μV from baseline < 100 ms in resting-state or ± 50 μV for event-related trials before and in an 20 ms interval following the TMS pulse, muscle pre-activation ± 100 μV from baseline from start of the trial to 100 ms before the TMS pulse, no clear MEPs in the TS and ICF conditions (defined as peak-to-peak amplitude < 50 μV), overlap of the MEP with the voluntary muscle activation in event-related trials, or trials with documented suboptimal coil placement.

Applied parcellations for defining the regions of interests (ROIs) of the hypothesis-driven motor learning network and the corresponding seeds

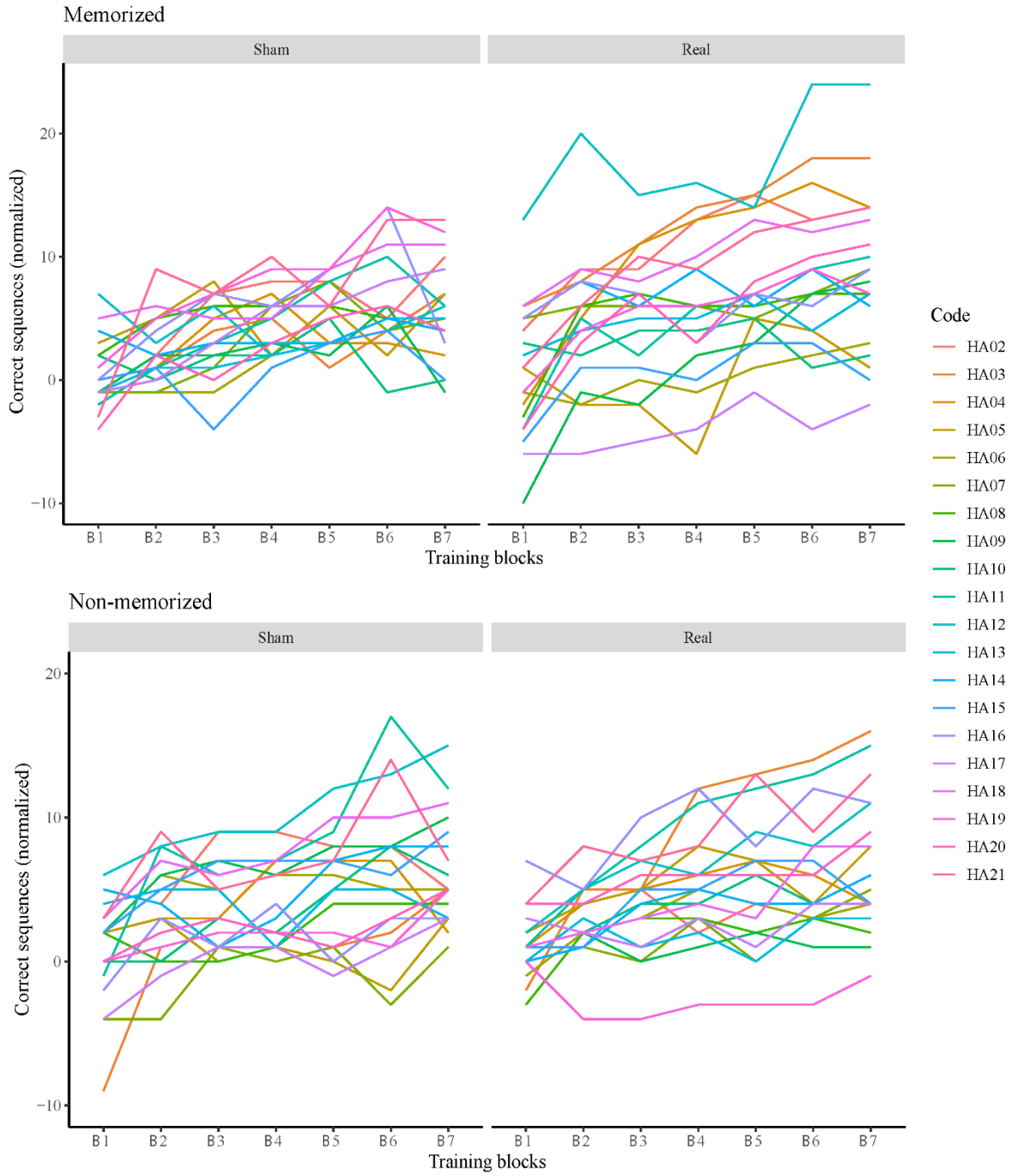
ROI	Side	Atlas	Name of ROI according to atlas	Reference
Primary motor cortex (M1)	Right	Brainnetome atlas	Upper limb	(Fan et al., 2016)
Dorsal premotor cortex (PMd)	Right	Brainnetome atlas	PMd	(Fan et al., 2016)
Ventral premotor cortex (PMv)	Right	Brainnetome atlas	PMv	(Fan et al., 2016)
Supplementary motor area (SMA)	Right	AAL	SMA	(Tzourio-Mazoyer et al., 2002)
Primary somatosensory cortex (S1,2,3)	Right	Brodmann atlas	Area 1, 2 and 3	(Rorden and Brett, 2000)
Dorsolateral prefrontal cortex (DLPFC)	Right	Brainnetome atlas	A9 and 46 (dorsal area of the middle frontal gyrus)	(Fan et al., 2016)
Motor area of the thalamus (THAL)	Right	Behrens atlas	Motor part	(Behrens et al., 2003)
Posterior parietal cortex (PPC)	Right	Brodmann atlas	Area 5, 7, 39 and 40. Contains inferior and superior parietal cortex	(Rorden and Brett, 2000)
Cerebellum (CB)	Left	Bruckner atlas	Network 2	(Buckner et al., 2011)
Striatum (STRIAT)	Right	Choi atlas	Network 2	(Choi et al., 2012)

Supplementary Table S4. Definition of hypothesis-driven ROIs of the assessed motor learning network.

9.2 Supplementary material study 4

Sequential finger tapping task - individual performance

To further investigate the results on the SFTT and appreciate the variance in performance the individual trajectories of the participants are indicated in the figure below.



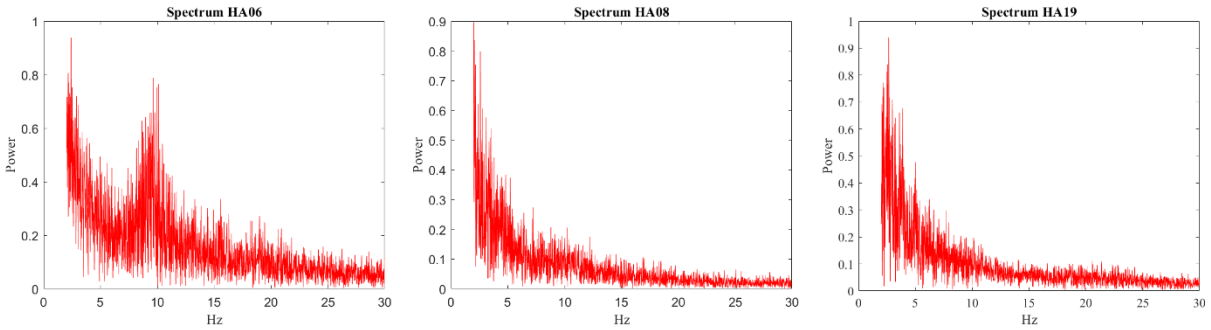
Supplementary figure 1. Individual performance of participants separated by task condition. Top figure indicates the memorized condition separated by sham and real stimulation. Bottom figure indicates the non-memorized condition divided by sham and real stimulation.

Peak frequency analysis

Peak frequencies in the theta range have been measured with the use of EEG. To appreciate the variance between the theta peak of every single electrode the individual data of 3 randomly chosen participants are shown, please see Supplementary table 2. In addition, a visualization of the individual EEG power spectrum is shown in supplementary figure 2.

Supplementary table 2. Peak frequencies in the theta range of single electrodes are shown for 3 randomly chosen participants of the study. The average theta peak and the standard deviation are shown for variance.

Code	Peaks single electrodes										
	F4	P4	Fp1	Fp2	F3	P3	Cz	Fz	Pz	Mean	SD
HA06	4.25	4.16	4.38	4.52	4.38	4.76	4.13	4.72	4.14	4.38	0.28
HA08	5.24	6.37	4.41	4.3	5.22	6.07	5.98	4.43	5.6	5.29	0.73
HA19	4.45	6.41	5.07	4.43	6.94	5.69	4.1	4.84	5.35	5.25	0.89



Supplementary figure 2. Examples of the individual EEG power spectrum per participant. Ranging from 2 Hz to 30 Hz.

Curriculum Vitae

Personal details

Name	Laurijn Rianne Draaisma
Address	77 Rue de Lausanne, Genève
E-mail	l.r.draaisma@gmail.com
Phone	+41 (0)774171578
Date of birth	2 June 1990
Nationality	Dutch

Education

2017 – Present	Doctoral student, EPFL program neuroscience (EDNE), Ecole Polytechnique Fédérale Lausanne (EPFL).
2013 – 2016	Research master Neuroscience & Cognition – at University Utrecht.
2010 – 2013	Bachelor of Science in Neuropsychology – at University Utrecht *Minor: Social neuroscience.*
2009 – 2010	University college Maastricht, Maastricht.
2002 – 2008	Pre-university education (VWO), St. Stanislas college Delft.

Articles and reports

2022 Wessel M.J., Draaisma L.R**.**, Hummel F.C. (2022) Mini-review: Transcranial Alternating Current Stimulation and the Cerebellum. *The Cerebellum.* https://link.springer.com/10.1007/s12311-021-01362-4

2022 Wessel M.J.*, Draaisma L.R.*, Durand-Ruel M., Maceira-Elvira P., Moyne M., Turlan J.-L., Mühl, A., Léger, B., Chauvigné, L., Park C., Koch P.J., Morishita T., Guggisberg A.G. & Hummel F.C. (in preparation). Multi-focal stimulation of the cortico-cerebellar loop during the acquisition of a novel hand motor skill in chronic stroke patients.

2022 Draaisma, L.R**.**, Wessel, M.J., Moyne, M., Morishita, T. & Hummel, F.C. (under revision), Targeting the fronto-parietal network using multifocal personalized transcranial alternating current stimulation to enhance motor sequence learning in healthy older adults. *Brain Stimulation*, DOI: 10.1101/2022.02.16.480660

2020 Wessel, M. J.*, Draaisma, L. R.*, de Boer, A. F. W., Park, C.-H., Maceira-Elvira, P., Durand-Ruel, M., Koch, P. J., Morishita, T., & Hummel, F. C., (2020) Cerebellar transcranial alternating current stimulation in the gamma range applied during the acquisition of a novel motor skill, *Scientific Reports*, 10(1), 11217. https://doi.org/10.1038/s41598-020-68028-9

2020 Draaisma, L. R.*, Wessel, M. J.*, & Hummel, F. C. (2020). Non-invasive brain stimulation to enhance cognitive rehabilitation after stroke. *Neurosci Lett*, 719, 133678. https://doi.org/10.1016/j.neulet.2018.06.047

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2019 Wessel, M. J*., Draaisma, L. R*., Morishita, T., & Hummel, F. C. (2019). The Effects of Stimulator, Waveform, and Current Direction on Intracortical Inhibition and Facilitation: A TMS Comparison Study. Frontiers in neuroscience, 13, 703.

2018 Draaisma, L.R., Wessel, M.J, & Hummel, F.C. (2018) Non-invasive brain stimulation to enhance cognitive rehabilitation after stroke. *Neurosci Lett*, *719*, 133678. https://doi.org/10.1016/j.neulet.2018.06.047

2016 Van Dellen, E., Bohlken, M.M., Draaisma, L., Tewarie, P.K., Van Lutterveld, R., Mandl, R., Stam, C.J., Sommer, I.E. (2016) Structural Brain Network Disturbances in the Psychosis Spectrum. *Schizophrenia Bulletin*. 782-789.

2015 L. R. Draaisma, I.O. Bergfeld, D. Hoffman & D. Denys (2015). Deep Brain Stimulation as a treatment for treatment-resistant depression: Efficacy and Underlying Mechanisms (Master thesis).

Employment history

2017 – Present	PhD student, UP-HUMMEL lab, Ecole Polytechnique Fédérale Lausanne (EPFL).
2017	Research assistant Biomedical Primate Research Centre, Rijswijk, The Netherlands
2016	Research assistant University Utrecht, department of experimental psychopathology, Utrecht, The Netherlands.
2015	Research assistant Biomedical Primate Research Centre, Rijswijk, The Netherlands
2006 - 2014	Various income generating jobs (Administration, Information services, bartender, cashier)

Conferences & Symposia

2020	"7th International Conference on Non-Invasive Brain Stimulation" – Online- Poster presentation "Cerebellar transcranial alternating current stimulation in the gamma range applied during the acquisition of a novel motor skill". Travel award
2019	"*Alpine Brain Imaging Meeting 2020"* – Champéry – 6-10 January. Poster presentation: "*The effect of stimulators, waveforms and current direction on intracortical inhibition and facilitation: a TMS comparison study."*
2018	*Stress in Health and Disease* – EPFL- Lausanne- 14 & 15 March
2017	*Neural implementations of learning models* – EPFL – Lausanne – 16 & 17 November

2017	CNP-retreat – Annecy – 23 & 24 October Poster presentation: Laurijn R. Draaisma, Maximilian J. Wessel & Friedhelm C. Hummel *Differential effects of waveforms and current direction on intracortical inhibition and facilitation*

Internships

2015	Research topic: The validation of a PTSD-animal model. Biomedical Primate Research Centre (BPRC), Rijswijk, The Netherlands Duration: 6 months
2013-2014	Research topic: Brain network analysis using fMRI scans. University Medical Centre Utrecht (UMC), department of psychiatry. Duration: 9 months

Computer experiences

MS office	Extensive
SPSS	Extensive
R	Extensive
Adobe	Extensive
Graphpad Prism	Moderate
Matlab	Basic

Languages

Dutch	Mother tongue
English	Fluent
French	B2 level
German	Basic knowledge

Other experiences

2018-2020	Captain of the CERN rugby team.
2012- 2017	Player in the Dutch national Rugby team.
2016	Runner-up at World championship qualifier tournament (Rugby, national team)
2015	Winner European Throphy cup (Rugby, national team)
2012-2016	Dutch National Champion Rugby (5 times)
2009	Extensive travel in Asia and Australia